Pediatric Cancer
Diagnosis, Therapy, and Prognosis

M.A. Hayat
Editor

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Volume 4
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To:
Robert and Nancy Cirasa

Although touched by technology, surgical pathology always has been, and remains, an art. Surgical pathologists, like all artists, depict in their artwork (surgical pathology reports) their interactions with nature: emotions, observations, and knowledge are all integrated. The resulting artwork is a poor record of complex phenomena.

Richard J. Reed, MD

One Point of View

All small tumors do not always keep growing, especially small breast tumors, testicular tumors, and prostate tumors. Some small tumors may even disappear without a treatment. Indeed, because prostate tumor grows slowly, it is not unusual that a patient may die at an advanced age of some other causes, but prostate tumor is discovered in an autopsy study. In some cases of prostate tumors, the patient should be offered the option of active surveillance followed by PSA test or biopsies. Similarly, every small kidney tumor may not change or may even regress. Another example of cancer or precancer reversal is cervical cancer. Precancerous cervical cells found with Pap test may revert to normal cells. Tumor shrinkage, regression, dormancy, senescence, reversal, or stabilization is not impossible. Can prosenescence therapy be an efficient alternative strategy to standard therapies for cancer prevention and treatment?

Another known example of cancer regression is found in pediatric neuroblastoma patients. Neuroblastoma shows one of the highest rates of spontaneous regression among malignant tumors. In addition to the well-known spontaneous regression in stage 4S disease, the high incidence of neuroblastoma remnants found during autopsy of newborns suggest that localized lesions may undergo a similar regression (Guin et al. 1969). Later studies also indicate that spontaneous regression is regularly seen in infants with localized neuroblastoma and is not limited to the first year of life (Hero et al. 2008). These and other studies justify the "wait and see" strategy, avoiding chemotherapy and radiotherapy in infants with localized neuroblastoma, unless MYCN gene is amplified. Infants with nonamplified MYCN and hyperdiploidy can be effectively treated with less intensive therapy. Infants with disseminated disease without MYCN have excellent survival with minimal or no treatment. Another example of spontaneous shrinkage and loss of tumors without any treatment is an intradurallipoma (Endoh et al. 1998).

Although cancers grow progressively, various lesions such as cysts and thyroid adenomas show self-limiting growth. Probably, cellular senescence occurs in many organ types following initial mutations. Cellular senescence, the growth arrest seen in normal mammalian cells after a limited number of divisions, is controlled by tumor suppressors, including p53 and p16, and so this phenomenon is believed to be a crucial barrier to tumor development. It is well-established that cell proliferation and transformation induced by oncogene activation are restrained by cellular senescence.

Metastasis is the main cause of death from cancer. Fortunately, metastasis is an inefficient process. Only a few of the many cancer cells detached from the primary tumor succeed in forming secondary tumors. Metastatic inefficiency varies depending on the location within an organ, but the malignancy may continue to grow preferentially in a specific tissue environment. Some of the cancer cells shed from the primary tumor are lost in the circulation due to hemodynamic forces or the immune system, macrophages, and natural killer cells.

Periodic rejection of a drug by FDA, which was previously approved by the FDA, is not uncommon. Most recently, the FDA ruled that Avastin should not be used to treat advanced breast cancer, although it remains on the market to treat other cancers, including colon and lung malignancies. Side-effects of Avastin include high blood pressure, massive bleeding, heart attack, and damage to the stomach and intestines.

Unwanted side effects of some drug excipients (e.g., propylene glycol, menthol) may also pose safety concerns in some patients. Excipients are defined as the constituents of the pharmaceutical formulation used to guarantee stability, and physicochemical, organoleptic, and biopharmaceutical properties. Excipients frequently make up the majority of the volume of oral and parenteral drugs. Not all excipients are inert from the biological point of view. Although adverse drug reactions caused by the excipients are a minority of all adverse effects of medicinal products, the lack of awareness of the possible risk from excipients should be a concern for regulatory agencies, physicians, and patients (Ursino et al. 2011). Knowledge of the potential side effects of excipients is important in clinical practice.

It is known that chemotherapy can cause very serious side-effects. One most recent example of such side-effects was reported by Rubsam et al. (2011). Advanced hepatocellular carcinoma (HCC) induced by hepatitis C virus was treated with sorafenib. It is an oral multikinase inhibitor that interferes with the serine/threonine kinases RAF-1 and B-Raf and the receptor tyrosine kinases of the vascular endothelial growth factor receptors and the platelet-derived growth factor receptor-beta. Although sorafenib is effective in regressing HCC, it shows serious side-effects including increasingly pruritic and painful skin changes (cutaneous eruption).

An example of unnecessary surgery is the removal of all the armpit lymph nodes after a biopsy when a sentinel node shows early stage breast cancer; removal of only the sentinel node may be needed. Limiting the surgery to the sentinel node avoids painful surgery of the armpit lymph nodes, which can have complications such as swelling and infection (such limited surgery is already being practiced at the Memorial Sloan-Kettering Cancer Research Center). Radiation-induced second cerebral tumors constitute a significant risk for persons undergoing radiotherapy for the management of cerebral neoplasms. High-grade gliomas are the most common radiation-induced tumors in children (Pettorini et al. 2008). The actual incidence of this complication is not known, although it is thought to be generally low.

Medical Radiation

Chromosome aberrations induced by ionizing radiation are well-known. Medical radiation-induced tumors are well-documented. For example, several types of tumors (sarcomas, meningiomas) can develop in the CNS after irradiation of the head and neck region (Parent 1990). Tumorigenic mechanisms underlying the radiation therapy of the CNS are discussed by Amirjamshidi and Abbassioun (2000) (See below).

Radiation therapy is commonly used to treat, for example, patients with primary and secondary brain tumors. Unfortunately, ionizing radiation has limited tissue specificity, and tends to damage both neoplastic and normal brain tissues. Radiation-induced brain injury, in fact, is a potential, insidious later cerebral side-effect of radiotherapy. Most commonly it consists of damage in small arteries and capillaries, resulting in secondary processes of ischemia.

After radiation therapy, imaging techniques (CT, MRI, SPECT) can be used to assess treatment response and detect radiation-induced lesions and recurrent tumors. Optical spectroscopy has also been used for detecting radiation damage (Lin et al. 2005). The F_{500}nm spectral peak allows accurate selection of tissues for biopsy in evaluating patients with new, contrast enhancing lesions in the setting of previous irradiation. This peak is highly correlated with a histological pattern of radiation injury. Deep lesions require a stereotactic biopsy to be conclusive. Also, much of the radiation effect is mediated by acute and chronic inflammatory cellular reactions. Biopsy samples supplement pathological differentiation of radiation effect from tumor progression. It should be noted that most of the biopsies show radionecrosis as well as scattered tumor cells.

Women treated with therapeutic chest radiation may develop cancer. This possibility becomes exceedingly serious considering that 50,000–55,000 women in the United States have been treated with moderate to high-dose chest radiation (~20 Gy). This possibility is much more serious for pediatric or young adult cancer patients, because these women are at a significantly increased risk of breast cancer and breast cancer mortality following cure of their primary malignancy (Martens et al. 2008). A recent study also indicates that such young women develop breast cancer at a young age, which does not appear to plateau (Henderson et al. 2010). In this high-risk population, ironically there is a benefit associated with early detection. In other words, young women with early stage breast cancer following chest radiation have a high likelihood for favorable outcome, although life-long surveillance is needed.

Presently, although approximately 80% of the children with cancer are cured, the curative therapy could damage a child's developing organ system; for example, cognitive deficits following cranial radiotherapy are well known. Childhood survivors of malignant diseases are also at an increased risk of primary thyroid cancer (Sigurdson et al. 2005). The risk of this cancer increases with radiation doses up to 20–29 Gy. In fact, exposure to radiation therapy is the most important risk factor for the development of a new CNS tumor in survivors of childhood cancer, including leukemia and brain tumors. The higher risk of subsequent glioma in children subjected to medical radiation

at a very young age reflects greater susceptibility of the developing brain to radiation. The details of the dose-response relationships, the expression of excess risk over time, and the modifying effects of other host and treatment factors have not been well defined (Neglia et al. 2006).

A recent study indicates that childhood brain tumor survivors are at an increased risk of late endocrine effects, particularly the patients treated with cranial radiation and diagnosed at a younger age (Shalitin et al. 2011). Among children with cancer, the application of radiotherapy, therefore, should not be taken lightly, and it should be administered only when absolutely necessary to successfully treat the primary tumor. When radiotherapy is administered, use of the minimum effective dose tends to minimize the risk of second CNS neoplasms (late effect). Prolonged follow-up of childhood cancer survivors (particularly those treated with radiation) is necessary because of the long period between treatment and the development of malignancy. This practice should be a part of the effective therapy of the primary disease.

It is well established that radiation doses are related to risk for subsequent malignant neoplasms in children with Hodgkin's disease. It has been reported that increasing radiation dose was associated with increasing standardized incidence ratio ($p = 0.0085$) in survivors of childhood Hodgkin's disease (Constine et al. 2008). Approximately, 75% of subsequent malignancies occurred within the radiation field. Although subsequent malignancies occur, for example, in breast cancer survivors in the absence of radiotherapy, the rise increases with radiation dose.

The pertinent question is: Is it always necessary to practice tumor surgery, radiotherapy, chemotherapy, or hormonal therapy or a combination of these therapies? Although the conventional belief is that cancer represents an "arrow that advances unidirectionally," it is becoming clear that for cancer to progress, it requires cooperative microenvironment (niche), including immune system and hormone levels. However, it is emphasized that advanced (malignant) cancers do not show regression and require therapy. In the light of the inadequacy of standard treatments of malignancy, clinical applications of the stem cell technology need to be expedited.

Prostate Cancer

There were an estimated 217,730 new cases of prostate cancer in the United States in 2010 with 32,050 deaths, making it the second leading cause of cancer deaths in men. Currently, there are more than 2,000,000 men in the United States who have had radical or partial prostate surgery performed. Considering this huge number of prostate surgeries and the absence of a cumulative outcome data, it seems appropriate to carefully examine the benefits of radical surgery, especially in younger men.

Clinical prostate cancer is very rare in men of the ages younger than 40 years. In this age group the frequency of prostate malignancy is 1 in 10,000 individuals. Unfortunately, the incidence of malignancy increases over the ensuing decades, that is, the chance of prostate malignancy may reach to one in seven in men between the ages of 60 and 79 years. Reactive or aging-related

alterations in the tumor microenvironment provide sufficient influence, promoting tumor cell invasion and metastasis. It has been shown that nontumorigenic prostate epithelial cells can become tumorigenic when cocultured with fibroblasts obtained from regions near tumors (Olumi et al. 1999).

Prostate cancer treatment is one of the worst examples of overtreatment. Serum prostate specific antigen (PSA) testing for the early detection of prostate cancer is in wide use. However, the benefit of this testing has become controversial. The normal cut-off for serum levels of PSA is 4 ng/ml, so a man presenting with a PSA above this level is likely to require a rectal biopsy, but only 25% of men with serum levels of PSA between 4 ng and 10 ng/ml have cancer (Masters 2007). The PSA threshold currently being used for biopsy ranges between 2.5 ng/ml and 3.4 ng/ml. Up to 50% of men presenting with prostate cancer have PSA levels within the normal range. It is apparent that screening of prostate cancer using PSA has a low specificity, resulting in many unnecessary biopsies, particularly for gray zone values (4–10 ng/ml). According to one point of view, the risks of prostate cancer over-detection are substantial. In this context, overdetection means treating a cancer that otherwise would not progress to clinically significant disease during the lifetime of the individual. Overdetection results in overtreatment. The advantages and limitations of PSA test in diagnosing prostate cancer were reviewed by Hayat (2005, 2008).

Androgen deprivation therapy (ADT) is an important treatment for patients with advanced stage prostate cancer. This therapy is carried out by blocking androgen receptor or medical or surgical castration. Although ADT is initially very effective, treated tumors inevitably progress to androgen-independent prostate cancer (AIPC), which is incurable. One possible mechanism responsible for the development of AIPC is modulation of the tissue microenvironment by neuroendocrine-like cancer cells, which emerge after ADT (Nelson et al. 2007).

Recently, Pernicova et al. (2011) have further clarified the role of androgen deprivation in promoting the clonal expansion of androgen-independent prostate cancer. They reported a novel linkage between the inhibition of the androgen receptor activity, down-regulation of S-phase kinase-associated protein 2, and the formation of secretory, senescent cells in prostate tumor cells. It is known that several components of the SASP secretome, such as IL-6, IL-8, KGH, and epidermal growth factor, are capable of transactivating androgen receptor under androgen-depleted conditions (Seaton et al. 2008). It needs to be pointed out that androgen deprivation therapy, used in high-risk patients with prostate cancer, may cause reduced libido, erectile dysfunction, fatigue, and muscle loss; osteoporosis is also a late complication. Therefore, periodic bone density scanning needs to be considered.

Recently, the FDA cleared the use of NADiA (nucleic acid detection immunoassay) ProsVue prognostic cancer test. This proprietary nucleic acid detection immunoassay technology identifies extremely low concentrations of proteins that have not been routinely used as a diagnostic or prognostic aid. It is an in vitro diagnostic assay for determining the rate of change of serum total PSA over a period of time. The assay can quantitate PSA at levels <1 ng/ml. This technique can be used as a prognostic marker, in conjunction with clinical

evaluation, to help identify patients at reduced risk for recurrence of prostate cancer for years following prostatectomy. It targets the early detection of proteins associated with cancer and infectious diseases. This technique combines immunoassay and real-time PCR methodologies with the potential to detect proteins with femtogram/ml sensitivity (10–15 g/ml). Additional clinical information is needed regarding its usefulness in predicting the recurrence.

A significant decrease in the risk of prostate cancer-specific mortality is observed in men with few or no comorbidities. Indeed, active surveillance in lieu of immediate treatment (surgery or radiation, or both) is gaining acceptance. Most men with prostate cancer, even those with high-risk disease, ultimately die as a result of other causes (Lu-Yao et al. 2009). Debate on this controversy is welcome, but narrow opinions and facile guidelines will not lead to facts and new information – men worldwide deserve it (Carroll et al. 2011). Automatic linking of positive diagnosis with treatment, unfortunately, is a common clinical practice. Unfortunately, even men who are excellent candidates for active surveillance in the United States often undergo some treatment. Deferment of treatment is advised in men with low-risk disease, especially of a younger age.

Active surveillance is proposed for patients with low-risk prostate cancer in order to reduce the undesirable effects of overdiagnosis. Prostate specific antigen serum level lower than 10 ng/L and Gleason score lower than seven are the main criteria to select patients for active surveillance. The correct use of these two criteria is essential to differentiate between aggressive and non-aggressive prostate cancer. Autopsy studies indicate that approximately one out of three men older than 50 years show histological evidence of prostate cancer (Klotz 2008). Thus, a large proportion of prostate cancers are latent, never destined to progress, or affect the life of the patient. It is estimated that the percentage of low-risk prostate cancer is between 50% and 60% of newly diagnosed cases. A large number of patients die having prostate cancer, but not because of this cancer (Filella et al. 2011).

First whole genome sequences of prostate tumors were recently published online in *Nature* journal (vol. 470: 214–220, 2011). This study revealed that rather than single spelling errors, the tumor has long "paragraphs" of DNA that seem to have broken off and moved to another part of the genome (rearrangement of genes), where they are most active. These portions of DNA contain genes that help drive cancer progression. The mutated genes involved include PTEN, CADM2, MAG12, SPOP, and SPTA1. This information may lead to the development of more efficient, less invasive ways to diagnose and treat this cancer. Such information, in addition, should lead to personalized therapeutics according to sequencing results of different gene mutations or chromosomal rearrangements. The urgent need of such studies becomes apparent considering the huge number of new cases of prostate problems reported every year.

In contrast to prostate cancer, cardiovascular disorders take the heavier toll of life. In other words, the risk of death for men in the United States between the ages of 55 and 74 years due to cardiovascular disease surpasses that of prostate cancer. Cardiovascular disease is the most common of the chronic non-communicable diseases that impact global mortality. Approximately,

30% of all deaths worldwide and 10% of all healthy life lost to disease are accounted for by cardiovascular disease alone.

In conclusion, initial treatment with standard surgery, irradiation, chemotherapy, or hormonal therapy, or combination of these protocols can result in both local and systemic sequelae. Therefore, surveillance for late recurrence and secondary primary malignancies is recommended for most cancer patients. Patients with breast, lung, prostate, colorectal, and head and neck cancers constitute the largest groups requiring long-term monitoring and follow-up care.

Eric Hayat

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Preface

It is recognized that scientific journals and books not only provide current information but also facilitate exchange of information, resulting in rapid progress in the medical field. In this endeavor, the main role of scientific books is to present current information in more detail after careful additional evaluation of the investigational results, especially those of new or relatively new diagnostic approaches and therapeutic methods and their potential toxic side-effects.

Although subjects of diagnosis, cancer recurrence including brain tumors, resistance to chemotherapy, assessment of treatment effectiveness, including cell therapy and side-effects of a treatment are scattered in a vast number of journals and books, there is need of combining these subjects in single volumes. An attempt is made to accomplish this goal in the projected multi-volume series of Handbooks.

In the era of cost-effectiveness, my opinion may be minority perspective, but it needs to be recognized that the potential for false-positive or false-negative interpretation on the basis of a single laboratory test in clinical pathology does exist. Interobserver or intraobserver variability in the interpretation of results in pathology is not uncommon. Interpretative differences often are related to the relative importance of the criteria being used.

Generally, no test always performs perfectly. Although there is no perfect remedy to this problem, standardized classifications with written definitions and guidelines will help. Standardization of methods to achieve objectivity is imperative in this effort. The validity of a test should be based on the careful, objective interpretation of the tomographic images, photo-micrographs, and other tests. The interpretation of the results should be explicit rather than implicit. To achieve accurate diagnosis and correct prognosis, the use of molecular criteria and targeted medicine is important. Equally important are the translation of molecular genetics into clinical practice and evidence-based therapy. Translation of medicine from the laboratory to clinical application needs to be carefully expedited. Indeed, molecular medicine has arrived.

There are many differences between adult and pediatric brain tumors beyond simple nomenclature; for example, pediatric tumors are often more sensitive to adjuvant irradiation and chemotherapy. Some pediatric tumors may need complete resection only to achieve a cure. It is pointed out that an experienced neurosurgeon should be aware of the difference between the adult tumors and pediatric tumors. It is emphasized, for example, that pediatric low-grade cancers need lower doses of anticancer drugs such as cisplatin/

etoposide. Refinements in clinical and molecular stratification for many types of childhood brain tumors to achieve risk-adapted treatment planning are discussed.

This is the fourth volume in the series, *Pediatric Cancer*. Brain tumors are the most common solid tumors of childhood, and remain the leading cause of cancer-related mortality in children. Molecular characterization of solid tumors is important for providing novel biomarkers of disease and identifying molecular pathways, which may provide putative targets for new therapies. Specifically, this volume discusses in detail molecular genetics, diagnosis, prognosis, and therapy of atypical teratoid/rhabdoid tumor (AT/RT). AT/RT is a highly aggressive embryonal CNS tumor, which is mainly found in children, with a peak incidence in infants younger than 3 years of age. In fact, these tumors are among the most common malignant neoplasms in children. Similarities of the AT/RT with some other CNS tumors (PNET and medulloblastoma) tend to misclassify this tumor, which is pointed out in this volume. Although AT/RT has overlapping histological features with some other tumors, one feature unique to most AT/RTs is the genetic abnormality in the INI1 gene on chromosome 22q11. Historically, outcomes for patients with the atypical AT/RT have been poor despite surgery and chemotherapy. Failure of standard treatments for early childhood paraspinal atypical teratoid/rhabdoid tumors is pointed out. Diagnosis of this tumor in the pineal region is explained. New strategies, including intensive multimodel therapy and high-dose chemotherapy with autologous stem cell transplantation have improved outcomes. Diagnosis of AT/RT type using imaging technology is included in this volume. Various therapies, including total resection followed by aggressive chemotherapy and radiation, for patients with this tumor are presented. Dissemination of this malignancy to the cerebral fluid is explained. A number of other pediatric tumors are described in this volume. The role of methylation in pediatric ependymoma is explained.

Treatments, including craniospinal radiation followed by adjuvant chemotherapy, of pediatric medulloblastoma are presented. Diagnosis and chemotherapy of children with acute lymphoblastic leukemia patients are explained. Diagnosis of bone marrow involvement in pediatric lymphoma patients is detailed. Ewing's sarcoma is a highly malignant connective tissue neoplasm formed by the proliferation of mesenchymal cells. Details of targeting molecular pathways involved and chemotherapy and surgery of patients with this neoplasm are included in this volume. Roles of apoptotic genes, MYCN gene, MDM2, and SNP309, P13K inhibitors, alternative splicing and micro RNAs, activated leukocyte cell adhesion molecule, inhibition by alu-like RNA in neuroblastoma are discussed in detail. In addition, diagnosis and treatment of other pediatric tumor types including adrenocortical tumors, supratentorial primitive neuroectodermal tumors, giant midline tumors, gastrointestinal stromal tumors, ependymomas, and intramedullary cavernoma, are discussed.

By bringing together a large number of experts (oncologists, neurosurgeons, physicians, research scientists, and pathologists) in various aspects of this medical field, it is my hope that substantial progress will be made against this terrible disease. It would be difficult for a single author to discuss

effectively the complexity of diagnosis, therapy, and prognosis of any type of tumor in one volume. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of the pediatric cancer. I hope these goals will be fulfilled in this and other volumes of this series. This volume was written by 91 contributors representing 13 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the reader in this important area of disease. I respect and appreciate the hard work and exceptional insight into the nature of pediatric tumors provided by these contributors. The contents of the volume are divided into seven subheadings: Neuroblastoma, Medulloblastoma, Leukemia, Lymphoma, Rhabdoid, Sarcoma, and Miscellaneous Tumors for the convenience of the readers. Diagnosis, biomarkers, therapy, and prognosis of these malignancies are discussed.

It is my hope that the current volume will join the previous volumes of the series for assisting in the more complete understanding and cure of globally relevant malignancies in children. There exists a tremendous, urgent demand by the public and the scientific community to address to cancer diagnosis, treatment, cure, and hopefully prevention. In the light of existing cancer calamity, government funding must give priority to eradicating these deadly children's malignancies over military superiority.

I am grateful to Dr. Dawood Farahi and Mr. Philip Connelly for recognizing the importance and necessity of providing the facilities for publishing up-to-date information regarding pediatric cancer. I thank my students for their assistance in preparing this volume.

Union, NJ, USA M.A. Hayat
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Part I

Neuroblastoma

Pediatric Neuroblastoma: Use of Hypermethylation of Apoptotic Genes as a Prognostic Factor

1

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Contents

Abstract

Neuroblastoma, the most common solid extracraneal tumor in childhood, develops from immature or de-differentiated neural-crest derived cells. Prognosis depends on the patient's age at diagnosis, tumor stage and MYCN oncogene amplification. Several established molecular parameters (DNA content, allelic loss in 1p and 11q and gain of genetic material in 17q) have been introduced as prognostic indicators, however, the molecular basis of NB development and progression remains poorly understood. Epigenetic mechanisms, such as DNA hypermethylation, are important regulators of gene expression and are frequently involved in silencing tumor suppressor genes. A clinically relevant methylation profile in NB has recently been studied using different screening techniques and hypermethylation of apoptotic genes such as *caspase-8*, have been identified as good prognostic indicators, emphasizing the potential use of epigenetic biomarkers for prognosis purposes.

Introduction

Neuroblastoma (NB), the most common extracraneal tumor in childhood, develops from immature or de-differentiated neural crest-derived cells. This tumor has an incidence of about ten per million children per year and is responsible for approximately 15% of all pediatric cancer deaths (Maris et al. 2007). In contrast to many other pediatric

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malignancies, NB exhibits contrasting patterns of clinical behaviors ranging from spontaneous remission to rapid tumor progression and death. Prognosis in NB fundamentally depends on age at diagnosis, histology, tumor stage and *MYCN* oncogene amplification status. Other molecular parameters such as tumor cell DNA content, gain of chromosome arm 17q, deletion of chromosome arm 1p and 11q have proved predictive of patient outcome. However, current knowledge on the molecular features of NB is not sufficient to explain the clinical heterogeneity observed. Recent studies have focused on identifying epigenetically modified genes in order to further understand NB pathogenesis and to identify new prognostic methylation markers. This review will focus on clinically relevant epigenetic alterations in NB and fundamentally on hypermethylation of apoptotic genes as prognostic indicators.

Neuroblastoma Overview

Neuroblastoma originates in the developing sympathetic nervous system, and primary neuroblastoma tumor is most frequently located in the abdomen, especially on the adrenal medulla. Other common locations are neck, thorax and pelvis. Signs and symptoms are highly heterogeneous and depend on the primary tumor location, as well as the presence of metastatic disease and paraneoplasic syndromes.

Patients with NB are diagnosed either by the coincidental palpation of an abdominal mass during a routine examination, or due to one of the following: local abdominal pain or symptoms related to compression of nerve roots or the spinal cord in case of localized NB or periorbital bruising and swelling, ecchymoses, paleness, or bone pain in case of metastatic disease. Most patients show elevated concentrations of urinary catecholamines and their metabolites. A definitive diagnosis of NB is based on a pathological examination of a primary tumor biopsy or an invaded bone marrow aspirate. (Brodeur 2003).

The main classification of the NB tumor family is determined by the degree of cellular differentiation within the tumor. Poorly differentiated tumors with a lot of neuroblasts are classified as neuroblastomas, while well-differentiated benign tumors with mature ganglion cells, increased stroma compartments, and sparse neuroblasts are described as ganglioneuromas. Ganglioneuroblastomas are an intermediary category with features of both neuroblastomas and ganglioneuromas. The Shimada classification, modified as the International Neuroblastoma Pathology Classification (Shimada et al. 1999), has been widely used to describe and predict neuroblastoma behaviour and prognosis. This classification system takes into account histologic features such as the degree of cellular differentiation, schwannian stroma and the mitosis-karyorrhexis index, in addition to the age of the patient.

Neuroblastoma tumors are divided into different stages according to the loco-regional extension of the primary tumor and the presence of metastases and their localization patterns. The classification system used in Europe is the revised Evans post-surgical international neuroblastoma staging system (INSS) (Brodeur et al. 1984). Recently this system has been replaced by the International Neuroblastoma Risk Grouping Staging System (INRGSS), which takes into account the extent of the disease at diagnosis as well as univocal image- defined risk factors (risk factors related to localized tumors) (Monclair et al. 2009). The short definitions of the four INRGSS stages are listed in Table 1.1.

In line with this classification, the INRG also segregates patients into four pre-treatment risk

Table 1.1 International Neuroblastoma Risk Group staging system

Stage	Description
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment.
L2	Loco-regional tumor with presence of one or more image-defined risk factors.
M	Distant metastatic disease (except stage MS).
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow.

Table 1.2 International Neuroblastoma Risk Group (INRG) consensus pre-treatment classification schema

INRG Stage	Age (months)	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	< 18			NA		Hyperdiploid	F Low
	< 12			NA		Diploid	I Intermediate
	12 to < 18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS	< 18			NA	No		C Very low
					Yes		Q High
				Amp			R High

categories based on age at diagnosis, International Neuroblastoma Staging System, tumor histopathology, DNA index and MYCN amplification (MYCNA) status (Cohn et al. 2009) (See Table 1.2).

Patients over 1 year with metastatic disease (stage M), infants with MYCNA (M or MS) and localized NB patients with MYCNA (L2) are included in the High Risk Protocol and receive an intensive treatment schedule including: intensive multi-agent induction chemotherapy, surgery, myeloablative chemotherapy followed by autologous stem cell reinfusion, radiotherapy and maintenance treatment with retinoic acid and immunotherapy to eradicate minimal residual disease. Nevertheless, high-risk patients frequently face fatal outcome. On the other hand, patients with localized disease and without MYCNA are generally treated only with surgery or with a standard treatment followed by surgery and stage MS patients without MYCNA, receive relatively nonintensive treatment or no treatment at all, and their outcome prospects are excellent.

Multiple biomarkers are involved in NB prognosis including MYCNA, DNA ploidy, ferritin levels, neuron specific enolase, loss of chromosomes 1p, 11q or gain of chromosome 17q, but the most important are both MYCNA and the patient's age at diagnosis. MYCNA is the main feature to risk stratification and is associated with a progression of the disease and poor survival. Amplification of the MYCN oncogene is present in approximately 20% of NB tumors (mostly in metastatic tumors) and was the first genetic marker for poor outcome (Brodeur et al. 1984). In all existing risk stratification systems, patients with MYCN amplified tumors are generally considered high-risk patients and treated accordingly. The inconvenience of this biological factor is that many metastatic NB do not show amplification of this gene, so in the absence of MYCNA, loss of heterozygosity of chromosome 11q was associated with a poor prognosis (London et al. 2005). Age at diagnosis is another important prognostic factor. Children less than 365 days (12 months) of age usually present a favourable prognosis, while prognosis of older children with metastatic disease is compromised. A recent study showed that a cut-off of 547 days (18 months) was a clinically more relevant outcome predictor than the 365 days limits (Cohn et al. 2009). This new age cut-off

will be used in future clinical trials except for patients with diploid, MYCN not-amplified tumors. For them, the 365 days cut-off will be maintained.

In about 25% of primary NB the short arm of chromosome 1 is deleted (White et al. 2005). This fact suggests the existence of one or more tumor suppressor gene(s) in this chromosome region. One of the most promising NB tumor suppressor genes in this region is CHD5 (Fujita et al. 2008). On the other hand, deletions of the long arm of chromosome 11 have been identified in 20% of primary NB (Attiyeh et al. 2005). Chromosome 11q deletions are often found in high stage tumors without MYCN amplification and an intact 1p chromosome. Recently, the critically deleted regions on 11q were refined and evidence was obtained that CADM1 is indeed a tumor suppressor gene (Michels et al. 2008). Chromosome 11q loss was shown to be associated with decreased progression-free survival (Attiyeh et al. 2005) and a chromosome instability phenotype with later disease onset (Carén et al. 2010).

Epigenetic Aberrations in Neuroblastoma

Epigenetic modifications, and particularly the methylation of cytosines 5′ of guanine residues (CpGs) in gene promoter regions, are essential regulatory mechanisms for normal cell development. Cytosine 5′ methylation to guanine residues (CpGs) in the gene promoter regions is considered a very important mechanism in the cell development regulation and has a direct relevance in cancer through different mechanisms. For example, the 5-methylcytosine is susceptible to deamination and thus the formation of point mutations (Espada and Esteller 2007). Moreover, the aberrant methylation of CpG-rich areas (defined as CpG islands) in gene promoter regions can, together with histone acetylation and deacetylation, modify nuclear chromatin conformation interfering in the transcriptional machinery and altering the relative gene expression (Laird 2003). This is how genetic factors as mutation, deletions, or chromosome rearrangements can cooperate with epigenetic mechanisms in the inactivation of important biological pathways.

Alterations in the methylation pattern are very common in cancer cells, indicating a direct influence on carcinogenesis. The aberrant methylation or demethylation is responsible for the deregulated expression of oncogenes or for the inactivation of tumor suppressor genes. CpG island hypermethylation in gene promoter regions leads to the binding of a family of proteins that remodels the higher-order structure of chromatin preventing transcription initiation (Esteller 2003). In contrast, hypomethylation of specific sequences may deregulate the expression of genes involved in crucial cellular functions. Thus, aberrant CpG methylation or demethylation can alter the expression of essential genes and may represent one of the ‘hits’ at the basis of the multistep hypothesis of cancer development (Park et al. 2011).

Examination of aberrant methylation in cancer has so far focused on adult tumors; thus the information on childhood malignancies is relatively limited. Until recently, NB was one of the pediatric malignancies for which only limited information on the methylation status of few genes was available. This scenario has now drastically changed and a large amount of clinically relevant information on methylation in NB is rapidly accumulating. Neuroblastic tumor samples could be clustered based on the methylation pattern of ten genes, allowing the identification of several clinically relevant groups of tumors (Alaminos et al. 2004). Global methylation studies have demonstrated that a methylator phenotype, characterized by the methylation of multiple CpG islands, is a hallmark of NB with poor prognosis (Abe et al. 2008).

In NB, several tumor suppressor genes have been shown to be silenced by aberrant hypermethylation of their promoters. Examples of such genes are CASP8 (van Noesel et al. 2003; Grau et al. 2010), RASSF1A (Michalowski et al. 2008), CCND2 (Alaminos et al. 2004), CD44 (Tang et al. 2004), MGMT (Lázcoz et al. 2007),

DCR2 (van Noesel et al. 2002) and TMS1 (Alaminos et al. 2004; Grau et al. 2011). Currently, about 75 genes are described as epigenetically affected in NB cell lines and/or NB primary tumors (Decock et al. 2011). These epigenetic alterations were either found using a candidate gene approach or based on the analysis of genome-wide screening techniques, and involve genes implicated in several cellular pathways such as cell cycle regulation, drug resistance, apoptosis, cell adhesion, cell invasion, etc.

Recently, the presence of methylation markers (DCR2 and RASSF1A) in serum of NB patients has been described as an indicator of prognosis and therapy efficacy (Misawa et al. 2009). Several groups have reported the clinical utility of circulating DNA in serum for genetic assessment of malignant tumors because serum DNA predominantly originates from tumor released DNA in patients with cancer. The detection of tumor-derived methylated genes in serum DNA has attracted attention as a novel marker because of their prognostic value and rapid accessibility as compared with tumor DNA.

DCR2 (decoy receptor 2) is a tumor necrosis factor-α receptor superfamily gene that is located on 8p21. DCR2 is ubiquitously expressed in normal tissue, where it prevents apoptosis (van Noesel et al. 2002). However, DCR2 expression was found to be silenced by aberrant methylation of its promoter regions in some cancers (Shivapurkar et al. 2004). In NB, there are studies focused on the detection of methylated-DCR2 in serum DNA. Their authors considered this a promise noninvasive assay for predicting prognosis and therapeutic efficacy in this tumor, especially in non-MYCN amplified cases (Yagyu et al. 2008).

The tumor suppressor gene RASSF1A is known to be frequently silenced by promoter hypermethylation in NB tumors and has been also explored in DNA serum samples (Misawa et al. 2009). The study of the methylation status of RASSF1A in serum samples from NB patients has the potential to become a prognostic outcome predictor.

Hypermethylation of Apoptotic Genes in Neuroblastoma

Based on these findings, the clinical relevance of promoter hypermethylation in the pathogenesis of NB has been demonstrated. The hypermethylation of apoptosis-related genes is particularly significant. NB shows the highest rate of spontaneous regression among pediatric tumors, and this phenomenon may reflect the activation of apoptotic/differentiation pathways. It is therefore not surprising that the level of expression of molecules involved in the apoptosis regulation has been screened as a prognostic factor in NB.

Apoptosis, the cell death program, plays a crucial role in the regulation of tissue homeostasis, and an imbalance between cell death and proliferation may result in tumor formation. In most cases, the apoptotic process is characterized by the proteolytic activity of caspases. The two main apoptotic pathways in which caspase activation occurs, are the intrinsic mitochondrial pathway and the extrinsic receptor mediated pathway; these two pathways are also interlinked (Elmore 2007). The intrinsic pathway is induced by cytotoxic signals such as DNA damage. These signals cause changes in the mitochondrial membrane, leading to an opening of the mitochondrial permeability transition pore, loss of the mitochondrial transmembrane potential and release of pro-apoptotic proteins such as cytochrome-C which are normally sequestered in the intermembrane space of the mitochondria. In the presence of (deoxy) adenosine triphosphate the cytoplasmic cytochrome-C clusters with the apoptotic protease activating factor-1 (APAF1). This adaptor protein contains a caspase recruitment domain (CARD) that allows binding with the CARD of procaspase-9. The formed apoptosome complex ultimately activates caspase-9, which then cleaves other targets (Elmore 2007).

On the other hand, the extrinsic pathway is initiated by binding of extracellular death signals with death receptors on the surface of the target cells. These death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily, which includes TNFR1 (DR1, CD120a, p55 or

p60), CD95 (DR2, APO-1 or FASR), DR3 (also TNFRSF25, APO-3, LARD, TRAMP or WSL1), TRAILR1 (DR4, APO-2 or TNFRSF10A), TRAILR2 (DR5, KILLER, TRICK2 or TNFRSF10B), DR6 (TNFRSF21), ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR). These receptors are characterized by a specific cytoplasmatic domain called death domain. By homotypic interaction, this death domain recruits specific adaptor proteins depending on the type of the stimulated receptor, and the final clustering of proteins results in the formation of the death-inducing signaling complex, which leads to activation of procaspase-8. Once caspase-8 is activated, the execution phase of apoptosis is triggered (Elmore 2007).

Apoptosis could play a key role in NB biology. For example, apoptosis may be involved in mediating spontaneous regression, one of the unique features of this tumor (Pritchard et al. 1994). In addition, defects in apoptosis programs may contribute to NB progression and chemotherapy resistance because killing of tumor cells by cytotoxic therapies is predominantly mediated through induction of apoptosis in target cells. Regarding the apoptotic pathways, the death receptors TRAILR1 and TRAILR2, the DcRs TRAILR3 and TRAILR4, CASP8 and APAF1 are described to be methylated in NB cell lines and primary tumors (Decock et al. 2011). Inactivation of caspase-8 by hypermethylation has become a hallmark of defective apoptosis in advanced NB disease, suggesting that this gene may act as a tumor suppressor. Indeed, it has been proposed that CASP8 is inactivated by methylation or deletion in MYCN amplified NB (Teitz et al. 2000).

The methylation status of CASP8 has been linked to MYCN amplification in some studies (Gonzalez-Gomez et al. 2003), but not in others (Fulda et al. 2006; Grau et al. 2010), and a direct effect of MYCN amplification on caspase-8 expression has not been described so far. On the other hand, a loss of expression of caspase-8 protein has been found in the majority of NB tumors (75%), irrespective of the disease stage (Fulda et al. 2006). Furthermore, in this last study, the caspase-8 expression was not correlated with many other variables of high-risk disease such as 1p36 aberrations, tumor stage, age at diagnosis, or tumor histology. Caspase-8 is absent in neural stem cells, so lack of caspase-8 expression may merely reflect the developmental status of the neuroblast at the time of malignant transformation. In any case, caspase-8 methylation status is a characteristic feature of aggressive NB, as some reports previously shown (Teitz et al. 2000).

Another apoptosis-related gene is TMS1 (PYCARD). TMS1 encodes an adaptor protein that promotes caspase-dependent apoptosis. The absence of TMS1 expression due to methylation in some tumors contributes to carcinogenesis and cancer development. In NB, some studies have reported the association of TMS1 methylation status with MYCN amplification, stage and risk, suggesting that the hypermethylation of this gene has an impact on NB progression (Grau et al. 2010; Alaminos et al. 2004). Furthermore, TMS1 hypermethylation has been described as a molecular marker in stage 4 tumors, but no evidence of methylation was found in 4S tumors, which undergo spontaneous regression (Alaminos et al. 2004). Similar results have been obtained in independent tumor sample series, so TMS1 epigenetic alterations may lead to an apoptosis blocking in tumor cells, and thus contribute to disease progression (Grau et al. 2010). The use of a combination of DNA-methylation biomarkers to show the effect of the methylation status on disease-free survival or overall survival has been described. Specifically, the simultaneous analysis of TMS1, APAF1 and CASP8 methylation status on primary NB tumor, which would be good prognostic indicator of disease progression (Grau et al. 2010).

TMS1 hypermethylation has been studied not only in primary tumors but also in disseminated NB disease (Grau et al. 2011). The presence of neuroblastic cells in bone marrow can be used to evaluate the response to treatment and alterations in certain tumor cells may confer a selective advantage over tumor dissemination process. This study reports a significantly poor prognosis in event-free survival in cases with hypermethylation of TMS1 gene. Although the authors could not confirm the presence of a specific methylation

profile in disseminated NB tumor cells, high accumulation of epigenetic events in those cells was found to be associated with a high risk of relapses, independently of MYCN amplification.

Another frequently methylated gene in NB is RASSF1A, a member of the RAS-association domain family of proteins. Due to its interaction with multiple partners, RASSF1A influences a diversity of signaling pathways, and is known to be implicated in the regulation of cell proliferation and apoptosis. RASSF1A is reported to be methylated in a very high fraction of NB patients, and there are many studies reporting the methylation status of RASSF1A in NB samples and the association of RASSF1A methylation with several clinical risk factors such as MYCN amplification, age at diagnosis and tumor stage (Misawa et al. 2009; Grau et al. 2010). Only approximately 7 kb upstream of RASSF1A, another promoter region frequently reported to be methylated in NB is zinc finger, myeloid, nervy and DEAF-1-type containing 10 (ZMYND10 (BLU)), which is related with stage, age at diagnosis and risk (Grau et al. 2010).

Future Perspectives

The results presented earlier clearly show that NB is a disease regulated not only by genetic mechanisms, but also by epigenetic ones. The screening studies focusing on candidate genes led to the discovery that frequently methylated DNA regions in NB can be found in apoptotic pathways or related to cell cycle regulation. Instead of the classical methodology for methylation analysis, some advanced technologies provide the possibility of investigating the epigenetic changes throughout the genome. Integrating this data with other data sources (miRNA expression profiles and proteome data) would reveal the epigenomic approach of NB. Understanding those kind of regulatory networks and the possible compensatory interactions of epigenetic mechanism in the NB biology may also be crucial for developing the appropriate therapeutic strategies and more efficient prognosis biomarkers.

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2 *MYCN* Nonamplified Neuroblastoma: Detection of Tumor-Derived Cell-Free DNA in Serum for Predicting Prognosis of Neuroblastoma

Shigeki Yagyu and Tomoko Iehara

Contents

Abstract

MYCN amplification (MNA) is the most powerful prognostic factor of neuroblastoma (NB) patients. In the high-risk non-MNA group, development of NB depends on factors other than MNA, such as genetic expression profiles, DNA methylation of tumor suppressor gene, and chromosomal loss and gain. On the other hand, many studies have demonstrated tumor-derived cell-free DNA in the serum of patients with malignancies, for a finding for molecular prognostic marker. We reviewed the clinical utility of cell-free DNA in the serum for the risk classification of patients with neuroblastoma. We discussed that detection of MNA, methylated DNA and chromosomal loss and gain in the sera. The serum based molecular analysis can provide noninvasively clinical information to determine for risk classification.

Introduction

Neuroblastoma (NB) is the most common extracranial tumor of childhood. The NB patients fall into two clinically distinct subtypes; a low-risk subgroup and a high-risk subgroup. These phenotypes are correlated with the age of onset (London et al. 2005), the extent of the disease (International Neuroblastoma Staging System) (Brodeur et al. 1993), pathological findings (International Neuroblastoma Pathological Criteria) (Peuchmaur et al. 2003), and genomic changes in NB tumors as represented by *MYCN*

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gene amplification (MNA) (Brodeur et al. 1984; Seeger et al. 1985). Above all, chromosomal aberrations and/or genetic abnormalities are the most important prognostic factors, and are strongly linked to the development and clinical behavior of neuroblastoma.

The patterns of genetic changes can predict the subtypes of NB (Brodeur 2003). The low-risk, favorable NBs have mitotic disorders, and are characterized by near triploid karyotypes with whole chromosomal gains. On the other hand, the high-risk, unfavorable NBs exhibit genetic instability, and are characterized by chromosomal structural changes, including deletion of 1p or 11q, unbalanced gain of 17q, and/or MNA. MNA is the most powerful prognostic factor identified so far, and is useful regardless of the tumor stage. In the recent studies reported in the International Neuroblastoma Risk Group (INRG) database, the 5-year event free survival of patients who had NB tumors with MNA was 53% for localized NB (Bagatell et al. 2009) and 29% for all NBs (Cohn et al. 2009), even with intensive, multimodal treatment. On the other hand, NB without *MYCN* amplification (non-MNA) falls into two clinically distinct subgroups: a low-risk subgroup with overall survival rates of more than 95% without any intensive therapy, and a high-risk subgroup with overall survival rates of less than 40% despite the use of dose-intensive, multimodal therapy. In the high-risk non-MNA group, the development of NB depends on factors other than MNA, such as the expression profiles of other genes (Ohira et al. 2005), aberrant hypermethylation of tumor suppressor genes (Banelli et al. 2005), and chromosomal loss of heterozygosity (LOH) (Attiyeh et al. 2005).

To determine the stratification of NB patients more precisely with regard to the treatment approach, a screen for these genetic aberrations should be performed before the initial treatment. Indeed, in the INRG staging system, routine assessment of MNA and 11q loss were required for the therapeutic stratification of NB (Cohn et al. 2009). Currently, genetic alterations in NB tumors are clinically evaluated by interphase dual-color fluorescence in situ hybridization (I-FISH), array comprehensive genomic hybridization (aCGH), PCR and multiple ligation probe amplification (MLPA) (Ambros et al. 2009). However, the evaluation of tumor-related genetic aberrations requires a fresh tumor sample, which is often difficult to obtain due to the frequent occurrence of life-threatening conditions in the patients with NB.

Recently, molecular techniques such as PCR have been improved to be able to detect small amounts of cell-free DNA in the serum and plasma of patients with various diseases, including cancers. Quantification of serum DNA has been proposed as a screening tool for the early detection of malignant tumors, and several groups have reported the clinical utility of circulating DNA in serum for the genetic assessment of malignant tumors, because the serum DNA predominantly originates from tumor-released DNA in patients with cancer (Leon et al. 1977; Sozzi et al. 2001). In our recent studies, we developed a system for evaluating genetic aberrations of NB using tumor-released cell-free DNA present in serum (Gotoh et al. 2005; Yagyu et al. 2008, 2011; Misawa et al. 2009). In this chapter, we outline the clinical utility of tumor-derived cell-free DNA for the evaluation of disease status. We also provide an update on the novel serum-based system for evaluating genetic aberrations for the risk stratification of patients with NB. Finally, we discuss our new approach for the risk stratification of NB using serum-based "less invasive" techniques.

Tumor-Derived Cell-Free DNA in Serum

Tissue and cell injuries take place under both normal and pathological conditions. It is expected, therefore, that intracellular material such as DNA may be released into the bloodstream. Indeed, cell-free DNA can be found in the serum of patients with various disorders, such as collagen diseases, pulmonary infarction, and cancer (Koffler et al. 1973). In particular, tumor-derived cell-free DNA has attracted attention as a novel genetic marker for cancer. The concentration of cell-free DNA in the serum of cancer patients tends to be

higher than that of non-cancer patients. Indeed, the mean serum DNA concentration in cancer patients was 180 ng/ml of serum, while that in the normal control patients was 13 ng/ml of serum, as detected by a radioimmunoassay (Leon et al. 1977). This is not only a result of the active release of cell-free DNA into the bloodstream from highly proliferative and/or apoptotic and/or necrotic cells in a bulky tumor (Anker et al. 1999), but also because the DNA fragments in the sera of cancer patients are generally stable due to the low DNase activity present in the serum (Tamkovich et al. 2006).

Further studies revealed that the level of cell-free DNA correlated with the disease status and prognosis of the patients. The serum DNA levels of the patients with metastatic tumors were significantly higher than those of patients with localized tumor (Nawroz et al. 1996), although tumor-derived cell-free DNA was also found in the serum of the patients with localized, early-stage tumors (Gotoh et al. 2005; Yagyu et al. 2008). Moreover, the serum DNA levels decreased during the course of treatment in responders, and increased in the patients who showed a lack of response to treatment (Leon et al. 1977; Sozzi et al. 2001; Gotoh et al. 2005; Yagyu et al. 2008). Interestingly, the survival rate of the cancer patients with higher serum DNA levels was poorer than that of patients with lower levels (Fournié et al. 1995).

Recent advances in molecular techniques have enabled the evaluation of tumor-related genetic aberrations using small amounts of cell-free DNA in the serum. Several groups have reported the usefulness of detecting tumor-related genetic alterations, such as *RAS* mutations (Anker et al. 1999), or *TP53* mutations (Kirk et al. 2005), microsatellite instability (Nawroz et al. 1996), gene amplification (Combaret et al. 2002; Gotoh et al. 2005), allelic gain and loss of oncogenes (Nawroz et al. 1996; Combaret et al. 2011; Yagyu et al. 2011), and aberrant promoter hypermethylation (Yagyu et al. 2008; Misawa et al. 2009). In NB, our group established a serum-based assay system for *MYCN* gene amplification, aberrant promoter hypermethylation, and chromosomal loss of 11q. These less invasive techniques are clinically useful for the preoperative risk stratification of NB patients, because genomic changes in the tumor correlate with the tumor behavior, survival outcome, and response to therapy of NB patients.

Detection of Amplified *MYCN* in the Sera of NB Patients

The *MYCN* oncogene can be detected in the serum of patients with MNA-NB. Combaret et al. performed a conventional PCR analysis targeting the *MYCN* gene using the sera of 102 patients with NB (32 patients with MNA, and 70 patients without MNA) and 72 individuals without cancer as controls (Combaret et al. 2002). *MYCN* sequences were detected in 31 of the 32 samples from the patients with MNA, 1 of 70 samples from the patients without MNA, and none of the samples from the 72 healthy controls. The authors of that paper also confirmed these results by quantitative real-time PCR, and revealed that the amount of *MYCN* DNA in the serum of MNA-NB patients was 25–600-fold higher than that in other individuals. They concluded that the existence of the *MYCN* gene in serum is strictly dependent on the *MYCN* status of the cancer cells, and the detection of the *MYCN* gene in the serum could be a potent biomarker for NB.

Our group confirmed these results in 87 NB patients (17 patients with MNA-NB and 70 patients without NB) (Gotoh et al. 2005). We simultaneously quantified the *MYCN* gene as a target and *NAGK* gene as a reference by real-time PCR, and evaluated the *MYCN* copy number to obtain the *MYCN*/*NAGK* ratio. The serum *MYCN*/*NAGK* ratio in the MNA group was significantly higher than the ratio in the non-MNA group. Notably, the sensitivity and specificity of the serum *MYCN*/*NAGK* ratio as a diagnostic test were both 100% when the serum *MYCN*/*NAGK* ratio cutoff was set at 10.0. Our group also reported that the serum-based *MYCN* status was an indicator of therapeutic efficacy. Among six MNA patients whose clinical courses were followed, the serum ratios decreased to within the normal range in the patients in remission (n = 3), whereas the ratios increased to high levels in the

patients who relapsed (n = 2) or failed to achieve remission (n = 1). These data strongly suggested that the serum-based quantitative *MYCN* status was a useful tool for preoperatively determining the stratification for therapy and for the evaluation of therapeutic efficacy during the course of treatment, when tumor cells were not available for a molecular analysis. Considering that it is often impossible to obtain tumor samples from patients with advanced NB for biological studies due to their life-threatening conditions, these data may have important clinical implications.

Indeed, most therapeutic regimens recommend primary radical resection for localized NB only when the tumor does not have MNA. Moreover, up-front surgical resection is not indicated for advanced localized NB, including the tumors with MNA. Therefore, knowing the preoperative serum-based *MYCN* status may provide a crucial advantage for decision-making, especially for localized NB. As mentioned above, circulating tumor-derived cell-free DNA could be detected in the serum of cancer patients regardless of the tumor stage. Therefore, it was of interest to determine whether the serum-based *MYCN* status was also useful to determine the risk classification for localized NB.

The possibility of determining the *MYCN* status of patients with NB from a blood sample would be especially useful for cases without tumor samples for a molecular analysis who have an unknown *MYCN* status.

Detection of Methylated DNA in the Sera of NB Patients

MNA is still considered to be the strongest prognostic factor for NB, and is routinely assessed for therapeutic stratification. However, there have been major concerns about using this marker, because some cases without MNA also still have a poor prognosis. It is therefore important to have additional biomarkers with prognostic value for the management of non-MNA cases of neuroblastoma. Recent studies have revealed that epigenetic alterations, such as silencing of tumor suppressor gene(s) by aberrant hypermethylation of the promoter, often play important roles in the pathogenesis and progression of NB. A positive correlation has been found between the hypermethylation of the promoters of these genes and a poor prognosis, thus suggesting that hypermethylation influences the phenotype of neuroblastoma.

Our group previously studied the aberrant hypermethylation in the promoter region of the *DCR2* gene (Yagyu et al. 2008). *DCR2* (decoy receptor 2) is a tumor necrosis factor alpha receptor superfamily gene, and is negatively associated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, because it lacks an intracellular death domain. In NB tumors, the methylation profile of *DCR2* has been found to be drastically different and independent of the *MYCN* status. Moreover, *DCR2* methylation was found to be associated with rapidly progressing tumors and a reduced overall survival. Using an established methylation-specific PCR analysis, the aberrant hypermethylation in the promoter region of *DCR2* gene could also be detected in the serum DNA, and strongly correlated with the expression in the tumor. A prognostic analysis revealed that *DCR2*-methylated NB patients, as detected by a serum-based assay, showed a significantly poorer 5-year event-free survival and overall survival than *DCR2*-unmethylated patients, especially in the non-MNA group. These observations indicated that the serum-based *DCR2* methylation status could be a potent biomarker for predicting the prognosis of patients with NB, especially the patients without MNA. Additionally, the serum-based *DCR2* methylation status can distinguish patients with a poor outcome within the non-MNA group, and may allow for a new type of risk stratification for patients with non-MNA NB in future trials.

The tumor suppressor gene *RASSF1A* is frequently silenced by promoter hypermethylation in various cancers. Indeed, *RASSF1A* is highly methylated in NB (93% of primary tumors and 100% of relapsed tumors, (Michalowski et al. 2008)). Based on these findings, our group also analyzed the methylation status of the *RASSF1A*

gene in matched tumor and pretreatment serum DNA samples (Misawa et al. 2009). We found that hypermethylation of *RASSF1A* was frequently detected in NB tumors (94%), which was in agreement with the previous studies (Michalowski et al. 2008). *RASSF1A* hypermethylation in tumors appears to be a relatively early event in NB tumorigenesis, as it is even detectable in early stage tumors. On the other hand, *RASSF1A* methylation was detected in the serum from only 25% of NB patients, and methylated *RASSF1A* was more frequently detectable in the serum of advanced NB patients than in those with early stage NB, although the *RASSF1A* methylation status in NB tumors was not significantly different between patients with advanced and early stage NB. These discrepancies in the sensitivities between serum-based and tumor-based methylation assays could be explained the smaller quantity of DNA released from a small tumor burden. Nevertheless, this serum-based *RASSF1A* methylation assay can provide important prognostic information to determine the appropriate risk classification.

Detection of Chromosomal Loss and Gain in the Serum DNA of NB Patients

Chromosomal gain and/or loss are frequently observed in NB, as mentioned above, and various studies have reported the clinical impact of chromosomal gain and/or loss in NB. Among the various unbalanced chromosomal aberrations, 17q gain is the most frequent chromosomal aberration, and correlates with a poor outcome (Lastowska et al. 1997). The loss of 11q is also a strong prognostic factor that can be used in addition to MNA (Attiyeh et al. 2005), and routine assessment of the 11q status, as well as MNA, is required for therapeutic stratification of NB in the INRG staging system (Cohn et al. 2009).

Some groups, including ours, have developed serum-based assays to detect chromosomal gain and/or loss in NB using various techniques. Combaret et al. reported a serum-based detection system for 17q gain (Combaret et al. 2011). They simultaneously quantified the gene dose of MPO (17q.23.1) and survivin (17q25) as targets, and p53 as a reference, by quantitative real-time PCR using 142 serum samples. They revealed that the serum-based determination of 17q gain had good specificity (94.4%) and 58.8% sensitivity in patients who were less than 18 months old ($P<0.001$), while this approach showed moderate specificity (71.4%) and 51.2% sensitivity in patients over 18 months of age. In a subset analysis according to the stage of NB, the sensitivity of serum-based 17q gain determination tended to increase with the stage of the disease. On the other hand, for metastatic NB, the sensitivity of the test never exceeded 60%, which is lower than the results achieved by the analysis of the serum-based *MYCN* status.

Previous studies have revealed the presence of a smallest region of overlap (SRO), which is a common region of deletion in all NB cases with 11q loss. By targeting some polymorphic markers in the SRO of 11q (most of which are located in 11q23), allelic loss could be detected using serum DNA as well as tumor DNA of NB patients (Yagyu et al. 2011). Using this technique, the sensitivity and specificity of the results between the serum- and tumor-based 11q loss analyses were both 100%, although a further study will be needed for confirmation of these findings because of the limited number of cases that were analyzed.

Clinical Application of Detection of Genetic Aberration Using Serum DNA

In a large-scale randomized trial of children with high-risk NBs (Matthay et al. 1999), the *MYCN* status was unknown in 27% of the children. In the clinical setting, some life-threatening cases with a huge mass or hepatomegaly (hepatic metastasis in stage 4s) were given chemotherapy and/or radiotherapy based on elevated tumor markers and positive MIBG scintigraphy, prior to tumor biopsy without evaluation of the *MYCN* status. The assay described above will be most useful when a primary tumor biopsy is not possible

and when genetic information will influence the risk grouping and treatment allocation of the NB patients. In Japan, infantile NB cases were formerly subclinically detected by mass-screening. Most of these cases showed a good prognosis and were recommended to undergo a reduced regimen, including the "wait-and-see" approach. However, we and others demonstrated that MNA was strongly correlated with a poor prognosis even in infantile, localized NB (Iehara et al. 2006; Bagatell et al. 2009). The serum-based MNA status has a significant implication for this group. Indeed, the serum-based MNA status of NB patients has considerable prognostic value, especially in cases less than 18 months of age (our unpublished data).

On the other hand, most of the infantile, localized NB cases did not have MNA. In the Cooperative German Neuroblastoma NB95 and NB97 trials, some localized cases without MNA did not receive chemotherapy after biopsy and showed spontaneous regression (Hero et al. 2008). Considering the clinical behavior of non-MNA NB, an early and non-invasive system for detecting genetic alterations besides MNA is needed to help select the appropriate therapy. In other words, combined preoperative assessments of MNA and 11q loss using serum DNA will make it possible to safely perform risk-adapted therapy according to the INRG staging system (Cohn et al. 2009). Particularly, preoperative serum-based MNA and 11q loss detection can be useful for cases that are in INRG stages L2 and MS, which have a wide range of clinical outcomes and potential therapeutic strategies depending on the existence of MNA and 11q loss.

In conclusion, significant advances in serum-based, less invasive molecular analysis can provide much better clinical information to determine the optimal therapeutic strategy for NB patients. Nevertheless, prospective validation in a large cohort will be needed to confirm the utility of these tools for assessing biological risk. Serum-based, surgery -free, rapid, sensitive, and specific genetic assessments have great potential to provide a personalized, risk-adapted therapy for patients with NB.

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Neuroblastoma: Role of MDM2 and SNP309 as Markers

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Contents

Abstract

MDM2 is a major negative regulator of p53 and in some tumors that present a wild type *p53* gene deregulated expression of *MDM2* could contribute to tumor development. In particular, in neuroblastoma, an extracranial pediatric tumor with unfrequent genetic inactivation of *p53*, deregulation of *MDM2* could significantly decrease the activity of p53, resulting in failure of p53-regulated functions such as cell cycle arrest, apoptosis and senescence. A single nucleotide polymorphism (SNP309,T>G change; rs 2279744) in the *MDM2* promoter increases the affinity for the transcription factor SP1, enhancing MDM2 expression and attenuating the activity of the p53 pathway. In this chapter, we review the role of *MDM2* and its SNP309 on neuroblastoma development and progression and discuss future pharmacological approaches based on the presence of this polymorphism.

Introduction

Neuroblastoma, a tumor arising from neuroectodermal precursor cells of the neural crest, represents the most common extracranial solid tumor in children, accounting for 8–10% of all childhood cancers but for 15% of all deaths due to pediatric malignancies (Maris et al. 2007). The clinical hallmark of neuroblastoma is its heterogeneity: age at diagnosis (Breslow and McCann 1971), clinical stage (based on International

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Neuroblastoma Staging System; Brodeur et al. 1993), and tumor histology (Shimada et al. 1999) are the most important factors for predicting the course of the disease and modulate the treatment accordingly. Age at diagnosis >1 year, advanced stage (3 and 4) and unfavorable histology are predictive of adverse outcome, but the response to treatment in patients with neuroblastoma is quite variable, probably reflecting differences in biological characteristics of tumor cells. Several biological markers related to outcome have been identified and they have further improved risk stratification. *MYCN* oncogene amplification, hemizygous deletions of chromosomal region 1p36, and unbalanced gain of 17q regions are the most common genomic aberrations in neuroblastoma (Maris and Matthay 1999). MYCN oncogene is amplified in 20% of cases and represents the most powerful marker of poor outcome (Komuro et al. 1993). In contrast with other malignancies, only 2–3% of neuroblastomas harbor mutations of the *p53* gene (Imamura et al. 1993). *p53* is a tumor suppressor gene activated by cellular stresses such as DNA damage, hypoxia, cold and heat shock that, primarily through its transcription activation function, is involved in many biological processes such as cell cycle arrest, apoptosis, and cellular senescence (Sharpless and DePinho 2002). Alteration of such processes has important implications for clinical behavior and response to treatment. *p53* also activates the transcription of the *MDM2* gene that encodes the major negative regulator of p53, thereby generating a negative feedback loop that leads to inhibition of p53 activity and proteasome-dependent protein degradation (Piette et al. 1997). Different studies in neuroblastoma cell lines and primary tumors have shown that the p53/MDM2 pathway is genetically intact, but that the function of p53 may be attenuated by aberrant expression/activity of MDM2 (Maris and Matthay 1999; Rodriguez-Lopez et al. 2001). In this regard, MDM2 expression can be enhanced by increased MYCN levels in tumors with *MYCN* amplification (Slack et al. 2005b), whereas in tumors with the 1p36 deletion expression of an activator of ARF (alternate reading frame) (which interacts with MDM2) is reduced (Bagchi et al. 2007), possibly enhancing MDM2 functional levels. A single nucleotide polymorphism (SNP309) in the first intron of *MDM2* causes an increase in the affinity for *SP1*, a transcription factor able to increase MDM2 mRNA and protein level and, consequently, an attenuation of the p53-regulated pathways that increase the risk for tumorigenesis (Bond et al. 2004). Because mutations of the *p53* gene are rarely found in neuroblastoma (Imamura et al. 1993), it is an intriguing possibility that a more aggressive neuroblastoma might develop in individuals harboring *MDM2* SNP309 variants that promote functional inactivation of p53.

Murine Double Minute Gene 2

MDM2 (murine double minute gene 2), also known as HDM2 in humans, is one of the most important negative regulator of tumor suppressor p53. *MDM2* was first described as one of the genes amplified in double minute chromosomes present in the spontaneously transformed Balb/c3T3 murine cell line 3T3DM (Cahilly-Snyder et al. 1987). MDM2 encodes a 90-kDa protein that comprises several distinct, highly conserved regions. The N-terminal domain harbors the main p53 binding interface. Two other notable regions of MDM2 are the central domain (amino acids ~200–300), often referred to as the acidic domain (AD), and the C-terminal RING (*r*eally *i*nteresting *n*ew *g*ene) domain (amino acids 438–478). The latter functions as an E3 ligase responsible for p53 ubiquitylation. Like other RING domain proteins, the MDM2 RING has intrinsic E3 ubiquitin ligase activity, in that it can promote the transfer of ubiquitin molecules from an E2 conjugating enzyme directly to lysine residues of target substrates (Deshaies and Joazeiro 2009). MDM2 functions as a homodimer or forms heterodimers with MDMX (also known as MDM4 and HDMX) through their RING fingers and both types of dimers are active as E3s (Lipkowitz and Weissman 2011). When wild-type p53 is activated by various stimuli such as DNA damage, MDM2 binds to p53 (that is in form of tetramer) at the N-terminus to inhibit the transcriptional

activation of p53 and directs the export of p53 away from its site of action in the nucleus to the cytoplasm, thanks to a central nuclear export signal and promote the degradation of p53 via ubiquitin-proteasome pathway generating a negative feedback loop.

Up to 50% of malignancies retain wild-type *p53* and in most of these there is increased MDM2 activity towards p53 as a consequence of amplification of *MDM2*, increased MDM2 expression due to promoter hyperactivity, gene polymorphisms, alterations in ARF activity or other mechanisms (Lipkowitz and Weissman 2011). Amplification and overexpression of *MDM2* is found in about 10% of all human tumors (Ganguli and Wasylyk 2003). In gliomas, for example, *MDM2* amplification identifies a subset of high-risk patients that do not have *p53* mutations (Slack et al. 2005a). In many soft tissue sarcomas, *MDM2* amplification and overexpression correlates with poor prognosis (Slack et al. 2005a). Studies of acute lymphoblastic leukemia and non-Hodgkin's lymphoma also demonstrate that *MDM2* overexpression is associated with poor survival and aggressive disease (Slack et al. 2005a). Thus, deregulation of *MDM2* gene expression likely contributes to the pathogenesis of a wide range of human tumors.

Murine Double Minute Gene 2 in Neuroblastoma

Different studies in neuroblastoma cell lines and primary tumors have shown that p53 is generally functional, accumulates in the nucleus in response to DNA damage and is efficiently degraded by MDM2 (Tweddle et al. 2001). Some reports strongly suggest that p53 function may be compromised as a consequence of aberrant MDM2 expression. The activity of MDM2 is critical in neuroblastoma, as the MDM2 ubiquitin ligase activity seems to be rate-limiting for p53 degradation (Isaacs et al. 2001). Consistent with the notion that MDM2 levels regulate p53 activity, a study examining etoposide-induced p53 activity in neuroblastoma cells suggested an important role for elevated MDM2 expression levels in the regulation of p53 translocation (Rodriguez-Lopez et al. 2001). Indeed, in SH-EP cells, which express high levels of MDM2, p53 accumulates in the cytoplasm upon exposure to etoposide. However, antisense oligodeoxynucleotide inhibition of *MDM2* restored p53 nuclear localization and apoptotic activation, suggesting that increased MDM2 expression levels can play a role in the inappropriate modulation of p53 function (Rodriguez-Lopez et al. 2001). Additionally, elevated MDM2 expression and the accompanying loss of p53 function are associated with multidrug resistance in some neuroblastoma cell lines (Keshelava et al. 2001). Together, these data demonstrate that the MDM2/p53 pathway is intact in neuroblastoma and suggest that deficiencies in p53 functions may be a consequence of aberrant MDM2 expression. In neuroblastomas with MYCN amplification, increased expression/activity of *MDM2* as an effector of *MYCN* (Slack et al. 2005a) could significantly decrease the activity of p53, resulting in failure to undergo appropriate cell cycle arrest and apoptosis. Recent studies (see below) show that MDM2 can be involved in neuroblastoma development if harbors single nucleotide polymorphism variants (such as SNP309) that increase its expression and promote functional inactivation of p53.

Single Nucleotide Polymorphisms in Genes Relevant for Neuroblastoma

In recent years, there has been an increasing interest in identifying and assessing the frequency of gene variants (polymorphisms) as a tool to predict inter-individual cancer risk and response to cancer therapies (Dong et al. 2008). A polymorphism is defined as a DNA sequence change that occurs in a significant proportion (more than 1%) of a large population (Olivier et al. 2002). The most common type of genetic variation is a single nucleotide polymorphism (SNP). The *p53* and *MDM2* genes both present several SNPs that appear to modify their expression/activity. *p53* is a tumor suppressor gene which is activated by cellular and genomic stresses such as DNA damage, oncogene mutation, hypoxia, cold and heat shock

and is involved in many biological processes such as cell cycle arrest, apoptosis, and cellular senescence (Sharpless and DePinho 2002). The effects of p53 are, mostly, transcription-dependent as it binds DNA in a sequence-specific manner to enhance the transcription of a number of genes, including *p21WAF1*, *MDM2* and *BAX*. *WAF1* inhibits G1 cyclin-dependent kinases, blocking cell cycle progression from G1 into S phase. In tumor cells, the selective pressure to delete or inactivate p53 is very high. This primarily occurs through amplification/overexpression of its inhibitors like *MDM2*, *MDM4* (*MDM2* family member) and loss or inactivation of upstream activators such as *p14ARF* (*p19ARF* in mice) and *p16INK4a* (Toledo and Wahl 2006). Different combinations of these events diminish or abolish wild type p53 levels and activity leading to defective apoptosis, uncontrolled proliferation, and cellular transformation. For the *p53* gene, a SNP has been identified within exon 4 at codon 72 causing an Arg>Pro substitution (Matlashewski et al. 1987). The p53-72R isoform appears to be more potent than the p53-72P isoform in inducing apoptosis, and among the proposed mechanisms that may be responsible for such an effect are increased mitochondrial localization and reduced affinity of the p53-72R isoform for the p53 inhibitor iASPP (Dumont et al. 2003; Bergamaschi et al. 2006).

Different studies in neuroblastoma cell lines and primary tumors have shown that the p53/MDM2 pathway is genetically intact, but that the function of p53 may be attenuated by aberrant expression/activity of MDM2 (Rodriguez-Lopez et al. 2001). In this regard, MDM2 expression can be enhanced by increased MYCN levels in tumors with *MYCN* amplification (Slack et al. 2005b), whereas in tumors with the 1p36 deletion expression of an activator of ARF (alternate reading frame) (which interacts with MDM2) is reduced (Bagchi et al. 2007), possibly enhancing MDM2 functional levels. In the first intron of *MDM2*, there is one of the two gene promoter enhancers (the other one is in the first exon; Bond et al. 2005). In humans, the first intron consists of a 524-nucleotide segment that includes two different single nucleotide polymorphisms (SNP; Bond et al. 2005). One of these, the SNP309 (a T>G change at nucleotide 309; rs 2279744), causes a fourfold increase in the affinity of the promoter for the transcription factor *SP1*, resulting in higher levels of MDM2 mRNA and protein, attenuation of the p53-regulated pathways, and increased risk for tumorigenesis (Bond et al. 2004).

Single Nucleotide Polymorphism 309 Incidence in Neuroblastoma and Disease Progression

The influence of *MDM2* SNP309 in neuroblastoma incidence and disease progression is a recent object of investigation. Until now, only few reports have investigated the relationship of *MDM2* SNP309 to neuroblastoma (Cattelani et al. 2008; Perfumo et al. 2008, 2009) and all are based on Italian cohorts of patients.

A first study performed by Cattelani et al. (2008) in a cohort of 239 primary and untreated neuroblastoma patients assessed whether the frequency of the SNP309 in neuroblastoma patients was associated with variables predictive of poor outcome such age at diagnosis >1 year, adrenal primary site, advanced clinical stage (3 and 4), *MYCN* amplification, and chromosome 1p status (deletion or imbalance). They found a significant association (P =0.016; two-sided Fisher's Exact Test) between heterozygous (T/G) and homozygous (G/G) variant genotypes at SNP309 and advanced clinical stage. Cumulative Kaplan Meier 5-year overall survival in neuroblastoma patients with the T/G and G/G variants of SNP309 was shorter than in those with the predominant T/T variant (P=0.046; log-rank test), suggesting that, in its homozygous (G/G) or heterozygous (T/G) form, the SNP309 might be a novel indicator of poor outcome in neuroblastoma.

A successive study performed by Perfumo et al. (2009) in a larger cohort of neuroblastoma patients (n=437) confirmed that the presence of the G/G or T/G SNP309 correlates with poor survival in particular in stage 4 disease.

The impact of the *MDM2* SNP309 genotypes on overall survival, event free survival and survival

after relapse was evaluated by the Cox regression model, while survival curves were obtained by the Kaplan–Meier method. Analyses were performed both on the whole cohort and after stratifying by stage at diagnosis, grouping patients with a localized disease (i.e., stage 1–3). Finally, a stratified analysis by patients' clinical and biological characteristics was also carried out, and differences in survival probabilities by *MDM2* SNP309 status were assessed by the log-rank test.

The results show that in stage 4 patients, the TG/GG polymorphism was correlated with a poorer outcome in particular among children with *MYCN* amplification. This effect was more evident for overall survival and survival after relapse than for event free survival, suggesting that the G allele is mainly associated with the progression of the disease instead of an increased risk of relapse.

All these studies strongly suggest that *MDM2* SNP309 may be an independent prognostic factor for neuroblastoma, whose impact is restricted to survival of stage-4 patients and is particularly evident in those with *MYCN* amplified tumors.

These results indicate that the *MDM2* SNP309 G allele is associated with significantly worse survival even in the presence of *MYCN* amplification. This observation suggests that targeting the MDM2/p53 circuit could represent an effective strategy for the treatment of such patients. In fact, the *MDM2* SNP309 G allele, which is predicted to achieve higher expression of MDM2, as observed in various cell line models (Bond et al. 2004), could attenuate the p53 response pathway possibly contributing to resistance to genotoxic chemotherapy in *p53* wild-type neuroblastoma cells. Hence, targeting MDM2 expression levels or the MDM2/p53 proteins interaction to obtain p53 stabilization could make neuroblastoma cells more sensitive to genotoxic drugs.

Conclusions

The biological characteristics of neuroblastoma suggest that targeting the molecular interaction of MDM2 and p53 may be an effective therapeutic strategy. As noted, the majority of neuroblastomas are *p53* wild type and therefore dependent on MDM2 regulation of p53 to prevent apoptotic cell death. In several other tumor models, inhibition of MDM2 leads to increased p53 activity and triggers an apoptotic stress response (Vassilev et al. 2004; Zhang et al. 2004). In neuroblastoma cell lines, it has been recently demonstrated that down-regulation of *MYCN* leads to decreased MDM2 expression, p53 stabilization and subsequent rapid cell death. Since MYCN also sensitizes cells to apoptotic stress, neuroblastoma should be particularly sensitive to targeted disruption of the MDM2/p53 interaction in vivo. The *MDM2* SNP309 G allele is associated with significantly worse survival even in the presence of *MYCN* amplification. This observation suggests that also in this case targeting the MDM2/p53 circuit could represent an effective strategy for the treatment of such patients. In fact, the *MDM2* SNP309 G allele, which is predicted to achieve higher expression of MDM2, as observed in various cell line models (Bond et al. 2004), could attenuate the p53 response pathway possibly contributing to the resistance to genotoxic chemotherapy in *p53* wild-type neuroblastoma cells.

MDM2 as a drug target is the topic of extensive reviews and is an active area of research. Notably, treatment of p53 wild-type neuroblastoma cells with the small molecule MDM2 antagonist nutlin-3 resulted in activation of the p53 pathway leading to cell cycle arrest and apoptosis (Van Maerken et al. 2006).

Hence, targeting MDM2 expression levels or the MDM2/p53 proteins interaction to obtain p53 stabilization could make neuroblastoma cells more sensitive to genotoxic drugs. The unique developmental biology of neuroblastoma suggests that targeting MDM2 could be an effective therapy, especially in the context of MYCN-driven tumors or in presence of MDM2 SNP309 polymorphism. The future evaluation of such approaches will advance our understanding of neuroblastoma pathogenesis and hopefully lead to important clinical advances for this highly fatal pediatric malignancy.

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Role of PI3K Inhibitors in Sensitizing Neuroblastoma Cells to Apoptosis

4

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Contents

Abstract

The phosphatidylinositol 3′-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is aberrantly activated in most cancers and represents a key mediator of cell survival, for example by antagonizing apoptosis signaling. Recently, increased phosphorylation of Akt was identified as a novel marker of poor prognosis in primary neuroblastoma specimens, indicating that PI3K/Akt presents a clinically relevant molecular target. A variety of pharmacological approaches have been developed over the last years to block distinct elements of the PI3K/Akt/mTOR axis. The current review will focus on the potential of PI3K inhibition to sensitize neuroblastoma for apoptosis induction and to overcome treatment resistance. Such a strategy may pave the avenue to more effective treatment options for children with neuroblastoma.

Introduction

The phosphatidylinositol 3′-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway represents one of the major signaling pathway that mediates cell survival of cancer cells by supporting proliferation, metabolism or angiogenesis and by blocking cell death (Shaw and Cantley 2006). Most human cancers exhibit aberrant signaling via this cascade, which has also been linked to poor prognosis and resistance to current treatment regimens (Yuan and Cantley 2008).

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Targeting abnormal PI3K/Akt/mTOR pathway activation has emerged over the last decade as a promising strategy for molecular cancer therapies and individual components of the signaling cascade have been tested for their suitability as cancer drug target (Garcia-Echeverria and Sellers 2008). As far as neuroblastoma is concerned, the most common extracranial solid tumor in childhood, aberrant activation of PI3K/Akt has recently been identified as a novel prognostic factor of poor outcome (Opel et al. 2007). This highlights the clinical relevance of the PI3K/Akt/mTOR signaling pathway in neuroblastoma. The current review discusses recent advances on the role of the PI3K/Akt/mTOR pathway in neuroblastoma.

Neuroblastoma

Neuroblastoma represents the most common solid malignancy in childhood that occurs outside the central nervous system and is also the most common tumor that arises in infants younger than 12 months (Maris et al. 2007). Neuroblastoma is considered to originate from cells derived from the peripheral neural crest (Maris et al. 2007). Several typical genetic as well as biological features have been identified in neuroblastoma including amplification of the oncogene *myc myelocytomatosis viral related oncogene*, *neuroblastoma derived* (*MYCN*) and allelic loss at chromosome 1p36 (Maris et al. 2007). While elevated levels of the neurotrophin receptor tropomyosin receptor kinase (Trk) B have been linked to poor prognosis of neuroblastoma, high expression of the neurotrophin receptor TrkA has been identified as a good prognostic indicator (Maris et al. 2007).

For many years, the prognosis of children with high-stage neuroblastoma remains still desperate despite aggressive treatment protocols (Maris et al. 2007). This poor outcome stands in sharp contrast to the tremendous advances that have been encountered for various other pediatric tumors, namely acute lymphoblastic leukemia. Based on the limitations of current treatment options for neuroblastoma, it will be critical to identify and characterize new molecular targets in order to improve the poor prognosis of children with this disease.

PI3K/Akt/mTOR Signaling

The PI3K/Akt/mTOR pathway is a key mediator of cell survival signals such as those delivered by growth factors, extracellular matrix proteins or cell-cell interactions (Shaw and Cantley 2006). Following the ligation of growth factor receptors on the cell surface by their respective ligands, transmembrane receptor tyrosine kinases (RTKs) become auto- and transphosphorylated and in turn recruit PI3K to the plasma membrane, where PI3K interacts with Akt to generate phospholipids (Shaw and Cantley 2006). In addition to this translocation process to the plasma membrane, distinct phospho-residues have been identified in Akt that are critical for the activation of Akt. These phosphorylation sites include both threonine 308 (Thr308) in the activation loop of the kinase and serine 473 (Ser473) at the C-terminus (Shaw and Cantley 2006). Phosphorylation of Akt in the kinase domain on Thr308 occurs via phosphoinositide-dependent kinase-1 (PDK-1) and results in partial activation of Akt (Shaw and Cantley 2006). In addition, phosphorylation at Ser473 by PDK-2 is necessary for full activation of Akt (Shaw and Cantley 2006). mTORC2 complex has been implicated in Ser473 phosphorylation of Akt, besides DNA-dependent protein kinase (DNA-PK), integrin-linked kinase (ILK), protein kinase C βII (PKCβII) and also Akt itself (Shaw and Cantley 2006). By comparison, PI3K/Akt signaling is negatively regulated by the tumor suppressor gene *phosphatase and tensin homologue deleted on chromosome 10* (*PTEN*), a dual-function lipid and protein phosphatase (Shaw and Cantley 2006). PTEN shuts off PI3K/Akt signaling by removing phospho-groups from PIP_3 at the D3 position.

Among its multiple functions, Akt can support the survival of cancer cells either directly by phosphorylating apoptosis signaling molecules or, alternatively, indirectly via changes in transcription factor activity or energy metabolism (Shaw and Cantley 2006). Examples of proteins that are direct targets of Akt-mediated phosphorylation include various apoptosis regulators such as Bad, Bax, caspase-9, Omi/high temperature requirement protein A2 (HtrA2) and acinus.

Akt-mediated phosphorylation of transcription factors such as NF-kappaB (NF-κB) and cAMP response element-binding (CREB) protein or members of the fork head transcription factors, including FOXO1, FOXO3a, FOXO4 and FOXO6 can have an impact on cell survival by indirectly altering expression levels of pro- and anti-apoptotic target genes. To this end, phosphorylation of fork head transcription factors via Akt favors its sequestration in the cytoplasm and prevents its nuclear localization, thereby suppressing the expression of pro-apoptotic target genes such as Fas ligand, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or Bim. Similarly, Akt can interfere with apoptosis signaling by phosphorylation and activation of other transcription factors, e.g., NF-κB.

Activation of the PI3K/Akt/mTOR Pathway in Neuroblastoma

Recently, aberrantly activation of PI3K/Akt has been identified as a novel predictor of poor outcome in primary neuroblastoma samples (Opel et al. 2007). The analysis of the phosphorylation status of Akt, the mTOR target S6 ribosomal protein and extracellular signal-regulated kinase (ERK) in a large panel of primary neuroblastoma specimens by tissue microarray revealed that phosphorylation of Akt at serine 473 (S473) and/or threonine 308 (T308), phosphorylation of S6 ribosomal protein as well as phosphorylation of ERK are frequent events in primary neuroblastoma. Of note, elevated phosphorylation of Akt turned out to be a new prognostic marker that indicates reduced event-free or overall survival of neuroblastoma patients. In contrast to Akt, the phosphorylation status of S6 ribosomal protein or ERK did not correlate with adverse prognosis, pointing to a distinct role of PI3K/Akt in the biology of neuroblastoma. Also, increased phosphorylation of Akt correlated with several parameters of advanced disease, including clinical markers such as advanced disease stage or age at diagnosis as well as biological features such as *MYCN* amplification, 1p36 aberrations, and unfavorable histology. Interestingly, monitoring Akt at T308 or both phosphorylation sites proved to be superior compared to the analysis of Akt at Ser473 in neuroblastoma specimens to predict the prognosis. Together, these findings demonstrate that Akt presents a clinically relevant, new target for therapeutic intervention in neuroblastoma. These findings were confirmed in a subsequent, independent study showing that phosphorylated Akt and mTOR are expressed in all primary neuroblastoma samples compared to lack of these markers in normal tissue of adrenal medullas (Johnsen et al. 2008). Moreover, the catalytic isoform of class IA PI3K, i.e., p110δ, as well as the regulatory isoform of PI3K, i.e., p85α, were reported to be expressed in primary neuroblastoma samples and cell lines compared with normal adrenal gland tissue (Boller et al. 2008). Selective targeting of these isoforms by RNA interference resulted in cell growth inhibition upon downregulation of p110α and in antiproliferative and cytotoxic effects following knockdown of p110δ in neuroblastoma cells (Boller et al. 2008), suggesting that both p110α and p110δ isoforms regulate neuroblastoma growth and survival.

Genetic Alterations

In principle, genetic lesions, epigenetic changes or deregulation of signaling cascades can cause abnormal activation of the PI3K/Akt/mTOR cascade in neuroblastoma. It is interesting to note that genetic inactivation of the tumor suppressor gene *PTEN* by either deletion or mutation have been reported to present rare events in neuroblastoma. Accordingly, the analysis of 45 primary neuroblastoma specimens and 12 neuroblastoma cell lines revealed homozygous deletions of *PTEN* in only a small proportion of primary tumors (2 of 41 tumors = 5%) (Munoz et al. 2004). In contrast, *PTEN* is frequently deleted in several other malignancies such as different types of carcinomas and glioblastoma. Alternatively, PTEN may be inactivated in neuroblastoma through promoter hypermethylation, which occurred in a substantial percentage of primary neuroblastoma (i.e., 25%) in one study (Hoebeeck

et al. 2008). The role of PTEN in the control of PI3K/Akt signaling in neuroblastoma was supported by data showing that ectopic expression of PTEN interferes with the Insulin-like growth factor (IGF)-I-conferred rescue from apoptosis in neuroblastoma cells (van Golen et al. 2001).

Furthermore, mutational activation of PIK3CA was reported to be an uncommon event in neuroblastoma (Dam et al. 2006), in contrast to various adult cancers that were described to harbor an activated version of the PI3K isoform PIK3CA. The analysis of 42 primary neuroblastoma samples and 27 cell neuroblastoma lines by sequencing for PIK3CA-activating mutations within the helical, C2 and kinase domain "hot spot" regions, where 80% of mutations have been described to cluster, showed that mutations in the PIK3CA gene occur in only 2 of 69 neuroblastoma (2.9%) (Dam et al. 2006). Thus, genetic changes in key components of the PI3K/Akt pathway are rare events in neuroblastoma.

There is emerging evidence that mutational activation of anaplastic lymphoma kinase (ALK), a tyrosine kinase receptor, may lead to stimulation of the PI3K/Akt pathway in neuroblastoma. Recently, activating ALK mutations have been identified in the germline in the case of hereditary neuroblastoma and may also occur somatically (George et al. 2008). Interestingly, silencing of ALK by RNA interference resulted in a significant suppression of Akt phosphorylation and survival of neuroblastoma cells (Osajima-Hakomori et al. 2005), supporting the notion that ALK may belong to the positive upstream regulators of Akt in neuroblastoma.

Receptor Tyrosine Kinase (RTK) Signaling

Aberrant activation of receptor tyrosine kinases (RTKs) can also contribute to the elevated activity of the PI3K/Akt pathway in neuroblastoma. RTKs are constituents of the plasma membrane and can bind their corresponding ligands such as growth factors via an extracellular domain (Shaw and Cantley 2006). In addition, they harbor an intracellular domain that facilitates the recruitment of signaling molecules (Shaw and Cantley 2006). RTK activity has been reported to be elevated in various human cancers among them neuroblastoma, which has been implicated in the activation of intracellular signaling cascades that promote cancer cell survival such as the PI3K/Akt/mTOR cascade (Shaw and Cantley 2006). In neuroblastoma, several growth factors have been linked to contribute to the abnormal stimulation of RTKs, including insulin-like growth factor (IGF), brain-derived growth factor (BDNF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF).

Primary neuroblastoma tumor specimens were shown to express IGF-1 receptor (IGF-1R) in the majority of cases (Weber et al. 2003). In addition, IGF-1R expression status was associated with PI3K/Akt and MAP kinase pathway activation (Kurihara et al. 2000), suggesting that signaling via the IGF-1 system may contribute to stimulation of the PI3K/Akt cascade. IGF-1 has also been linked to phosphorylation of fork head transcription factors via PI3K/Akt in a neuroblastoma cell line (Schwab et al. 2005), providing an additional connection between the IGF-1 system and PI3K/Akt signaling. Another piece of evidence that RTK stimulation via IGF-1R contributes to PI3K/Akt activation comes from a recent study showing that addition of IGF-1 triggers Akt phosphorylation in a PI3K-dependent manner in various neuroblastoma cell lines (Opel et al. 2007). A large body of data exists demonstrating that IGF-I receptor activation results in inhibition of apoptosis in neuroblastoma (Opel et al. 2007).

BDNF belongs to the neurotrophin family of growth factors, which are critical regulators of development and survival of the sympathetic nervous system. Since neuroblastoma and the sympathetic nervous system share a similar origin from the neural crest, it is not surprising that this growth factor plays also an important role in the biology of neuroblastoma. TrkB is the corresponding tyrosine kinase receptor to BDNF that promotes proliferation and survival following BDNF ligation, at least in part, via engagement of the PI3K/Akt/mTOR pathway.

The TrkB/ BDNF receptor/ ligand system and subsequent activation of PI3K/Akt signaling has

been implicated in the chemoresistant phenotype of advanced neuroblastoma. For example, overexpression of TrkB or addition of BDNF triggered phosphorylation of Akt and concomitantly decreased drug-induced cell death (Ho et al. 2002; Jaboin et al. 2002). Reduction of expression levels of Bim, a pro-apoptotic BH3-only protein of the Bcl-2 family, may contribute to the BDNF-conferred rescue of neuroblastoma cells from paclitaxel-induced cell death (Li et al. 2007). Chemoprotection by BDNF may also involve inactivation of glycogen synthase kinase (GSK)-3β. Furthermore, BDNF has been shown to stimulate angiogenesis during hypoxic conditions in neuroblastoma cells via induction of vascular endothelial growth factor (VEGF) expression in a PI3K/Akt/mTOR-dependent manner (Nakamura et al. 2006).

Expression of EGF receptor-1 (EGFR1) has been reported in most cases of primary neuroblastoma specimens as well as in neuroblastoma cell lines (Ho et al. 2005). Both PI3K/Akt and MAPK signaling pathways were stimulated upon the addition of EGF as the corresponding ligand resulting in enhanced proliferation. Vice versa, the addition of ZD1839, a pharmacological inhibitor of human epidermal growth factor receptor 1 (HER1), inhibited phosphorylation of Akt in neuroblastoma cells with little effects on MAPK signaling (Ho et al. 2005). These findings underscore the hypothesis that PI3K/Akt signaling contributes to EGF-stimulated proliferation in neuroblastoma.

There is also evidence that VEGF contributes to the survival of neuroblastoma cells via stimulation of PI3K/Akt signaling. For example, activation of Akt upon exposure to VEGF resulted in increased expression levels of survivin, a protein of the IAP family of proteins that supports cell cycle progression while inhibiting apoptosis in a PI3K-dependent fashion (Beierle et al. 2005). Furthermore, gland-derived vascular endothelial growth factor/prokineticin-1 (EG-VEGF/Prok-1), a key factor during development that engages differentiation and growth of enteric neural crest cells, was shown to support tumor progression of neuroblastoma via activation of Akt signaling.

Targeting the PI3K/Akt/mTOR Axis in Neuroblastoma

Since enhanced signaling via the PI3K/Akt/mTOR survival cascade represents a characteristic feature of multiple human malignancies including neuroblastoma and since this aberrant activation has been linked to treatment resistance and poor prognosis (Yuan and Cantley 2008), components of the PI3K/Akt/mTOR cascade have attracted considerable attention over the last decade as potential cancer drug targets. In principle, the cascade can be blocked at different levels, e.g. upstream by inhibiting RTKs, PI3K and Akt or, alternatively, downstream at the level of the mTOR complexes mTORC1 and mTORC2. Approaches to interfere with pathway activation include small-molecule kinase inhibitors and antagonistic antibodies directed against receptor tyrosine kinases anchored to the plasma membrane and harboring extracellular domains suitable for antibody-directed targeting.

Abnormal activity of several RTK has been shown to commonly occur in neuroblastoma, thus providing the basis for the use of agents that block RTKs signaling in order to dampen signal transduction via the PI3K/Akt/mTOR cascade. Several small-molecule kinase inhibitors and antagonistic antibodies have been developed to target the IGF-1R. A proof-of-principle experiment demonstrated that the blockage of IGF-1R expression in neuroblastoma cells by intratumoral delivery of an IGF-1 Rantisense construct caused tumor regression in a mouse xenograft model of neuroblastoma (Liu et al. 1998). Furthermore, several antagonistic monoclonal antibodies to the human IGF-IR were evaluated in neurobastoma. For example, the IGF-IR antibody EM164 inhibited IGF-I-, IGF-II-, and serum-stimulated proliferation and reduced survival of various neuroblastoma cell lines (Maloney et al. 2003). The fully human IGF-1R antibody SCH 717454 (19D12) displayed anti-tumor activity against neuroblastoma tumor xenografts when it was tested in the Pediatric Preclinical Testing Program (PPTP) (Kolb et al. 2008). NVP-AEW541, a small-molecule IGF-IR inhibitor,

prevented the IGF-II-triggered stimulation of Akt, enhanced apoptosis and reduced neuroblastoma growth in vivo (Tanno et al. 2006). Also, chemosensitization to cisplatin-mediated apoptosis was achieved in the presence of NVP-AEW541. These results underline that therapeutic blockage of the IGF-1R/IGF-1 system represents a relevant approach in neuroblastoma.

Another approach is to target PI3K/Akt/mTOR signaling at the level of PI3K. The PI3K family of lipid kinases comprises several members. As far as tumor biology is concerned, the four class I isoforms, i.e. p110α, p110β, p110γ and p110δ, are considered to be most relevant (Garcia-Echeverria and Sellers 2008). A series of initial studies in neuroblastoma using the wide-range PI3K inhibitor LY294002 demonstrated that PI3K inhibition reversed the IGF-I–mediated inhibition of chemotherapy-induced apoptosis (Opel et al. 2007), indicating that IGF-I inhibits chemotherapy-induced apoptosis in a PI3K-dependent manner. In *MYCN*-amplified neuroblastoma, PI3K inhibition caused decreased MycN protein levels, leading to growth inhibition and apoptosis in cell lines and decreased tumor mass in a *MYCN*-driven neuroblastoma mouse models (Chesler et al. 2006).

Recently, a series of new PI3K inhibitors have been developed, including selective class I PI3K inhibitors such as GDC-0941 as well as dual class I PI3K/mTOR inhibitors such as PI-103 and NVP-BEZ235 (Garcia-Echeverria and Sellers 2008). Importantly, inhibition of PI3K by PI-103 has recently been demonstrated to provide a new strategy to sensitize neuroblastoma cells to death receptor- or chemotherapy-induced apoptosis.

PI-103 was shown to synergize with various chemotherapeutics including doxorubicin, etoposide, topotecan, cisplatin, vincristine and taxol to trigger apoptosis in neuroblastoma cells (Bender et al. 2011). Calculation of the combination index demonstrated a high level of synergistic interaction. Mechanistic studies revealed that PI-103 cooperated with doxorubicin to reduce Mcl-1 expression and Bim_{EL} phosphorylation and to upregulate Noxa and Bim_{EL} levels. This shifted ratio of pro- and anti-apoptotic Bcl-2 proteins resulted in increased Bax/Bak conformational change, loss of mitochondrial membrane potential, cytochrome c release, caspase activation and caspase-dependent apoptosis. While Mcl-1 knockdown enhanced doxorubicin- and PI-103-induced apoptosis, silencing of Noxa, Bax/Bak or p53 reduced apoptosis, underscoring the functional relevance of the doxorubicin- and PI-103-mediated modulation of these proteins for chemosensitization. Furthermore, Bcl-2 overexpression inhibited Bax activation, mitochondrial perturbations, cleavage of caspases and Bid, and apoptosis, confirming the central role of the mitochondrial pathway for PI-103-mediated chemosensitization (Bender et al. 2011). Interestingly, the broad-range caspase inhibitor zVAD.fmk did not interfere with Bax activation or mitochondrial outer membrane permeabilization, whereas it blocked caspase activation and apoptosis. These results placed mitochondrial events upstream of caspase activation. Importantly, PI-103 and doxorubicin cooperated to induce apoptosis and to suppress tumor growth in patients'-derived primary neuroblastoma cells and in an in vivo neuroblastoma model, underlining the clinical relevance of the results.

Furthermore, PI-103 cooperated with the death receptor ligand TRAIL to synergistically induce apoptosis in a panel of neuroblastoma cell lines (combination index < 0.1)(Opel et al. 2011). In addition, PI-103 and TRAIL acted in concert to suppress clonogenic survival and to reduce tumor growth in a neuroblastoma in vivo model. Similarly, genetic silencing of PI3K significantly increased TRAIL-mediated apoptosis, whereas genetic or pharmacological blockage of mTOR failed to potentiate TRAIL-induced apoptosis. Molecular studies showed that the combined treatment with PI-103 and TRAIL enhanced cleavage of Bid and the insertion of tBid into mitochondrial membranes. Additionally, PI-103 decreased expression of Mcl-1, XIAP, and cFLIP, thereby promoting Bax/Bak activation, mitochondrial perturbations, and caspase-dependent apoptosis. Knockdown of Bid or Noxa or overexpression of Bcl-2 rescued neuroblastoma cells from PI-103- and TRAIL-induced apoptosis, whereas Mcl-1 silencing potentiated apoptosis.

In addition, Bcl-2 overexpression inhibited cleavage of caspase-3, caspase-8, and Bid pointing to a mitochondria-driven feedback amplification loop (Opel et al. 2011). Together, these findings demonstrate that targeting PI3K presents a novel and promising strategy to sensitize neuroblastoma cells for chemotherapy- or TRAIL-induced apoptosis. Thus, these results will likely have important implications for the development of new approaches to target the PI3K/Akt/mTOR signaling pathways in neuroblastoma.

Akt has also been evaluated as a therapeutic target in neuroblastoma. For example, phosphatidylinositol ether lipid analogues, which block the translocation of Akt to the plasma membrane and subsequent Akt activation by preventing the binding of PIP_3 to the PH domain of Akt, were reported to sensitize neuroblastoma cells to etoposide- or cisplatin-mediated cell death and to reverse the protection from chemotherapy that was imposed by BDNF (Li et al. 2005). Genetic manipulation of Akt by expression of a dominant-negative Akt construct further validated inhibition of Akt as an effective approach to counteract the BDNF-mediated protection against chemotherapy (Li et al. 2005). Furthermore, Akt inhibition has been reported to increase the sensitivity of neuroblastoma cells to the anti-proliferative effects of the IGF-IR inhibitor NVP-AEW541 (Guerreiro et al. 2006), demonstrating that a dual blockade of receptor tyrosine kinases such as IGF-IR and Akt cooperatively suppresses neuroblastoma growth. Recently, perifosine, a small-molecule inhibitor of Akt, has been reported to inhibit the activation of Akt and proved to be an effective cytotoxic agent in neuroblastoma cells in vitro and in vivo (Li et al. 2010). Furthermore, perifosine-mediated inhibition of Akt increased the sensitivity of neuroblastoma cells to chemotherapy and attenuated BDNF-conferred chemoresistance. There is also evidence that Akt rather than mTOR may regulate the production of VEGF under hypoxic conditions in neuroblastoma cells, since specific inhibition of Akt by A-443654 was more effective to inhibit hypoxia-driven VEGF expression than rapamycin, an inhibitor of mTOR (Kurmasheva et al. 2007).

Inhibitors of mTOR represent an additional approach to interfere with elevated PI3K/Akt/mTOR activation in human cancers (Garcia-Echeverria and Sellers 2008). Examples of mTOR inhibitors include rapamycin (sirolimus) that is derived from Streptomyces hygroscopicus and several rapamycin derivatives, e.g., CCI-779 (temsirolimus), RAD001 (everolimus) and AP23573. Rapamycin exhibited low or intermediate antitumor activity against neuroblastoma xenografts in vivo when it was tested by the Pediatric Preclinical Testing Program (Houghton et al. 2008). By comparison, rapamycin and temsirolimus were described to display anti-proliferative activity especially in neuroblastoma cell lines with *MYCN* amplification, leading to inhibition of tumor growth and angiogenesis accompanied by downregulation of MycN protein, cyclin D1 and VEGF expression levels (Johnsen et al. 2008). Furthermore, rapamycin cooperated with anticancer drugs to suppress proliferation and tumor growth, to trigger cell death and to shut down angiogenesis (Marimpietri et al. 2007). However, inhibition of mTOR may not be sufficient to block angiogenesis in neuroblastoma, since dual blockade at the level of mTOR and Akt was shown to be more effective to block VEGF production than treatment with rapamycin alone (Kurmasheva et al. 2007). Thus, inhibition of both upstream and downstream elements of the PI3K/Akt/mTOR cascade will likely be necessary for therapeutic intervention in neuroblastoma.

Conclusions

The PI3K/Akt/mTOR pathway is a major signaling cascade that is aberrantly activated in neuroblastoma and confers a poor prognosis, highlighting the clinical relevance of this pathway as a cancer drug target in this childhood malignancy. There are a number of pharmacological strategies currently available to interfere with distinct elements of the PI3K/Akt/mTOR axis. In order to translate the knowledge on this signaling cascade from the laboratory to medical application, it will be pivotal to determine which molecular targeted agents are best suited for neuroblastoma, both as monotherapy or in combination regimens. In

addition, it will be critical to identify the subgroups of neuroblastoma patients that will likely benefit from this treatment strategy. In the long run, therapeutic targeting of the PI3K/Akt/mTOR pathway can pave the avenue to the design of more effective treatment options for cancers that depend on aberrant PI3K/Akt/mTOR activation.

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Regulation of Neuroblastoma Cell Differentiation by Retinoic Acid: Role of Alternative Splicing and micro-RNAs

5

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Contents

Abstract

High-risk metastatic neuroblastoma tumors have a poor prognosis even after aggressive multimodal therapeutic protocols. Retinoic Acid (RA) treatments were included in these multimodal therapies because its ability to induce differentiation of neuroblastoma cells *in vitro* and it appears to reduce the risk of tumor recurrence as established in a large multicentre clinical assay. The present article deals with the molecular actions of RA on neuroblastoma cells. We want to discuss novel actions of RA at the post-transcriptional level (alternative splicing, translational control of specific mRNAs, microRNA regulation) that in addition to the well-characterized actions of RA at the transcriptional level contribute to neuroblastoma cell differentiation. We propose that RA acts coordinately over the multiple regulatory layers of gene expression and therefore must be considered as a global regulator of gene expression in neuroblastoma cells.

Introduction

Neuroblastoma is the most common extracranial solid tumor in childhood. The tumors originate from aberrant development of primordial neural crest cells, and show a high grade of heterogeneity, ranging from mild tumors spontaneously differentiating to benign ganglioneuromas to high-risk aggressive metastatic tumors. The 5-year

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survival rate of children suffering high-risk neuroblastoma is now around 40%, despite the improvements in multimodal therapeutic protocols (for review, see Maris 2010). Neuroblastoma cell lines can be induced to differentiate *in vitro* by several agents, including retinoic acid (RA), the biologically active form of vitamin A. Administration of RA to neuroblastoma cells *in vitro* leads to proliferative arrest and neuronal differentiation as judged by their morphology and the expression of neuronal markers (Pahlman et al. 1984). In addition to inhibit cell proliferation and induce differentiation, long-term RA treatment decreases the biological aggressiveness of neuroblastoma cells *in vitro*, by reducing their migratory, invasive and metastatic abilities (Voigt and Zintl 2003; Meseguer et al. 2011; Escamilla et al. 2012). All these results prompted the introduction of RA and its derivatives into the therapeutic protocols for neuroblastoma patients. In children with high-risk neuroblastoma, treatment with a retinoid called 13-cis-retinoic acid (isotretinoin) reduces the risk of recurrence after high-dose chemotherapy and stem cell transplant, as has been demonstrated in a large multicentre clinical assay (Matthay et al. 1999, 2009). Nevertheless, the molecular bases of RA therapeutic actions are largely unknown. During the last years we have studied the molecular mechanisms through which RA induces cell differentiation in human neuroblastoma cell lines (Lopez-Carballo et al. 2002; Masia et al. 2007; Laserna et al. 2009; Meseguer et al. 2011). In the present paper we show that in addition to its well-known actions at the transcriptional level, novel actions of RA at the post-transcriptional level also contribute to RA-induced differentiation of neuroblastoma cells.

Retinoic Acid as a Signaling Molecule: Transcriptional Activation and Activation of Signaling Pathways by Retinoic Acid

In addition to its well known actions on the visual cycle, vitamin A (retinol) plays crucial roles for early embryonic development and organogenesis and for the homeostasis of the skin, the immune system, the male reproductive organs and other body tissues in the adult. Dietary retinol is the only source of vitamin A in the organism, and it has to be converted to its active form as RA by two consecutive dehydrogenation enzymatic reactions. The actions of RA are mediated mainly by the Retinoic Acid Receptor (RAR), belonging to the nuclear hormone receptors ruperfamily that normally act as a heterodimer with another nuclear receptor, the Retinoid X receptor (RXR). RXR itself can bind retinoid derivatives *in vitro* and appears to play an important role in retinoid signaling. As other nuclear hormone receptors, RAR and RXR act primarily as ligand-gated transcription factors, regulating the transcriptional activity of target genes that have specific DNA binding sequences (called RAREs) in their promoter regions. Transcriptional actions of RAR and RXR involve interplay of protein interactions with co-repressor and co-activator complexes that are strictly regulated by the binding of the ligand to the C-terminal domain of the receptor, and which finally results in local alterations of the chromatin structure and the recruitment of RNApol II transcription machinery to the promoter regions of their target genes (Mark et al. 2009; Theodosiou et al. 2010; Duong and Rochette-Egly 2011).

Nevertheless, transcriptional actions are not the only actions of RA, and as in the case of steroid receptors, transcription-independent actions (also known as non-genomic actions, Hammes and Levin 2007) have been reported. We first show several years ago that RA-induced activation of phosphatidyl-inositol-3-kinase (PI3K) pathway, one of the main intracellular signaling pathways, is a prerequisite for RA-induced neuroblastoma cell differentiation. When PI3K is blocked with a specific inhibitor, the morphological and molecular changes associated to differentiation (neurite extension and gene expression changes) do not occur (Lopez-Carballo et al. 2002). Since then many other authors have shown evidences of the activation of PI3K and other signaling pathways like the MAP kinases ERK and p38, in many different cell types and functional contexts, and nowadays transcription-independent actions are regarded as an important feature of RA signaling.

PI3K activation by RA occurs very rapidly, and phosphorylation of Akt, a downstream target for PI3K activation is detected within 5 min of RA treatment. Activation of PI3K by RA appears truly transcription-independent, because phosphorylation of Akt could be detected after treating the cells with actinomycin D, that blocks new gene transcription, or with Cycloheximide, that inhibits newly synthesized proteins (Masia et al. 2007). An arising issue in transcription-independent actions of steroid receptors is the involvement of *classical* nuclear receptor or the requirement for new *non-canonical* receptors distinct of those contributing to the transcriptional actions. In the case of RA, we have demonstrated the implication of the *classical* nuclear receptor RAR in the activation of PI3K by RA, by using a genetic strategy. Embryo fibroblasts from wild type mice (MEFs) readily activate PI3K upon RA treatment. However, PI3K activation by RA do not occur in MEFs lacking the three genes for RAR, derived from RARα, β, γ triple floxed mutant mouse embryos (Altucci et al. 2005). When a functional RA receptor like RARα is reintroduced into those RAR-null cells by means of a retroviral vector, the ability to activate PI3K by RA is restored, demonstrating the involvement of RAR in transcription-independent actions leading to PI3K activation (Masia et al. 2007).

PI3K is an enzyme composed of two subunits; a 85 KDa regulatory subunit (p85) and a 110 KDa catalytic subunit (p110). Activation of PI3K by RA relies in a physical association between RAR and the two subunits of the PI3K. PI3K activity could be detected in RAR immunoprecipitates obtained from RA-treated neuroblastoma cells, but not in those obtained from untreated cells. Surprisingly, an association between the regulatory subunit p85 and RARα was detected both in untreated as well as in RA-treated neuroblastoma cells, suggesting that RARα forms a stable complex with p85. Activation of RAR by RA results in the recruitment of the catalytic subunit p110 to that RARα-p85 complex, resulting in the enzymatic activity observed in the immunoprecipitates. Although the interaction between p85 and RARα appears to be constitutive, a direct protein-protein interaction could not be detected neither in co-transfection/co-immunoprecipitation experiments, nor in GST-pulldown *in vitro* binding experiments (Masia et al. 2007), nor in the yeast two-hybrid system (Castrillón Y,García-Gimeno A and Barettino D, unpublished results), suggesting that a yet unknown scaffold protein might mediate the interaction between RAR and p85 as part of the signaling complex.

PI3K is an enzyme acting on plasma membrane phospholipids, whereas RAR is mainly located in the nucleus. These facts open the question of where the activation of PI3K by RAR could take place within the cell. We have constructed a chimeric RARα version including the myristoylation domain of *Src* kinase and this *Myr*-RARα is targeted to the plasma membrane and accumulates in the lipid rafts fraction, membrane domains containing sphingolipids and cholesterol that are enriched in signaling proteins. In these conditions, a high activity of PI3K pathway could be detected upon transfection of *Myr*-RARα to COS7 cells or to MEF-RAR-null cells. However, activation takes place independently of RA treatment, suggesting that plasma membrane location favours activation of PI3K by RAR. By a combination of conventional biochemical fractionation and immunoprecipitation techniques we could show that RA treatment increases the presence of RAR and the RAR-p85 complex in plasma membrane in SH-SY5Y neuroblastoma cells as well in NIH-3T3 cells (Masia et al. 2007). This suggest that binding of RA to its receptor RAR could contribute to PI3K activation at the plasma membrane by facilitating the translocation of a small fraction of RAR in complex with p85 to the membrane. Once the RAR-p85 complex is located at the plasma membrane, the interaction with the catalytic subunit p110 might occur, leading to PI3K activation. Attempts to demonstrate that hypothesis by means of confocal microscopy using GFP-RARα fusion proteins were not successful, probably because the amount of RAR present at membrane fractions is below the detection limits. Recently, it has been shown that in MCF7 cells although most of RARα is located in the nucleus, a small pool of RARα is present in the lipid rafts fraction, where appears to interact with G protein αq to rapidly activate p38 MAP Kinase in a transcription-independent

manner. In these cells it could be possible to detect RARα protein outside of the nucleus by immunofluorescence microscopy, co-locating with the lipid rafts protein flotillin. Moreover, using a very sensitive *in situ* Proximity Ligation Assay (PLA) for microscopy, these authors could show that the number of RARα-Gαq complexes rapidly increase upon the addition of RA (Piskunov and Rochette-Egly 2011). All these evidences support a mechanism for transcription-independent actions of RA in which both the location of a RAR pool at plasma membrane and the interaction with proteins involved in signaling cascades might play relevant roles.

Retinoic Acid Treatment Modifies the Phosphorylation Pattern of Nuclear Proteins

As a first approach to understand the physiological role of the transcription-independent signaling response in the context of the induction of neuroblastoma cell differentiation by RA, we wanted to identify the proteins that become rapidly phosphorylated as a consequence of RA treatment. We have restricted our study to nuclear proteins, because our interest was to confirm whether the rapid signaling response would play a role on the gene expression changes occurring during RA-induced differentiation. For this purpose we have used a proteomic approach, in which nuclear phosphoproteins from control and RA-treated neuroblastoma cells were enriched by affinity chromatography. The differences between the two phosphoprotein populations were investigated after tryptic digestion using a quantitative proteomic assay (iTRAQ™ assay; isolated Tags for Relative and Absolute Quantification) that combines isobaric peptide labeling, separation by multi-dimensional nano-liquid chromatography and tandem mass spectrometry (Laserna et al. 2009). We could find 63 different protein species that have an altered phosphorylation pattern as a consequence of RA treatment. Among them, 36 protein show increased phosphorylation and the other 26 were de-phosphorylated as consequence of the treatment. The results obtained show that the changes in phosphorylation induced by RA occurred mainly in two families of nuclear proteins: (i) those related to chromatin dynamics involved in transcriptional regulation and (ii) those related to mRNA processing and in particular mRNA splicing. The involvement of rapid signaling response in chromatin remodeling and transcriptional regulation of steroid receptor target genes was already reported (Vicent et al. 2006) and participation of rapid transcription-independent actions in nuclear receptor phosphorylation and co-factor recruitment has been recently reported also for RAR (Bruck et al. 2009). However, our results underscore novel functions for the rapid transcription-independent signaling elicited by RAR in the regulation of mRNA processing, because a large percentage of the identified proteins participate in RNA processing in a wide sense (SR proteins, RNA binding proteins, hnRNPs, splicing factors, RNA helicases, etc.). Geneontology categories were assigned to the identified proteins, and with respect of biological process, the proteins whose phosphorylation is increased as a consequence of RA treatment, a 68% belong to RNA metabolism (GO5), 41% to RNA splicing (GO7), 27% to regulation of transcription (GO7) and 13% to RNA splicing, via transesterification reactions with bulged adenosine as nucleophile (GO9). Among those proteins with decreased phosphorylation as effect of RA treatment 63% belong to RNA metabolism (GO5), 38% to regulation of transcription (GO7), 31% to RNA splicing (GO7) and 15% to RNA splicing, via transesterification reactions with bulged adenosine as nucleophile (GO9). The presence of a considerable number of RNA-binding proteins involved in mRNA processing and in particular mRNA splicing among those whose phosphorylation was modified by RA treatment uncovered the involvement of nuclear proteins engaged in mRNA processing in wide sense (splicing, transport, translational regulation, etc.) in the cellular response to RA. Therefore, we proposed as hypothesis that RA, through the rapid transcription-independent activation of signaling pathways, could regulate mRNA processing, as part of a cellular response elicited by the nuclear receptor RAR.

Regulation of Alternative Splicing by Retinoic Acid

Many evidences support the idea that signaling pathways could regulate alternative splicing, by phosphorylating RNA-binding proteins that are constituents of the spliceosome and/or function as splicing regulatory factors (Stamm 2008). We first examined whether RA treatment of neuroblastoma cells had any effect on the regulation of mRNA splice site selection in vivo using the adenoviral E1A minigene splicing reporter. When transfected to neuroblastoma cells this construct generates a splicing substrate containing competing splice sites allowing changes in splice site usage to be detected from the relative amounts of the different mRNA species, analyzed by RT-PCR. The results show that RA treatment altered the relative amounts of the different spliced mRNA forms, resulting in a remarkable increase in the percentage of the 10S form as consequence of RA treatment. Treatment of the cells simultaneously with RA and the specific PI3K inhibitor LY294002 abolished this change, but RA-induced increase in the form 10S is not affected by inhibition of the ERK MAP kinase pathway by the specific MAP-ERK kinase (MEK) inhibitor U0126. Therefore, we can conclude that RA treatment of neuroblastoma cells altered the regulation of splice site selection, through PI3K-dependent but ERK MAP kinase-independent mechanisms (Laserna et al. 2009). Among the proteins whose phosphorylation is affected by RA treatment, we could find the serine/arginine-rich (SR) protein family, which are important regulators of mRNA splicing (Long and Caceres 2009; Zhong et al. 2009). Splice site efficiency and specificity is determined by critical protein-protein interactions among pre-mRNA binding proteins. We could show that RA treatment promotes the interactions among splicing factors SFRS1 (SF2/ASF) and U1-snRNP-70K protein in immunoprecipitation experiments (Laserna et al. 2009). This result is in good agreement with previous reports indicating that binding of U1 snRNP-70K protein to the 5′-splice site appear to rely in an interaction with SFRS1 (SF2/ASF) which is enhanced by phosphorylation of the SR protein (Xiao and Manley 1997).

However, splicing reporters are artificial genes and we wanted to test whether RA treatment could influence alternative splicing regulation in native genes. We use Affymetrix exon microarrays which allow the analysis of not only the changes in mRNA levels, but also changes in transcript variants generated by alternative splicing (Meseguer S. and Barettino D., unpublished results). After treating neuroblastoma cells with RA for 0.5, 6 and 24 h, we could classify the genes in four groups: A first group included genes not affected by RA treatment, that have similar expression levels as the control and low probability of alternative splicing. The second group was composed of genes differentially expressed by RA that have low probability of alternative splicing. A third group included genes differentially expressed by RA treatment that have also a high probability of alternative splicing. The fourth group included some genes whose overall expression level is not changed as consequence of RA treatment, but show high probability of alternative splicing. The third group could be representative of what was called co-transcriptional alternative splicing, first described by O'Malley and co-workers for steroid nuclear receptors, a mechanism resulting from co-transcriptional coupling of RNA transactivation and alternative splicing (Auboeuf et al. 2002, 2004, 2007). Cotranscriptional splicing regulation relies on transcriptional co-activators bound to nuclear hormone receptors, therefore acting only on receptor target genes. The fourth group could reflect regulation of alternative splicing independently of transactivation that could be a result of transcription-independent activation of signaling-pathways as described above (Fig. 5.1, for examples). Nevertheless validation experiments in which the effects of signaling pathways inhibitors on individual splicing events could be tested are required to confirm the proposed mechanisms. RA-dependent changes in alternative splicing have been described recently during RA-induced neural differentiation of for embryonic carcinoma cell lines P19 and N/tera2 (Alam et al. 2010; Apostolatos et al. 2010; Wakamatsu et al. 2010).

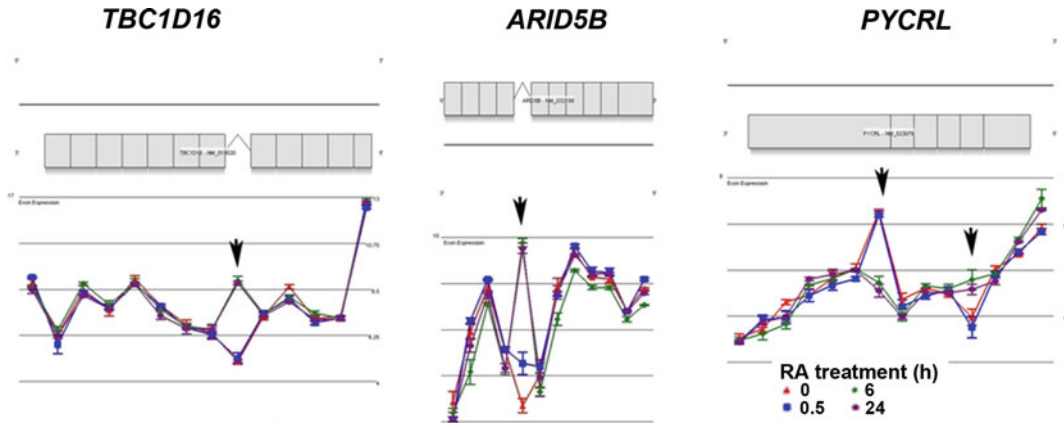

Fig. 5.1 Examples of RA-induced alternatively splicing in neuroblastoma cells. SH-SY5Y cells were treated with 1 mM RA during 0, 0.5, 6 and 24 h, and total RNA was extracted. Transcriptome analysis was performed with Affymetrix Human Gene 1.0ST microarrays, following the instructions of the manufacturer. For each treatment time 4 independent samples were analyzed. The results were analyzed and represented using Partek Genomic Suite software. The average values (+/− standard deviation) obtained for the different probes along each transcript were represented. On top of each graph schematic representations of the mRNAs exon structures are shown. The genes show a high probability of alternative splicing and a low probability of differential expression as compared to the control (RA 0 h), and correspond to the fourth group indicated in the text. The values for 0 h. RA (control) are shown in *red*, those for 0.5 h RA in *blue*, those for 6 h RA in *green* and those for 24 h RA in *magenta*. The *arrows* show evidence of RA-induced exon inclusion (genes *TBC1D16* and *ARID5B*) and more complex pattern showing RA-induced exon skipping and RA-induced exon inclusion on the same gene (gene *PYCRL*)

The question that arises is how these changes in alternative splicing could contribute to neural differentiation. Neural tissues have an extremely complex pattern of alternative spliced transcripts and a growing series of neural-specific or -enriched splicing factors regulating neuron-specific alternative exons have been described, like Nova-1/2, Fox-1/2, PTBP2, or the neural specific SR protein nSR100 (Jensen et al. 2000; Underwood et al. 2005; Boutz et al. 2007; Coutinho-Mansfield et al. 2007; Calarco et al. 2009). RA-dependent regulation of the levels and/or activities of these neural-specific factors as well as those of the general splicing regulatory factors could contribute to the establishment of alternative splicing patterns related with neural differentiation.

Retinoic Acid-Induced Translational Control

In addition to the well-characterized actions of SR proteins in splicing, several post-splicing activities have been described for a subset of shuttling SR proteins, including regulation of mRNA export and translation (Long and Caceres 2009; Zhong et al. 2009). To analyze a possible influence of the activated signaling pathways on SR protein-dependent translation, we used a translation reporter based on the fibronectin gene. The reporter contained a Luciferase coding sequence harboring the Exonic Splicing Enhancer as present in the fibronectin extra domain A (EDA's ESE; pLCS-EDA) or a mutant version of this ESE (pLCS-EDAmt) that does not bind the SR protein SFRS1 (SF2/ASF) (Sanford et al. 2004). After transfection to neuroblastoma cells, the relative luciferase activity from pLCS-EDA over that obtained from pLCS-EDAmt in a parallel experiment would indicate a specific effect of the SR proteins on mRNA translation. RA treatment produced a modest but consistent increase in the translation of the reporter (~1.7-fold). This stimulation could be abrogated both by an inhibitor of the PI3K/Akt pathway as well as by an inhibitor of the ERK-MAP Kinase pathway (Laserna et al. 2009). The results suggest that RA treatment may affect the regulation of translation of specific mRNAs through specific phosphorylation of

members of the SR protein family. The SR protein SFRS1 (SF2/ASF) promotes translation initiation by enhancing the phosphorylation of eIF4E-BP1, a competitive inhibitor of cap-dependent translation, and therefore suppressing its activity (Michlewski et al. 2008). RA treatment resulted in an increase in the phosphorylation of 4E-BP1 that could be abolished by simultaneous treatment with the PI3K inhibitor LY294002, but not by treatment with the inhibitor of the ERK MAP kinase pathway U0126. As expected, LY294002 abolished the activation of Akt, mTOR and p70S6 kinases as consequence of RA treatment. However, treatment with U0126 did not affect the activation of the three components of the PI3K/Akt pathway by RA (Laserna et al. 2009). Therefore, in spite of the good correlation between mTOR activity and the phosphorylation of 4E-BP1, additional mechanisms depending on the activity of the ERK MAP kinase pathway appear to contribute to the establishment of a RA-dependent mRNA translation control.

Post-Transcriptional Regulation of Gene Expression by Retinoic Acid Through micro-RNAs

MicroRNAs (miRNAs, miRs) are an emerging class of small non-coding endogenous RNAs that are involved in multiple biological processes. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC) to regulate gene expression by targeting the 3′-untranslated region (3′UTR) of mRNAs with consequent translational repression and/or target mRNA degradation (Bartel 2009; Chekulaeva and Filipowicz 2009). This mode of action demonstrates the great regulatory potential of miRNAs, since a unique mRNA can be targeted by diverse miRNAs and conversely each miRNA may have hundreds of different target mRNAs. In vertebrates, the highest variety of miRNAs is expressed in the brain than in any other tissue (Miska et al. 2004; Lau and Hudson 2010), suggesting an important role in nervous system development. Conversely, deregulation of miRNA action contributes to different pathological processes, including cancer. In recent years miRNAs have been established as important regulators of tumor development, progression and metastasis, and have demonstrated to be useful for tumor diagnosis and classification (Lovat et al. 2011). Moreover, miRNA regulation might represent a new avenue for cancer treatment in a near future. Several lines of evidence support a role for miRNAs in neuroblastoma pathogenesis (Stallings 2009, and references therein), and the usefulness of miRNA profiles for neuroblastoma diagnostics, classification and prognosis has been recently reported (De Preter et al. 2011, and references therein).

We have studied the changes in the pattern of expression in 667 different human miRNAs upon RA treatment of SH-SY5Y neuroblastoma cells through miRNA profiling with TaqMan RT-PCR Low Density Arrays (Meseguer et al. 2011). 452 miRNAs were expressed above detection level. From them, 42 specific miRNAs change significatively their expression levels (26 upregulated and 16 downregulated) during RA-induced differentiation. This suggests miRNAs as an additional post-transcriptional regulatory layer under RA control. The closely related miR-10a and -10b showed the most prominent expression changes. Loss of function experiments with antisense anti-miRs antagonists could show that miR-10a and -10b contribute to the regulation of RA-induced differentiation. RA-induced neurite outgrowth was impaired in cells with reduced levels of miR-10a or -10b, and the expression of several neural differentiation markers like tyrosine kinase receptors *NTRK2* (*trkB*) and *RET*, *GAP43*, neuron-specific enolase (*ENO2*), medium-size neurofilament protein NEFM and the enzyme tyrosine hydroxylase (TH) was abrogated or severely impaired after suppression of miR-10a or -10b. Conversely, the downregulation of the members of the ID gene family, *ID1*, *ID2* and *ID3* was abolished in RA-treated cells transfected with anti-miR-10a and anti-miR-10b. However, miR-10a and -10b did not appear to play a relevant role in RA-induced proliferation arrest, because the strong reduction of the incorporation of ^{3}H-Thymidine (to approximately 30% of the control values) and the decrease in the percentage of cells in S- phase (to 50% of the control) induced by RA treatment, was equivalent in neuroblastoma cells transfected with anti-miR-10a

and anti-miR-10b. Overexpression of miR-10 and -10b by transfecting synthetic precursor pre-miRs could not trigger full differentiation itself and although the mRNA levels of *RET*, *NTRK2*, *GAP43* and *ENO2* or the protein levels of NEFM and TH were slightly enhanced by transfection of pre-miR-10a and -10b, the attained expression levels for all the markers analyzed were far below those obtained by RA treatment. Therefore, miR-10a and-10b appeared to be necessary but not sufficient for full neural differentiation, and consequently additional actions of RA must contribute to differentiation (Meseguer et al. 2011).

It has been shown that long-term RA treatment reduces the biological aggressiveness of neuroblastoma cells, by reducing their migratory and invasive abilities (Voigt and Zintl 2003; Escamilla et al. 2012). In transwell assays we could show that suppression of miR-10a or -10b in anti-miR transfected cells abolished the reduction in migration induced by RA in SH-SY5Y cells. In similar assays cell invasion through Matrigel was increased upon RA treatment, and knockdown of miR-10a or-10b results in further increase of the invasive potential of neuroblastoma cells. To assess the contribution of miR-10a or -10b to the effects of RA treatment in the metastatic potential of neuroblastoma cells we employed the chicken chorioallantoic membrane (CAM) assay. Neuroblastoma cells readily disseminate to the lungs when transferred to the upper chorioallantoic membrane of 10-day-old chicken embryos, and as expected RA treatment reduced significantly the presence of human cells in the chicken lungs. However, when RA treatment was administrated to cells transfected with anti-miR-10a the observed RA-dependent reduction in lung metastasis was abolished (Meseguer et al. 2011). Although some reports indicate that miR-10a and -10b have a role in cancer pathogenesis and metastatic spread (Ma et al. 2007; Veerla et al. 2009; Weiss et al. 2009), our findings, together with other study (Moriarty et al. 2010), give support to the idea that miR-10a and -10b could act as tumor suppressor genes that opposite to tumor malignancy. This apparent controversy could be explained by the fact that in different cellular contexts, the same miRNA may exhibit diverse functions, depending on the repertoire and stoichiometry of its direct mRNA targets. In fact, association of lower expression of miR-10a and lower Overall Survival has been reported for a subgroup of neuroblastoma tumors having 11q deletions (Foley et al. 2011).

Through a combination of bioinformatic analysis and molecular biology experiments we could show that the important splicing factor SFRS1 (SF2/ASF) is a target for miR-10a and -10b regulation. miR-10a and -10b are new players in the complex regulation of SFRS1 protein through a mechanism involving enhanced mRNA cleavage. In addition, we showed how changes in miR-10a and -10b expression levels may influence some molecular activities in which SFRS1 is involved, such as translation enhancement of certain mRNAs and alternative splicing (Meseguer et al. 2011). As indicated above, activation of signaling pathways by RA treatment results in rapid changes in the phosphorylation pattern of SR proteins, including SFRS1 and subsequently, changes in alternative splicing selection and an increase of the translation of mRNAs containing SFRS1 binding sites take place (Laserna et al. 2009). In this context, the reduction in SFRS1 levels trough miR-10a and -10b regulation could be interpreted as the closing of the feedback regulatory loop of RA on the activities of SFRS1. In a recent paper, it has been shown that miR-10a and -10b could target *NCOR2* (Foley et al. 2011), a nuclear co-repressor that interacts with unliganded RAR and other nuclear receptors to keep their target genes repressed in the absence of the ligand (Chen and Evans 1995). Downregulation of repressive *NCOR2* might result in potentiating RAR-dependent transactivation.

Retinoic Acid as a Global Regulator of Gene Expression

The picture emerging from all these results is that both transcriptional as well as transcription-independent actions elicited by RAR are integrated and converge at multiple levels in the

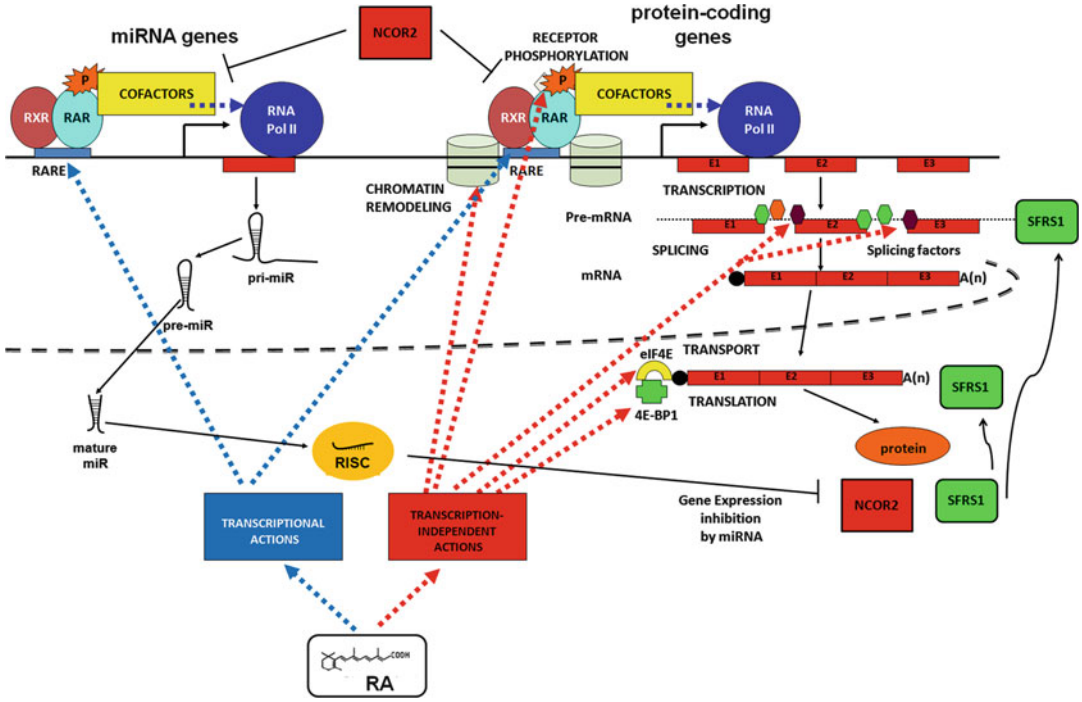

Fig. 5.2 Retinoic Acid as a global regulator of gene expression. Transcriptional as well as transcription-independent actions elicited by RA through its receptor RAR are integrated and converge at multiple levels in the regulation of gene expression during neuroblastoma cell differentiation. The classical transcriptional actions of RA contribute to differentiation through changes in the patterns of expression of protein-coding genes. Transcription-independent actions contribute to transcriptional activation through phosphorylation of the RAR receptor and of important chromatin proteins. In addition the activation of signaling pathways by RA also contributes to the regulation of alternative splicing by phosphorylating splicing regulatory factors as SFRS1. Moreover SFRS1 also appears to be involved in enhancing translation of a subset of mRNAs, through the activation of the PI3K-mTOR pathway. On top of that, RA regulates the expression of specific microRNA genes, another regulatory layer under the control of RA, with actions on transcriptional regulation via downregulation of RAR corepressor *NCOR2* and alternative splicing and translation regulation via the long-term downregulation of *SFRS1*

regulation of gene expression during neuroblastoma cell differentiation (Fig. 5.2). Transcription-independent actions contribute to transcriptional activation through phosphorylation of the RAR receptor and of important chromatin proteins. In addition the activation of signaling pathways by RA also contributes to the regulation of alternative splicing by phosphorylating splicing regulatory factors. Moreover through the activation of the PI3K-mTOR pathway RA activity could result in the enhanced translation of a subset of mRNAs. On top of that, microRNA regulation by RA appears as another regulatory layer under the control of RA, with actions on transcriptional regulation via downregulation of RAR corepressor *NCOR2* and alternative splicing via the long-term downregulation of *SFRS1*.

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Neuroblastoma: Role of Activated Leukocyte Cell Adhesion Molecule

6

Maria Valeria Corrias, Michela Croce, and Marina Fabbi

Contents

Abstract

Neuroblastoma is a rare sympathetic nervous system neoplasia with a broad spectrum of clinical presentations. Prognosis depends on age, stage, genetic and histological features. However, in spite of favorable prognostic factors, the event-free survival of some neuroblastoma patients with localized disease may be poor. Since the Activated Leukocyte Cell Adhesion Molecule (ALCAM/CD166), involved in nervous system development and neuritis extension, has been linked to tumor progression and metastasis in several tumor types, we studied its expression in neuroblastoma cell lines and primary tumors from patients with localized neuroblastoma. Neuroblastoma cell lines display various levels of ALCAM surface expression, which can be dynamically regulated by metalloprotease-mediated shedding. More importantly, ALCAM is expressed also in neuroblastoma primary tumors and diverse patterns of subcellular localization can be observed. In patients with localized disease and favorable prognostic factors, high levels of ALCAM membrane expression, together with low expression in the cytoplasm and neuropil area, were significantly associated with relapse, suggesting that high ALCAM membrane expression may represent a new negative prognostic factor in these patients. In conclusion, assessment of ALCAM subcellular localization may represent a useful tool to identify patients at high risk of relapse that could benefit from a more careful follow-up.

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Introduction

Activated Leukocyte Cell Adhesion Molecule (ALCAM/CD166) is a member of the Immunoglobulin gene superfamily belonging to the subgroup with five extracellular immunoglobulin-like domains (VVC2C2C2), which mediates cell-cell clustering through homophile (ALCAM-ALCAM) and heterophile (ALCAM-CD6) interactions (Swart 2002). In adult tissues ALCAM expression is limited to subsets of cells, whereas in several human tumors, including melanoma, prostate, breast, bladder and colorectal cancer, alterations in expression of ALCAM have been reported, as reviewed by Ofori-Acquah and King (2008).

ALCAM role in tumor progression and metastasis has been well documented in several tumor types. In melanoma tumors, high levels of ALCAM membrane expression correlated with the vertical growth phase of tumor progression. Indeed, amino-terminally truncated ALCAM molecules, unable to support homotypic cell clustering, increased spontaneous lung metastasis in a transplant tumor model, indicating that suppression of surface ALCAM adhesive functions was required to mobilize cells from primary tumors, as reviewed in van Kempen et al. (2000). In glioblastoma, Kijima et al. (2011) demonstrated that ALCAM can identify cancer progenitor cells in tumor specimens and that high frequency of ALCAM-expressing cells is a negative prognostic marker. In addition, ALCAM is involved in the regulation of glioblastoma cell motility, as siRNA-mediated down-regulation of ALCAM membrane expression significantly enhanced tumor cell invasion. In epithelial ovarian cancer (EOC) cells, Piazza et al. (2005) showed that ALCAM is expressed at the cell surface and is internalized following soluble ligand engagement. Moreover, Rosso et al. (2007) demonstrated that ALCAM is released from EOC cells by a metalloprotease (ADAM)17/TACE-dependent mechanism leading to the generation of a soluble ALCAM form (sALCAM). Therefore, the perturbation of ALCAM-ligand interaction is relevant to EOC cell motility. Indeed, Mezzanzanica et al. (2008) showed that the loss of EOC cell anchorage is accompanied by a loss of ALCAM expression at the membrane level, and that the decreased/lost membrane expression of ALCAM correlated with a poorer outcome of EOC patients. Tachezy et al. (2011) found that ALCAM expression is an unfavourable prognostic marker also for pancreatic neuroendocrine tumor patients. In patients with colorectal cancer, Weichert et al. (2004) found ALCAM expression both at membrane and cytoplasmic levels; however, only membrane expression significantly correlated with worse patient survival. Conversely, in breast cancer patients, Burkhardt et al. (2006) showed that ALCAM cytoplasmic, rather than membrane, overexpression correlated with disease progression. Taken together, these findings strongly suggest that dynamic changes of ALCAM expression may be relevant for the progression of different tumor cells.

ALCAM has a physiological role in the central nervous system development. Ott et al. (2001) demonstrated that it is fundamental for motor axon growth and guidance to their targets. Afterward, Weiner et al. (2004) showed that ALCAM promotes fasciculation of multiple axonal populations, while Buhusi et al. (2009) demonstrated its pivotal path finding activity during formation of the retinocollicular maps. Moreover, Wade et al. (2012) recently showed that ALCAM enters the axonal retrograde transport route and co-operates with NGF signalling, widening the range of its functions from adhesion molecule to modulator of growth factor signalling in the nervous system.

Neuroblastoma (NB) is a rare sympathetic nervous system neoplasia with a broad spectrum of clinical presentations, varying from aggressive disease (stage 4) to spontaneous maturation and even regression (stage 4S). As reviewed by Maris (2010), prognosis of NB patients depends on age, stage, histological and genetic features, such as *MYC-N* amplification. As reported by Cohn et al. (2009), patients with localized disease have good prognosis; however, some of them relapse and may eventually die of the disease. Thus, the search for new prognostic markers able to identify patients at risk of relapse is warranted.

Based on the above considerations, and on recent data by Wierzbicki et al. (2008) indicating that ALCAM represents an antigenic target for

NB immune recognition, we have investigated ALCAM expression in human neuroblastoma cell lines and primary tumors from patients with localized NB. We also evaluated whether different ALCAM subcellular localization associated with different outcomes of patients with localized NB.

Expression and Localization of Activated Leukocyte Cell Adhesion Molecule in Neuroblastoma Cell Lines

First, we analysed a panel of 13 human neuroblastoma cell lines for cell surface ALCAM expression. ALCAM was expressed at various levels in different cell lines, ranging from very low as GI-LI-N to high levels as GI-ME-N. No relationship was found between cell surface ALCAM and *MYC-N* amplification or chromosome 1p deletion status. Moreover, surface expression levels were diverse, irrespectively of the relative expression of mRNA, as assessed by semi-quantitative PCR.

Since ALCAM membrane expression may be influenced by its proteolytic cleavage, we then evaluated the expression of the metalloprotease ADAM17/TACE, supposed to generate ALCAM soluble form, as described by Rosso et al. (2007) in ovarian carcinoma cells. ADAM17/TACE was indeed expressed in NB cells both at mRNA and protein levels, and most importantly, it was able to generate the 65 kDa soluble form from the full-length ALCAM molecule. It is interesting to note that in NB cell line conditioned media, as reported in Corrias et al. (2010), ADAM17/TACE generated two soluble ALCAM forms of approximately 95 and 65 kDa, demonstrating that dynamic control of surface ALCAM expression actually occurs also in neuroblastoma cells.

Second, we analysed the pattern of ALCAM distribution in human NB cells following all-trans retinoic acid (ATRA)-induced differentiation. In ATRA-treated NB cell cultures no increase of sALCAM in the conditioned media nor changes in ADAM17/TACE protein expression were detected. However, after ATRA treatment, surface ALCAM expression was evident on the neuritis and dendrites of differentiated NB cells, becoming particularly strong on the neuritis after 7 days of treatment (Fig. 6.1). It is noteworthy that surface ALCAM expression appeared higher on the neuritis than on the cell body membrane at that time point, suggesting that in differentiating NB cells ALCAM re-localized into the growing neuritis. As expected, the acquisition of this ATRA-induced phenotype was independent of ADAM17/TACE activity.

Expression and Localization of Activated Leukocyte Cell Adhesion Molecule in Tumor Samples

Since different NB cell lines, that derive from high risk tumors, exhibited various levels of surface ALCAM expression, and because ATRA-treated NB cells, resembling more differentiated low risk tumors, re-localized membrane ALCAM into neuritis, we decided to investigate whether also in primary NB tumors different patterns of ALCAM localization could be observed.

Preliminary immuno histochemical analysis performed on frozen primary NB tumors showed indeed that ALCAM could be expressed at different levels and in different cellular compartments (Fig. 6.2), indicating that a correlation between ALCAM and clinical and pathological characteristics could be explored. We therefore performed immuno histochemical analysis of paraffin embedded archival samples from a cohort of resectable stroma poor NB tumor specimens. As described in Corrias et al. (2010), ALCAM could localize at the body membrane, in the cytoplasm and in the neuropil area formed by non-myelinated neuritis and dendrites.

Correlation Between Localization of Activated Leukocyte Cell Adhesion Molecule and Relapse in Patients with Localized Neuroblastoma

Mezzanzanica et al. (2008) showed in EOC tumors that loss of ALCAM membrane expression occurred together with its cytoplasmic re-localization. Moreover, this re-localization correlated with a worse prognosis. Thus, we evaluated whether

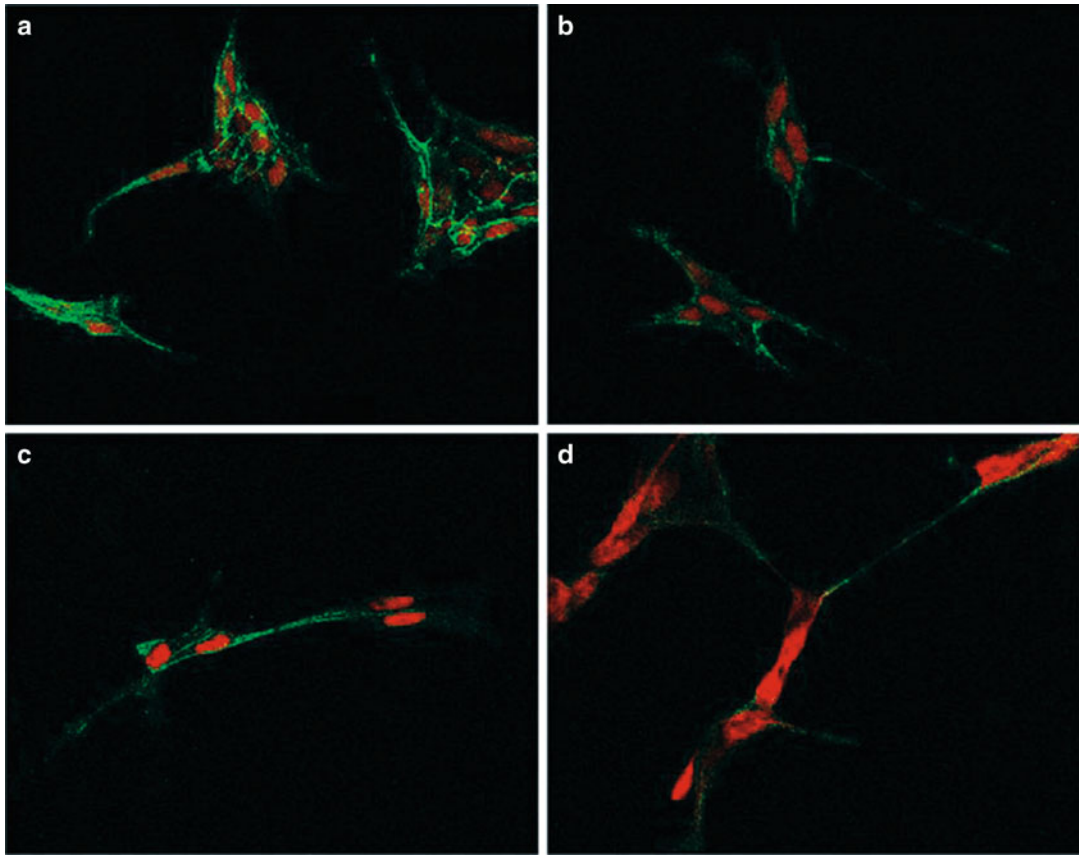

Fig. 6.1 ALCAM re-localization following treatment with retinoic acid. ALCAM expression and localization in differentiated SH-SY-5Y NB cells. Untreated cells (**a**) and cells treated for 24 (**b**), 48 (**c**) hours or 7 days (**d**) with 10 μM ATRA were stained with the anti-ALCAM I/F8 scFv followed by Alexa488-conjugated goat anti-mouse according to Piazza et al. (2005). Nuclei were counterstained with propidium iodide. Immunofluorescence was visualized by confocal microscopy (original magnification 600×)

different subcellular localizations of ALCAM expression were related to different risks of relapse. As demonstrated by Navarro et al. (2006), prognosis of NB patients with localized disease and normal *MYC-N* copy number is good, unless they present unfavorable histological features. However, among patients with localized disease and favorable histology, few local relapses can be observed. Thus, ALCAM expression was evaluated in 23 tumor specimens from patients with localized disease and favorable histology that had or not had experienced relapse. Precisely, 11 specimens were from patients that relapsed and 12 specimens were from patients that never relapsed. An event-free survival (EFS) analysis was then performed using the pattern of ALCAM expression to stratify the patients. As shown in Fig. 6.3, low expression in the neuropil area significantly associated with worse EFS ($P<0.0001$). It is interesting to note that all the patients that showed low ALCAM expression in the neuropil area had high expression in the cell body membrane, suggesting that ALCAM re-localization into neuritis has a protective effect.

In conclusion, NB cell lines, usually derived from highly proliferating tumors, display various levels of ALCAM surface expression, which can be dynamically regulated by ADAM17/TACE metalloprotease activity, which generates two different ALCAM soluble forms in the conditioned media. When NB cell lines were treated with retinoic acid that reduces their proliferation

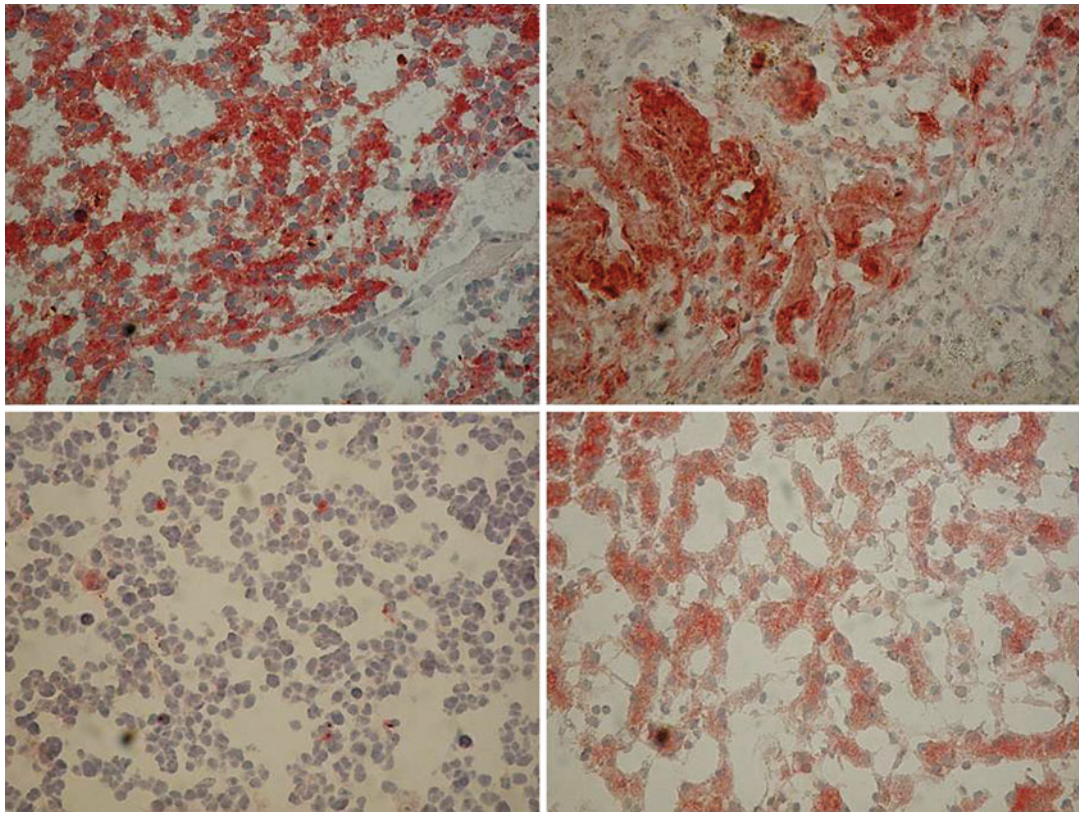

Fig. 6.2 Expression of ALCAM in cryostat sections from NB primary tumors. Immuno histochemical analysis of ALCAM expression was performed on 5 µm thick cryostat sections from NB tumors. Aceton fixed sections were stained with the anti-ALCAM I/F8 scFv, according to Piazza et al. (2005) and a peroxidase-labelled dextran polymer conjugated anti-mouse antibody. Slides were counterstained with Mayer's hematoxylin. Magnification 40×

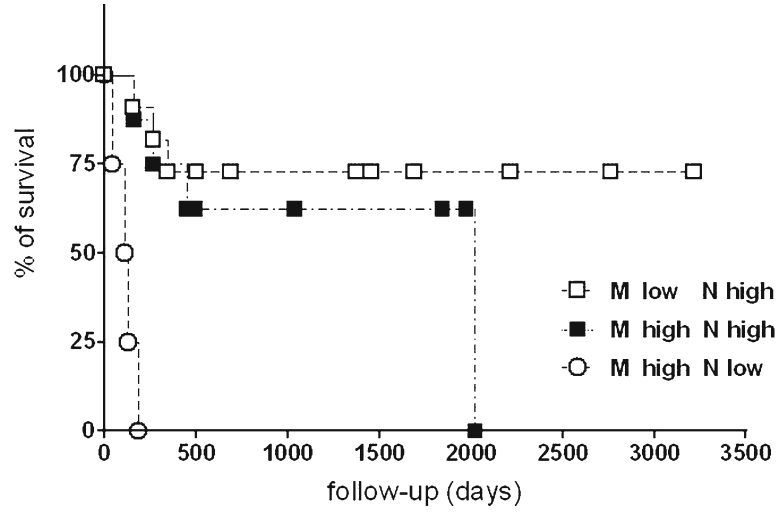

Fig. 6.3 ALCAM expression and event-free survival of patients with localized NB. Patients were stratified according to ALCAM expression (low or high) in membrane and neuropil area

and induces a more differentiated phenotype (see Clagett-Dame et al. 2006 for review), ALCAM re-localized into the growing neuritis. More importantly, ALCAM expression also occurs in NB primary tumors, showing diverse patterns of subcellular localization, involving the membrane, the cytoplasm and the neuritis. In a small but highly uniform cohort of patients with localized NB, favorable histology and normal *MYC-N* copy number, low ALCAM expression in the neuropil area and high levels in the body cell membrane significantly associated with relapse, suggesting that ALCAM expression may represent a new prognostic factor for these patients.

It could be speculated that the presence of high ALCAM levels in the neuropil area limit NB cell motility through a homophile interaction. Since neuropil is formed by non-myelinated dendrites and neuritis, and ATRA-differentiated NB cell lines showed strong ALCAM expression on neuritis, ALCAM staining in the neuropil identify more differentiated NB tumors, which have better prognosis. Furthermore, elevated ALCAM expression on the cell body of neuroblasts may support local tissue invasion, by acting as path finding molecule. Developmental studies by Ott et al. (2001), Weiner et al. (2004) and Buhusi et al. (2009) have indeed demonstrated that ALCAM favours neuritis outgrowth towards their targets. In this regard it is interesting to note that van Kempen et al. (2000) found that high ALCAM membrane expression in melanoma tumors, which share the same neuro-ectodermal origin as NB, associated with increased tumor progression. Similarly, Weichert et al. (2004) reported that membrane ALCAM overexpression associated with shorter survival time also in colorectal cancer patients.

Apparently conflicting results were reported by Mazzanzanica et al. (2008) and Jezierska et al. (2006) in ovarian and breast cancer patients, respectively. In fact, membrane ALCAM overexpression represented a good prognostic factor in these tumor types. Likely, this contradictory role depends on the fact that in these tumors ALCAM may increase cell to cell interaction rather than act as a path finding molecule. This holds true also in glioblastoma cells, where down-regulation of ALCAM by siRNA resulted in increased tumor cell invasiveness, as shown by Kijima et al. (2011). The function of ALCAM may indeed vary according to the cell type and to stimuli from the microenvironment, spanning from cell-cell adhesion to modulation of signalling in the nervous system, as reported by Wade et al. (2012). In conclusion, assessment of ALCAM subcellular localization may represent an easy and useful tool to identify, in the group of NB patients with localized disease and favorable histology, those at risk of relapse that could benefit from a more careful follow-up.

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Neuroblastoma: Inhibition by Alu-Like RNA

7

Manuele Castelnuovo, Tullio Florio, Giorgio Dieci, Ranieri Cancedda, and Aldo Pagano

Contents

Abstract

Neuroblastoma (NB) is one of the most frequent pediatric solid tumors characterized by remarkable cell heterogeneity within the cancer nodules. We have recently documented that the synthesis of a pol III-transcribed non-coding (nc) RNA (NDM29) strongly restricts NB development by promoting cell differentiation, a drop of malignancy processes and a dramatic reduction of the tumor initiating cells (TICs) fraction in the NB cell population. Importantly, the overexpression of NDM29 also confers to malignant NB cells an unpredicted susceptibility to the effects of antiblastic drugs used in NB therapy. Thus, treatments able to induce NDM29 expression within cancer cells, making the elusive cancer stem cells more vulnerable to the anticancer drugs, may be used synergistically with traditional cytotoxic therapy to obtain a more complete tumor eradication.

Introduction

Neuroblastoma (NB) is one of the most frequent pediatric solid tumors, accounting for about 15% of all pediatric cancer deaths and often developing in children younger than 3 years (Park et al. 2008). NB originates from the sympathoadrenal lineage, derived from the neural crest, and shows a clinical behavior markedly heterogeneous (Brodeur 2003). When tumors develop in children under 1 year of age the prognosis is often favorable and spontaneous differentiation of NB

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cells or regression of NB nodules may occur. On the other hand, NBs occurring in patients over 1 year of age tend to grow more aggressively and frequently have a fatal outcome (Brodeur 2003).

NBs show significant heterogeneity also as far as cell composition within tumor nodules, since they may derive from different neural crest lineages at different stages of differentiation. Importantly, differentiation levels are directly related with a favorable prognosis (Walton et al. 2004; George et al. 2001). Similar phenotypical diversity is also observed among (and even within) NB cell lines. According to (Ross et al. 1995) the differentiation state of cultured NB cells correlates with three main phenotypes that can be identified in vitro:

1. N-type (cells that grow as sphere-like aggregates, showing poor adherence to the substrate and form a morphological point of view display short neuritic processes),
2. S-type (cells with a large flat cell body growing with a significant substrate-adherence), and
3. I-type (cells displaying an intermediate phenotype, being mildly adherent to the substrate and showing marked stem-like traits; I-type cells are thought to give origin to both S-type and N-type cells (Ross et al. 1995).

Several in vitro and in vivo experimental data suggest that I-type NB cells represent the tumor initiating/stem-like cell population, providing tumors with the malignant potential. On the other hand, N-type and S-type cells display modest capacity to originate tumors in vivo (Walton et al. 2004). Indeed, experimental evidence was recently provided to support this concept. It was shown that cell lines enriched in N-type (BE(2)-M17, SH-SY5Y) or S-type cells (LA1-5 s, SH-EP1) are either non tumorigenic or able to develop tumors in less than 25% of inoculated mice (SH-SY5Y), while percentages ranging from 60 to 80% are reached by injection of I-type rich cell lines (BE(2)-C, SK-N-LP, CB-JMN, SK-N-ER) (Ross and Spengler 2007). Accordingly, the hypothesis that NB cell phenotype defines the malignant state of the nodules and exerts the most relevant influence on the prognosis, was supported by several studies, whereas classical genetic alterations identified in NB, such as the amplification status of Myc-N or its expression level, are now considered less predictive of tumor fate (Walton et al. 2004). Interestingly, this observation fit well with the cancer stem cell hypothesis stating that the relative abundance of tumor initiating/stem-like cancer cells (hereafter referred to as tumor initiating cells, TICs) in a tumor mass defines its malignant potential and may have prognostic significance. As far as NB is concerned, it is reasonable to hypothesize that the relative amount of I-type cells in NB nodules might represent an index used to predict the evolution of tumor.

Since NB is still one of the most challenging tumors to treat, a better understanding of the molecular and differentiation characteristics of NB may open the path to novel therapeutic strategies, effective for each tumor subset that, combined with a precise tumor risk classification, are required to improve the remission rate, or, at least, quality of life of the patients. In this context the concept that the differentiation state of NB cells is responsible of the clinical evolution of the tumor, may open significant new perspectives from a therapeutic point of view. The availability of drugs able to promote NB I-type cell differentiation would constitute an important advancement in the treatment of the more aggressive NB types. On the other hand, the identification of molecular markers to unambiguously select and classify the distinct stages of differentiation of NB masses (often it is difficult to histologically classify NB for the different biological and prognosis properties) would constitute a further significant advancement to perform a correct diagnosis and therapeutic approach for the highly heterogeneous NB histotypes.

We recently identified novel roles for several small nuclear (sn)RNA-like transcripts (Castelnuovo et al. 2010; Massone et al. 2011a, b), whose synthesis is driven by RNA polymerase (pol) III- type 3 promoters, in the regulation of gene expression (Pagano et al. 2007). These transcriptional units share consensus sequences for different regulatory elements including a TATA box, a Proximal Sequence Element (PSE) and, often, a Distal Sequence Element (DSE) that synergistically promote their synthesis (Dieci et al. 2007).

Among all the new transcripts identified, the 29th transcription unit in our collection (hereafter referred to as Neuroblastoma Differentiation Marker 29 or NDM29 (Castelnuovo et al. 2010) shows a canonical Alu/7SL-derived sequence structure and maps in humans in an oncosuppressive portion of 11p15.3, a genomic region whose deletion has been shown to be involved in NB development (De Preter et al. 2005; Amid et al. 2001). We provide in vitro and in vivo evidence that the transcription of NDM29 RNA restricts NB cell malignancy promoting the acquisition of a highly differentiated phenotype and a dramatic reduction of TIC content in the cell population. Notably, we also demonstrate that NDM29 RNA expression confers to drug-resistant NB cells the susceptibility to different chemotherapics, suggesting that the chemical induction of NDM29 RNA expression may represent a possible innovative therapeutic approach for NB. Moreover, the quantification of the expression levels of NDM29 may represent a relevant marker for NB prognosis and for a better prediction of the fate of cancer nodules.

NDM29 Is a Novel Alu-Like Pol III-Transcribed Non-Coding RNA

Eukaryotic genomes contains hundreds of small genetic elements that share the property of being recognized and transcribed by the RNA polymerase (Pol) III machinery, to produce a variety of small, abundant non-protein-coding (nc) RNAs (tRNAs, 5S rRNA, U6 snRNA and many others) (Dieci, et al. 2007; Orioli et al. 2012). Most Pol III-transcribed genes contain gene-internal promoter elements, but a significant number of them lack internal promoters and their transcription is driven by upstream-located *cis*-acting elements (forming together the so-called type 3 promoters (Dumay-Odelot et al. 2010).

We recently performed a bioinformatic search on the human genome sequence revealing the existence of several putative RNA polymerase (Pol) III transcription units displaying a combination of two basal upstream promoter elements typical of type 3 promoters: a Proximal Sequence Element (PSE) followed, approximately 30 bp downstream, by a TATA box (or a TATA-like element) and often preceded, at variable distances, by a Distal Sequence Element (DSE) (Pagano et al. 2007). One of these transcription units (named NDM29, for neuroblastoma differentiation marker 29) maps in the human genome in an oncosuppressive portion of 11p15.3, a genomic region whose deletion has been shown to be involved in neuroblastoma development [De Preter et al. 2005). In particular, NDM29 maps in the first intron of the Achaete Scute homologue 3 gene (ASCL3; RefSeq: NC_000011.8), coding for a member of the basic helix-loop-helix (bHLH) protein family in humans (Jonsson et al. 2004). Figure 7.1a shows the chromosomal location and context of NMD29, while Fig. 7.1b reports the sequence of its upstream promoter elements, as compared to the sequence of the same elements present in the promoter region of the human H1 gene (that was originally used as query for NDM29 computational identification (Pagano et al. 2007).

To verify whether NDM29 is a pol III transcription unit, it was tested as template for in vitro transcription, using a HeLa cell nuclear extract (Dignam et al. 1983). In vitro transcription of a plasmid-borne NDM29 construct containing the upstream PSE and TATA box, followed by the putative transcribed region, produced two specific RNAs of large size (>300 nt) (Fig. 7.1c). Such a pattern was difficult to interpret, presumably because of the lack of canonical Pol III terminators in the NDM29 transcription unit. The ability of the NDM29 promoter region to direct efficient transcription by Pol III was thus further verified by in vitro transcription of fusion constructs in which the 5′-flanking region of NDM29 was fused to the coding sequence of the 7SK RNA gene, a well characterized Pol III gene (Murphy et al. 1989). As shown in Fig. 7.1d, the NDM29 upstream region, containing the TATA-like and PSE elements (lanes 2–3) was almost as efficient as the native 7SK upstream promoter (lane 5) in directing Pol III-dependent transcription; moreover, removal of the PSE resulted in a significant decrease in transcription efficiency (lane 4). As recently outlined, NDM29 RNA gene can be

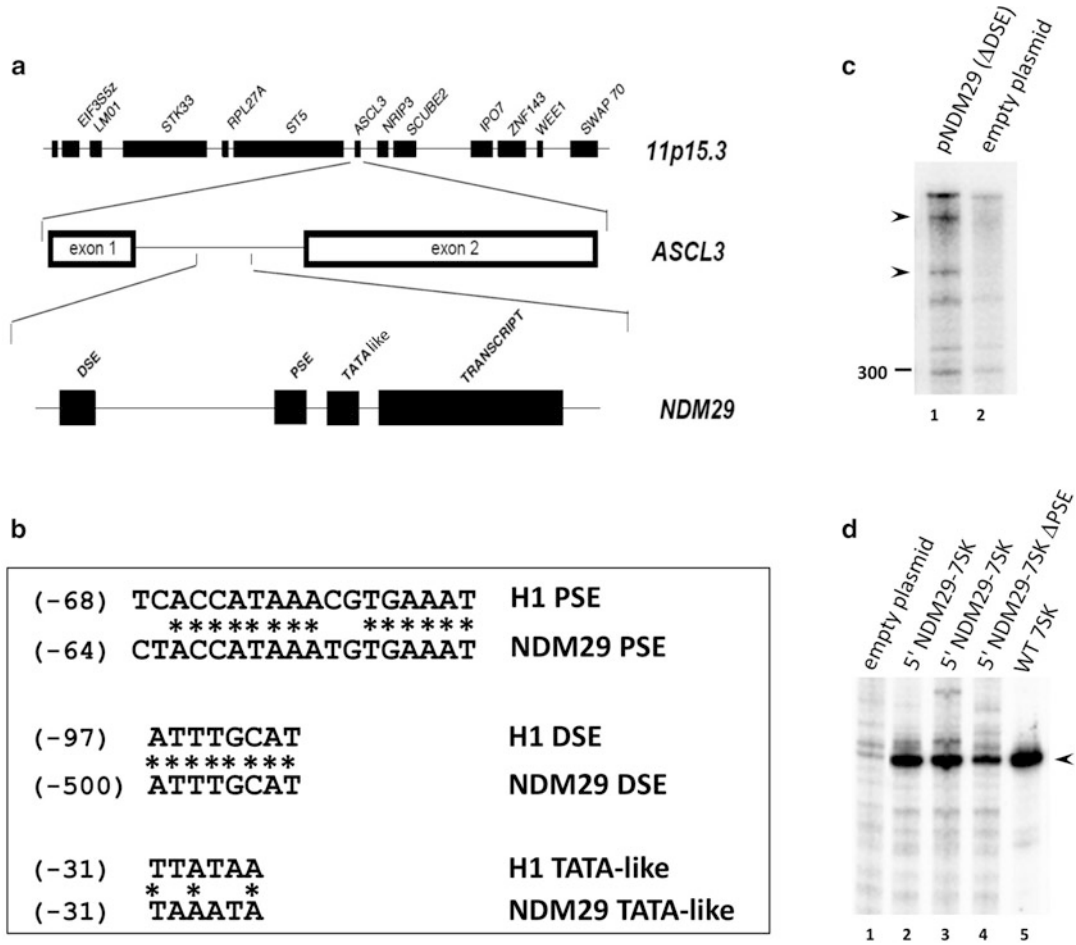

Fig. 7.1 NDM29 genomic location, promoter elements and in vitro transcription directed by the NDM29 promoter. (**a**) Schematic representation of NDM29 chromosomal localization. The oncosuppressive region mapping in 11p15.3 is reported according with De Preter et al. 2005 *BMC Genomics*. The organization of NDM29 promoter and transcription unit is schematically illustrated by the bottom panel. (**b**) The sequences of NDM29 upstream promoter elements are shown in comparison with sequences of the corresponding elements from the H1 gene promoter (Myslinski et al. 2001, 29: 2,502–2,509). (**c**) In vitro transcription of native NDM29 transcription unit. Transcription products generated from either plasmid-borne pNDM29 transcription unit lacking the DSE (lane 1) or empty pNEB193 vector DNA (lane 2) were radiolabeled during synthesis, gel fractionated, and directly visualized. Migration positions of a radiolabeled RNA size marker loaded in parallel is indicated at right. Arrowheads indicate pNDM29-specific transcripts. (**d**) In vitro transcription of NDM29-7SK fusion constructs. Reactions were programmed with empty vector DNA (lane 1); a fusion of the upstream region of NDM29, comprising either the TATA and PSE elements (lanes 2–3) or the TATA-like element only (lane 4), with the 7SK RNA coding sequence; the 7SK coding region precede by its natural promoter (lane 5)

classified as a conserved AluJb sequence preceded by a unique sequence at the 5′end (Berger and Strub 2011). The Alu portion does not contain A and B boxes, typical Pol III internal promoter elements specifically recognized by TFIIIC (Orioli et al. 2012). Its transcription thus solely relies on upstream located PSE and TATA elements, as is also the case for the recently discovered, AluJB-containing transcription unit referred to as 21A (Pagano et al. 2007).

NDM29 Expression Promotes NB Cell Differentiation

Highly proliferating cells, such as HeLa (cervical cancer), HEK293 (Human Embryonic Kidney) and primary cultures of fibroblasts, express high levels of NDM29, while low proliferating cells, such as SHSY5Y, SKNBE2 cells and primary cultures of human gliomas, express low levels. Thus, we hypothesized that NDM29 RNA might play a role in cell cycle control. To test our hypothesis, three plasmid constructs harboring different regions of NDM29 transcription unit [(i) the whole NDM29 transcription unit; (ii) the NDM29 promoter region lacking the transcribed portion; (iii) the NDM29 type 3 minimal promoter followed by the transcribed portion] were transiently transfected in SKNBE2 and HeLa cells. Forty-eight hours after transfection, proliferation rate was significantly decreased in cells harboring the transcribed portion of NDM29 transcription unit, whereas it was essentially unchanged in cells transfectecd with the plasmid including the promoter region only. This result supported the involvement of NDM29 in cell proliferation control. To better investigate the mechanisms involved in such effects, we generated permanently-transfected SKNBE2 cell lines constitutively expressing either basal or increased levels of NDM29 RNA: i) mock (M) cells, transfected with the pEGFP-N1 plasmid, express the NDM29 RNA at basal level; ii) Stable 2 (S2) cells express a 2.2-fold higher level; iii) Stable 1 (S1) cells express a 5.4-fold increased level of NDM29 RNA. Analysing these three cell lines, we showed an inverse relationship between the expression of NDM29 RNA and the cell proliferation rate. Indeed, we determined a duplication time of 30 h for M cells, of 39 for S2 cells, and 48 h for S1 cells. Given the well-established correlation between cell proliferation and differentiation, we also measured, in the three cell lines, the expression level of matrix metalloprotease 9 (MMP9) a known differentiation marker of neuroblastoma cells. The S2 cells, presenting a 2.2-fold increased synthesis of NDM29 RNA, showed a 3-fold increased expression of MMP9 with respect to M cells, whereas S1 cells showed a 75-fold increased synthesis of this ncRNA, consistent with a direct correlation between differentiation level and synthesis of NDM29 RNA.

To rule out the possibility that the phenotype of NDM29 over-expressing cells might be the resultant of a selection occurred during the clonal cell line generation, we silenced the expression of NDM29 RNA in transfected cell lines using a NDM29-specific silencing RNA. When we measured the expression of MMP9 in M, S2 and S1 cells silenced with the same construct, we observed that in all the cell types silencing NDM29 expression was accompanied by the reduction of MMP9 synthesis, thus confirming the correlation between the expression of the NDM29 RNA and cell differentiation. On the whole, these findings indicate that a slight increase in NDM29 RNA could delay the cell cycle progression and promote a modest level of cell differentiation, while high levels of NDM29 RNA result in a more significant increase doubling time and differentiation state of the cells. To better investigate changes associated to cell cycle, we determined, by propidium iodide-based FACS analysis, the distribution of the cells in the different cell cycle phases. The slow-cycling cell population (S1) was characterized by the lower percentage of cells in G0-G1 and increased percentage of cells in S Phase compared to the M cell population (mock transfected), suggesting a delayed M phase exit and a prolonged/delayed DNA synthesis. The S2 cell population, characterized by intermediate cell cycle duration, presented a distribution of cells among the different cell cycle phases intermediate between the ones of the S1 and M cell populations. Interestingly, cell morphology in the three cell lines mirrored the changes in the cell cycle. S1 cells were approximately 5-fold bigger than M cells and displayed a neuron-like phenotype characterized by a rich network of neuritic processes. On the contrary, S2 cells presented a much less differentiated phenotype and displayed a smaller number of relatively short neuritic processes.

To monitor the expression of other markers of neuron lineage, such as neurofilament 68 (a marker of NB N-type cells), tyrosinase (characteristic of the S-type neuroblastoma cells) and c-Kit (a marker of the I-type neuroblastoma stem-like cells), we performed Real Time RT-PCR analysis, confirming that the acquisition of neuron-like phenotype by SKNBE2 cells was strictly correlated to the extent of their NDM29 expression. In particular, NDM29 RNA expression resulted in a directly and proportionally increased synthesis of neurofilament 68 and an inversely proportional decreased synthesis of c-Kit (a marker of TICs). These observations suggest the existence of a reduced "stemness potential" in NDM29-overexpressing cells. An additional proof of the acquirement of a neuron-like phenotype by NDM29-overexpressing SKNBE2 cells, came from the measurement of their excitatory properties through the determination of the A-type voltage-dependent current of potassium channels in patch clamp experiments. Inactivation time-course of the outward K^+ current elicited in M and S1 cells could be described by a single exponential curve, detectable in NDM29-transfected but absent in pMock cells. The gaining of the fast inactivation properties of the voltage-dependent potassium channels, promoting the activation of transient component of the A-type current, is a significant part of the functional synapse excitatory properties, thus confirming that the expression of NDM29 in NB cells favors the acquisition of a functional neuron-like phenotype.

Following the preliminary observation that S1 cells adhere to the substrate more efficiently than S2 and M cells, we seeded S1, S2 and M cells in glass chambers slides where, in standard conditions, SKNBE2 cells are poorly adhese. After 24 h culture, cells were stained with DAPI, the nuclei detectable in ten randomly-chosen microscope fields counted, and average number determined for each cell line. Increased cell adhesion was observed in a proportional way with the expression of NDM29 RNA. The increased anchorage-dependent growth for S1 (and to a lesser extent for S2) was confirmed when the three cell populations were seeded on fibronectin- and laminin-coated or uncoated (negative control) dishes. Cells were then centrifuged in upside down position and nuclei stained. Altogether these experiments demonstrate that the expression of NDM29 RNA leads to the acquisition of neuron-like differentiated properties, suggesting a strong reduction of the malignant potential in NDM29-overexpressing cells.

The Synthesis of NDM29 RNA Restricts Neuroblastoma Malignant Potential

Based on specific molecular markers expression level in vitro and on the acquirement of an anchorage-dependent cell growth, we hypothesized a possible restriction of malignant potential of NDM29-overexpressing cells. In order to test this hypothesis we evaluated the clonogenic potential in methylcellulose of cells stably overexpressing different levels of NDM29. We found that the clonogenic potential of NB cells is inversely proportional to the expression of NDM29 RNA (Fig. 7.2). Since we considered that the newly acquired behavior might be accompanied by inhibition of tumor formation capacity in vivo, we verified the possible NDM29-dependent restriction of tumorigenic potential in a mouse model of human neuroblastoma xenografts. As shown in Fig. 7.2b we found that the frequency of tumors formed is indeed inversely proportional to the expression level of NDM29 in xenotransplanted cells. This result pointed toward an oncosoppressive activity of NDM29 RNA. Notably, since the frequency of tumors formed by a population of cancer cells is a direct measure of the number of tumor initiating/cancer stem cells (TICs), this experiment demonstrated that the antitumor effects induced by NDM29 expression targets the TIC fraction of NB cells.

Thus, the most intriguing aspect of the anticancer properties of NDM29 RNA is the capacity to drive differentiation/maturation of cells toward a neuron-like phenotype, previously shown in vitro (see above). We investigated in vivo the differentiative properties of this small RNA, analysing of the cytoarchitecture of the tumor nodules formed by NDM29-overexpressing and control cells, subcutaneously injected in NOD-SCID mice. Our results confirmed that NDM29

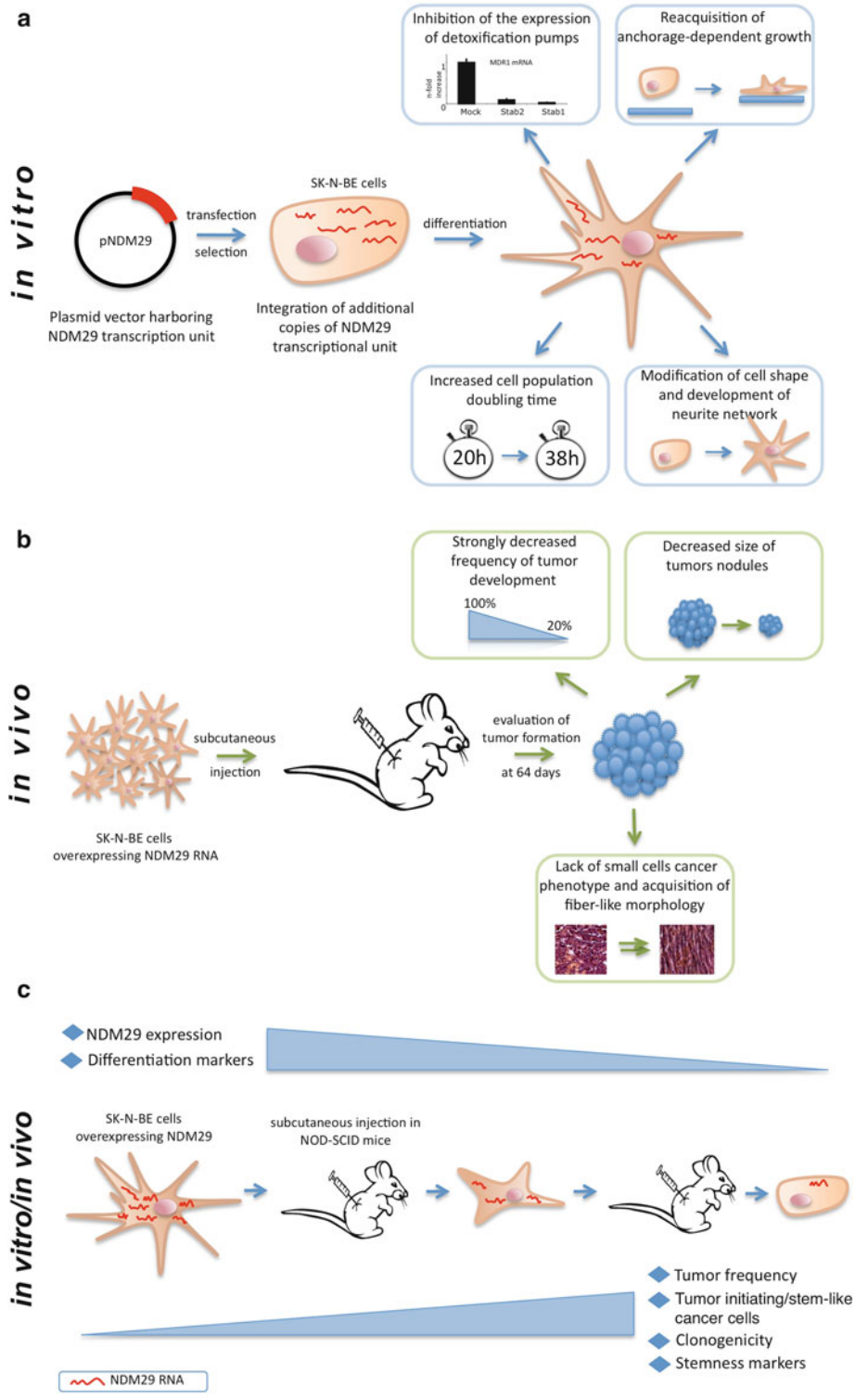

Fig. 7.2 (**a**) NDM29 RNA overexpression leads to neuroblastoma cell differentiation. (**b**) NDM29 RNA restricts neuroblastoma malignancy in vivo. (**c**) Effects of the progressive enrichment of TICs (Tumor Initiating Cells) in NDM29A RNA overexpressing cells

expression influence tumor formation supervising the differentiation stage of cancer nodules. In fact, the nodules formed by NDM29-overexpressing cells (1) express low level of genes associated to stemness traits (c-Kit), (2) strongly synthesize proteins associated to neuron-like differentiated phenotype, and (3) show a dramatic modification of the cell phenotype as they lack small cancer cell, whereas acquire a morphology of well-oriented, large, fiber-like cells (Fig. 7.2). These data thus confirmed in vivo that the antitumor effect associated to the synthesis of NDM29 RNA previously described in vitro is dependent on increased cell differentiation.

To further corroborate this relevant finding, the diminished tumor formation capacity associated to NDM29-overexpression was strengthened by demonstrating the decrease of NDM29 expression, the increase of malignant potential and the inhibition of expression of differentiation markers consequent to the enrichment in tumor initiating/stem-like cells (TICs) in this mouse model (Fig. 7.2c). Therefore, besides the direct correlation between NDM29 expression and NB cells differentiation, these experiments demonstrate in vivo that NB nodules at different maturation stages and with variable malignant potential, can be formed solely depending on the expression level of NDM29 ncRNA. In this context the fact that the expression of NDM29 RNA reflects the differentiation stage of the nodules might help to better understand NB heterogeneity and suggest the determination of its transcription level in tumor masses to predict their malignant potential and, possibly, to select the most appropriate therapy.

NDM29-Dependent Increase of Tumor Susceptibility to Anticancer Drugs

The anticancer effect of NDM29 RNA expression suggests that its controlled expression in tumor masses might be relevant for cancer therapy. Since we observed that NDM29-driven differentiation converts small tumor cells into fiber-like poorly malignant cells, we speculated that the overexpression of NDM29 RNA might also increase the susceptibility to antiblastic treatments by the possible downregulation of MDRs expression. Indeed, this class of proteins is associated to very malignant/stem-like stages of differentiation of cancer cells, in which they contrast the action of anticancer drugs. To test this hypothesis we treated NDM29-overexpressing cells (or the respective controls) with cytotoxic drugs, such as doxorubicin and cis-platinum, to test the potential antiproliferative and cytotoxic effects of these drugs. Using the MTT assay, we showed a clear sensitization to both doxorubicin and cis-platinum cytotoxicity as a consequence of NDM29 RNA synthesis as compared to mock-transfected cells (Fig. 7.3).

Next, we measured the expression level of MDR1 (to which the efflux of cis-platinum and doxorubicin has been associated) in S1, S2 and M cells in order to assess if this different susceptibility has to be ascribed to the lack of detoxification potential evidencing a reduced resistance of S1 cells to both cis-platinum and doxorubicin. This effect was strongly associated to the lower expression of MDR1 in NDM29-overexpressing cells that may prevent the efflux of the drugs causing their intracellular accumulation and cell toxicity. These results were particularly relevant since NDM29-overexpressing, slowly proliferating cells are expected to be more refractory to the action of antimitotic cytostatic drugs such as cis-platinum and doxorubicin. On the contrary, our results clearly show that the expression of NDM29 RNA makes cells more vulnerable to these chemicals through the inhibition of MDR1 expression. In this context, the observation that the synthesis of MDR1 is at the basis of the chemoresistance of TICs and/or cells with stem-like features, suggests to consider the pharmacological induction of NDM29 synthesis as possible treatment for cancer nodules, constituting the starting point for a novel therapeutic approaches to flank the more traditional antiblastic therapies. Indeed, our in vivo results show that the expression of NDM29 RNA exerts its effects also on slowly proliferating TICs that usually are refractory to the antitumor therapy. This observation suggests that the pharmacological induction of NDM29 RNA expression in cancer nodules might be used to increase TIC vulnerability to the anticancer drugs, and the synergic

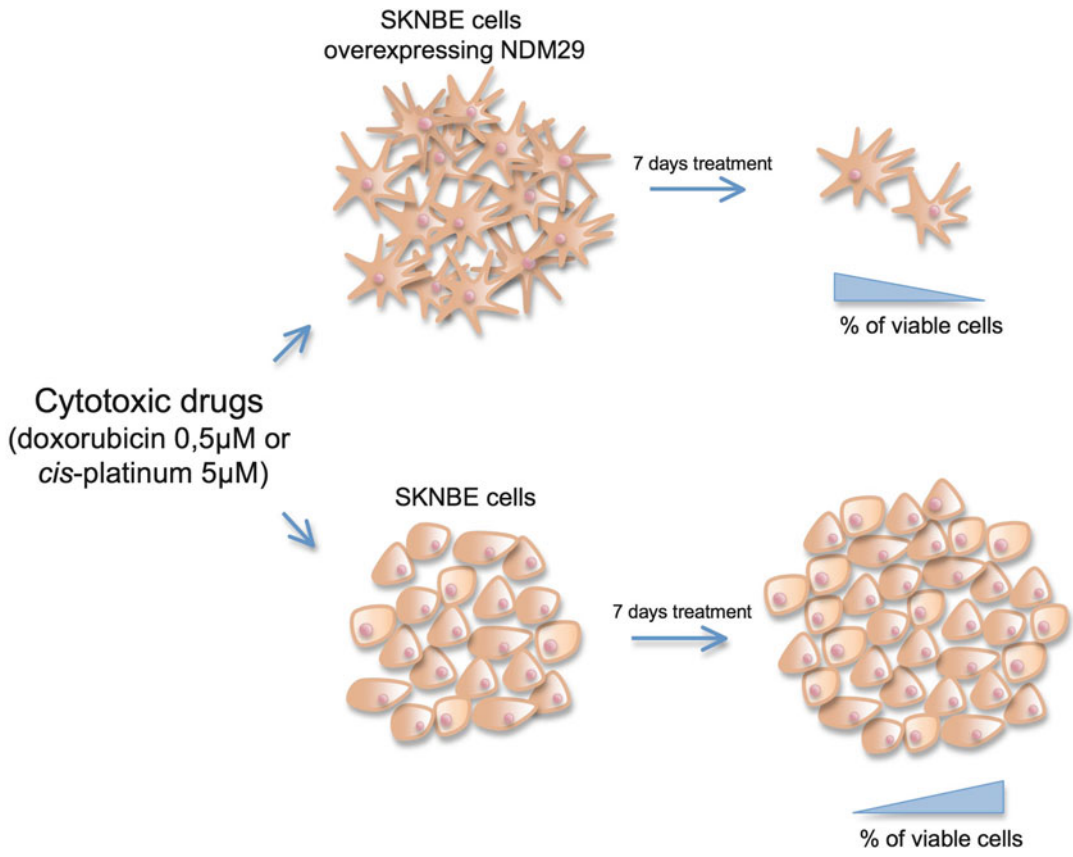

Fig. 7.3 Effects of NDM29 RNA expression on susceptibility of neuroblastoma cells to antiblastic drugs. The experimental setting is based on time course analysis on cells constitutively expressing different levels of NDM29 RNA

administration of traditional cytotoxic drugs could better target all tumor cell types to eventually induce tumor relapse.

In conclusion, in the past few years, the number of newly identified non-protein coding RNAs increased dramatically, although the issue of their transcriptional regulation and biological meaning is still largely underappreciated. However, it is now evident that there are vast, complex populations of transcripts that are short and/or non-polyadenylated. We report the biological role of NDM29, a novel snRNA-like transcription unit whose pol III-dependent transcriptional activity is inversely related to NB cell proliferation. We demonstrated that overexpression of NDM29 induces differentiation of NB cells abolishing their tumorigenic potential. This is the first report demonstrating the differentiative role of a ncRNA and, more importantly that the activation of its synthesis may be used as innovative therapy for highly malignant tumors, opening a completely novel scenario in the therapeutic approach of tumors. Importantly the differentiation induced by NDM29 seems to primarily affect tumor initiating/cancer stem cells, nowadays believed to represent an ineludible drug target to prevent tumor relapses. Thus, treatments able to induce NDM29 expression within cancer cells may be used synergistically with traditional antiblastic therapy to render the elusive cancer stem cells more vulnerable to the anticancer drugs. The second relevant issue coming out from our study is the use of NDM29 expression level for NB staging. In fact NB cell differentiation occur in a NDM29 dose-dependent manner. Thus the measure of NDM29 synthesis in NB explants may be used as prognostic index for this cancer type.

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8 Children with High Risk Neuroblastoma: Prophylactic and Therapeutic Treatment with Docosahexaenoic Acid

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Contents

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Abstract

Despite intense multimodal treatment of neuroblastoma consisting of surgery, chemotherapy, radiotherapy, and stem cell rescue, long-term survival is only 50% in the high-risk group. We therefore need to improve existing treatment protocols and search for new medications.

Inflammation drives cancer growth, and targeted therapy that dampens inflammatory responses is anti-proliferative. The inducible COX-2 enzyme that converts the omega-6 fatty acid arachidonic acid (AA) to various inflammatory prostaglandins is up-regulated in neuroblastoma tissue. Non steroidal anti-inflammatory drugs (NSAIDs) that inhibit prostaglandin synthesis have profound growth inhibitory effects on neuroblastoma cells in preclinical models.

Omega-3 fatty acids oppose the effects of omega-6 fatty acids and have been implicated in cancer treatment and prevention. Omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are precursors of anti-inflammatory compounds. From DHA and EPA resolvins and protectins are produced, which are potent pro-resolving lipid mediators essential for the clearance of inflammatory cells and mediators at an injured site.

This chapter will discuss the toxicity of DHA to neuroblastoma cells both *in vivo* and *in vitro* as well as discuss the effects of DHA in clinical trials of various cancers. *In vivo*, DHA is able to delay time to tumor development

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and reduce tumor growth in neuroblastoma xenograft models. *In vitro*, DHA acts by inducing mitochondrial-dependent apoptosis of neuroblastoma cell lines. In addition, clinical studies show that DHA acts in synergy with chemotherapy.

In summary, this chapter shows that omega-3 fatty acids such as DHA are possible new agents for neuroblastoma prevention and treatment, and suggests that these compounds be tested in clinical trials as adjuvant therapy to chemotherapeutic drugs in children with neuroblastoma.

Introduction

The conventional therapy of neuroblastoma has presently a 70% success rate at best. One of the novel treatment modalities that has been explored concerns fatty acids and their metabolism. Fatty acids, once thought of as solely an energy source in our bodies, have proven to be highly active molecules. They can act as ligands in signal transduction, as transcription factors that regulate protein synthesis, and as membrane components that regulate the fluidity, permeability, and dynamics of cell membranes.

In addition, fatty acids are precursors to a wide range of different lipid mediators that regulate inflammatory responses and metabolic pathways. Most fatty acids can be synthesized in the body, but not all. The essential precursors of all omega-3 fatty acids (linolenic acid; LNA), and of all omega-6 fatty acids (linoleic acid; LA), must be obtained from the diet. Thus dietary habits, and especially intake of fat, affect the body more than just influence weight and waist circumference. Dietary habits affect the system as a whole, evendown to gene-level, and both the amount of fat and the kind of fat we eat can have profound and significant effects on our health.

The overall impact of proper daily fat intake for neuroblastoma patients remains to be investigated, but the data presented here show that it can be of significance. By inhibiting the omega-6 while enhancing the omega-3 metabolic pathways, neuroblastoma growth *in vitro* and *in vivo* is reduced, as shown by *in vitro* studies on human neuroblastoma cells as well as by animal studies. In addition, this chapter proposes possible mechanisms responsible for the observed effects, and suggests how lipid mediators and enzyme inhibitors of the metabolic pathways of interest can augment the effect of cytostatic drugs.

Precursors and Production of Eicosanoids and Docosanoids

"Eicosa" and "docosa" are Greek words meaning 20 and 22, respectively. Eicosanoids and docosanoids are small and short-lived hormone-like molecules formed from fatty acids that regulate numerous processes in the body. They consist of 20 or 22 carbon backbones with various side chains and differently positioned double bonds. The main groups of eicosanoids are prostaglandins (PGs), tromboxanes (TXs), and lipoxins, and the main groups of docosanoids are resolvins and protectins.

The precursors of all eicosanoids and docosanoids are the following polyunsaturated fatty acids (PUFAs): Arachidonic acid (20:4, n-6, AA), Eicosapentaenoic acid (20:5, n-3, EPA), and Docosahexaenoic acid (22:6, n-3, DHA). These fatty acids are in turn the result of desaturation of LA and LNA by Δ5- and Δ6-desaturases, and of elongation by elongases. The omega-6 and the omega-3 fatty acids compete for the same desaturases and elongases, but the omega-3 family members are the preferred substrates. However, because the conversion from LA and LNA is low, PUFAs are best obtained from the diet. Once consumed, they are incorporated into phospholipids of cell membranes and the distribution is tissue-dependent. For example, the nervous system, retina, and testes are especially enriched in DHA, while most other tissues have a surplus of AA. When needed, these fatty acids are released from the cell membrane by the enzyme phospholipase A2 (PLA2). The PLA2-activity is tightly regulated by Ca^{2+} and phosphorylation, and is increased in response to factors such as

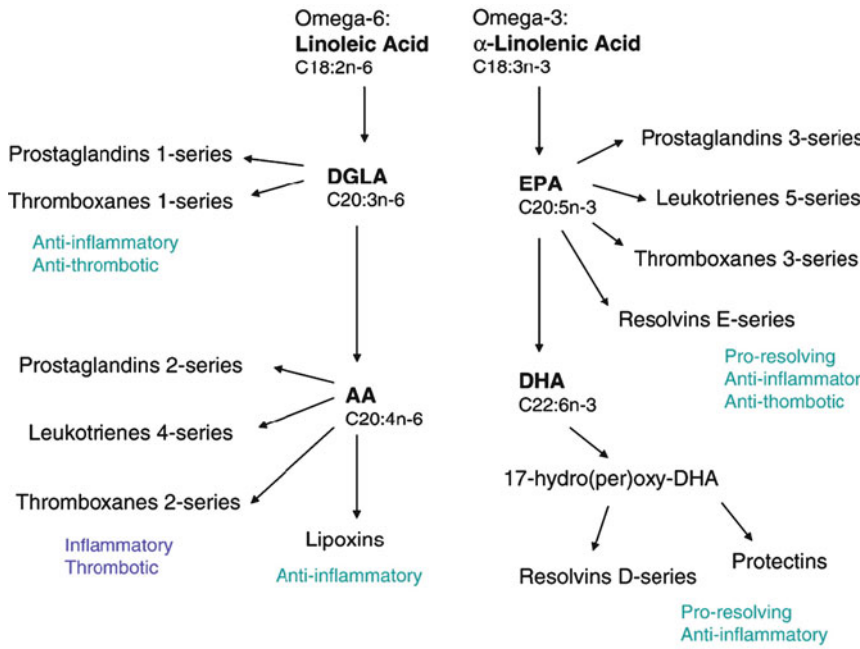

Fig. 8.1 The wide range of compounds formed from metabolism of omega-3 and omega-6 fatty acids and their general effects. Fatty acids give rise to an enormous range of compounds with various effects. The omega-3 and omega-6 fatty acids cannot be synthesized in the human body but must be obtained from the diet. Hence, the balance of the different eicosanoids and docosanoids and the microenvironment that they form are mainly due to dietary habits. Abbreviations: *DGLA* dihomo-γ-linolenic acid, *AA* arachidonic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid

inflammatory stimuli. The free fatty acids are then available for conversion to a panel of different lipid mediators by the enzymes cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 monooxygenase.

AA is converted by COX to PGs of the 2- series and to TXs (collectively termed prostanoids). It can also be converted by LOX to leukotrienes (LTs) of the 4-series, lipoxins, hydroperoxy eicosatetraenoic acids (HpETEs), hydroxyl eicosatetraenoic acids (HETEs), and hepoxilins. Furthermore, AA is converted by cytochrome 450 or cytochrome 450-induced radical oxygen species (ROS) to epoxygenase products (EETs), AA ω/ω-1 hydroxylase products (HETEs), LOX-like products (HETEs), and primary free radical oxidation products (HpETEs) (Biondo et al. 2008). EPA is converted by COX to PGs of the 3-series, and by LOX to LTs of the 5-series, and to lipoxins. LOX can also convert both EPA and DHA to resolvins of the E- and D-series, respectively. Furthermore, LOX can convert DHA to protectins, which will be discussed more in detail below. See Fig. 8.1 for a summary of the metabolic pathways discussed so far.

Eicosanoid Signaling and Biological Effects

Generally, eicosanoids that are formed from the omega-6 fatty acid AA are pro-inflammatory, while eicosanoids and docosanoids formed from the omega-3 fatty acids DHA and EPA are anti-inflammatory. The reality is not as simple as stated here, however; the anti-inflammatory lipoxins that are formed from AA are a good example of an exception to this rule. The role of eicosanoids as inducers and regulators of inflammatory response has been thoroughly

studied for decades. Recently, the docosanoids have been studied more extensively, and it has become evident that resolution of inflammation, a process previously thought to occur passively, is governed by the production of docosanoids—that is, the resolvins (Serhan et al. 2008).

Docosahexaenoic Acid (DHA)

The one essential omega-3 fatty acid is LNA, from which both EPA and DHA are formed. EPA and DHA contain different numbers of double bonds, but the first double bond from the methyl end of the carbon chain is always situated between carbons number three and four. LNA can primarily be found in leafy green vegetables, walnuts, and canola oil. By the enzymes Δ-6-desaturase, Δ-5-desaturase and elongase, LNA is converted to EPA and DHA. However, this conversion only takes place to a limited extent, and occurs more in women than in men. The primary source of EPA and DHA for humans is fatty fish such as salmon, herring, and mackerel. These fish are rich in omega-3 fatty acids because they consume photosynthetic and heterotrophic microalgae of the genus Schizochytrium that produce EPA and DHA, which become increasingly concentrated in organisms as they move up the food chain.

DHA (*all-cis*-docosa-4,7,10,13,16,19-hexaenoic acid) has a chain length of 22 carbons that contains six double bonds, which makes it the longest chain and most unsaturated fatty acid commonly found in biological systems. In the human body, it is either acquired from the diet or it is derived from EPA via docosapentaenoic acid (DPA) as an intermediate; a pathway known as Sprecher's shunt. In humans, DHA is especially enriched in neural tissue. It comprises 40% of the PUFAs in the brain, 60% of the PUFA in the retina, and 50% of the weight of the neurons' plasma membrane. It is esterified into phospholipids embedded in cell membranes, especially of phosphatidylethanolamine (PE) and phosphatidylserine (PS), preferably in sn-2 position (Piomelli et al. 2007).

In membranes containing DHA, the packing is distorted by steric restrictions associated with the presence of multiple rigid double bonds; that is, the bent shape of this fatty acid prevents a perfect fit in the membrane. This is thought to significantly alter many basic membrane properties including acyl chain order and fluidity, phase behavior, elastic compressibility, permeability, fusion, flip-flop, and protein activity (Stillwell and Wassall 2003). Once taken up by cells or released from the cell membrane, DHA acts as a ligand to certain nuclear receptors, such as the PPAR-γ (Gani and Sylte 2008) and the RXR receptor (Lengqvist et al. 2004). In conjunction with these receptors that act as transcription factors, DHA helps regulate various biological functions ranging from lipid metabolism and homeostasis to cell differentiation and cell death (Berquin et al. 2008). Many of the receptor-mediated effects of DHA are still unexplored.

In addition, DHA has been shown to influence signal transduction of a variety of pathways. For example, DHA activates the Jak/Stat pathway; downregulates protein kinase C, Ras, ERK and NF-κB; sustains phosphorylation of EGFR; and influences the Bcl-2 family of proteins regulating cell growth (Berquin et al 2008). Furthermore, DHA can modulate the translation machinery by reducing intracellular Ca^{2+} stores (Jude et al. 2006).

Areas of Application of Docosahexaenoic Acid

In 1970 the pioneers of omega-3 fatty acid research, Dr. Dyerberg and Dr. Bang from Denmark, visited Greenland on an expedition to understand how the Inuits could eat a high-fat diet and still have one of the lowest death rates from cardiovascular disease in the world. Their discovery that the Inuits had favorable blood lipids resulted in a publication in *Lancet* in 1971 (Bang et al. 1971). Not until some years later had Dr. Dyerberg and Dr. Bang analyzed all blood samples on an old gas chromatogram and found two fatty acids, DHA and EPA. This was the birth of omega-3 fatty acid research. Since then, mainly through dietary studies, DHA has been associated in beneficial ways with an enormous range of human afflictions including cancer, heart disease, rheumatoid arthritis, asthma, lupus, alcoholism, visual acuity, kidney

disease, respiratory disease, peroxisomal disorders (Zellweger's Syndrome), dermatitis, psoriasis, cystic fibrosis, schizophrenia, depression, neurologic and brain development, malaria, multiple sclerosis, and even migraine headaches. In fact, it is difficult to find any human disorder where omega-3 fatty acids have not been tested.

The common denominator that might explain the beneficial effects of omega-3s and DHA in particular in this great variety of diseases and symptoms is its anti-inflammatory properties. Until recently, it was unknown how DHA exerted these anti-inflammatory effects. One postulated reason is that DHA replaces AA in cellular membranes, and hence less AA is available for conversion by COX and LOX to pro-inflammatory eicosanoids. Furthermore, DHA competes with AA for binding sites on the COX enzyme, and is actually the preferred substrate. These indirect mechanisms for inhibiting inflammatory responses seem reasonable and have proven to be correct, but a huge step was taken towards understanding DHA's beneficial effects when resolvins and protectins were identified Serhan et al. (2002).

Resolvins of the D-series are produced from DHA by the enzymes 5-LOX and 15-LOX, or by a COX-enzyme that has been acetylated by aspirin. Protectins are also formed via LOX-mediated pathways. These lipid mediators, and also resolvins of the E-series (EPA-derived), powerfully clear inflammation by clearing neutrophils and macrophages from inflammatory sites. Actually, they are essential for resolution of an inflammatory response, a process that was formerly believed to occur passively (Serhan et al. 2008). These newly discovered substances are currently in clinical testing (as reported by Resolvyx Pharmaceuticals at www.resolvyx.com). However, many of the mechanisms behind the positive effects observed by DHA and EPA are still elusive.

Docosahexaenoic Acid in Cancer Prevention

What we known about the role of omega-3 fatty acids in cancer development is almost exclusively based on epidemiological observations. There are many challenges in interpreting data of this sort due to heterogeneity in study design and the fact that subjective dietary questionnaires often are used instead of biomarkers. However, a small to moderate reduction in cancer risk, or no effect, is most often the result of such studies.

An observational study that supports the theory of cancer prevention in children by omega-3 fatty acids was carried out among the native Inuit population of Alaska (Lanier et al. 2003). In this study, childhood cancer incidence was analyzed from 1969 to 1996, and apart from the increased number of hepatocellular carcinomas in this population due to Hepatitis B infection (a phenomenon that disappeared after initiation of a vaccination program), the rate of childhood cancer was significantly lower compared to a North American population. Specifically, the incidence of neuroblastoma was reduced tenfold (0.9/million *vs* 7,9/million). The Inuit population of Alaska pursue a lifestyle where fish and seal meat are the main commodities, and their DHA levels are several-fold higher than in Caucasians, where the omega-3/omega-6 ratio has dropped dramatically over the past decades (Simopoulos 2006).

In the Japanese population, whose traditional diet includes much fish, the incidence of certain cancers such as breast cancer has increased along with a more "westernized" food consumption and lifestyle. Since this observation was made, several studies have pointed out that omega-3 fatty acid consumption is associated with decreased cancer risk of the breast, prostate, colon and kidneys as summarized by (Berquin et al. 2008).

There is a large, randomized, double-blind and placebo-controlled study ongoing called VITAL (VITaminD and OmegA-3Trial (Manson et al. 2011). This study aims to investigate DHA, EPA (Omacor® fish oil, 1 g/day), and Vitamin D (cholecalciferol, 2,000 IU/day) in the primary prevention of cancer and cardiovascular disease among 20,000 US citizens aged over 50 years. The treatment period will be 5 years. The study is one of the first and largest studies where cancer prevention will be studied by intervention and not by observation only. We await the results with anticipation.

Animal studies on DHA supplementation as cancer prevention have shown that a DHA-enriched or fish oil-enriched diet can inhibit the formation of not only neuroblastoma (Gleissman et al. 2010b; Barnes et al. 2012), but also papillomas, mammary carcinogenesis, carcinogenesis of the large and small intestine, and carcinogenesis of the lung. DHA-enriched diets can also reduce formation of aberrant crypt foci, metastatic colon cancer carcinoma, sarcoma, and prostate cancer.

The Fat-1 transgenic mouse model provides strong evidence that DHA and DHA-derived compounds may have significance in cancer development (Kang et al. 2004). These mice carry a gene which encodes a desaturase that catalyzes conversion of omega-6 to omega-3 fatty acids, a feature that is lacking in most mammals, including humans. In this mouse model where the omega-3/omega-6 ratio is increased, melanoma formation and growth, colitis-associated colon cancer growth, prostate cancer growth and breast cancer growth were all reduced compared to tumor growth in non-transgenic animals.

Docosahexaenoic Acid in Cancer Therapy

For obvious ethic reasons, DHA is never given as single therapy to humans with a tumor burden, but is combined with adequate cytostatic drugs. The use of DHA as an adjuvant to conventional therapy has proved to be efficient. DHA can potentiate the anticancer effects of both chemo- and radiotherapy. (The mechanisms behind the observed synergistic effects are discussed in the next section.)

One therapeutic study in breast cancer patients where DHA was combined with epirubicine, cyclophosphamide, and 5-fluorouracil emphasizes that an inter-individual uptake and incorporation of DHA alters the treatment response (Bougnoux et al. 2009). Patients were supplemented with DHA daily during the chemotherapy cycles and could then be divided into high and low incorporating groups based on the DHA levels in plasma and red blood cells. The high incorporating group was characterized by longer overall survival and delayed time to tumor progression compared to the low incorporating group.

In another study, non small cell lung cancer (NSCLC) patients were given DHA and EPA together with their first-line therapy (platinum-based regimens such as carboplatin in combination with vinorelbine or gemcitabine). Patients in the supplemented group had an increased response rate, greater clinical benefit and greater 1-year survival compared with the control group (Murphy et al. 2011).

In some settings, supplementation with DHA may not alter treatment response, but can still be beneficial since it improves the nutritional status and quality of life of patients. DHA sometimes even reduces unwanted side-effects of conventional treatment. In a study on NSCLC patients the supplemented group reported significantly higher on the quality of life parameters, physical and cognitive function, global health status and social function than the control group. The intervention group showed a higher Karnofsky Performance Status and tended to have a higher physical activity (van der Meij et al. 2012).

The aim of another study on patients with lung cancer was to investigate the effect of EPA and DHA on inflammatory condition, and oxidative and nutritional status. A significant increase of body weight in the supplemented group was observed. Levels of inflammatory biomarkers differed significantly between the supplemented and placebo groups and progressively decreased during chemotherapy in the supplemented group, evidencing anti-inflammatory action. Concerning oxidative status, plasma reactive oxygen species levels increased in the placebo group. These anti-inflammatory and anti-oxidative actions could be considered a preliminary goal in anti-cachectic therapy, as cachexia is a common problem among cancer patients (Finocchiaro et al. 2011).

There are currently more than 30 ongoing clinical trials in the USA where DHA or omega-3s are being tested for cancer prevention, support, or therapy (as reported by National Cancer Institute at http://www.cancer.gov/clinicaltrials). As single therapy, DHA has been given to animals

in three different studies. In two, the animals were xenografted with SK-N-BE(2) or SK-N-SH neuroblastoma cells (Gleissman et al. 2010b; Barnes et al. 2012), and in the other with BxPC-3 pancreatic cancer cells. In the latter study, DHA was also combined with curcumin, a dual COX-2 and 5-LOX inhibitor. Tumor growth was inhibited by DHA as single therapy, and the inhibitory effect increased with the combination of DHA and curcumin. DHA as singel therapy does have effect on tumor burden, but is not efficient enough to be used as such.

Mechanisms of Action of Docosahexaenoic Acid in Cancer Cells

Apoptosis/Autophagy

DHA induces dose-dependent apoptosis of cancer cells (Lindskog et al. 2006; Gleissman et al. 2009). Serini et al. (2009) have reviewed several suggestions of mechanisms that seek to explain this phenomenon, including both the intrinsic and the extrinsic pathways. DHA modifies the expression of proteins of the Bcl-2 family by increasing the levels of the pro-apoptotic proteins Bak and Bcl-xS and reducing those of the anti-apoptotic proteins Bcl-2 and Bcl-xL (Manna et al. 2008). DHA induces cytochrome *c* release from mitochondria and mitochondrial membrane depolarization. DHA causes downregulation of Wnt/Beta-catenin signalling and inhibits syndecan-1 of the MEK-Erk pathway. Furthermore, DHA induces autophagy through p53/AMPK/mTOR signalling.

Oxidative Stress

The initial event in the oxidative metabolization of all PUFAs is abstraction of hydrogen. This occurs at an increased rate when the internal redox balance of a cell is seriously disturbed and the production of initiators, such as radical oxygen species (ROS), cannot be sufficiently suppressed, a common scenario in tumorcells. The most common ROS are hydroxyl radicals, superoxide radicals, alkoxyl radicals, peroxyl radicals, singlet oxygen, ozone, anions, and hydrogen peroxide.

Intracellular accumulation of ROS leads to disruption of the mitochondrial membrane potential, to release of cytochrome *c* with consecutive activation of the caspase cascade, and, ultimately, to programmed cell death through apoptosis as discussed above. The glutathione (GSH) system (GSSG/2GSH) is considered to play a central role in maintaining cellular redox balance by scavenging radicals formed by oxidation. Since DHA is a highly unsaturated PUFA, it is susceptible to peroxidation and can cause accumulation of a surplus of ROS that cannot be scavenged by the cancer cells. Addition of anti-oxidants to cells incubated with DHA diminishes the toxic effects, strengthening this theory (Lindskog et al. 2006).

Potentiation of Cytostatic Drugs

The effect of combined treatment of DHA with cytotoxic drugs or radiation seems to be a potential way to clinically apply DHA in cancer treatment. DHA in combination with doxorubicin, irinotecan, cisplatin, melphalan and vincristine on neuroblastoma cell survival shows additive or synergistic interactions (Lindskog et al. 2006). A few different mechanisms whereby DHA enhances the effects of chemotherapeutic drugs have been suggested and summarized (Biondo et al. 2008; Siddiqui et al. 2011) and include:

- DHA acts on membrane-associated signal transduction, such as decreased Ras-, PI3K/AKT- and Her-2/neu- signaling, and changes lipid raft composition
- DHA-peroxidation stimulates formation of oxygen free radicals
- DHA inhibits chemotherapy-induced NF-κB activation
- DHA enhances drug uptake by altering membrane properties and decreasing production of MDR proteins
- DHA induces apoptosis by modulating the effects of pro- and anti-apoptotic proteins in the Bcl-2 family of proteins
- DHA affects several other intracellular targets including cyclooxygenase-2, peroxisome

proliferator-activated receptor gamma, mitogen-activated protein kinase, and AKT.

Inhibition of COX-2 and PGE_2

DHA is incorporated into cell membranes at the expense of AA, which leads to less formation of AA-derived PGE_2, a lipid mediator that has been shown to drive tumor growth. Several studies have demonstrated that supplementation of omega-3 PUFA to cells, animals, and humans reduces the AA-derived eicosanoids.

COX-2 and microsomal PGE synthase 1 (mPGES-1), the enzymes responsible for converting AA to PGE_2, are highly expressed in neural tumors and inhibition of these enzymes has profound effects on the survival of these tumors (Baryawno et al. 2008). The effect of combining DHA with the COX-2 specific inhibitor celecoxib in neuroblastoma cells and other various human cell lines show that these compounds induce synergistic cytotoxicity. Mechanisms seem to be both COX-2-dependent, such as blockage of COX-2 and inhibition of AA metabolism, and COX-2 independent, such as induction of heat shock proteins and modulation of NF-kB activity and steroid receptors. In addition, DHA may also induce cytotoxicity by binding to catalytic sites of elongases and desaturases (Larsson et al. 2004).

Cytotoxic Intermediates of DHA Metabolism

When DHA is converted to resolvins and protectins, two specific intermediates are formed, namely 17-hydroperoxy-DHA(17-HpDHA) and 17-hydroxy-DHA (17-HDHA). When comparing DHA, 17-HpDHA and 17-HDHA with respect to tumor cell toxicity, 17-HpDHA displays the highest cytotoxic potency. Furthermore, DHA and 17-HpDHA, but not 17-DHA, induces apoptosis in neuroblastoma cells (Gleissman et al. 2009). Hence, DHA probably has the capacity to induce cytotoxicity in neuroblastoma cells by the intracellular formation of hydroperoxy fatty acids as described above and as hypothesized by Siddiqui et al. (2008) in a review on DHA's many oxidation products.

If a non-stereospecific DHA-derived hydroperoxy fatty acid is exogenously supplied to neuroblastoma cells it causes apoptosis through several apoptotic hallmarks including nuclei condensation, DNA fragmentation, poly- (ADP-ribose) polymerase cleavage, and increased activity of caspase-3. In addition hydroperoxy fatty acids cause release of cytochrome *c*, increased Bcl-2 expression, and attenuation of mitochondrial membrane potential. These data indicate that DHA hydroperoxides induce apoptosis in human neuroblastoma cells, which is be mediated by the mitochondrial (intrinsic) pathway.

Adhesion and Angiogenesis

Two of the first steps required for tumor establishment are adhesion and angiogenesis. DHA can inhibit adhesion, probably by down-regulating Rho GTPase, inhibiting cytoskeleton reorganization, and reducing ICAM-1 and VCAM-1 protein expression. DHA has also been shown to decrease TNFα-induced monocyte rolling, adhesion, and transmigration. These effects might also be applicable to tumor cells in the process of adhering to tissue sites. Furthermore, DHA has been shown to reduce angiogenesis, probably by decreasing levels of vascular endothelial growth factor, platelet-derived growth factor, and platelet-derived endothelial cell growth factor (Victory et al. 2007).

Metastatic Spread

In a laboratory setting, DHA inhibits invasion through matrigel of both urinary bladder and pancreatic cancer cells. It does so by down-modulation of Granzyme B, a serine proteinase with extracellular functions that promote invasion (D'Eliseo et al. 2011).

In a mouse model of human breast cancer cell metastasis to bone, it has been shown that a diet enriched in DHA and EPA prevents the formation of osteolytic lesions in bone, indicating suppression of cancercellmetastasis to bone. DHA and EPA also significantly attenuate the migration/invasion of breast cancer cells in culture. One proposed mechanism is that DHA and EPA significantly inhibit the expression of CD44 protein and mRNA by a transcriptional mechanism. Aberrant increased expression of CD44 is associated with generation of cancer stem cells, which contribute to metastasis of breast cancer cells. Furthermore, in the mice fed with a fish oil diet, the levels of CD44 mRNA and protein in the tumors where reduced (Mandal et al. 2010).

Can Docosahexaenoic Acid Be Given to Children with Neuroblastoma?

The information on pharmacokinetics of DHA in children is limited. However, DHA supplementation to children has been done in different studies with doses up to several grams per day, resulting in increased plasma levels and without much adverse side effects. Some studies report but a few gastrointestinal problems such as diarrhea after high doses of DHA. This problem is adjustable by dose-titration. The fear of bleeding due to DHA-supplementation seems to be uncalled-for. DHA does increase bleeding time, but not to an extent that has any clinical implication. DHA supplementation effectively alters plasma lipid composition in children, an important prerequisite for any attempt to validate these experimental findings in children with neuroblastoma (Lien 2009).

In addition to being toxic to cancer cells, DHA can protect healthy nervous tissue via the downstream products protectins (Farooqui 2012). This is of particular interest when treating cancers of the nervous system such as neuroblastoma and also medulloblastoma, another childhood cancer of the nervous system with poor outcome. In this case, DHA may work as both a sword and a shield (Gleissman et al. 2010a), which would be highly useful since treatment often gives severe sequele.

Summary

DHA is a safe compound that exerts anti-proliferative effects on cancer cells and works in synergy with chemotherapeutic drugs. It is easy to administer and monitor. We therefore suggest that DHA should be evaluated as an adjuvant to chemotherapy during treatment of neuroblastoma in clinical trials. In some settings, DHA may increase treatment efficacy by increased tumor cell killing and prolonged survival of patients. DHA may also reverse adverse side effects and malnutrition, which could allow intensified or prolonged treatment if necessary. In other settings DHA may be inert, but as far as studies have shown it will not counteract current conventional treatment.

Epidemiological observations show that DHA-intake may reduce neuroblastoma incidence as well as other cancer forms. This is of high interest when it comes to nutrition in early childhood and pregnant women. Because DHA acts anti-inflammatory and pro-resolving via resolvins, the aspect of cancer-associated inflammation is highly interesting. Other anti-inflammatory agents used in cancer treatment, such as COX-2 inhibitors that have been reported to increase the risk of severe side-effects, might be replaced by these newly identified lipid mediators. Resolvins work as agonists of resolution instead of antagonists of inflammation, which indicates that less toxic side effects due to inhibition of prostaglandin and thromboxane synthesis can be expected.

In conclusion, we are beginning to understand the physiology and molecular basis of omega-3 fatty acids and thereby gradually encompass the full clinical potential of these compounds. Hopefully, this understanding can quickly pass on into the clinic, and possibly save lives and enhance quality of life for numerous neuroblastoma patients.

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Part II

Medulloblastoma

Overview of Treatment of Pediatric Medulloblastoma

9

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Contents

Abstract

Medulloblastoma is the most common brain tumor in children. Its current treatment consists of surgery, irradiaton, and chemotherapy with their significant short term and long term toxicity. Intensity of current therapy, determined by clinical risk stratification of the tumor (age of patient, extent of tumor resection and presence of metastasis), results in a markedly different overall survival rate depending on different risk groups (average-, high-risk and young patients). The potentials of present therapeutic approach seems to have reached its plateau. Recently, a new molecular stratification of medulloblastoma was introduced defining four different subgroups based on the affected molecular pathway in tumorigenesis of medulloblastoma (SHH, Wnt, Group C, and D). This new classification presumably will open new possibilities of individualized therapy and application of new drugs, based on the important molecular alteration of individual medulloblastoma cases. Here we summarize the current therapeutical approaches and existing new therapeutic attempts. Although there already are several new experimental attempts to treat medulloblastoma beyond conventional therapy, a really effective new therapy is still to be discovered.

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Introduction

The survival rates of malignant diseases improved rapidly during the second half of the last century. More than 70% of children with malignant diseases are cured by combination of surgery, radiotherapy, and chemotherapy. However, the survival of children with brain tumors increased only slightly inspite of the development of advanced neurosurgery and radiotherapy. This review discusses the present immuno-, chemo- and molecular therapy of pediatric medulloblastoma.

Incidence

Tumors of the central nervous system (CNS) are the second most common childhood malignant diseases after hematological malignancies. The incidence is similar in different developed countries: in Europe it is 26 per million, and in the USA it is 32.4 per million. However, the highest incidence is registered in Hungary and the Scandinavian countries. In Hungary the incidence was 37.41 per million between 1999 and 2008 (data of the Hungarian Pediatric Cancer Registry). Medulloblastomas comprise ~ 20% of all primary CNS tumors occurring in patients younger than 18 years of age, although the tumor may rarely occur in older patients. In childhood, medulloblastomas have a bimodal distribution, peaking at 3–4 years of age, and then again between 8 and 9 years of age (Packer et al. 1999).

Staging and Prognostic Factors

Historically, first Chang's clinical staging system of medulloblastoma was based on the size of the tumor and presence of metastases (brain, spinal fluid, spinal subarachnoid space and outside of the cerebrospinal axis) used. Later, it was modified; age of patient, histology of primary tumor and extent of surgical ablation of the tumors, confirmed by early postoperative MR examination were proved to be also important prognostic factors. Younger age, <3 years, large/anaplastic histology and residual tumor after surgery were shown to be unfavourable prognostic factors (Packer et al. 1999). However, there are controversial data regarding the significance of postsurgical residual tumor.

Behaviour of tumors depends on molecular biology of the tumor cells. As the biology of medulloblastoma is being understood in more detail, new risk factors can also be taken into account to predict the prognosis more precisely. In the past several genetic and molecular factors were described to have an independent prognostic role (deletion of chromosome 6q, 17p, overexpression or mutation of ERBB2, PDGFR, RAS/MAPK, MYCC, MYCN, trkC receptor, γ and β cathenin and p53) (Gottardo and Gajjar 2008). However, these results occasionally turn out to be rather conflicting. They presumably will loose their importance in the light of more recent molecular approaches.

Presently, the most complete examination of the biologic alterations in brain tumors is the RNA microarray analysis. These procedures differentiated four distinct molecular subgroups with aberrantly regulated signaling pathways: Wnt-group, SHH-group, group C, and group D. According to recent data, Wnt- and SHH-group tumors have a favourable outcome. Group C and D are characterized by the overrepresentation of pathways involved in neuronal development and point to a worse prognosis. However, further examinations are needed to confirm these observations. An advantage of this subgrouping system is that these subgroups consisting of genetically, clinically, and transcriptional distinct variants can be distinguished by immunohistochemical methods. Group Wnt, SHH, C, and D are characterized by the presence of CTNNB1, GLI1, NPR3, and KCNA1 proteins respectively by immunohistochemistry (Taylor et al. 2011). This makes it widely applicable in clinical laboratories. It may help not only in more reliable classification of patients into appropriate risk groups and decide the intensity of chemotherapy, but also in selecting patients for targeted molecular therapy. Recently, new markers were described within these groups. FSTL-5 is a marker of poor prognosis in non-Wnt/non-SHH medulloblastoma.

Its negativity delineates a subgroup with good prognosis in this otherwise poor prognostic group (Remke et al. 2011). It is possible that further molecular characters will be revealed and the four groups will be extended by new subclusters, helping to make a more exact prognosis. However, the biologic (molecular) stratification cannot overcome the problem, because medulloblastomas are heterogeneous tumors constantly evolving: different mutations appear and compete for the greatest selective advantage. Hence, the targeted, selective inhibitors may ablate only one subpopulation of cells. However, the targeted therapy combined with chemotherapy may improve the presently stagnating survival rates.

Chemotherapy

General Considerations

The pioneer of the adjuvant chemotherapy in medulloblastoma, which is the most frequent brain tumor in childhood, was H. J. Bloom. The first results which proved the better survival with adjuvant chemotherapy after surgery and radiotherapy, than surgery or radiotherapy alone were published by international, randomized study of SIOP (International Society of Pediatric Oncology) and CCG (Children's Cancer Study Group) (Gottardo and Gajjar 2008). More drugs and/or higher doses resulted in better survival; however, toxicity of treatment and late side effects also increased.

The chemotherapy schedules of primary therapy for medulloblastoma used worldwide apply similar groups of drugs (alkylating agents, platina analogues, topoisomerase inhibitors, vinca alkaloid, folate antagonist) with varying agenda, doses, and combination. Initially, vincristine and the nitrosoureas were applied, which are still used in several protocols for the treatment of medulloblastoma. The usefulness of vincristine was recently challenged, as vincristine could not be detected in the spinal fluid in measurable concentrations after 1.5 mg/m^2 i.v., bolus injection (Kellie et al. 2002). However, it is included in most protocols up to now, especially as concomitant therapy during irradiation. Some protocols apply ifosfamide alternating with cyclophosphamide (Kortmann et al. 2000). Application of methotrexate is limited, not only because of its potential side effects, but also because it can be used only before radiotherapy. Some protocols described rather good results with this drug when irradiation was not applied. The alkylating oral drug dibromdulcitol was an excellent substance for the treatment of medulloblastomas because of its favourable pharmacokinetic properties in the spinal fluid and its tolerable toxicity. Later, in spite of good clinical results the drug became unavailable. This seemed to be partially substituted by temozolomide. However, in recent studies of limited cohort of patients with relapsed tumor, application of temozolomide did not prove its superiority to other regimens (Bartels et al. 2011).

Optimal timing of adjuvant chemotherapy compared to time of irradiation was also examined. Administration of chemotherapy before irradiation was found to be less effective, than chemotherapy following irradiation (Kortmann et al. 2000). In some trials preoperative chemotherapy was administered to patients with large tumor without histological confirmation or after biopsy only. The aim was to decrease the tumor size, making possible its total ablation. The feasibility and safety of the preoperative chemotherapy was proved by pilot trials; however, it is not applied in wider chemotherapeutic trials (Schuler et al. 1993). An important obstacle of the chemotherapy is the blood-brain barrier despite the fact that the tumour breaks through the barrier when growing. There were several trials for increasing the possibility of passing the barrier but without any provable result.

Intrathecal Therapy

Beyond conventional intravenous or oral application of chemotherapeutic agents, direct intrathecal or intraventricular administration is also important in medulloblastoma. By this route drug concentration in CSF is higher than that by intravenous application only, as we find it with methotrexate.

In an overview by Conroy et al. (2010) 126 candidate drugs were examined concerning their possible intratechal administration, 99 were immediately rejected due to their irritant nature, neurotoxicity, overt lack of tumor specific activity or the need of their previous enzymatic activation in the liver. Surprisingly, methotrexate, a widely used drug in medulloblastoma, based on authors' criteria was also rejected due to its overt neurotoxicity. 12 drugs were found to be possible candidates for further examination (only Ara-C is currently in use).

Chemotherapy and Risk Stratification

To avoid the unnecessarily intensive chemotherapy staging of tumors is essential. Aggressiveness of therapy depends on the age of the patient (less than 3 years of age regarded as young patient), extent of tumor resection (residual tumor is less than 1.5 cm^2 in early, within 72-h postoperative MRI regarded as completely resected tumor), metastatic pattern of the tumor and whether the tumor is primary or recurrent. Patients with localized completely resected disease older than 3 years are regarded as average risk patient. Patients older than 3 years, with incomplete resection or presence of metastasis are high risk patients. Patients, less than 3 years old, at whom radiotherapy is usually not applied due its serious side effects on developing brain, compose the group of young patients.

Common elements of the therapy are the repeated, different combination of several drugs, along with parallel intrathecal administration. In case of high risk patients, or where radiotherapy could not be applied, high dose chemotherapy with stem cell rescue is also applied in several treatment schedules to achieve a better survival. Maximal survival resulted by chemotherapy was achieved by application of high dose chemotherapy with stem cell rescue, albeit this better survival costed higher price in term of short and long term possible serious side effects. Rationality of longer duration of less intensive therapy, like maintenance therapy is clear, however, its benefit in practice is not yet proved.

Aim of chemotherapy is slightly different in average risk, high risk, very young and recurrent patients. At average risk patients, where chemotherapy adds less survival benefit beyond surgical resection and irradiation, its present application aims not only to achieve a better survival, but try to decrease the dose of irradiation, to avoid its serious side effects on cognitive and hormonal functions. At high risk patients, with incomplete surgical removal and/or CNS metastases, the chemotherapy has a very important additional role, achieving highly significant survival benefit by its more aggressive application with high dose chemotherapy and stem cell rescue. At very young patients irradiation could not be applied at all or only locally in a reduced dose. Therefore, aggressive chemotherapy should substitute irradiation completely or help patients to reach the appropriate age for irradiation without early progression or recurrence. In patients with recurrence application of chemotherapy is hindered by usually highly exhausted bone marrow function and questioned by poor survival rate despite aggressive treatment. The latter suggests not to use any kind of therapy, which might result in pointless long term hospitalization with decreased quality of life.

Due to these concerns chemotherapy will be discussed by risk groups.

Average Risk Patients

In medulloblastoma of average risk patients, older than 3 years, with localized disease with no residual tumor after surgery the best therapeutic results are achieved with the least aggressive treatment. In these cases primary treatment was posterior fossa booster (54 Gy) and cerebrospinal irradiation (36 Gy). However, due to its side effects (neurocognitive, hormonal), dose of craniospinal irradiation was attempted to reduce (24 Gy). Although the side effects remarkably decreased, the survival rate also diminished (Thomas et al. 2000). The first significant step in decreasing irradiation was adding of postirradiation chemotherapy, consisting of vincristine, cisplatin, lomustine. Due to its success, it became a

gold standard in average risk patients. PNET III trial also proved the superiority of additional chemotherapy with different drug combinations: irradiation alone was compared to irradiation plus combined chemotherapy (vincristine, carboplatin, cyclophosphamide and etoposide). Five-year EFS were 60% vs 75% respectively (Taylor et al. 2003). Different timing of irradiation was also examined by application of preirradiation chemotherapy. HIT 91 German trial, although the different inclusion criteria (M1 patients were also included) partially biased the results, showed that administration of preirradiation chemotherapy, postponing timing of irradiation results inferior survival rate (5-year EFS: 78% vs 65%) (Kortmann et al. 2000).

Best results (more than 80%) of chemotherapy were achieved by vincristine, CCNU, cisplatin (German HIT) or high-dose chemotherapy with 4-times repeated high-dose cyclophosphamide, cisplatin, and vincristine and stem cell rescue (St Jude Medulloblastoma 96). 5-year EFS were 84% and 83% respectively (Kortmann et al. 2000; Gajjar et al. 2006). Probably, these are the plateau that by chemotherapeutic drugs could be reached. Further improvement could only be expected by the co-administration of new molecularly targeted drugs.

Young Patients

One of the most problematic groups is of the young patients less than 3 years of age. In this group radiotherapy should be omitted in its original dose due to its serious side effects on neurocognitive, hormonal and motoric functions. To overcome this problem several different approaches were tried in different study groups. The rational was to avoid irradiation as long as possible. Some studies clearly aimed to gain time with less aggressive therapy, and in case of progression or relapse a highly intensive therapy was started to provide them long term cure later. Other studies administered more intensified chemotherapy from the beginning. Therefore, in this age group to compare effectiveness of the studies, overall survival (OS) but not event free survival (EFS) should be regarded. In addition, the superiority of a study is also highly determined by its acute toxicity and long term CNS side effects (e.g., high dose methotrexate v.s. irradiation).

One of these therapeutic approaches administers systemic intensive chemotherapy followed by high dose chemotherapy with stem cell rescue. Irradiation was applied only in relapsed patients. This is the method of US groups (Head Start I-II). Head Start I and II consisted of repeated administration of vincristine, cisplatin, cyclophosphamide and etoposide followed by high dose chemotherapy using carboplatin, thiotepa and etoposide and stem cell rescue. Overall survival was 70%. Irradiation could be completely omitted at localized completely resected patients in 71%. However, the limitation of this therapy was its relatively high toxic death rate (19%) (Dhall et al. 2008).

In a different approach, a French Group (BB SFOP) gives slightly toxic, somehow regarded as maintenance chemotherapy for 16 months. Aggressive chemotherapy, like high dose chemotherapy with stem cell rescue, was started only in case of relapse or progression. 5-year OS and EFS of patients with localized non-residual disease were 73%, and 29%. Hence, we can conclude that in more than 40% of cases second line therapy had to be started with high efficacy. 5-year OS of metastatic patients with French approach was very poor, 13% (Grill et al. 2005).

The German HIT study group chose another approach by 6 months administration of high dose methotrexate (5 g/m^2) with concomitant intraventricular methotrexate. High dose chemotherapy with stem cell rescue was not applied. Their method was more toxic to the developing brain than other chemotherapeutic approaches, but less toxic compared to irradiation. MR signs of leukoencephalopathy without clinical symptoms were shown in more than 80% of cases. 5-year OS in nonresidual localized cases was 93%, and EFS was 82% (Rutkowski et al. 2005).

The British approach (UKCCSC/SIOP) applied conventional chemotherapy with vincristine + carboplatin, vincristine + methotrexate (8 g/m^2), vincristine + cyclophosphamide and

cisplatin in seven repeated 42-day cycles followed in case of suspected residual tumor or tumor spread in cerebrospinal fluid (CSF) by age-adopted radiotherapy (20 Gy booster with 25 Gy CSI). This approach did not show superiority to other trials in overall survival (Grundy et al. 2010).

One interesting observation was first published by a German group and later confirmed by UKCCSG/SIOP Group, that desmoplastic/nodular medulloblastoma has a higher survival benefit (20–50%) compared to classical subtype (Rutkowski et al. 2009; Grundy et al. 2010). Other groups did not confirm this observation, which could be attributed to their incoherent histological differential diagnosis between classic and desmoplastic subtypes.

At this age group basic chemotherapy of residual and metastatic tumors was highly similar to that in localized tumors (only some exceptions, such as, in Head II, high dose methotrexate was added to basic therapy). Irradiation or high dose chemotherapy usually could not be omitted, except for the German HIT group. OS of non-metastatic tumors with local residue was much poorer compared to that in non-residual localized cases: French 41% vs. 73%, Head Start 57% vs 79%, German HIT 56% vs 93% (no high dose chemotherapy no irradiation), but better than in primary metastatic cases, at whom it was only 13–38% (Grill et al. 2005; Dhall et al. 2008; Rutkowski et al. 2009). In conclusion, there are significant differences in survival among study groups for young patients. The best survival was achieved by the German HIT group.

High Risk Patients

This group of patients, older than 3 years old with residual tumor or primary metastasis carries the most difficulties, as by conventional chemotherapy and irradiation alone low survival rate could be achieved. Historically, irradiation alone, beyond surgical resection of primary tumor achieved 25–40% EFS (Gottardo and Gajjar 2008). One of the first study by administration of concurrent vincristine to irradiation and additional cycles of vincristine, lomustine and cisplatin reported by Packer et al. (1994) achieved 67% of 5-year EFS in a little series of patients (n = 15). Different CCG studies compared timing of irradiation, by administration of preirradiation or neoadjuvant chemotherapy or postirradiation chemotherapy. These studies confirmed the importance of primary irradiation followed by chemotherapy. European studies (PNET III and HIT 91) also could not confirm the superiority of preirradiation chemotherapy to postirradiation chemotherapy (Kortmann et al. 2000; Taylor et al. 2003). Several study group introduced high dose chemotherapy with autologous stem cell rescue for intensifying therapy for these patients. This approach and most of the intensified concomitant chemoirradiation therapy resulted in the best EFS in this group. St. Jude protocol applied topotecan upfront window followed by 6 weeks of craniospinal radiotherapy (36.0–39.6 Gy), then four courses of cyclophosphamide based high dose chemotherapy with stem cell rescue during 16 weeks. 5-year EFS was 70% (Gajjar et al. 2006). More intensive concomitant chemoirradiation therapy means weekly addition of carboplatin to vincristine to irradiation followed by further cycles of cyclophosphamide and vincristine. This approach in COG99701 study resulted in 66% 4-year EFS (Jakacki et al. 2007). Prolonged survival in the future may be achieved by administration of intensive concomitant chemoirradiation with drug-combination followed by high–dose chemotherapy and a long term low dose maintenance therapy with cytostatic drugs or biologically targeted therapy. Maintenance therapy consisted of cyclophosphamide, etoposide, thalidomide, isotretionin and celecoxib after high dose chemotherapy in a little cohort of patients with metastatic medulloblastoma (two with anaplastic, one with desmoplastic medulloblastoma) (Choi et al. 2008). One of these patients had metastates of anaplastic medulloblastoma, after high dose chemotherapy at the start of metronomic therapy. This patient still has a stable disease after 33-month of follow-up.

Therapy in Relapse

Treatment in recurrence or progression is highly ineffective in terms of long term survival in medulloblastoma. Most of the patients are ineligible

for further curative therapy because of heavy pretreatment or disseminated recurrrence with quick progression, or inoperable state. In selected patients, at whom curative intent was established high dose chemotherapy with stem cell rescue has been applied in most cases. However, their survival is still disappointing. The survival is highly determined by whether the patient is radiotherapy naive at time to recurrence. Another important factor in terms of survival is the extent of disease right before application of high dose chemotherapy with stem cell rescue. Patients in complete or partial remission have a better 3-year EFS than with stable or progressive disease (67% vs 16%) (Sung et al. 2007) Based on a retrospective overview of recurrent medulloblastoma and PNET, long term OS of patients who receive curative irradiation at time of recurrence beyond high dose chemotherapy is 70–80%, without irradiation is less then 20% (Butturini et al. 2009). Toxicity is relatively high among patients with recurrence or progression. In one study, the treatment related mortality due to toxicity was 30% (Rosenfeld et al. 2010). In an Italian trial, five patients with recurrent medulloblastoma among other CNS tumors received 28 Gy local reirradiation in the affected area with concomitant metronomic temozolomide. Although all patients were in complete remission at the end of treatment, OS was poor with 20% (Padovani et al. 2011).

In conclusion, taken the fact, that only a little percentage of patients is suitable for second line treatment, then results of second line therapy are even more disappointing. In most cases of relapse or progression the realistic aim of therapy is prolongation of survival with long term low dose therapy to slow down progression and maintain a good quality of life. There are some promising results in little cohort of patients with long term anti-angiogenic or metronomic therapy, which has to be confirmed in larger patient population. Anti-angiogenic therapy is based on the observation that inhibition of microvessels of a tumor hinders the progression. First trial of anti-angiogenic or metronomic therapy introduced by Kieran: thalidomide, cox-2 inhibitor celecoxib and 3-weekly alternating administration of low dose daily etoposide or cyclophosphamide were given to only one patient, with a recurrent medulloblastoma among several other patients with CNS tumors. This patient is still alive 10 years after recurrence. Based on this observation a new study was opened, in which beyond these four drugs, bevacizumab, fenofibrate and intraventicular etoposide and liposomal Ara-C were also applied. At 2 years the EFS survival of seven patients was 69%, which is outstandingly good compared to previous results (Peyrl et al. 2011). Beyond efficacy, another main advantage of this treatment for patients is that these drugs can be taken in an outpatient manner, which makes possible to live their normal daily life without hospitalization. A Phase 2 study is still ongoing to confirm this result in a larger cohort of patients.

Novel Treatment Approaches

General Aspects

There is an active search for new therapeutic approaches in the treatment of medulloblastoma. Before the new molecular stratification, which will presumably bring new therapeutic agents in use against medulloblastoma, there were also several attempts on molecular basis to improve survival of medulloblastoma. Most of these cases are single or highly limited series of patients, which makes difficult to draw long term consequences based on them. Common feature of these treatments was their transient success, which finally turned into progression. The background of progression could be the mutation of targeted receptors or other involved proteins. Therefore, at this moment, these could be regarded as supplementation of conventional therapy but not substitution. The patient group, which can benefit from these also has to be exactly determined.

Molecular Therapy

Presently, targeted treatment only in SHH group exists, where tumor proliferation is attributed to the lack of inhibition of Smo protein by loss of function of inhibitory Patched protein or activating mutation of SMO. SMO inhibitors, as cyclopamine,

IPI-926 and the orally available GDC-0449 inhibit hedgehoge pathway. The latter went through a Phase 1 study, resulting in a 3-month long transient, well-defined remission achieved in a 26-year old patient with proven Ptch1 mutation (Rudin et al. 2009).

Promising treatment of medulloblastoma could be the administration of retinoic acid (RA). RA has been shown to have anticancer efficacy in a variety of cancers. RA is commonly used in the treatment of certain childhood cancers such as neuroblastoma. It is a drug with protean effects including cytodifferentiation, apoptosis, and inhibition of angiogenesis. RA has been shown in preclinical models to cause apoptotic cell death in medulloblastoma by promoting BMP-2 transcription. This results in the production of soluble BMP-2 protein that induces p38 MAP kinase phosphorylation and ultimately apoptosis (Spiller et al. 2008). Recently, a phase 3 trial has been opened to treat medulloblastoma with RA.

Another promising therapy is inhibition of histone deacetylase (HDAC). Acetylation of histones' amino terminal tails by histone acetyltransferase relaxes chromatin for transcription, and removal of acetyl groups by HDAC represses transcription. Histone hypoacetylation and inappropriate transcriptional repression are hypothesized to be a key contributor to the development of human cancers. HDAC inhibitors have been shown to cause pleiotropic effect on human cancer cells, including apoptosis, cell cycle arrest, and differentiation. Valproic acid an anticonvulsive drug, widely used is one of the recently discovered HDAC inhibitors. There is one published Phase 1 study in pediatric CNS tumors. Treatment was well-tolerated; however, response was not observed among the three patients with medulloblastoma (Su et al. 2011).

Long known possible targets of medulloblastoma are somatostatin receptors (SSTR). Several attempts were made to bind radioactive isotope to its agonist, octreotide or its derivates, providing localized, targeted radiotherapy, which did not result in survival advantage better than that with conventional irradiation. Somatostatin analogues themselves through SSTR receptors may inhibit cell proliferation. Medulloblastoma expresses high amount of SSTR based on Octreoscan examination. Only one patient has been reported to receiving octreotide alone without radioactive isotope with long term survival (Glas et al. 2008).

Another possible target group is the different type of receptor tirozin kinases. There are several drugs targeting these receptors which are also expressed in a certain part of medulloblastoma (ErbB2, PDGFR) (Gottardo and Gajjar 2008). Their increased expression usually holds worst survival and increased metastatic ability. There is a Phase I study with combination of temozolomide and ErBB tirozin kinase inhibitor erlotinib, which shows good tolerability, but lack of effectiveness (Jakacki et al. 2008). This may be partially caused by their hindered penetration through blood brain barrier.

Wolff et al. (2011) elaborated personalized and targeted therapy approach in pediatric brain tumors, which means that after recurrence, beyond reevaluation of previous therapy and toxicities, a tumor sample was taken and series of molecular pathways possibly involved in disease progression were examined by the so-called morphoproteomics approach. Based on these findings individualized chemo- and molecularly targeted therapy was started. This method was applied in two patients with recurrent medulloblastoma (one patient received sorafenib, and another patient estrogen receptor antagonist, fulvestrant). In both cases individualized chemotherapy was also applied. Application of targeted molecular therapy did not result in any advantage compared to conventional chemotherapy in terms of longer survival in these cases.

Immunotherapy

Immunotherapy is a specific approach to treat cancer that was present in adult patients with several types of other cancers. Central nervous system has long been considered a relatively immunologically privileged site. Therefore, it was initially unclear, if a potent immuneresponse was inducible against brain tumors, but studies have demonstrated that immune effector cells can infiltrate the CNS and induce efficient immune responses against intracranial tumors. However, pediatric evidence is very scarce.

There are several different approaches to exploit immunotherapy against medulloblastoma cells. Only some of them were introduced in small series of pediatric patients with limited success. In the last decades of twentieth century lymphokine-activated killer (LAK) cells were directly administered intrathecally with coadministration of human recombinant IL-2 for patients with recurrent disseminated medulloblastoma. Some of the limited number of patients showed long term survival without recent larger confirmation of these findings (Silvani et al. 1994).

Another immunotherapeutic method, which already has been investigated in human patients with recurrent medulloblastoma is based on dendritic cell-based tumor vaccination. Dendritic cells are the antigen presenting cells, which are limited in their presence in CNS. Autologous mature dendritic cells loaded with tumor lysates derived from autologous, resected medulloblastoma cells were injected subcutaneously in five patients with medulloblastoma. Although the treatment was safe, no response was observed in any patient (Ardon et al. 2010).

In conclusion, outcome of medulloblastoma with present therapeutic tools (surgery, irradiation, chemotherapy) seems to reach its plateau, with differences in three main risk groups of patients (average, high risk, and young patients). The novel molecularly-based re-stratification of patients (Group SHH, Wnt, C, and D) may improve their survival by more precise classification, possibly by introducing new molecularly targeted drugs. Although there are several new theoretical attempts to treat medulloblastoma beyond conventional therapy, a really effective therapy is still to be discovered.

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10 Event-Free Survival of Children with Average-Risk Medulloblastoma: Treatment with Craniospinal Radiation Followed by Adjuvant Chemotherapy

Eugene I. Hwang and Roger J. Packer

Contents

Abstract

Medulloblastoma is the most common malignant brain tumor in children. Risk stratification for medulloblastoma separates average and high-risk disease based on age, degree of surgical resection, metastatic status and histopathologic appearance. In children with average-risk medulloblastoma, evolving therapy with surgical resection, craniospinal and focal radiotherapy, and chemotherapy has improved long-term survival to approximately 85%. However, significant long-term adverse effects are more apparent and occur in a majority of survivors. Studies to improve cure rates while decreasing long-term morbidity are ongoing. Knowledge of the molecular sub-categories of medulloblastoma is rapidly emerging and may lead to refined risk criteria and improved treatment strategies through biological targeting.

Introduction

Medulloblastoma is a small round blue-cell tumor categorized as a primitive neuroectodermal tumor (PNET) arising in the cerebellum. It is the most common malignant brain tumor in childhood, comprising almost 20% of all pediatric brain tumors, and has an incidence of 0.51 per 100,000 person-years in patients 0–19 years of age (CBTRUS 2011). Medulloblastoma incidence displays a bimodal distribution at a young age with peaks between 3–5 years and 8–9 years,

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with almost two-thirds of patients diagnosed at less than 10 years old (Rickert and Paulus 2001).

Children with medulloblastoma frequently present with symptoms of elevated intracranial pressure due to obstructive hydrocephalus from tumor growth. Early morning vomiting and headache (often worse when lying down), ataxia, irritability, and lethargy are the most common initial complaints and are exhibited by a majority of these children. Parinaud's syndrome, characterized by paralysis of upward gaze and pupillary reaction to accommodation but not light, can occur as a result of pressure on the dorsal midbrain from obstructive hydrocephalus.

Diagnosis of Medulloblastoma

Staging

Although a computed tomography (CT) scan is often the initial imaging modality demonstrating a posterior fossa mass, magnetic resonance imaging (MRI) is the preferred radiologic tool for a more detailed evaluation. Because medulloblastoma has a propensity for dissemination along the neuraxis, a spinal MRI will also be obtained to evaluate for metastatic disease. This is typically completed either prior to surgical resection or at least 10 days after resection to avoid postoperative artifact. Medulloblastoma can also occasionally (<1%) involve extracranial organs such as bone marrow, lungs, and the lymphatic system; localizing symptoms in any of these areas requires evaluation. Other imaging modalities such as positron emission tomography (PET), single photon emission computed tomography (SPECT), diffusion tensor imaging (DTI), and magnetic resonance spectroscopy (MRS) may aid in the initial assessment, but are not required to adequately assess extent of disease. Current staging also requires examination of cerebrospinal fluid (CSF) to detect microscopic dissemination because of the greatly increased sensitivity in detecting leptomeningeal disease when a spine MRI is combined with cytologic CSF examination (Fouladi et al. 1999).

Histopathology

Classical medulloblastoma consists of sheets of densely packed cells with scant cytoplasm and occasional Homer-Wright rosettes, which contain abundant fibrillary material. Medulloblastoma is immunopositive for synaptophysin, vimentin, and neuron specific enolase (NSE). The World Health Organization (WHO) recognizes four distinct histopathological subtypes of medulloblastoma, including desmoplastic/nodular medulloblastoma, medulloblastoma with extensive nodularity (MBEN), anaplastic medulloblastoma, and large-cell medulloblastoma. Desmoplastic/nodular medulloblastoma is identified by neuronal differentiation with islands of pale, reticulin-free zones. Medulloblastoma with extensive nodularity occurs in infants and is identified by the dominance of lobular architecture and a marked decrease in the inter-nodular reticulin content. Anaplastic medulloblastoma is characterized by diffuse nuclear pleomorphism and high mitotic activity. Large-cell medulloblastoma, representing approximately 4% of medulloblastomas, features large areas of necrosis and high mitotic activity with cells that have large, round, and pleomorphic nuclei. This subtype may overlap with the anaplastic variant, and indeed these types are often considered together in many clinical analyses (Louis et al. 2007). Each morphological subtype is associated with distinct clinical behavior. For instance, medulloblastoma with extensive nodularity is thought to be a good prognostic indicator. Desmoplastic/nodular histology had previously likewise been associated with improved outcomes, although more recent studies showed only equivalency with classical medulloblastoma (Gajjar et al. 2006) except for a clear benefit in very young children (Rutkowski et al. 2005). Large-cell/anaplastic tumors are the most aggressive tumors, with a hazard ratio of treatment failure of 3.9 times that of other patients (Gajjar et al. 2006). The results of a recent cooperative group study have also suggested that anaplastic medulloblastomas have worse outcomes, leading to the inclusion of these patients in high-risk arms in subsequent studies (Packer et al. 2006).

Risk Stratification

The currently accepted clinical risk stratification has evolved from a series of cooperative group studies that have demonstrated improved survival for specific subsets of patients with medulloblastoma.

Age

The age of the patient is important in determining risk, as studies have shown that children less than 3 years of age have an inferior 5-year progression free survival (PFS) when compared to older children (32 ± 10% vs. 61 ± 7%) (Zeltzer et al. 1999). This is thought to be potentially secondary to both underlying biologic differences as well as differences in treatment, as radiotherapy is often avoided or delayed in infants and young children due to concerns of unacceptable toxicity.

Metastatic Status

Metastases are present in up to 30% of patients on initial presentation (Yao et al. 1997) and are usually graded in the modified Chang staging system: M0 if there is no evidence of disseminated disease; M1 if malignant cells are noted in the CSF; M2 with gross nodular seeding in the brain; M3 with noted gross nodular seeding of the spine; M4 with extraneuraxial metastasis. Patients with M0 disease have considerably improved 5-year PFS when compared with patients with M1 or M2+ disease (70 ± 5% vs. 57 ± 10% vs. 40 ± 8%, respectively) (Zeltzer et al. 1999).

It is therefore critical to obtain accurate MRI-based imaging to assess for the presence of gross metastatic disease. CSF analysis with cellular pathologic analysis must either be undertaken prior to resection or after 10–14 days (Fouladi et al. 1999) to evaluate for microscopic metastatic disease. This is typically obtained from lumbar spinal fluid because of increased sensitivity over ventricular CSF in detecting metastatic disease.

Surgical Staging

Surgical intervention in medulloblastoma is an essential portion of both the diagnostic and therapeutic plan. The Chang staging system was initially published in 1969 and used to divide medulloblastoma into higher and lower-risk disease. It described the size and invasiveness of the primary tumor at surgery, labeled the "T(umor)" stage, and the evidence of "M(etastatic)" spread. Subsequent pediatric studies demonstrated that the extent of surgical resection was a more accurate predictor of high-risk disease than the Chang system (Laurent et al. 1985). After resection is attempted both the neurosurgeon's assessment of residual tumor amount and the post-resection MRI collaborate to estimate residual disease burden. Post-operative imaging is preferably performed within 48–72 h of resection to minimize the impact of post-surgical artifact in obscuring this assessment. Current clinical staging considers a remaining disease burden of cumulatively less than 1.5 cm^2 as a favorable prognostic factor, likely because of the enhanced efficacy of subsequent radio- and chemotherapy.

Average Risk Disease Definition

Therefore, patients are currently considered to have "average" or "low" risk disease if they are older than 3 years of age, have a gross-total or near-total resection with less than 1.5 cm^2 residual disease with no evidence of metastasis, and lack anaplastic/large-cell histologic features. This population accounts for approximately two-thirds of the total incidence of children with medulloblastoma.

Treatment for Average-Risk Medulloblastoma

Therapy for children with average- or low-risk medulloblastoma centers around complete surgical resection, risk-adapted radiation therapy, and chemotherapy.

Surgical Resection

Since the early 1900s, surgical removal as described by Harvey Cushing has been a standard in the treatment of children with medulloblastoma. At that time, complete radical resection without adjuvant therapy yielded survival times of 17 months, whereas patients with gross residual disease lived only 6 months. Initial operative mortality was as high as 42%. As both radiological and surgical technique developed, the morbidity and mortality of the operation was reported to lessen (Sutton et al. 1996). However, because of the close proximity of these tumors to the cerebellum and occasionally the brainstem, resection continues to carry a significant risk of morbidity, including posterior fossa mutism syndrome. This syndrome usually develops over 48–72 h although can occur within the first week after resection, and is associated with mood lability, decreased or impaired speech, ataxia and hypotonia. Posterior fossa mutism is most common when tumors involve the midline of the cerebellum and occurs in nearly a quarter of children with medulloblastoma. Although once thought to resolve completely in most patients, new evidence indicates that difficulties may persist or even be permanent (Huber et al. 2006).

In the current era, surgical extirpation remains a clear prognostic variable in the outcome of pediatric medulloblastoma, as patients with M0 disease who have residual disease >1.5 cm^2 demonstrate inferior 5-year PFS of 54%, versus 78% with complete resection (Zeltzer et al. 1999).

Radiation Therapy

Post-operative radiation therapy has been utilized in the treatment of medulloblastoma for decades, with the goal of purging residual gross and microscopic disease. Because of the metastatic potential of medulloblastoma, craniospinal irradiation was initially added in 1953, with doses of 5,000 rad to the posterior fossa and 3,500 rad to the remaining neuraxis, becoming a mainstay of treatment in the 1970s. This addition was singularly effective, resulting in an improved 5-year EFS of 59–68% (Bailey et al. 1995)in patients with localized disease, eventually evolving to 36 Gy to the neuraxis with posterior fossa doses of 54–59.6 Gy.

However, radiation-induced long-term adverse events resulted in significant morbidity and even mortality in children with medulloblastoma. Survivors experience multiple long-term adverse effects, including secondary malignancy, vasculopathy, endocrinopathies, hearing loss, and cognitive impairment (Merchant et al. 2011). Given these significant long-term morbidities, attempts were made to reduce the dose of radiotherapy. To this end, children with localized medulloblastoma were randomized to receive craniospinal irradiation at either the standard 36 Gy or a reduced-dose of 23.4 Gy (Deutsch et al. 1996), each without chemotherapy. This study was suspended when analysis revealed a higher relapse rate, especially outside of the posterior fossa, in children who received the reduced dose of radiotherapy, although this difference was less marked with longer follow-up. Subsequently, the International Society of Pediatric Oncology (SIOP) and German Society of Paediatric Oncology (GPO) attempted to compensate for decreased radiation doses by augmenting therapy with pre-irradiation chemotherapy. This study randomized "low" risk children to either standard 35 Gy or reduced-dose 25 Gy CSI in addition to chemotherapy with vincristine, procarbazine, and methotrexate for 6-weeks prior to irradiation (Bailey et al. 1995). The only group which fared significantly worse was the group which first received pre-irradiation chemotherapy followed by reduced-dose CSI; this was thought to be secondary to delay in definitive radiotherapy with an ineffective chemotherapy schedule. These studies combined to demonstrate that CSI dose reduction alone or following pre-irradiation chemotherapy was not an effective strategy to maintain disease control in children with non-disseminated medulloblastoma.

Chemotherapy

Chemotherapy was initially utilized in salvage regimens of recurrent medulloblastoma. Single-agent cyclophosphamide, platinum agents

(cisplatin or carboplatin), and methotrexate, among others, demonstrated encouraging responses (Packer 1990). This led to combination therapy such as the 8-in-1 regimen (vincristine, lomustine, hydroxyurea, procarbazine, cisplatin, cyclophosphamide, cytarabine, and methylprednisolone) which showed activity in the majority of relapsed tumors tested in small studies. Because of these responses, chemotherapy was subsequently explored as an adjuvant therapy in newly diagnosed patients, including in average-risk patients.

In this risk group, chemotherapy has been explored as an adjuvant therapy that can delay or reduce the dose of radiation, as well as improve outcomes. As stated earlier, initial attempts to reduce the dose of craniospinal radiation dose from 36 to 23.4 Gy resulted in a 15% worsening of 5-year EFS from 67% to 52% (Thomas et al. 2000). However, in a subsequent CCG study, the addition of adjuvant chemotherapy in the context of a CSI reduction to 23.4 Gy demonstrated the preservation of efficacy for average-risk patients, maintaining an 81%±2% 5-year EFS (Packer et al. 1999). This study highlighted the utility of chemotherapy in preserving survival with decreased irradiation. Other studies demonstrated similar survival rate maintenance with alternate chemotherapy regimens, including 4 cycles of post-radiotherapy high-dose chemotherapy and stem-cell rescue (Merchant et al. 2008). With these encouraging results, the most current COG study is randomizing children with average-risk medulloblastoma to receive the now-standard CSI of 23.4 Gy versus an additional reduction to 18 Gy in the context of post-radiotherapy chemotherapy. This study is also examining the novel question of reducing the size of the posterior fossa boost field. The concurrent European trial, SIOP-PNET-4 is investigating the utility of hyperfractionated radiotherapy in comparison to reduced-dose CSI (23.4 Gy) in conjunction with chemotherapy. The final efficacy and morbidity analyses of these two trials is pending.

The optimal timing of chemotherapy in average-risk patients has also undergone scrutiny. The goal of pre-irradiation chemotherapy is reduction of tumor burden, potentially leading to a decreased radiation field and reduced patient exposure without compromising survival. Besides the previously mentioned SIOP II study, the HIT ’91 trial reiterated the inferiority of outcomes in average-risk patients if radiotherapy was delayed with chemotherapy (Kortmann et al. 2000). Thus, post-radiation chemotherapy has become the standard of care in average-risk patients. The most common agents include vincristine, cisplatin, cyclophosphamide, etoposide, lomustine, and methotrexate. Radiosensitization with concomitant chemotherapy has also been investigated, particularly with either carboplatin or, more commonly, vincristine. This approach has gained acceptance as an effective component of pharmacologic care, and vincristine radiosensitization serves as a backbone in the most current average-risk clinical trials.

Biology of Medulloblastoma

The failure of standard treatment regimens to cure up to 20% of average-risk patients and the significant long-term adverse sequelae resulting from that treatment highlight the need for improvements in therapeutic options. Comprehension of the basic biological and molecular constitution of medulloblastoma has recently increased, which may aid in improving the currently accepted clinical risk stratification criteria and offer alternate therapeutic options. Although pathologically medulloblastomas have been linked to PNETs arising outside of the cerebellum in the brain, recent analysis has demonstrated the molecular dissimilarity of these two entities (Pomeroy et al. 2002). Genetic changes such as loss of genetic material from chromosome 17p (Pan et al. 2005) and gain or loss of 6q (Pfister et al. 2009) may alter patient outcomes irrespective of traditional staging. Amplification and expression of many cellular proteins may also have a predictive impact on risk stratification, including TRK-C (Grotzer et al. 2000), myc c or n expression (Ryan et al. 2012), WNT/β-catenin (Cho et al. 2011), platelet-derived growth factor (PDGF), ERBB2, Sonic hedgehog (SHH), Notch signaling pathway, p53 (Tabori et al. 2010), and orthodenticle homeobox 2 (OTX2), to name a few. Each of these identified pathways may also provide potential therapeutic

targets in medulloblastoma, and inhibitors of some of the abovementioned pathways are currently in clinical trials.

Molecular studies have also demonstrated the existence of at least four subtypes of medulloblastoma distinct from standard histopathological subtypes(Northcott et al. 2011) involving (1) Sonic hedgehog or (2) WNT pathway activation, (3) Group C tumors with c-myc activation and (4) group D tumors. In multiple retrospective analyses, these non-overlapping subtypes seemed to predict outcomes of patients with medulloblastoma more accurately than the traditional criteria (Cho et al. 2011; Northcott et al. 2011). As an example, Northcott et al. noted a decrease in survival in children with Group C medulloblastoma, irrespective of metastatic stage. This suggests that intensification of treatment may be required for patients with Group C medulloblastoma regardless of conventional risk stratification, although prospective validation of these data are still required. Conversely, tumors with WNT pathway activation, thought to be present in up to 20% of medulloblastoma, are predicted to have a more favorable outcome regardless of standard risk categorization (Ellison et al. 2005).

The current cooperative group studies are thus incorporating tissue analysis to evaluate molecular features in order to improve understanding and stratification of medulloblastoma. Investigation into agents that can target specific molecular abnormalities is also underway. For example, a subgroup of medulloblastomas have SHH pathway activation. Pre-clinical evidence demonstrated positive responses of medulloblastoma specimens when exposed to agents designed to block the SHH pathway, leading ultimately to clinical trials exploring the feasibility of this approach.

Long-Term Sequelae

More children with medulloblastoma are being cured of disease, making long-term adverse effects more evident. Chronic toxicity from chemotherapy and surgical resection is not uncommon, and radiation is likewise a major cause of permanent impairment.

Neurocognitive deficits can be both pervasive and severe in long-term survivors. CSI with focal boost radiotherapy will result in ongoing and progressive IQ deficits in children treated for average-risk medulloblastoma. Children exposed to CSI doses of 36 Gy experienced deficits of greater than 20 IQ points in the years after completion of treatment, with the majority of survivors requiring special education assistance during schooling (Mulhern et al. 1998). This decline is less pronounced both in older children and with the advent of dose reduction in average-risk patients, although significant cognitive impairment persists with most patients experiencing a decline of 4.3 IQ points per year over a 3-year period post-therapy (Ris et al. 2001). IQ decline is not the only measure of functional impairment experienced by these children; attention, memory, processing speed and nonverbal abilities are all impacted and influence the potential independence of survivors. In a recent study, despite risk-adapted craniospinal irradiation of 19.8 Gy in children less than 10 years old, few long-term survivors were reported to have social independence and productivity (Massimino et al. 2012). This neurotoxicity, while best described in conjunction with radiation, is also associated with high-dose intrathecal and intravenous methotrexate.

Other long-term morbidities, especially endocrinopathies, vasculopathy, and second malignancy are well described. Growth hormone deficiency, especially in pre-pubertal children, and impaired vertebral growth results in decreased height velocity and ultimately shorter stature. Although some reservations remain regarding growth hormone replacement due to a fear of tumor recurrence, multiple studies have shown the safety of this approach (Rohrer et al. 2010). Thyroid and sexual hormone dysfunction also may occur, although are less common. Survivors are also at risk for vascular deterioration, leading to an increased incidence of stroke (Bowers et al. 2006). With the strategy of chemotherapy intensification, other chemotherapy-induced toxicities may become more apparent over time such as platinum-induced ototoxicity or etoposide-induced risk of second malignancy. Further, the neuropsychological impact of both tumor

and treatment is complex. Body image issues, socialization, depression, and other behavioral problems are also common in survivors. In summary, for those children who survive medulloblastoma, treatment-related effects – particularly neurologic deficits (71% of survivors) and endocrinopathies (52% of survivors) (Frange et al. 2009) – persist throughout adulthood, impeding the realization of life-goals.

In conclusion, the treatment of average-risk medulloblastoma has undergone significant evolution and improvement over the recent decades. However, the proportion of children who are not cured in addition to the significant long-term treatment-related adverse effects emphasize the need for continued refinement of therapy. The current treatment approach of maximal surgical resection followed by CSI with focal boost and subsequent chemotherapy has improved the number of survivors but has also highlighted these toxicities. The most exciting recent advances stem from an enhanced understanding of the molecular biology of medulloblastoma. This new knowledge will potentially lead to improved risk-stratification and more tailored therapies, improving cure while reducing the negative impact on survivors.

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Part III

Leukemia

Risk of Childhood Acute Lymphoblastic Leukemia: Identification of Inherited Susceptibility

11

Amy L. Sherborne and Richard S. Houlston

Contents

Abstract

Acute lymphoblastic leukemia (ALL) is the major paediatric cancer in developed countries. It has long been speculated that common genetic variation influences the development of this haematological malignancy, however until recently evidence for this hypothesis has been lacking. The advent of genome-wide association studies (GWAS) has allowed the search for this class of susceptibility allele to be conducted on a genome-wide basis. Such analyses have identified novel disease genes for ALL and underscore the importance of polymorphic variation in B-cell development genes as determinants of leukemia risk. Furthermore these data indicate that a significant difference in the risk of an individual developing ALL can be attributed to heritable genetic factors.

Introduction

Acute lymphoblastic leukemia (ALL) is the major paediatric cancer in economically developed countries, with precursor B-cell (BCP-ALL; MIM 613065) accounting for approximately 70% of all childhood ALL (Stiller and Parkin 1996). In contrast to many other haematological malignancies, evidence for a familial risk to ALL is weak. Data from the Swedish family-cancer database does, however, lend some support to an excess risk in relatives of patients (Hemminki and Jiang 2002). Although rare (<5% of ALL), direct evi-

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dence for inherited genetic susceptibility is provided by the high risk of ALL associated with Bloom's syndrome, neurofibromatosis, ataxia telangiectasia and constitutional trisomy 21. While evidence linking an environmental exposure to risk of childhood ALL has largely been inconsistent (as reviewed in Belson et al. 2007), epidemiological data for an infectious etiology is persuasive, albeit indirect (Greaves 2006). Implicit in a model of ALL having an infectious etiological basis is that ALL is likely to represent a rare sequelae of infection with germline variation influencing host response.

Models of Inherited Susceptibility and Candidate Gene Studies

Modest familial relative risks are compatible with a wide range of genetic models of inheritance. However, the absence of families segregating ALL argues against the role of high penetrance susceptibility to the disease being the norm. Hence it is probable that the risk of ALL attributable to genetic variation is due to the co-inheritance of multiple low-risk variants, as outlined in the polygenic model (Fig. 11.1). Under this model variants conferring relative risks of 1.1–1.5 could make an important contribution to the overall inherited risk. Although such alleles have small effects individually, they could contribute significantly to disease susceptibility in the general population. Furthermore, by acting in concert they have the capacity to generate a high risk of ALL in a subset of the population.

While it has long been speculated that common polymorphic variation contributes to the susceptibility of ALL, evidence for common low risk alleles has only just emerged. The search for risk loci for ALL has until recently centred on association studies of candidate genes, where the frequencies of polymorphic variants, single nucleotide polymorphisms (SNPs), are compared in cases and controls. Most of these studies have

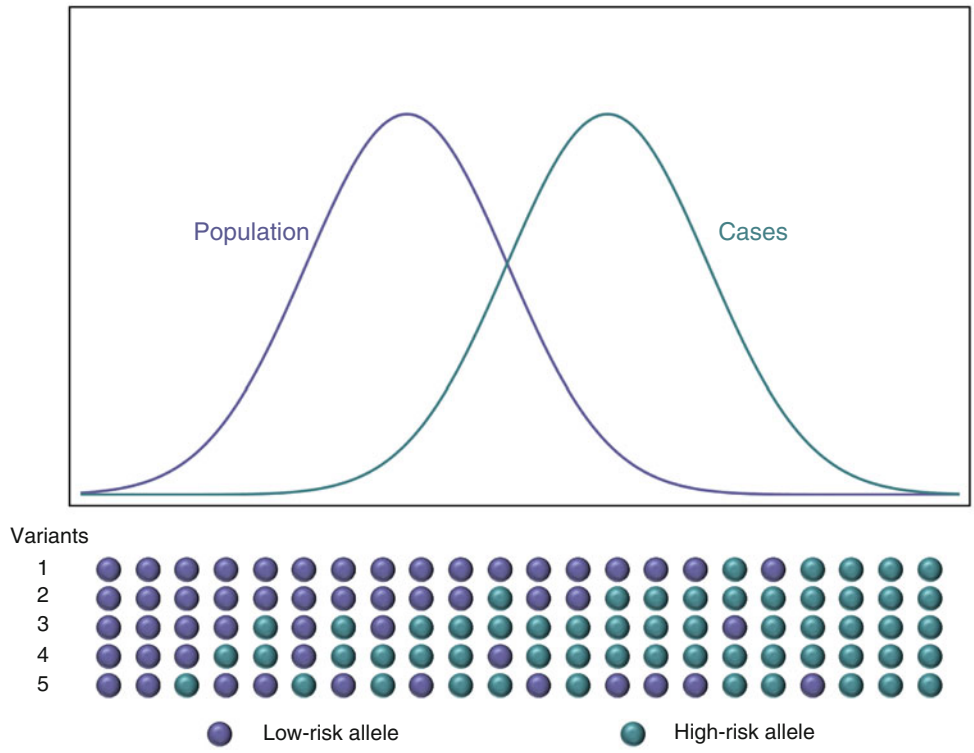

Fig. 11.1 The polygenic model of susceptibility to cancer

evaluated only a restricted number of polymorphisms, such as those influencing methylation or carcinogen metabolism. Although numerous associations have been proposed from such candidate gene analyses conducted over the past 20 years, no definitive susceptibility alleles have been unequivocally identified (Sinnett et al. 2000; Ye and Song 2005; Bolufer et al. 2006; Pereira et al. 2006; Guha et al. 2008). As with many other diseases, positive associations have been reported for various polymorphisms but few of the initial positive results have been replicated in subsequent studies (Vijayakrishnan and Houlston 2010). The inherent statistical uncertainty of studies involving just a few hundred cases and controls seriously limits the power available to reliably identify genetic determinants conferring modest but potentially important risks. Furthermore, without a clear understanding of the biology of predisposition the definition of what truly represents a candidate gene is inherently problematic, making an unbiased approach to loci selection highly desirable.

Genome-Wide Association Studies

Following completion of the Human Genome Project, more than 20 million SNPs have been catalogued in addition to smaller numbers of insertion/deletion and copy number variations. The high resolution LD maps and comprehensive sets of tagging SNPs (tagSNPs) available through the HapMap, coupled with the development of highly efficient analytical platforms, have allowed genome wide association studies (GWAS) to be conducted efficiently and cost effectively. This approach is unbiased and does not depend on prior knowledge of the function or involvement of any gene in disease causation. Furthermore, the strategy offers the prospect of identifying important variants in previously unstudied genes and non-coding regions of the genome.

Recent GWASs of ALL have vindicated the hypothesis of common susceptibility to ALL, identifying SNPs at four novel risk loci7p12.2 (*IKZF1*, rs4132601), 9p21.3 (*CDKN2A*, rs3731217) 10q21.2 (*ARID5B*, rs7089424) and 14q11.2 (*CEBPE*, rs2239633) (Table 11.1) (Papaemmanuil et al. 2009; Trevino et al. 2009; Sherborne et al. 2010; Vijayakrishnan and Houlston 2010). Intriguingly, excluding *CDKN2A*, none of the genes implicated by these GWAS scans have previously been evaluated in targeted association studies, emphasizing that the candidate gene approach was severely limited by inadequate knowledge of tumor biology.

Contribution of GWAS Findings to the Understanding of ALL Development

The SNP genotyped in GWAS are not generally candidates for causality, and enumeration of the causal variant at a specific locus can pose a significant challenge. While fine-mapping and resequencing is required to identify functional variant(s) the associations identified for ALL implicate a number of genes in tumor aetiology.

The strongest association signal for ALL was attained at 7p12.2 with rs4132601, which maps to the 3′ region of the Ikaros family zinc finger 1 (*IKZF1*) gene. Ikaros proteins are master regulators of lymphocyte development (Fig. 11.2) and differentiation, and play a pivotal role in CD4 versus CD8 T-cell lineage commitment decisions (Harker et al. 2002). In homozygous mutant mice deleted for the N-terminal zinc finger DNA binding domain of *IKZF1*, loss of expression leads to arrest of lymphocyte development at its earliest

Table 11.1 ALL susceptibility loci identified through genome-wide association studies

SNP	Chr	MAF	Gene	P_{trend}	OR (95% CI)
rs4132601	7p12.2	0.28	*IKZF1*	1.20×10^{-19}	1.65 (1.58–1.81)
rs3731217	9p21.3	0.14	*CDKN2A*	3.01×10^{-11}	0.71 (0.64–0.78)
rs7089424	10q21.2	0.32	*ARIDB5*	6.69×10^{-19}	1.65 (1.54–1.76)
rs2236933	14q11.2	0.48	*CEBPE*	2.88×10^{-7}	1.34 (1.22–1.45)

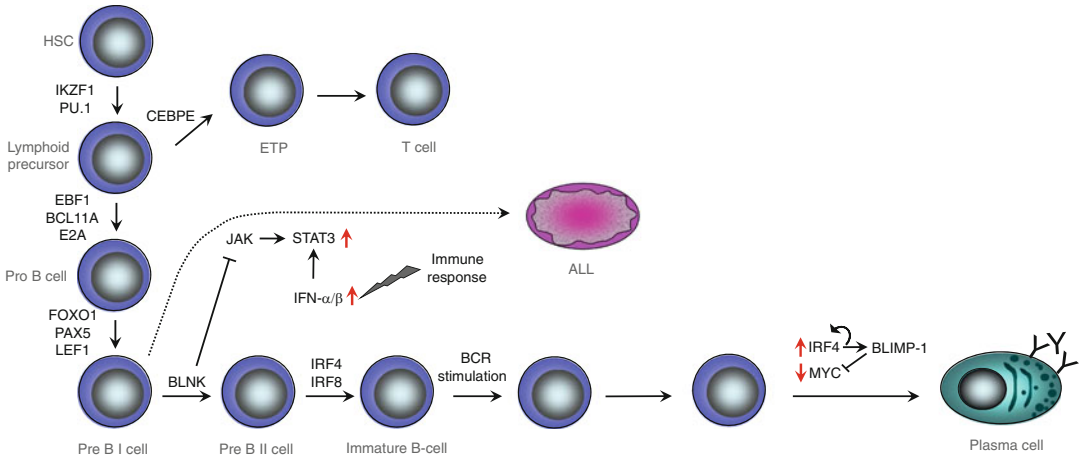

Fig. 11.2 Genes regulating lymphoid development

recognizable stage followed by rapid development of leukemia (Georgopoulos et al. 1994).

The region of association defining the 9p21.3 association encompasses the *CDKN2A* and *CDKN2B* tumor-suppressor genes and the non-coding antisense RNA encoded by *CDKN2BAS*. *CDKN2A* encodes both p16 (INK4A), a negative regulator of cyclin-dependant kinases, and p14 (ARF1), an activator of p53. *CDKN2A* and *CDKN2B* are frequently inactivated in multiple hematological malignancies. Moreover, mono- or biallelic deletion of *CDKN2A* is one of the most frequent genetic events in both childhood BCP and T-ALL (Mullighan and Downing 2009). Perhaps not surprisingly the association between 9p21.3 risk genotype and ALL is generic and not confined to a specific form of ALL.

The association at 10q21.2 implicates the AT rich interactive domain 5B (*ARID5B*) gene in the etiology of ALL. While *ARID5B* has not been extensively studied, evidence for *ARID5B* having a role in defining B-cell lineage is supported by data from homozygous knockout mice, which display decreased bone marrow cellularity and reduced numbers of B-cell progenitors (Lahoud et al. 2001).

The 14q11.2 association with ALL annotates the gene encoding CCAAT/enhancer-binding protein, epsilon (*CEBPE*). CEBP is a suppressor of myeloid leukemogenesis. *CEBPE*, along with other CEBP family members, is occasionally targeted by recurrent IGH translocations in BCP-ALL suggesting opposing functions of CEBP dysregulation in myeloid and lymphoid leukemogenesis and a role in susceptibility to ALL (Akasaka et al. 2007).

Given the biological heterogeneity of ALL, variants are likely to have differential effects on ALL risk depending on cell lineage and phenotype. This is well illustrated by the primary impact of variation defined by the 7p12.2, 9p21.3, 10q21.2 and 14q11.2 risk variants for B-lineage leukemia. Furthermore, subtype analysis of B-precursor ALL provides strong evidence that variation at 10q21.2-*ARID5B* is highly associated with the risk of developing hyperdiploid ALL (Sherborne et al. 2010). Given that the frequency of many ALL subgroups is small, identifying differential effects will only be realistically possible through multi-center pooled analyses.

A role for specific human leukocyte antigen (HLA) variants in the etiology of ALL has been extensively studied over the last 30 years, but no unambiguous association has been identified. It has recently been demonstrated that it is possible to use SNP variation within the 6p21 region to accurately predict alleles at key class I (*HLA-A*, *HLA-B*, and *HLA-C*) and class II (*HLA-DRB1*, *HLA-DQA1*, and *HLA-DQB1*) loci with better than 90% accuracy (Leslie et al.

2008). Information from GWAS has also allowed the role of variation in specific loci such as the MHC to be comprehensively examined. Through such analyses it has been possible to demonstrate that major histocompatibility complex-defined variation in immune-mediated response is unlikely to be a major risk factor for B-cell precursor-ALL (Hosking et al. 2010).

Identifying Functional Variants and the Heritability of ALL

Validated tagSNPs are highly unlikely to directly impact on ALL risk. Identifying a functional variant from a tagSNP that is statistically associated with disease is challenging. Although blocks of LD allow the efficient survey of the genome, they hamper fine mapping of the disease-associated region. Different ethnic groups are likely to have different LD block patterns and they can, therefore, be used to refine the location of a disease susceptibility locus prior to fine mapping genotyping and functional analyses.

While the risks of ALL associated with the identified SNPs are modest, with relative risks of 1.2–1.7 per allele as predicted by the polygenic model, their contribution to ALL incidence is high as the alleles are common within the population. Moreover, the risk of ALL increases with increasing numbers of variant alleles carried by an individual. It is also likely that known loci may carry additional, as of yet unidentified, risk variants, potentially including low-frequency variants with larger influences on disease risk.

The testing of SNPs individually for an association in GWAS necessitates the imposition of a very stringent *P*-value to address the issue of multiple testing. While this reduces false positives, real associations may be missed and therefore any estimate of the total heritability will be negatively biased. By considering all typed SNPs simultaneously it has been calculated that 24% of the total variation in ALL risk can be ascribed to common genetic variation (Enciso-Mora et al. 2012). These findings suggest common variation rather than a restricted number of associations, influence ALL and provide further support for a polygenic basis for susceptibility to the disease. It is, therefore, likely that additional common low risk variants remain to be discovered and should be eminently harvestable in new larger GWAS or through further pooling of additional existing datasets. How much of the unaccounted heritable risk is truly embodied in a long tail of association is currently unknown but will impact on our ability to fully understand the genetic and ultimately biological basis of ALL predisposition.

Incorporating Non-genetic Risk Factors into Risk Models

The risk of developing ALL, like many other cancers, will undoubtedly be determined by complex interactions between genetic, environmental factors and chance. Epidemiological studies have so far provided indirect evidence that ALL may have an infective basis although no specific infectious agent has been implicated (MacMahon 1992; Greaves and Alexander 1993; Kinlen 1995). There is also consistent data supporting birth weight as a risk factor for ALL possibly operating through association with high IGF2 levels and the latter's impact on stem/progenitor cells (Robison et al. 1987). There is little robust evidence linking either pre- or post-natal environmental exposures to risk of childhood ALL, with ionizing radiation being the one notable exception (Mahoney et al. 2004).

Ethnic differences in the risk of ALL are well recognized. Thus, in assessing the interplay between inherited and non-genetic risk factors, analyses using different population cohorts with different incidence rates are likely to be highly informative. This is supported by recent studies of ALL in a Thai population (Vijayakrishnan et al. 2010) and in a black population (Yang et al. 2010) suggesting that 7p12.2 and 10q21.2 variation may contribute to racial differences in ALL risk.

Type 1 diabetes (T1D) is an autoimmune disease for which infectious triggers of disease onset have been sought with increasing evidence pointing

to enteroviruses. Co-morbidity between ALL and type 1 diabetes has been reported from cohort analyses performed in Sweden (Shu et al. 2010), which is especially interesting as variation in *IKZF1* appears to be a determinant of risk for both diseases, albeit reciprocally. Although such observations are intriguing, the robust identification of interactions between genetic variants and environmental risk factors will be contingent on very large datasets, realistically something which can only be achieved through multi-center collaboration.

Conclusions and Future Challenges

Recent studies have provided the first unambiguous evidence that common genetic variation contributes to the risk of developing ALL and implicate genes involved in transcriptional regulation and differentiation of B-cell progenitors as the biological basis of predisposition to B-cell malignancy. Furthermore, their identification provides novel insight into disease causation of these two major haematological malignancies.

The power of recent GWAS to identify common alleles conferring risks of 1.5 or greater (such as the 7p12.2 variant) is high. Hence, there are unlikely to be many additional SNPs with similar effects for alleles with frequencies greater than 0.3 in populations of European ancestry. Tagging SNPs employed for GWAS capture on average approximately 80% of common SNPs in the European population, but only approximately 10% of SNPs with minor allele frequencies of 5–10% are tagged at this level, limiting power to detect this class of susceptibility allele. While coverage of the genome offered by current arrays is generally high, some chromosomal regions cannot be readily typed due to inadequate tagging or technological constraints. GWAS-based strategies are not configured optimally to identify low frequency variants with potentially stronger effects or identify recessively acting alleles. It is, therefore, highly likely that a large number of disease-causing variants remain to be discovered through next generation arrays and through throughput sequencing projects.

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Pediatric Acute Lymphoblastic Leukemia: Role of BIM Protein in Prednisolone-Induced Apoptosis

12

Nan Jiang, Grace Shimin Koh, and Allen Eng Juh Yeoh

Contents

Abstract

Acute lymphoblastic leukemia (ALL) is a heterogeneous cancer characterized by abnormal accumulation of immature blasts in the bone marrow. Glucocorticoids such as prednisolone (PRED) have been widely used in the treatment of pediatric ALL and the resistance to PRED is associated with unfavorable outcome in patients. We have identified BIM to be an important regulator of PRED-induced apoptosis, and its expression level may have prognostic value. By understanding the molecular basis of PRED-induced apoptosis, we hope that improved treatment strategies can be defined.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer, accounting for more than 30% of the newly diagnosed childhood cancer cases annually throughout the world (Pui et al. 2004). The intensity of modern chemotherapy for children with ALL is tailored according to risk groups defined by both clinical and laboratory features, in order to minimize long-term toxicity from overtreatment on one hand and relapse from under treatment on the other hand. Currently, the event-free survival (EFS) rates

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for childhood ALL has reached 80% in most developed countries. However, a significant remaining 20% relapse despite current intensive chemotherapy regimens.

Several study groups have evaluated a variety of early response estimates as prognostic factors for treatment stratification in childhood ALL. This includes Day 8 prednisolone (PRED) response, Day 15 bone marrow blast count and minimal residual disease levels. Conventional cytogenetic abnormalities like t (9;22) and new genetic alterations identified by whole genome technology such as the deletion/mutation of IKZF1 present at diagnosis predicts a poorer outcome in ALL.

Since 1986, the widely adopted German Berlin-Frankfurt-Munster (BFM) clinical trials have consistently shown that patient early response to PRED is one of the most important prognostic factors (Schrappe et al. 2000).In the BFM protocols, therapy for all patients starts with 7 days of PRED monotherapy and one intrathecal dose of methotrexate on Day 1. The absolute number of leukemic blasts in the peripheral blood on Day 8 is then determined. PRED poor responders had a total blast count of ≥1,000/μl on Day 8; and patients who exhibit a PRED good response have favourable outcome (Schrappe et al. 2000). This provided a powerful yet simple tool to pick up a group of high risk patients, comprising of 10% of all patients, who had a significantly poorer outcome. More importantly, this suggested that resistance to therapy is already present at initial diagnosis and detection of early resistance to therapy allow us to reliably stratify patients so as to improve eventual outcome.

PRED is a form of glucocorticoid (GC). Although GCs has been the most effective drug used in the treatment of ALL for more than 50 years, the molecular basis of GC sensitivity and resistance remains largely unknown. Resistant mechanisms have been postulated to involve mutations affecting glucocorticoid receptors (GR), defects in the GC response genes or their cross talks distal to the binding of GR. Besides drug resistance, GC therapy also has severe side effects like severe weight gain especially with high doses. Therefore, there is a need for a deeper understanding of GC-induced apoptosis and its signal transduction pathways in order to optimize its value in the treatment of ALL.

Prednisolone-Induced Apoptosis

The process of GC-induced apoptosis can be arbitrarily divided into three stages: initiation, decision and execution stages (Fig. 12.1) (Distelhorst 2002).

First, GC enters the cell by passive diffusion and binds to glucocorticoid receptors (GR). The GC-GR complex then undergoes conformational changes and translocates into the nucleus. In the nucleus, the GC-GR complex dimerizes and binds to glucocorticoid response elements (GREs) causing transactivation and transrepression.

The gene that encodes for the GR is located on chromosome 5q31 and is a transcription factor. Much research has provided evidence that GCs induce apoptosis by the transrepression of the GR interaction with transcription factor AP-1 (activating protein-1), which regulates expression of genes involved in cell growth, differentiation and transformation. There are also evidence that GC-induced apoptosis involves the transactivation of the GR through NF-κB. This differs from other apoptosis inducers as GC-induced apoptosis is initiated at a transcriptional level involving the multi-catalytic proteasome and calcium. In addition, the cross talks between GR and other signaling pathways add to the complexity of apoptosis induction. However, the specific genes that mediate cell death in response to transactivation and transrepression have not been clearly identified. It is also unclear if both pathways are required in mediating cell death in ALL cells (Helmberg et al. 1995; Chapman et al. 1996).

The decision stage in GC-induced intrinsic apoptosis involves the mitochondria and caspase cascade. This is also where the regulatory role of Bcl-2 family members is indispensable. GC-induced apoptosis has been reported to be both positively and negatively regulated by the Bcl-2 family members. The balance between pro- and anti-apoptotic signals decides if the cell survives or dies.

The most well-studied anti-apoptotic Bcl-2 family member is BCL-2. Although the inhibitory

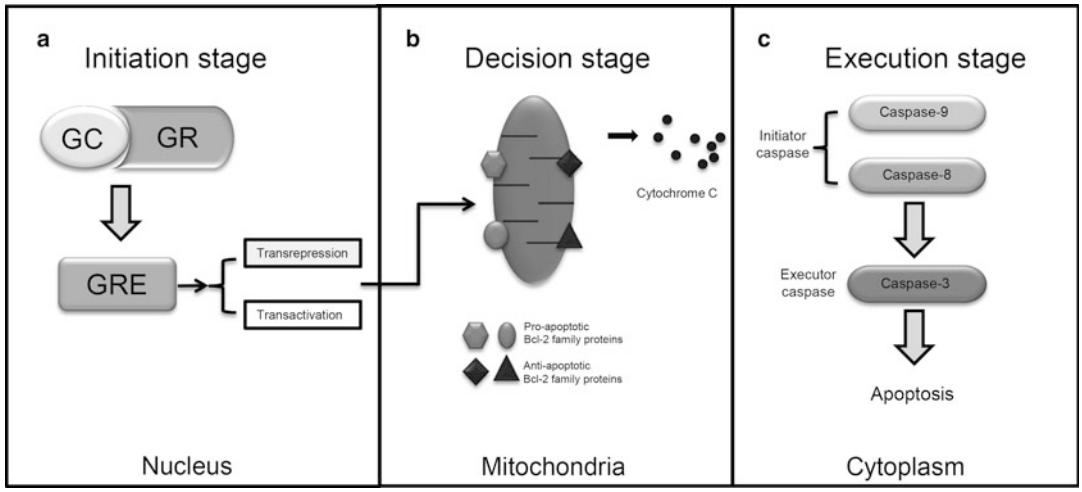

Fig. 12.1 Schematic of glucocorticoid (*GC*) induced apoptosis. GC-induced apoptosis can be arbitrarily divided into three stages: initiation stage, decision stage and execution stage. (**a**) In the initiation stage, GC enters the cell and binds to glucocorticoid receptors (*GR*). The GC-GR complex is activated and translocates into the nucleus. In the nucleus, the GC-GR complex binds to the glucocorticoid response elements (*GRE*), initiating transactivation and transrepression. (**b**) GC-induced apoptosis is reported to be positively and negatively regulated by the Bcl-2 family members. In the decision stage, the balance between pro- and anti-apoptotic signals of Bcl-2 family members decides if the cell survives or dies. Cytochrome c is released and further activates caspases. (**c**) In the execution stage, the activated caspases will execute the final step of apoptosis. There are two types of caspases, initiator and effector caspases. Initiator caspases including caspases -8 and -9, are first activated; they will further cleave effector caspases such as caspase-3. A number of morphologic changes will happen due to the cleavage of caspase-3 such as cell shrinkage, membrane blebbing, and the formation of apoptotic bodies

mechanisms of BCL-2 are not clear, it is believed to act via the outer mitochondrial membrane to affect mitochondrial function (Kroemer and Reed 2000). Experiments showed that after dexamethasone treatment, the apoptosis induced in *bcl-2*$^{-/-}$ knockout mice is accelerated (Veis et al. 1993). In contrast, BCL-2 overexpression was reported to inhibit apoptosis (Camilleri-Broet et al. 1998). *Bcl-2* antisense oligonucleotides also induce apoptosis in drug-resistant cells in various hematological cell lines (Keith et al. 1995). Another important anti-apoptotic member in the Bcl-2 family, MCL-1(which was identified to have putative GREs within the promoter regulatory regions), has also been well studied for its anti-apoptotic roles in GC-induced apoptosis (Lynch et al. 2010).

The involvement of pro-apoptotic members of the Bcl-2 family in GC-induced apoptosis have also been investigated including BAX, BAK, BID, BAD, and BIM (Gross et al. 1999). These pro-apoptotic members transmit death signals generated from the initiation stage to the execution stage during apoptotic signaling. Mice that lack BAX and BAK, BIM, PUMA or NOXA are GC resistant. However, the relative importance of each Bcl-2 family member in GC-induced apoptosis is still unknown as they appear to be cell type dependent.

In the execution stage, the cleavage of caspases will induce the final step of apoptosis leading to the cell's death in a programmed manner. Caspases represent a family of proteases that cleave substrates at aspartate residues. There are two types of caspases: initiator and effector caspases. The initiator caspases include caspase-8 and -9, which when activated, will further cleave effector caspases such as caspase-3 and -7. A number of morphologic changes will result from the cleavage of caspase-3 and -7 such as cell shrinkage, membrane blebbing and the formation of apoptotic bodies. The caspase cascade can be activated in two ways; (1) caspase-9 dependent and caspase-3 independent and (2) caspase-9 independent and caspase-3 dependent. The dominant pathway of GC-induced apoptosis in ALL

cells is caspase-9 dependence. Although the activation of caspases during apoptosis is a down-stream effector event, the signal that triggers this caspase cascade remains unclear. GC may induce either the extrinsic or intrinsic apoptotic pathways or both. In the extrinsic apoptotic pathway, the initiator caspase-8 is activated and will further activate effector caspase, caspase-3, by either a mitochondria-dependent or a mitochondria-independent pathway. However, the role of the extrinsic apoptosis pathway in GC-induced apoptosis remains inconclusive.

The Mechanism of Prednisolone Resistance

GC resistance has deleterious impact on treatment outcome of ALL patients. Although many mechanisms of resistance have been proposed, most of them remained uncertain. To arbitrarily classify the mechanism of GC resistance, it can be separated into "upstream" and "downstream" mechanisms (Sionov et al. 2008). The factors involved in "upstream" mechanisms include: GR, its ligand and GR-associated proteins. The "downstream" mechanisms involve the genes or proteins that respond to GC-GR signals and the ones that can regulate this signal.

Regarding the upstream GC resistance mechanism, several studies have tried to link the resistance of GC with the low expression or mutation of GR proteins (Sionov et al. 2008). The nuclear-transfer-deficient receptor was also reported to contribute to GC resistance (Moalli and Rosen 1994). It is proposed that these receptors may have mutations in nuclear localization signals. Some other resistance mechanisms include the modification of GR by mono-ADP ribosylation or phosphorylation, overexpression of GRβ and genetic polymorphisms. Until now, no conclusive data has shown a clear correlation between GR expression or modification to GC resistance in ALL.

In our study (Jiang et al. 2011a), we have analyzed the presence of GR mutations in 72 ALL patients and none of them carried any GR mutation. This may indicate that GR mutation is not the main cause of PRED resistance observed in clinical settings, but it may be more frequently observed *in vitro*. It also highlights the possibility that defects down-stream of GR activation could be the major cause of GC resistance at the initial induction phase. Defects in downstream signaling components such as GC-regulated genes will cause resistance to GC. Using gene microarray, the GC-treatment induced genes were identified to be involved in many important regulation processes such as transcription, mRNA splicing and protein synthesis (Obexer et al. 2001).

The role of Bcl-2 family members in GC resistance has been extensively investigated (Kfir-Erenfeld et al. 2010) and several clinical studies have established that Bcl-2/Bax ratio is associated with GC resistance. However, a clear correlation in the expression levels of BCL-2 or Bcl-2 family members with GC resistance is still lacking in childhood ALL. Since Bcl-2 family members play indispensable role in GC-induced apoptosis, further investigation is needed to increase current understanding on the mechanism of GC-induced resistance in ALL cells.

BIM, the Main Regulator of Bcl-2 Family Members in Prednisolone-Induced Apoptosis

GC-induced apoptosis involves many genes and proteins, making it difficult to elucidate the mechanism of GC-induced apoptosis. The role of key proteins from the Bcl-2 family has been the major focus of many investigations in the last few decades.

GC-induced apoptosis could be positively and negatively regulated by members of the Bcl-2 protein family, commonly termed as "Bcl-2 rheostat" (Schmidt et al. 2004). Currently, the widely accepted hypothesis is that anti-apoptotic members such as BCL-2, BCL-XL and MCL-1 act on the outer mitochondrial membrane to preserve the mitochondrial integrity by inhibiting pro-apoptotic members such as BIM. This inhibits BAX and BAK activation and subsequent apoptosis (Kfir-Erenfeld et al. 2010).

In our recent study (Jiang et al. 2011b), we validated five members of the Bcl-2 family

(BCL-2, MCL-1, BCL-XL, BID and BIM) and an inhibitor of apoptosis family member, SURVIVIN, in four ALL cell lines that respond differentially to PRED treatment (three PRED-sensitive and one PRED-resistant). These genes were identified to be differentially expressed between PRED poor response and PRED good response patients based on the microarray data generated by 45 paired ALL bone marrow samples at diagnosis (D0) and after 7 days of PRED treatment (D8) (35 PRED good responders and ten PRED poor responders) among all the other Bcl-2 family members (Yeoh et al., unpublished data). Only BIM protein was validated to be upregulated in PRED-sensitive cells but not in PRED-resistant cells. Co-treatment with Ru486, a GR antagonist, inhibited the elevation of BIM, indicating that the upregulation of BIM was GR-dependent. Transfecting specific siRNAs to silence the expression of BIM showed that when BIM was silenced, PRED-induced apoptosis was significantly inhibited. Similarly, the upregulation of BIM by GCs treatment has been confirmed in other types of leukemic cells (Kfir-Erenfeld et al. 2010). Taken together, the role of BIM in PRED-induced apoptosis is indispensable. BIM may play the main regulatory role in the "decision stage" of GC-induced apoptosis that control the downstream "execution stage" which involves the release of cytochrome c and caspase cascade activation.

GC-induced BIM upregulation may be a GC-specific pro-apoptotic process in the signaling pathways of a cancerous cell. The exact mechanism by which GC activates BIM is not clear. It is known that the promoter of the *bim* gene does not contain a consensus GRE (Wang et al. 2003). Thus, it is hypothesized that the presence of a GR-dependent upstream regulator could control BIM activation and the subsequent apoptotic signals. Many studies suggest that BIM could be regulated by a dynamic "Kinome" which is influenced by GC and may be cell type dependent (Kfir-Erenfeld et al. 2010). Future studies focusing on this area to elucidate the upstream regulation mechanism of BIM will shed light on the understanding of GC resistance mechanisms and have the potential to reverse the GC-resistance in the clinic.

BIM Is Identified as a Prognostic Gene Using Whole Genome Expression Studies

To improve the quality of life for pediatric patients with ALL, there is a requirement for prognostic biomarkers that can reflect the activation or inactivation of certain molecular pathways in treatment regiments which will increase our chances to understand the disease progression and to enhance our ability to predict outcome. Currently, we are able to identify potential biomarkers systematically using modern technologies such as gene microarray, proteomics tools or transcriptome analysis.

Using global gene profiling (GEP), several prognostically important subtypes of childhood ALL such as *ETV6-RUNX1*, *BCR-ABL*, *TCF3-PBX1*, *MLL* rearrangement, hyperdiploidy could be identified and differentiated from each other by their gene expression signatures (Yeoh et al. 2002). Large panels of genes that respond to GC treatment have been revealed, providing valuable information regarding resistance mechanisms and involvement of pathways for further functional investigations.

Many studies have found that BIM has prognostic value in clinical settings. Schmidt et al. (2006) identified that BIM was a frequently upregulated Bcl-2 family member in 13 pediatric ALL patients after 24 h GC treatment using GEP, while Bachmann et al. (2010) reported that GC resistance attributes to epigenetic silencing of the *bim* gene in pediatric ALL biopsies and xenografts established in immune-deficient mice from direct patient explants. Based on gene microarray data using our patients' bone marrow samples, we have shown that only *bim* was found to be upregulated at both gene and protein expression levels among all the Bcl-2 family members screened. BIM expression was found to be highly predictive of PRED response (ROC area under the curve = 0.81; $p = 0.032$) in paired bone marrow samples and is independent of molecular subtype. Patients whose BIM protein expression levels fail to be upregulated at Day 8 compared to Day 0 have significantly poorer EFS (60%) than

those patients whose BIM protein expression levels were upregulated (92%). Despite a relatively small sample size, we have demonstrated that BIM stands out as a promising prognostic biomarker of PRED response at Day 8 in B-lineage ALL patients (Jiang et al. 2011b). Further validation is needed on larger cohorts of patients to confirm the prognostic value of BIM protein within the first 7 days of GC induction therapy. This promises earlier diagnosis to identify slow responders or drug-resistant patients.

Gene expression profile should also be considered as a diagnostic tool to provide a clearer picture of early response genes. Great effort has been dedicated to understand the genetic profiles and significant progress has been made in this area recently. Flotho et al. (2007) have investigated the correlation between gene signature and minimal residual disease. Low expression of CASP8AP2 has been identified to be able to predict a low event-free survival and a higher rate of leukemia relapse (Flotho et al. 2006). MCL-1 may also be an important regulator of GC-induced apoptosis. Rapamycin, an mTOR inhibitor, can modulate MCL-1 to reverse GC resistance in lymphoid malignancies (Wei et al. 2006). Therefore, finding a solution to overcome steroid resistance and directly using genomic information at the "bench" will facilitate diagnosis and patients stratification at the "bedside" for childhood ALL management. It also opens up the opportunities for novel drug development based on the biomarkers identified through a hypothesis-driven approach to make personalized therapy possible.

Targeting Bcl-2 Family Members to Induce Apoptosis in ALL Cells

It is well known that resistance to GC therapy is associated with unfavorable outcome in ALL. Moreover, acquired resistance towards GC is also a huge challenge for relapsed ALL. To re-sensitize GC-resistant cells to therapy requires novel therapeutic strategies.

BIM can bind and sequester all anti-apoptotic Bcl-2 family members with high affinity and has greater potential to be an apoptosis-inducer compared to other BH3-only proteins, such as BAD and NOXA that bind to limited number of anti-apoptotic members (Youle and Strasser 2008). However, it is observed that upregulation of BIM alone was not sufficient to induce apoptosis because it requires posttranslational activation or interaction with other proteins (Puthalakath and Strasser 2002).

A novel group of chemicals commonly termed "BH3-mimetics", that mimic the BH3 domain and has been used in the treatment of various cancers (Chonghaile and Letai 2008). Several chemicals have been identified to be BH3-mimetics including Obatoclax (GX15-070), ABT-737 and Gossypol. ABT-263, a member of this family, is currently used in Phase I clinical trials for many cancer types. Studies showed that BH3-mimetics may have a unique way to induce cell death by mimicking BH3-only proteins such as BIM or PUMA and regulating the interaction between Bcl-2 family members (Chonghaile and Letai 2008). It is proposed that the high expression of anti-apoptotic members of the Bcl-2 family in malignant cells is the main reason why BH3-mimetics are effective (Wei et al. 2006). Indeed, Bonapace et al. (2010) reported a complete re-sensitization of multidrug-resistant childhood ALL cells to GCs with subcytotoxic concentrations of Obatoclax, a putative antagonist of Bcl-2 family members, through induction of autophagy-dependent necroptosis. Mason et al. (2009) reported that ABT-737 is highly effective as a single agent against most primary chronic lymphocytic leukemia (CLL) samples, and is synergistic with a range of cytotoxic chemotherapy agents.

However, there are conflicting evidence concerning the correlation of Bcl-2 family members expression level and sensitivity to BH3-mimetics; several cell types were found to be less sensitive to the treatment using BH3-mimetics. The reason for their resistance is largely unknown. In addition, some BH3-mimetics may have other apoptotic induction mechanisms besides regulating Bcl-2 family members alone. Studies have shown that known prognostic factors cannot predict the responsiveness of leukemic cells to BH3-mimetics (Mason et al. 2009). Therefore,

it would be greatly beneficial if the synergism between different BH3-mimetics and BH3-mimetics with conventional chemotherapeutic drugs is tested before use in the clinic.

In conclusion, glucocorticoids, like PRED, are important medications in the treatment of pediatric ALL and resistance to GC is associated with unfavorable outcome. Among all the Bcl-2 family members, BIM, a pro-apoptotic BH3-only protein, plays an essential regulatory role in PRED-induced apoptosis in pre-B ALL cells. BIM was up-regulated after PRED treatment in PRED-sensitive cell lines and primary bone marrow samples of PRED good response patients both at gene and protein expression levels. This has potential prognostic value in clinical settings. BH3-mimetics may re-sensitize resistant patients by rational combination in the future.

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13 Infectious Complications of Antineoplastic Chemotherapy in Children with Acute Leukemia or Solid Tumors

Elio Castagnola, Malgorzata Mikulska, Francesca Ginocchio, and Claudio Viscoli

Contents

Abstract

Infections represent important complications during chemotherapy for pediatric malignancies. The incidence of infections varies according to the type of underlying disease, being higher in children with acute leukemia/lymphoma than in those with solid tumors, and to the presence or not of important risk factors like severe granulocytopenia, severe mucositis and indwelling central venous catheters. Fever of unknown origin is the most frequent diagnosis, especially during neutropenia, but bacteremia and invasive fungal disease are the most feared. Bacteremia is the most frequent diagnosis in granulocytopenic patients, followed by lower respiratory tract infections and infections localized at the gastrointestinal tract. Central venous catheter-related infections are most frequently diagnosed in absence of granulocytopenia. Management of infections in cancer patients require an intensive, and sometime aggressive, diagnostic approach. The use of anti infective prophylaxis is not well studied in children, with the exception of Pneumocystis pneumonia, and many management strategies adopted in this field are derived form experiences in the adults. Empirical antibacterial therapy is a cornerstone of management of febrile cancer patients, especially in presence of granulocytopenia. This task is becoming very difficult in the present era of increasing infections due to antibiotic-resistant pathogens. Therefore, beyond the results of clinical trials, meta-analyses and guidelines,

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each single center must identify at the local level he most frequently isolated pathogens and their sensitivity to antibiotics in order to adopt the better strategy.

Introduction

Infections represent a well-known complication of antineoplastic chemotherapy. Their occurrence may cause delay of chemotherapy, with alteration of the antineoplastic program and dose-intensity, or even cause the death of a patient that could heal from his/her neoplasia. Therefore a correct management of infections in cancer patients is pivotal for the good management of the underlying disease. This is particularly true in an era of increasing in bacterial resistance to antibiotics and need to reduce the cost of health systems. Cancer patients probably represent the best example of how both a disease and its treatment can impair the complex immunologic network in charge of maintaining the integrity of our body and defend it from infection from both the external and the internal environment. In the last years a growing amount of evidence showed that cancer patients are not all the same, with important differences related with drugs and schedules of administrations of antineoplastic schemes. Moreover, infections in cancer patients have often been considered nosocomial infections, despite the fact that these patients are often cared for as outpatients or even on a home-care basis. Finally, in the last years we are facing the great problem of bacterial resistance to antibiotics, and this fact could change the possibility of treatment of cancer patients in the next future. The following text will give information regarding epidemiology (etiology, proportions and rates of infectious complications according to the underlying disease) and clinical features of infections in neutropenic and non neutropenic patients that determinates the most effective prophylactic and/or therapeutic approach, tailored according to the underlying disease. These data are necessary not only for clinical purposes, but may represent also a useful "summary" of the present knowledge and a "benchmark" for future studies.

Definitions Useful for the Management of Infectious Complications in Children Receiving Antineoplastic Chemotherapy

Infections represent one of the most important complications of antineoplastic chemotherapy. Febrile episodes during the course of chemotherapy, and especially in neutropenic periods, must be considered infectious in origin until proved otherwise and are generally classified according to the presence or absence of a microbiological or clinical documentation. Infectious complications in neutropenic cancer patients are generally classified as (1) microbiologically documented infections (MDI) with bacteremia (Table 13.1), (2) MDI without bacteremia (isolation of a significant pathogen from a well defined site of infection (e.g., urine, respiratory secretions obtained with sterile procedures or abscess aspiration), (3) clinically documented infections (CDI), in the presence of a clinical picture clearly and objectively infectious in nature, but without microbiological proof and (4) unexplained fever or fever of unknown origin (FUO), when both clinical and microbiological proofs are lacking, but the clinical course is compatible with an infection (Viscoli and Castagnola 2010). Central venous catheters (CVC) are another well-known cause of infections in cancer patients. The main types of catheters used in cancer patients are: partially (Hickman-Broviac; Groshong and others), totally (Port-a-Cath) implanted catheters and peripherally inserted central catheters (PICC). Some of these catheters may present a valve in the distal or in the proximal part of the device and may present one or more (generally two) lumens or reservoirs. All catheters require maintenance procedures when not in use and the frequency of the procedure and the material employed vary according with catheter type. The definitions of three types of CVC-related infections are reported in Table 13.1.

Another important classification regards invasive fungal diseases (IFD) that represent an important challenge in cancer patients, but that it is frequently complicated to diagnose because of

Table 13.1 Definitions of bacterial and fungal infections in pediatric cancer patients

Type of infection	Definition
Bacterial infections	
Bacteremia	Isolation of a bacterial or fungal pathogen from at least one blood culture, in the presence of clinical signs of infection, including at least one of the following conditions:
	Fever (>38 °C)
	Systolic blood pressure <60 mmHg
	Signs of localized infection (inflammation) in a major organ/system.
	For coagulase-negative staphylococci, corynebacteria other than *Corynebacterium jeikeium*, and other common skin contaminants, at least two sets of positive blood cultures (at least one bottle for each set) are required, unless the same pathogen is concomitantly isolated from another site of infection.
Polymicrobial bacteremia	Isolation of two or more pathogens in a single blood culture or in at least two separate blood cultures obtained 24 h apart
CVC-related infections	
Infection of the "emergence"	Cellulitis from the cuff to the skin emergence of the partially tunneled CVC[a]
Infection of the tunnel or pocket	Cellulitis or abscess around the tunnel (Hickman-Broviac type etc.) or involving the port pocket[a]
CVC-related bacteremia	CVC-associated bacteremia without any evidence of skin and soft tissue infection
Fungal infections	
Invasive fungal disease (IFD)	
Proven IFD	Positive culture from a sterile site and/or histopatholgical evidence
	Only for disseminated cryptococcosis – the presence of cryptococcal antigen in the cerebrospinal fluid
Probable IFD	Presence of 1 host factor + 1 clinical criterion + 1 mycological criterion
Possible IFD	Presence of 1 host factor + 1 clinical criterion
Host factors	Neutropenia temporally related to the onset of IFD
	Prolonged use of corticosteroids at a mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks
	Allogeneic hemopoietic stem cell transplant
	Treatment with other recognized T cell immunosuppressants, such as cyclosporine, TNF-α blockers, specific monoclonal antibodies such as alemtuzumab, or nucleoside analogues during the past 90 days
	Inherited severe immunodeficiency
Clinical criteria	For pulmonary aspergillosis, presence of one of the following lesions on the CT scan:
	Dense, well-circumscribed lesions(s) with or without a halo sign
	Air-crescent sign
	Cavity
	NB. Fever is not a predisposing condition but a clinical feature that is nonspecific for IFD.
Mycological criteria	Presence of molds in sputum or bronchoalveolar lavage
	Indirect tests for detection of antigens or cell-wall constituents like galactomannan (in serum, bronchoalveolar lavage or cerebrospinal fluid) or 1-3-β-D-glucan in serum.

[a]All pocket or tunnel infections can subsequently be a cause of bacteremia

clinical signs and symptoms and difficulties in performing invasive procedures due to severe thrombocytopenia or other life threatening conditions frequently present in cancer patients. Moreover, fungi frequently do not grow in culture and therefore only a descriptive diagnosis (e.g., "infection due to filamentous fungi") can often be made based on histological or morphological examination. To overcome these difficulties a set of definitions has been proposed with three levels of certainty of diagnosis, as "proven," "probable," and "possible" IFD to be used in epidemiological and interventional clinical studies, but in spite of these recommendations they have become a common language also in the everyday clinical practice (Table 13.1) (Viscoli and Castagnola 2010). The definition of "probable" infection is determined by the concomitant presence of a host factor, a clinical criterion plus a mycological criterion, and *Aspergillus* galactomannan antigen detection by means of an ELISA test is now widely used as a mycological criterion for the diagnosis of invasive aspergillosis in cancer patients. Data in children are a smaller number and in general with a lower quality than in adults. However, an index >0.5 in serum may be considered as highly suggestive of the presence of invasive aspergillosis in the right clinical context (e.g., persistent febrile neutropenia and abnormal CT scan), especially in the leukemic patient (Castagnola et al. 2010a). The detection of galactomannan antigen has been demonstrated predictive of the presence of invasive aspergillosis also when detected in bronchoalveolar lavage (using an index >1) in children (Desai et al. 2009), and in cerebrospinal fluid at least in adults (Viscoli et al. 2002). Unfortunately the assay may present false positive results, mainly originating from piperacillin-tazobactam treatment (Machetti et al. 2005) or some formulated milks in infants (Gangneux et al. 2002). The performance of 1-3-beta-D-glucan for the diagnosis of IFD in children is at present not evaluable. The clinical criteria must be consistent with the mycological findings, if any, and must be temporally related to current episode and indicate the presence of clinical findings "compatible" with a fungal etiology (e.g., pulmonary nodules for invasive aspergillosis, or small, target-like abscesses [bull's-eye lesions] in liver or spleen, or progressive retinal exudates on ophthalmologic examination for disseminated candidiasis) after reasonable attempts for exclusion of an alternative cause. These definitions, mainly derived in the adult setting, have been demonstrated to be useful also for studies in the pediatric population (Castagnola et al. 2006).

Epidemiology of Infections

The risk of infectious complications is given by the sum of the underlying disease that may cause per se modifications in the defense mechanisms (e.g., absence of normal leukocytes in peripheral blood due to leukemic infiltration of bone marrow or stasis of secretions because of solid tumors), the disruption of skin barrier by invasive diagnostic procedures or positioning of venous access, the toxicity of chemotherapy (neutropenia, lymphocytopenia, mucositis), and maybe the presence of some impairment of innate immunity (e.g., levels of mannose binding lectin or peculiar toll-like receptor polymorphisms). Many, if not all of these factors may be present simultaneously and/or in sequence (and even be repeated in process of time) during the course of antineoplastic chemotherapy and therefore it could be difficult to weight the role played by any single factor in determining the risk of an infectious complication in a given patient and in a given time of the antineoplastic treatment. Therefore, the knowledge of epidemiology of infections during the whole course of chemotherapy and/or at least in particular conditions, when many of the risk factors might be present altogether, is pivotal for planning management strategies. In order to obtain a reliable scenario data should be reported not only in terms of proportions of events over the total number of patients and/or treatment cycles (= how frequent?) that gives a rough information since it does not take into account the duration "of the period at risk", but also as cumulative risk (= how many patients have at least one episode, and when?) even if it do not evaluate episodes occurring after the first one (i.e., repeated episodes), and rate (= how often) calculated as

the number of episodes occurring during the whole period at risk, and reported for example as episodes/1,000 person-days at risk.

Neutropenia is the most well known risk factor. Historical studies showed that the presence of signs and symptoms of infection is clearly related with the absolute neutrophil count. Fever frequently represents the first and sometime only sign of an infection (even of a severe one) while the presence of more evident signs of an infection (especially if localized) are associated with the presence of an adequate number of granulocytes (PMN). These observations led to the identification of cut-off values of granulocyte count associated with an increased risk of severe infectious complications and with the need for starting empirical antibacterial therapy in case of fever (Viscoli and Castagnola 2010): PMN count <500/cmm or <1,000/cmm but rapidly falling below 500/cmm, while the World Health Organization identifies four levels of granulocytopenia: level 0 (no granulocytopenia) with more than 2,000 cells/cmm, level 1 (1,500–1,900 cells), level 2 (1,000–1,400), level 3 (500–900 cells) and level 4 (below 500 cells). Many experts in this field believe that the risk of infection increases significantly when the granulocyte count decreases under 1,000 cells, because neutropenia is not a static concept, but a dynamic one, although 500/cmm is the critical point. As a confirm to this "fluid" definition of neutropenia a pediatric survey on fever during neutropenic periods performed adopting the absolute number of 1,000 PMN/cmm (Castagnola et al. 2007) reported that severe infectious complications (e.g., bacteremia or invasive mycosis) were observed also in a small group of patients with PMN count never dropping below 500/cmm, suggesting the presence of a "gray zone" that should be carefully monitored. More recently, in adult patients it has been proposed a new index (D-index, or c-D-index) that evaluated the area under the curve of the neutrophil count (combining depth and duration of neutropenia) for the risk of severe infections (Portugal et al. 2009). The index seems to be effective in predicting the risk of late IFD (associated with other data like CT-scan or determination of serum galactomannan antigen) but not early bacteremias. No data are presently available in the pediatric population. Until recently, the majority of data on the incidence of infections were derived from clinical trials of prophylaxis or therapy of febrile neutropenia (mainly performed in adults) and from studies focused on specific underlying disease or infection. In these cases the information obtained is partial since it refers only to subgroups of patients or infections and do not consider episodes occurring out of the clinical trial (both in the patients enrolled and in those who do not fulfill inclusion criteria). In the last years, some studies have been performed on the epidemiology of infectious complications (mainly, but not only bacteremias and IFD) in children treated for acute leukemia or solid tumors . Few reports studied the incidence of infections during granulocytopenia (Castagnola et al. 2007), while CVC-related infections were analyzed in a large series of patients (Viscoli and Castagnola 2010; Pinon et al. 2009). Mucositis is frequently present in febrile neutropenic children and many studies clearly showed that the incidence and severity of infections is associated with the severity of chemotherapy-induced mucositis even after adjusting for severity and duration of neutropenia (Viscoli and Castagnola 2010). Tables 13.2, 13.3 and 13.4 report the available data on the epidemiology of infections in children with cancer in different settings (Viscoli and Castagnola 2010; Pinon et al. 2009; Castagnola et al. 2010b, 2011a; Flynn et al. 2003). All these data clearly show that the incidence (rates and proportions) of infectious complications, especially the most severe ones (i.e., bacteremias and IFD), is strictly associated with the aggressiveness of antineoplastic chemotherapy. As for CVC-related infections, their frequency is higher in partially implanted and double lumen devices, compared to totally implanted and single lumen, respectively. Bacteremias represent the most frequent CVC-related infectious complication, while other types of CVC-related infections are less frequently reported. The number of CVC manipulations, which depends on the aggressiveness of antineoplastic chemotherapy and the consequent need of supportive care, represents the most important risk factor for the development of CVC-related infections. Few data are available

Table 13.2 Proportions and mean rates (episodes/1,000 patient-days at risk) of infectious complications during neutropenic periods observed in children receiving antineoplastic chemotherapy

	Overall		Bacteremias		IFD		FUO	
Patients' group	%	Rate	%	Rate	%	Rate	%	Rate
Primary episode of infection during neutropenia								
AL-NHL aggressively treated	48	39.44	14	7.17	4	2.62	76	28.16
Auto-HSCT	58	37.76	11	5.08	3	0.73	77	27.96
ALL 1st phase of therapy	25	13.62	4	0.54	4	1.01	78	10.09
ST aggressively treated	32	24.71	6	1.49	0.4	0.10	85	21.03
Central nervous system tumors	27	18.62	11	1.97	1	0.84	74	13.82
Gentle	11	6.19	0	0	1	0.62	90	5.57
Standard	30	27.02	8	2.16	0	0	88	23.9
Auto-HSCT	48	31.02	17	5.17	3	2.07	57	17.58
AL-NHL/HD not aggressively treated	21	12.78	8	1.06	0	0	76	9.59
ST not aggressively treated	22	14.70	7	0.96	4	0.64	71	10.55
Maintenance AL/NHL	9	5.03	0	0	0	0	100	5.03
Further infections during the same neutropenic period (=secondary episodes)								
AL-NHL aggressively treated	38		29		13		53	
Auto-HSCT	5	–	60	–	0		20	–
ALL 1st phase of therapy	17		0		25		50	
ST aggressively treated	7		13		0		61	
ST not aggressively treated	2		0		0		100	

AL acute leukemia, *ALL* acute lymphoblastic leukemia, *HD* Hodgkin disease, *HSCT* hematopoietic stem cell transplant, *NHL* non-Hodgkin lymphoma, *ST* solid tumor

Table 13.3 Epidemiology of infectious complications observed during the whole course of antineoplastic chemotherapy in children

	Bacteremias			IFD			
Patients' group	% of treatments	Rate	Cumulative risk (%)	% of patients	Rate	Cumulative risk (%)	Notes
Solid tumors							58% of episodes diagnosed in neutropenia
Aggressive treatment	32	1.17	–	3	0.05	–	
Less aggressive treatment	2	0.15	–	0	0	–	CVC-related infections 39% (99% bacteremias, 1% IFD)
High-risk Neuroblastoma							57% of episodes in neutropenia
1st line	36	1.86	45 at day 365	5	0.18	–	CVC-related bacteremias 54%
Rescue	17	1.72	39 at day 250	0	0	–	
HSCT	16	4.32		0	0		
ALL							
1st diagnosis	33	2.45	51	9	0.64	12	Neutropenia was present in 91% of bacteremias and in 79% of IFD
			13 at day 30			7 at day 30	CVC-related infections 23% (86% bacteremias, 14% IFD)
			23 at day 60			9 at day 60	
Relapse	26	3.74	46	15	2.10	32	
			16 at day 30			7 at day 30	
			26 at day 60			13 at day 60	

(continued)

Table 13.3 (continued)

Patients' group	Bacteremias			IFD			Notes
	% of treatments	Rate	Cumulative risk (%)	% of patients	Rate	Cumulative risk (%)	
Acute leukemias							
ALL 1st diagnosis aggressive	21	1.10	–	5	0.25	–	Neutropenia at diagnosis of infection in 87% of bacteremias and 82% of IFD
ALL 1st diagnosis less aggressive	17	0.87	–	2	0.11	–	CVC-related bacteremias 29%
ALL relapse	34	2.30	–	10	0.31	–	
AML 1st diagnosis	55	3.08	–	9	0.39	–	
AML relapse	29	1.95	–	44	0.71	–	
Children receiving antineoplastic chemotherapy or HSCT	37	2.52		6	0.46		Neutropenia in 52% of bacteremias and 73% of IFD

– data not reported
Multicenter studies: beware of possible local peculiar conditions
Bacteremias in maintenance ALL only CVC related
ALL acute lymphoblastic leukemia, *AML* acute myeloid leukemia, *CVC* central venous catheter, *HSCT* hematopoietic stem cell transplant, *IFD* invasive fungal disease

on the incidence of infectious complications in oncological surgery, but is clear that the type of underlying disease, and therefore the type and duration of surgical procedure the presence of other sites of infection, and the surgical procedure classified as contaminated or infected seem to be more important than previous antineoplastic chemotherapy for determining this risk (Viscoli and Castagnola 2010). Unfortunately, no data are available for the epidemiology of these specific complications in children. Finally, differences in rates of infectious complications have been also observed according with the state of the neoplastic disease: the presence of an active disease was associated with a rate of 12.9/1,000 days at risk in acute leukemias and 8.2 in solid tumors, while in patients in complete remission the rate was 5.7/1,000 days at risk in acute leukemias and 6.0 in solid tumors (Viscoli and Castagnola 2010). Additionally, the state of remission or presence/progression of the underlying disease is an important factor also for the prognosis of severe infectious complications like invasive aspergillosis (Viscoli and Castagnola 2010).

Clinical Features and Etiology

The knowledge of the etiology of "documented" infections is another cornerstone for the implementation of management strategies. For the purpose of starting empirical antibiotic therapy, fever is usually defined as an axillary temperature greater than 38 °C at three different times within a 12 h period, or as a temperature greater than 38.5 °C in a single measurement. A prospective survey of febrile episodes during neutropenic periods showed that the majority of them were classified as fever of unknown origin (Castagnola et al. 2007). When an infection was clinically or microbiologically documented, an isolated bacteremia (independently from the presence of septic shock) was the most frequent diagnosis, followed by infections of the lower respiratory tract or the gastrointestinal tract (including oral mucositis). All other localizations had a frequency <5% and some of the most severe clinical pictures in immunocompetent hosts (like infections of the central nervous system or endocarditis) had frequencies by far <1% even in presence

Table 13.4 Epidemiology of different vascular access devices-related infections in children with cancer

Type of device	Type of infection	% of devices or episodes	Rate/1,000 days at risk	Comments
Broviac	Bacteremias			Relative risk of infection 3.0 hematological malignancy vs. solid tumor
Double lumen		49	1.40	
Single lumen		31	0.46	
Valved		31	0.84	
	Bacteremias	12	0.46	Type of device (partially vs. totally implanted, number of lumen (double vs. single) and site of insertion (jugular vs. subclavian) as risk factors for bacteremia, age <3 years for tunnel infection
	Tunnel infection	3	0.19	
	Bacteremias	–		Device removal because infection 25% port, Broviac 9%
Port			1.8	
Hickman-Broviac			3.3	
Non tunneled short-term			5.2	
Port	Bacteremias	10	0.86	Higher risk of recurrent bacteremias in patients with ports (OR 10, 95% CI 3.1–33.3)
Hickman-Broviac		20	1.18	
Port	Bacteremias	8	0.09	Included literature review on port infections: proportion 2–60% of the devices, rate 0.1–1.43 episodes/1,000 catheter days
	Pocket/tunnel infection	0.2	0.02	
Broviac	Infections during neutropenia			
	Bloodstream	3	–	
	Tunnel	0.4		
	Exit-site	0.7		
Broviac	Infections within 30 days in devices inserted during neutropenia	2	–	No prophylaxis administered

Broviac	Infections within 30 days from insertion			
	Surgical wound	1.4	0.48	
	Bacteremias	1.7	0.56	
Broviac	Bacteremias related to malfunction			
	Malfunction before infection	1.4	–	
	Infection before malfunction	3.3	–	
Totally implanted	Bacteremias		0.7	Cumulative risk of infection higher in partially implanted; in totally implanted the majority of episodes occurred in the first 2 weeks from insertion while in partially implanted the risk persisted throughout the CVC placement course
Partially implanted			4.8	
Peripherally inserted central venous catheter (PICC)	Bacteremias	10	0.63	
	Local infection	2	0.1	

Sometimes differences in definitions: CVC associated or in presence of CVC

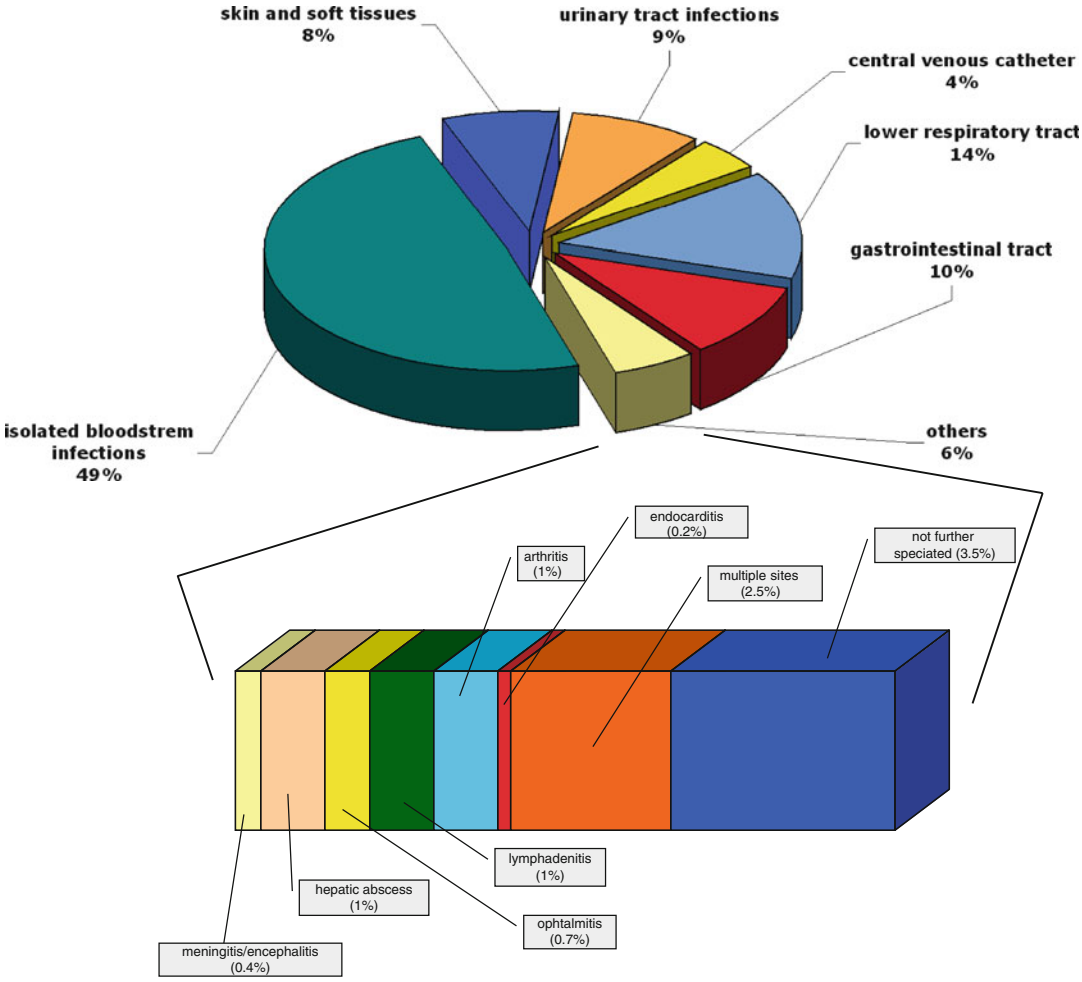

Fig. 13.1 Localizations of infections in neutropenic cancer children

of severe and prolonged granulocytopenia (Fig. 13.1) (Viscoli and Castagnola 2010; Castagnola et al. 2006; Ammann et al. 2010; Girmenia et al. 2010; Gorschlüter et al. 2002; Pagano et al. 2002; Yadegarynia et al. 2003; Yilmaz et al. 2008).

Bloodstream Infections in Neutropenic Patients

The clinical picture of bacteremia may be represented by isolated fever, but petechial or ecchymotic skin lesions, hyperventilation with respiratory alkalosis, sensory loss or changes in the mental status, hypotension, unexplained oliguria, thrombocytopenia with an increase in fibrinogen and coagulative disorders are all possible signs of infection that can accompany or precede fever. These signs and symptoms in a neutropenic patient may represent the first manifestation of a severe infection even in presence of normal temperature or hypothermia. Also patients that are particularly debilitated or receiving high-dose steroids can develop severe sepsis without fever. Therefore any modification of the clinical status, especially in a neutropenic patient, should warrant careful clinical monitoring.

In neutropenic patients bacteremias are more frequently due to Gram-positives, mainly

coagulase-negative staphylococci, but in the last years the incidence of infections due to Gram-negatives has increased and in some centers they have become the most frequently documented cause of bacteremia, both in neutropenic and non neutropenic patients (Velasco et al. 2006; Anatoliotaki et al. 2004); moreover Gram-negatives have been reported as the most frequent cause non bacteremic episodes (Yadegarynia et al. 2003). Anaerobic bacteria represent less than 1% of the positive blood cultures in cancer patients and active hematologic malignancy receiving aggressive chemotherapy or conditioning regimen for HSCT (with severe abdominal mucositis), or extended abdominal surgery are the most important conditions present in these cases (Zahar et al. 2005; Blairon et al. 2006). Septic shock is reported in near 3% of episodes of febrile neutropenia 1/2 cases is associated with bacteremia, near 1/3 with clinically documented infections and less than 1/5 with microbiologically documented infections without bacteremia (Malik et al. 2001).

Pneumonia

Pneumonia, acquired from either the respiratory tract or the bloodstream, is one of the most severe infections in cancer patients, especially in presence of neutropenia. Thus the presence of cough or thoracic pain in a neutropenic patient should be promptly evaluated with a lung CT scan, since the sensitivity of chest x-ray for the diagnosis of infectious pulmonary lesions is poor, especially in neutropenic patients (Viscoli and Castagnola 2010). Bacterial pathogens account for most of the pulmonary infiltrates that appear as segmental shadows that do not disrupt the normal anatomic borders of the lung or patchy infiltrates or dense, localized areas of consolidation, and in many cases are also detectable in blood cultures. During neutropenia, a picture of acute respiratory distress syndrome (ARDS) and septic shock was typical for viridans streptococcal bacteremia, although it may represent a typical organ dysfunction of septic shock due to any type of pathogen. In contrast, fungi cause the majority of focal infiltrates. Early fungal lesions in a neutropenic patient may be represented by a halo-sign (i.e., a macronodule, ≥1 cm in diameter, surrounded by a perimeter of ground-glass opacity). The evolution of the lesion is toward aspecific nodules (that are the most frequently observed lesions in pediatrics (Burgos et al. 2008) and to a crescent of air in a cavity if the patient is no longer neutropenic. These radiological features are suggestive of pulmonary aspergillosis, but other filamentous fungi like Zygomycetes (now identified as Glomeromycetes) cause similar lesions, even if pulmonary zygomycoses was more frequently associated with the presence of multiple (>10), bilateral lesions and severe pleural effusion. Therefore, the knowledge of the epidemiology of IFD in any single center is mandatory in order to evaluate the risk of non-*Aspergillus* mold infections. The observation of a cavitary lesion in a neutropenic patient must raise also the suspicion of a bacterial etiology rather than a mycosis due to filamentous fungi, especially in presence of positive blood cultures (e.g., *S. aureus* or Gram-negative rods, mainly *Klebsiella-Enterobacter-Serratia* (KES) group or *Pseudomonas*). Finally it must be remembered that in patients with pulmonary aspergillosis a (too) rapid recovery of the granulocyte count may be associated with the development of severe complications like pneumothorax or fatal hemoptysis. Tuberculosis due to *M. tuberculosis*, although not common in cancer patients, may present as either localized pulmonary disease or devastating miliary infection in patients receiving antineoplastic chemotherapy or HSCT (Al-Anazi et al. 2007). In developed countries, the rate of tuberculosis in cancer patients is 90 per 100,000, i.e., a nine times greater than in the general population, with higher incidence in foreign-born patients and in racial and ethnic minorities. In endemic areas its incidence has been reported to be 7–8% (Mishra et al. 2006), with the highest frequency in patients with acute non-lymphoblastic leukemia. Alveolar or interstitial infiltrates with decreased blood oxygen saturation in non-neutropenic patients are typical for *Pneumocystis jiroveci* that cause diffuse, hypoxemic pulmonary disease in cancer patients, with attack rates in patients not receiving

prophylaxis of 6.5–43% in acute lymphoblastic leukemia, 4–25% in rhabdomyosarcomas, and near 1% in Hodgkin disease and primary or metastatic central nervous system tumors; some cases have also been described after autologous HSCT. The clinical picture is characterized by subacute onset of fever, cough and dyspnea. CT scan frequently shows a bilateral ground-glass attenuation, but nodular infiltrates and sometimes pleural effusion or pneumothorax may also be observed. Similar lesions might be caused also by viruses such as CMV or influenza. As the outpatient care increases, the possibility of a neutropenic patient acquiring pneumonia due to typical community pathogens such as *S. pneumoniae*, *Mycoplasma*, *Chlamydia or Legionella* cannot be excluded. Etiological molecular diagnosis of many pathogens, including intracellular bacteria and viruses, can be performed on pharyngeal swab, sputum samples or broncho-alveolar lavage fluid. In invasive aspergillosis, serum galactomannan may be positive in neutropenic patients, while in the non-neutropenic hosts galactomannan in bronchoalveolar lavage has a much higher sensitivity.

Infections Localized at the Gastrointestinal Tract

Severe mucositis represents an important complication of intensive chemotherapy. The damage is more evident in the oral mucosa, but can involve the whole gastrointestinal tract. Intestinal mucositis is extremely important in the pathogenesis of bacteremia in the neutropenic cancer patient, since it is supposed to be the initial step of bacterial translocation, i.e., the mechanism through which intestinal bacteria migrate into the mesenteric lymphatic system and may invade the bloodstream. As a consequence in addition to fever, neutropenic patients may present gastrointestinal signs and symptoms such as abdominal pain, nausea, vomiting or diarrhea, with an overall incidence of abdominal infections near 20%. Neutropenic enterocolitis is a necrotizing cellulitis of the bowel walls that determines a severe clinical syndrome characterized by fever, severe abdominal pain, either constipation or diarrhea, sometimes hemorrhagic, evolving to acute abdomen and septic shock, which develops in about 3–6% of adults receiving aggressive treatment for acute leukemia. CT and ultrasound demonstrate thickening (>4 mm) of intestinal mucosa typically (but not exclusively) localized at the cecum (the thickening of the mucosa with disappearance of the intestinal lumen may differentiate neutropenic enterocolitis from appendicitis). Gram-negatives, often in combination with anaerobes, are main agents of perianal cellulitis with painful abscess formation. Bacterial gastroenteritis due to classic enteric pathogens (*Salmonella*, *Shigella*) is a very rare event in patients with acute leukemia, involving less than 1% of acute enteritis following chemotherapy. Viruses such as rotavirus, adenovirus or norovirus (frequently hospital acquired) have been identified as cause of severe diarrhea in pediatric oncology units and are sometimes associated with the development of bacteremias and other severe medical complications (Rayani et al. 2007). *Clostridium difficile* and *Helicobacter pylori* have also been reported as a possible cause of gastrointestinal disease with different degrees of severity in cancer children. Chronic disseminated candidiasis, also called hepatosplenic candidiasis, is a rare (no more that 2% of patients with acute leukemia, especially lymphoblastic) complication of candidemia in neutropenic patients, and radiological lesions in liver, spleen or kidneys can be observed only after recovery from neutropenia. The clinical signs include fever, elevated alkaline phosphatase and abdominal discomfort with or without organomegaly. *Strongyloides stercoralis* should be remembered as another possible cause of diarrhea, and sometimes of a disseminated infection, especially in patients from endemic areas. Finally, hepatotropic viruses, mainly HBV and HCV, may represent important problems in endemic areas and/or in the presence of an inadequate pre-transfusion blood screening. Therefore, the possibility of harboring chronic or latent infections in children coming from endemic regions should not be overlooked.

Skin and Soft Tissue Infections

In presence of skin infection initial erythema and infiltration are frequently rather mild and atypical but, if left untreated, infiltration and abscess formation progresses and may involve extensive areas, with development of necrosis and gangrene. Because the lesions caused by different organisms are similar, a needle aspiration or biopsy should be performed as early as possible in the course of the infection to establish a definitive diagnosis. Ecthyma gangrenosum is a distinct clinical entity, occurring in patients with profound neutropenia and is associated with bacteremia due to *P. aeruginosa*, even if scattered skin lesions without bacteremia may sometime occur. The lesion begins as a painful, well circumscribed, bright red macule, often with a surrounding ring of pallor. As the lesion becomes larger over the next 24 h, a sharp margin and deepening cherry-red center appears, which may form a bulla. The sharply marginated lesion eventually becomes black. Biopsy shows numerous gram-negative bacilli in deep subcutaneous veins. Much less commonly, skin lesions resembling ecthyma gangrenosum might be due to a mold infection, diagnosed by the presence of hyphae in the biopsy sample. The isolation of filamentous fungi from blood cultures in a patient with skin lesions is suggestive of a *Fusarium* infection. Skin involvement may also occur in disseminated aspergillosis or zygomycosis and are represented by nodular, necrotic, crusted, or ecchymotic lesions with surrounding erythema or cellulitis.

CVC-Related Infections

Infections related to the presence of central venous catheter are important complications (Table 13.4) mainly related with the type of device and the frequency of manipulations, but their role as a cause of infection is mainly recognized in non-neutropenic patients (Viscoli and Castagnola 2010; Pinon et al. 2009). Clinical features of CVC-related bacteremias are typically represented by spiking fever and chills soon after catheter manipulation, or persistently positive blood cultures also in an afebrile patient, in absence of endocarditis or deep-venous thrombophlebitis. Pain frequently represent the first symptom of subcutaneous tract infection also in a neutropenic patient and is frequently followed by erythema and edema around the tunnel, especially in presence of bone marrow recovery. Infections at the exit site of an implanted catheter are usually indolent, often without fever. These infections are generally due to Gram-positive cocci (especially coagulase-negative staphylococci) that are actually isolated in more than 50% of the episodes. Gram-negative rods can also cause CVC-related bacteremias, in a proportion of cases varying from 25% to 40% of the episodes. Gram-positive cocci usually come from skin or hub contamination, while Gram-negative rods (especially KES group *Citrobacter*, *Achromobacter*, and *Pseudomonas* non-*aeruginosa*) are supposed to be more frequently related with not correct procedures of catheter management. Polymicrobial infections are not rare (8–49% of the episodes) with a predominance of Gram-negatives among the isolated pathogens while fungi (mainly *Candida* sp.) are usually isolated in no more than 10% of cases (Viscoli and Castagnola 2010). It is a general rule that in patients with an indwelling CVC at least one sample for blood culture should be drawn percutaneously and the other from the device (in patients with multi-lumen devices it should be recommendable to obtain two samples from each lumen) in order to identify the possibility that the CVC represent the source of the infection. The majority of children with cancer and chemotherapy have an indwelling venous catheter, and considering the low prevalence of positive blood cultures (15–20%) in neutropenic subjects, and given that most children find venipuncture unpleasant (and sometimes technically difficult), it seems reasonable to perform blood cultures initially only from the CVC, and to draw peripheral blood cultures only if a CVC-related infection is strongly suspected, reducing patients' discomfort.

Other Clinical Pictures

Otitis presents with a painful, discharging ear with increasing pain on tugging on the pinna and an edematous, pale external auditory canal full of moist desquamated debris. Extension of the otitis to the middle ear may lead to seventh cranial nerve palsy. Sinusitis should be suspected in presence of headache or pain over a paranasal sinus or referred pain in the upper dental arch, but some patients present only with fever. Localization in the paranasal sinuses may frequently present with facial swelling or nasal discharge. Infections of the central nervous system are rare in neutropenic cancer patients, signs and symptoms may be limited to pain in the neck, a confusional state or delirium. Another possible presentation include sudden onset or relapse of seizures, focal neurological abnormalities that depend on the part of brain involved in the inflammatory process, and signs of increased intracranial pressure (headache, vomiting, bradychardia), associated with fever or neck stiffness. A lumbar puncture for culture, antigens, and PCR for DNA/RNA detection should be performed provided that no increased intracranial pressure is present. Infections of the genitourinary tract are observed in 10% of infectious episodes in pediatric oncology patients (also in absence of neutropenia), even if no urinary catheters are used. The utility of routine urinalysis and urine cultures at the initial evaluation of all febrile neutropenic children remains debatable, but seems reasonable in neutropenic patients with signs or symptoms of an urinary tract infection, a urinary catheter in place or an abnormal urinalysis. The presence of genital necrotic lesions in a febrile neutropenic female should raise the suspect of a local (but frequently also disseminated) infection due to *P. aeruginosa*.

Management of Infectious Complications

Evaluation of the Risk of Presence of Severe Infections in Patients with Febrile Neutropenia

In the last years many studies have focused on identifying, based on clinical and laboratory data available at the onset of fever, neutropenic cancer patients with low probability of a serious infection, with the aim of individualizing treatments. A recent retrospective study in children identified six parameters, of which four were reproducible but none was able to be validated, showing the difficulty in identifying standardized decision rules in the management of a condition with numerous clinical variables (Macher et al. 2010).

Prophylaxis

In any setting, the decision of administering antiinfective prophylaxis must generally be based on the answers to four major questions: (1) can you easily treat the event you are trying to prevent if it happens? (No: prophylaxis, Yes: no prophylaxis); (2) is it a severe event? (Yes: prophylaxis, No: no prophylaxis); (3) does the prophylaxis have adverse effects, including development of resistance? (No: prophylaxis, Yes: no prophylaxis); (4) is the prophylaxis effective, i.e., which is the number of patients needed to treat (NNT) to prevent one event? (low: prophylaxis, high: no prophylaxis) (Castagnola et al. 2011b). The NNT that is considered acceptable is debatable and depends on frequency of the disease in the controls, consequences of the treatment (efficacy vs. toxicity and/or drug interactions), costs (of prophylaxis and the treatment of the disease if not prevented) and selection of resistances (Castagnola et al. 2011b). In cancer children the efficacy of antibacterial prophylaxis for the prevention of febrile neutropenia was evaluated in only one randomized, double blind, placebo-controlled trial (Castagnola et al. 2003), which showed a statistically significant protective effect in children with acute leukemia/lymphoma (−17%), but not in solid tumors, with a NNT of six patients. Analyses of studies performed in adults receiving fluoroquinolones showed a NNT of five patients to prevent a febrile episode, of 33 to prevent one death and of 23 to prevent an infection-related death (Castagnola et al. 2011b). However, it must be stressed that no study evaluated the effects of repeated cycles of prophylaxis administered during the whole course of antineoplastic chemotherapy, and that the widespread

use of antibacterial prophylaxis induces the development of resistance, circumstance that is becoming one of the major concerns (Caselli et al. 2010). No satisfactory study evaluated the efficacy and effectiveness of antifungal prophylaxis in children (Castagnola et al. 2011b). In adults, the efficacy of prophylaxis with oral posaconazole (−6% of events) or nebulized liposomal amphotericin B + fluconazole (−10% of events) compared with other drugs or placebo was demonstrated in reducing proven/probable IFD during repeated periods at risk following chemotherapy for acute leukemia. Unfortunately, at present the pediatric dosage of posaconazole remains unknown, since it was evaluated only in 12 children aged 8–17 years, and concerns have been raised about pharmacokinetics parameters of posaconazole that could be of great importance in pediatrics (need for a fat meal, or at least supplemental food or acid drink, possible need for refracted daily dosing, avoidance of proton-pump inhibitors or administration through a nasogastic tube). No data are available for nebulized liposomal amphotericin B, while it is known that the compliance with nebulization systems may be lower in younger children than in adults. Recently, a systemic antifungal prophylaxis with liposomal amphotericin B at 2.5 mg/kg twice weekly resulted feasible and safe in high-risk paediatric cancer children, compared with a historical control group (Bochennek et al. 2011). A proficient training of the individuals performing CVC manipulations, both in hospital and in the patients' home probably represent the most cost-effective procedure for reducing indwelling CVC-related infections.

Isolation Procedures

Isolation might represent another important action for the prevention of infections in children with cancer. However, evidence-based indications for interventions with non-pharmacological tools are still lacking in the pediatric hematology-oncology literature. Guidelines on standard precautions, as well as precautions to avoid transmission of specific infectious agents, have been recently summarized (Caselli et al. 2011), and should be implemented in any clinical setting involving cancer patients.

Colony-Stimulating Factors and Immunoglobulins

Colony-stimulating factors are used with the aim of facilitating more dose-intense treatments and decreasing treatment-related complications. In children with acute myelogenous leukemia, G-CSF did not significantly modify the incidence of febrile episodes or severe infections (Viscoli and Castagnola 2010). The fact that a very short time (median 3 days) occurs between onset of neutropenia and fever (Castagnola et al. 2007) raises some question about the possibility for G-CSF to be pragmatically effective in the prevention of febrile neutropenia. In some phases of acute leukemia treatment, production of immunoglobulins (Ig) is reduced. Since maintenance of normal Ig levels is important to prevent infections in patients with primary immunodeficiency, the same approach has been used in patients with secondary iatrogenic Ig defects. Studies performed in adults with chronic lymphocytic leukemia showed a reduction of infectious episodes (bacterial, but not viral or fungal) occurring in patients with less than 600 mg/dl of IgG, with monthly administration of low doses of Ig, provided the treatment could be performed for at least 6 months (Viscoli and Castagnola 2010). Therefore, since many controversial issues concerning cost, scarce availability and optimum dosage of Ig treatment still remain, it seems reasonable to suggest to administer Ig only in patients with very low levels and with more than two recent severe infections (Viscoli and Castagnola 2010).

Therapy

Fever during neutropenia must always be considered a medical emergency and should always be considered as due to infection, unless otherwise proven. The use of empirical antibacterial therapy

in febrile neutropenic cancer patients has been demonstrated to significantly improve survival, and it is now considered one of the cornerstones of the supportive care in cancer. In the last years it has been demonstrated that not all cancer patients are the same and therefore different antibacterial approaches (e.g., oral vs. intravenous) are feasible in different patients' groups, e.g., solid tumors vs. acute leukemias. All the guidelines indicate the feasibility of front-line intravenous monotherapy with the use of anti-*Pseudomonas* beta-lactams (ceftazidime or cefepime or piperacillin-tazobactam or a carbapenem) in high-risk patients and the use of oral therapy with ciprofloxacin + amoxicillin-clavulanate in low risk patients (mainly with solid tumors) (Viscoli and Castagnola 2010).

The initial use of an aminoglycoside as a part of intravenous therapy should be limited to the combination with ceftriaxone to give coverage of *Pseudomonas*, or in presence of peculiar, local epidemiology. In case of empirical administration of an aminoglycoside, its discontinuation is recommendable after 3 days of therapy (three doses) in patients with fever of unknown origin. In all cases the empirical use of glycopeptides, both as initial treatment or in presence of persistent fever, is not recommended unless clinical signs suggestive of infection due to methicillin-resistant Gram-positives are present (e.g., skin and soft tissue infection, CVC- related infection etc.) (Viscoli and Castagnola 2010). There are no epidemiological or clinical reasons to suggest a different approach in the pediatric population. Above all, it should be stressed that any single center must be aware of the pathogens most frequently isolated in its patient population, and of their resistance patterns, in order to adopt the most reliable management strategies, including the optimal choice of antibiotics for febrile neutropenia.

The administration of empirical antifungal therapy is a common practice in the treatment of persistently febrile neutropenic cancer patients. The rationale for this practice was based on old autopsy studies showing IFD undetected during life and on two small randomized studies, which enrolled less than 200 patients altogether. These studies were not double blind or placebo-controlled and actually did not demonstrate an unequivocal advantage of an empirical antifungal therapy. In both studies the statistical power was very small, especially for subgroup analyses. Except for the initial studies which used as primary endpoint persistence of fever and survival, the more recent ones used a very controversial composite clinical endpoint, which included 5 criteria (defervescence, no discontinuation for toxicity, treatment of baseline fungal infections, prevention of breakthrough fungal infections and survival) (Viscoli and Castagnola 2010). Many drugs have been tested for this indication (liposomal amphotericin B, caspofungin, voriconazole, fluconazole), but only recently a pediatric study was published (liposomal amphotericin B vs. caspofungin) (Maertens et al. 2010). In general, no drug has been demonstrated significantly more effective than the control and differences were mainly based on lower toxicity. In the last years, awareness has grown that the empirical approach has resulted in a tremendous overtreatment of just a symptom (fever). More recently a study in adults showed the feasibility of clinically driven approach, called pre-emptive therapy, i.e., the administration of antifungal therapy in a persistently febrile neutropenic patient with either a radiological result (e.g., chest CT scan) or a microbiological result (e.g., *Aspergillus* galactomannan in serum or BAL fluid, glucan detection in serum, cytological detection of fungal hyphae or positive culture on sputum or BAL fluid) (Girmenia et al. 2010). At present no study on pre-emptive approach has been performed in pediatrics. Indications for the treatment of documented infections, especially due to resistant pathogens, and/or syndromes due to localized infections are beyond the scope of the present document.

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Pediatric Leukemia of Natural Killer Cells: Diagnosis and Multi-Agent Chemotherapy

14

Ashwin Patel and Somsuvra Ghatak

Contents

Abstract

Natural killer (NK) cell-derived neoplasms are a group of uncommon, heterogeneous and highly aggressive malignancies mainly seen in Asian and South American populations. The involvement of children is less common compared to adults and hence exclusive pediatric data is lacking and many management decisions are based on adult data. They are classified by the World Health Organization (WHO) into extranodal NK/T-cell lymphoma (nasal and extranasal) (ENKL) and aggressive NK cell leukemia (ANKL). NK cell lymphoblastic lymphoma and chronic lymphoproliferative disorder of NK cell are included in provisional category. While myeloid/NK cell leukemia is considered as a type of AML and Hematodermic NK cell neoplasm is considered dendritic cell neoplasm. NK cell neoplasm is characterized by polymorphic neoplastic infiltrate, angioinvasion and/or angiodestruction, cytoplasmic azurophilic granules, $CD2^+$/surface $CD3^-$/cytoplasmic $CD3\varepsilon^+$/$CD56^+$ phenotype and Epstein-Barr virus (EBV) infection. Cytogenetic study shows recurrent aberrations of 6q, 11q, 13q, and 17p. T cell receptor (TCR) genes are always in germline configuration. Mutations of p53, C-KIT, FAs gene and B-catenin are frequently seen. NK-cell neoplasms are often resistant to conventional combination chemotherapy due to p-glycoprotein expression and associated multidrug resistance. L-asparaginase based chemotherapy gives better outcome. The prognoses of both localized and advanced stages of

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NK-cell malignancies are worse than majority of other lymphoid malignancies. Stem cell transplantation is recommended for the advanced/relapsed disease. Studies are currently underway to assess the safety, efficacy and outcomes of chemotherapy, molecular pathway inhibitors and monoclonal antibodies.

Introduction

Natural killer (NK) cells constitute <5% of peripheral blood lymphocytes that mediate lysis of tumor cells and bacteria- or virus-infected cells and the production of immunomodulatory cytokines. NK cells are derived in the bone marrow from $CD34^+$ hematopoietic progenitor cells (HPCs) through the intermediate developmental stages of lymphoid stem cells, bipotential T/NK progenitor cells and committed NK progenitor cells (Liang and Graham 2008). Morphologically, mature NK cells are large granular lymphocytes (LGLs), with germ line T cell receptor (TCR) and immunoglobulin gene configurations. NK cells have abundant pale cytoplasm with azurophilic granules containing cytolytic molecules such as perforin, granzyme B, and TIA-1. NK cells show a variable expression of the T lineage-associated antigens, such as CD2, CD7 and CD8. They are typically negative for surface-CD3 and myeloperoxidase (MPO) and express cytoplasmic CD3ε chain. CD16, CD56, and CD57 are NK-associated markers, out of which, CD56 (neuronal cell adhesion molecule [N-CAM]) is the most consistently expressed. However, CD56 can also be expressed on cytotoxic T lymphocytes, neural/neuroendocrine tissues and skeletal muscle (Siu et al. 2002; Liang and Graham 2008).

Neoplasms of the NK cells and NK-like T cells are uncommon and are prevalent in Asian and Central and South American populations (Schwartz et al. 2008). The peripheral T-cell and NK/T-cell neoplasms comprise 5–10% of all Non-Hodgkin lymphoma (NHL) in Western countries and 15–20% of NHL in Asia. EBV infection is believed to be responsible for NK cell neoplasm as EBV encoded RNA (EBER) in a clonal episomal form is consistently demonstrated by in situ hybridization (ISH) (Siu et al. 2002). In fact, demonstration of EBV in the neoplastic cells is a diagnostic requisite in WHO classification of NK-/T-cell lymphoma (Chan et al. 2008).

Classification of NK Cell Neoplasm

In the 1994 Revised European-American Lymphoma (REAL) classification, NK cell malignancies were included within the categories of large granular lymphocytic leukemia and angiocentric lymphoma (Harris et al. 1994). Their distinct nature was formally acknowledged at a slide workshop on nasal and related extranodal angiocentric T/NK cell lymphomas by Jaffe et al. (1996), who classified NK cell neoplasm into three categories: precursor NK cell leukemia, nasal and nasal-type NK cell lymphoma, and aggressive NK cell leukemia. The new World Health Organization (WHO) classification encompasses three distinct entities of NK cell neoplasms: extranodal NK/T-cell lymphoma (nasal and extranasal) (ENKL) and aggressive NK cell leukemia (ANKL) (Chan et al. 2001). Aggressive NK cell leukemia and extranodal NK cell lymphoma, nasal types originate from mature NK cells (Liang and Graham 2008). Chronic lymphoproliferative disorder of NK cell and NK/lymphoblastic lymphoma are included in provisional category. While myeloid/NK cell leukemia is considered as a type of AML and Hematodermic NK cell neoplasm is considered a dendritic cell neoplasm.

Extranodal NK/T-cell lymphoma (angiocentric lymphoma in the REAL classification) constitutes most cases of NK cell neoplasms. The term "nasal" is used when the primary site of involvement is the nasal cavity and the upper aerodigestive tract, while "extranasal" or "nasal-type" represent the counterpart of nasal NK cell lymphomas and involve any other part of the body. Nasal NK cell lymphoma must be excluded by CT scan and/or multiple biopsies from nasal septum before diagnosing extranasal type.

An aggressive NK cell leukemia is an uncommon, aggressive, systemic proliferation of NK cells with more than 30% blasts of NK cell origin with rapidly declining clinical course and poor prognosis.

Hematodermic neoplasm (blastic NK cell lymphoma) is now considered a dendritic cell neoplasm rather than NK cell neoplasm due to following observations (1) This tumor is $CD4^+$/$CD56^+$ and also expresses CD68 and CD123 (the interleukin [IL]- 3 receptor) (Chan et al. 2001) . CD 123 is dendritic cell marker (2) absence of EBV, TIA-1, granzyme B and perforin (all NK cell markers). Skin infiltration of hematodermic neoplasm, NK cell lymphoma and myelomonocytic leukemia are CD4+/CD56+ but skin infiltration of hematodermic neoplasm lacks angiodestruction and necrosis unlike NK cell lymphoma while skin infiltration of Hematodermic neoplasm is esterase/peroxidase/T cell lymphoma one antigen negative unlike skin infiltration due to AML-M4.

Myeloid/NK-cell precursor acute leukemia is CD 56+/CD 33+ and is now considered as AML and not NK cell neoplasm. This leukemia affects young population and has frequent extramedullary infiltration. Precursor NK-cell acute lymphoblastic leukemia and chronic NK-cell lymphoproliferative disorders are included in probable category of NK cell neoplasm. Myeloid/NK-cell precursor acute leukemia and Precursor NK-cell acute lymphoblastic leukemia have poor prognosis. Chronic NK-cell lymphoproliferative disorder is less aggressive and has an indolent course in concordance with the T-cell large granular lymphocytic leukemia.

Chronic lymphoproliferative disorder of NK-cells, previously designated as chronic NK-cell lymphocytosis, chronic NK-large granular lymphocyte lymphoproliferative disorder and NK-cell LGL lymphocytosis, are characterized by a persistent (≥6 months duration) increase in peripheral blood NK-cells ($>2\times109$/L) without an identifiable cause. Since reactive and neoplastic proliferations are difficult to distinguish, it is currently a provisional entity within the WHO classification. NK-cells are surface CD3-, cytoplasmic CD3ε +; CD16 + with weak CD56 expression. Markers of cytotoxic T cells such as TIA1, granzyme B and granzyme M are positive. The expression of CD2, CD7, and CD57 may be reduced or absent while aberrant co-expression of CD5 and CD8 can be seen. Expression of the KIR family of NK cell receptors is either restricted to one isoform or completely lacking. Immunoglobulin and TCR genes are in germ line configuration and karyotype is normal in most cases. In contrast to aggressive NK-cell leukemia, EBV is negative. There is expression of Fas ligand by the neoplastic cells with elevated serum levels (Lim et al. 2009).

Clinical Presentation

ENKL shows a predilection for Asians, Mexicans, Central and South Americans. It represents 3.3% of all non-Hodgkin's lymphoma in Japan, 6% in Hong Kong, 8% in Korea and 5% in Taiwan (Suzuki 2005). The median age is 53 years and the male to female ratio is about 3:1 (Cheung et al. 2003).

Nasal NK/T-cell lymphoma is the predominant histologic type of primary lymphoma of the nasal cavity in the Asian population. The patients present with a mass or progressive ulceration and destruction of the midline facial structures involving the nasal cavity, nasopharynx, paranasal sinuses, tonsils, hypopharynx and larynx. The common symptoms include nasal discharge, nasal obstruction, purulent rhinorrhea, epistaxis and local swelling of the nasal bridge. In patients with more advanced disease, there may be erythema, swelling of the face, proptosis, nasal septal perforation and impairment of extraocular movement. Erosion of the floor of the nasal cavity may result in the destruction and perforation of the hard palate. In about 10% of patients, hemophagocytic syndrome complicates the disease characterized by fever, rapidly developing pancytopenia, hemophagocytic histiocytes in the bone marrow, coagulopathy, and rapid deterioration in liver function. Lymph node involvement at presentation is uncommon and there is rare involvement of distant sites such as the skin, liver, lung, gastrointestinal tract and testis. Thus, most patients (~82%) have an early stage disease (stage I/II) at presentation (Siu et al. 2002).

Extranasal NK cell lymphomas ("non-nasal" or "nasal-type" NK/T cell lymphomas) represent the counterpart of nasal NK cell lymphomas

involving sites other than the nasal cavity and nasopharynx. The median age of presentation and male predominance is similar. Most patients have involvement of multiple anatomic sites at presentation, in the absence of superficial lymphadenopathy. The predominant sites are skin, upper aerodigestive tract (such as palate and larynx), gastrointestinal tract, salivary glands, spleen, testis, soft tissues, (especially muscle), central nervous system, lungs and liver. Isolated nodal involvement is highly unusual. The primary sites of non-nasal NK-cell lymphomas are also the areas where nasal NK-cell lymphomas tend to disseminate (Cheung et al. 2003). Therefore, the diagnosis of extranasal NK cell lymphoma requires the exclusion of nasal involvement at presentation. The skin lesions may be nodules, plaques with ulceration and necrosis or diffuse erythematous swelling. Intestinal perforation due to necrosis may be seen unlike bowel obstruction in conventional intestinal lymphomas. Common systemic symptoms include fever, malaise and weight loss. Additional sites of the disease are often identified on staging. Hemophagocytic syndrome presents with rapidly developing pancytopenia. The disease is highly aggressive. Chan et al. have reported only 10% 2-year disease-free survival (Chan et al. 1997). The worse prognosis may be because most patients were in stage III or IV, had higher International Prognostic Index, lactate dehydrogenase levels (LDH), low hemoglobin and platelet levels compared with nasal NK/T-cell lymphoma (Cheung et al. 2003).

Aggressive NK cell leukemia, first characterized by Imamura et al. (1988) is often designated as "leukemia/lymphoma" because of the concomitant features of both forms. This is a catastrophic, systemic disease and is more prevalent in Asians than in Caucasians (Chan et al. 2001). The disease is characterized by more than 30% of neoplastic NK cells in the peripheral blood or bone marrow. In contrast to ENKL, there is no gender preference and early age of presentation (median being the third decade). Patients typically are very ill at presentation with fever, liver dysfunction, hepatosplenomegaly, systemic lymphadenopathy, leukemic blood picture and sometimes accompanied by a reactive hemophagocytosis. Coagulopathy and multiorgan failure are common. Serum lactate dehydrogenase and Fas ligand are elevated. In contrast to ENKL, skin lesions are uncommon (Cheung et al. 2003; Ishida and Kwong 2010).

Chronic NK- lymphocytosis (CNKL) is characterized by a chronic expansion of mature looking peripheral blood NK cells ($\geq$600/mL) without lymphadenopathy or organomegaly for $\geq$6 months. The median age of presentation is 60.5 years (range, 7–77 years) without sex predominance. CNKL has a chronic, indolent course. It can present with neutropenia, pure red cell aplasia, vasculitis and fever with unknown origin. Most patients have an indolent course but some may progress with increasing lymphocytosis and/or worsening of cytopenias. Rarely, patients may transform to an aggressive NK-cell disorder (Greer and Mosse 2009). CNKL may be reactive to viral infections or underlying solid tumors, careful search for infection and solid tumor is recommended (Suzuki 2005).

Diagnostic Work-Up

The diagnoses of NK cell neoplasm requires: expression of at least one NK cell marker (CD56, CD16, or CD57); lack of expression of surface CD3, B-cell antigens (CD19 and CD20), MPO and other lineage markers; and/or germline configuration of TCR and Ig genes. EBV status provides the supportive evidence. It is important to distinguish T-cell neoplasms, myelomonocytic neoplasms and myeloid neoplasms with CD56 expression and hematodermatic neoplasm from true NK cell neoplasms (Liang and Graham 2008).

Examination of the nasal tumor by nasal panendoscopy and biopsy of every involved or suspicious area should be done in a case of suspected NK cell lymphoma. The specimen should be as sizeable as possible to avoid including just the necrotic areas, because tumor zonal necrosis is characteristic of NK-cell lymphomas. The specimens should not be fixed in formalin and should be sent fresh to the laboratory for cryostat

sectioning or flow cytometric analysis. This will enable the detection of surface CD3, which distinguishes between T and NK cell lymphomas. NK cell lymphoma lacks surface CD3 while T cell neoplasm shows surface CD3. Surface CD3 is positive in NK cell neoplasm if the tissue is preserved. However, if fresh tumor biopsies are not available, TCR gene rearrangement study helps. TCR genes are germline in NK-cell lymphomas but are clonally rearranged in T-cell lymphomas (Ishida and Kwong 2010; Kwong 2011).

The histological features are similar for nasal and extranasal NK cell lymphoma irrespective of the involved sites. The tumors are characterized by mucosal ulcerations, dense abnormal lymphoid cells infiltration, angiocentric and angiodestructive growth and fibrinoid changes in the blood vessels, coagulative necrosis and apoptosis. Tumor cells may be predominantly small (8% of cases); mixed small and large cells (49% of cases); or predominantly large cells (43% of cases) with moderate amount of pale to clear cytoplasm, irregular or elongated nuclei, granular or vesicular chromatin, and inconspicuous, small nucleoli. Mitotic figures are frequent. Giemsa-stained cytologic preparations show azurophilic granules in the cytoplasm. In some patients, inflammatory cells comprising of small lymphocytes, plasma cells, histiocytes and eosinophils are also seen (Siu et al. 2002; Cheung et al. 2003).

Extranodal NK/T-cell lymphoma are $CD2^+$, surface $CD3^-$, cytoplasmic $CD3^+$ and $CD56^+$ (Ho et al. 1990). Nasal $CD3^+/CD56^-$ lymphomas are also currently categorized as NK/T-cell lymphoma if they express cytotoxic molecules and harbor EBV. If cytotoxic molecules and EBV are negative, they are labeled as peripheral T-cell lymphoma unspecified. The clinical and histologic features of the $CD56^-$ subgroup of NK/T-cell lymphoma are indistinguishable from the $CD56^+$ subgroup. The T-cell receptor genes are in germline configuration in NK cell neoplasms and EBV is nearly always positive. EBV association is strong in Oriental patients, but less consistent in Caucasians. The most commonly observed cytogenetic changes in nasal type NK cell lymphoma are del (6) (q21–q25), del (17) (p12–p13), del (13) (q14–q34), and gain of 1p32-pter (Cheung et al. 2003).

Radiologic imaging of NK cell lymphomas is an essential initial evaluation for staging. Computerized tomographic (CT) scan is better for detection of bony involvement; magnetic resonance imaging (MRI) is superior for soft tissue infiltration. Positron emission tomography (PET) is very useful for other systemic sites. NK cell lymphomas are moderately 18- fluorodeoxyglucose (FDG) avid, with an approximate standardized uptake value of about 5–10. A routine lumbar puncture is not necessary as CNS involvement is uncommon (Ishida and Kwong 2010).

The nasal and upper aerodigestive tract must be thoroughly examined and biopsies taken for patients presenting with the disease at non-nasal sites to exclude nasal NK cell lymphoma with involvement of distant organs. PET scans are useful to detect occult tumors.

An increase in circulating plasma EBV DNA is observed, possibly due to the release of viral DNA following apoptosis of proliferating tumor cells. Therefore, the quantification of circulating plasma EBV DNA in plasma or whole blood by real-time quantitative polymerase chain reaction (Q-PCR) may be a potential surrogate tumor marker in NK cell neoplasm (Ishida and Kwong 2010; Kwong 2011). EB virus encoded RNA-1 in situ hybridization should always be done from bone marrow in NK cell lymphoma as its presence suggests marrow infiltration and poor outcome. CD 56 + cells are known to be lost in marrow and hence immunohistochemical stain for CD 56 is not useful to demonstrate marrow involvement (Lee et al. 2007) .

The leukemic cells may be typical large granular lymphocytes or lymphocytes with enlarged, convoluted nuclei, condensed chromatin, distinct nucleoli, and abundant pale blue cytoplasm containing fine or coarse azurophilic granules. In tissue specimens, the neoplastic infiltrate is diffuse, destructive and permeative. The lymphoid cell population appears monomorphous, but can sometimes be polymorphous. Apoptotic bodies, areas of zonal cell death, necrosis, angioinvasion and angiodestruction are common (Liang and Graham 2008).

Immunophenotypically, these leukemias are similar to extranodal NK/T-cell lymphomas

(CD56 $^{+}$, CD2^{+}, CD7^{+}, cytoplasmic CD3ε $^{+}$, surface CD3^{-}). CD16 is expressed in half of the aggressive NK-cell leukemias, unlike extranodal NK/T-cell lymphomas. The T- cell receptor gene is in germline configuration and EBV is always expressed (Siu et al. 2002). Like ENKL, the most common cytogenetic abnormalities observed in aggressive NK-cell leukemia are deletion of 6q21–q25 and loss of 17p13 (Greer and Mosse 2009).

In CNKL, bone marrow infiltration by small lymphocytes is interstitial or intrasinusoidal. Occasionally, patients present with slow progressive increase of peripheral blood NK cells with organ involvement. EBV is not usually associated with CNKL. The circulating neoplastic cells are morphologically large granular lymphocytes. Moderate amount of pale cytoplasm containing ≥3 azurophilic granules are also observed. The NK cells are CD2+, surface CD3–, cytoplasmic CD3ε+, CD16+ and CD56+ with variable expression of CD57. Bright, uniform CD94 and decreased CD161 expression may be seen. Cytotoxic markers including TIA-1, granzyme B and granzyme M are positive. Altered expression of NK-associated KIR (killer-immunoglobulin like receptors), C-type lecithin-like receptors and natural cytotoxicity receptors have also been described.

To summarize, diagnosis of NK cell neoplasm depends on following data (although not all are required to make a complete diagnosis): (1) a thorough clinical history and physical examination (2) expression of CD56 and the lack of expression of T cell specific antigens. Absence of surface CD3 and presence of cytoplasmic CD3 (3) expression of cytotoxic granule proteins, such as TIA-1, granzyme, and/or perforin; (4) in situ hybridization for EBER to demonstrate EBV positivity; and (5) germline configuration of T cell receptor/Vb chain (Hasserjian and Harris 2007).

Treatment Protocols

The treatment strategy for NK cell neoplasm has not been fully standardized due to recent recognition of these disease entities, their low frequencies and lack of controlled trials. For nasal NK cell lymphomas, combined-modality therapy (radiation and chemotherapy) serves as the best treatment regimen. On the other hand, chemotherapy is the mainstay of treatment for patients with non-nasal NK cell lymphoma and aggressive NK cell leukemia/lymphoma. Unlike conventional lymphomas, anthracyclines are less effective (Kwong 2011). Frontline high dose chemotherapy and hematopoietic stem cell transplantation (HSCT) needs to be evaluated on an individual basis (Kwong 2009). Novel treatment strategies under investigation such as chemotherapy, inhibitors of molecular pathways and monoclonal antibodies are needed to improve the outcome of patients with advanced diseases.

Nasal NK Cell Lymphoma in the Upper Aerodigestive Tract

ENKL is uncommon in children and hence there is little data for children compared to adults. However from the data available, management and outcome of pediatric ENKL is comparable with adults (Wang et al. 2009). Since more than 70% of NK-cell lymphomas develop in the nasal area, much of the clinical data exists for the nasal NK-cell lymphomas. Cure occurs in approximately half of the patient population (Ishida and Kwong 2010). For localized stage I/II nasal NK cell lymphoma, radiotherapy used to be considered as the primary treatment. However, systemic failure occurred in at least 30% of patients which was attributed to the sub-clinical dissemination. Primary chemotherapy was associated with treatment failure in about 40% of localized stage I/II nasal NK cell lymphoma. Therefore, combined chemotherapy and radiotherapy is preferred.

In contrast to other lymphomas that are usually treated with 30 Gy, the radiation dose is typically 50–54 Gy here. A smaller radiation dose has been associated with inferior outcomes when used alone, even after a complete response (CR) has been reached. The radiation field should include the adjacent areas, such as the paranasal cavity, nasopharynx, palate, tonsils, larynx, or any involved cervical lymph nodes, depending on the primary lesions. Optimal margin should be at least 1 cm from the tumor. However, an intensity-modulated radiation therapy (IMRT) with helical

tomography in addition to MRI gives more promising results than 3D conformal radiation therapy (3D-CRT) in terms of a significantly better planning target volume (PTV) coverage and equivalent or slightly better organs at risk (OAR) avoidance. Whether the dose of radiotherapy can be decreased with concomitant chemotherapy or radio-sensitizer is not known. Early initiation of radiation, concomitantly or sequentially with chemotherapy, gives better results. Adverse effects of radiation include dermatitis, oral mucositis, taste disturbance and nasal bleeding and discharge. Massive bleeding with mortality due to rapid necrosis of tumors has been reported occasionally.

A phase I/II study of concurrent radiotherapy (50 Gy) and three courses of dexamethasone, etoposide, ifosfamide, and carboplatin (DeVIC) in patients with a newly diagnosed stage IE or contiguous IIE localized nasal natural killer (NK)/T-cell lymphoma showed 77% complete response (CR) with a 2-year overall survival (OS) of 78% compared to 45% with the historical control of radiotherapy alone. The most common grade 3 non-hematologic toxicity was mucositis (30%) with no mortality (Yamaguchi et al. 2009).

A phase II trial of concurrent chemoradiotherapy (CCRT) followed by three cycles of etoposide, ifosfamide, cisplatin, and dexamethasone (VIPD) was conducted in newly diagnosed stages IE to IIE nasal ENKL. The overall response rate and the CR rate were 83.3% and 80.0% respectively. The estimated 3-year progression-free and overall survival rates were 85.19 and 86.28% respectively. Only one patient experienced grade 3 toxicity during CCRT (nausea), whereas neutropenia was seen in 12 out of 29 patients (Kim et al. 2009). Controlled trials of concurrent chemoradiotherapy compared with radiotherapy alone or sequential chemotherapy and radiotherapy are required before drawing definite conclusions.

Unlike other lymphomas, very late relapses of early-stage nasal NK cell lymphoma have been described up to 30 years. Half of the relapses arose from local failure and the rest presented with dissemination or distant lesions. Therefore, life-long follow up is advisable (Kwong 2011).

The use of non-invasive diagnostic technique such as [18F]-fluorodeoxyglucose positron emission tomography (FDG-PET) and the detection of EBV-positive lymphoma cells by EBV-encoded RNA (EBER) in-situ hybridization (ISH) in bone marrow, a site relatively insensitive to FDG-PET, will enable more precise tumor staging and facilitate therapy monitoring. However, more systemic approach is to be adopted for distant or disseminated relapse rather than using simple local radiotherapy (Ishida and Kwong 2010).

In nasal NK-cell lymphoma, responses to radiotherapy or chemotherapy might be affected by the site of the primary lesion. For instance, patients with nasal natural killer (NK)/T-cell lymphoma (N-NKTL) were associated with a lower overall response (54% versus 89%) and higher persistent or progressive disease after initial chemotherapy (46% versus 11%; P=0.000) as compared to those with Waldeyer ring NK/T-cell lymphoma (WR-NKTL). The 5-year overall survival and progression-free survival rates were 67 and 56% for N-NKTL and 65 and 47% for WR-NKTL, respectively. Patients with stage II WR-NKTL showed favorable prognosis compared to those with stage II N-NKTL. Compared with radiotherapy alone, patients with early-stage WR-NKTL that received radiotherapy and chemotherapy showed a superior progression-free survival and improved overall survival (Li et al. 2009). However, treatment options for stage I/II NK-cell lymphomas at different sites in the upper aerodigestive tract are not markedly different (Ishida and Kwong 2010). The destruction of the orbital bones or palate can lead to functional disabilities, persistent discomfort or cosmetic problems. Reconstructive plastic surgery may be needed.

Localized NK-Cell Lymphomas at Other Areas

Skin is the second most common site of involvement for limited-stage NK-cell lymphomas following upper aerodigestive area. The condition is typically characterized by multiple lesions, resistance to cyclophosphamide, adriamycin, vincristine and prednisolone (CHOP) therapy with the survival usually less than a year. Persistent CR has been achieved through a combination of INF-α, dexamethasone and narrow-band ultraviolet B phototherapy for skin lesions (Tai et al. 2009).

Chemotherapy for Localized NK-Cell Lymphoma at Sites Other Than Upper Aerodigestive Area

Chemotherapy is the mainstay of treatment for advanced stage NK cell lymphomas. Oncologists frequently used the CHOP or CHOP like regimens as they are frequently used for Non-Hodgkin's lymphoma but CR rate was <20%. The reason may be high expression of the multidrug resistance 1 (MDR-1) gene, leading to high levels of ABC transporters such as P-glycoprotein in NK lymphoma cells and thereby facilitating the active export of many chemotherapeutic drugs including anthracyclines. Hence, non-anthracycline containing regimens may actually be more effective in these patients.

Dose-intensified CHOP (DI-CHOP) (1,250 mg/m^2 of cyclophosphamide, 75 mg/m^2 of doxorubicin, 1.4 mg/m^2 of vincristine (maximum 2 mg) on day 1 and 100 mg/day of prednisolone for 5 days) and involved-field radiotherapy (44 Gy, within 4–6 weeks after the DI-CHOP) improved the results with the CR rate of 76% and the 3-year OS rate of 67%. The combination of methotrexate, etoposide, an alkylating agent (cyclophosphamide or ifosfamide) and a corticosteroid (prednisolone or dexamethasone) [ifosfamide 1.5 g/m^2 (days 1–3), methotrexate 30 mg/m^2 (days 3–10), etoposide 100 mg/m^2 (days 1–3) and prednisolone 120 mg (days 1–5)] with or without subsequent radiation showed a CR rate of 55–65% in limited-stage disease. High-dose radiotherapy at 50 Gy with a range of 15–61 Gy, at a dose per fraction of 1.8–2 Gy is considered to be equally beneficial in younger patients and adolescents, but with substantial late complications (Ishida and Kwong 2010).

Advanced-Stage Nasal and Non-nasal NK Cell Lymphoma

The SMILE regimen (comprising of methotrexate 2 g/m^2 on day 1, ifosfamide 1,500 mg/m^2, etoposide 100 mg/m^2 and dexamethasone 40 mg from days 2 to 4, and L-asparaginase 6,000 U/m^2 every other day from day 8 to day 20, repeated every 28 days) was designed to address the limitations of MDR phenotypes. Neoplastic NK cells lack asparagine synthase activity and hence L-asparaginase has been shown to induce apoptosis of tumoral NK cells in vitro. Phase I and II studies have confirmed the effectiveness of the SMILE regimen in refractory and relapsed NK/T-cell lymphoma patients, approximately half of whom were in stage IV disease (Ishida and Kwong 2010; Kwong 2011). The overall response rate was 67% and the complete response rate was 50% in the Phase I study, while that in Phase II was 74 and 38% respectively. Because of the profound myelotoxicity, granulocyte colony-stimulating factor support is required.

Unpublished data by Tse and Kwong (2010) showed that 100% of patients with stage I-II disease achieved a durable complete remission when treated with a total of six courses of SMILE chemotherapy supplemented with field radiotherapy after the initial three courses. For patients with advanced-stage disease, a total of six courses of SMILE chemotherapy are usually given. Allogeneic hematopoietic stem cell transplantation should be considered to maintain the remission. High dose chemotherapy with autologous hematopoietic stem cell rescue does not appear to offer substantial benefit. CNS prophylaxis is helpful in NK cell prognostic index group III/IV lymphoma (Kim et al. 2010).

Salvage Treatment of NK-Cell Lymphomas

Patients with relapsed or refractory disease have a very poor outcome. The administration of L-asparaginase in monotherapy or in combination chemotherapy has resulted in favorable responses. Small retrospective studies have observed very good response and survival rates in patients treated with L-asparaginase, combined with vincristine, vinblastine, dexamethasone or methotrexate. A multi-centric open-label French prospective phase II trial in 19 patients confirmed the excellent activity of L-asparaginase-containing regimens with CR in 61%. The median OS and response duration were 1 year. The main adverse

events were hepatitis, cytopenia, and allergy. A significantly better outcome was probably due to the absence of anti-asparaginase antibodies and the disappearance of Epstein-Barr virus serum DNA (Jaccard et al. 2011). Various L-asparaginase preparations (Escherichia coli-derived, Erwinia-derived and pegylated forms) appear to have similar treatment results (Kwong 2011).

Aggressive NK-Cell Leukemia/ Lymphoma

Few treatment successes have been reported for aggressive NK-cell leukemia/lymphoma, considering its rarity and devastating prototype (Patel et al. 2010). Treatment results of anthracycline-based regimens have been unsatisfactory. Only three of 13 patients achieved CR and extension of survival for several weeks only (Ishida and Kwong 2010). L-asparaginase containing chemotherapy with or without allogeneic hematopoietic stem cell transplantation (HSCT) had resulted in prolonged survivals in a few cases, although the approach still needs to be validated (Ishida and Kwong 2010).

Hematopoietic Stem Cell Transplantation (HSCT)

Because of unsatisfactory outcome in advanced staged ENKL, primary-refractory or relapsed patients and aggressive leukemia, the role of autologous and allogeneic HSCT has been explored. Prospective studies are lacking and most reports are retrospective (Ishida and Kwong 2010).

Autologous HSCT

A recent retrospective multicentric analysis compared NK cell lymphoma patients receiving an autologous HSCT at a median of 8.5 months from diagnosis with a historical control group, treated with chemotherapy or radiotherapy only (Lee et al. 2008). The disease status before HSCT was the most important factor affecting the outcome. Patients with early-stage disease had better outcome than those with advanced or refractory disease but the OS did not differ. Therapy-related mortality was 8.5% in the HSCT group, mainly due to infections. HSCT confers a survival benefit in patients who attained CR on post-remission consolidation therapy. Therefore, for patients in CR with high NK/T cell lymphoma International Prognostic Index (NKIPI), HSCT should be considered.

On the contrary, patients with early stage disease limited to the upper aerodigestive area are potentially curable with combined radiotherapy and chemotherapy. The addition of autologous HSCT would probably not improve survival. The role of autologous HSCT in relapsed or advance stage in CR needs to be explored. Although conditioning regimens CBV (etoposide, carmustine and cyclophosphamide), and BEAM (carmustine, etoposide, cytarabine and melphalan) have been frequently used as they are extensively used in NHL and Hodgkin's disease, the optimal conditioning before HSCT will need to be defined (Ishida and Kwong 2010; Kwong 2011).

Allogeneic HSCT

A review of case reports indicates that the majority of patients who received allogeneic HSCT had nasal NK-cell lymphoma and, at the time of transplantation, 69% had recognizable or refractory diseases. Half of the patients were alive after HSCT, with 25% transplantation-related mortality and the rest 25% mortality from progressive lymphoma. In one of the largest retrospective series till date, 32% of the patients with active disease at allogeneic HSCT showed 40% 2 year OS. A graft-versus lymphoma effect was evident when a patient relapsed after allogeneic HSCT achieved a durable remission with discontinuation of immunosuppression. Thus, theoretically, reduced-intensity allogeneic HSCT may significantly reduce treatment-related complications while conserving the benefits of graft-versus lymphoma effect. The potential benefit of allogeneic over autologous HSCT is theoretical graft-versus-lymphoma effect, which is of particular significance as NK-lymphoma

cells express EBV viral antigens, which may be targeted by donor derived cytotoxic T cells. Heterogeneity of these retrospective trials affect accurate interpretation: differences in donor source (such as HLA-matched siblings, unmatched donors, or cord blood), conditioning regimens (presence or absence of total-body irradiation), and timing of HSCT (at remission, during relapse or refractory disease) (Ishida and Kwong 2010; Kwong 2011). Further international collaborative trials are required to decide the optimal role of allogeneic HSCT in NK cell malignancies.

Novel Treatment Approaches

High P-glycoprotein expression by NK cell neoplasm contributes to the resistance to anthracycline based treatment regimens. Novel agents that bypass P-glycoprotein may improve prognosis. However, new studies are hampered by the rarity of the diseases, the geographic variation and the lack of international cooperation (Greer and Mosse 2009).

Gemcitabine, a pyrimidine analogue, and the purine analogues pentostatin, fludarabine, cladribine and forodesine have shown activity as single agents in peripheral NK/T-cell lymphomas and are currently being combined with other agents. Preclinical and clinical studies on pralatrexate, a novel folate analog inhibitor of dihydrofolate reductase, designed to have high affinity for the reduced folate carrier type 1, have demonstrated greater intracellular accumulation and high response rate in peripheral T-cell lymphomas (Greer and Mosse 2009). Mucositis, the dose-limiting toxicity for pralatrexate could be abrogated with folic acid and vitamin B12 supplementation. The combination of pralatrexate followed by gemcitabine has exhibited synergistic activity in vitro and is currently in clinical trials.

Histone deacetylase (HDAC) inhibitors induce histone hyperacetylation and chromatin remodeling and can modify gene expression in cancer cells by changing the conformation of DNA and thus alter gene interaction with transcription factors. Vorinostat and romidepsin (depsipeptide) have been shown to increase histone acetylation and to restore expression of tumor-suppressor and/or cell-cycle regulatory genes—inducing cell-cycle arrest and apoptosis in cutaneous T-cell lymphoma (CTCL). Panobinostat is well tolerated and down regulates genes affecting angiogenesis in CTCL patients thereby inducing clinical responses. Recent data indicates that HDAC inhibitors may up regulate IL-2 receptor expression on malignant T cells, resulting in enhanced susceptibility to killing by agents targeting IL-2, such as denileukin diftitox (Greer and Mosse 2009).

The activation of nuclear factor (NF)-kappaB/Rel proteins plays an important role in the development/progression of B and T cell lymphoid malignancies. NF-κB influences the activation of genes that encodes anti-apoptotic proteins and proteins that affect cell-cycle progression and is mediated by the ubiquitin-proteasome pathway. Bortezomib inhibits the canonical nuclear factor (NF)-κB pathway by preventing proteasome mediated degradation of inhibitor of κBα (IκBα) as well as the alternative processing pathway of the p-100 subunit (Greer and Mosse 2009).

EBV-positive ENKL cell lines, Hank-1, NK-YS, and NK-L, have high Fas surface expression but are resistant to Fas-mediated apoptosis induced by anti-Fas antibodies. Co-treatment of Hank-1 with cycloheximide, a protein synthesis inhibitor, markedly sensitized cells to Fas-mediated apoptosis, activated caspase 8 and downregulated c-FLIP(L) (cellular FLICE inhibitory protein long form) (Greer and Mosse 2009).

Four courses of a farnesyl transferase inhibitor tipifarnib has been found to improve elevated pulmonary artery pressure and erythroid differentiation after disrupting the NK-receptor signaling pathway in large granular lymphocyte leukemia which has poor hematopoiesis (Epling-Burnette et al. 2008).

Various monoclonal antibodies against NK cell or NK-related cells are now available, although there is no ubiquitous marker similar to CD20 in B-cell lymphomas. Alemtuzumab, a humanized anti-CD52 antibody, is highly active in T-prolymphocytic leukemia, but the level of CD52 expression varies widely in T/NK-cell neoplasms. Case reports have suggested responses to bevacizumab, a monoclonal antibody against

VEGF, in angioimmunoblastic T-cell lymphoma with a prominent vascular component. Ongoing trials are investigating the role of denileukin diftitox, alemtuzumab and bevacizumab with combination chemotherapy regimens for NK/T-cell lymphomas (Greer and Mosse 2009).

A phase I dose-escalation trial of siplizumab, a humanized monoclonal antibody to CD2 in patients with T-cell malignancies, resulted in decreased expression of CD2 and depletion of CD4+, CD8+ T cells and NK cells. Although initial responses were encouraging, 13.7% of the patients developed EBV-positive lymphoproliferative disease and the trial was terminated. Another phase I study demonstrated the potential efficacy and tolerance of KW-0761, a defucosylated humanized anti-CC chemokine receptor 4 (CCR4) antibodies, in patients with relapsed CCR4-positive adult T-cell leukemia-lymphoma (ATLL) or peripheral T-cell lymphoma (PTCL). CCR4 receptor is also expressed by a subset of NK cell neoplasm and hence KW 0761 may be useful in NK cell neoplasm also (Greer and Mosse 2009).

NK cell neoplasm is EBV positive but antiviral drugs are not effective as thymidine kinase is not expressed. Arginine butyrate, a short chain fatty acid, induces EBV thymidine kinase expression so that anti-viral drugs can act on these cells. Arginine and gancyclovir have been used in refractory cases with significant anti-tumor activity in 66% of cases (Perrine et al. 2007).

Prognosis

Ann-Arbor staging doesn't correlate well with prognosis in NK cell neoplasm due to various reasons: (1) NK cell lymphomas are almost exclusively extranodal (2) Ann-Arbor staging system is based on the concept of contiguous lymphatic spread and designed mainly for Hodgkin lymphoma and may not always be accurate for NK-cell lymphomas and (3) Ann-Arbor staging system does not take into account the tumor burden. Therefore, a T-staging system, originally designed for sinonasal B-cell lymphoma has been adopted to overcome this problem by taking into account the extent of local tumor involvement (Robbins et al. 1985). T1 denotes confinement to the nasal cavity. T2 indicates extension to the maxillary antra, anterior ethmoid sinus or hard palate. T3 indicates extension to posterior ethmoid sinus, sphenoidal sinus, orbit, superior alveolar bone, cheeks, or superior buccinators space. T4 indicates involvement of the inferior alveolar bone, inferior buccinators space, infratemporal fossa, nasopharynx, or cranial fossa. A number of prognostic models have been designed in this regard, including the International Prognostic Index (IPI), a clinical tool developed by oncologists to aid in predicting the treatment outcome in malignant lymphomas of different grades and subtypes. The index depends on factors like patient's age, stage of the lymphoma, whether or not it is in organs outside the lymph system, performance status of the patient and the serum level of LDH. It allows the doctors to plan treatment better than they could just base on the type and stage of the lymphoma. It also gives patients information about the outlook for their future. The good prognostic factors include:

- Age 60 or below
- Stage I or II (Ann Arbor)
- No lymphoma outside of lymph nodes, or lymphoma in only 1 area outside the lymph nodes
- Performance Status: Able to function normally
- Serum LDH is normal

The poor prognostic factors, on the other hand, include:

- Age above 60 years
- Stage III or IV (Ann Arbor)
- Lymphoma is in more than 1 organ of the body outside of lymph nodes
- Performance Status: Needs a lot of help with daily activities
- Serum LDH is elevated

Each poor prognostic factor is assigned 1 point. Patients without any poor prognostic factors would have a score of 0, while those with all the poor prognostic factors would have a score of 5. The index divides patients into four risk groups:

- Low (0 or 1 poor prognostic factors)
- Low intermediate (2 poor prognostic factors)
- High intermediate (3 poor prognostic factors)
- High (4 or 5 poor prognostic factors)

Patients with IPI of one or less have been shown to have a better overall survival. Two other prognostic models based on the IPI concept have also been proposed. Considering B symptoms, stage, LDH level and regional lymph node involvement, stage I/II nasal NK cell lymphomas were found to be better stratified into different risk groups (Kwong 2011). In another prognostic model, non-nasal type, stage, performance status and number of extranodal involvement were found to be significant in predicting outcome (Suzuki et al. 2010).

In conclusion, NK cell tumors are uncommon, aggressive, and heterogeneous group of disorders with varying geographical prevalence and dismal clinical outcome. They are clinically subdivided into nasal NK-cell lymphoma, non-nasal NK-cell lymphoma and aggressive NK-cell leukemia/lymphoma. The diagnosis is based on the morphology, immunohistochemistry, demonstration of EBV and cytogenetic study. The management requires high dose local radiotherapy and non-MDR-dependent drugs due to P-glycoprotein expression. L-asparaginase based regimens like SMILE appear to be promising. Autologous and allogeneic HSCT have been recommended in selected patients with a poor prognosis. The novel agents that bypass P-glycoprotein expression or that target molecular pathways or surface receptors are under investigations.

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Part IV

Lymphoma

Pediatric Lymphoma Patients: Cytomegalovirus Infection

15

Samah A. Loutfy

Contents

Abstract

Cancer care has improved and intensified over recent decades, and as patients with cancer survive longer, various infectious complications have been more pronounced. Pediatric cancer patients are at high risk of infectious complications because they are immunologically immature. Human cytomegalovirus (HCMV) is a persistent pathogen, can cause life threatening infection in immunocompromised patients, such as bone marrow and organ transplant recipients, persons with AIDS, and patients with hematological malignancies (leukemias and lymphomas). Despite Previous studies before application of sensitive molecular methods, demonstrated association of CMV with fever and hepatitis in children with malignancy, CMV has not been extensively studied in pediatric cancer patients. Very limited data are available in the literatures related to symptomatic CMV infection and its clinical relevance on outcome of diseases in pediatric cancer patients especially children with hematological malignancies. These studies are relevant as new advanced diagnostic techniques are now available for detection of the virus in different clinical specimens, new advances in the management of CMV infection and disease have been developed, and the performance of prospective clinical trails of antiviral agents has been evaluated. Therefore, the aim of this review is to shade a light on some of these data that are available

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in the literatures, and relevance of future studies concerned activity of CMV in pediatric lymphoma patients.

Cytomegalovirus Background

Human cytomegalovirus (HCMV) is a DNA virus of the betaherpesviridae family, with a diameter of 200 nm, linear double stranded DNA is approximately 240 kb in size. It is the largest member of the herpesvirus family. It has unique long (UL) and short sequences (US), both of which are bounded by homologous repetitive sequences. It encodes about 200 open reading frames. Until now only 33 structural proteins and some infected cell proteins are known. CMV replicative cycle has been divided into three independent times periods-immediate-early (IE: defined 2–4 h post infection), early (E: 8–24 h post infection), and late (L 12–36 h post infection) based on the appearance of different classes of CMV-specific proteins during each interval. Expression of both E and late genes is dependent on IE gene expression (Sinclair and Sissons 2006). The first CMV infection in humans was probably recorded in the year 1881 by Ribbert (Vancíková and Dvorák 2001). Various strains of CMV, that can consequently infect the same patient, exist.

CMV infection is distributed worldwide, with geographic differences explained by socioeconomic differences of exposure. In developing countries where poor hygiene and overcrowdness, and low socioeconomic status, children acquire infection early in life, and seroprevalence approaches 100% by early adulthood. In contrast, in developed countries, the seroprevalence of CMV approximates 50% in young adults (Wang et al. 2011). Sources of virus include oropharyngeal secretions, urine, cervical and vaginal secretions, spermatic fluids, breast milk and blood. Vertical spread is transplacental. An important route of infection is iatrogenic-solid organ (SOT) and bone marrow transplantation (BMT) and blood transfusion (Vancíková and Dvorák 2001). Except for a mononucleosis-like illness in some persons, infection with CMV rarely causes disease in immunocompetent individuals. Therefore, CMV disease is restricted to the immunocompromised host (Vancíková and Dvorák 2001; Loutfy and Mansout 2000).

CMV has evolved several strategies to avoid its elimination and eventually hides itself in a silent state, referred as "viral latency" with absence of any detectable production of infectious virus, but kept the ability of viral genome to reactivate under specific stimuli. There is a possibility that CMV reactivation occurs routinely in normal, healthy virus carriers, but this is unlikely to present a problem in the immunocompetent, due to a robust CD8+ cytotoxic T-lymphocytes (CTL) response to the virus. Consistent with this is the observation that the T-cell repertoire of healthy seropositive individuals contains a strikingly high frequency of CTLs that recognize CMV epitopes. It is also still unclear whether any increased frequency of cells reactivating CMV from latency results from immunosuppression per se; reactivation itself could be stimulated greatly by numerous cytokines elicited by other infections, allogeneic stimulation, transplant rejection or graft-versus-host disease-all of which often result in, or are treated by, immunosuppression (Sinclair and Sissons 2006).

In vivo studies have demonstrated a particularly strong relationship between CMV and DCs and showed that persistence of CMV is associated intimately with the normal program of myeloid-cell differentiation; it is the changes in the internal cellular environment that accompany differentiation that promote virus reactivation.

Therefore, future studies will be needed to define precisely the biochemical triggers responsible for myeloid DC differentiation as these also appear to promote the switch from viral latency to reactivation (Sinclair and Sissons 2006).

Cytomegalovirus Infection in Patients with Hematological Malignancies

Cytomegalovirus continues to be a significant cause of morbidity and mortality in immunocompromised hosts, including those with human immunodeficiency virus (HIV) infection and patients following allogeneic stem cell transplantation

(SCT) or organ transplantation. The virus causes such CMV related diseases as pneumonia, enterocolitis, and retinitis, Ganciclovir and foscarnet are effective drugs for treating CMV caused disease, but they have various side effects, including pancytopenia and renal dysfunction. Moreover, inappropriate dosage regimens can lead to the appearance of drug resistant virus strains (Ikewaki et al. 2003).

T-cell function plays a crucial role in maintaining CMV in the latent stages and in controlling CMV infection. Therefore, patients with impaired cellular immunity such as leukemia or lymphoma are at higher risk for developing CMV antigenemia (A) and disease (D) due to insufficient lymphoid control (both humoral and cellular) (Han 2007; Torres et al. 2006). Furthermore, T-cell depleting agents (e.g. alemtuzumab) and aggressive chemotherapy (e.g. hyper-CVAD, and acute leukemia induction) appear to increase the risk of CMV infection and disease (Wade 2006). Reports about incidence of these infections in lymphoma patients have been limited to a few case reports, small case series, and postmortem studies (Torres et al. 2006; Han 2007). Epidemiological studies of CMV infection in cancer patients is important not only clinically for risk assessment and the timely diagnosis and treatment of the infection to allow better management of underlying cancers but also scientifically for better understanding of the virus-host interaction (Han 2007).

Incidence

In the absence of effective antiviral prophylaxis, the incidence of CMV infection among patients with hematological malignancy ranges from 5 to 75% (Wade 2006). An Early prospective surveillance study from the University of Maryland Cancer Center reported an incidence of CMV infection in patients with acute leukemia and ranged from 32 to 58% (Wade 2006). Non-SCT patients had an overall positivity rate of 9.3%, and those with lymphoid hematologic malignancies (CLL, lymphoma and ALL) were affected more than those with myeloid hematologic malignancies (13.6% versus 3.9%, $P<0.001$) (Han 2007; Ljungman et al. 2008). Investigators at the Medical Anderson Cancer Center have reported a series of retrospective studies on the incidence of CMV disease among patients receiving conventional therapy (Torres et al. 2006). Those investigators have reported an overall increase in CMV gastrointestinal disease and CMV pneumonia among patients with lymphoma and acute leukemia. These diseases were associated with high dose of cytarabine, fludarabine, or cyclophosphamide, and increased patient age. CMV attributable mortality for these patients ranged from 30% in lymphoma to 57% in leukemia and up to 90% in those undergoing HSCT (Torres et al. 2008; Wade 2006). Faderl and his co-workers have reported that CMV viremia was detected in 15% of patients with lymphoid malignancy who were treated with alemtuzumab and rituximab (Faderl et al. 2003). Viremia developed a median of 28 days after starting therapy (Wade 2006). Torres et al. (2008) have reported that incidence of CMV pneumonia in 20% of lymphoma patients was mainly in NHL (16% NHL versus 4% HL).

In contrast, another study (Chemaly et al. 2005) has reported that CMV pneumonia is less common among patients with lymphoma (1%: 1.2% in NHL versus 0.6% in HL patients) than among patients with leukemia (2.9%), or patients who have undergone autologous HSCT (2%), solid organ transplantation (17–90%), or allogeneic HSCT (7–20%). They reported that median time from diagnosis of lymphoma to onset of CMV pneumonia was 469 days (range 27–4,682 days) in patients with NHL and 135 days (range 40–275 days) in patients with Hodgkin disease ($P=0.020$).

Epidemiology in Pediatric Lymphoma Patients

Generally, pediatric cancer patients are different from their adults in spectrum of oncologic diagnosis, intensity of chemotherapeutic regimens, and incidence of co-morbid medical conditions preceding diagnosis of cancer (Koh and Pizzo

2011). They reported that some risk factors exacerbate immunocompromise state in pediatric cancer patients enhancing their susceptibility to infectious complications like: alterations in central nervous system function or decreased levels of awareness, obstruction of a hollow viscus, depressed nutritive states this besides maturity of immune system is related to age (Jones et al. 1996). They reported that mortality rate due to CMV pneumonia was higher among lymphopenic patients highlights the important role of lymphocytes in controlling viral infections (Nguyen et al. 2001).

Yee-Guardino et al. (2008) have added, however, that β-herpesviruses are known to be an important pathogens in immunocompromised patients, and they have not been extensively studied in children with malignancies. Children with leukemia have been reported to have a high frequency of active CMV infection (range, 27–46%). But a relatively low frequency of serious CMV disease (range, 3–5%) (Yee-Guardino et al. 2008).

Torres et al. (2008) have reported that CMV disease specific mortality rate reaches up to 30% in lymphoma patients. In our previous report, CMV infection have been detected in 34% of pediatric lymphoma patients (Loutfy et al. 2010). Most of CMV infection was among NHL patients of B subtype. This might be as reported previously due to exposure to more selective suppressive chemotherapy such as methotrexate, corticosteroids and cyclosporine that leads to diminished T cell function with disappearance of CD8 cytotoxic population (Chemaly et al. 2005). Recently, a retrospective study has been performed in Taiwan, showing that 29.9% of their hematological malignancy adult patients suffered from CMV viremia with a mortality rate of 43.8% (Wang et al. 2011).

Severity of CMV Disease

Serious CMV disease is especially high among patients with impairments in their cell mediated immunity. Disease manifestation varies in severity depending on degree of host immunosuppression. In patients with hematological malignancies CMV infection can cause a wide variety of disease manifestations, including fever, cytopenia, esophagitis, enterocolitis, hepatitis, cystitis, pneumonitis, retinitis, encephalitis, marrow suppression and disseminated disease. (Nguyen et al. 2001; Wade 2006). Pneumonitis, gastro-intestinal disease and retinitis are serious complications of CMV reactivation in patients with non-Hodgkin's lymphoma (Ducancelle et al. 2004). At MDACC, they observed that the frequency of serious CMV disease and of CMV pneumonia in particular among patients with hematological malignancies, escalated steadily during 1990s (Nguyen et al. 2001). Table 15.1 summarizes some of the most common clinical manifestations in patients with hematological malignancies.

Table 15.1 Clinical manifestations of CMV infection in patients with hematological malignancies

Clinical manifestations	Type of malignancy	Patients diagnosed/ patients reviewed (%)	Diagnostic test	Reference
Leukocytosis	Hematological malignancies	5/32 (15.6)	Real time PCR	Wang et al. (2011)
Neutopenia		13/32 (40.6)		
Lymphopenia		24/32 (84.4)		
Pneumonia	Adults with leukemia	61/2,136 (2.9)	Cell culture, IHC, histopathology	Nguyen et al. (2001) and Torres et al. (2008)
	Hematological malignancies	16/25 (64)		
Gastrointestinal	Hematological malignancies, and solid tumors	47/236,113	Cell culture, in situ hybridization, IHC, histopathology	Torres et al. (2006)
		15/47 were lymphoma		
Retinitis	CLL	Case report	PCR	Church et al. (2007)

Risk Factors Associated with CMV Disease

Some risk factors showed to be associated with CMV viremia and not only have an impact on outcome of cancer disease but also may be used in combination to identify patients at the highest risk of CMV disease in whom early intervention might be of greatest value (Meyers et al. 1990). They demonstrated that seropositive patients, older patients, patients with acute graft-versus-host disease were more likely to develop CMV pneumonia than were patients without these characteristics.

In the study of Wang et al. (2011), univariate analysis showed that mechanical ventilation, leukocytosis, hypoalbuminemia, and lack of appropriate early treatment were associated with higher mortality among patients with underlying diseases (hematological malignancy and solid tumors) suffering from CMV viremia. In the multivariate analysis, mechanical ventilation, leukocytosis, and lack of appropriate antiviral therapy were independent risk factors for mortality associated with CMV viremia in cancer patients. This indicates that CMV viremia had poor outcomes in cancer patients.

In another study, multiple logistic regressions identified complete remission and long duration of lymphopenia (>3 months) as independent factors associated with fatal CMV pneumonia in lymphoma patients (Torres et al. 2008). In addition, in their autopsy series they demonstrate other common factors that may be used to identify patients at risk of fatal infection, among these predictors, herpes simplex virus infection/reactivation that seemed to be a marker of presumptive cellular immunosuppression preceding the onset of CMV pneumonia in the study patients. Other previous studies have reported that HHV6 infection is one of the major contributions for induction of an immunosuppression state in patients with BMT and solid organ transplantation associated with active replication of CMV in blood compartment and affects both clinical picture and prognosis in those patients (Loutfy et al. 2010).

Chemaly et al. (2005) have reported some predictors of death due to CMV pneumonia in lymphoma patients on univariate analysis included, a high APACHE II (higher Acute Physiology and Chronic Health Evaluation II) score (>16), this may be as reported by Wang et al. (2011) due to leukocytosis which is a criterion of systemic inflammatory syndrome and have higher APACHE II score (Chemaly et al. 2005; Wang et al. 2011). Admission to ICU, lack of antiviral therapy, and development of toxicity to antivirals are other predictors of death due to CMVp. Using multivariate analysis, predictors of death due to CMVp were a high APACHE II score (>16) at onset of CMVp and development of toxicity to antivirals. Patients with high APACHE II score (>16) at onset of CMVp had 15.5 times the risk of dying of CMVp compared to patients with low APACHE II scores (Chemaly et al. 2005).

In an earlier study done by Torres et al. (2006), they have reported that mortality rate with CMV disease in lymphoma patients was 29%, they have identified several risk factors can predict fatal outcome of CMV antigenemia and or/CMV disease in such patients by univariate analysis included, admission to ICU, mechanical ventilation level of LDH, high antigenemia burden (median 133 infected cells /1,000,000 WBC's), active lymphoma disease (progressive disease), relapsed patients, advanced lymphoma stage (III/IV), and antiviral related toxicity. On multivariate analysis only antiviral related toxicity was independent predictor of fatal outcome of CMV antigenemia/or disease in lymphoma patients.

As regards age, sex, and ethnicity and their association with CMV viremia in lymphoma patients. Seroprevalence of CMV is age-dependent, ~about 58.9% of individuals at age of 6 and older are infected with CMV while 90.8% of individuals at age of 80 and older are positive for HCMV (Staras et al. 2006). In the study done by Wang et al. (2011) they observed that mean age of CMV viremic patients with solid organ malignancies was significantly younger than those with hematological malignancies (63 years vs 71.8 years, P=0.03). Torres et al. (2006) have reported that the majority of CMV viremic

patients were men. The median age was 60 years (range 17–87 years). These authors showed in the autopsy study that the median age of patients with CMV pneumonia in lymphoma patients was 43 years (15–76 years). Also, it has been reported that Asian patients with lymphoma and myeloid and other hematological diseases had significantly higher CMV antigenemia rates than whites. This may be explained by higher rates of CMV antibody among Asians and blacks than whites. This suggests the role played by host factors in CMV antigenemia rates and viral burden (Han 2007).

Association with Other Herpes Viruses

In our previous report, it has been observed that both CMV and HHV6 were present in 47% of pediatric NHL cases (Loutfy et al. 2010). Previous studies have addressed explanations for such observations which could be due to: (1) immunosuppression from both NHL disease and its treatment may predispose patients to higher risk of coinfection, (2) An immunomodulating effect of HHV6 since it can induce production of interleukin -1β and tumor necrosis factor- alpha, suppress T lymphocyte function due to reduced interleukin-2 synthesis, and suppress bone marrow by inducing interferon -alpha production. (3) HHV-6 can directly infect CD4+ T-cells and induce apoptosis, thus altering key immune activation molecules pathways and subsequently disturbing the cytokine network. (4) HHV-6 can also infect thymic epithelial cells, hematopoietic stem cells, and natural killer cells, which are critical for immune maturation and protection against cancer and viral infections.

All these factors could contribute to pathologic effects of CMV as a result of HHV6 reactivation, and also create an environment suitable for persistence of HHV6 latency (Wang et al. 2006). In addition, it has been reported that the combination of both HHV6 and CMV infection after organ transplantation was more likely to be associated with CMV disease than with CMV infection alone (Loutfy et al. 2010). Furthermore, our study extended to demonstrate adverse impact of presence both herpes viruses (HHV6, CMV) in pediatric lymphoma patients, as 70% of those patients showed clinical manifestations of severe chest infection and were associated with more frequent episodes of febrile neutropenia (median 3 episodes), long duration of febrile neutropenia > 10 days, absolute neutrophil count (ANC) of < 0.8, thrombocytopenia (plt < 96), and low Hb concentration (Hb < 9.1). However, these data are limited by interference of lymphoma treatment which could aggravate suppressive effect of presence of both herpes viruses (Loutfy et al. 2010).

A few clinical studies have investigated whether there is an association between CMV and Epstein-Barr virus (EBV) reactivation in the blood compartment of immunosuppressed patients. While they have been found that reactivation of each virus occurred independently, others have shown an association between CMV infection and the serologic profile of EBV reactivation. In vitro studies have shown as well that there might be an association between CMV and EBV (Bauer et al. 2007).

Laboratory Diagnosis for CMV Infection/Disease in Pediatric Lymphomas

Early and accurate diagnosis and reliable methods for monitoring CMV infection are essential for managing adult T- cell leukemia-lymphoma patients (Ikewaki et al. 2003).

The conventional methods for the diagnosis of CMV infection/disease are viral isolation by viral culture, serology which includes CMV specific antigen and antibody detection, molecular method for detection of viral DNA from blood and clinical specimens. Although serology is sensitive and specific, results are not helpful in immunocompromised cases because, (1) not rapid due to the need to obtain a convalescent serum sample 10–14 days after initial sample, (2) in certain types of immunocompromised patients, the ability to mount an IgM response may be impaired; therefore, IgM is not reliable for diagnosing active infection (Drew 1992).

Viral isolation done by either tissue culture or shell vial is the most specific diagnostic test and

till now was regarded the gold standard, but it is labor intensive and take time (24–48 h) till the results are available. Hence, other rapid methods such as detection of pp65 antigen from peripheral blood leukocytes (antigenemia assay) and CMV DNA are preferred for diagnosis (Jain et al. 2011). A valuable feature of the CMV antigenemia assay is that it is rapid (4–5 h), quantitative, antigenemia became positive 8± 7 days before onset of symptoms while antibody response observed 4 ± 9 days after onset of symptoms. Therefore, antigenemia test is useful in monitoring infection and antiviral treatment in immunocompromised patients, because high levels of antigen are frequently found in patients with CMV disease and low levels correlate with asymptomatic infections (Loutfy and Mansout 2000). However, there is disadvantage to this method, it couldn't distinguish between primary and reactivated infection (Vancíková and Dvorák 2001).

Molecular methods considered to be the relevant diagnostic methods for detection CMV DNA in various samples. PCR is highly sensitive and specific method that is now being applied in a quantitative or semi-quantitative manner. It has the ability to detect minute amounts of nucleic acid in various clinical samples, and can detect the onset of CMV viremia 1-2 week prior to culture and antigenemia tests. However, its inherent sensitivity poses a problem because latent CMV genomes, which are present in leukocytes of practically all seropositive individuals, may be amplified (Razonable et al. 2002).

Quantitation of CMV DNA

Quantitation of CMV DNA in plasma and other biological samples is very useful for rapid diagnosis of infection and effective monitoring clinical course of disease and response to therapy. Therefore, it can be used as an early indicator of development antiviral resistance as CMV DNA in the plasma tend to persist longer after therapy than pp65 antigens (Razonable et al. 2002). Preliminary data suggests that various clinical manifestations, such as prolonged fever, pneumonitis, heart failure, and retinitis, existed in immunocompromised patients with heavy viral burden (Han 2007). Difference between viral load among symptomatic patients when compared with asymptomatic patients in kidney transplant patients (KR) was reported. In study from Kuwait, they reported that median viral load (4.7log10 copies/ml) of symptomatic KR was significantly higher than that found among asymptomatic KR (2.2 log 10 copies/ml) (Madi et al. 2007). Such data are not available in the literatures, particularly in patients with malignancies with CMV infection and or /disease (Wang et al. 2011). However, as we mentioned before regarding data reported concerned antigenemia rate which is one of the risk factors for development of fatal outcome of CMV disease in lymphoma patients (median number of CMV infected cells per 1,000,000 WBCs was higher in patients with CMV-disease compared to those with antigenemia (median 18 vs 5 cells). Meyers et al. (1990) have reported that CMV viremia had a higher positive predictive value before the onset of CMV disease, particularly prior to pneumonia and gastrointestinal diseases. Prevention of the progression of CMV infection from asymptomatic excretion to symptomatic CMV disease depends on a number of factors like: (1) the interval between the first excretion and the onset of clinical disease, they reported that the median interval between CMV viremia and occurrence of CMV disease was 14 days which is sufficiently long to allow initiation of antiviral chemotherapy, (2) Rapid and higher test sensitivity, and (3) disease prevalence in seropositive patients. These data when combined with viral load might help clinician in the early identification of patients at high risk for fatal outcome due to CMV viremia and increase opportunity of early intervention in the course of infection before the onset of disease.

All possible definitions that related to diagnosis of CMV infection and disease have been published for application in immunocompromised patients and summarized in Fig. 15.1. They recommended that CMV syndrome which can cause fever and bone marrow suppression (neutropenia and thrombocytopenia), these symptoms can be associated with other causes in stem cell transplant recipients, including human

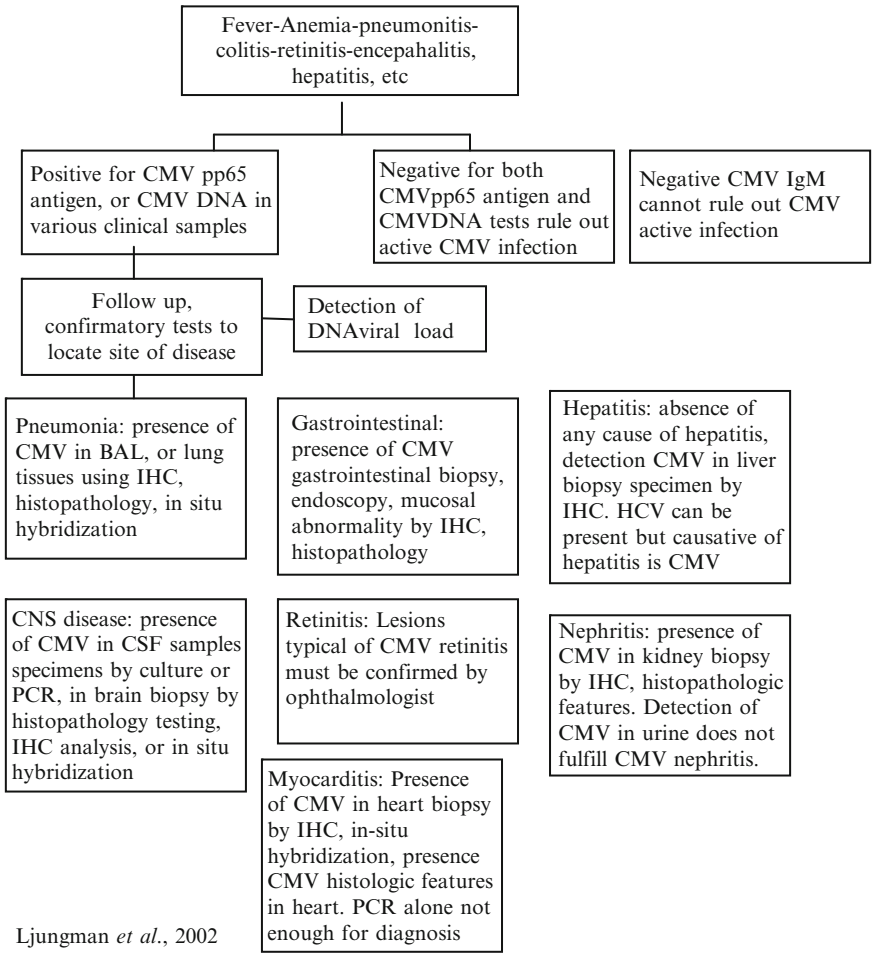

Fig. 15.1 Systematic approach for diagnosis of CMV infection/disease in immunocompromised host (Ljungman et al. 2002). *ICH* immunohistochemistry

herpesvirus 6 (HHV6). Therefore, term CMV syndrome can be used but at least after testing HHV6 and it is important that cases of CMV syndrome be differentiated from cases of end organ disease (Ljungman et al. 2002).

Treatment of CMV Infection/Disease in Immunocompromised Patients

The choice of antiviral drugs depends on; individual patient, risk of progression of CMV disease, and risk of side effects of chosen drug (Ljungman et al. 2008). Three major therapeutic approaches are currently employed to manage CMV infections and diseases; (1) prophylactic, (2) preemptive, and (3) disease treatment.

Prophylactic treatment. This strategy of treatment is started in the absence of detectable virus or disease, aimed to prevent CMV infection or reactivation in patients at risk of subsequently developing disease. The potential benefit of the prophylactic treatment is to prevent infection during 3–6 months after transplantation. Although this can decrease early CMV disease, the mortality rate was not changed because intercurrent

infections and high incidence of late CMV disease (Yeung et al. 2009). Ganciclovir administered intravenously (DNA polymerase inhibitor) for 2 weeks or for at least 3 months following transplantation, reduces risk of CMV disease but didn't improve survival as long lasting treatment was found to be associated with neutropenia and secondary bacterial and fungal infections (Ljungman et al. 2008). Valganciclovir is an effective oral formulation for treatment of CMV infection, is devoid of adverse side effects related to the use of i.v. ganciclovir, cidofovir and foscarnet (Cvetković and Wellington 2005). Foscarnet can be used as an alternative to i.v. ganciclovir in case of marrow suppression or development of resistance (Yeung et al. 2009). Immunoglobulins have no value in prophylaxis.

Preemptive treatment. This strategy was first documented in 1990s, requires the administration of antiviral drug only when patient develops laboratory evidence of CMV infection. In this regard, pre-emptive therapy is usually guided by routine monitoring of CMV infection such as the presence of viral DNA or antigens in the blood prior of the development of symptoms (Wang et al. 2011). Such strategy showed some advantages include: (1) target patients who are at high risk of developing infection and disease, (2) reduce antiviral toxicity, (3) reduce the chances of the emergence of drug resistant mutants, and (4) reduce the cost of treatment (Ljungman et al. 2008). Either i.v. ganciclovir or foscarnet can be used for first line preemptive therapy. Cidofovir can be considered for second line pre-emptive therapy with careful monitoring of renal toxicity. Valganciclovir might be used in place of i.v. agents in low risk patients (Ljungman et al. 2008). Clinical risk factors for CMV disease need to be well defined so that prophylactic and preemptive strategies can be targeted rationally (Nguyen et al. 2001)

Treatment of symptomatic CMV infections and diseases. In case of symptomatic infection, those are patients with CMV viremia (CMV DNA in blood) and showing symptoms compatible with CMV (fever with or without bone marrow suppression) but without signs of CMV end organ diseases, which should be carefully assessed. In SCT, i.v., ganciclovir or foscarnet can be administered as first line of treatment. In patients receiving alemtuzumab, valganciclovir is used in addition to ganciclovir and foscarnet (Ljungman et al. 2008). Failure of preventive strategies leads to development of CMV disease; such disease can develop anytime after SCT from early neutropenic phase up to several years after transplantation. Combination of i.v., gancyclovir and high dose of immunoglobulin is used for treatment of CMV pneumonia. No data support the administration of immunoglobulin for treatment of manifestations of CMV diseases other than pneumonia. Foscarnet might be used in place of ganciclovir. Cidofovir or combination of foscarnet and i.v. ganciclovir can be used as second line of therapy (Ljungman et al. 2008). The development of new antiviral drugs seems very promising, because some of them are able to prevent immunopathogical events triggered by the virus. In addition, they are unlike those targeted CMV DNA polymerase and therefore, suppress active viral replication but do not eliminate the virus (Ducancelle et al. 2004). Maribavir, CMV UL97 kinase inhibitor, does not target DNA polymerase, is considered one of the most promising anti CMV drugs in clinical development. Lobucavir, adefovir-dipivoxil and antisense oligonucleotides are under clinical development (Vancíková and Dvorák 2001). Table 15.2 demonstrates some of the recommendations for the management of CMV diseases in immunocompromised hosts.

Adoptive immunoprophylaxis. It is not standardized for routine use. Several groups have studied the usefulness of adoptive transfer of CMV-specific T cells or vaccination with CMV-primed DC (dendritic cells) (Ljungman et al. 2008). These technologies seem not to be associated with significant toxicity but their effectiveness needs to be further assessed in controlled trials.

Anti CMV Drug Resistance

Antiviral drug-resistant CMV mostly emerges in highly immunocompromised patients such those with AIDS and bone marrow or solid

Table 15.2 Treatment of CMV infection/disease in immunocompromised host

Drug	MOA	Dose	Beneficial role
Ganciclovir (GCV)	Nucleoside analogue	Induction: 5 mg/kg q 12h i.v. , 2–3 weeks, then for pts at risk of relapse 6 mg/kg once a day i.v. for 5 days per week Immunoglobulin (500 mg/kg) every other day for first 2 weeks, then weekly for pneumonia	Prophylaxis, preemptive therapy and treatment of CMV disease
Valganciclovir	Nucleoside analogue	Oral: 900 mg /day for 3 weeks	Prophylaxis, preemptive and treatment of CMV disease
Cidofovir	Nucleoside analogue	i.v. 5 mg/kg infusion once/week for 2 weeks. Then 3 mg/kg once every 2 weeks	Poor results for preemptive therapy and treatment of CMV disease, nephrotoxic
Foscarnet	Pyrophosphate analogue	Induction: 60 mg/kg q 12 h i.v. Maintenance: 90 mg/kg once a day i.v. for 5 days per week for 3 weeks	Second line for preemptive therapy or treatment of CMV disease in case of resistance to GCV or neutropenia

Reusser (2002)

organ recipients with a high systemic CMV load (Drew 2000). Drug-resistant CMV infections have rarely been reported in other clinical settings. However, Erice et al. (1989) highlighted the risk of drug-resistant CMV emerging in patients with blood malignancies. These authors were the first to describe ganciclovir-resistant isolates and one of these isolates was recovered from a patient with chronic lymphocytic leukaemia. Rise in the viral load during first week of antiviral therapy is not an indication of viral resistance (Ljungman et al. 2008), but usually does emerge after several weeks of antiviral therapy. Drug resistance might be clinical or viral. Clinical resistance depends on host factors, but viral resistance is due to mutations in the viral genome. The simultaneous recurrence of multiple strains has been observed in immunocompromised patients (Baldanti et al. 1998). The presence of antiviral resistance can be determined by either phenotypic or genotypic assay. DNA sequencing can be used to screen for the most commonly seen mutations in ganciclovir-resistant strains of CMV (Ljungman et al. 2008). Such assays should be performed to allow selection of correct second line antiviral therapy. Understanding how the CMV genotype changes in the presence of antiviral therapy changes will help to determine the best strategy for long-term anti-CMV treatment.

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Diagnosis of Bone Marrow Involvement in Pediatric Lymphoma Patients: FDG PET/CT Versus Bone Marrow Biopsy

16

Gang Cheng

Contents

Abstract

Bone marrow infiltration (BMI) is common in patients with Hodgkin's disease (HD) and non Hodgkin's lymphoma (NHL), often indicates poor prognosis. Accurate assessment of BMI is critical for staging and selection of proper therapeutic options in lymphoma patients. Up to now, bone marrow biopsy (BMB) is an integral part of initial work-up in these patients, although it has a high false negative rate. In recent years, FDG PET/CT has established as a highly accurate imaging tool in the assessment of Hodgkin's disease and non-Hodgkin's lymphoma. Multiple studies have found that FDG PET/CT in the initial diagnosis work-up detects more bone marrow involvement in lymphoma patients thus are more sensitive and more accurate than bilateral bone marrow biopsy performed at the iliac crest. At the same time, it has been shown that BMB performed based on findings of FDG PET/CT significantly decreased false negative findings and improved accuracy of BMB. In this review, we discuss the value of FDG PET/CT in identifying BMI and in guiding BMB in the initial evaluation of pediatric lymphoma patients.

Introduction

Lymphoma is a common malignancy. Based on the data from the American Cancer Society in 2011, lymphoma is the fifth most common cancer in males and the seventh most common cancer in

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females, with predicted 75,190 new cases (8,830 cases for HD and 66,360 cases for NHL) and 20,620 deaths in 2011. It represents the ninth leading cause of death in male and sixth leading cause of death in female. Bone marrow involvement in Hodgkin's disease (HD) or aggressive non-Hodgkin's lymphoma (NHL) indicates advanced stage of disease and also poor prognosis. However, bone marrow infiltration (BMI) is not uncommon and can occur in patients at seemly early stage of disease. The incidence of lymphoma bone marrow involvement ranges approximately 5–21% in HD patients (Moulin-Romsee et al. 2010; Cheng et al. 2011), 30–50% in NHL patients (Schaefer et al. 2007a; Cheng et al. 2011), and as high as 50–80% in low-grade NHL (Pelosi et al. 2008a). To guide therapeutic planning and to minimize side effects and toxicity when aggressive treatment can be avoided, bone marrow biopsy (BMB) has been performed routinely for decades as an integral part of initial work up in lymphoma patients to assess bone marrow status, and has been considered as a "gold standard".

However, BMB is not a perfect procedure for this purpose. A positive finding on BMB confirms the status of bone marrow infiltration, but a negative BMB finding is less informative. It has been recognized for years that BMB is associated with a high false negative rate. BMB is most commonly performed blindedly at the unilateral or bilateral iliac crest, either anteriorly or posteriorly. Traditionally, this selection of biopsy site is because of the convenience of the procedure and easy access to bone marrow, without considering whether there is evidence of BMI at the biopsy site. At the same time, only limited sampling of small amount of the bone marrow is obtained, because of an invasive nature of the procedure. The blinded selection of biopsy sites and a small specimen make sampling error inevitable, leading to a high false negative rate.

Pathologists were among the first to recognize a high false negative rate (the number of false negative cases on BMB/total positive cases, FNR) of BMB in the assessment of lymphoma patients (Wang et al. 2002). Levis et al. (2004) reported that positive bone marrow involvement was limited to only one of the two specimens of BMB in 35% of cases in a study involving 1,161 HD patients. Similarly, among NHL patients with positive bone marrow involvement of lymphoma, 30% had positive BMI only on one side biopsy specimens (and up to 50% in diffuse large cell lymphoma) (Juneja et al. 1990). The fact that bilateral iliac crest BMB detects more lesions than unilateral BMB confirms potential false negative findings of unilateral as well as bilateral BMB, as demonstrated in numerous studies. For example, Menon and Buchanan (1979) reported that bilateral BMB increased the yield of positive marrow lesions by 26%, as compared with unilateral examination, in a series of 145 patients with HD and NHL.

18F-Fluoro-2-Deoxyglucose (FDG) Positron Emission Tomography and Computed Tomography (PET/CT) Is a Valuable Imaging Modality for Lymphoma

18F-Fluoro-2-Deoxyglucose (FDG) positron emission tomography (PET) is a valuable imaging tool in the evaluation of multiple malignancies. A unique aspect of FDG PET imaging is that it is a functional study independent on morphological changes. FDG is actively transported into a cell via glucose transporters, converted into FDG-6-phosphate by hexokinase, which is trapped within the cell because FDG-6-phosphate cannot be further metabolized. In general, tumor cells have higher expression level of glucose transporters and are metabolically more active, thus will demonstrate as increased FDG activity on a PET imaging (Cheng et al. 2011).

Available data indicate that FDG PET has become a valuable imaging modality in the evaluation of lymphoma, either in adults or in pediatric patients (Cheng et al. 2012). The value of FDG PET in the evaluation of bone marrow involvement in lymphoma patients has been recognized more than 10 years ago (Moog et al. 1998). More recent studies confirmed this finding and provided clear evidence that FDG PET outperforms BMB with more additional positive

findings of bone marrow lesions on the initial evaluation of HD or NHL patients (Fuster et al. 2006; Schaefer et al. 2007a; Pelosi et al. 2008b; Ribrag et al. 2008; Cheng et al. 2011; Purz et al. 2011). The application of FDG PET staging led to changes of clinical management ranging from 8 to 45% in adults lymphoma patients (Allen-Auerbach et al. 2008), and in 10–23% in pediatric patients (Depas et al. 2005), often with upstaged diagnosis due to additional findings of BMI on PET. FDG PET (now more commonly, PET/CT) is now widely with high sensitivity and specificity in the evaluation of lymphoma. In addition to initial diagnosis, FDG PET has been used successfully for response assessment, prognosis prediction, and for detection of residual lesions or recurrence.

In our own study (Cheng et al. 2011), we evaluated BMI in 54 pediatric patients with pathologically proven lymphoma (31 HD, 23 NHL) and 13 of them had BMI. FDG PET/CT revealed additional BMI in six patients who were false negative on BMB, while BMB revealed only one additional case with BMI who was false negative on FDG PET/CT imaging. The overall sensitivity of FDG PET/CT was much higher than that of BMB in detecting BMI by lymphoma (92% versus 54%; $p<0.05$) (Cheng et al. 2011). Our data was similar to previous reports. For example, Fuster et al. (2006) reported that FDG PET had a sensitivity and specificity of 86 and 99% respectively, in contrast to 57 and 100% by BMB, in detecting BMI in lymphoma patients. Schaefer et al. (2007a) found that FDG PET/CT upstaged up to 42% of all cases of lymphoma as regarding to uni- or multifocal BMI. More recently, Purz et al. (2011) reported a retrospective study involving 175 pediatric patients with newly diagnosed classical HD, and found that FDG PET scans correctly detected all 45 cases with BMI out of 175 patients without false positive or false negative findings, achieved 100% sensitivity and 100% NPV in the diagnosis of BMI. In contrast, BMB detected only seven cases among all 45 patients with BMI with a sensitivity of 16% and NPV of 77%.

We noticed that there was a meta-analysis of the value of FDG PET in the assessment of BMI in lymphoma patients, which demonstrated a discrepancy and variable effectiveness of FDG PET for identifying BMI (Pakos et al. 2005). After careful examination of this analysis, we noted that the majority of data cited in this meta-analysis were obtained from old style instruments, on PET-alone machines without corresponding CT images, and many of them did not even have attenuation correction, which is now totally obsolete. These factors could have negative impact on accurate interpretation of FDG PET studies thus on the conclusions as derived from this meta-analysis. Current PET machines are coupled with an integrated CT scanner to obtain complementary PET and CT images and are equipped with multiple artifact correction capabilities, which significantly improve the accuracy of diagnosis. Table 16.1 is a short list of recent published data on the application of FDG PET/CT versus BMB in the assessment of BMI in lymphoma patients, and only studies on the initial diagnosis were included. Three FDG PET/CT studies (Ribrag et al. 2008; Moulin-Romsee et al. 2010; Pelosi et al. 2011) are included in Table 16.1 for the general patient population of lymphoma, and three available PET or PET/CT studies for pediatric patients (Kabickova et al. 2006; Cheng et al. 2011; Purz et al. 2011) are also included. In either general patient population or in pediatric patients, FDG PET outperforms BMB in detecting BMI in the initial evaluation of lymphoma. While BMB and FDG PET both are very specific in detecting BMI in lymphoma patients, FDG PET has an important advantage over BMB, i.e., FDG PET is much more sensitive in this regard (thus less false negative findings), especially for pediatric patients. These studies show that FDG PET or PET/CT had a sensitivity ranging 69–100% (92–100% for pediatric patients) and an accuracy ranging 91–100% (98–100% for pediatric patients), while BMB had a sensitivity ranging 0–60% and an accuracy ranging 78–90%, while the specificity was similar for PET and BMB.

On FDG PET/CT, tumor infiltration of the bone marrow in lymphoma patients is often manifested as focal or multifocal increased FDG uptake (Fig. 16.1). Multiple studies (including our own experience) indicated that multifocal

Table 16.1 Performance of FDG PET/CT versus BMB in detecting BMI in the initial diagnosis of lymphoma

	Studies	Case number	Total BMI	BMI incidence	Accuracy PET	Sensitivity PET	Specificity PET	Accuracy BMB	Sensitivity BMB	Specificity BMB
General patient population	Pelosi et al. (2011)	337	87	26%	91%	69%	99%	90%	60%	100%
	Moulin-Romsee et al. (2010)	83	18	22%	100%	100%	100%	87%	39%	100%
	Ribrag et al. (2008)	47	10	21%	98%	90%	100%	85%	30%	100%
Pediatric patients	Purz et al. (2011)	175	45	26%	100%	100%	100%	78%	16%	100%
	Cheng et al. (2011)	54	13	24%	98%	92%	100%	89%	54%	100%
	Kabickova et al. (2006)	55	8	15%	100%	100%	100%	85%	0%	100%

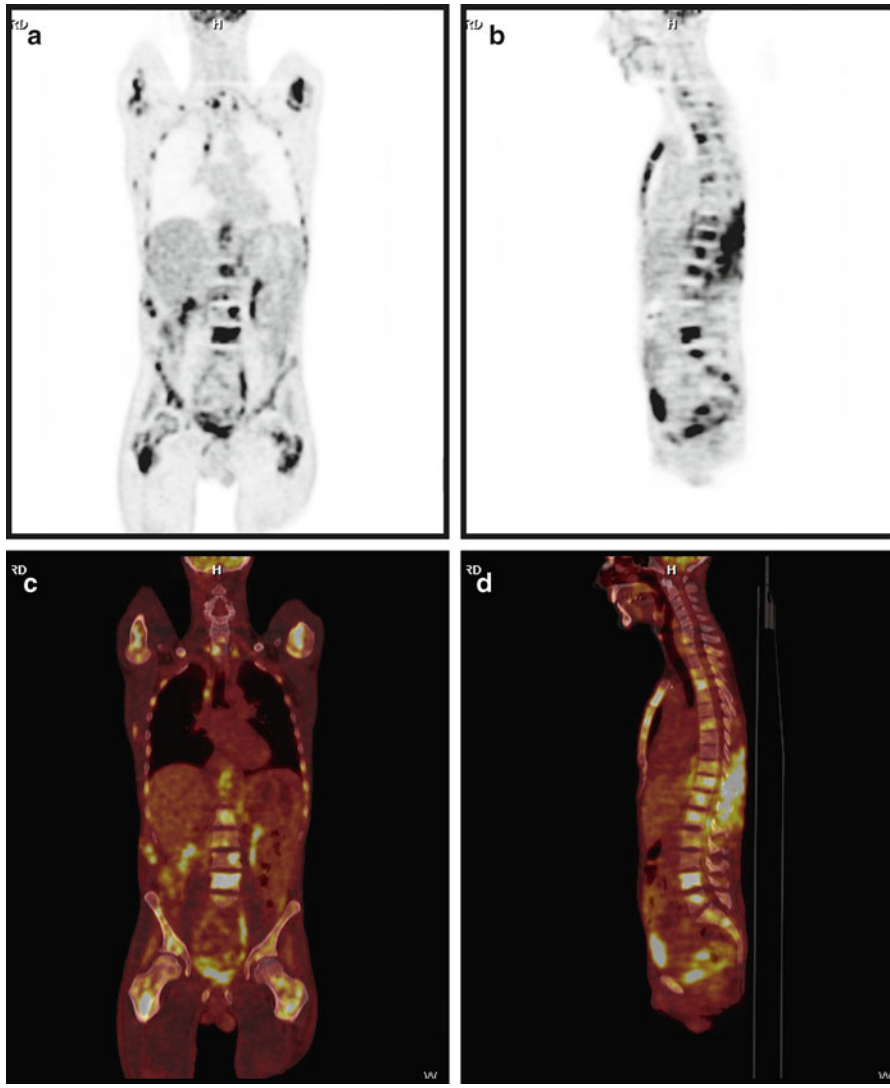

Fig. 16.1 FDG PET/CT was performed in a 54-year old male for initial staging of NHL and revealed numerous foci of FDG-avid bone marrow lesions, characteristic of bone marrow infiltration of lymphoma. FDG-avid soft tissue tumor masses are also noted in the posterior back and in the mediastinum. (**a**, **b**) coronal and sagittal view of PET images; (**c**, **d**) coronal and sagittal view of fused PET/CT image

intense FDG uptake in bone marrow without other clinical explanation is very specific for the diagnosis of BMI (Schaefer et al. 2007a; Moulin-Romsee et al. 2010; Cheng et al. 2011), as confirmed by post FDG PET biopsy. It has to be recognized that focally increased FDG uptake on a PET scan is, by itself, not specific. Many other etiologies (such as osteomyelitis or fracture) can cause similar changes. However, multifocal FDG uptake on a PET scan can be a very specific finding for BMI in appropriate clinical settings: if the patient has no other clinical history (for osteomyelitis, or fracture, etc.), and if the patient has a recently diagnosed lymphoma, and the multifocal FDG uptake on a PET imaging has a typical pattern suggestive of BMI. It is more common for a lymphoma patient to have multifocal lesions of BMI on FDG PET imaging although occasionally a patient may have only one or two bone marrow lesions. Purz et al. (2011) recently

reported in the study involving 175 pediatric HD patients that the majority (32 out of 45 patients) had three or more skeletal lesions on FDG PET imaging.

Several factors may contribute to high accuracy of FDG PET/CT in the identifying BMI in lymphoma patients. First, FDG PET is a functional study independent on morphological changes. It is well known that FDG PET is more accurate than CT imaging in detecting bone marrow lesions. A recent report provided clear evidence that in some cases, morphologic changes on a CT scan occurs after resolution of FDG PET abnormalities (after successful treatment) (Gemmel et al. 2012). Second, PET allows convenient whole body scan and allows assessment of the majority of the whole skeleton system. Third, simultaneous CT images provide important information for anatomic correlation, which is very helpful in the differential diagnosis (for example, to rule out fractures or acute inflammatory disease).

High FNR of BMB Is Due To Sampling Error

The focal or multifocal localization of bone marrow infiltration on FDG PET/CT indicates heterogeneous nature of BMI in lymphoma patients, and that this heterogeneous nature of BMI is likely the underlying cause of high false negative rate of BMB. BMB is a well-established method with sound technique and successful application in soft tissue biopsies, as demonstrated in years of clinical practice. However, if the biopsy missed the location of malignancy, sampling error occurs and false negative finding is inevitable.

In fact, data from bone marrow pathological findings provided strong evidence of the heterogeneous nature of bone marrow involvement in lymphoma patients, indicating a negative effect of inadequate sampling on the diagnosis. Bone marrow aspiration is less accurate than BMB in detecting BMI. For example, Moid and Depalma (2005) reported that among 20 cases of HD patients with a positive bone marrow trephine biopsies, bone marrow aspirate was positive on only one case. Similar findings were reported by others (Subramanian et al. 2007). However, iliac BMB is far from perfect. It is well-recognized that bilateral iliac BMB is more accurate than unilateral BMB in detecting bone marrow lesions in lymphoma and other malignancies (a discrepancy between the left and right side biopsy specimen was identified in 39% for HD samples, 9.2% for NHL samples, 29% for sarcoma samples, 23% for carcinoma samples) (Wang et al. 2002). The fact that an additional biopsy site leads to additional positive finding of bone marrow lesions on BMB indicates focal rather than diffuse pattern of marrow infiltration by lymphoma. Similarly, additional positive finding of bone marrow lesions on BMB can be achieved by increasing the size of biopsy specimen. For example, Campbell et al. (2003) reported that in patients with diffuse large cell lymphoma, 35% of BMB biopsies were positive for BMI if the length of biopsy specimen were ≥ 20 mm, in contrast to only 20% positive finding for BMI if the specimen length were < 20 mm.

A common feature to these techniques (increasing the size of BMB specimen, or by adding additional biopsy site, or by changing bone marrow aspirate to biopsy) to increase accuracy of pathologic examination is increasing the size or tissue volume of bone marrow specimen to be examined. However, the size of BMB specimen is limited, due to invasive nature of biopsy. No matter what biopsy method employed, only a very limited volume of the bone marrow can be directly examined. Because lymphoma infiltration of bone marrow is non uniform, it is easy to understand the underlying reason for high false negative rate of BMB.

While the lymphoma infiltration of bone marrow is often multifocal, it has to be realized that these lesions are often localized in red marrow region regions (including the ribs, spine, sternum, clavicles, scapulas, pelvic bones, the proximal humeri and proximal femurs) in a rather random pattern. FDG PET/CT imaging provides clear evidence that the iliac crest (the site of blinded BMB) is frequently spared even

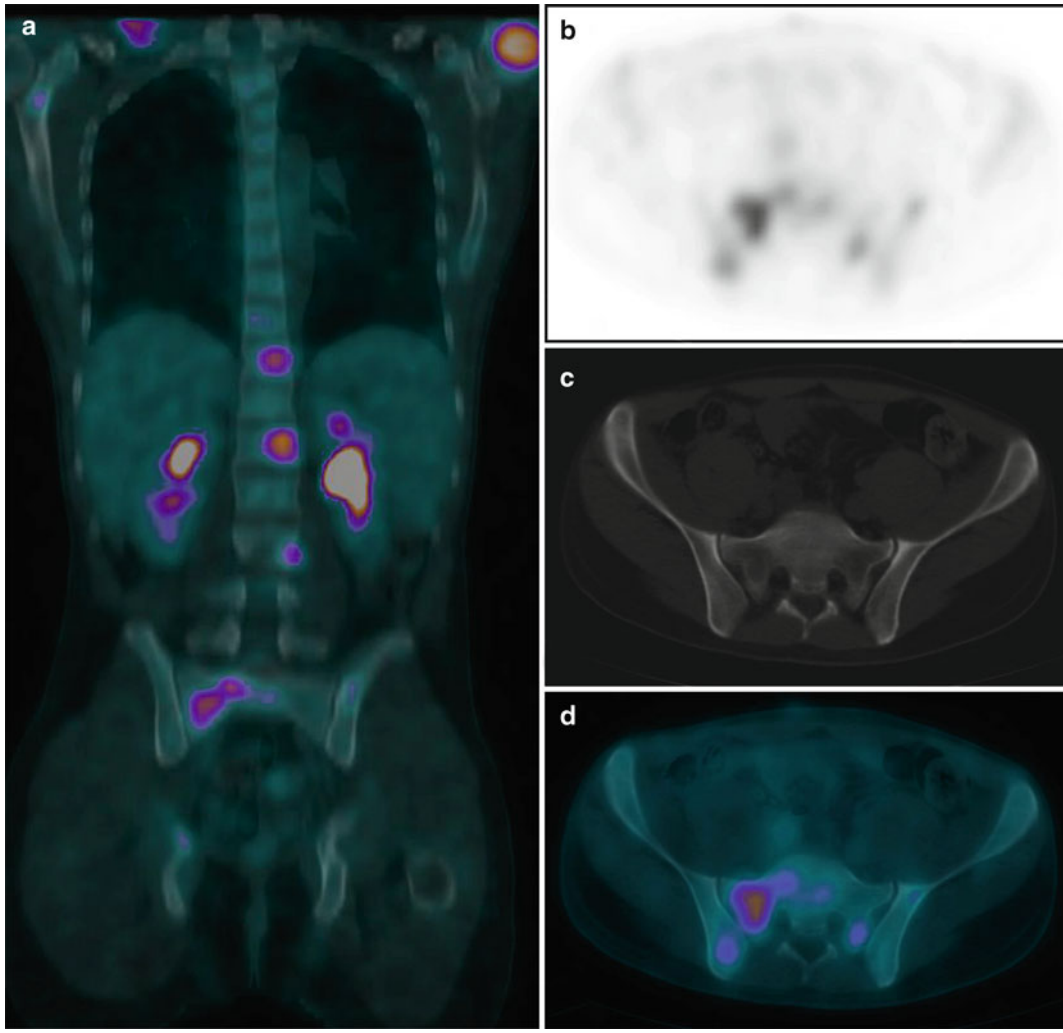

Fig. 16.2 FDG PET/CT was performed in a 22-year old male for initial staging of Hodgkin's lymphoma and revealed multiple foci of bone marrow infiltration including the right scapula, the left humerus, multiple thoracolumbar vertebrae, and the sacrum. FDG-avid soft tissue tumor masses in the *right lower neck* and the mediastinum are not shown. Blind BMB performed on bilateral anterior iliac crests were negative as there was no FDG-avid lesion in the anterior iliac crests. All FDG-avid lesions resolved on repeat PET imaging after chemotherapy. (**a**) coronal fused PET/CT image; (**b**-**d**) transaxial images of PET, CT, and PET/CT, respectively, showing BMI in the sacrum but not in the anterior iliac crests

though there are multiple lesions of BMI elsewhere in the skeleton (Ribrag et al. 2008; Moulin-Romsee et al. 2010; Cheng et al. 2011). An example is provided on (Fig. 16.2). These data indicated that the high false negative rate of BMB is due to sampling error (more specifically, due to blinded selection of the biopsy site), rather than the technique itself.

The High FNR of Iliac BMB Can Be Avoided by Targeted BMB

The high FNR of iliac BMB is not observed in soft tissue biopsy. Upon analyses of the biopsy yielding (the percentage of positive findings among all biopsies) of soft tissue biopsy versus BMB in the same group of pediatric patients with

lymphoma, we found that soft tissue biopsy had a very high yield (91.9% of HD cases and in 96.0% of NHL cases) while the yield of blinded BMB in the same patients was low (only in 6.5% of HD cases and in 20.0% of NHL cases, with an overall false negative rate of 46.2%) (Cheng et al. 2011). This discrepancy can only be explained by sampling error: while soft tissue biopsy sites were chosen based on findings from either physical examination or structural diagnostic imaging tests that suggest a high likelihood of malignancy (Weiss et al. 2008), there was no selection of biopsy site for BMB, as BMB is performed on predetermined site, regardless of potential chance of tumor involvement.

It was interesting to note that the FNR of BMB was low if there was evidence of bone marrow infiltration in the iliac crest on FDG PET imaging. For example, Moulin-Romsee et al. (2010) analyzed 83 cases of HD patients who underwent FDG PET/CT imaging for initial evaluation. All seven cases with positive BMB on the iliac crest had abnormal FDG uptake on FDG PET scan, while BMB was false negative in other 11 cases with bone marrow lesions on FDG PET/CT, because in these 11 patients, BMI was found in regions other than the iliac crest (the biopsy site) on FDG PET. Similarly, we noticed that BMB had a higher yielding in patients with abnormal FDG uptake in the biopsy sites. For all 54 pediatric HD or NHL patients, BMB was positive for malignancy in 85.7% of patients with abnormal FDG PET findings in the biopsy site of the iliac crests, and was positive only in 2.1% of patients with normal FDG PET findings in the biopsy sites. In another ward, except for one case that was false negative on FDG PET/CT, all other cases with positive BMB had focal lesions of abnormal FDG uptake at the biopsy site (Cheng et al. 2011). In addition, it has been reported that targeted BMB performed in regions with suspicious bone marrow involvement (based on abnormal FDG PET findings) is almost always positive (Schaefer et al. 2007a; Muslimani et al. 2008). For example, Schaefer et al. (2007a) reported none of the 18 targeted BMB procedures in lymphoma patients was negative for BMI if performed based on abnormal FDG PET findings, although some of these patients had negative blind iliac BMB.

As discussed above, FDG PET/CT outperforms BMB in detecting BMI. Then the question is BMB still needed? There is no doubt that BMB has a role in confirming a diagnosis of a malignancy that cannot be replaced by any other study. However, in patients undergoing BMB, most likely these patients already had a soft tissue mass biopsy and had a pathologically proved diagnosis of lymphoma. Whether these patients benefit another biopsy of the bone marrow remains controversial and should be further evaluated (Quereux et al. 2009), especially now that FDG PET/CT provides better diagnosis in this regard. However, it is becoming clear that BMB has little additional value of FDG PET/CT in detecting BMI in lymphoma patients (Cheng et al. 2011). While it remains to be determined if these patients should have a BMB procedure, it is more clear as regard to when and where the biopsy should be performed, if it is performed anyway.

Since FDG PET/CT detects more BMI in lymphoma patients, and since BMB performed at the site with focally abnormal FDG uptake on a PET/CT imaging has a high accuracy to reveal malignancy, it is reasonable to recommend that BMB should be performed in the region of bone marrow with abnormal FDG uptake, and for this purpose, BMB should be performed after FDG PET/CT. It is important that BMB is performed based on the findings of FDG PET/CT so that the findings on bone marrow pathology can best reflect the tumor status of a patient. We believe that BMB should no longer be performed in a blinded area of the iliac crest and should not be used as a screening technique. If BMB is performed in selected regions in selected patients with high risk of bone marrow involvement, its finding will be more valuable in guiding our clinical practice.

The Value of FDG PET/CT in Post-Therapy Bone Marrow Evaluation Remains to Be Defined

Please note that above discussion is limited to initial diagnostic evaluation of lymphoma. Whether these findings apply to interim evaluation or post-therapeutic follow up remain to be determined. It has to be emphasized that focal increased FDG uptake on a FDG PET scan is a

nonspecific finding (i.e., not specific for malignancy) although the pattern of multiple focal abnormality can be specific enough to represent BMI under appropriate clinical settings. Two factors complicate the evaluation of lymphoma patients who had received treatment. The first is chemotherapy. Most chemotherapy will significantly compromise the immune system and make patients prone to infection, and infection of any kind is a common cause of false positive findings on a FDG PET. The second is that these patients on chemoradiation therapy will likely receive supportive treatment such as granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF). These growth factors stimulate proliferation (and the metabolism) of the bone marrow, leading to increase background FDG uptake (Salaun et al. 2009). On FDG PET imaging, these patients often have significantly increased and diffuse FDG uptake throughout the bone marrow, making it difficult to detect occult bone marrow lesions, although a negative finding on FDG PET may still have a good negative predictive value. Still, available data indicate that FDG PET/CT is highly accurate and outperforms CT alone or CT in combination with iliac BMB in lymphoma patients after the end of treatment and at further follow-up, with overall sensitivity, specificity, PPV and NPV of 100, 91, 85, and 100%, respectively (66 patients with Hodgkin lymphoma were analyzed, and all patients with positive FDG lesions had surgical biopsy for histopathologic confirmation) (Schaefer et al. 2007b). However, data is limited regarding to FDG PET/CT performance in this patient group, and further evidence is needed to establish the clinical value of FDG PET/CT in this regard.

FDG PET Has Been Used Experimentally in Guiding Biopsy in Real Time

In addition to select a biopsy site based on findings of FDG PET/CT findings, we are seeing some pioneering work in recent years to perform percutaneous bone marrow biopsy under direct guidance of FDG PET/CT imaging. This is because that some lesions (especially bone marrow metastases) do not have distinctive structural abnormalities on CT or ultrasound. Klaeser et al. (2010) performed percutaneous PET/CT-guided bone biopsies to histologically verify the etiology of hypermetabolic bone lesions in patients with breast cancer, non-small cell lung cancer, cervical cancer, soft tissue sarcoma, and osteosarcoma. The procedure was performed with patients repositioned according to the findings in PET-CT, and the biopsy needle being adjusted based on a subsequent single-bed PET-CT acquisition of the region concerned and with the guidance of repetition of a single-bed PET-CT acquisition before sampling. The procedure was technically successful in all 20 patients, reached a definite histological diagnosis in 95% of cases without any complications or adverse effects (Klaeser et al. 2010). In addition, percutaneous PET/CT-guided biopsy has been performed in other locations with potential malignancy (including liver lesions, the spleen, pancreas, presacral soft tissues, retroperitoneal lymph nodes) (Tatli et al. 2010). While PET/CT-guided bone biopsy seems to be a promising alternative to conventional techniques to accurately target metabolically active bone lesions, more research work is needed to establish its clinical value.

Conclusion

Current literatures indicate that FDG PET/CT is more accurate in the initial evaluation of bone marrow involvement, and detects substantially more bone marrow lesions with similar specificity as compared with BMB, in the initial diagnosis of lymphoma patients. As it becomes wide available in clinical practice, FDG PET/CT is changing the evaluation workup for lymphoma patients, with high positive and negative predictive values in the evaluation of BMI. BMB performed at the predetermined regions of the iliac crests has a high false negative rate, thus a negative BMB does not exclude BMI. The high FNR of BMB is due to sampling error because the blinded biopsy sites (the iliac crests) may have no lymphoma involvement. Although BMI is often multifocal with frequent involvement of

the spine and pelvic bones, in approximately one third of cases with positive findings on FDG PET, the iliac crests are not involved at all. The FNR of BMB can be significantly decreased if BMB is performed at selected regions based on FDG PET findings.

Based on available data and our own experience, we believe that BMB should no longer be regarded as the "gold standard" in the initial evaluation of BMI in HD and aggressive NHL patients, because it has high FNR, and biopsy from one or two iliac crests cannot accurately reflect the status of the bone marrow as a whole. FDG PET/CT should be employed as a first-line study and should be performed in all patients, to evaluate the bone marrow status as well as to evaluate other parts of the body. BMB should be performed in selected patients rather than as a screening examination. If BMB is planned, BMB should be performed after FDG PET/CT, and that the biopsy site should be selected according to FDG PET/CT findings, i.e., to biopsy bone marrow with abnormal FDG uptake, so as to minimize false negative finding of BMB.

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17 Survivors of Childhood Hodgkin's Lymphoma After Treatment: Subsequent Solid Tumor Malignancies Based on Gender and Radiation Dose

Sughosh Dhakal, Paul Youn, Michael T. Milano, and Louis S. Constine

Contents

Abstract

Over the past few decades, dramatic advances in the treatment of childhood cancers have created a large and growing population of long-term survivors. Although many pediatric cancers can now be cured, the aggressive therapies employed in the recent era are associated with increased risks of a variety of adverse health effects, including subsequent malignant neoplasms (SMNs). Due to characteristics inherent to both the biology and treatment of Hodgkin's lymphoma (HL), including the relatively high cure rate, a significant number of SMNs occur in HL survivors. The elevated risk of malignancy persists for decades after cure of HL is obtained, with no apparent plateau in risks over time. The cumulative mortality from second cancers exceeds deaths due to HL beyond 15–30 years after therapy for HL. The topic of SMNs after HL is broad and long-term data continue to accumulate. This chapter will specifically discuss second malignancies in survivors of childhood HL, focusing on gender and radiation dose.

Introduction

Over the past few decades, dramatic advances in the treatment of childhood cancers have created a large and growing population of long-term survivors. In fact, it was recently estimated that one in every 640 young adults between the ages of 20 and 39 was a cancer survivor (Hewitt et al. 2003).

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Although many pediatric cancers can now be cured, the aggressive therapies employed in the recent era are associated with increased risks of a variety of adverse health effects, including subsequent malignant neoplasms (SMNs). Several large cohort studies conducted in North American and Europe have identified at least a sixfold increase in the risk of developing a new primary cancer in childhood cancer survivors relative to the general population (Inskip and Curtis 2007; Olsen et al. 1993).

Due to characteristics inherent to both the biology and treatment of Hodgkin's lymphoma (HL), including the relatively high cure rate, a significant number of second malignancies occur in HL survivors (Inskip and Curtis 2007), with the elevated risk of malignancy persisting for decades after cure of HL is obtained and no apparent plateau (Hodgson et al. 2007; Ng et al. 2002). Due to the significant incidence of second cancer, the cumulative mortality from second cancers exceeds deaths due to HL beyond 15–30 years after therapy for HL (Ng et al. 2002).

Both radiation therapy (RT) and alkylating chemotherapy for HL increase the risks of SMN. Typical mantle radiation fields for HL encompass the thoracic mediastinal and cervical lymph nodes, resulting in radiation exposure to the breasts, normal lung, esophagus, and thyroid. In the era before chemotherapy, many patients received more comprehensive nodal radiation, encompassing the infradiaphragmatic lymph nodes in the para-aortic chain and pelvis. With a combined modality (RT and chemotherapy) approach to HL therapy, with less comprehensive nodal coverage, the infradiaphragmatic lymph nodes are only treated in patients with infradiaphragmatic disease, representing less than 10% of stage I-II HL. These patients are at increased risk of gastrointestinal malignancies. While the topic of SMN after HL is broad and long-term data continue to accumulate, this chapter will specifically discuss second malignancies in survivors of childhood HL focusing on gender and RT dose.

Incidence of Subsequent Malignant Neoplasms by Gender

In contrast to adults, in whom the risk of SMN is reportedly gender neutral or greater in men (van Leeuwen et al. 2000; Hancock et al. 1993), several studies have reported an increased incidence of SMN in girls with HL (Olsen et al. 1993; Tarbellet al. 1993), which has been attributed to the greater occurrence of subsequent breast cancer. A pooled analysis of 5,925 children with HL from 16 population-based international cancer registries calculated an overall observed/expected ratio for all SMN of 7.7 (95% CI, 6.6–8.8) with a value of 6.3 for males and 8.8 for females. However, when evaluating only solid-tumor, non-gender-specific SMN, there was no significant difference, with observed to expected values of 6.6 (95% CI, 4.8–8.9) for females and 5.2 (95% CI, 3.8–6.9) for males (Metayer et al. 2000).

A recent report by Constine et al. (2008) from five institutions that included 930 children and adolescents treated for HL between 1960 and 1990 provided further evidence to support the increased incidence in SMN in female survivors of HL; however, it suggests that this difference is not due entirely to the differences in incidence of breast cancer and that there may be inherent differences between the genders in regards to susceptibility to SMN. After a mean follow-up of 16.8 years (maximum 39.4 years), the investigators found that SMNs occurred in 102 (11%) patients, with a 25-year actuarial rate of 19%. With 15,154 patient-years of follow-up, the expected number of solid tumors in the general population would be 7.18, resulting in a standardized incidence ratio (SIR) of 14.2 and an absolute excess risk (AER) of 63 cases per 10,000 years. Of the 102 children who developed a SMN, 30 (29%) were male and 72 (71%) were female (ratio, 0.42:1), The SIR of 19.93 (95% CI, 15.65–25.32) for females, was significantly greater than the 8.41 (95% CI, 5.68–12.03; $p<0.0001$) SIR for males (Fig. 17.1). While there were 29 cases

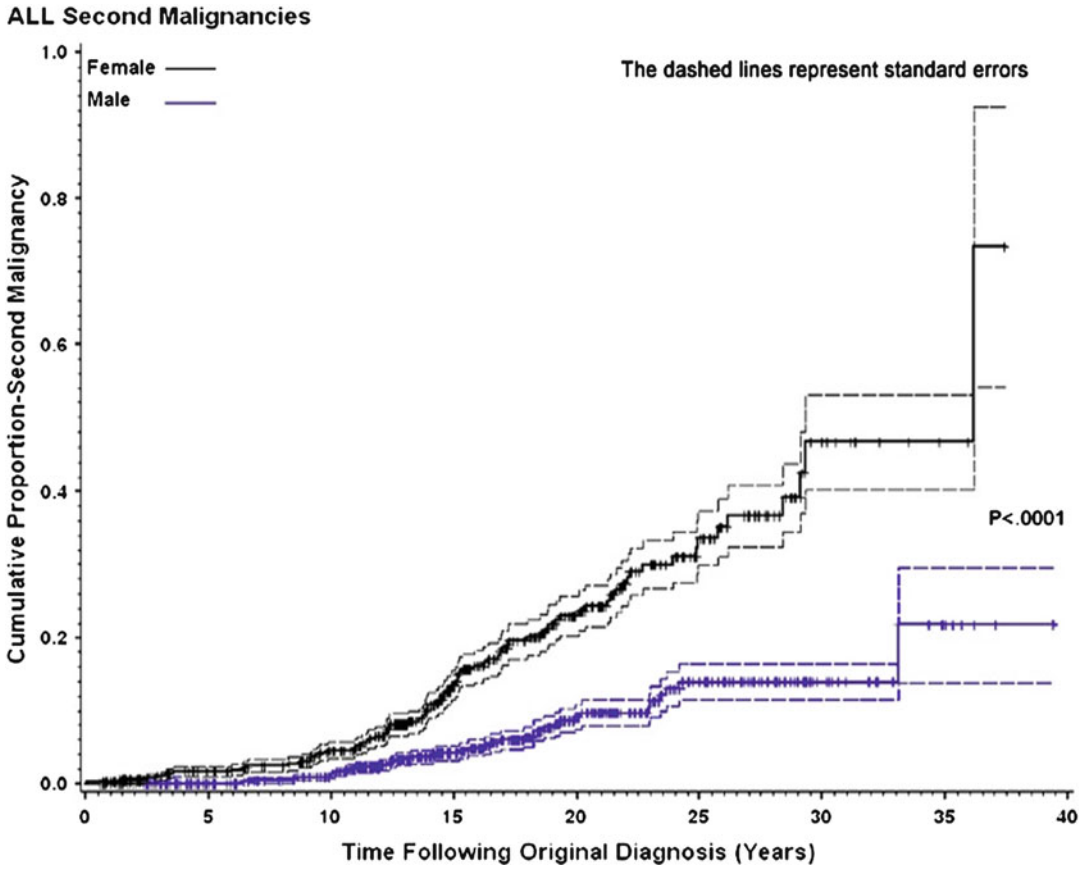

Fig. 17.1 Cumulative proportion of second malignancies according to gender, with standard errors (Reprinted with permission from Constine et al. 2008)

of breast cancer, other cancer types also occurred more frequently in females. If breast cancer was excluded from the risk analysis (Fig. 17.2), the SIR for females fell from 19.93 to 15.14 (95% CI, 9.71–22.0) ($p = 0.0012$ compared with males). In addition, if thyroid cancer was excluded from the risk analysis, the SIR for females fell from 19.28 to 17.28 (95% CI, 13.31–22.48), whereas the risk for males fell from 8.41 to 7.44 (95% CI, 4.90–10.86), which remains significantly different ($p < 0.001$). This data suggests that females may be inherently more vulnerable to SMNs than males.

In contrast, other reports have not demonstrated an increased risk of SMNs for females treated for HL. Among 1,641 children in five Nordic countries, the SIRs for females and males were 8.9 (95% CI, 6.2–12) and 6.5 (95% CI, 4.3–9.6), respectively (Olsen et al. 1993). Similarly, two large single institution studies conducted in the US, one from Stanford University Medical Center reporting on 694 children (Wolden et al. 1998), and one from Roswell Park Cancer Institute reporting on 182 patients (Green et al. 2000) both provided statistically insignificant difference in SIRs between females and males of 15.4 (95% CI, 10.6–21.5) and 10.6 (95% CI, 6.6–16), and 10.16 (95% CI, 5.56–17.05) and 9.39 (95% CI, 4.05–18.49), respectively. A third trial from Harvard also did not demonstrate a difference in relative risk (RR) of SMN according to gender (Ng et al. 2002).

Breast Cancer

Mantle RT fields expose the medial breast tissue to radiation, resulting in a significantly increased risk of breast cancer. Breast cancer accounts for

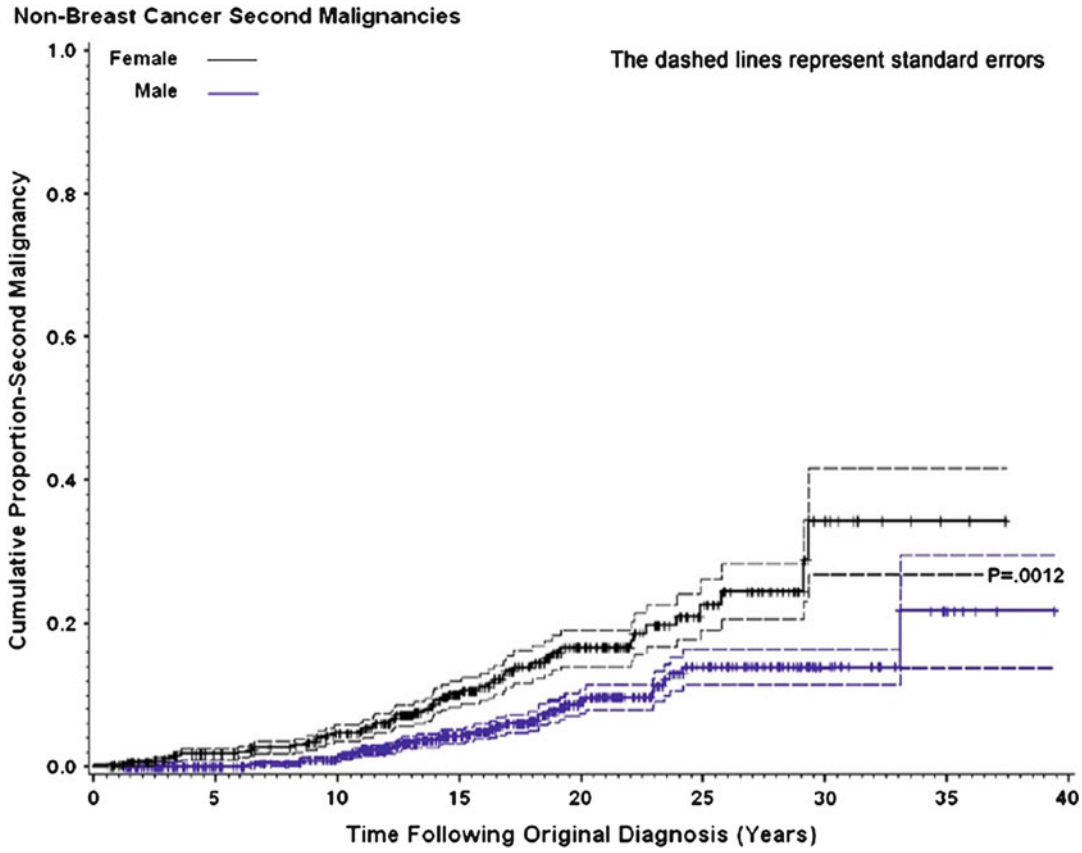

Fig. 17.2 Cumulative proportion of second malignancies according to gender, with standard errors, after excluding breast cancer (Reprinted with permission from Constine et al. 2008)

the largest absolute risk of SMNs among female survivors of HL. Radiation dose, chemotherapy administration, age at HL diagnosis, and treatment influence risk of breast cancer.

Radiation Dose

Earlier data suggested a roughly linear increase in breast cancer risk after exposure to RT in the low-dose range (Hildreth et al. 1989). Several more recent studies have analyzed RT dose after therapy for HL and provided quantitative information concerning RR and AER of breast cancer. van Leeuwen et al. (2003) conducted a case-control study of patients from the Netherlands who had been diagnosed with HL before age 41. They identified 48 cases of breast cancer and 175 matched control subjects treated for HL (of which 172 received RT). The RT dose was estimated to the area of the breast where the case patient's tumor had developed and to a comparable location in the matched control subjects, in order to examine the association between individually estimated RT doses at the precise site of subsequent breast tumor development and breast cancer risk. The risk of breast cancer increased statistically significantly with RT dose, with patients who received 38.5 Gy or more having an RR of 4.5 (95% CI,1.3–16) times that of patients who received less than 4 Gy.

Of note, analysis of patients' RT dose on risk of SMN must be done with attention to potential confounding factors, the most important of which appears to be ovarian functional status. For example, patients in this study who received both chemotherapy and RT had a statistically significantly lower risk of breast cancer than those treated with

RT alone (RR = 0.45, 95% CI, 0.22–0.91). The RR for developing breast cancer in patients who received 38.5 Gy of more versus less than 4 Gy was 12.7 (95% CI, 1.8–86) for the subset of patients who received RT alone, but 0.45 (95% CI, 0.22–0.91) for those who received chemotherapy as well as RT. Reaching menopause before age 36 was associated with a strongly reduced risk of breast cancer (RR = 0.06, 95% CI, 0.01–0.45). Therefore, while breast cancer risk increases with increasing RT dose up to at least 38.5 Gy, chemotherapy was associated with a substantial risk reduction. It would appear that an alteration of the hormonal milieu brought about by early menopause is protective because it reduces exposure of RT-damaged breast cells to the stimulating effects of ovarian hormones.

Travis et al. (2003) conducted a similar population-based matched case-control study with a cohort of 3,817 female HL patients diagnosed at age 30 years or younger. Breast cancer occurred in 105 patients with HL, who were matched to 266 patients with HL but without breast cancer. A RT dose of 4 Gy or greater was associated with a RR of 3.2 (95% CI, 1.4–8.2), compared with the risk in patients who received lower doses and no alkylating agents, while the RR was 8.0 (95% CI, 2.6–26.4) with a dose of more than 40 Gy. Again, ovarian function greatly affected the RR of subsequent breast cancer. Treatment with alkylating agents alone resulted in a RR of 0.6 (95% CI, 0.2–2.0) of breast cancer, and patients who received both an alkylating agents and radiotherapy had a RR of 1.4 (95% CI, 0.6–3.5). The RR of breast cancer decreased with increasing number of alkylating agent cycles (P = .003 for trend). Similarly, a RT dose of 5 Gy or more to the ovaries was protective against breast cancer compared with those who received lower doses (RR of 0.4; 95% CI, 0.1–1.1).

A third case-control study conducted as part of the Childhood Cancer Survivor Study included 6,647 women who were 5-year survivors of childhood cancer (all initial cancer types included), of whom 120 patients (65% of whom were initially diagnosed with HL) were identified with breast cancer and matched with 464 control patients (Inskip et al. 2009). A linear dose-response model fit the data well and demonstrated a slope of 0.27 per Gy (95% CI, 0.10–0.67) (Fig. 17.3). The estimated RRs were 6.4 at 20 Gy and 11.8 at 40 Gy. Again, the risk associated with breast irradiation was sharply reduced among women who received 5 Gy or more to the ovaries, with an excess odds ratio per Gy of 0.36 for those who received ovarian doses less than 5 Gy and 0.06 for those who received higher doses. Given the well-established increased risk of breast cancer in female children treated for HL, the Children's Oncology Group has developed and studied a gender-specific, single modality chemotherapy regimen for high-risk HL patients that appears to maintain efficacy without RT, although follow-up is somewhat limited (Kelly et al. 2011).

Age at Diagnosis of HL

Two single institution retrospective cohort studies have demonstrated a strong relationship between age at diagnosis of HL, until the age of 30–35, and RR of breast cancer compared with the general female population. Data from Harvard on 1,319 patients demonstrates a continuous downward trend in the RR of breast cancer as age at diagnosis of HL increased, with a RR of 111.4 for those diagnosed before age 15, 31.1 for those diagnosed from ages 15–19, and an approximate decrease by half for the RR thereafter for every 5 year interval until a RR of 3.7, which is of borderline significance in women diagnosed between age 30 and 35 years. After age 35, the RR was not significantly increased (Ng et al. 2002). Data from Stanford on 885 patients is strikingly similar, with a RR of 33 for patients diagnosed before age 20, RR of 19 for patients treated between ages 20 and 24, and a RR of 7.3 for patients treated between the ages of 25 and 29. There was no increase in the RR after age 30 (Hancock et al. 1993).

Hodgson et al. (2007) conducted an international population-based retrospective cohort study using multivariate modeling to describe the age at HL diagnosis and attained age on both the RR and AER. Second malignant neoplasm risk was found to be strongly dependent on age at HL diagnosis and attained age. Consistent with the

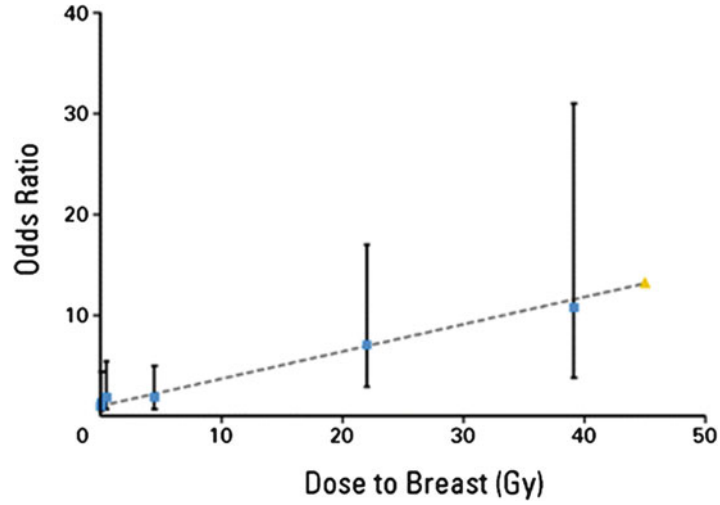

Fig. 17.3 Breast cancer risk by radiation dose to the breast (Reprinted with permission from Inskip et al. 2009)

previous two studies, the RR of female breast cancer declined significantly with attained age; however, the baseline risk with advancing age led to a significant increase in AER, which suggests that the increased risk of breast cancer persists with increasing attained age. This was particularly notable for younger patients, as the AER at an attained age of 60 was 212 (95% CI, 108–401), 94 (95% CI, 61–140), and 24 (95% CI, 6.9–46) for women diagnosed at ages 20–29, 30–39, and greater than 40, respectively. At an attained age of 50, similar values were 139 (95% CI, 88–212), 61 (95% CI, 46–79), and 16 (95% CI, 4.5–30) for women diagnosed at ages 20–29, 30–39, and greater than 40, respectively. This study was based on analyses that simultaneously evaluated both age at HL diagnosis and attained age, which is important because the variables are highly correlated. Without adjustment for age at HL diagnosis, the AER for breast cancer decreased instead of increased with attained age.

Breast Cancer Survival After HL vs. De Novo Breast Cancer

Two recent reports have evaluated outcomes of patients who develop breast cancer after HL compared with those who have de novo breast cancer. A recent multi-institutional matched case-controlled study of 253 women with breast cancer and a history of RT for HL (HL-BC) and 741 patients with sporadic breast cancer (BC-1) similarly found inferior outcomes for HL-BC patients. Specifically, although HL-BC patients were more likely to be detected by screening mammography (40% vs. 33%) at an earlier stage (stage 0 or 1; 61% vs 42%) and to be postmenopausal at diagnosis (51% vs 31%), breast cancer–specific mortality had an elevated risk (adjusted HR, 1.6; 95% CI, 0.7–3.4), although this was not statistically significant. Of note, survivors of HL were more than four times more likely to be diagnosed with metachronous contralateral breast cancer (adjusted HR, 4.3; 95% CI, 1.7–11.0; $p < .01$). The most striking finding of this study was a near double risk of death from any cause among the HL-BC patients (adjusted HR, 1.9; 95% CI, 1.1–3.3) (Elkin et al. 2011).

Another similar U.S. population-based analysis by Milano et al. (2010) included 298 HL survivors who developed breast cancer and 405,223 women with a first or only breast cancer. It revealed significantly worse outcomes for HL-BC patients in several outcome measures. Fifteen year OS among patients with HL-BC was significantly inferior to that of patients with BC-1 both for localized (48% versus 69%; $p < .0001$) and regional/distant (33% versus 43%; $p < .0001$) BC. Additionally, HL-BC patients had a significantly increased sevenfold risk ($P < .0001$) of death from cancers other than breast cancer

when compared with patients with BC-1. Ten year cause-specific survival was similar for patients with HL-BC and BC-1 with regional/distant disease, but it was inferior for patients with localized breast cancer (82% vs. 88%, respectively; P = .002). Of note, patients with HL-BC also experienced a two- to fourfold greater risk of cardiac death. The investigators hypothesized that these inferior outcomes were due to a combination of patient susceptibility, treatment-induced factors, and limitations in treatment options for breast cancer after HL.

Of note, two recent reports provided a pathologic evaluation and molecular profile of breast cancer specimens of survivors of HL compared with women who had de novo breast cancer and suggest that subsequent breast cancers may have quantitatively different biology. For example, the overall frequency of microsatellite alterations in the post-HL breast cancers was 4.2-fold greater than in the de novo specimens ($P = .16$), suggesting widespread genomic instability (Behrens et al. 2000). In another series, BC after HL as compared with de novo BC had a gene expression profile characterized by high proliferation and more aggressive tumor type (Broeks et al. 2010).

Lung Cancer

Lung cancer is the second most common SMN after HL, with a substantially increased3- to 20-fold relative risks compared with the general population (Dores et al. 2002; Ng et al. 2002; Swerdlow et al. 2000, 2001; Travis et al. 2002; van Leeuwen et al. 1995). Clearly established risk factors for lung cancer after treatment of HL include RT and alkylating chemotherapy (Travis et al. 2002), both of which demonstrate a dose-dependent response. However, cigarette smoking is by far the greatest risk factor; in fact, it appears that virtually all HL patients who develop lung cancer had a history of tobacco abuse (Travis et al. 2002).

The RR of lung cancer among pediatric HL survivors is similar to that of adult HL patients, ranging from 5.1 to 27.1, while AER is smaller in children (Bhatia et al. 2003; Castellino et al. 2011; Dores et al. 2002; Green et al. 2000; Metayer et al. 2000; Wolden et al. 1998). However, the incidence of second lung cancer is low among pediatric survivors. In a population-based long-term follow-up study by Dores et al. (2002), only four out of 157 solid tumors occurred in the lung. Similarly, the most recent analysis on the Childhood Cancer Survivorship Study HL cohort showed seven out of 277 second cancers occurring in the lung (Castellino et al. 2011).

Radiation Dose

Ionizing radiation is a well-known pulmonary carcinogen (UNSCEAR 2000). However, quantification of the carcinogenic effect of radiation specific to lung cancer is challenging due to multiple confounding agents such as chemotherapy and smoking (Swerdlow et al. 2001). A case-control study of 61 lung cancer patients and 120 control survivors among women treated with RT for breast cancer reported the RR of lung cancer associated with initial RT for breast cancer of 1.8 with excess RR of 0.20 per Gy to the ipsilateral lung (Inskip et al. 1994).

Specific to HL treatment, van Leeuwen et al. (1995) conducted a case-control study from a cohort of 1,939 patients treated in the Netherlands. They identified 30 patients with lung cancer following HL and 82 matched control subjects. They used estimates of RT dose (<1, 1–5, 5–8, and >9 Gy) to the ipsilateral lobe of lung and reported dose and reported a statistically significant increase in risk of lung cancer with increasing RT dose and an RR of 9.6 for patients who received 9 Gy or more compared with those who received less than 1 Gy.

A more recent report by Travis et al. (2002) is the largest and most comprehensive study that describes the relationship between RT and chemotherapy as well as smoking in the occurrence of excess lung cancer among HL survivors. In this large case-control study among 19,046 HL patients from international population-based cancer registries, they identified 222 HL survivors who developed lung cancer and 444 matched controls without lung cancer. A RT dose of 5 Gy or

more without chemotherapy was associated with increased lung cancer risk (RR = 5.9). Patients aged 40 or younger who were treated with RT had a RR of 3.8. There was a RT dose-dependent increase of risk with a peak RR of 8.6 in the 30–39 Gy dose range. It also reported dose-dependent risk with receipt of chemotherapy and effects appeared to be additive when patients received both chemotherapy and RT. Finally, smoking increased lung cancer risk more than 20-fold and appeared to multiply the risk from HL treatment. The authors estimated that of lung cancers after HL, ~63% are due to the combined effect of treatment and smoking, ~24% are due to smoking, ~10% are due to treatment alone (including RT and/or chemotherapy), and the remaining ~3% are due to causes unrelated to tobacco use or therapy. However, it is important to note that this comprehensive study was not specific to pediatric population as it did not do a separate analysis on patients treated before age 21.

Gender

Analysis of gender-related lung cancer risk among pediatric cancer survivors is challenging. Both gender-related smoking patterns and duration of exposure to tobacco affect risk of lung cancer and may act as confounders in lung cancer risk analysis subsequent to previous cancer treatment. In a study of atomic bomb survivors, Pierce et al. (2003) attempted adjusting for smoking, which removed a large female:male ratio of 1.6 in radiation relative risk due to the interaction between sex and smoking level. In the study by Milano et al. (2011) which compared disease characteristics of subsequent lung cancer among HL survivors versus first primary lung cancer in the general population, there was no difference in gender distribution between the two populations.

Lung Cancer Survival After HL versus De Novo Lung Cancer

A population-based analysis by Milano et al. (2011) that compared outcomes of 187 HL survivors who developed non small cell lung cancer and 178,431 patients with a first primary lung cancer from SEER data revealed significantly worse outcomes in the HL cohort. The multivariable Cox proportional hazard analysis showed that survival of HL cohort was inferior with hazard ratio of 1.60, 1.67, and 1,31 for localized, regional, and distant stage diseases respectively. However, only three out of 187 HL survivors were diagnosed of HL before age 20. Therefore, whether pediatric HL survivors with subsequent lung cancer would have inferior survival compared to age-matched patients with first primary lung cancer could not be answered with this analysis.

Subsequent Breast and Lung Cancer Risk in Modern RT Era

The above studies are most relevant to patients treated for HL decades ago in an era when much larger RT fields and doses were administered. It is almost certain that the reduced exposure of various organs to RT in the modern era will decrease subsequent neoplasms and other late effects. However, these data are informative for the tens of thousands of HL survivors treated decades ago, especially with data outlined above suggesting that their elevated risk of SMN and other late effects may be lifelong.

Although only time will tell to as what extent modern RT fields will reduce second cancer risk, Koh et al. (2007) recently performed a comparison of traditional mantle field versus involved-field radiotherapy for HL to quantify the reduction in normal tissue does and estimate the reduced risk of subsequent malignancies. In this study, organ-specific dose-volume histograms were generated for 41 patients receiving an antiquated dose and volume of 35 Gy to a traditional mantle field, 35 Gy IFRT, and a contemporary dose and volume of 20 Gy IFRT. Reduction of volume from 35 Gy mantle RT to 35 Gy IFRT resulted in a decrease in the mean RT dose to the breast and lung of 9.0 Gy to 3.2 Gy, and 14.7 Gy to 11.2 Gy, respectively, but given its equivalent inclusion in both fields there was no decrease in dose to the thyroid. Reducing dose from 35 Gy IFRT to

20 Gy IFRT resulted in an expected reduction in dose by the same proportion, with a value of 1.8 Gy to the breast and 6.4 Gy to the lung.

The use of 35 Gy IFRT rather than 35 Gy mantle field corresponded to a decrease in predicted excess relative risk (ERR) of approximately 65% for female breast and lung cancer and 35% for male lung cancer, while reduction of dose from 35 Gy IFRT to 20 Gy IFRT reduced predicted EER by an additional approximately 40%. The reduced dose and subsequent estimated risk of SMN for both breast and lung cancer was largely attributed to the omission of the axillary fields in IFRT, although the more superior placement of the inferior border in IFRT also contributed to the decreased lung dose and SMN risk.

Gastrointestinal Cancers

It is well established that HL survivors are at increased risk of developing SMN in the gastrointestinal (GI) system, with a RR of 1.7–6.0 compared with the general population (Birdwell et al. 1997; Dores et al. 2002; Ng et al. 2002; Swerdlow et al. 2000; van Leeuwen et al. 2000). The risks are higher for children with 7.2- to 35-fold increased incidence (Bhatia et al. 2003; Birdwell et al. 1997; Constine et al. 2008; Dores et al. 2002; Green et al. 2000; Metayer et al. 2000; van Leeuwen et al. 2000).While GI cancers are grouped together in many reports, several studies separately analyzed individual sites in GI system. The majority of the cancers occurred in the stomach or large intestine, while relatively few cases were observed in the esophagus, small intestine, and pancreas. However, the RRs in these sites were dramatically elevated due to low incidences in the general population. The latency period for SMN development is 10–20 years following the initial treatment, and increased risk seems to persist past 20 years (Metayer et al. 2000). Increased risk of second GI cancers leads to higher mortality from GI cancers compared with the general population. In a long-term follow-up study of 5-year survivors of HL from Childhood Cancer Survivor Study, Castellino et al. (2011) reported that GI system cancers werethe most common cancer cause of deaths along with breast cancer.

Age at treatment is strongly associated with risk of developing GI cancer. In 25-year follow-up studies of 32,591 long-term survivors of HL from 16 international population-based cancer registries, Dores et al. (2002) showed that pediatric HL survivors who were treated before age 20 had relative risk of 10.0, significantly higher compared with adult survivors whose risk is less than twofold in a large population-based analysis. Metayer et al. (2000) analyzed outcomes of 5,923 pediatric survivors from nine population-based cancer registries, including the effect of age at treatment within the pediatric survivors. Children who were treated before age of 16 had a RR of 19.3, while survivors who were treated between ages 17–21 seemed to have lower RR of six. There was also a threefold difference in AER between the two groups.

Cancer treatment is an important risk factor in development of GI cancer. Both chemotherapy and RT are known to be associated with an increased risk of GI cancer. While some have reported marginally increased risk due to RT, (Swerdlow et al. 2000) others found it highly significant (Birdwell et al. 1997; Dores et al. 2002; van Leeuwen et al. 2000). In general, combined modality treatment was associated with higher risk compared with RT alone. This pattern may suggest that the role of chemotherapy and RT is additive. However, there has been no conclusive study in this matter. Though not specific to GI cancer, RT field size was associated with second cancer risk. In a single institution long-term follow-up study, Ng et al. (2002) showed significant excesses of solid organ tumors following exposure to large radiation fields, such as total nodal irradiation on para-aortic/pelvic radiation. In an analysis of 930 children for HL, Constine et al. (2008) reported that 77% of second cancers, including GI cancers, occurred within the RT field. In a more recent analysis of the British Childhood Cancer Survivor Cohort, children who received abdominal or pelvic RT for selected childhood cancers, including HL, had 3.3-fold increased risk of developing cancers in the digestive system (Reulen et al. 2011).

Specific to stomach cancer, van den Belt-Dusebout et al. (2009) conducted a nested case-control study with a cohort of 5,142 HL or testicular cancer survivors to evaluate the roles of RT dose in the development of stomach cancer. Forty-two patients who developed stomach cancer were compared with 126 matched controls whose mean RT dose to the stomach was estimated. They found that RT increases the risk of stomach cancer by 8.8-fold. Use of the para-aortic field was associated with a higher risk compared with mantle field only. There was a dose-dependent escalation of risk where patients whose estimated stomach dose was higher than 20 Gy had a 9.9-fold increased risk compared with those who received less than 11 Gy. Estimated RR was 0.84 per Gy. On the other hand, no study has evaluated the relation between RT dose to colon and subsequent colon cancer risk.

In general, GI cancer incidence is reportedly higher in males than females. This pattern is site-dependent as stomach and esophagus cancers are highly associated with behavioral risk factors including tobacco and alcohol which may also have strong gender relations. Overall, it does not appear that one gender has a higher RR of GI cancer than the other (Birdwell et al. 1997; Dores et al. 2002). In the pediatric population, comparison of RR becomes more challenging as these values become inflated due to the low number of observed cases and low background incidence.

Thyroid Cancer

Thyroid cancer is another common SMN among children who were treated for HL. Relative risks of children developing thyroid cancer compared with the general population range from 13- to 53-fold (Bhatia et al. 2003; Constine et al. 2008; Dores et al. 2002; Green et al. 2000; Wolden et al. 1998), which is higher when compared with adult HL survivors (Dores et al. 2002; Ng et al. 2002; Swerdlow et al. 2000; van Leeuwen et al. 2000). While early studies based on single or multi-institution databases reported higher RRs due to a small number of reported cases, recent population-based studies with more than 20 years of followup repeatedly reported statistically significantly elevated RR as well as AER (Dores et al. 2002). The majority of cancers occur 10 years after diagnosis with a median time of 14–16 years for thyroid cancer (Bhatia et al. 2003; Constine et al. 2008). This is similar to the latent interval of 13–16 years for second thyroid cancer in an analysis of all childhood malignancy survivors (Sigurdson et al. 2005). Risks are elevated 10 years past diagnosis and remain elevated up to 30 years after diagnosis (Bhatia et al. 2003; Dores et al. 2002; Metayer et al. 2000), suggesting the role of cancer treatment in thyroid carcinogenesis.

Age at treatment has a strong association with risk of developing thyroid cancer (Bhatia et al. 2003; Dores et al. 2002; Metayer et al. 2000). Children who were treated before age 21 have significantly higher RR and AER compared with adult survivors whose RR is less than fivefold in most large-scale series (Dores et al. 2002; Ng et al. 2002; Swerdlow et al. 2000; van Leeuwen et al. 2000). Within the pediatric population, children who were treated before age 10 were found to have even higher RR and AER (Bhatia et al. 2003; Metayer et al. 2000). According to Bhatia et al. (2003), compared to children who were diagnosed with HL after age 10, there was a 3.7- and 1.6-fold increased risk of thyroid cancer among children who were diagnosed at age less than five, and between 5 and 10 years, respectively. Such a finding suggests that younger children whose thyroids are still developing have higher sensitivity to the carcinogenic effects of ionizing radiation.

Radiotherapy to the neck has been recognized as the main risk factor of second thyroid cancer. In a pooled analysis of seven cohort studies, including atomic bomb survivors and populations who received RT for benign diseases of the head and neck, Ron et al. (1995) suggested linear relation between the doses to the thyroid and cancer risk. While thyroid doses as low as 9 cGy were found to be associated with a fourfold increased risk of malignant tumors, Tucker et al. (1991) showed that doses greater than 200 cGy were

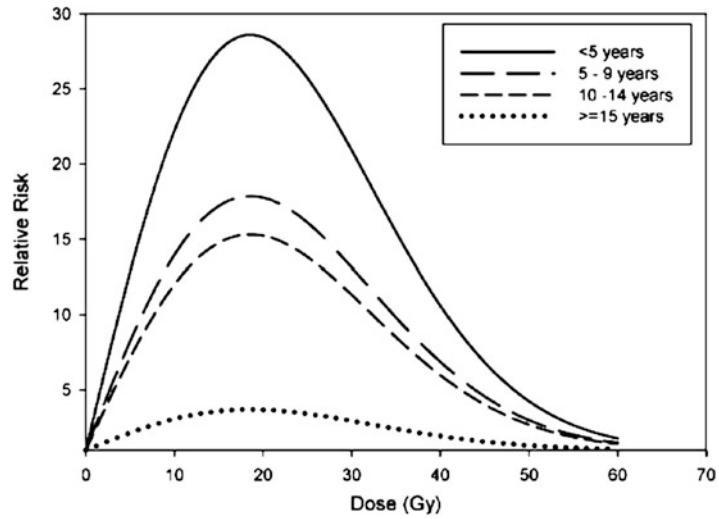

Fig. 17.4 Observed relative risk of thyroid cancer as a function of mean radiation dose to the thyroid gland for categories of dose and fitted values (Reprinted with permission from Bhatti et al. 2010)

associated with a 13-fold increased risk in an analysis of 9,170 childhood cancer survivors. However, a flattening of the dose-response curve was observed in higher doses (>10 Gy) used for treatment of HL. In a more recent nested case-control study from the Childhood Cancer Survivor Study, Sigurdson et al. (2005) confirmed a similar trend with a linear increase of thyroid cancer risk in the low dose range to 20 Gy with a peak in risk in the 20–29 Gy range with an odds ratio of 9. However, it demonstrated a decreasing risk at doses above 30 Gy, consistent with the cell-killing hypothesis of high dose RT. This analysis also showed the ascending slope of the dose-response curve was steeper for children who were diagnosed with cancer at less than 10 years of age. These trends were confirmed in a subsequent update from the Childhood Cancer Survivor Study (Bhatti et al. 2010) (Fig. 17.4). The higher risk of thyroid cancer observed in the 20–29 Gy range suggests that children, particularly those treated at younger ages, will continue to be at significant risk of thyroid cancer despite the effort to decrease RT dose and field size in the treatment of HL.

It is well established that females within the general population have approximately a three times higher incidence rate of thyroid cancer compared with males. Following this pattern, there was increased incidence in female survivors of childhood HL (Bhatia et al. 2003; Constine et al. 2008; Dores et al. 2002; Green et al. 2000; Metayer et al. 2000; Wolden et al. 1998). In Metayer et al. (2000), six of 3,188 male survivors had thyroid cancer while 16 of 2,737 female survivors developed thyroid cancer. In a smaller series by Bhatia et al. (2003), males and females had an incidence rate of 0.9% and 2.3% respectively. However, females do not seem to have increased AER compared with males. In a multiple regression analysis by Bhatia et al. (2003), RR of thyroid cancer in male children with HL was 1.7 compared with females, though not statistically significant. A similar trend is seen in a population-based analysis by Metayer et al. (2000) where males and females had relative risk of 18.1 and 12.6, respectively.

Bone and Soft Tissue Sarcoma

Bone and soft tissues are another common site of second cancer development after HL. The reported RR of sarcoma ranges from 10 to 14.9 (Bhatia et al. 2003; Castellino et al. 2011; Metayer et al. 2000; Wolden et al. 1998). Common histologies include malignant fibrous histiocytoma, osteosarcoma, spindle cell sarcoma, and undifferentiated soft tissue sarcoma (Wolden et al. 1998). Latency from most series ranges from 10 to 15 years

following treatment, while the significance of elevated RR past 20 years is questioned due to low number of observed cases (Bhatia et al. 2003; Metayer et al. 2000).Children are reported to have higher risk of developing sarcoma compared with adult survivors. Metayer et al. (2000) noted that children who were diagnosed between age 10–16 years have the greatest risk of sarcoma. Both alkylating agents and RT have been reported as risk factors. In particular, there was dose-dependent increase of sarcoma risk (Le Vu et al. 1998) and many of the sarcomas occurred within the radiation field such as in the thoracic vertebral body, clavicle, and scapula (Wolden et al. 1998).

In conclusion, although dramatic progress has been made in the treatment of pediatric HL, SMNs have remained a concern for the ever increasing cohort of long-term survivors. Female gender clearly increases risk of SMN, although it is not entirely clear whether all of the excess risk is attributable to the increased risk of breast cancer. Increased RT dose has unequivocally been shown to increase the risk of SMN in nearly all sites, with the notable exception of subsequent thyroid cancer, which displays a peak within the relative low dose range of 20–29 Gy.

Increasing awareness of SMNs continue to influence treatment strategies used in pediatric HL, with shorter courses of chemotherapy and decreasing RT dose and field size. There is a clinical need for improvements in screening guidelines for long-term survivors and for effective treatment strategies for those who develop SMNs. This is especially the case for the cohort of patients treated in previous eras, who are at an increased risk of developing SMNs relative to contemporary patients, and, for certain cancers, appear to have poorer outcomes compared to patients with similarly staged *de novo* cancers.

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Part V

Rhabdoid

Early Childhood Paraspinal Atypical Teratoid/Rhabdoid Tumor: Failure of Standard Treatments

18

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Contents

Abstract

AT/RT involving the spine and the spinal cord is extremely rare and can occur anywhere along the spinal axis with only few case reports in the literature. As far as spinal AT/RT is concerned these tumors present between 7 months to 17 years of age, with a mean age of 6.5 years with a male preponderance. Usually the location of these tumors is either intramedullary or extramedullary, determining their clinical presentation and course of the disease. Para-spinal AT/RT has many similarities with brain and spinal locations including rapid and fatal clinical course, histopathological features and lack of definitive treatment protocol. Because of the rarity of the para-spinal AT/RT, the present reported literature is not enough to make any concrete conclusions and there is a need for prospective studies to further understand the biology of the disease and also to develop optimal treatment approaches for children with para-spinal AT/RT.

Introduction

Atypical teratoid/rhabdoid tumor (AT/RT) is a highly aggressive and uncommon tumor of the central nervous system, primarily affecting infants and young children with a rapidly progressive course and a fatal outcome (Rorke et al. 1996; Howlett et al. 1997; Warmuth-Metz et al. 2008; Yano et al. 2008; Squire et al. 2007; Chi et al. 2009). AT/RT involving the spine and

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the spinal cord is extremely rare and can occur anywhere along the spinal axis with only few case reports in the literature (Stabouli et al. 2010; Niwa et al. 2009; Tinsa et al. 2008; Yano et al. 2008; Zarovnaya et al. 2007; Moeller et al. 2007; Rorke et al. 1996; Warmuth-Metz et al. 2008; Howlett et al. 1997; Cheng et al. 2005; Bambakidis et al. 2002; Tamiya et al. 2000; Rosemberg et al. 1994; Kodama et al. 2007; Tanizaki et al. 2006). Paraspinal location of AT/RT is extremely rare thus making concrete conclusions difficult (Agrawal et al. 2009).

Clinicopathological Features

As far as spinal AT/RT is concerned these tumors present between 7 months and 17 years of age, with a mean age of 6.5 years with a male preponderance. Usually the location of these tumors is either intramedullary or extramedullary, determining their clinical presentation and course of the disease (Tinsa et al. 2008). In a reported case of paraspinal AT/RT the patient was 18 months female who presented with paraspinal mass, pain, and later development of lower limb weakness (Agrawal et al. 2009).

Histological appearance of the paraspinal AT/RT is similar to their counterparts in brain or spinal cord (Agrawal et al. 2009; Yano et al. 2008; Rorke et al. 1995, 1996). On routine hematoxylin/eosin (HE) staining, the tumor contains rhabdoid cells with or without primitive neuroectodermal tumor like cells showing epithelial, mesenchymal, glial or neuronal differentiation (Agrawal et al. 2009; Yano et al. 2008). It is important to recognize the rhabdoid element as this is associated with significantly worse prognosis than primitive neuroectodermal tumor (PNET) or medulloblastoma (MB). (Agrawal et al. 2009) AT/RT is characteristically positive for epithelial membrane antigen (EMA), vimentin, smooth-muscle antigen and negative for germ cell markers (Rorke et al. 1996). The inactivation of the INI-1 gene is hall mark of these tumors (Yano et al. 2008) and inactivation of the INI1 may be associated with a poor prognosis, even if the rhabdoid component of the tumor is not apparent on histological or immunological studies (Tamiya et al. 2000). However details of these investigations were not available for the reported case paraspinal AT/RT (Agrawal et al. 2009).

Imaging

On plain computerized tomography paraspinal AT/RT was recognized as a large, well-defined, isodense, lobulated mass in the left paraspinal area, with erosion of the lumbar vertebra (Agrawal et al. 2009). MRI imaging findings of spinal AT/RT include heterogeneity, isointensity/hypointensity to cord on T2-weighted images. Inhomogeneous enhancement, calcification, cyst formation, and hemorrhage are frequently seen. It can also appear as diffuse spinal cord enlargement. Typical enhancement pattern consists of a wavy bandlike rim of strong enhancement completely surrounding a central cystic or necrotic area (Warmuth-Metz et al. 2008). However because of paucity of reported cases details regarding these investigations are grossly lacking in literature.

Treatment

Currently there is no effective therapy for life-saving and avoidance of neurological deficit in children with paraspinal AT/RT (Agrawal et al. 2009). The most appropriate treatment is total tumor resection and chemotherapy, however the results are not encouraging (Agrawal et al. 2009). The purpose of surgery is to make a diagnosis and to reduce the tumor burden. Early surgery for decompression of the spinal cord is of primary importance for children with paralysis (Yano et al. 2008; Agrawal et al. 2009). Rarity of the disease makes the treatment protocol difficult, (Shonka et al. 2011) but it has been shown that aggressive therapy can prolong the natural history in a subset of children; however prospective multi-institutional and national clinical trials designed specifically for AT/RT are needed (Holden et al. 2004).

Prognosis

The prognosis of AT/RT occurring in the spine is not different from AT/RT occurring in other locations and survival is poor (Agrawal et al. 2009; Rorke et al. 1996; Howlett et al. 1997; Warmuth-Metz et al. 2008; Yano et al. 2008). Despite the use of aggressive chemotherapy, radiotherapy, or both; children rarely respond to treatment and the mean survival duration is 1 year (Tinsa et al. 2008; von Hoff et al. 2011; Bhattacharjee et al. 1997; Chi et al. 2009; Agrawal et al. 2009).

In conclusion, para-spinal AT/RT has many similarities with brain and spinal locations including rapid and fatal clinical course, histopathological features and lack of definitive treatment protocol. Because of the rarity of the para-spinal AT/RT, the present reported literature is not enough to make any concrete conclusions and there is a need for prospective studies to further understand the biology of the disease and also to develop optimal treatment approaches for children with para-spinal AT/RT (Stabouli et al. 2010).

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Cerebral Tumor with Rhabdoid Features

19

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Contents

Abstract

Rhabdoid tumors have been reported to arise from various locations such as the central nervous system, soft tissues, liver, retroperitoneum, pelvis, and skin, in addition to the kidneys. These tumors with rhabdoid cell components have been reported to be aggressive lesions. Atypical teratoid/rhabdoid tumors (AT/RT) are the most common cerebral tumors with rhabdoid features. Recently, new disease categories, such as rhabdoid meningioma and rhabdoid glioblastoma, have been reported. These tumors are difficult to distinguish radiologically. Histological and immunohistological examinations are useful for making an accurate diagnosis. Recent investigations have revealed that the lack of INI-1 gene expression is the most characteristic feature in AT/RT and malignant rhabdoid tumor. We recently reported a frontal brain tumor with rhabdoid features that could not be categorized in any of the current classifications, and referred to this tumor as a cerebral tumor with extensive rhabdoid features. We present several types of cerebral tumors with rhabdoid features and discuss the differences among them.

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Introduction

Malignant rhabdoid tumor (MRT) was first described as a subtype of Wilms' tumor of the kidney that presented with rhabdomyosarcomatoid features (Beckwith and Palmer 1978).

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However, ultrastructural studies revealed that these tumor cells had not differentiated into rhabdomyoblasts (Fung et al. 1981). Rhabdoid tumors have been reported to arise from various locations: the central nervous system (CNS), soft tissues, liver, retroperitoneum, pelvis, and skin, in addition to the kidneys (Parham et al. 1994). In the CNS, such lesions are commonly called atypical teratoid/rhabdoid tumors (AT/RT); however, some authors differentiate between AT/RT and MRT (Tekkök and Sav 2005). Recently, several cerebral tumors with rhabdoid features other than those of AT/RT or MRT have been reported, such as rhabdoid meningioma and rhabdoid glioblastoma (Perry et al. 1998; Wyatt-Ashmead et al. 2001). These tumors contain various populations of rhabdoid cells, which have eccentrically placed nuclei containing vesicular chromatin, prominent eosinophilic nucleoli, and abundant cytoplasm with eosinophilic globular cytoplasmic inclusions. Genetic studies have revealed that the deletion of chromosome 22q and the inactivation of the INI1/hSNF5 tumor suppressor gene on chromosome 22q11.2 were characteristic features in AT/RT and MRT (Makuria et al. 2008; Biswas et al. 2009; Tekkök and Sav 2005). As described above, new tumor entities with rhabdoid features have been reported, and a new tumor that cannot be categorized using the current classifications has been found. In this chapter, we describe several types of cerebral tumors with rhabdoid features and report our case with a lesion that we have referred as a "cerebral tumor with extensive rhabdoid features", and discuss the differences among them.

Atypical Teratoid Rhabdoid Tumor

Atypical teratoid rhabdoid tumor (AT/RT) is a rare, highly malignant tumor that accounts for 1.3% of all primary pediatric tumors of the CNS (Rickert and Paulus 2001). AT/RT is defined as typically containing rhabdoid cells, often with primitive neuroectodermal cells and with differentiation along mesenchymal, epithelial lines (Rorke et al. 1996). It usually occurs in children under the age of 5 years, although several adult cases have also been reported (Mirone et al. 2009; Raisanen et al. 2005). AT/RT usually occurs in the posterior fossa. Dang et al. (2003) reported that 52% of these tumors were located in the posterior fossa and 39% were supratentorial. AT/RT tend to occur off-midline and to have calcification and cyst formation (Biswas et al. 2009; Lee et al. 2009). The solid component of the tumors had a homogeneous iso-intensity on T1 and T2-weighted images in one series, whereas, another study showed that more than half of the tumors had mixed intensities on T1 and T2-weighted images. Moderate to strong enhancement was found in most cases (Lee et al. 2009; Warmuth-Metz et al. 2008). A band-like rim of the central cyst or necrotic area was strongly enhanced in some cases. Leptomeningeal seeding was often detected (Lee et al. 2009). The optimal treatment has not been elucidated. Because of the young age of the patients, high-dose radiation therapy is not suitable. Various chemotherapeutic regimens have been reported (Biswas et al. 2009). Rorke et al. (1996) reported that the median time-to-progression and overall survival were 4.5 and 6 months, respectively. A recent study showed that the chemotherapeutic response and radiation therapeutic response were 58 and 38%, respectively, and that the 2-year progression-free and overall survival rates were 53 and 70% respectively (Chi et al. 2009).

Histologically, AT/RT have varying proportions of rhabdoid cells. Rhabdoid cells are characterized by abundant eosinophilic cytoplasm, globular inclusion, and an eccentrically placed vesicular nucleus with open chromatin and prominent nucleolus. Rhabdoid cells exist often with undifferentiated PNET component consisting of small cells with a high nuclear/cytoplasmic ratio and oval hyperchromatic nuclei and with differentiation along mesenchymal, epithelial lines (Mohapatra et al. 2010). Immunohistologically, AT/RT is usually positive for epithelial membrane antigen (EMA), smooth muscle actin (SMA), and vimentin and occasionally positive for synaptophysin, cytokeratin, glial fibrillary acidic protein (GFAP) and neurofilament (Mohapatra et al. 2010; Rorke et al. 1996). The MIB-1 index is relatively high, and the mean MIB-1 index was

45% in one series (Mohapatra et al. 2010). Electron microscopic examination revealed whorled bundles of intermediate filaments filling some or most of the perikaryon (Rorke et al. 1996). The characteristic genomic feature seen in AT/RT is the inactivation of the INI-1 gene (Haberler et al. 2006). Monosomy or deletion of chromosome 22 is detected in 75–90% of AT/RT lesions (Bruch et al. 2001; Raisanen et al. 2005).

MRT of the central nervous system was first reported by Briner et al. (1985). The concept of AT/RT was constructed by Rorke et al. in 1996; however, some authors have reported that MRT is not the same disease as AT/RT (Tekkök and Sav 2005). According to Tekkök's review, local recurrence or subarachnoid spread were reported in more than two-thirds of patients after a mean period of 6.9 months, and these patients died after a mean period of 8.9 months after their diagnosis (Tekkök and Sav 2005). MRT is characterized by medium to large round or polygonal cells with prominent eosinophilic cytoplasm and eccentric and round nuclei with prominent nucleoli. MRT is usually immunoreactive for vimentin, EMA and cytokeratin and is often immunopositive for SMA, S-100, and synaptophysin. MRT shows the deletion of region 11.2 of the long arm of chromosome 22 (22q) and the inactivation of the INI1/hSNF5 tumor suppression gene (Tekkök and Sav 2005; Mirone et al. 2009).

Most cases of AT/RT (and primary CNS MRT) occur in children; however, several adult cases have been reported (Mirone et al. 2009; Raisanen et al. 2005). Adult and childhood AT/RTs have been shown to have similar cytologic, architectural, and genetic features (Raisanen et al. 2005). Mirone et al. reviewed 21 cases of cerebral adult AT/RTs (including MRTs) and reported a mean survival time of 38 months. Although leptomeningeal dissemination is also common in adults, adult cases seem to have a relatively good prognosis. This difference may be due to the greater radicality of surgical resection for the tumor and higher-dose radiation therapy, which cannot be tolerated by children. Some cases reportedly exhibited a normal chromosome 22q (Mirone et al. 2009).

Rhabdoid Meningioma

Rhabdoid meningioma was first described in 1998 (Kepes et al. 1998). Rhabdoid meningioma is classified as a WHO grade 3 meningioma and exhibits aggressive progression with a high rate of proliferation (Kim et al. 2007). Two series have been published (Perry et al. 1998; Kim et al. 2007). Perry's study included eight men and seven women with a median age of 50 years (range, 13–73 years) at the time of first surgery and 53 years (range, 13–73 years) at the time that the rhabdoid features became apparent. Kim's study included four men and 11 women with a mean age of 52 years (range, 22–75 years). Rhabdoid meningioma tends to have cystic components, prominent peritumoral edema, and bone involvement (Kim et al. 2007). In Perry's study, 87% of the patients had recurrence, 13% had extracranial metastasis, and 53% died (one patient died from pulmonary embolism on postoperative day 2). The median time until death was 5.8 years after the initial operation and 3.1 years after the first appearance of rhabdoid morphology (Perry et al. 1998). In another study, additional radiation therapy was performed after the operation in all the cases except one case, and only one recurrence (7%) and no extracranial metastases or deaths were found during a follow-up period ranging from 11 to 39 months (Kim et al. 2007).

Rhabdoid meningioma is characterized by loosely cohesive sheets of cells with abundant eosinophilic cytoplasm, eccentric nuclei and hyaline, and frequent fibrillar paranuclear inclusion (Perry et al. 1998). The components of conventional meningiomas were present in the majority of tumors, and rhabdoid components often became increasingly prominent with subsequent recurrence (Perry et al. 1998). Electron microscope revealed paranuclear whorls of intermediate filaments, frequently with entrapped lysosomes and mitochondria (Perry et al. 1998). The MIB1 index ranged from 0.8 to 43% (median, 6.3%) (Perry et al. 1998). The tumor cells are immunopositive for vimentin and EMA and are usually

negative for GFAP. The deletion of 22q was identified in 10 of the 14 (71%) cases, and nuclear INI-1 expression was retained in all 16 cases (Perry et al. 2005).

Rhabdoid Glioblastoma

Rhabdoid glioblastoma is a recently reported entity in which an epithelioid glioblastoma is associated with a rhabdoid component (Fung et al. 2004; Kleinschmidt-Demasters et al. 2010; Lath et al. 2003; Wyatt-Ashmead et al. 2001). The typical radiological pattern is a ring-enhanced mass with extensive edema, similar to a conventional glioblastoma. Glioblastoma is one of the most malignant brain tumors. Rhabdoid glioblastoma is highly aggressive with a potential for early recurrence, and patients usually die within several months (Fung et al. 2004; Kleinschmidt-Demasters et al. 2010; Lath et al. 2003; Wyatt-Ashmead et al. 2001). Whether rhabdoid glioblastomas should be treated with surgery followed by aggressive multiagent chemotherapy and cranio-spinal radiation, like other malignant rhabdoid tumors, or treated like typical glioblastomas remains uncertain (Lath et al. 2003). Tumor cells with the rhabdoid phenotype are strong immunostained with vimentin and EMA, and a subpopulation is GFAP immunopositive. The glial component shows prominent mitotic activity and extensive tumor necrosis with GFAP immunopositivity (Fung et al. 2004; Kleinschmidt-Demasters et al. 2010; Lath et al. 2003; Wyatt-Ashmead et al. 2001). The MIB-1 index ranged from 18 to over 30%. Ultrastructurally, the tumor cells contain cytoplasmic whorls of intermediate filaments (Wyatt-Ashmead et al. 2001). Monosomy of 22 and polysomy of 22q were found in two and one cases, respectively, in three examined cases (Kleinschmidt-Demasters et al. 2010; Wyatt-Ashmead et al. 2001). The expression of INI -1 was found in the nucleus of glioblastoma cells without rhabdoid features, but not in a subset of cells with the strongest rhabdoid features (Kleinschmidt-Demasters et al. 2010).

Rhabdoid Melanoma

Rhabdoid melanoma was first described in 1992 (Bittesini et al. 1992). The extent of the rhabdoid changes in the neoplastic cells vary. In a large series of Chang et al., 48% of the samples were composed exclusively of rhabdoid cells, whereas in 52% of them, the rhabdoid cells constituted less than 25% of the tumor (Chang et al. 1994). The presence of rhabdoid features in melanoma is more common in metastatic melanoma than in primary lesions (Magro et al. 2006). Chang et al. reviewed 31 specimens from 29 patients with metastatic melanoma with rhabdoid features. Seventeen patients were men and 12 patients were women, ranging in age from 24 to 78 years. Preexisting cutaneous melanomas had been observed in 25 cases (Chang et al. 1994). Rhabdoid melanoma shows significant histologic, immunohistochemical and ultrastructural heterogeneity (Gavino and Gillies 2008). It is immunopositive for vimentin, but has diverse immunoreactivity patterns for S-100 and frequently loses HMB-45 expression (Magro et al. 2006; Gavino and Gillies 2008). Rhabdoid melanoma is less immunoreactive for S-100 protein than nonrhabdoid melanoma. No difference in staining with HMB-45 has been noted between rhabdoid melanoma and nonrhabdoid melanoma (Chang et al. 1994). The loss of S-100 and /or HMB-45 expression in the cells of rhabdoid melanoma is thought to be a manifestation of the process of dedifferentiation (Gavino and Gillies 2008). Rhabdoid melanoma appears to have a no more aggressive behavior than conventional melanoma (Gavino and Gillies 2008).

Metastatic Tumor from Carcinoma with Rhabdoid Features

Some carcinomas with rhabdoid features include lung tumors, stomach, jejunum, colon, and so on (Tamboli et al. 2004; Amrikachi et al. 2002). Metastatic cerebral tumors of these primary lesions are different diagnoses of cerebral tumors with rhabdoid features. Tamboli et al. (2004)

reviewed 32 patients with rhabdoid lung tumors who ranged in age from 25 to 82 years. There were 20 men and 12 women. The amount of rhabdoid cells ranged from 10 to 90%. Rhabdoid lung tumors are usually immunopositive for vimentin and epithelial markers. After a mean follow-up period of 8.3 months, 60% of the patients had died, with a mean survival period of 8.1 months (Tamboli et al. 2004). Amrikachi et al. (2002) reviewed 16 cases of gastrointestinal rhabdoid tumors; the patients in this series were 11 men and five women, ranging in age from 52 to 84 years. All the cases were immunopositive for vimentin, and cytokeratin or EMA was positive in 15 of the 16 cases. Electron microscopy showed whorls of intermediate filaments in esophagus, stomach and small intestine tumors (Amrikachi et al. 2002). Seventy five percent of the cases died within 10 months, and 50% died within 3 months (Amrikachi et al. 2002). The prognosis of tumors with rhabdoid features was reported to be poor, independent of their locations (Tamboli et al. 2004; Amrikachi et al. 2002).

Cerebral Tumor with Extensive Rhabdoid Features

We recently reported a 32-year-old pregnant female with a frontal tumor (Ohba et al. 2009). Systemic examinations revealed no other lesions. The tumor was hypercellular and contained a diffuse sheet of various sizes of eosinophilic cells. These cells had a few prominent, eccentrically placed, variously sized, hyperchromatic nuclei. The tumor was immunohistologically reactive for vimentin, EMA, SMA, and BAF47/INI-1 and negative for GFAP, neurofilament protein, S-100, and HMB-45. The MIB-1 index was 4.2%. The deletion of 22q was not detected by the CGH analysis. Chemotherapy and radiotherapy were administered, and the patient is still alive after more than 7 years. Radiological examinations have lowered the possibility of undetected metastatic tumors and rhabdoid meningiomas. Histologically, this tumor had no components containing PNET-like cells, meningiomas, glioblastomas, melanomas, or carcinomas of other origins. Immunohistologically, rhabdoid glioblastoma and AT/RT (MRT) were excluded. The tumor could not be characterized using the current classification. We therefore referred to this tumor as a "cerebral tumor with extensive rhabdoid features".

Discussion

Tumors with rhabdoid features are uncommon; however, rhabdoid cells are found in several types of tumors. In addition to histological examination, immunohistological examination and genetic investigation are often useful to make an accurate diagnosis. In this paper, we described the clinical, radiological, histopathological, and genetic features of several tumors with rhabdoid features and discuss the differences among them.

Radiological Features

It is very difficult to distinguish a tumor with rhabdoid features from other tumors with rhabdoid features using radiology because cerebral tumors with rhabdoid features generally have similar radiological features: cyst formation, homo to heterogeneous images, moderate to strong enhancement, ring enhancement, and remarkable edema. The presence of calcification may help to distinguish AT/RT and rhabdoid meningioma from other lesions. Leptomeningeal dissemination is a key feature associated with AT/RT (Lee et al. 2009; Tekkök and Sav 2005). Rhabdoid meningioma is an extra-axial tumor that is usually attached to the dura mater and sometimes invades bone tissue, unlike the other tumors described here. The presence of other primary lesions is often useful for diagnosing metastatic tumors.

Histological Features

Rhabdoid cells are characterized by abundant eosinophilic cytoplasm, globular inclusion, and an eccentrically placed vesicular nucleus with open chromatin and prominent nucleolus. The proportion of rhabdoid cells varies even among

lesions of the same disease type. In AT/RT, rhabdoid cells often exist with an undifferentiated PNET component and with differentiation along mesenchymal, epithelial lines (Mohapatra et al. 2010). Many tumors with rhabdoid features have an original neoplastic cell component in addition to the rhabdoid cells. Rhabdoid glioblastoma has the classical histological features of a high-grade astrocytoma (Kleinschmidt-Demasters et al. 2010). A conventional meningioma component is present in the majority of rhabdoid meningiomas (Perry et al. 1998). Rhabdoid melanoma usually retains melanocytic attributes (Chang et al. 1994). Other carcinomas with rhabdoid features are usually composed of rhabdoid cells and carcinomatous neoplasm (Tamboli et al. 2004). The lesion in our reported case consisted of only rhabdoid cells, without other variable components.

Immunohistochemical Features

Tumors with rhabdoid features are usually immunopositive for vimentin and EMA (Mohapatra et al. 2010; Perry et al. 1998; Rorke et al. 1996). AT/RT (and MRT) are usually positive for EMA, SMA, and vimentin and are occasionally positive for synaptophysin, cytokeratin, GFAP and neurofilaments (Mohapatra et al. 2010; Rorke et al. 1996). Rhabdoid meningioma is immunonegative for GFAP (Perry et al. 1998; Kepes et al. 1998). The glial component of rhabdoid glioblastoma is immunopositive for GFAP (Lath et al. 2003). Rhabdoid melanoma shows diverse patterns of immunoreactivity to S-100 and frequently loses HMB-45 expression. Our case was immunohistologically reactive for vimentin and EMA and negative for GFAP, neurofilament protein, S-100, and HMB-45. The MIB-1 index is usually remarkably high in tumors with rhabdoid features and moderately high in rhabdoid meningioma but was not so high in our case.

Ultrastructure

Ultrastructurally, rhabdoid tumors show three patterns. The most common pattern is that of whorled filamentous bodies with entrapped lipid and organelles. The second pattern is that of intermediate filaments packed in elongated, gently curved bundles. The third form is characterized by a filamentous pattern composed of twisted sheaves of intermediate filaments, resembling tonofilaments of squamous epithelial cells (Gavino and Gillies 2008; Haas et al. 1981).

Chromosome 22 and INI-1 Expression

The loss of INI-1 expression is the most characteristic feature of AT/RT and MRT (renal and extra-renal). Monosomy or deletion of chromosome 22 is detected in 75–90% of AT/RT (Bruch et al. 2001; Raisanen et al. 2005), and all AT/RT cases stain negatively for INI-1 protein (Biswas et al. 2009; Mohapatra et al. 2010). Neoplasms with rhabdoid features that are not associated with the suppression of INI-1 protein or gene include carcinomas, melanomas, meningiomas, and gliomas (Perry et al. 2005). All the cases of rhabdoid meningioma showed the INI-1 expression. The expression of INI-1 was found in the nuclei of glioblastoma cells without rhabdoid features, but not in a subset of cells with the most rhabdoid features (Kleinschmidt-Demasters et al. 2010). INI-1 expression was detected in composite rhabdoid tumors, such as melanoma and carcinoma (Perry et al. 2005), as well as in our case. Perry et al. (2005) suggested that composite rhabdoid tumors were genetically distinct from MRT and AT/RT and retained INI-1 expression.

Treatment

The optimal treatment for AT/RT has not been elucidated. If possible, surgery followed by chemotherapy and radiotherapy are often performed. Because most patients are young, there is reluctance to perform radiation therapy. Surgery with additional radiation therapy is recommended for rhabdoid meningioma. Whether rhabdoid glioblastomas should be treated like other MRTs or treated like typical glioblastomas remains uncertain (Lath et al. 2003). In our case, radiation

therapy and chemotherapy were performed after tumor removal, and these treatments appeared to be effective (Ohba et al. 2009).

Prognosis

Tumors with rhabdoid cell components have been reported to be aggressive lesions. Rhabdoid meningioma is classified as WHO grade 3, whereas conventional meningioma is WHO grade 1. Glioblastoma and malignant melanoma are potentially aggressive. The occurrence of metastasis to the brain from a primary carcinoma represents a severe disease stage. Although the prognosis of AT/RT has been reported to be poor (Rorke et al. 1996), a recent study showed a better outcome than that of previous reports (Chi et al. 2009). Compared with them, our case responded well to chemotherapy and radiation therapy and had a good outcome (Ohba et al. 2009). The MIB1 index of this case was not high, which might explain the good outcome. The factors that define the prognosis remain uncertain, and further investigations are needed to identify these factors.

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Atypical Teratoid/Rhabdoid Tumor of the Pineal Region: Diagnosis

20

Hidehiro Takei and Lauren A. Langford

Contents

Abstract

Atypical teratoid/rhabdoid tumor (AT/RT) is a highly malignant embryonal tumor most commonly occurring in young children, with the highest incidence in the first 2 years of life. The ratio of supratentorial to infratentorial localization of this tumor is reported to be 1.4:1. Supratentorial AT/RTs are located mostly in the cerebral hemispheres and affect more predominantly older children and adults, in contrast to AT/RTs occurring in the posterior fossa. AT/RTs of the pineal region are much rarer, and there have been several single case reports of both children and adults in the literature. There are no known symptoms or signs at presentation, or radiological features that are specific to AT/RTs of the pineal region, but a rapid progression of symptoms reflecting their aggressive biological behavior is common. The prognosis is dismal.

Histologically, AT/RTs are very cellular tumors and show marked regional heterogeneity, with primitive neuroectodermal tumor (PNET)-like, rhabdoid, epithelial, and mesenchymal components in variable proportions. Immunohistochemically, AT/RTs are polyphenotypic with constant expression of vimentin and variable expression of epithelial membrane antigen, smooth muscle actin, glial fibrillary acidic protein, cytokeratins, synaptophysin, and neurofilament protein. Loss of expression of INI1 is sensitive and specific marker for AT/RTs.

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Histological differential diagnosis of AT/RTs of the pineal region includes so-called "malignant small blue cell tumors" (e.g., supratentorial PNETs, pineoblastoma), choroid plexus carcinoma, tumors with prominent rhabdoid features involving this region (e.g., rhabdoid meningioma, rhabdoid glioblastoma), and metastatic tumors. In addition to careful histological evaluation, a panel of immunohistochemical markers, including INI1, as well as detailed clinical information is crucial for this differential.

Introduction

Atypical teratoid/rhabdoid tumor (AT/RT) is a rare, highly malignant central nervous system (CNS) neoplasm of unknown histogenesis with characteristic rapid progression and high potential for widespread dissemination throughout the CNS. It predominantly affects young children with the highest incidence in the first 2 years of life, while it rarely occurs in adults with less than 30 cases reported to date (Samaras et al. 2009; Takei et al. 2010). According to the current World Health Organization (WHO) 2007 classification of CNS tumors, this unique neoplasm constitutes one of three major embryonal tumor entities, all of which correspond to WHO grade IV (Judkins et al. 2007).

AT/RTs are immunophenotypically and genetically distinct from and are resistant to standard therapy for the other embryonal tumors; CNS primitive neuroectodermal tumors (PNETs) and medulloblastomas. Histologically, AT/RT is named for the presence of characteristic rhabdoid cells, which usually compose the tumor in part, with a variable combination of PNET-like, mesenchymal spindle-shaped and epithelial-type components. Although its unique similarity with the malignant rhabdoid tumor of the kidney is known, AT/RTs composed purely of rhabdoid cells are uncommon. Given the frequent overlapping histological features, the histological distinction from PNETs and medulloblastomas is sometimes very difficult, especially in small specimens. In keeping with their histologic diversity, they exhibit a broad spectrum of immunoreactivity (i.e., polyphenotypic), with frequent positivity for glial fibrillary acidic protein (GFAP), epithelial membrane antigen (EMA), cytokeratins, smooth muscle actin (SMA), and vimentin. The most distinctive and diagnostically almost pathognomonic feature of AT/RT is its association with the inactivation of the *SMARCB1*/*hSNF5*/*INI1*gene, located on chromosome 22q11.2, in the vast majority of cases. Thus, immunohistochemical study using an antibody against *INI1* gene product or fluorescence in situ hybridization (FISH) to identify loss of the *INI1* locus is the current routine work-up for diagnostic confirmation of AT/RT. Given its much worse prognosis, necessitating intensive therapy that differs markedly from the treatment of PNETs and medulloblastomas, this diagnostic confirmation is crucial.

In two large series of pediatric cases (a total of 73 cases) in the US, the ratio of supratentorial to infratentorial localization is 1.4:1 (Hilden et al. 2004; Tekautz et al. 2005). Supratentorial tumors are located mostly in the cerebral hemispheres and less frequently in the ventricular system and pineal region, and more predominantly affect older children as well as adults (Samaras et al. 2009). In contrast, posterior fossa, including cerebellum, cerebellopontine angle, and brainstem, is the most common primary tumor site among patients younger than 3 years.

The pineal gland is a small (5–8 mm) midline structure and embryologically develops during the second month of gestation as a diverticulum in the diencephalic roof of the third ventricle. It is located directly below the splenium of the corpus callosum and is flanked by the posterior and habenular commissures in the rostal portion of the midbrain. The "pineal region" includes a pineal gland, posterior wall of the third ventricle, tela choroidea, and velum interpositum. The primary pineal region tumors are derived from cells located in these anatomic structures, and have male and childhood predominance. The most common tumors are germ cell tumors and pineal parenchymal tumors. In older adults, metastatic tumors should be considered. It is known that AT/RTs can rarely arise in this region and that they

are much rarer than those occurring in the cerebral hemispheres and posterior fossa. There have been several single case reports in the literature since the first case of "malignant rhabdoid tumor" of the pineal region was described in 1994 (Muller et al. 1994). In this chapter, we review those reported cases in the literature, including our case (Takei et al. 2010).

Clinical Features

Both pediatricas well as adult cases of AT/RT of the pineal region have been reported in the literature. However, of those reported, there are only three adult cases (Sugita et al. 1999; Ingold et al. 2006; Takei et al. 2010). No symptoms or signs at presentation specific to AT/RTs of the pineal region are known, but a rapid progression of symptoms reflecting their aggressive biological behavior is common. Tumors involving the pineal region that compress adjacent structures result in typical clinical symptoms, and one of the most common presentations includes headache, nausea, and vomiting, caused by aqueduct compression and resultant obstructive hydrocephalus, for which a shunt surgery should be considered. It is also commonly associated with bilateral papilloedema. Tumors compressing or directly infiltrating into the mesencephalic tectum, including the superior colliculus and adjacent oculomotor and Edinger-Westphal nuclei, result in dorsal midbrain syndrome (also known as Parinaud's syndrome), characterized by upgaze paralysis and papillary and oculomotor nerve paresis.

Leptomeningeal dissemination is commonly seen in AT/RTs of the pineal region as is those occurring in other locations. Metastasis beyond the CNS from the pineal region has been reported to date in the following two patients. A hematogenous metastasis to lung, which was histologically diagnosed 18 months after the pineal tumor surgery, was described in a 27-year-old man (Sugita et al. 1999). A case of abdominal seeding along a ventriculoperitoneal shunt catheter, which was found at autopsy approximately 7 months after the initial diagnosis, was reported in a 45-year-old woman (Ingold et al. 2006).

The first-line treatment is surgery; however, deep localization and diffuse infiltration into the surrounding vital structures usually precludes total gross resection of this tumor. The prognosis of AT/RTs of the pineal region is very poor despite intensive adjuvant chemotherapy (and radiation therapy) after surgery. Adult patients appear to have longer survival than pediatric patients probably due to adjuvant aggressive chemoradiation therapy available in adults, which would not be tolerated by young children.

Radiological Features

There are no specific imaging characteristics for intracranial AT/RTs (Parmar et al. 2006). No detailed magnetic resonance imaging (MRI) features of AT/RTs of the pineal region have been described in the literature. The MRI features of our case occurring in 33-year-old woman (Fig. 20.1a, b) are similar to those described in supratentorial AT/RTs, which are characterized by a large and lobulated lesion with heterogeneous signal intensity on T1 and T2-weighted images and heterogeneous enhancement, resulting from cystic change, necrosis, and/or hemorrhage (Cheng et al. 2005; Meyers et al. 2006; Parmar et al. 2006; Tez et al. 2008). Leptomeningeal disseminated foci can be detected.

Histopathological Features

No cytological or histological features unique to AT/RTs of the pineal region have been described. Cytomorphologically, the smears of AT/RTs stained with hematoxylin and eosin (H&E) are hypercellular with primitive-appearing neoplastic cells, which are characterized by a high nuclear/cytoplasmic (N/C) ratio, speckled chromatin, and small nucleoli, admixed with intermediate-sized, rhabdoid cells in varying proportions (Parwani et al. 2005). The rhabdoid cells are characterized by eccentrically placed nuclei, single prominent nucleoli, and granular-to-fibrillary, brightly eosinophilic cytoplasm with or without globoid "inclusions" (Fig. 20.2a). Apoptosis, mitosis and necrosis are commonly seen.

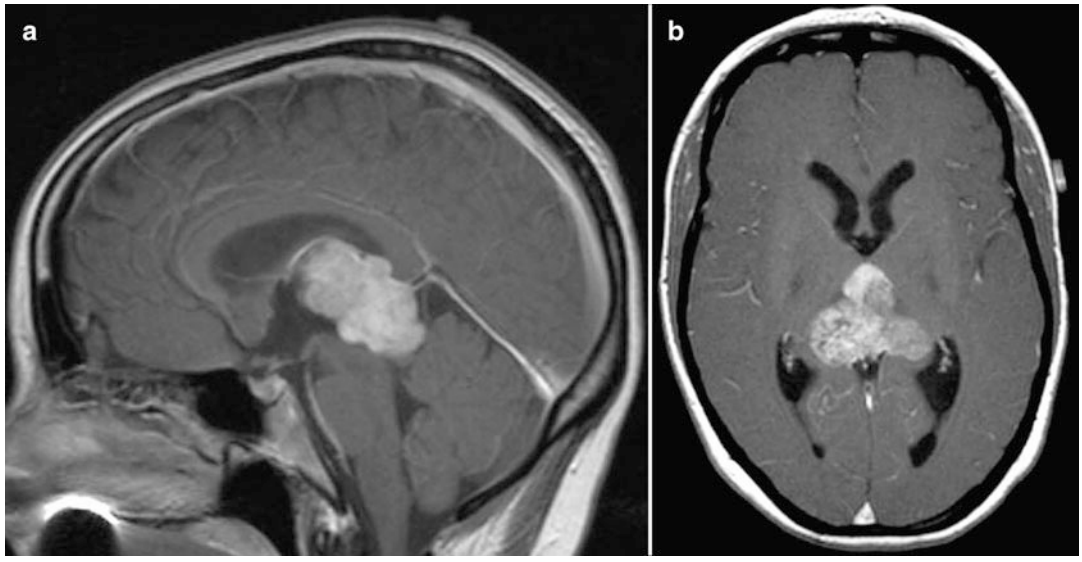

Fig. 20.1 MRI features, T1-weighted image with gadolinium contrast enhancement. (**a**) Sagittal section, (**b**) Transverse section. Heterogeneously enhancing, lobulated mass is present in the pineal region

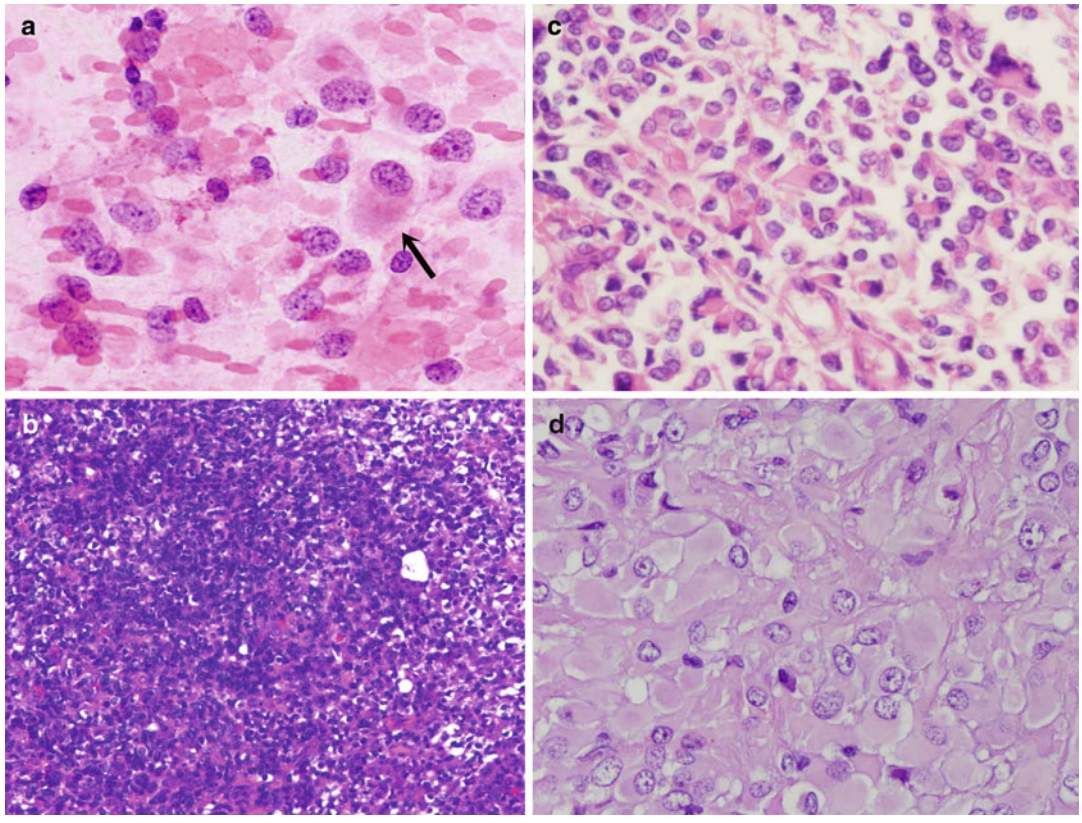

Fig. 20.2 (**a**) Cytologic touch imprint: Rhabdoid cells with eccentrically placed nuclei and granular-to-fibrillary, brightly eosinophilic cytoplasm present (*arrow*) (H&E stain). Histological features (H&E stain): (**b**) A mixture of primitive neuroectodermal tumour (PNET)-like (malignant small *blue* cells), rhabdoid, epithelial-like components as well as cells with clear vacuolated cytoplasm. (**c**) Rhabdoid cells admixed with PNET-like cells. (**d**) Uncommon AT/RT composed exclusively of rhabdoid cells

Histologically, AT/RTs are very cellular tumors that show marked regional heterogeneity, with PNET-like, rhabdoid, epithelial, and mesenchymal components in variable proportions (Fig. 20.2b, c). There is often a fibrovascular stroma separating lobules and sheets of tumor cells. The cellular morphology varies from smaller PNET-like cells (so-called malignant small blue cells) indistinguishable from tumor cells of medulloblastomas/PNETs, characterized by hyperchromatic nuclei, scant cytoplasm, and a high N/C ratio, to large cells with eosinophilic, pale, or clear cytoplasm and large round to oval nuclei with or without prominent nucleoli. Tumor cells with clear cytoplasm are commonly seen. Characteristic rhabdoid cells with eccentric reniform nuclei, frequent prominent nucleoli, and eosinophilic discrete cytoplasm with or without intracytoplasmic hyaline globular inclusions, are usually present. Brisk mitotic activity and tumor necrosis is common. Spindle tumor cells having a sarcomatoid appearance are also seen, often as a minor component. In general, a PNET-like component is a more common feature than other components in AT/RTs (Ertan et al. 2009). AT/RTs composed exclusively of rhabdoid cells are uncommon (Fig. 20.2d). An unusual adult case of pineal AT/RT composed of rhabdoid tumor cells and chondroid formation without any other mesenchymal components was reported (Sugita et al. 1999). Given the pineal location, the distinction from very rare immature teratomas remains blurred in this particular case, although commonly used immunohistochemical markers for germ cell tumors were all negative.

Immunohistochemical Profile

The immunohistochemical profile of AT/RTs reflects their morphological heterogeneity. Immunoreactivity for a range of neuroectodermal markers, mesenchymal, and epithelial markers are known to be present, but of note is that the tumors are consistently negative for the germ cell markers. Vimentin is consistently expressed in this tumor, and labels hyaline globular intracytoplasmic inclusions of the rhabdoid cells. Expression of EMA and SMA is frequently observed. GFAP, cytokeratins, synaptophysin, and neurofilament protein are also known to be expressed, depending on the different cellular composition of this tumor (Fig. 20.3a–c). Loss of expression (absent nuclear staining in tumor cells) of INI1, the product of *SMARCB1/hSNF5/INI1*gene, is a sensitive and specific marker for AT/RTs (Fig. 20.3d). For the interpretation of INI1 immunohistochemistry results, given the negative nuclear staining, it is important to insure that normal components within the tumor such as endothelial cells preserve nuclear expression, as positive internal control. It has been recently reported that claudin-6, a key component of tight junctions, is expressed by most AT/RTs, but not by most other brain tumors, most importantly PNETs/medulloblastomas (Birks et al. 2010).

In the diagnosis of AT/RTs, a panel of immunohistochemical markers, including vimentin, EMA, SMA, GFAP, cytokeratins, and synaptophysin, is likely to help confirm the diagnosis in the context of the appropriate morphological appearance. Claudin-6 may be a useful positive marker and can be included in the panel. INI1 immunostaining can be utilized in those cases having indeterminate histological features, or in small biopsies that may not be representative of the morphological heterogeneity typical of AT/RTs, since negative staining is intuitively not as desirable an end-result as compared with positive staining (Takei et al. 2007). The latter examples are very commonly encountered in pineal region tumors, in general, given the difficult location of surgical access.

Molecular Pathology

Most AT/RTs exhibit monosomy 22 or deletions of the chromosome 22q11 locus containing the *SMARCB1/hSNF5/INI1*gene, and less commonly show mutations of the *SMARCB1/hSNF5/INI1* gene. This gene encodes a component of the SWI/SNF chromatin remodeling complex that has an important role in transcriptional regulation (Imbalzano and Jones 2005).

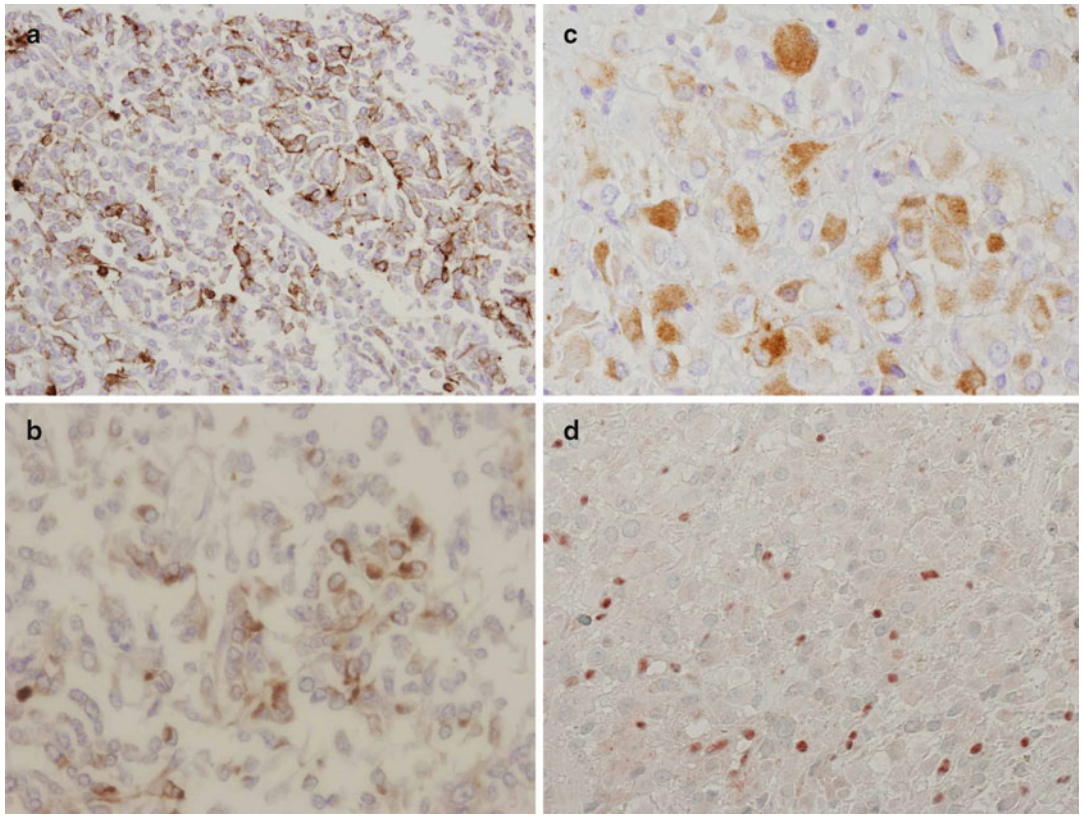

Fig. 20.3 Immunohistochemical features: (**a**) Epithelial membrane antigen, (**b**) Glial fibrillary acidic protein, (**c**) Neurofilament protein, (**d**) INI1, The tumour nuclei are negative, while the internal controls (i.e., endothelial cells and inflammatory cells) are positive

Immunohistochemical staining with anti-INI1 antibody (i.e., nuclear immunonegativity) correlates with these molecular alterations.

Differential Diagnosis

The differential diagnosis of AT/RTs of the pineal region includes so-called "malignant small blue cell tumors", choroid plexus carcinoma (CPC), tumors with prominent rhabdoid features involving this region, and metastatic tumors.

Malignant small blue cell tumors as a differential diagnosis include pineoblastomas and supratentorial PNETs involving the pineal region. Pineoblastoma is a highly malignant primitive embryonal tumour of the pineal gland, preferentially affecting young children, and is composed of primitive cells resembling PNETs/medulloblastomas. This tumor is distinct from PNETs in other locations in its photosensory differentiation, characterized by Flexner-Wintersteiner rosettes and fleurettes. Since AT/RTs usually contain PNET-like tumor cells as a major component, these tumors are considered to be the most important differential diagnoses, especially in children. In addition, this histological differential is particularly difficult or sometimes impossible in small biopsies, unless typical rosette formation is found. Rare pineoblastomas contain pigmented epithelium and mesenchymal components such as skeletal muscle and cartilage and are referred to as 'pineal anlage tumors' (Nakazato et al. 2007), which can further complicate the differential diagnosis. Immunohistochemically, pineoblastomas express not only neuronal, but also photosensory markers (e.g., retinal S-antigen), as do

some PNETs/medulloblastomas (Janss et al. 1996; Kramm et al. 1991). No studies to test photosensory marker immunoreactivity on AT/RTs have been reported to date. As mentioned above, INI-1 immunohistochemical staining (i.e., nuclear immunonegativity) is of great help for this differential diagnosis.

CPCs arising in the posterior third ventricle can involve the pineal region. CPCs and AT/RTs are known to share numerous clinical, radiological, and pathological (i.e., histological and immunohistochemical) features. Prominence of rhabdoid tumor cells favors AT/RT over CPC. However, histopathologic distinction between these tumors using routine immunohistochemical panels is often very difficult or may be impossible, especially if the AT/RT shows predominant epithelial differentiation, or if the CPC exhibits minimal epithelial differentiation. In general, significant immunoreactivity for SMA and EMA is a feature of AT/RT, while transthyretin (prealbumin) labeling favors CPC, although the expression is variable in this malignant choroid plexus tumor. Given that INI1expression is preserved in the majority of CPCs and is lost in AT/RTs, this immunohistochemical marker is particularly important for this differential diagnosis.

Tumors with prominent rhabdoid features that can involve the pineal region include rhabdoid meningiomas and rhabdoid glioblastomas, although they are much less commonly encountered as a differential diagnosis. Given the age incidence, this differential diagnosis is probably more important in older children and adults. Histologically, it is very important to find a subpopulation of an otherwise classic meningioma or glioblastoma, respectively. Moreover, a PNET-like component is not a feature of these tumors. Immunohistochemically, glial and neuronal markers are usually negative in rhabdoid meningiomas. Of note is that cytogenetic evidence of monosomy 22 cannot exclude a rhabdoid meningioma, since this is the most common cytogenetic alteration in meningiomas. INI1 nuclear expression is preserved in reported cases of rhabdoid meningioma (Wakabayashi et al. 2005; Buccoliero et al. 2010). In addition, cases of rhabdoid glioblastoma with cytogenetical evidence of monosomy 22 have been reported (Wyatt-Ashmead et al. 2001; Kleinschmidt-DeMasters et al. 2010). Interestingly, it is recently reported that two cases of rhabdoid glioblastoma show focal loss of INI1 protein nuclear immunoexpression and no expression of claudin-6, in contrast to AT/RTs, and it is suggested that claudin-6 immunohistochemical staining may be a good discriminator of AT/RTs from rhabdoid glioblastomas (Kleinschmidt-DeMasters et al. 2010).

AT/RTs with predominant epithelioid/rhabdoid components can be mistaken as metastatic carcinoma or melanoma, especially in older adults. Carcinomas with rhabdoid features have been reported in many anatomic sites, including stomach (Ueyama et al. 1993) and lung (Shimazaki et al. 2001; Yilmazbayhan et al. 2005). Some pulmonary cases were reported to show neuroendocrine differentiation immunohistochemically (Shimazaki et al. 2001). Malignant melanomas are known to show plasmacytoid features, and rare cases of rhabdoid phenotype have been reported (Abbott et al. 2004; Gavino and Gillies 2007). Characteristic polyphenotypic immunohistochemical profile described above supports the diagnosis of AT/RT rather than metastatic tumors. Interestingly, complete loss of S-100 protein and/or HMB-45 expression in some cases of rhabdoid melanoma has been reported (Gavino and Gillies 2007), while a single case of AT/RT with few HMB-45 immunoreactive melanin-containing tumors cells in an 8-year-old girl was reported (Tekkok and Sav 2005). Although younger age favors AT/RT rather than metastasis, clinical and radiological information are crucial for this distinction.

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Part VI

Sarcoma

Ewing's Sarcoma Family of Tumors: Targeting Molecular Pathways and the Race for a Cure

21

Atif A. Ahmed, Glenson Samuel, and Joy M. Fulbright

Contents

Abstract

Ewing's sarcoma family of tumors (ESFT) is a malignant tumor of bone and soft tissue in children and adolescents that is characterized by molecular alterations that most commonly involve the *EWS* gene on chromosome 22. It is an aggressive tumor, with metastases present at diagnosis in 20–25% of cases. Modern treatment regimens for localized disease have resulted in significant improvement in survival. However, the presence of metastasis is associated with mortality in 70–80% of patients. New insights into the pathogenesis and proliferative mechanisms of human cancers have led to the identification of a number of proteins acting as messenger molecules and modulators of tumor growth. Targeting these molecules in ESFT has the potential effect of controlling tumor growth. This chapter summarizes the current research on molecular pathogenesis, proliferation pathways and new investigational pharmacologic agents that are under development in the race for the cure of this malignancy.

Introduction

Ewing's sarcoma family of tumors (ESFT) is an aggressive malignancy that affects the growing bones and soft tissues of children and young adults. It is regarded as the second most common sarcoma of bone and commonly affects children in the second decade of life. It constitutes about

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3% of all pediatric malignancies and 40% of pediatric bone cancers. The disease, which is predominantly seen in Caucasians and is rare in other races, is not predisposed to by any environmental or familial factor, and does not have any known association with any syndrome or genetic disease. The median age at diagnosis is 15 years and approximately three cases occur each year per one million Caucasian patients younger than 21 years (Paulussen et al. 2001). Although ESFT is a primary bone cancer, a substantial proportion of these tumors arise from extraskeletal sites. Patients with extraskeletal ESFT have a higher mean age, but also have a bimodal distribution, being more commonly found in those older than 35 years and less than 5 years compared with skeletal tumors (Applebaum et al. 2011).

ESFT presents with pain and later a swelling from a localized mass that appears on imaging studies as a destructive diaphyseal bony lesion that also extends to the soft tissues. The pelvic bones, long extremities and ribs are, in decreasing frequency, the most commonly affected sites. However this tumor can arise in any bone or soft tissue location in the human body, including deep seated visceral organs. Patients may also present with nonspecific signs because of tumor location or with general signs of inflammation such as fever, anemia and leukocytosis. No blood, serum, or urine test can specifically identify ESFT. Serum and urine catecholamine levels are always normal (Bernstein et al. 2006).

Patients may also present with metastasis that can occur in 20–25% of cases before diagnosis. ESFT metastasizes most commonly to the lungs, bone and bone marrow. The presence of metastasis is the most important prognostic factor and patients with metastatic tumor have poor survival. About 30–40% of those with localized disease and 80% with metastatic disease die as result of disease progression (Bacci et al. 2007).

Molecular Insights into Tumor Pathogenesis

The term "Ewing's sarcoma family of tumors" is coined to describe closely related group of tumors that were previously considered separate entities: Ewing's sarcoma, peripheral primitive neuroectodermal tumor and Askin tumor. These tumors exhibit similar histologic, immunophenotypic and molecular signatures and hence grouped as one family. Histologically they are characterized by a "small round blue cell" morphology (Fig. 21.1) that may also reveal limited neural and/or epithelial differentiation. The cell of origin is not definitively known but is probably a primitive mesenchymal cell or neuroectodermal stem cell that has retained some ability for multi-lineage differentiation. Recent evidence has suggested that the cell of origin may actually be a bone marrow associated mesenchymal stem cell (MSC) that undergoes transformation from a spindle cell morphology to a rounded cell that is characteristic of ESFT (Riggi et al. 2005; Lin et al. 2011).

The primary genetic event that drives this cellular malignant transformation is a chromosomal translocation that involves *EWS*, a gene located in chromosome 22q12 (Riggi et al. 2008). *EWS* is fused to the DNA binding domain (DBD) of one of five ETS family transcription factors, that include *FLI1*, *ERG*, *ETV1*, *ETV4*, and *FEV* (Arvand and Denny 2001). Ewing's sarcoma family tumors (ESFT) are commonly associated with the chromosomal translocation t (11;22) (q24;q12) that generates the *EWS-FLI1* fusion gene in >85% of cases and *EWS-ERG* in 5–10% of cases. In the remainder, *EWS* is fused to other ETS members such as *ETV1*, *ETV4*, or *E1AF* (Giovannini et al. 1994). The evidence, that the induction of EWS-FLI1 and other chimeric EWS/ETS proteins in mesenchymal progenitor cells is the primary initiation genetic event in the tumorigenicity of ESFT, comes from in vitro studies on MSC lines. There was a change of morphology and immunophenotype of the MSC (positive for CD10, CD13 and vimentin) to a rounded cells that have the morphologic and immunophenotypic features of ESFT with upregulation of CD54, CD99, CD117 and CD271. The malignant transformation of the MSC towards ESFT is accompanied by ability of the cells to have invasive properties (Miyagawa et al. 2008).

EWS-FLI1 fusion protein is a transcription factor that can bind DNA and cause altered expression of down-stream genes. EWS-FLI1

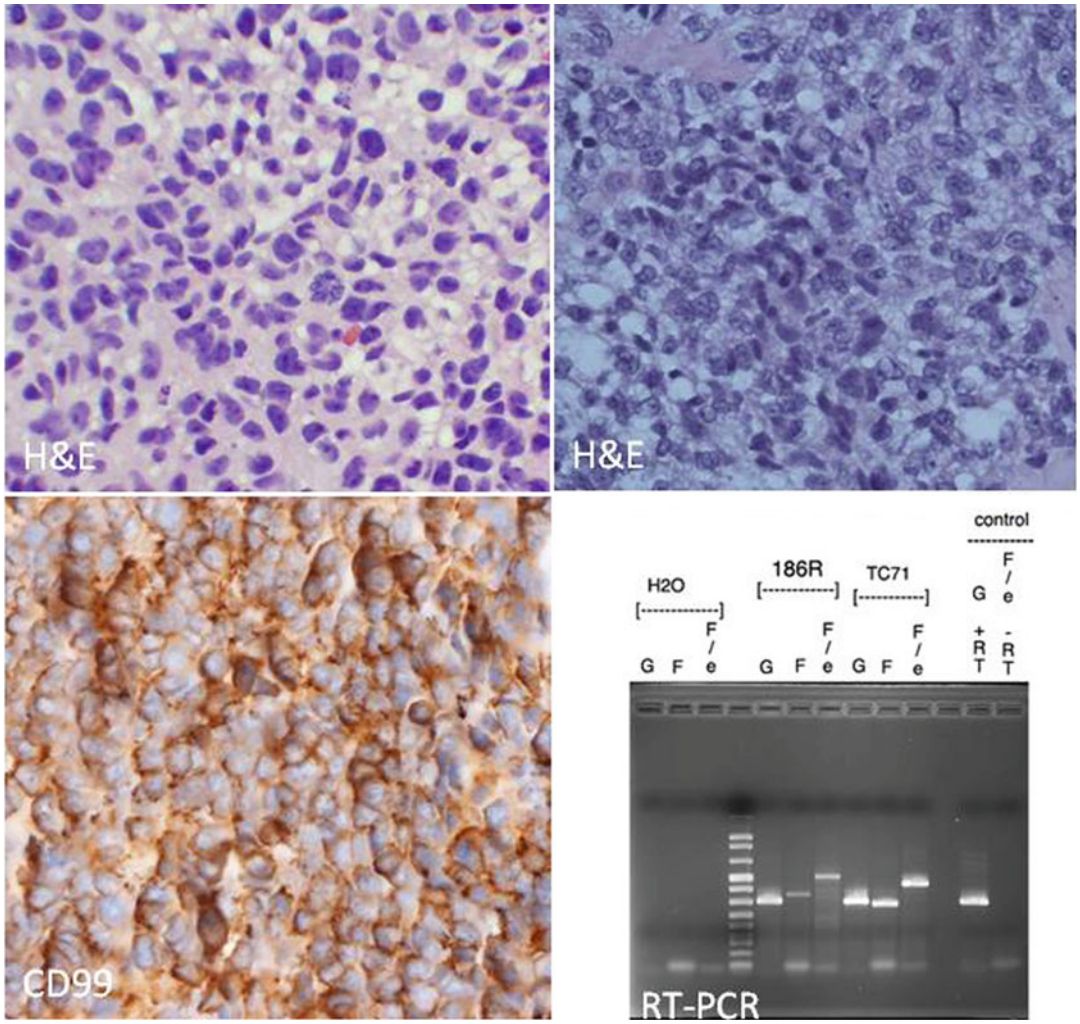

Fig. 21.1 The diagnosis of Ewing's sarcoma family tumor is suspected from the presence of the characteristic small round cell morphology on conventional H&E stains and is confirmed by the diffuse membranous immunohistochemical positivity for CD99. Molecular methods that detect the presence of EWS-FLI1 fusion gene by reverse transcription polymerase chain reaction (RT-PCR) are also important in confirming the diagnosis

interacts with the transcription factor CBP and results in promoter interactions with the AP-1 (fos/jun) transcription complex and is thus important for transcriptional regulation of several genes that mainly influence the production of cell cycle proteins, growth factors and apoptosis molecules. There is up-regulation of *MYC*, *IGF1*, *GLI1*, *TOPK*, *NKX2.2*, *DAX1*, *EAT-2*, *EZH2*, *MK-STYX*, *PLD2*, *MMP-3*, *FRINGE*, *ID2*, and *CCND1* along with repression of *TGFBR2* and *CDKN1A* (Jedlicka 2010). It has also been suggested that other mutations may occur in conjunction with *EWS-FLI1* oncogenetic transformation in a cooperative mechanism that allows for cell growth and proliferation (Lin et al. 2011). Comparative genomic hybridization studies have revealed the presence of altered copy number of numerous other genes including gains and deletions (Toomey et al. 2010). Gene expression studies have also yielded valuable information regarding gene signatures and their differences in localized versus metastatic disease (Pinto et al. 2011).

Pathologic Diagnosis

Details of tumor pathogenesis and biologic characteristics have led to valuable information that is also used in the pathologic diagnosis of this tumor. Currently, the diagnosis of ESFT is achieved through combination of histology and results of ancillary tests. The histology is that of an undifferentiated cellular tumor that may have overlapping features with other small round cell tumors, particularly solid alveolar rhabdomyosarcoma, lymphoblastic lymphoma or undifferentiated neuroblastoma. The tumor is composed of diffuse sheets of small cells with central nuclei and scant cytoplasm. The nuclei are round to oval with smooth contours and inconspicuous nucleoli and exhibit a low mitotic rate. The small amount of cytoplasm may appear clear or vacuolated due to accumulation of glycogen that can be identified with the periodic acid shift (PAS) special stain. Immunohistochemical tests are widely used to arrive at the diagnosis and help exclude other tumors in the differential diagnosis. Immunohistochemical stain with antibodies against CD99 reveal a diffuse membranous staining that is characteristic for ESFT (Fig. 21.1). An immunohistochemical test for Fli1, the partner in EWS-FLI1 translocation, can also be used in the diagnosis of ESFT and yields a nuclear staining pattern. Combination of histologic morphology and characteristic positive staining with PAS, CD99 and/or Fli1 is highly sensitive and specific for the diagnosis of ESFT (Pinto et al. 2011). Molecular testing to detect the characteristic chromosomal translocations is helpful to confirm the diagnosis. These molecular translocations can be detected by RT-PCR on fresh, frozen or paraffin-embedded formalin-fixed tissue (Fig. 21.1). The *EWS* (also called *EWSR1*) gene can also be detected by fluorescent in situ hybridization using specific probes (Lewis et al. 2007). In less than 5% of cases, characteristic *EWS-ETS* translocations are not identified and hence, gene sequencing of the *EWS* gene may confirm the diagnosis if necessary.

Pathobiologic Proliferation Pathways

EWS-FLI-1 fusion and other dysregulated genes lead to over-expression of several cellular signaling pathways that mediate cellular proliferation and tumor growth. Insulin-growth factor pathway and its members, IGF1 and IGFR1 have been found to be over-expressed in ESFT cell lines and tissues (Scotlandi et al. 1996). This may explain the surge in ESFT incidence during the pubertal growth spurt. The ubiquitous PI3K-AKT pathway and its members are protein kinases that are also identified in ESFT, both in vivo and in vitro studies (Ahmed et al. 2011). NF-Kappa B is a transcription factor that regulates genes involved in inflammation and cell proliferation and dysregulation of NF-kappa B has been identified in ESFT cell lines (Ahmed et al. 2011). Similarly the vascular endothelial growth factor (VEGF) and its family of proteins play a role in tumor angiogenesis and proliferation and have been identified in ESFT cell lines and tissues (Nagano et al. 2010). The expression of these molecules and several other proliferation pathway members can be detected on human tumor tissues using conventional immunohistochemical techniques (Fig. 21.2).

CD99 is upregulated during the cellular malignant transformation of ESFT and is commonly used as an immunohistochemical marker for confirming the diagnosis of ESFT as shown above (Fig. 21.1). CD99 (also known as MIC2, and recognized by the antibodies 12E7, HBA71, and O13) is a 32-kDa integral membrane glycoprotein that plays a key role in several biological processes, including cell adhesion, migration, and apoptosis, maintenance of cellular morphology and regulation of intracellular membrane protein trafficking. However the function of CD99 in ESFT is currently unknown. There is some evidence that CD99 is required for ESFT transformation (Rocchi et al. 2010). CD99 can affect ESFT cell growth, tumorigenesis and metastatic ability and may prevent neural differentiation of tumor cells. CD99 may affect the modulation of

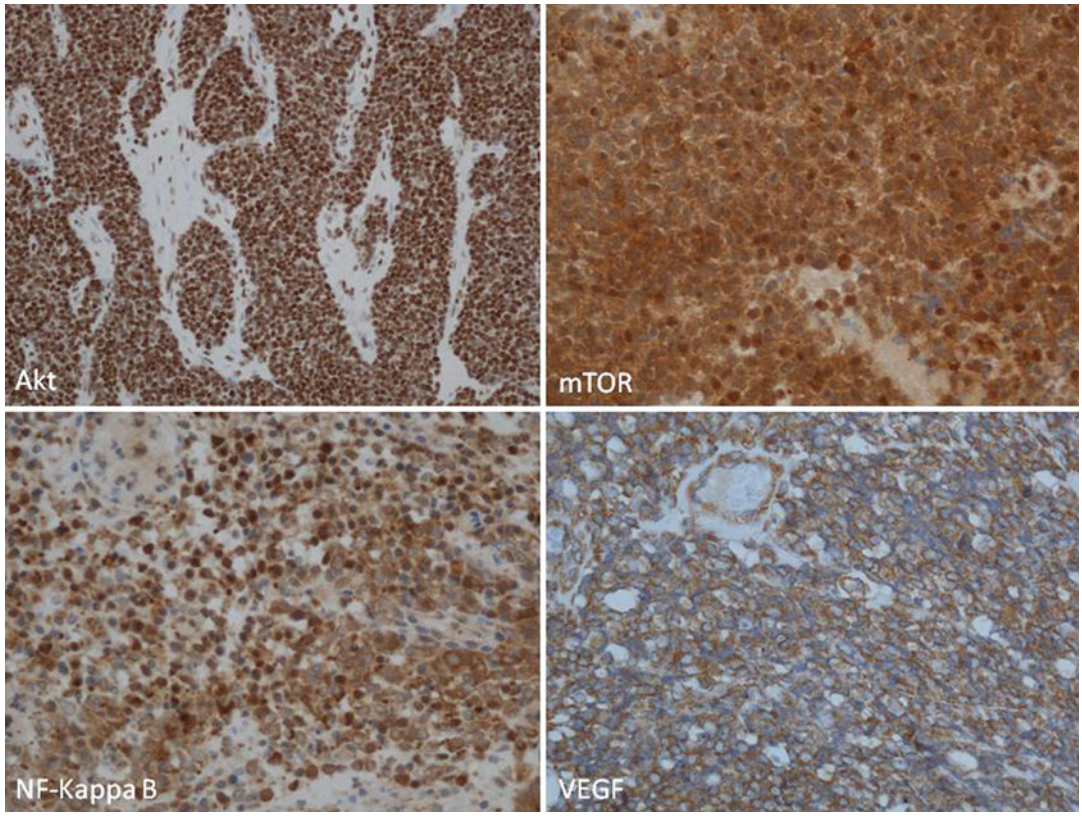

Fig. 21.2 Akt, mTOR, NF-kappa B and VEGF are over-expressed in the majority of Ewing's sarcoma tumor samples. Over-expression of proliferation pathway members and signaling molecules can often be detected by routine immunohistochemistry as shown

several intracellular pathways, including PI3K/Akt and RAS/MAPK which are activated by EWS-FLI1. CD99 seems to inhibit the activation of ERK1/2 and thus counter-act the effect of EWS-FLI1 on MAPK pathway activation. There is evidence that CD99 knockdown inhibits Akt while increases ERK1/2 phosphorylation, which in turn seems to modulate the neural differentiation of ESFT tumor cells.

Several other related pathways and molecules such as C-kit, caveolin-1, and E-cadherin are over-expressed and play an indirect role in tumor cell proliferation through their interaction with main proliferative pathways. Similarly, basic fibroblast growth factor and receptor molecules and platelet-derived growth factor receptors are over-expressed in ESFT. Furthermore, basic fibroblast growth factor may cause stimulation of EWS-FLI1 fusion protein activity (Girnita et al. 2000; Bozzi et al. 2007). Targeting these proteins and other proliferation pathways by small interfering RNAs and monoclonal antibodies is the subject of fervent research in attempts to discover new therapies for ESFT tumors that are resistant to conventional treatment (McAllister and Lessnick 2005).

Current Treatment Regimens

The current approach to treatment of Ewing's sarcoma is a combination of chemotherapy, surgery and radiation. For localized disease, chemotherapy with compressed cycles of ifosfamide/ etoposide alternating with vincristine/ adriamycin/cyclophosphamide or VIDE (vincristine/ifosfamide/doxorubicin/etoposide) per the Euro-Ewing's group, given prior to and post local

control, is the standard of care. With this treatment regimen, the overall survival is approximately 70% (Ladenstein et al. 2010). Patients with metastatic disease have worse outcomes, though patients with isolated lung metastasis fare better, with overall survival ranging from 9 to 41%. Independent poor prognostic factors include presence of ≥2 bone metastases, primary tumor volume >200 ml, age older than 14, pulmonary metastasis and bone marrow metastasis (Ladenstein et al. 2010). The question of whether autologous transplant improves survival in patients with metastatic disease continues to be evaluated.

Novel Therapeutic Targets

Given the poor outcomes of patients with metastatic or high risk Ewing's sarcoma, methods to improve outcomes have been the focus of extensive research. One area being explored is drug resistance and how to overcome it. Another large area of research, as alluded to previously, is targeted therapy. The targets of these therapies are some of the pathway members discussed previously that are over-expressed in ESFT. Several of these therapeutic targets are currently being evaluated in pre-clinical and/or early clinical trials (Table 21.1). Examples of such therapeutic targets and their applications in clinical trials are listed below.

Table 21.1 Targeted therapy currently being evaluated in pre-clinical and clinical trials for the treatment of Ewing's sarcoma

Target	Example of targeting agents
IGFR-I	Small molecule inhibitors (OSI-906, BMS-554417)
	Monoclonal antibodies (IMC-A12, R1507)
mTOR	Rapamycin, Sirolimus, Ridaforolimus
VEGF	Bevacizumab, Sunitinib, Cediranib
Multiple kinases	Sorafenib, Imantinib
Aurorakinase inhibitor	PF-03814735
Hedgehog pathway	Arsenic Trioxide, ZIO-101
EWS-FLI 1	YK-4-279
PARP 1	BMN673
	Olaparib
Dual (P13K and MTOR)	BEZ235

Ewing's Sarcoma-FLI1

Due to EWS-FLI1's role in the pathogenesis of Ewing's sarcoma there has been a lot of interest in targeting EWS-FLI1 itself, but unfortunately it has proven difficult to move these therapies from bench to bedside due to the pharmacokinetics of these targeting agents (Herrero-Martin et al. 2011). Extensive work has also been done looking at blocking downstream targets and to inhibiting binding of transcription proteins to EWS-FLI1. An example of downstream targeting work is that De Vito et al. (2011) performed on the miRNA expression in human mesenchymal stem cells and ESFT cell lines. This work demonstrated a difference in the miRNA signature and revealed a decrease in let-7a expression in ESFT cell lines compared to mesenchymal stem cells, thus implicating let-7a as a potential treatment target. Mice with Ewing's sarcoma treated with let-7a miRNA had a decrease in tumor burden along with a change in gene expression. This implies that let-7a miRNA plays a significant role in Ewing's sarcoma, and can also be a target for Ewing's sarcoma therapy (De Vito et al. 2011).

Another way to target EWS-FLI1 is to target the proteins that are involved in its transcription. YK-4-279 blocks RNA helicase A binding to EWS-FLI1 and thus induces apoptosis in ESFT cell lines. This treatment has led to inhibition of growth in xenograft mouse models (Erkizan et al. 2009). YK-4-279 has been evaluated in a clinical trial at the University of Texas MD Anderson Cancer Center (Subbiah and Anderson 2011).

Another very promising downstream target that is being tested in clinical trials is poly(ADP-ribose) polymerase (PARP 1). Brenner et al. (2012) published data demonstrating that EWS-FLI increases PARP 1 expression which in turn promotes more EWS FLI expression. Xenograft Ewings sarcoma models demonstrated sensitivity to PARP 1 inhibitors. This sensitivity is further increased when PARP 1 inhibitors are combined with Temazolamide.

Vascular Endothelial Growth Factor (VEGF)

The pathogenesis of Ewing's sarcoma is partly contributed to by blood vessel formation. There are two specific processes that are part of blood vessel formation: angiogenesis and vasculogenesis. Angiogenesis is the growth of new vessels off of preexisting vessels and vasculogenesis refers to the initial formation of the vasculature. Production of vascular endothelial growth factor (VEGF) and engagement of its receptors is thought to contribute to the development of new vessels in Ewing's sarcoma (Stewart and Kleinerman 2011). EWS-FLI1 also contributes to vessel production as it upregulates production of VEGF-A mRNA and protein levels (Nagano et al. 2010). In a preclinical trial using an ESFT model it was noted that VEGF was produced and secreted in 6/6 ESFT cell lines tested. High VEGF expression was also noted in 18 of 30 patients' tumor samples. Expression of VEGF correlated with microvessel density (MVD) (Dalal et al. 2005). High expression in tumor samples also correlated with patient's survival. Patients with high level of expression had decreased survival compared to those with low level of expression (Ahmed et al. 2011).

Preclinical studies have demonstrated that by blocking VEGF A production (with a VEGF-A-targeted siRNA) (Nagano et al. 2010) and inhibiting VEGF with an antibody (rhuMAb) (Dalal et al. 2005) there is significant tumor shrinkage. These preclinical studies, along with others, have led to the use of anti-angiogenesis/vasculogenesis agents in phase 1 trials. Despite the evidence that antivascular therapy has efficacy, it is likely not going to be curative alone. In future studies, it will be important to evaluate how antivascular agents improve outcomes in combination with traditional chemotherapy or other targeted therapy (Stewart and Kleinerman 2011).

Insulin Growth Factor Receptor (IGFR)

It has been shown that most ESFT tumor cells express an increased level of the insulin growth factor- 1 receptor (IGF-1R) or insulin growth factor- 2 receptor (IGF-2) ligands (Grimberg and Cohen 2000). The IGF-1R is a tetrameric tyrosine kinase receptor which is seen in both normal and neoplastic cells. The activation of IGF-1R is by the engagement of growth factor ligands IGF-1 and IGF-2, which then results in the autophosphorylation of these receptors. There are six IGF binding proteins which serve to regulate the activity of the IGF-1R by either promoting or inhibiting signaling by the binding of these IGF ligands in the circulation (Grimberg and Cohen 2000). The IGF-1R converts extracellular signaling intracellularly, which then mediates the proliferation, growth and survival of cells. Activation of the IGF-1R leads to activation of several signaling pathways, such as the phosphatidylinositol 3 phosphate kinase/Akt/mammalian target of rapamycin (mTOR) pathway. When activated, these pathways may lead to the promotion of an oncogenic phenotype. There has been evidence that the IGF-R1 signaling is critical in the biology of the ESFT. It has also been shown that malignant transformation by the pathognomonic *EWS-FLI1* fusion gene is dependent on IGF-1R (Toretsky et al. 1997). The *EWS-FLI1* fusion gene also promotes the activation of the IGF-R1 by downgrading the expression of the insulin growth factor binding protein-3 (IGFBP-3). There have been several studies demonstrating that drugs targeting the IFG-R1 inhibitors may elicit growth arrest in Ewing's sarcoma cells in xenograft models (Scotlandi et al. 2005).

Recently there have been several clinical trials using monoclonal antibodies against the IGFR-1 receptor. So far the results of these trials have not been as promising as expected (Ho and Schwartz 2011; Subbiah and Anderson 2011). There are current trials that are designed to improve the efficacy of IGF-R1 inhibitors by combining them with other targeted therapy involved in the pathway, such as mTOR inhibitors (Subbiah and Anderson 2011).

The Mammalian Target of Rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that controls cap-dependent

translation. It plays an integral role in the regulation of multiple signaling pathways that control cellular growth, division, metabolism and angiogenesis (Bjornsti and Houghton 2004). mTOR is activated in response to nutritional and environmental conditions and plays a role in the transduction of proliferative signals mediated through the phosphoinositide-3-kinase (PI3K)/Akt pathway. mTOR and members of the PI3K-AKT pathway are identified in ESFT, both in vivo and in vitro studies (Ahmed et al. 2011).

Rapamycin is a macrocyclic lactone antibiotic, and the first compound to demonstrate specific mTOR inhibition. Deforlimus (also known as AP23573) is a non-prodrug analog of rapamycin that has also been shown to have mTOR activity inhibition. mTOR inhibition may have direct effects on cancer cell proliferation and survival, or indirectly by inhibition of the hypoxia inducible factor 1α (HIF1α) which reduces tumor induced vascular endothelial growth factors (VEGF) (Faivre et al. 2006). Rapamycin has demonstrated in multiple tumor models an anti-angiogenic activity through decreasing the density of vessels. The anti-angiogenic activity of rapamycin has been linked to a decrease in the production of VEGF and inhibition of vascular endothelial cells to stimulation by VEGF (Guba et al. 2002).

In both adult and pediatric cancer cell lines, including those derived from rhabdomyosarcoma, neuroblastoma, glioblastoma, osteosarcoma, and Ewing's sarcoma, rapamycin has resulted in inhibition of cell growth (Bjornsti and Houghton 2004; Mateo-Lozano et al. 2003). mTOR inhibitors are currently being studied as single agents or part of combination therapy in the treatment of solid tumors and specifically for Ewing's sarcoma in combination with IGF-R1 inhibitors (Subbiah and Anderson 2011).

In conclusion a better understanding of the pathogenesis of Ewing's sarcoma has led to the development of new targeted therapies for this disease. Targeted therapies may be combined with current chemotherapeutic regimens to improve outcomes. The incorporation of other targeted therapies to prevent resistance is the next step in the cure of this malignancy.

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Ewing's Sarcoma: Current Concepts in Chemotherapy and Surgical Control

22

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Abstract

Ewing sarcoma is a relatively rare tumor of childhood and constitutes 3% of all pediatric malignancies. It is pathologically a malignant round cell tumor, CD99 positive on immunohistochemical staining and characterized by a unique translocation, t(11;22). The most common presenting symptoms are persistent pain and swelling. Imaging of primary site and biopsy are essential for confirmation of diagnosis and staging work-up includes bone marrow biopsy, bone scan and CT scan of chest. A comprehensive multidisciplinary approach incorporating multiagent chemotherapy along with surgery and or radiation therapy is the standard of care. Outcome of patients with localized disease has improved considerably but remains dismal for those with metastatic and recurrent disease.

Introduction

Ewing sarcoma (ES) is the second most common primary malignant bone tumor of childhood and adolescence. It constitutes 3% of all pediatric cancers. From a dismal 5 year survival rate of 10% in the 1960s, the reported 5 year survival rates have now improved to 65–70% (Grier et al. 2003). Management is multidisciplinary and combination chemotherapy forms the treatment backbone. Local therapy in the form of surgery and or radiotherapy is essential for a curative outcome. Advances in techniques of limb sparing

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surgery and radiotherapy have greatly contributed to quality of life of the survivors. In this chapter we will briefly dwell on epidemiology, diagnosis and prognostic factors before discussing current concepts in chemotherapy and surgical control.

Epidemiology

Ewing sarcoma occurs most commonly in the age group 10–20 years, and is extremely rare in patients younger than 5 years of age. The peak incidence in males is between 10 and 14 years of age; in females, it is 5–9 years (range: 1–80 years) with a frequency of approximately 2.9 per million in children under 20 years of age. Approximately 250 patients are diagnosed with ES each year in the USA (Gurney et al. 1975). As with many pediatric solid tumors, males are slightly more affected than females. It is distinctly rare in African-American children and uncommon in Chinese.

Biology

Ewing sarcoma probably originates from unique mesenchymal stem cells capable of multilineage differentiation along osteogenic, adipogenic, or neurogenic lines. The term Ewing sarcoma family of tumors includes a group of malignant round cell tumors arising from bone and soft tissue and includes the peripheral primitive neuroectodermal tumors (PNET). A specific chromosomal translocation has been observed in 85% of ES, the t(11;22)(q24;q12) and its variant in another 10% (Delattre et al. 1994). The EWS-FLI-1 translocation results from the apposition of the N-terminal portion of the EWS gene (located at 22q12) with the C-terminal FLI-1 gene (11q24) of the erythroblast transformation sequence (ETS) transcription factor family. Less common chimeric pairings include EWS-ERG (5–8% incidence) and EWS-ETV1, EWS-EIAF, and EWS-FEV, each occurring in less than 1% of reported cases (May et al. 1993).

Diagnosis

Clinical Features

Pain is the most common initial symptom of ES and may subsequently be followed by a palpable mass. Pain is usually localized, intermittent or variable in intensity, and is often mistaken for ‘growing pains’ or sports-related injuries that occur in this age group. When diagnosed at a later stage with advanced or metastatic disease, constitutional symptoms such as malaise, low-grade fever or anemia may be observed. The symptoms frequently mimic those of other conditions such as osteomyelitis or sciatica (radicular pain in pelvic or spinal lesions). The duration of symptoms before presentation usually varies from few weeks to months depending on the location of the tumor, being longer for tumors arising in the pelvis and abdomen. ES affects the axial and appendicular skeleton almost equally. It typically occurs in flat bones and diaphysis of long bones, though other areas may also be affected. The most common primary bony sites, in descending order, include the pelvis (26%), femur (20%), tibia/fibula (18%), chest wall (Askin tumor, 16%), upper extremity (9%), and spine (6%); any bony site can be involved and, less frequently, ES can originate in extraskeletal soft tissues such as calf muscle and orbit.

Imaging

The initial imaging investigation in a suspected case of ES is usually a radiograph in two planes (AP and lateral). The imaging features of ES may be quite variable but usually include a primary lytic bone lesion with periosteal reaction typically described as “onion skin ’ and an associated soft tissue mass, frequently larger than the bony component of the tumor. There may, however, be several other radiological presentations, including a ‘sclerotic lesion resulting from bone necrosis or reactive bone deposition, a perpendicular type of periosteal reaction, a predominantly

periosteal tumor resulting in a "sauceristation" of the cortex of the long bone, or a purely intramedullary tumor with minimal soft tissue mass. Spinal ES may have a "vertebra plana" or minimal bony involvement with a large epidural component.

CT scan is helpful in defining osseous details particularly for tumors of pelvis, spine, scapula, sacrum and ribs. MRI is useful in delineation the soft tissue mass, its relation with the neurovascular bundle, presence of skip lesions and marrow involvement (Frouge et al. 1988). Fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET) is a highly sensitive staging tool for the detection of metabolically active disease in bone, soft tissue and bone marrow.

A biopsy is required to confirm the diagnosis. Core needle biopsy carried out as an outpatient or day care procedure is performed in the majority of patients, though an incisional biopsy may sometimes be required. Usually the soft tissue component is biopsied and the biopsy tract is placed in line with the planned definitive surgical incision, so as not to violate tissue planes and neurovascular structures, and should be excised during definitive surgery. The biopsy is best performed by an experienced orthopedic surgeon, working as part of the oncology team performing the definitive surgery (Mankin et al. 1996).

Pathology

Microscopically, ES consists of densely packed homogeneous small round to oval-shaped cells arranged in sheets. Because the histologic and immunophenotypic features of ES may overlap with the other small round-cell tumors of childhood, an expanded panel of immunohistochemical studies may be necessary. Neuroblastoma is immunoreactive for NSE, S-100 but is negative for vimentin and immunoreactive for neurofilament protein. Lymphoblastic lymphoma is strongly immunoreactive for CD99, but unlike ES, it is also immunoreactive for leukocyte common antigen (CD45) and or TdT and other lymphoid markers. Rhabdomyosarcoma may also be immunoreactive with antibodies to CD99; however, staining is usually focal, weak, and cytoplasmic, and unlike ES, rhabdomyosarcoma is immunoreactive for myogenin, myoD1, desmin, and actin. The differential diagnoses therefore, may range from osteomyelitis to small cell osteosarcoma, rhabdomyosarcoma, primary bone lymphoma, metastatic neuroblastoma and Langerhan cell histiocytosis.

Staging

Staging work-up of ES is essential before starting treatment. The disease is metastatic in about 25% of patients at the time of diagnosis. The most common metastatic sites are the lungs, the skeletal system, and the bone marrow, or combinations thereof. Locoregional lymph node involvement is rare. In addition to imaging of primary site, investigations should include computed tomography (CT) scan of the chest and 99 m-technetium whole-body radionuclide bone scans or PET CT scan and bilateral bone marrow biopsy. There is no universally accepted staging available for ES at this time, and most treatment protocols use presence or absence of metastases at diagnosis as the main tool to plan treatment strategy. As, ES rarely spreads to the lymph nodes and, by definition, is high grade, therefore the TNM (tumor, node, and metastasis) and AJCC (American Joint Committee on Cancer) staging systems for primary tumors of bone and soft tissue, do not apply very well to this disease (Juergens et al. 2009).

Prognostic Factors

Several clinical and biologic characteristics help to prognosticate patients with ES. These include the age of the patient, primary tumor location, tumor volume, presence or absence of metastases, and response to therapy. The most important prognostic factor is the presence or absence of metastases at the time of diagnosis. Approximate 5-year survival rates for patients with localized disease are 70%, compared to 25% for those who

have overt metastases at diagnosis. Patients with isolated lung metastases fare better than the others. For patients with localized disease, those with axial primary tumors have a worse treatment outcome than those with extremity lesions. In addition, patients with small primary tumors (<100 mL) fare better than those with larger tumors. The poorer prognosis of large primary tumors, and those involving the pelvis and spine, is at least partly attributable to the difficulty in achieving wide negative resection margins, and higher rates of local failure after radiotherapy for large lesions. Patients with poor histological response to chemotherapy do worse than those with minimal or no residual tumor. Older age (>10 years) has been linked to a poor prognosis in some reports, but not others.

Treatment Principles

The treatment of ES involves a multidisciplinary approach. As it is a systemic disease from the onset, therapy includes a backbone of multiagent chemotherapy combined with surgery and or radiotherapy for local control. The disease is not only very chemosensitive but is also highly radiosensitive. This responsiveness makes local therapy of ES a controversial topic.

The treatment is aimed at achieving two major goals, local control and eradication of systemic disease. To achieve these, most protocols consider three phases of treatment: (1) Induction chemotherapy, which is aimed at achieving rapid initial cytoreduction, eradicating micrometastatic disease, facilitating limb salvage procedures and assessing the response to chemotherapy; (2) Local control, using surgery, irradiation, or both, usually after 9–12 weeks of induction chemotherapy and (3) Continuation therapy, using similar chemotherapeutic agents as induction to eradicate systemic disease and prevent recurrences.

Chemotherapy Current Concepts

Before the era of chemotherapy, fewer than 10% of patients with Ewing sarcoma survived despite the well known radiosensitivity of this tumor as described by Ewing (1921). Most of the patients succumbed to distant metastasis, thus, highlighting the need for systemic therapy. Historically, the role of chemotherapy in Ewing sarcoma was first demonstrated in the early 1960s, when Sutow and Sullivan (1962) and Pinkel (1962) independently reported experiences with the chemotherapeutic agent cyclophosphamide. Subsequently, Hustu et al. (1968) reported that the combination of cyclophosphamide, vincristine and radiotherapy resulted in sustained responses in five patients with ES. Thereafter, Rosen et al. (1974) from Memorial Sloan-Kettering Cancer Center used the VACD regimen (vincristine, actinomycin D, adriamycin and cyclophosphamide) in combination with radiotherapy resulting in long-term survival of 12 patients. These early studies demonstrating the beneficial effect of chemotherapy, laid the foundation of the modern era multidisciplinary approach to ES. Further improvements in the survival of patients with ES came through systematic organized clinical trials by various cooperative groups, including the North American Intergroup Ewing Sarcoma Study (IESS), European UK Children's Cancer Study Group (UKCCSG) and the German–Dutch–Swiss Cooperative Ewing's Sarcoma Studies (CESS) groups. The clinical lessons learnt from these studies are discussed below.

The first IESS trial (1973–1978) by Nesbit et al. (1990) showed an unequivocal survival advantage with regimen using doxorubicin in addition to VAC chemotherapy in terms of 5 year event-free survival (60% from the earlier 24%). It also showed that inclusion of doxorubicin in every cycle is superior to the use of doxorubicin alternating with actinomycin D, even when the cumulative doses of both the drugs in the two schedules remained the same. The addition of prophylactic whole lung radiotherapy was also shown to be beneficial, although not as much as the addition of doxorubicin. The IESS-II trial (1978–1982) demonstrated the advantage of increasing doxorubicin intensity early in the course of therapy. This trial proved that intermittent high dose therapy with VAC plus doxorubicin (dose increased by 150% in the initial weeks of therapy) was superior to continuous moderate dose therapy improving overall survival from 77

to 56% (Burgert et al. 1990). Since then, many multi-institutional collaborative trials both within and outside the United States have confirmed the clinical benefit of VACD-based regimen.

The European cooperative groups, through independent single-group studies by the UKCCSG and CESS groups, evolved a different approach. Based on the presence or absence of metastases, and pretreatment radiographically-determined tumor volumes (100 or 200 ml), the CESS group classified patients into standard and high risk groups. Poor histological response to initial chemotherapy was also identified as a poor prognostic factor. Both the CESS and UKCCSG adopted a chemotherapy design in which four drugs were given at once, and this evolved from VACA (vincristine–doxorubicin–cyclophosphamide–actinomycin), to VAIA (substituting ifosfamide for cyclophosphamide) (Paulussen et al. 2001a). In later studies these group incorporated etoposide to VAIA (EVAIA), and to the current VIDE (omitting actinomycin). The only randomised controlled trial in this series, the EICESS-92, found no difference between VACA and VAIA for standard risk patients with ES, and a slight advantage (although statistically insignificant) for EVAIA over VAIA in patients with high-risk localised or metastatic tumors (Paulussen et al. 2008).

As discussed above, another breakthrough in the management of ES came with the addition of etoposide and or ifosfamide to preexisting regimen. Both these drugs have been found to be very effective against this tumor because of their synergistic antitumor effect and especially with fractionated administration. European trials ET-1 and ET-2 clearly showed the advantage of the addition of these agents in patients with non-metastatic disease (Craft et al., 1997; 1998.) Later on, the North American Intergroup Ewing trial (INT-0091 - POG-8850/CCG-7881) showed that VACD/IE regimen was superior to the standard VACD (5-year EFS 69% vs. 54% respectively, $p=0.005$) for patients with localized disease especially with large tumors and pelvic primaries. This study also demonstrated that the benefit of intensive chemotherapy was not only limited to its systemic effects, but was also advantageous for local control (Grier et al. 2003).

The next phase of trials was aimed at improving survival rates by increasing dose intensity or dose density of chemotherapy. Advancements in supportive care and use of growth factors have made it feasible to intensify treatment without increasing treatment related morbidities. One can increase dose intensity either by increasing the doses per cycle, or decreasing the interval between cycles. The first Intergroup study INT-0154, used an experimental regimen, VDC–IE, in increased doses, thus, decreasing the length of treatment from 17 cycles (48 weeks) to 11 cycles (30 weeks) to maintain same total cumulative drug doses. This regimen was associated with more treatment related toxicity without improvement in survival (Granowetter et al. 2009). The other approach of reducing the interval between cycles while maintaining the same dose per cycle was tested by the Children's Oncology Group in AEWS-0031 study. In this study, patients with non-metastatic ES received alternating courses of VAC and IE provided in a 'dose-dense' fashion every 2 weeks or as a standard 3-week interval. This dose intensification and interval compression has the theoretical advantage of allowing less time for recovery of partially resistant cells. Interval compression provided a 25% increase in dose intensity of all agents without an increase in toxicity. Overall and event-free survival were both improved in the interval-compressed group (event-free survival 79% vs 70% at 4 years, $p=0.023$) (Womer et al. 2008). The regimen of alternating VDC–IE every 2 weeks has now become standard of care for the North American patients with ES.

The EUROpean Ewing tumor Working Initiative of National Groups (EURO-EWING) 99 protocol, has taken a different approach, providing 6 cycles of VIDE (vincristine, ifosfamide, doxorubicin, and etoposide) to all patients (Juergens et al. 2006). Thereafter, the patients are risk stratified depending on tumor volume, presence (and location) of metastatic disease, and histological response to chemotherapy. For patients with poor histological responses, or large tumors treated with radiation, or lung metastases, it compares VAI with busulfan–melphalan megatherapy. Outcome data are not yet available from this ongoing trial.

Standard of Care: Non-Metastatic Disease

Currently in the United States chemotherapy for ES includes vincristine-doxorubicin-cyclophosphamide alternating with ifosfamide and etoposide every 2 weeks. Typically, 4–6 cycles of chemotherapy are given before local therapy. Additional cycles of the same combination chemotherapy are continued after local therapy to a total duration of 30 weeks.

Metastatic Disease

Patients with disseminated disease at diagnosis often respond well to the same type of systemic chemotherapy as that used for localized disease. Standard treatment options include conventional or high doses of vincristine, doxorubicin, and cyclophosphamide alternating with ifosfamide and etoposide with or without actinomycin D (VDCA or VDC/IE). Chemotherapy regimen incorporating higher doses of alkylating agents, and more intensive multiagent combinations generally require aggressive supportive care. After 4–6 cycles of chemotherapy, primary as well as metastatic sites are reassessed for response. If there is progression of disease, there is hardly any chance of cure and these patients are treated either on experimental arms or undergo palliative therapy. Those who have good response to initial chemotherapy, receive local therapy usually radiation to both primary and metastatic sites and followed by chemotherapy with similar agents for 48 weeks. The COG recently completed a study adding metronomic anti-angiogenic therapy with vinblastine and celecoxib to this backbone, the results of this trial are still awaited.

Role of Autologous Stem Cell Transplant

In view of the exquisite chemosensitivity of ES and with the aim of improving survival of patients with high risk disease, investigators have tried the approach of high-dose chemotherapy with autologous stem cell rescue. Published studies have not shown any survival advantage of this approach. However it is clearly associated with an increased incidence of treatment-related leukemia and myelodysplastic syndromes (t-AML/MDS) (Meyers et al. 2001). The ongoing European EuroEWING-99 trial is testing this approach for the first time in a randomized fashion. Patients with localised tumors and a poor response to initial VIDE chemotherapy, or with lung metastases at diagnosis, are randomly assigned to either further chemotherapy (vincristine, dactinomycin and ifosfamide) or busulfan–melphalan megatherapy with autologous stem cells. In a few years from now, we may have a first answer to this long-running controversy.

Surgery and Local Control

Local control in the form of surgery and or radiotherapy is an essential component of successful treatment of ES. There is a general agreement now for surgical treatment as the choice of local therapy. This is largely due to a trend towards better oncological outcome (Rodríguez-Galindo et al. 2008), but also due to apprehensions for complications such as musculoskeletal growth disturbances, and radiation induced second malignancies. Better outcomes with surgery have often been attributed to selection bias, however this is the preferred modality in patients amenable to limb salvage surgery. The advancements in limb salvage surgery and prosthetic technology are responsible for making surgery a safe and effective local therapy for ES. Indeed, because of the heterogeneity of the disease, biologic and treatment variables, it is difficult to analyze the exact role of local control modality in the treatment of ES. Hence, the choice of local treatment has to be individualized to the patient through a multimodal approach and involving the patients or their parents in this decision.

The principle of surgical resection of ES is resection with wide margins (removal of tumor with a cuff of normal tissue covering it all around). For soft tissue margins this usually means removal of 2 cm cuff of normal tissue or presence of good

anatomical barrier including fascia or articular cartilage (Kawaguchi et al. 2004) and for osteotomy a 3–5 cm from the level of radiological involvement is recommended. After effective neoadjuvant chemotherapy, smaller margins on bone may also be acceptable (Kawaguchi et al. 2004). Joint sparing resections may also use open physeal cartilage as margin. This approach while being oncologically sound, helps save the nearby joint at the same time. Some workers have advocated the use of computer navigation for accurate resection with safe margin based on imaging findings while preserving as much bone as feasible (Kim et al. 2010). Similarly, distraction of growth plate is also being done preoperatively to enable preservation of the physis while retaining good margins for excision. Similar principles of resection are followed for other sites including chest wall and soft tissue ES. An intraoperative frozen section from the bone marrow should be sent for confirmation of negative margins at the osteotomy site. Reconstruction of large segmental defects following resection is a challenging task. These patients are young, and often have significant growth remaining. Hence an ideal reconstruction should be durable, compensate for the loss of growth of the involved limb, result in the function and appearance of the limb as close to normal as possible, be compatible with early rehabilitation, be cost effective and readily available. Obviously, there is no single ideal method of reconstruction, and it has to be chosen keeping in mind the requirements of the patient. Use of megaprosthesis is a common mode of reconstruction as it has a predictable functional outcome, allows early rehabilitation, allows for intra operative flexibility in the length of the reconstruction required, is non biological and is unaffected by adjuvant chemotherapy or radiotherapy. However, the main disadvantage of megaprosthesis is the vulnerability to wear and tear leading to loosening or breakage in the long term. Furthermore, the reattachment of tendons to the prosthesis is another factor compromising the functional outcome. Improvements in prosthetic technology (rotating platform design, hydroxyapatite coated collar and stem, porous tantalum and compression osteointegration technology) hold promise in overcoming these limitations of this useful method of reconstruction (Palumbo et al. 2011). Availability of expandable prosthesis has minimized the problem of limb length discrepancy in young children with significant remaining growth, as they can be lengthened non-invasively.

Biological methods are also used for reconstruction after segmental resection of long bones. They depend on bone healing for rehabilitation, which is subject to effects of adjuvant therapy and is associated with a long rehabilitation time. Allografts offer the advantage of good reattachment of tendons for optimal function, particularly at sites such as proximal tibia, proximal femur and proximal humerus. However, the availability of cadaveric grafts is limited, and the issues of infection, graft fracture, non union and osteoarthritis are reasons for concern. Resected tumor may be extracorporeally treated (radiotherapy/liquid nitrogen/pasteurization) and reimplanted to reconstruct the defect. However, the indications are limited and the post operative histopathological input is suboptimal (Puri et al. 2010). Vascularised autografts are also used for intercalary defects, arthrodesis, or as a growing osteoarticular grafts. They unite more predictably and show earlier hypertrophy compared to non vascular autografts. Hence, surgical treatment of ES in children, is associated with good outcome in terms of survival and function. These surgeries are complex and demand detailed planning for resection as well as reconstruction.

Role of Radiotherapy

Current protocols tailor local treatment to the individual patient with the goal of maximizing local control without compromising functional outcome. Patients who lack a function-preserving surgical option because of tumor location or extent, and those who have clearly unresectable primary tumors following induction chemotherapy are appropriate candidates for RT. Radiotherapy is also indicated in the patients who have positive margins in the post surgical specimen on histopathological examination. Role of radiotherapy in patients with macroscopic viable

tumor in the resected specimen following neoadjuvant chemotherapy is controversial.

The RT dose is an important factor in local control, particularly for large tumors, where most of the current protocols employ 45 Gy in 25 fractions to the initial clinical target volume, followed by a 10.8 Gy boost in six fractions to the site of original bony disease and any residual soft tissue disease that remains following chemotherapy. For patients undergoing adjuvant RT, doses of 45–55 Gy depending on presence of microscopic or gross residual disease. Conventional RT schedules usually consist of once daily RT doses of 1.8–2.0 Gy per fraction (Arai et al. 1991). Patients with a limited number of lung metastases or small volume metastatic disease at other site benefit from radiotherapy at these sites.

Recurrent Disease

Relapse in ES usually occurs in the first 5 years from diagnosis, although late relapses are also known to occur. The recurrence site, prior treatment, and relapse-free interval determine subsequent treatment choices. The likelihood of response to chemotherapy increases with longer duration of relapse free survival and the chemotherapy regimen depends upon initial agents used. Patients who have not previously received ifosfamide and etoposide may respond to this regimen. Salvage chemotherapy regimens include combination chemotherapy such as cyclophosphamide-topotecan (response rates 40%) (Saylors et al. 2001), and temozolomide-irinotecan (Wagner et al. 2004). The role of gemcitabine-taxotere or trabectedin, a novel marine-derived antimitotic compound that binds the minor groove of DNA, have shown promise in soft tissue sarcomas and warrant further consideration in ES (Maki et al. 2007). Management of local recurrence usually includes surgery (and possibly an amputation if the local recurrence involves an irradiated extremity), radiotherapy, or both. Prognosis of distant recurrence remains poor.

Late Effects

Successful outcome of patients with non-metastatic ES has shifted the focus to minimising late effects in long term survivors. Some of the important late effects are musculoskeletal growth disturbances, second malignancy, endocrine dysfunction, cardiomyopathy, pulmonary fibrosis and sterility.

The most devastating late complication of treatment is therapy related second malignancy. These include secondary leukemias wherein radiotherapy, alkylating agents and etoposide are all implicated, and sarcomas often arising in the field of radiotherapy. The cumulative incidence of second malignancy in most large series is about 2% (Paulussen et al. 2001b). Anthracycline induced cardiotoxicity is an important late effect impacting quality of life of survivors of ES. The incidence of chronic cardiotoxicity is most closely related to the cumulative dose of anthracycline administered. Regardless of its timing, chronic cardiomyopathy generally begins as asymptomatic diastolic or systolic dysfunction, and progresses to heart failure, which may be fatal. Steinherz et al. (1991) reported an incidence of 23% echocardiographic abnormalities with median cumulative dose of 450 mg/m2 at 7 years. Hence cumulative dose of doxorubicin is usually limited to less than 450 mg/m2. The alkylating agents cyclophosphamide and ifosfamide are associated with infertility, especially male infertility, therefore sperm cryopreservation should be offered to post-pubertal boys prior to the institution of chemotherapy. In addition, ifosfamide can cause a persistent renal tubular electrolyte loss and, less commonly, a decrease in glomerular function, again in a dose-dependent fashion.

Late effects of radiotherapy are related to field and the dose received by normal tissue and skeletal maturity, of the patient. Younger children are at greatest risk for radiation-induced arrest of bone growth. The largest series with longest follow-up includes 266 survivors of EFT treated at St Jude Children's research Hospital, the National Cancer Institute, and the University of Florida

showed an estimated cumulative risk of 6.5% for a secondary sarcoma at 20 years. All the secondary sarcomas occurred near or at the primary site of the ES and within the primary irradiated field.

Conclusion

In conclusion, poor prognosis of patients with extensive metastatic and recurrent disease warrants newer therapeutic strategies. One of the most promising drug targets is the insulin-like growth factor-I receptor (IGF-1R). Activation of this receptor is essential for EWS-FLI-1-induced malignant transformation of ES (Benini et al. 2001). Given the biological rationale for targeting IGF-1R in ES, at least seven antibodies and an equal number of small molecules targeting IGF-1R are in development. Initial results of this drug in few patients are encouraging. At the genetic level, ES is defined by the presence of EWS–ETS gene in more than 95% cases. Thus, inhibition of signaling pathway and downstream of EWS–FLI1 may reverse the malignant phenotype of ES (Kovar et al. 1996). At this time, no antisense-based or siRNA-based therapies are in clinical trials for ES. The other likely target may be CD99 due to its ubiquitous expression in ES cell line. In vitro studies have shown that CD99MIC2 binding and silencing by specific antibodies is associated with rapid tumor cell death, and is more effective with conventional chemotherapeutic drugs. Because of high-level expression of CD99MIC2 in hematopoietic stem cells and several cell types in the gonads and the pancreas in humans, clinical trials using anti-CD99MIC2 antibodies have not yet been attempted. Other potential targets include the Wnt signaling pathway, the Hedgehog signaling pathway, the p53 pathway and therapy against activated molecules like mTOR, MAPK, PI3K/Akt, EGFR, VEGF. Improved understanding of complex tumor biology may provide a roadmap for successful development of biologically targeted therapies. Integration of these therapeutic inventions in clinical practice may shift the paradigm towards more individualized and effective therapy.

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Pediatric Epitheloid Finger Sarcoma: Diagnosis and Treatment

23

Felix Stang, Peter Mailänder, and Frank Siemers

Contents

Abstract

The epithelioid sarcoma (ES) is a rare, high-grade malignant soft tissue tumor, which shows preference for distal parts of the extremities, particularly the hand. It differ from benign lesions (e.g. ganglion) in ways of that are often non apparent until there is recurrence. Regional lymph node involvement and distant metastases (lungs) are often apparent. Treatment of the ES includes the primary radical surgical excision, sometimes combined with sentinel lymph node biopsy and radiation or chemotherapy. Amputation of the affected fingers is necessary in all cases of recurrence but might be avoided in initial surgery with respect to pediatric patients and maximal function. Consequently, soft tissue defects must be treated following plastic surgical principles. Close oncological follow-ups are inevitable, since there can be tumor recurrence even years after resection of the primary tumor.

Introduction

Approximately 5% of soft tissue sarcomas emerge at the hand. Beside the synovial sarcoma and malignant fibrous histiocytoma, the epithelioid sarcoma (ES) is a prevalent histologic form and was first described in 1961 by Laskowski as "sarcoma aponeuroticum" (Sobanko et al. 2009). In 1970, Enziger described the ES as a sarcoma simulating a granuloma or carcinoma (Enzinger 1970). Epithelioid sarcomas, a subcategory of

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non-rhabdomyosarcomas, are very rare, high-grade tumors that constitute less than 1% of all soft tissue sarcomas (which represent itself only about 8% of all malignant tumors), however, the ES is the most common soft tissue sarcoma of the hand, mostly located on the dorsal fingers. The overall incidence for ES in 2005 was reported to be 0.041 per 100,000 persons (Jawad et al. 2009).

Aetiopathogenesis

The precise cellular origin of the ES is still unclear. Some authors suggest a role for fibroblasts (Fisher and Horvat 1972), but clinically, the site of the ES, the frequency of recurrence and the existence of nodal metastases favor a synovial origin – a hypothesis followed by other authors (Patchefsky et al. 1977). Recent immunohistochemical analysis suggest the ES being a mesenchymal neoplasm capable of partial epithelial transformation (Laskin and Miettinen 2003). Several cytogenetic findings have been described in literature, some of them involving chromosome 22 with translocation t(8;22)(q22;q11) (Lualdi et al. 2004) or t(6;8)(p25;q11.2) and add(7)(p15) (Feely et al. 2000). Another reported frequent property of these tumors is the loss or inactivation of the tumor suppressor gene SMARCB1/INI1, a member of the SWI/SNF chromatin remodeling complex\ located on chromosome 22q11.2 (Hornick et al. 2009; Raoux et al. 2009). However, a characteristic anomaly for the ES has not yet been identified. The ES is usually occurring between the age of 15–35, males are disproportionately affected with a ratio approaching 2:1 (Sobanko et al. 2009).

Clinical Features and Diagnosis

The ES impresses initially as a tan-white, well-circumscribed and slow growing, painless nodular formation, palpable in the deep soft tissues, subcutis or dermis (Sobanko et al. 2009). Therefore the ES is likely to be confused with a variety of other benign conditions of the hand (s.b.) and it might be present for months to years before the patient seeks medical advises(Bos et al. 1988; de Visscher et al. 2006). Later, the nodules may become larger, fixed and adherent to tendons and aponeuroses and eventually invading adjacent structures such as neurovascular bundles and bones, causing pain or paresthesiae, dysfunction or stiffness. The local progress is by continuity and later ulcerations of the lesions are commonly seen (Prat et al. 1978).

Table 23.1 Differential diagnosis of the epithelioid sarcoma

Benign conditions	Malignant conditions
Dupuytrens disease	Synovial sarcoma
Granulomatous processes	Fibrosarcoma
Rheumatoid nodules	Fibrous histiocytosarcoma
Nodular tendosynovitis	Squamous-cell carcinoma

Several benign differential diagnosis have to be taken into consideration, such as Dupuytrens disease (Rhomberg et al. 2002), granulomatous processes (Chase and Enzinger 1985; Enzinger 1970; Spillane et al. 2000), rheumatoid nodules or nodular tendosynovitis (Bos et al. 1988; Patchefsky et al. 1977). Other malignant conditions could be a synovial sarcoma (Bos et al. 1988; Prat et al. 1978), fibrosarcoma (Bos et al. 1988), fibrous histiocytosarcoma or a squamous-cell carcinoma (Callister et al. 2001; Daimaru et al. 1987) (Table 23.1).

In order to confirm the final diagnosis of ES by histopathological examination, a biopsy of a subcutaneous tumor mass can be obtained. Prior to a biopsy, an X-ray imaging should exclude periostal bone affection. A magnetic resonance imaging (MRI) is then preferred due to its possibility to visualize soft tissue details and the tumors circumference. When the diagnosis of ES is confirmed, an oncologic staging with CT of the thorax and abdomen should follow, a positron emission tomography might help to detect rare distant muscle metastasis (Sakamoto et al. 2008).

Histology and Immunohistochemistry

Microscopic examination reveals some distinguished characteristics of the “distal type” of ES: A nodular arrangement of the tumor cells with

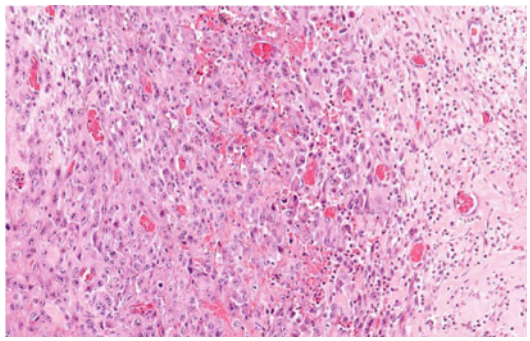

Fig. 23.1 HE-staining of the ES: large round, oval or polygonal cells with eosinophilic, well defined cytoplasma and a centrally placed nucleus

foci of central degeneration, necrotic debris or microcalcification; large round, oval or polygonal cells with eosinophilic, well defined cytoplasma and a centrally placed nucleus – being analogous to epithelial cells (therefore "epithelioid" sarcoma) and fusiform cells arranged at the periphery of the nodules (Halling et al. 1996) (Fig. 23.1). Mitotic activity is almost identified in all cases and an inflammatory infiltrate often surrounds the tumor (Halling et al. 1996).

Some other morphologic variants of the ES have been described, including the "fibroma-like" variant consisting of deceptively bland fibrohistiocytic and myoid cells arranged in a fibroma-like or dermatofibroma-like pattern with an affinity for osseous involvement (Mirra et al. 1992). The "proximal type" of ES is characterized by a predominantly large-cell, epithelioid cytomorphology, marked cytologic atypia, frequent occurrence of rhabdoid features, and lack of a granuloma-like pattern in most cases (Guillou et al. 1997). An "proximal type" ES features cyst formation and hemorrhage mimicking a cavernous angiosarcoma (von Hochstetter et al. 1991). Immunohistochemical analyses show a positive response in up to 75% for vimentine, cytokeratin, epithelial membrane antigen, CD 34, smooth muscle action, muscle-specific actin and desmin (Daimaru et al. 1987; Miettinen et al. 1999). Diagnostically, p63 and keratin 5/6 can distinguish cutaneous squamous cell carcinoma (positive) from ES (usually negative) (Laskin and Miettinen 2003).

Treatment

Until now, the ES is still an only surgically curable disease (de Visscher et al. 2006). A local resection of the tumor alone is often inadequate due to a high risk of recurrence. A wide excision at the fingers is often associated with an amputation, a problem especially in pediatric patients, but it is inalienable in the case of tumor relapse. Sentinel lymph node biopsy can identify patients who may benefit from systemic therapy (Callister et al. 2001) and therapeutic axillary lymph node dissection should follow in the presence of lymph node metastasis (de Visscher et al. 2006; Spillane et al. 2000). Some authors perform routinely axillary lymph node eradication (Maduekwe et al. 2009).

Optimal function in the pediatric hand remains a major goal, consequently, soft tissue defects must be treated following plastic surgical concepts (Muller et al. 2008).

Halling reports, that surgical resection of small metastatic lesions may result in increased long term survival (Halling et al. 1996). Radiotherapy proofed to be efficient in addition to surgery in soft tissue sarcomas, but studies of ES are of limited conclusion due to a small study population and limited follow up (Russell et al. 1981). However, radiotherapy is advised to be extended to the forearm in all cases of insufficient tumor extirpation, local recurrence or palliative treatment (de Visscher et al. 2006). Adjuvant chemotherapy (including ifosfamide and doxorubicine) is also often used in addition to surgery in the case of metastatic disease and it can be useful as a neoadjuvant therapy in order to reduce tumor size before surgical resection, but its role is less clear in nonmetastatic ES (Ross et al. 1997). Systemic chemotherapy can provide satisfactory palliation in patients with epithelioid sarcoma, but due to the aggressive nature of this disease, responses to chemotherapy are of short duration (Jones et al. 2012). The distinguished roles of chemotherapy and radiation still remain unclear and additional studies with a large study population and long term follow up will be useful to address the role of adjuvant therapy in the treatment of ES (Sobanko et al. 2009).

Regarding the hyperthermic limb perfusion, there are currently no specific studies for its role in the treatment of ES.

Prognosis

The development of ES is quite unpredictable. Even an R0-resection still has a high risk of relapse due to tumor growth along the tendon sheets within 1-2 years of treatment. Local recurrence can be found in up to 85% (de Visscher et al. 2006; Enzinger 1970; Prat et al. 1978). Additionally, metastasis can be found in up to 50% (Chase and Enzinger 1985; de Visscher et al. 2006; Spillane et al. 2000), with the most frequent sites being lymph nodes (up to 65%) and lungs (25%) (Callister et al. 2001; de Visscher et al. 2006; Halling et al. 1996), other rare sites for metastasis such as skin, scalp, brain, digestive tract, liver, kidneys and musculoskeletal system are described (Prat et al. 1978; Raoux et al. 2009; Sakamoto et al. 2008). Lymph node involvement seems to be less evident in pediatric patients than in adults (Casanova et al. 2006). The general outcome tends to be worse when the ES was associated with lesions proximal to the elbow or knee (Callister et al. 2001; Guillou et al. 1997; Jawad et al. 2009). Poor data records do not allow a reliable prognostic conclusion, but small tumors (<5 cm) without metastasis might have a beneficial prognosis with R0-resection (Fig. 23.2). The overall 5-year survival rate in the literature varies between 21% and 92%, the overall 10-year

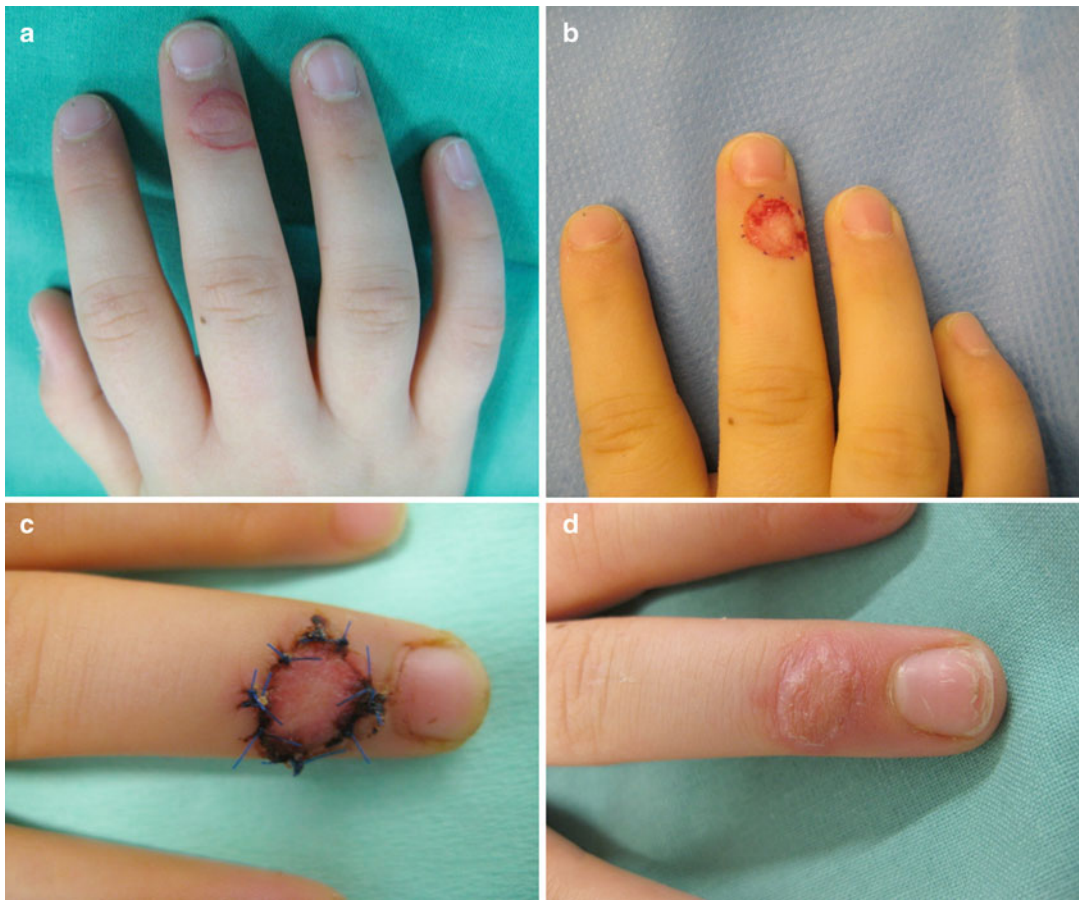

Fig. 23.2 (**a**) Epithelioid sarcoma located at the dorsum of the middle finger. Two-step surgical excision and split skin grafting in order to avoid amputation of parts of the finger in an 11-year old girl (**b**, **c**). Two years follow up without signs of recurrence (**d**)

survival rate between 21% and 87% (Casanova et al. 2006; de Visscher et al. 2006; Halling et al. 1996). The median postmetastatic survival was reported to be 8 months (de Visscher et al. 2006). The SEER database (Surveillance, Epidemiology, and End Results) shows only young age (< 16 years), negative lymph nodes, or local stage of disease and operability of primary lesion independently predict survival in patients with ES (Evidence level II) (Jawad et al. 2009).

In conclusion, epithelioid sarcomas are in general very rare malignant tumors, showing preference for distal parts of the upper extremity. Since they have an indolent and sometimes slow growth rate, they are often confounded with benign lesions. Therefore, every unclear tumor of pediatric hands should be admitted to a specialized center with hand surgical and oncological experience; detailed preoperative diagnosis in terms of x-ray, MRT, ultrasound and possibly biopsy should be performed in advance. Good coordination between hand-surgeon, pediatric oncologist, pathologist, and radiotherapist is mandatory to plan a successful multimodality treatment for these patients (Laskin and Miettinen 2003).

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Part VII

Miscellaneous Tumors

Diagnosis and Prognosis of Pediatric Patients with Adrenocortical Tumors

24

Gaetano Magro, Giada Maria Vecchio, and Rita Alaggio

Contents

Abstract

Pediatric adrenocortical tumors (ACTs) only rarely occur in children. Unfortunately their clinical behavior is often unpredictable and their diagnosis (*benign* versus *malignant*) is still challenging because the pathological criteria of malignancy used in adults (Weiss system) are not always useful in children. Among the several pathological scoring systems, that proposed by Wieneke et al. seems to be the most reliable and reproducible for prognostic purposes in daily practice. Although seemingly straightforward, the identification/definition of the key pathological features of this system is still controversial, resulting in pathological reports that are different not only among general pathologists but even among expert pediatric pathologists. A review of the literature on pediatric ACTs and our personal experience with 20 cases are provided, discussing confusing and/or challenging pathological problems, especially those with clinical impact. General guidelines, including histological illustrations, are provided in order to offer pathologists a practical approach for a correct identification of predictors of clinical outcome.

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Introduction

Adrenocortical tumors (ACTs) are neoplasms that only rarely occur in pediatric patients (age <20 years). It has been estimated that they

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represent about 0.2% of all pediatric malignancies and 5–6% of all adrenal tumors, with an incidence of 0.2 new cases per one million children per year. The male-female ratio is 2:1 (Humphrey et al. 1983; Stratakis and Chrousos 1997; Plowman 1997) and the age incidence curve is characterized by two peaks, the first being younger than 3 years and the second during adolescence (Ribeiro et al. 2000; Wieneke et al. 2003). Notably there is a high incidence in southern Brazil, wherein it is about 12–18 times the incidence reported in the United States and Europe (Mendoca et al. 1995; Sandrini et al. 1997; Ribeiro et al. 2000; Pianovski et al. 2006).

ACTs in children may occur sporadically or as a component of some multisystemic constitutional pediatric syndromes, such as Beckwith–Wiedemann syndrome (BWS), multiple endocrine neoplasia (MEN) type 1, Carney's complex, congenital adrenal hyperplasia, urinary tract abnormalities, hemihypertrophy syndrome, and familiar cancer syndrome, including Li-Fraumeni and SBLA syndrome (S, sarcoma; B, breast and brain neoplasms; L, leukemia, laryngeal and lung carcinoma; A, ACC) (Lynch et al. 1978; Libè and Bertherat 2005; Soon et al. 2008; Ribeiro et al. 2010). However, only a minority of children with ACTs has one of these constitutional syndromes. ACTs can be discovered incidentally at autopsy or during unrelated diagnostic radiographic methods or surgical evaluation. Unlike in adult patients, however, most cases (~90%) of pediatric ACTs are detected because of symptoms or signs related to hormonal dysfunction, such as virilization, Cushing syndrome or feminization (Patil et al. 2002; Wieneke et al. 2003; Michalkiewicz et al. 2004). Notably, some patients may present with a mixed endocrine syndrome: virilization and Cushing's syndrome, or feminization and Cushing's syndrome (Michalkiewicz et al. 2004; Ribeiro et al. 2010). A minority of patients, without clinical evidence of endocrine dysfunction, present with non-specific symptoms, including abdominal mass, abdominal pain, and fever (Wieneke et al. 2003). ACTs are usually divided into adenomas and carcinomas, the former being most frequent in clinical practice than the latter. However this distinction is often difficult, due to the lack of reliable clinico-pathological features that correlate with patients' outcome. Neither gender nor age seem to independently predict clinical outcome (Wieneke et al. 2003). Although a functional tumor may be indicative of poor outcome in adults, the same has not been confirmed in children (Wieneke et al. 2003). Similarly, tumor size and weight alone are not independent prognostic factors, even if the likelihood of malignant behavior increases for neoplasms with a diameter >10.5 cm and a weight >500 g (Cagle et al. 1986). In the past some authors (Sandrini et al. 1997) proposed a four-tiered staging system for childhood ACTs based on the possibility of tumor excision at diagnosis (totally excised tumor; microscopic or macroscopic residual tumor; distant metastases). Although it was relatively helpful in identifying patients with good (Stage I) and poor (Stage IV) prognosis, however, predicting outcome for patients with intermediate stages (Stage II–III) of disease is much more difficult. On the contrary, some authors (Wieneke et al. 2003) dealing with 84 pediatric patients with ACTs, showed that an increased tumor stage does not correlate with a poor clinical outcome.

Unfortunately, the clinical behavior of ACTs in children is unpredictable even if it is based on pathological findings. This is mainly due to the fact that a correct pathological diagnosis, i.e. a distinction between benign and malignant tumors, is still challenging because the histological criteria considered to be predictive of the biological behavior of the conventional ACTs in adults (Weiss 1984), are not always useful when applied in pediatric tumors (Cagle et al. 1986; Wieneke et al. 2003). In this regard, some authors reported that ACTs in children have a more favorable outcome than in adults (Mendoca et al. 1995; Wieneke et al. 2003). Infact it is largely known that a significant number of pediatric patients, with tumors which would have been classified as "*malignant*" based on a high Weiss score, experienced a benign clinical course without local recurrence or death from disease (Wieneke et al. 2003; Dehner and Hill 2009). This clinical evidence, as similarly observed for adult oncocytic ACTs (ie, tumors composed of >90% of neoplastic

cells with clear-cut features of oncocytes) (Bisceglia et al. 2004; Wong et al. 2011), raised the question as to whether the Weiss system could be applicable to the subset of pediatric tumors. Accordingly, the most important practical problem regarding pediatric ACTs still concerns their diagnosis and biological behavior.

Prognostically-Related Pathological Features in Pediatric Adrenocortical Tumors

Wieneke et al. (2003) suggested that Weiss scoring system be modified when evaluating pediatric ACTs. They proposed the categorization of these tumors into three different prognostic groups, "*clinically benign category*", "*clinically malignant* category", and "*clinically intermediate or indeterminate for malignancy category*" on the basis of a combined assessment of nine, macroscopic and microscopic, parameters that resulted to be statistically significant for a potential malignant behavior: (i) tumor size (>10.5 cm); (ii) tumor weight (>400 g); (iii) extension into periadrenal soft tissues and/or adjacent organs; (iv) invasion into vena cava; (v) capsular invasion; (vi) tumor necrosis; (vii) mitotic rate (>15 mitoses per 20 high power fields); (viii) atypical mitoses; (ix) vascular invasion (Table 24.1).

Four of these parameters are related to invasive tumor properties: (i) vascular invasion (sinusoidal and/or venous invasion); (ii) capsular invasion; (iii) vena cava invasion; (iv) extra-adrenal (soft tissues and/or adjacent organs) infiltration. Two other parameters are related to cytological features, namely the number of mitotic figures per 20 high power fields (>15 per 20 high-power fields), and the presence of atypical mitoses (defined by abnormal chromosome spread, tripolar or quadripolar forms, circular forms or indescribably bizarre). One parameter is related to tumor structure, namely tumor necrosis (focal to confluent, coagulative necrosis of tumor cells, not just degenerative changes) and the remaining two parameters are tumor size (greatest dimension in centimeters) and tumor weight (in grams). With application of these pathological criteria, pediatric ACTs are classified as "*clinically benign*" if ≤2 of the unfavorable parameters are present; tumors with ≥4 unfavorable parameters are regarded as "*clinically malignant*", and, lastly, tumors with three unfavorable criteria are classified as "*clinically intermediate or indeterminate for malignancy*" (Table 24.1).

A comparison between the scoring system used for adults (Weiss system) and that used for children (Wieneke system) reveals the following considerations: (i) both systems share a selection of several histological features that are associated with a more aggressive biologic behavior; (ii) four parameters, such as capsular invasion, vascular invasion, tumor necrosis and atypical mitoses, are similarly used in both systems; (iii) although mitotic count is included in both systems, modified cutoff values are used for pediatric ACTs (≥15 per 20 high power fields versus ≥5 per 50 high power fields in adults); (iv) unlike Weiss system, that proposed by Wieneke et al. includes, among the unfavorable parameters, tumor size and weight, invasion into vena cava and extra-adrenal infiltration; (v) three parameters, such as nuclear grade, percentage of clear cells, and percentage of diffuse growth pattern, used in Weiss system, are excluded from Wieneke system.

Table 24.1 Correlation between Wieneke scoring system and expected clinical outcome. Practical approach of classification of three cases fulfilling parameters for benign, indeterminate and malignant categories

Case no.	Tumor size (cm)	Tumor weight (g)	Extra-adrenal extension	Invasion into vena cava	Capsular invasion	**Necrosis**	Mitotic rate	Atypical mitosis	Vascular invasion	Wieneke system/ category
1	7	50	Absent	**Present**	Absent	**Present**	<15	Absent	Absent	**2**/Benign
2	5	80	Absent	Absent	Absent	**Present**	<15	**Present**	**Present**	**3**/indeterminate
3	4.5	25	**Present**	Absent	**Present**	**Present**	<15	Absent	**Present**	**4**/malignant

The terms written in bold highlight the presence of unfavorable parameters which have to be summed for final score

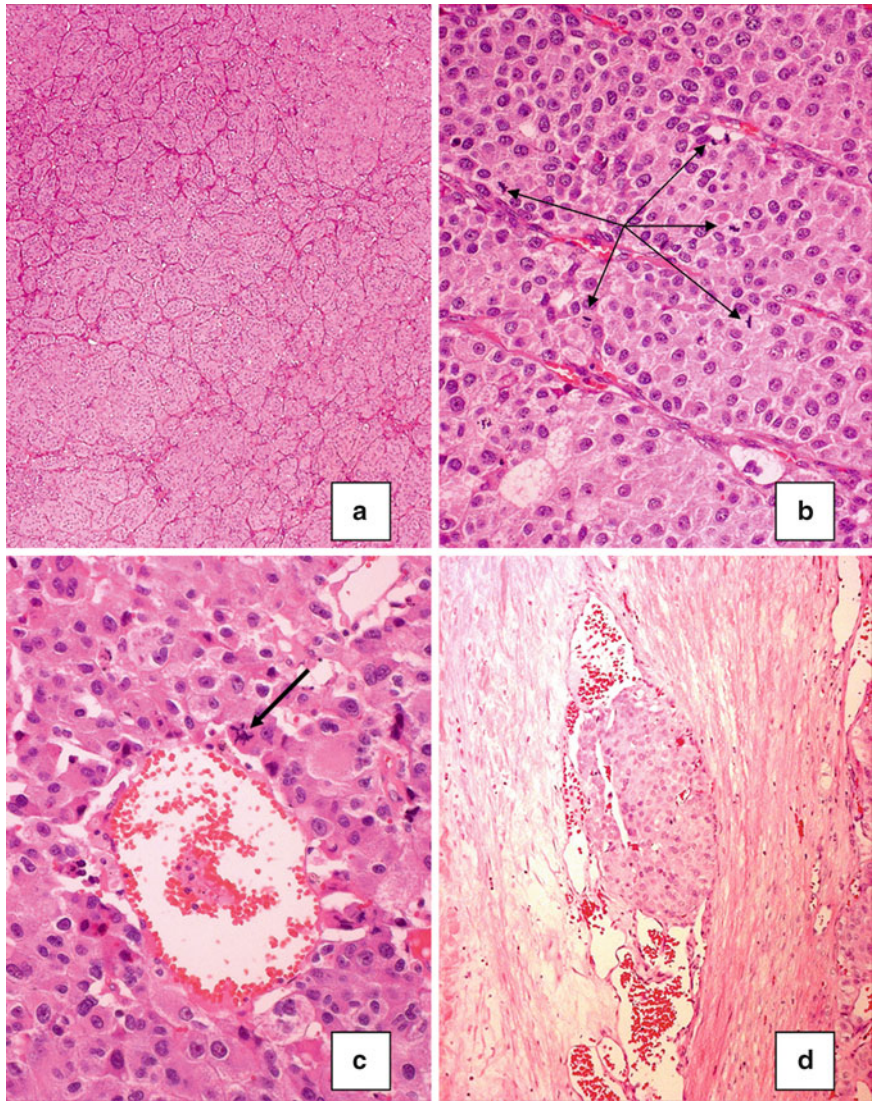

Fig. 24.1 (**a**) Most ACTs exhibit "nested" growth pattern and polygonal cells with eosinophilic cytoplasm. (**b**) High power magnification showing numerous typical mitoses (*arrows*) and (**c**) one atypical mitosis (*arrow*). (**d**) Vascular invasion is observed in the context of tumor capsule

In the original study by Wieneke et al. (2003) histologic features that were associated with a more aggressive biological behavior were capsular invasion, vascular invasion, tumor necrosis, increased mitotic activity (>15 mitoses per 20 high power field) and atypical mitoses (Figs. 24.1 and 24.2). In addition other features that have been found to be statistically predictive of patient outcome were extra-adrenal infiltration and invasion into the vena cava. Notably, vena cava invasion, tumor necrosis and increased mitotic activity (>15 mitoses/20 high power field) were independently indicators of a worse clinical outcome based on multivariate analysis. However the authors stated that none of the unfavorable parameters identified can be used alone as a predictor of malignancy (Wieneke et al. 2003). Infact tumor necrosis, increased mitotic activity, and atypical mitotic figures were also documented in patients who experienced a good clinical outcome without evidence of recurrence or metastatic disease (Wieneke et al. 2003).

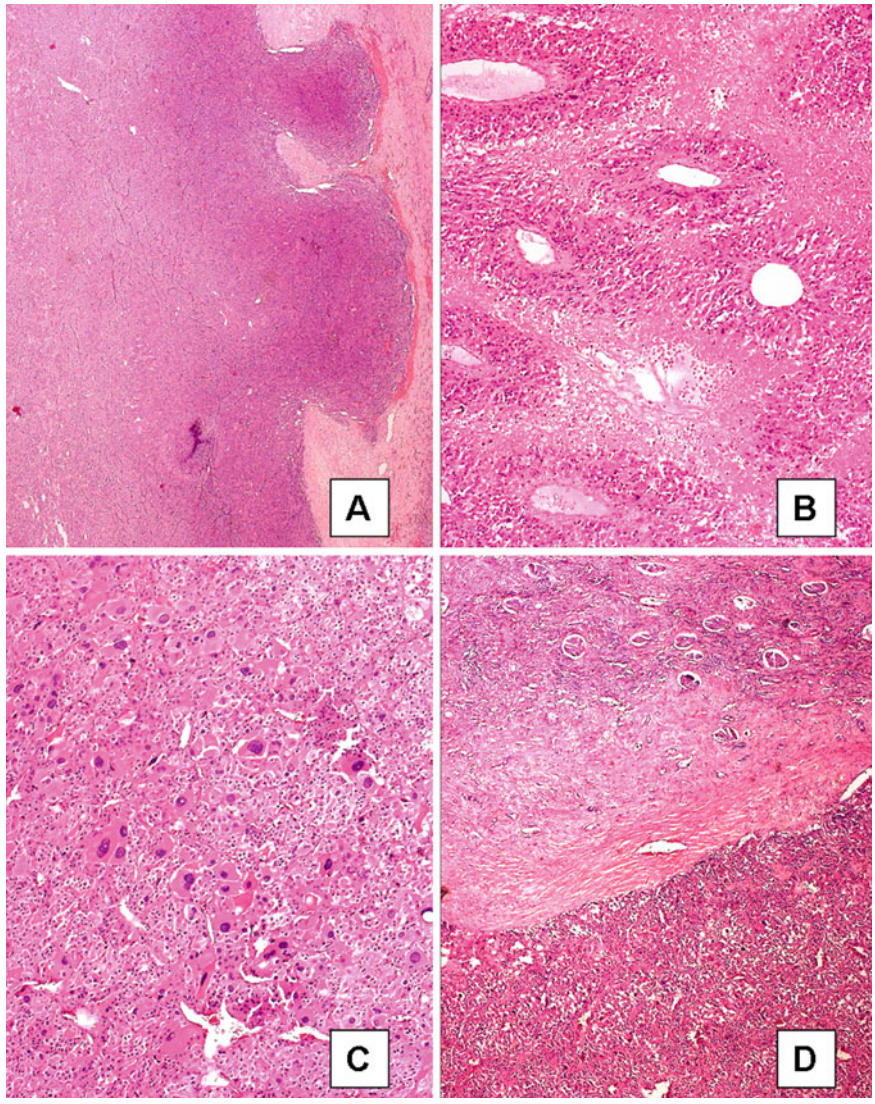

Fig. 24.2 (**a**) Capsular invasion, (**b**) confluent tumor necrosis with periteliomatous arrangement of neoplastic cells, (**c**) pleomorphic, bizarre, large cells, and (**d**) infiltration of renal parenchyma are seen

Since 2003 there are no available studies on the diagnostic utility of Wieneke system for pediatric ACTs. The prognostic value of this scoring system has been recently confirmed by the Italian Pediatric Rare Tumor (TREP) study project that included, as co-authors, two of us (GM, RA) (Magro et al. 2012). Wieneke system was found to be a simple and reproducible diagnostic tool helpful for prognostic categorization of patients. Infact the vast majority of tumors categorized as "*benign*" consistently lacked or had only one Wieneke parameter, whereas most cases of tumors classified as "*malignant*" had a Wieneke total score of 6 or higher. In that study, dealing with 20 cases of ACTs, all 12 patients with tumors classified as "*benign*" are still in complete remission with no relapse, whereas 4 out 7 patients with tumors classified as "*malignant*" died of disease, 2 are alive with metastases and only 1 is in first complete remission (Magro et al. 2012). The unique patient of the series with a tumour classified as "*intermediate/indeterminate for malignancy*" is having a benign clinical course.

All tumors were mostly composed of polygonal cells with abundant eosinophilic cytoplasm, with focal nests of intermingling clear cells (always <30% of the entire tumor) identified in a minority of cases. The growth pattern was predominantly of diffuse, nested (Fig. 24.1a), or trabecular type, with only rare examples exhibiting pseudo-glandular features. Although nuclear grade (Furhman nuclear grades I–IV) was variable, in most cases neoplastic cells showed II–III nuclear grade. Only rarely were bizarre or monster cells with marked nuclear pleomorphism (Fig. 24.2c) encountered, as typically seen in pure oncocytic ACTs of adults (Bisceglia et al. 2004). Among the unfavorable parameters included in the Wieneke scoring system, capsular and vascular invasion, tumor necrosis, and periadrenal infiltration were concurrently present in the majority of pediatric neoplasms with malignant clinical outcome (Figs. 24.1 and 24.2) (Magro et al. 2012). A high mitotic rate, as defined by Wieneke et al. (2003) (>15 mitoses per 20 high power field), although highly suggestive of malignancy (not found in benign tumors), was of low sensitivity. Interestingly, despite myxoid stromal changes is not a histological criterion included in the Wieneke' scoring system, TREP study found that their identification, in the form of small foci, similarly to observed in adults (Papotti et al. 2010), may be a potential morphological marker indicative of malignant behavior, being observed in most malignant tumors and only in a minority of benign tumors (Magro et al. 2012). However we admit that further studies on a larger number of cases are needed to confirm these results. Although tumor weight (>400 g) has been shown to be prognostically relevant in pediatric ACTs (Wieneke et al. 2003; Dehner and Hill 2009), TREP study failed to confirm these results (Magro et al. 2012). In fact the weight of most malignant tumors ranged from 25 to 170 g. As previously emphasized (Wieneke et al. 2003), TREP study confirmed that morphological features selected for evaluating adult ACTs (Weiss 1984), such as severe nuclear pleomorphism, clear cell component, and diffuse growth pattern, are histological features that can be found in both benign and malignant tumors, and thus lacking statistically significant correlation with patient outcome (Magro et al. 2012).

Although seemingly straightforward, the application of the Wieneke scoring system, as similarly observed in adults for Weiss system, may have a major limitation in the daily practice, related to its potentially low reproducibility due to inter-observer variability among pathologists, not only at the different institutions but even within the same institution. Infact the assessment of histological features, such as vascular and capsular invasion, tumor necrosis, mitotic count, and nuclear atypia, may be subjective in terms of both identification (absent/present) and/or quantification, especially depending on the expertise of pathologist and on the adequacy of tumor sampling. In this regard, pathologist should be aware that the greater the number of tumor sections examined (*one paraffin inclusion* × *cm*, *with special reference to the capsule/invasive front*) the greater the chance of identifying unfavorable histological parameters. Accordingly, pediatric ACTs, especially those of large dimension, should be approached cautiously, prompting a careful search for histological features, including mitotic activity/atypical mitoses and tumor necrosis, which, more than others (ie, capsular/vascular invasion; periadrenal soft tissue infiltration), can be under-recognized or over-looked. In this regard, we would like to stress that if only one of the abovementioned features is missed, there is the risk of understaging the patient. For example, patients who would have been placed in the category "*clinically benign*" (score = 2), with the additional recognition of at least one unfavorable parameter, they should be included into the "*intermediate category*", and, similarly, patients with score = 3 (*clinically intermediate*) should be over-staged in the category "*clinically malignant*".

Molecular Markers in the Diagnosis and Prognosis of Pediatric Adrenocortical Tumors

Several molecular biomarkers, largely studied in other endocrine tumors, including p53, c-erbB2, Bcl-2 and Ki67, have also been assessed in

pediatric ACTs with disappointing and conflicting results (Sbragia et al. 2005, Orhan et al. 2006). Since the loss or deregulation of HLA class II antigens have been documented in a great variety of malignant tumors, some authors have evaluated whether this event is similarly observed in ACTs. Interestingly, molecular studies showed different median expressions of the levels of *LA-DRB1*, *HLA-DPB1*, *HLA-DRA*, and *HLA-DPA1* mRNA in adrenocortical carcinomas versus adenomas in pediatric patients (West et al. 2007). These molecular findings seem to support the evidence that most cases of adult adrenocortical carcinomas lack the immunohistochemical expression of major histocompatibility class II genes, a finding which has been proposed as a potential marker of malignancy (Marx et al. 1996). Apart from the HLA class II antigens, the immunohistochemical expression of some matrix metalloproteinases (MMP), especially MMP2, has been shown to be independent predictors of clinical aggressiveness in adult ACTs (Volante et al. 2006). Infact the most interesting finding was a restricted MMP2 expression to malignant tumors, with a high specificity but low sensitivity. In addition, MMP2 expression in more than 20% of neoplastic cells correlated with a more a aggressive clinical outcome (Volante et al. 2006). Unfortunately, these results were not confirmed by TREP study on a series of 20 cases of pediatric ACTs, in which both benign and malignant neoplastic cells failed to express HLA class II antigens, while MMP2 was focally to diffusely detected in both benign and malignant tumors (Magro et al. 2012). All these biological findings further support the idea that although pediatric ACTs seem to be similar histologically to their adult counterparts, they have distinctive molecular pathways responsible for cancer onset and progression.

Conclusions

Among the systems used for classification of ACTs in pediatric patients, we recommend the use of Wieneke scoring system for assessing the likelihood of the biologic behavior. The reliability of the criteria included in this system is largely dependent on the accurate pathological assessment. Accordingly, we strongly recommend that a diagnosis of "*borderline or malignant ACT*" in children should be rendered only after an extensive tumor sampling, a meticulous mitotic count, and a careful search for vascular/capsular invasion, tumor cell necrosis and atypical mitosis. If these general guidelines are disregarded, there is the risk of misdiagnosis, with the possibility of understaging the patient. Conversely, we emphasize that a diagnosis of "*adrenocortical carcinoma*" cannot be achieved based on a single morphological parameter but mostly on a combination of multiple features suggestive, but not conclusive, of malignancy. This means that although the diagnostic performance of Wieneke system is relatively high in daily practice, it does not reach a sensitivity and specificity of 100% when evaluating a single patient's diagnosis. Although some immunohistochemical and molecular markers have obtained promising results, however, none of them has been validated enough to be considered as a novel biomarker helpful in the prognostic categorization of children with ACTs.

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Supratentorial Primitive Neuroectodermal Tumor: Therapy

25

Barry Pizer and James Hayden

Contents

Abstract

Supratentorial primitive neuroectodermal tumors (StPNET) are a group of highly aggressive cancers that account for 2–3% of all brain tumors in childhood arising within the pineal gland and at non-pineal sites. These tumors share many histological features with their infratentorial counterpart (medulloblastoma) as well as a propensity for leptomeningeal dissemination and accordingly they have traditionally been treated along similar lines. More recently, however, it has been shown that the biological mechanisms underlying the development of StPNETS and medulloblastoma differ significantly. As with medulloblastomas, StPNETs are both radio and chemo sensitive although the outcome for patients treated using similar regimens of multi-model therapy with surgery, radiotherapy and chemotherapy in clinical trials is consistently significantly inferior. Young children under the age of 3 years have a dismal prognosis. Large multicentre trials specifically for patients with StPNETs are now required, including the identification of clear prognostic clinical and biological prognostic factors. In future studies should aim to exploit StPNET biological targets to develop novel disease specific therapies to improve prognosis.

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Introduction

The term primitive neuroectodermal tumors (PNETs) applies to a group of highly cellular malignant embryonal tumors of unknown etiology accounting for approximately 20% of central nervous system (CNS) neoplasms of childhood. This tumor type was first described by Bailey and Cushing (1925) who coined the term 'medulloblastoma' as a tumor of primitive origin and composed of small round blue cells that predominantly arose in the posterior fossa of young children. The concept of PNETs was originally proposed by Hart and Earle (1973) in recognition of the similar histological characteristics of cerebellar PNETs (medulloblastoma) and tumors occurring in the supratentorial region of the brain (supratentorial PNETs [StPNETs]) such as those arising from the cerebral hemispheres and pineal gland. Although the concept of PNET was generally accepted and was included in previous versions of the World Health Organization (WHO) classification of CNS tumors (Kleihues et al. 1993; Kleihues and Cavenee 2000), there is now major doubt as to whether PNETs arise from a common cell of origin, with increasing evidence of differences between the molecular characterization of medulloblastoma and StPNETs (see below). In this respect, nowadays the terms StPNETs and MB distinguishing these tumor groups are preferred and indeed this distinction is recognized in the current version of the WHO classification (Louis et al. 2007). StPNET and MB do, however, share common clinical features such as sensitivity to both radiotherapy and chemotherapy, and a propensity to leptomeningeal dissemination via the CSF pathways.

StPNETs are rare, comprising ~2–3% of CNS tumors in childhood and as a group are around five times less common than MB (Bruno et al. 1981; Gaffney et al. 1985; Dai et al. 2003). It is a disease of early childhood with a mean age of presentation of 5.5 years, (Jakacki et al. 1995; Louis et al. 2007). Supratentorial PNETs rarely arise in adulthood. A review in 2008 found only 57 reported cases worldwide of StPNETs in adults (Ohba et al. 2008). The majority of StPNETs arise in the cerebral hemispheres principally in the frontal, parietal and temporal lobes. About 20% arise in the pineal region, where they are known as pineoblastomas.

Histology and Biology

Morphologically StPNETs are similar to medulloblastoma. They are classified as WHO grade IV aggressive embryonal tumors composed of undifferentiated or poorly differentiated cells with high nuclear to cytoplasmic ratios. The nuclei are typically large and round. The tumor cells may also exhibit differentiation along neuronal, astrocytic, muscular or melanocytic lines. Neuronal markers such as synaptophysin and neurofilament are therefore characteristically positive, and markers such as glial fibrillary acidic protein (GFAP) may also be positive depending on the degree of astrocytic differentiation. Homer Wright rosettes are typically present but vary in frequency. The diagnosis of StPNET poses a challenge for the neuropathaologist as, depending on the degree of differentiation, these tumors may resemble other brain tumors. In the SIOP PNET 3 study (see below) for example, 12% of StPNET diagnoses made institutionally were not confirmed at central pathological review and the diagnoses changed to anaplastic astrocytoma, atypical teratoid rhabdoid tumor (ATRT), anaplastic oligodendroglioma and anaplastic ependymoma (Pizer et al. 2006). Immunohistochemical testing for INI1, retained in StPNETs and absent in ATRTs, now forms part of the mandatory diagnostic work up of these tumors. Recently, a variant of StPNETs has been proposed based on histopathological features that have been found to be associated (see below) with a specific molecular characterization. The embryonal tumor with abundant neuropil and true rosettes (ETANTR) was first proposed by Eberhart et al. in recognition of a group of tumors that exhibited prominent ependymobalstic rosettes, neuronal differentiation and a neuropil background which occurs in young children under the age of 6 years and is associated with a very poor prognosis (Eberhart et al. 2000; Pfister et al. 2009).

A review of the biology of StPNETs is the subject of Chapter 10, Volume 9 in Tumors of the Central Nervous System (Hayden and Pizer) The

key messages from this review is that there are clear differences between the biology of StPNETs and medulloblastoma suggesting differing molecular mechanisms involved in their respective pathogenesis. Furthermore, there is now compelling recent evidence that there are significant biological differences between pineoblastomas and StPNETs occurring elsewhere in the CNS.

Presentation

StPNETs usually present with symptoms and signs associated with the mass effect of the tumor including headaches, seizures and hemiplegia, although signs and symptoms of raised intracranial pressure and hydrocephalus may feature. Pineoblastoma usually presents with symptoms and signs of hydrocephalus due to obstruction of CSF pathways at the level of the aqueduct, and sometimes Parinaud's syndrome, with paralysis of upwards gaze. Non-pineal StPNETs are usually large at presentation with around half being greater than 5 cm in diameter at diagnosis.

MRI scanning shows these tumours are typically large with heterogeneous signals on both T1 and T2 weighted images due to the variable presence of calcification, cystic change and blood within the StPNET tumor mass. Compared with glial tumors, StPNETs are typically hyperintense on diffusion weighted images (DWI), and fluid-attenuated inversion recovery (FLAIR) sequences may be used to differentiate necrotic areas (hyperintense) from cystic regions (hypointense). In accordance with other intracranial tumors, StPNETs have been shown to exhibit high choline and low N-acetyl aspartate (NAA) on MR spectroscopy (MRS) (Chawla et al. 2007).

In common with medulloblastoma, whole neuraxis MRI scanning and lumbar puncture for CSF cytology are required for staging using the modified Chang score (Zeltzer et al. 1999). A significant minority of patients have metastatic spread at the time of presentation, although is variable in different reports. For example, in the PNET 3 and CCG 921, patients with metastatic disease comprised 21 and 16% respectively (Cohen and Packer 1996; Pizer et al. 2006). The proportion of patients with metastatic disease at diagnosis appears to be greater in 'infants' than in older children and young adults. Metastatic spread outside the CNS is extremely rare although has been reported, particularly at the time of relapse (Gaffney et al. 1985).

Prognostic Factors

Whereas certain prognostic factors are clear and based on a high level of evidence in MB, there is a distinct lack of data on prognostic factors in StPNETs. There is strong evidence to suggest that younger children with StPNET e.g. those age less than 3 years do worse than older children. The reasons for this are not totally clear but include the avoidance of cranial spinal radiotherapy (CSRT) and also radiotherapy to the primary tumor site in younger children, possibly a higher rate of metastatic disease in younger patients and possibly age related differences in biology. There does appear to be strong evidence that at least for 'older' children treated with CSRT that the outlook for pineoblastomas is superior to that for StPNETS occurring at other sites (discussed below). However, the influence of factors such as the presence of metastatic disease, the extent of resection, histological features and biological characteristics are currently unclear in respect of their association with prognosis. The paucity of data with respect to prognostic factors and the determination of optimal therapy in StPNET reflect the rarity of these tumors. This fact together with the previous acceptance of the concept of PNETs resulted in the inclusion of patients with StPNET into trials for MB, particularly studies of high-risk disease. There is thus a lack of studies in both 'infants' and older children specifically designed for StPNETs.

General Principles of Management

Diagnostic and staging investigations at diagnosis are similar to those for MB i.e. whole neuroaxis MRI and lumbar puncture for CSF cytology to determine the presence of metastatic disease. It is widely accepted that surgery plays an

important part in the management of StPNET. This assumption is based on the general principles of management of malignant CNS tumors, evidence for the importance of complete or near-complete resection in MB and the observation that the majority of StPNETS, certainly in older children, relapse in a localized fashion (Timmermann et al. 2002; Pizer et al. 2006) stressing the importance of local tumor control. However, as with other prognostic factors in StPNET, the evidence base for the importance of the degree of surgical excision behind this widely accepted principle is limited. In the CCG 921 trial, however, there was a trend toward better outcome for tumors with less than 1.5 cm^2 of residual disease, but this difference did not reach statistical significance (Albright et al. 1995).

It is of note that for StPNET rate of complete resection is generally lower than that for MB. For example in the SIOP PNET 3 (Pizer et al. 2006) and HIT 91 (Timmermann et al. 2002) studies referred to below, the proportion of patients having complete tumor resection was 45.6 and 33.3% respectively. This reflects the large size and frequent complex and critical area associations of StPNETs limiting the ability to obtain complete surgical clearance. Despite this, most national and international groups recommend that the tumor should be excised as completely as possible. It is likely that with recent advances in neuro-oncological surgery such as the routine use of computer-assisted navigation that the rate of resection will improve, possibly with an increasing use of second look surgery to obtain a complete excision. The use of intra-operative MRI may also improve resection rates.

Supratentorial PNET is clearly a radiosensitive tumor, although a small proportion of patients appear to have tumors that are radio-resistant and progress during RT. As with medulloblastoma, RT is considered a fundamental aspect of treatment particularly in 'older' children. Although the number of patients with StPNET presenting with a radiological or cytological evidence of metastatic disease is lower than seen in MB and the observation that patients with StPNET tend to relapse in a localized fashion, it is widely accepted that for at least for children aged over the age of 3–5 years, that cranial spinal radiotherapy (CSRT) with a boost to the site of the primary tumor is standard practice.

The adverse effects of RT must however, clearly be taken into account when planning therapy and clinical trials for patients with StPNET. The long-term effects associated with CSRT are well reported in medulloblastoma, particularly the severe and often devastating neuro-psychological sequelae seen in younger children. Although the systematic study of long-term effects of CSRT in StPNET is not as well described, it is likely that such neurological damage is least as important in StPNET as in medulloblastoma and probably more so given the fact the tumor boost is delivered to the supratentorial brain rather than the posterior fossa. In a recent Childhood Cancer Survivor Study incorporating 818 adult survivors of childhood CNS tumors, radiation doses ≥ 30 Gy to the temporal region were associated with a greater propensity for memory impairment, whilst radiotherapy to the frontal lobe was associated with physical performance limitations (Armstrong et al. 2010). In addition, survivors who had received temporal lobe radiotherapy reported dose dependent higher rates of poor general health and social functioning. Thus the risk to benefit balance of the use of RT, particularly CSRT, is the major determinant influencing treatments used in StPNET. To avoid these risks, a few select centers are approaching the treatment of children with CNS tumours with proton beam radiation therapy (Greco and Wolden 2007). The unique controllable physical nature of proton beams allows extremely conformal dose distributions and at least on radiobiological grounds results in a reduced dose of radiotherapy to normal CNS structures. The number of proton beam therapy facilities is increasing rapidly in both Europe and North America. It is quite possible that over the next 10 years or so proton beam radiotherapy will become the standard of a care for children with StPNET as well as most other CNS tumours in childhood. The introduction of proton beam therapy should of course be accompanied by a systematic evaluation of the utility of this form of radiotherapy both with respect to tumour control but also with regard to

the potential of proton beam therapy to result in less neuropsychological and other sequelae.

Supratentorial PNET is a chemosensitive tumor and chemotherapy is now used in nearly all treatment protocols. There is, however, a paucity of classical single agent phase 2 studies in this disease, although there is clear evidence from Phase 3 studies of responsiveness to combination chemotherapy used in a neo-adjuvant setting prior to radiotherapy and in younger children treated with chemotherapy-based regimens, (Pendergrass et al. 1987; Cohen and Packer 1996; Kuhl et al. 1998). For example, in the pilot HIT 88/89 study a response rate of 57% was observed in those aged 3–17 years (67% in MB) (Kuhl et al. 1998). Despite this, there is as yet little evidence that chemotherapy adds significantly to improving survival in those patients who are treated with CSRT.

With respect to younger children treated with radiotherapy free approach, a high response rate to induction chemotherapy was observed in the Headstart I and II trials, in which 18 of 22 evaluable patients had a complete or partial response (Fangusaro et al. 2008) In another study, 21 children under the age of 36 months with an StPNET received two courses of vincristine and cyclophosphamide (Duffner et al. 1993). In 6/21 (29%) a complete or partial response was observed and an additional 9/21 (43%) were described as having stable disease. Given the different treatment approaches for 'older' children, that generally included CSRT, and for 'younger' children e.g. those aged less than 3–5 years, these are described separately in the following paragraphs. In addition data and strategies with respect to pineoblastomas and patients with relapsed StPNETs are discussed.

Treatment for 'Older' Children with StPNETs

The generally accepted standard therapy is to remove the tumor as completely as possible, although this may be problematical as discussed above. Craniospinal radiotherapy is delivered with dose of 35–36 Gy with a boost to the primary tumor of approximately 20 Gy, giving around 55–56 Gy to the area of the primary tumor. Conventional intensity chemotherapy is generally administered according to combinations of drugs used in high-risk medulloblastoma protocols. As discussed above, there is a lack of large multi-center studies specifically for StPNETs; instead, they are generally treated with protocols designed for children with high-risk MB. The largest series comes from the SIOP PNET 3 (Pizer et al. 2006), HIT 88/89 and 91 (Timmermann et al. 2002) and CCG-921 (Cohen and Packer 1996) studies with 66, 64 and 55 patients respectively. All three studies use the strategy described above.

The SIOP PNET 3 study was designed to determine whether 10 weeks of moderately intensive chemotherapy (vincristine, cyclophosphamide, carboplatin, etoposide) given after surgery and before RT would improve the outcome for patients with primitive PNETs (medulloblastoma and StPNET) compared with RT alone. Patients with a histological diagnosis of StPNET and no radiological evidence of metastatic disease were initially eligible for randomization to either chemotherapy followed by CSRT 35 Gy with a boost of 20 Gy or CSRT alone. In respect of the increasing recognition that StPNET were high-risk tumors, randomization for this group closed early and there were too few patients to determine the benefit of chemotherapy. Sixty-eight patients with StPNET aged 2.9–16.6 years (median 6.5 years) were included in the analysis (chemotherapy + RT: 44, RT alone: 24). Fifty-four patients (79%) had a non-pineal and 14 (21%) a pineal site. At a median follow-up of 7.4 years, for all patients 5-year overall survival (OS) and event free survival (EFS) was 48.3 and 47.0% respectively. There was no statistically significant difference in OS or EFS according to whether chemotherapy was received. This is in contrast to the improved EFS for patients with standard risk medulloblastoma treated on the PNET 3 trial with the same chemotherapy (Taylor et al. 2003). For StPNETs, OS ($p=0.05$) and EFS ($p=0.03$) were significantly better for patients with pineal primary sites. The EFS for pineal tumors was 92.9% at 3 years and 71.4% at 5 years and for

non-pineal primaries 40.7% at 3 years and 40.7% at 5 years. The effect of tumor size was shown to be the only statistically significant factor in a multivariate analysis an observation that requires investigation in future studies.

In the HIT88/89 study patients were treated with pre-RT chemotherapy (ifosfamide, etoposide, methotrexate, cisplatin and cytarabine) whereas in the HIT 91 studies patients were randomized between this therapy and immediate RT followed by eight courses of 'maintenance' chemotherapy with cisplatin, CCNU and vincristine. For all patients, 3-year progression free survival (PFS) was 39% (Timmermann et al. 2002). The only significant prognostic factor was the dose and volume of RT although a statistically non-significant trend was seen for improved survival in pineal disease, with a 3 year PFS for pineal tumors of 64% and for non-pineal StPNETs of 34%.

In the CCG-921 study for high-risk PNETs, 55 patients aged 1.5–19.3 years with StPNETs were randomized to receive CSRT followed by 8 cycles of CCNU, vincristine and prednisolone or 2 cycles of 8-in-1 chemotherapy followed by CSRT and then eight further cycles of 8-in-1 (Cohen and Packer 1996). Three-year Kaplan-Meier estimates of OS and PFS rates for StPNET patients were 57 ± 8% and 45 ± 8%, respectively. PFS rates for children with pineal PNETs were 61 ± 13%, respectively, and were significantly superior to the PFS rate of 33 ± 9% for the other S-PNETs ($p < 0.03$). On univariate analysis, other prognostic factors that influenced PFS included metastasis ($p < 0.03$: M0: 50 ± 9% v M1-4: 0%, $p < 0.03$) and age (1.5–2 years: 25% ± 3 years and above: 53 ± 9%, $p < 0.02$). In both HIT 91 and CCG-921, neither chemotherapy arm in each study showed a benefit over the other arm.

Thus these larger series have shown a higher survival for older patients with non-metastatic pineal tumors (60–70%) than those with non-pineal StPNETs (30–40%). Suggested reasons for an improved survival for patients with pineal disease include earlier presentation, smaller tumor size at diagnosis due to pineal tumors presenting with CSF obstruction, and possible biological differences including differences in response to treatment. However, as shown in the PNET-3 study where only one of seven patients with M2/M3 disease survived, the apparent survival advantage for patients with pineal tumors may only apply to those with non-metastatic disease (Pizer et al. 2006). Other prognostic features for StPNETs have not yet been clearly determined. Whereas the CCG-921 showed a detrimental effect of metastatic disease, this was not demonstrated in patients entered into the HIT-91 or PNET-3 studies. Likewise, as discussed above, the significance of the extent of surgical resection is unclear.

An important observation in all of these largest multicenter studies (Timmermann et al. 2002; Hong et al. 2004; Pizer et al. 2006) is that the predominant pattern of relapse was local to the original tumor site. This is in marked contrast to the principally distant pattern of relapse consistently seen in medulloblastoma. This localized pattern of relapse in StPNET would indicate that local control is one of the major challenges in the treatment of StPNETs. As discussed, complete surgical excision is frequently not achieved in this tumor group.

Planning RT for StPNET presents several technical challenges that may contribute to local failure. There may be concern about the long-term effects of irradiating a large amount of supratentorial brain particularly for younger children, resulting in the use of 'tight' margins. Planning technology and imaging are, however, improving, and together with advances in surgery may contribute to a reduced rate of local relapse.

In order to improve survival particularly for patients with non-pineal PNETs a variety of new approaches to treatment have been investigated. These include the use of myeloablative chemotherapy for both pineal and non-pineal StPNETs (Strother et al. 2001; Gururangan et al. 2003). For example, Gururangan et al. (2003) evaluated the usefulness of a treatment regimen that included high dose chemotherapy (HDCT) with autologous stem-cell rescue (ASCR) in patients with newly diagnosed pineoblastomas. Six children and six adults (four with metastatic disease) were initially treated with surgery and induction chemotherapy. All but two patients underwent RT. At the time of reporting, nine patients were alive

and recurrence-free at a median of 62 months from diagnosis, including three patients with metastatic disease and two infants who did not receive radiotherapy. The 4-year PFS and OS were 69% and 71% respectively.

The St Jude group has reported on a study (1996–2003) of risk-adapted CSRT and a local tumor boost dose and subsequent HDCT supported by ASCR (Chintagumpala et al. 2009). Sixteen patients with StPNET were enrolled. Patients with average-risk disease (no metastases and residual tumor ≤ 1.5 cm^2) received 23.4 Gy CSRT and those with high-risk disease (metastases and/or residual tumor > 1.5 cm^2), 36–39.6 Gy. The tumor bed received a total of 55.8 Gy. Subsequently, all patients received 4 cycles of high-dose cyclophosphamide, cisplatin, and vincristine with stem cell support. Seven patients had pineal StPNET. Encouraging survivals were observed for this limited number of patients. The 5-year EFS and OS estimates for all patients were $68 \pm 14\%$ and $73 \pm 13\%$ respectively. The 5-year EFS and OS was $75 \pm 17\%$, for the eight patients with average-risk disease and $60 \pm 19\%$ for the eight with high-risk disease. This study showed that HDCT with ASCR after CSRT may permit a reduction in the dose of CSRT in average-risk patients.

With respect to RT, there is considerable interest in Europe investigating alternative fractionation of RT, for example, hyperfractionated (twice daily) RT that potentially can increase the therapeutic index of RT e.g. allowing a higher dose to be delivered to rapidly proliferating tumours whilst maintaining a similar dose to normal CNS in respect of late effects. Hyperfractionated radiotherapy generally uses twice daily administration of 1 Gy fractions. Such RT may also be 'accelerated' in which a higher dose per fraction is given, potentially increasing tumour cell kill by reducing tumour cell proliferation between fractions.

In this respect so called hyperfractionated accelerated radiotherapy (HART) has been investigated by the Milan group who have demonstrated extremely favourable outcomes for patients treated using a regimen based on HART for patients with metastatic medulloblastoma. The Milan group's initial experience in using this protocol in children with StPNET was reported by Massimino et al. (2006). Fifteen consecutive patients (3 pineal; 12 non-pineal) received pre-radiation chemotherapy consisting of high-dose methotrexate, etoposide, cyclophosphamide, and carboplatin followed by CSRT with HART plus a focal boost to the primary site. After RT, maintenance with vincristine/lomustine or consolidation with high-dose thiotepa followed by ASCR was given. Six patients were had no evidence of disease after surgery. Of those with residual disease after surgery, two had central nervous system spread. Of nine evaluable patients, two had a complete response, two a partial response, four developed stable disease and in one progressive disease occurred after chemotherapy. Because of rapid progression in two of the first five patients, high-dose thiotepa was systematically adopted after HART in the subsequent ten patients. Three-year PFS, EFS, and OS were 54, 34, and 61, respectively. The use of high-dose thiotepa possibly improved the outcome (3/10 vs. 3/5 relapses). To date (June 2010) this protocol has been used in a total of 27 patients, two of which were under 3 years of age (Massimino – personal communication). This included seven pineal and 20 non-pineal tumours. Complete surgical excision was achieved in only nine patients. The updated response rate to pre-RT chemotherapy was 67%. For this larger group, 3-year PFS, EFS, and OS were 65%, EFS 49%, OS 66% respectively. The improved outcome for patients receiving high-dose thiotepa appeared to be confirmed (3 year PFS 68% v 33%, $p = 0.05$).

A further approach has been to exploit the potential for certain chemotherapy drugs to act as radio-sensitizers when given concomitantly with RT. Such an approach using carboplatin give with RT has been used in the Children's Oncology group study 99701 to treat (not treated) high risk PNETs in which following surgery patients received 36 Gy CSRT with boosts to the primary site and sites of metastatic disease (Jakacki et al. 2008). During RT, patients received 15–30 doses of carboplatin (30–45 mg/m^2/dose) given 1–4 h prior to each fraction, as well as weekly vincristine. All patients received adjuvant chemotherapy with six monthly courses of cyclophosphamide

and vincristine. Very encouraging early results have been reported for using this approach in patients with high risk medulloblastoma. With regard to StPNETs, early presented but unpublished data described 28 patients (24 non-metastatic, four metastatic) with a centrally reviewed diagnosis of a non-pineal StPNET. 3-year OS and EFS was 52 ± 10% and 49 ± 10% respectively. This compares to 86 ± 7% and 76 ± 9% for patients with pineoblastomas treated on the same regimen (p = 0.007 and 0.01 respectively). Three of the four patients with metastatic disease were alive at the time of the report. Extent of surgical resection (gross or near total resection versus other) did not appear to impact outcome.

Treatment for 'Younger' Children with StPNETs

Young patients appear to have a particularly poor prognosis, possibly as a result of biological differences or an underutilization of adequate doses of RT. Furthermore, in recognition of the observed poor prognosis and severe neuro-psychological sequelae in very young children with malignant brain tumors, from the 1980s onwards, a number of national collaborative groups developed so-called 'baby brain' protocols. These used prolonged administration of chemotherapy in an attempt to a delay or in some cases avoid the use of RT to the developing brain. In the Pediatric Oncology Group (POG) study, children under 36 months of age were treated postoperatively with two 28-day cycles of cyclophosphamide plus vincristine, followed by one 28-day cycle of cisplatin plus etoposide. This sequence was repeated until the disease progressed or for 2 years in 132 children 24 months of age and for 1 year in 66 children 24–36 months of age at diagnosis. Following this, the patients received radiation therapy i.e. at 3 years of age. Reported in 1993, this landmark study included 36 patients with StPNET with only eight (27%) having a complete tumor resection (Duffner et al. 1993). The outcome was poor for the 36 StPNET patients, with 2 year OS and PFS of 21 and 19% respectively.

A sub-analysis of the POG study was undertaken for 11 infants with pineoblastomas was reported in 1995 (Duffner et al. 1993). The age at diagnosis ranged from 1 month to 35 months. Four had positive CSF cytology and three had metastatic disease at diagnosis demonstrated on myelography. Following 2 cycles of cyclophosphamide and vincristine one patients had a partial response, five demonstrated stable disease, and five developed progressive disease. All children ultimately failed chemotherapy at between 2 and 11 months, and six receiving radiation relapse or progression following failure on chemotherapy. All children died, with survival following diagnosis ranging from 4 months to 13 months.

The UK baby brain study for children under 3 years of age used alternating blocks of myelosuppressive and non-myelosuppressive chemotherapy every 14 days for an intended duration of 1 year. Radiotherapy was withheld unless imaging showed progressive disease. Reported recently, this study included 11 children with StPNET, only one of which had evidence of metastatic disease at diagnosis (Grundy et al. 2010). Initial surgery consisted of complete resection in two, partial resection in five and biopsy only in four patients. Radiotherapy was given electively to two patients and to four children following relapse. These patients faired very poorly with all children dying with a maximal survival time of just over 1 year.

A sub-analysis of the CCG-921 examined the outcome for eight infants with pineal PNETs less than 18 months of age were treated with 8-in-1 chemotherapy without RT (Jakacki et al. 1995). All developed progressive disease with a median of 4 months from the start of treatment.

High dose chemotherapy has been used in an attempt to improve on the poor outcome of young children with StPNET. Fangusaro et al. (2008) reported on the so-called Head Start series of trials designed for young children with malignant brain tumors. This approach is based upon limiting the use of RT. After surgery, intensive chemotherapy after surgery followed by consolidative myeloablative chemotherapy with ASCR. In Head Start I and II (1991–2002), 43 children with StPNET were treated with 5 cycles of

chemotherapy (vincristine, cisplatin, cyclophosphamide, and etoposide). In patients on HS II with disseminated disease, high-dose methotrexate was added to the chemotherapy regimen. If the disease remained stable or in response, patients received a single cycle of high-dose myeloablative chemotherapy followed by ASCR. Five-year EFS and OS were 39% (95% CI: 24%, 53%) and 49% (95% CI: 33%, 62%), respectively. Non-pineal StPNET patients faired significantly better than those patients with pineal StPNETs. In this series, metastasis at diagnosis, age, and extent of resection were not significant prognostic factors. Sixty percent of survivors (12 of 20) were reported as being alive without exposure to radiation therapy.

Treatment at Relapse

It is clear that relapse of StPNET in patients that have received prior CSRT carries a dismal prognosis when treated with conventional chemotherapy and/or surgery. A number of groups have investigated the role of myeloablative chemotherapy with ASCR in order to cure such patients. Early studies using this approach did report a small number of patients with relapsed StPNET surviving after HDCT. For example, Graham et al. (1997) reported two survivors out of three patients with recurrent StPNET treated with cyclophosphamide and melphalan-based HDCT (Graham et al. 1997). In another series, Broniscer et al. (2004) reported one out of eight previously irradiated patients with relapsed StPNET surviving following a HDCT (carboplatin, thiotepa and etoposide) regimen. Such reports, however, tended to study survival of patients from the time of HDCT and in this respect, report a selected group of patients who were able to get to the point of treatment with HDCT following relapse. The UK relapsed PNET study included a HDCT-based strategy but reported survival from the time of relapse. Five patients with StPNET were included in the study, only one of whom reached the point of receiving HDCT with all patients dying of progression within 1.7 years of relapse (Pizer et al. 2011).

There is a paucity of data with respect to strategies for salvaging patients who relapse having not received prior focal or CSRT. Clearly a small proportion of these patients may be cured by an approach that includes RT, although such patients tend to relapse early after initial treatment and there must be serious concern about the severity of long-term sequelae of survivors given focal supratentorial RT or CSRT in these patients. Investigators have explored the use of HDCT for patients with relapsed StPNET not treated with prior RT. The study by Broniscer et al. (2004) referred to above, included nine patients who were treated at relapse who had not previous received RT. Of these nine patients, four died of disease and one of toxicity. Of the four surviving patients (EFS range 44–123 months), three had involved field RT and one child CSRT following HDCT.

Conclusions

Supratentorial PNETs remain a high risk tumour group, particularly for young children and the non-pineal group in older children. Progress in this tumour has been hampered by the paucity of studies for StPNETs that reflects the rarity of these tumours and the inclusion of StPNETs into studies designed for high-risk medulloblastoma. With increasing recognition of biological differences between StPNET and medulloblastoma and clear differences in response to treatment and outcome, the time has come to conduct multicentre studies specifically designed for patients with StPNET. Such studies should not only investigate conventional approaches to treatment but also novel approaches including biologically directed therapy.

Such tumour specific studies will also help define the prognostic factors for StPNETs. Whilst it seems clear that for older children the outcome is superior for patients with pineal PNETs than for non-pineal tumours there is a distinct lack of clarity with respect to other prognostic factors. For example, whether there is a benefit for chemotherapy in addition to CSRT and the prognostic significance of factors such as complete

resection of tumour, metastatic disease and biological factors is at the present time uncertain. Prognostic factors in young children are equally poorly defined although it is of note that the advantage for patients with pineal disease in the older age group is not present in younger children treated with chemotherapy only approaches.

Novel approaches to RT including the use of HART should be further investigated in order to confirm whether using higher doses of CSRT may improve outcome, whist maintaining a level of neuropsychological sequelae associated with conventional RT. In addition, the role of proton beam therapy needs to be systematically investigated as it is introduced into practice. As well as defining prognostic factors, further group wide studies should continue to explore the role of HDCT. The observation from the St Jude's group that a proportion of patients with so called average-risk disease obtain reasonable survival rates when treated with intensive chemotherapy and low dose CSRT (23.4 Gy) is interesting and should be further investigated, if possible in a randomised controlled trial. Whether treatments other than conventional chemotherapy such as HDCT or biologically based approaches can obviate the need for CSRT is an area worthy of discussion. Other approaches include the use of intrathecal chemotherapy, particularly in young children which again may provide an alternative means of sterilising the neuroaxis of tumor cells (Fleischhack et al. 2005). In the modern era, it is also clear that all treatment studies for StPNET should include a systematic assessment of the quality of survival, particularly given the severe neuropsychological and other sequelae likely to be associated with these tumours.

Finally, there is clearly a need to improve our knowledge of the biology of StPNETs using large cohorts of patients preferably linked to clinical trials to improve our knowledge of the biology of this poor risk group of patients, in order to define biologically based prognostic factors and to help develop new and less damaging therapeutic approaches.

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Giant Pediatric Midline Tumors of the Posterior Fossa: The Combined Transventricular and Supracerebellar Technique and other Approaches

26

Elvis J. Hermann and Joachim K. Krauss

Contents

Abstract

Huge pediatric midline tumors of the posterior fossa involving the fourth ventricle and the tectal region are challenging and difficult to approach. One important postoperative side effect is the occurrence of cerebellar mutism. During the last decades survival rates increased remarkably, not only due to technical innovations in surgery but also due to improved postoperative adjuvant therapy options like radiation therapy and chemotherapy in an interdisciplinary therapy setting. We present our experience with a combined transventricular/telovelar and supracerebellar/infratentorial approach in a total of five pediatric patients which allows removal of huge pediatric midline tumors of the posterior fossa without vermian splitting and thus reducing the risk of postoperative cerebellar mutism.

Introduction

Intracranial tumors are the most common solid tumors in children. About 60–70% of these tumors are located in the posterior fossa, including astrocytomas, medulloblastomas, ependymomas and other entities. The reason why these tumors prosper in the posterior fossa is not known. Most of these tumors achieve remarkable size before diagnosis. Huge midline tumors of the posterior fossa involving the fourth ventricle and the tectal region remain a challenge in contemporary neurosurgery, in particular in children.

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The aim of tumor resection is to remove as much tumor as possible while preserving neurological function. Complete tumor resection, especially in malignant tumors, offers better conditions for adjuvant therapy options and in some cases even cures are possible. As survival rates increase, postoperative quality of life becomes increasingly important (Steinbok et al. 2003; Steinlin et al. 2003).

Splitting the cerebellar vermis has been a common surgical procedure to remove huge midline tumors of the posterior fossa as reported already by Dandy (1966) and by Kempe (1970). This approach, however, may not only result in neurological deficits like ataxia and cerebellar mutism but also in neuropsychological and behavioral alterations (Dailey et al. 1995; Dietze and Mickle 1990; Grill et al. 2004; Kempe 1970; Levisohn et al. 2000; Steinlin et al. 2003). The combined transventricular/telovelar and supracerebellar/infratentorial approach which allows removal of huge pediatric midline tumors of the posterior fossa without the need for vermian splitting has several advantages as compared to other techniques (Hermann et al. 2008).

Surgical Technique

The combined transventricular/telovelar and supracerebellar/infratentorial approach for removal of giant pediatric midline tumors is performed in a semi-sitting position with the head fixed in a standard three-pins Mayfield clamp. Rigid fixation is obtained by securing the master screw of the clamp only incrementally. Evoked potentials (somatosensory evoked potentials and acoustic evoked brainstem potentials) are used for intraoperative monitoring during positioning of the patient and during tumor resection. In order to detect intraoperative air embolism as early as possible patients are monitored by transthoracic ultrasound. An intravenous line is located with its tip in the atrium to allow the removal of air once it may have entered the venous system despite utmost efforts to avoid it. Extraventricular drains are not implanted pre- or postoperatively as a routine procedure.

A linear skin incision is made in the midline extending from 4 cm above the inion down to the spinous process of the second cervical vertebra. A medial suboccipital osteoplastic craniotomy with opening of the foramen magnum extending above the transverse sinus is then performed. Additionally, partial or complete resection of the first cervical lamina may be done to enlarge the working area providing a greater angle of exposure from the obex up to the Sylvian aqueduct. The semi-sitting position provides additional value in such scenarios.

The transventricular/telovelar approach starts by mobilizing the tonsils bilaterally to gain access to the cerebellomedullary fissure. This natural corridor is covered by two thin membranous layers, the tela choroidea and the inferior medullary velum, which have to be dissected to open the fissure. First, the tela choroidea is opened on one side between the vermis and the tonsil. Great care has to be taken to protect the PICA (posterior inferior cerebellar artery) and its branches. The tonsil and the vermis are retracted gently with spatulae (Fig. 26.1a). Finally, the inferior medullary velum is dissected creating a wide access to the cavity and the floor of the fourth ventricle from the obex to the aqueduct and to the lateral recesses on both sides.

After debulking the tumor via the transventricular/telovelar approach first, the cerebellum descends mildly along the axis of gravity and the upper portion of the tumor can be approached via the infratentorial/supracerebellar route working through the corridors between the cerebellar veins draining to the tentorium (Fig. 26.1b). Utmost care is taken to preserve these veins. Thus, also the rostral tumor portions in the peritectal region extending way up to the thalami are exposed with ease and the tumor can be resected completely.

Material and Methods

The combined approach as described here is particularly useful in large pediatric posterior fossa tumors which cannot be removed completely by either a transventricular/telovelar or a infratentorial/supracerebellar approach. We have

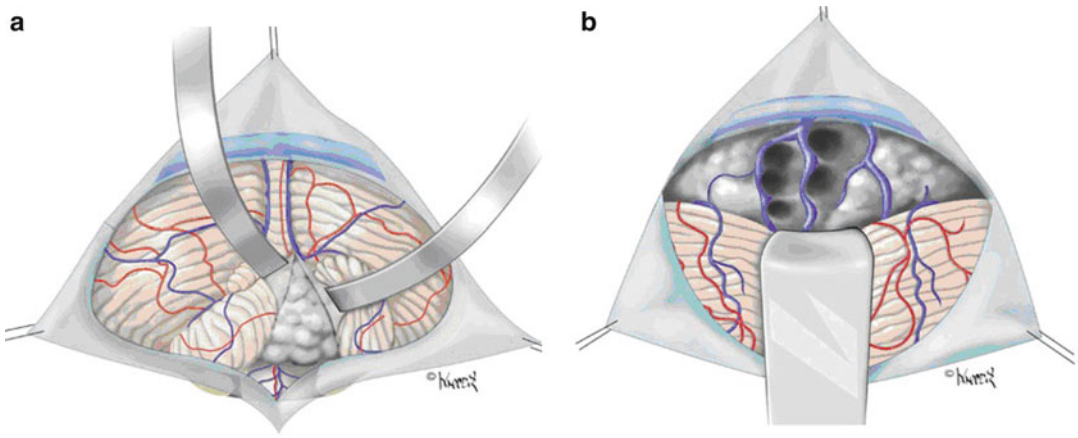

Fig. 26.1 (**a**, **b**) Illustration of the combined surgical approach showing the transventricular/telovelar approach with preservation of the vermis (**a**) and the infratentorial/supracerebellar approach (**b**) with preservation of the bridging veins (From Hermann et al. 2008, Neurosurgery. With permission from Wolters Kluwer Health)

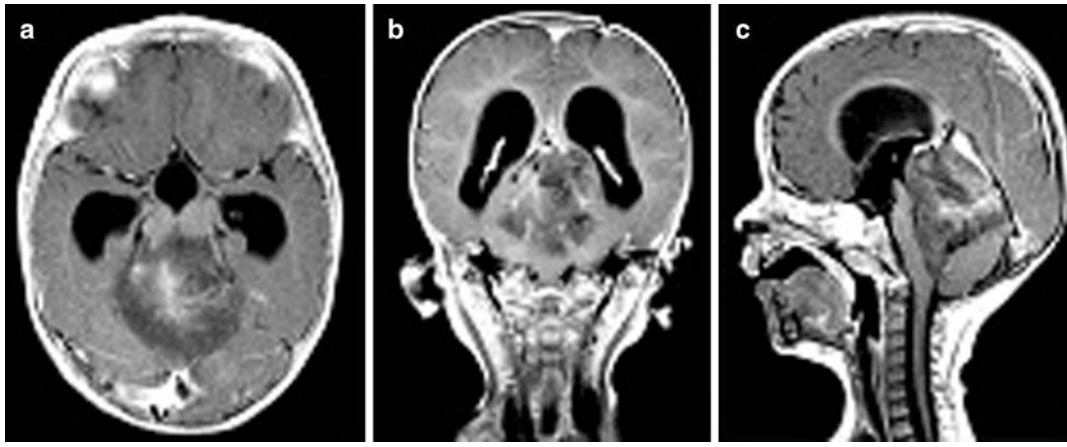

Fig. 26.2 (**a–c**) Axial (**a**), coronal (**b**) and sagittal (**c**) contrast enhanced MR scans of a 1-year-old boy who presented with headache, nausea and vomiting, showing a huge tumor within the fourth ventricle extruding above the peritectal region to the thalami compressing the brainstem and the cerebellum. Note, that the cerebellar vermis is compressed and dislodged but not infiltrated by the tumor. Histological diagnosis was atypical teratoid/rhabdoid tumor (From Hermann et al. 2008, Neurosurgery. With permission from Wolters Kluwer Health)

demonstrated its application previously in four children with huge midline tumors filling the fourth ventricle and extruding beyond the rostral midbrain well above the midbrain in the peritectal region compressing and dislodging the cerebellar vermis (Hermann et al. 2008). Here we report extended follow-up and its use in an additional patient. We operated on three boys and two girls. Mean age at surgery was 3.9 years (range, 1–5 years). Histopathological diagnosis was low grade astrocytoma in two children, and a malignant tumor in the other three. The three children with malignant tumors underwent adjuvant radiation and chemotherapy postoperatively. All patients had pre-/and postoperative magnetic resonance imaging. Figure 26.2a–c demonstrates

Table 26.1 Summary of clinical data, operative time, postoperative therapy, and outcome in five patients who underwent tumor resection by the combined transventricular/telovelar and supracerebellar/infratentorial approach

Case no.	Age (year)/ sex	Operative time (min.)	Histopathological diagnosis	Postoperative complications	Adjuvant therapy	Outcome
1	1/M	283	Atypical teratoid/ rhabdoid tumor	Transient alternating strabismus	Radiotherapy and chemotherapy	Alive and well at 63 months postoperatively
2	4/F	255	Pilocytic astrocytoma	None	None	Alive and well at 62 months postoperatively
3	5/M	240	Medulloblastoma	Transient aggravation of gait ataxia	Radiotherapy and chemotherapy	Alive and well at 18 months postoperatively
4	8/F	265	Pilocytic astrocytoma	Transient diplopia	None	Alive and well at 24 months postoperatively
5	1.5/M	495	Glioblastoma	Transient pneumocephalus	Radiotherapy and chemotherapy	Alive and well at 6 months postoperatively

a typical example of a tumor which may be approached ideally by the combined transventricular/telovelar and the infratentorial/supracerebellar approach (case no.1).

Results

Clinical data, operative time, postoperative therapy and outcome are summarized in Table 26.1. Our aim to preserve the cerebellar vermis was achieved in all five cases. There were no new permanent neurological deficits postoperatively and during follow-up (6 months up 63 months). Complete tumor resection was achieved in patient 1 with a huge atypical teratoid/rhabdoid tumor, in patient 2 with a large pilocytic astrocytoma and in patient 5 with a large glioblastoma. Near-total tumor removal was performed in the two other cases (3 and 4). A small tumor remnant invading the floor of the fourth ventricle was shown by postoperative MRI after resection of a huge medulloblastoma, and a small tumor remnant was noted in a pilocytic astrocytoma. Figure 26.3a–d shows MR scans from patient 1 at follow-up 18 months postoperatively.

Discussion

Since survival rates in pediatric brain tumors increased considerably over the past few years not only due to better diagnostic and better technical and surgical tools but also due to improved adjuvant therapy options, quality of life becomes more and more important especially in long-term survivors. Pediatric tumors of the posterior fossa within the fourth ventricle are challenging. One of the most important steps in the interdisciplinary concept of treatment is complete or nearly complete tumor removal. Tumor removal may be achieved by a transvermian route, a relatively fast and straight forward technique, or by techniques preserving the vermis. Huge tumors extending through the superior medullary velum up to the peritectal region and rostrally up to the transverse fissure are rare but they require special therapeutic considerations, in particular with regard to achieve complete tumor removal while preserving function. Especially for this group of patients the combined approach described offers certain advantages. In the following we summarize the important steps, the assets and drawbacks of the classical transvermian approach, the transventricular/telovelar approach and the infratentorial/supracerebellar approach.

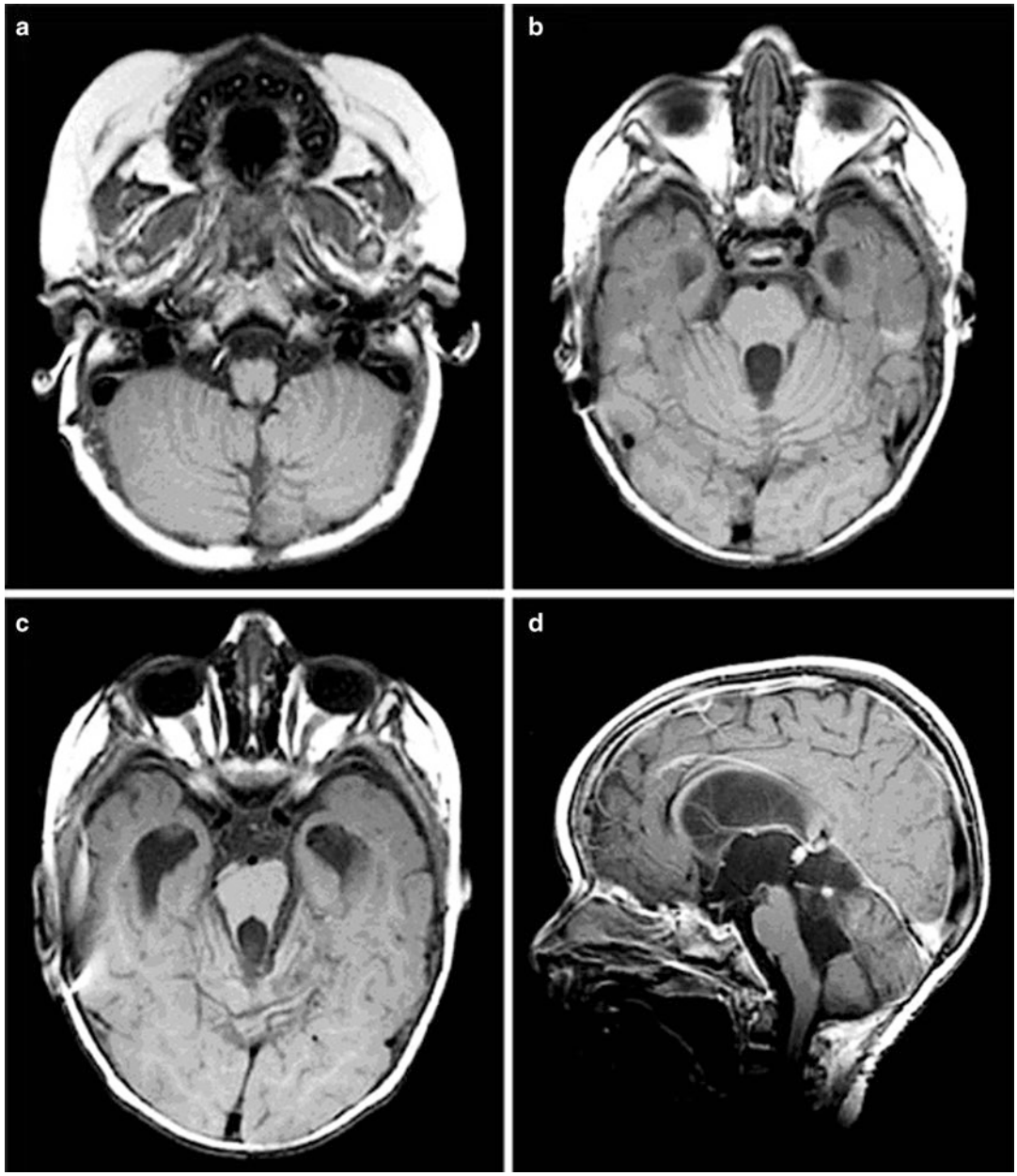

Fig. 26.3 (**a**–**d**) Contrast enhanced MR scans 18 months after operation and adjuvant therapy. The axial scans (**a**–**c**) show vermian preservation and the sagittal scan (**d**) demonstrates no tumor recurrence. (From Hermann et al. 2008, Neurosurgery. With permission from Wolters Kluwer Health)

Transvermian Approach

The cerebellar vermis is located in the medial, cortico-nuclear zone of the cerebellum. The vermis is the unpaired, median portion of the cerebellum that connects the two hemispheres. The traditional approach to resect tumors of the fourth ventricle is splitting the inferior part of the vermis as described 1966 by Dandy and 1970 by Kempe. They reported no or only minimal neurological impairment after vermian splitting.

Newer data, however, show that the transvermian approach is associated with an increased incidence of neuropsychological and psychiatric problems (Steinlin et al. 2003). The so-called "cerebellar cognitive affective syndrome" is characterized by impairment of executive functions such as planning, verbal fluency, abstract reasoning and working memory, difficulties with spatial cognition and personality changes. These behavioural changes were clinically prominent in patients with lesions in the posterior lobe of the cerebellum and the vermis (Schmahmann and Sherman 1998). Dysregulation of affect was also described by lesions involving the cerebellar vermis (Levisohn et al. 2000). Both superior and inferior vermian incisions have been associated with cerebellar mutism (Dietze and Mickle 1990; Dailey et al. 1995; Sinha et al. 1998). The precise cause and the neuroanatomical substrate for the cerebellar mutism syndrome after posterior fossa tumor resection remain elusive. Ersahin et al. (1996) found involvement of the vermis in more than 90% of 46 cases (seven own cases and 39 reviewed cases from the literature) with cerebellar mutism after resection of large midline tumors of the posterior fossa. Other investigators argued against direct operative vermis injury or resection as the sole mechanism for cerebellar mutism (Pollack et al. 1995; Dailey et al. 1995; Siffert et al. 2000). Both recovery of speech and ataxia may be incomplete (Steinbok et al. 2003). Also, impairment of intellectual skills was associated with vermian splitting (Grill et al. 2004). We suggest that vermian splitting should be abandoned whenever possible. Remarkably, also in autistic children hypoplasia of the vermis was shown as a common denominator (Courchesne et al. 1994; Hashimoto et al. 1995; Kaufmann et al. 2003).

Transventricular/Telovelar Approach

The cerebellomedullary fissure is formed rostrally by the tonsils and the biventral lobules, and caudally by the medulla oblongata, the tela choroidea and lateral recess (Rhoton 2000a, b). Exposure is obtained by retracting the cerebellar tonsils and opening the tela choroidea and the inferior medullary velum without vermian incision (Matsushima et al. 1992, 2012; Kellog and Piatt 1997; Mussi and Rhoton 2000). An anatomic study using the endoscope also demonstrated the benefits of this approach allowing complete visualization of the fourth ventricular cavity without splitting the vermis (Di Ieva et al. 2012). Using this approach, most tumor entities involving and filling the fourth ventricle can be approached like medulloblastomas, ependymomas, plexustumors and other entities.

To our knowledge there are no reports of neurological deficits in the literature following selective opening of the velum or of the tela, unless adjacent structures such as the posterior inferior cerebellar artery are injured. Rhoton described occlusion of veins around the tonsils, on the lower vermis, near the inferior part of the roof of the fourth ventricle including the vein of the cerebellomedullary fissure, without neurological impairment (Rhoton 2000a, b). This approach offers a natural corridor through noneloquent arachnoid planes and enables the safe resection of the tumor part filling the fourth ventricle. Mutism was absent by approaching posterior fossa tumors using the transventricular/telovelar approach without splitting the vermis (Kellog and Piatt 1997; Hermann et al. 2008).

Comparison of the Transventricular/Telovelar Approach and the Transvermian Approach

The transventricular/telovelar approach to resect tumors filling the fourth ventricle provides additional exposure to the lateral recesses and the foramen of Luschka without the necessity to incise any part of the cerebellum (Tanriover et al. 2004). The transvermian approach with an incision through at least the lower third of the vermis afforded only a modest increase in the surgeon's working angle as compared to the telovelar approach when accessing the rostral half of the fourth ventricle.

Deshmukh compared the telovelar approach with and without C1 posterior arch removal and the transvermian approach. He found that the additional removal of the C1 arch offers a larger

working area than the transvermian approach obviating the sole advantage of the transvermian approach to access the rostral half of the fourth ventricle (Deshmukh et al. 2006).

The Infratentorial/Supracerebellar Approach

While Krause described this approach first in 1926 to expose lesions in the peritectal region, Stein refined and popularized the technique for removal of pineal tumors in 1971. Yasargil (1995) developed the paramedian approach, and van den Bergh (1990) described a modified paramedian approach, the so-called "lateral paramedian approach". When Ammirati et al. (2002) compared these approaches by dissections performed on cadaveric heads, he found that the midline variant was the best option to expose the posterior incisural space and the paramedian and the lateral variants were superior to reach the posterior and the anterior part of the middle incisural space. The more lateral the approach, the more anterior and multiangled was the gain in exposure. Typical tumor entities reached by this approach are for example pilocytic astrocytoma of the cerebellar hemispheres, tumors arising from the pineal region and medulloblastomas.

Importance of Preservation of the Cerebellar and Vermian Bridging Veins

The creation of a corridor between the tentorium and the tentorial surface of the cerebellum is common for all these described approaches. Occasionally, some vermian or hemispheric bridging veins may be sacrificed for tumor removal. Few authors described no complications after closure of such bridging veins (Bruce and Stein 1993). Others, described complications such as cerebellar infarction and hemorrhage following dissections of bridging veins (Fain et al. 1994; Page 1977; Yamamoto et al. 1996). The paramedian approach offers certain advantages concerning the venous drainage of the cerebellum. One or two hemispheric bridging veins may be sacrificed using this approach (Rhoton 2000a, b), but important collateral pathways can be preserved avoiding complications. Hemispheric bridging veins usually drain into the tentorial sinus after forming one common stem. Cutting the common stem near to the tentorial surface allows preserving all possible collateral pathways (Ueyama et al. 1998).

The semi-sitting position helps to avoid brain retraction during operation because the cerebellum will move downward due to gravity (Yokoh et al. 1987). Tumor exposure can be usually achieved without or only with slight retraction of the cerebellum and the dural sinuses. Another advantage is the relatively bloodless operating field reducing the need for using suction during tumor resection. Sanai et al. (2010) reported on their experience with eight cavernous malformations and arteriovenous malformations (AVMs) of the posteroinferior thalamus and posterolateral midbrain in the semi-sitting position obtaining a satisfactory upward-viewing angle with the supracerebellar-supratrochlear approach and an increased downward-viewing angle with the infratentorial-infratrochlear approach. One limitation, however, is that the need of rigid skull fixation in the semi-sitting position makes it not suited for infants during the first months of life due to the thinness of the skull.

The Combined Approach Preserving the Vermis

The combined transventricular/telovelar and infratentorial/supracerebellar approach offers various advantages. In the first step, resection of tumor parts within the fourth ventricle using the transventricular/telovelar route is achieved with ease. In the second step, the rostral tumor at the peritectal region and inside the posterior third ventricle is removed by the infratentorial/supracerebellar route. One can then alternate between these routes during the surgery any time to achieve maximum protection of surrounding healthy tissue on one hand and maximum tumor resection on the other hand. The combined approach allows complete preservation of the cerebellar vermis. Remarkably, no patient suffered from postoperative cerebellar mutism.

Piatt and Kellogg (2000) described a similar approach in two patients, but they were discouraged because of complications. In one case postoperative swelling of the cerebellum and hemorrhage occurred secondary to sacrifice of vermian veins and the patient died 6 days postoperatively despite reoperation. The authors believed that an unusual and unfavorable pattern of venous collateralization was the cause of this complication. In the second case postoperative swelling of the right cerebellar hemisphere was seen after an unilateral cerebellomedullary fissure approach in combination with the infratentorial/supracerebellar approach sacrificing the precentral cerebellar vein. Seven months postoperatively, the patient had recovered completely. The authors suggested that concurrent sacrifice of veins in the two surgical corridors was responsible for the complications.

An interesting alternative approach would be to combine the transventricular/telovelar approach with an occipital interhemispheric transtentorial approach. As far as we know, however, this combined approach has not been reported yet.

In conclusion, the combined transventricular/telovelar and infratentorial/supracerebellar approach offers a unique possibility to remove safely huge pediatric midline tumors. It is essential to select tumors which are suitable for this operation strategy, namely tumors mushrooming into the supracerebellar space and filling the fourth ventricle, as opposed to tumors growing within the vermis and compressing the fourth ventricle. Special expertise and careful protection of the venous drainage of the cerebellum is required to avoid severe complications.

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Pediatric Gastrointestinal Stromal Tumor: Pathology, Genetics, and Therapy

27

Atif A. Ahmed and Vivekanand Singh

Contents

Abstract

Gastrointestinal stromal tumors (GIST) are distinctive stromal tumors with unique histologic, immunophenotypic and genetic features that usually occur in adults but are rarely found in the pediatric population. Few predisposing conditions are identified, namely Carney syndromes and type 1 neurofibromatosis. Patients usually present with tumors in the stomach that may often be multi-nodular. Current literature indicates that pediatric gastrointestinal tumors may be grouped into two categories. Type 1 tumors (adult type) that usually occur in older children and young adults show characteristic *KIT* and *PDGFR* gene mutations. Type 2 tumors display wild type *KIT* and *PDGFR* genes and are associated with Carney triad or Carney Stratakis syndrome. Type 2 tumors may be associated with germline mutations in succinate dehydrogenase enzyme gene. Pediatric GISTs have slow disease course and patients have good prognosis despite presence of metastasis to peritoneal cavity, lymph nodes and liver. Because they are often negative for *KIT* and *PDGFR* mutations, they are not amenable to tyrosine kinase inhibitors that target these proteins. However recent research into signaling pathways indicates that these tumors may be responsive to new targeted therapy that opens the chance for cure.

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Introduction

It is now well established that pediatric gastrointestinal stromal tumors (GIST) are derived from distinctive cells that show characteristics of the interstitial cells of Cajal, the gut pacemaker cells (Kindblom et al. 1998). The tumor cells can further differentiate into four major histologic categories based on presence of certain phenotypic features: differentiation towards smooth muscle or neural elements, dual differentiation toward smooth muscle and neural elements, and lack of differentiation toward either cell type (Suster 1996). Gastrointestinal stromal tumors are common in adults with median age at diagnosis of 60 years and are rare in children (Benesch et al. 2009). Tumors in children have emerged as a separate entity with unique clinical, biologic and genetic features that are distinct from those in adults (Pappo et al. 2011).

Clinicopathologic Features

Pediatric GIST comprise roughly 2% of all GIST, and usually occur in the second decade of life (Kaemmer et al. 2009). Unlike adult tumors, pediatric GIST exhibit a marked female predominance. Although they may involve any portion of the gastrointestinal tract or the omentum, mesentery, and retroperitoneum, they are most common in the stomach where they may affect any part of the stomach wall. They comprise the majority of primary non-epithelial neoplasms of the stomach (Prakash et al. 2005).

Pediatric GIST may be single or multiple and often have nodular growth pattern without a true capsule. Frequently they present as multiple nodules within the stomach, and measurement of the tumor's greatest dimension can be difficult to determine. Most are exophytic subserosal lesions while others are endophytic polypoid submucosal masses that are prone to surface ulceration and bleeding. Tumors have soft or rubbery consistency and larger tumors may undergo cystic degeneration, hemorrhage or necrosis (Miettinen et al. 2005).

Gastrointestinal stromal tumors have a wide range of histologic appearances in adults and children. Two basic cell morphologies are recognized: spindled and epithelioid (Fig. 27.1a, b) (Miettinen et al. 2005). Some of the spindle cell tumors may show features suggestive of neural differentiation including nuclear palisading and/or a plexiform growth pattern. Some tumors may contain "skeinoid fibers" which are small aggregates of eosinophilic filamentous material scattered in-between the tumor cells. Epithelioid GIST occur more commonly in the antrum and most often consist of round vacuolated or clear cells. Regardless of histologic type, the degree of cellularity varies greatly. Pleomorphism is uncommon and usually associated with malignant potential, but mild pleomorphism may occasionally occur in benign tumors. Mitotic rate is also variable (Miettinen et al. 2005).

The most important immunohistochemical marker that highlights the tumor cells is CD117 (KIT), which is found in the vast majority (>90%) of GIST (Fig. 27.1c) (Fletcher et al. 2002). More than 80% of cases also express CD34. Smooth muscle actin is demonstrable in about 25% of cases, and desmin in 5%; both are indicative of smooth muscle differentiation. S100 may also be positive in tumors with neuronal differentiation (Newman et al. 1991). Other commonly reported markers include vimentin, protein kinase C (theta), nestin, bcl2 and h-caldesmon, while some cases express p53, PGP9.5, NSE, Leu-7, and synaptophysin (Newman et al. 1991). A recently characterized immunostain, DOG1, has been found to be a sensitive marker for the diagnosis of gastrointestinal stromal tumors (Fig. 27.1d) and may be positive in cases that did not stain with CD117 or CD34 (Miettinen and Lasota 2011).

Molecular Genetics

The molecular basis for KIT protein (CD117) overexpression is most commonly through activating mutation of the *KIT* gene (Hirota et al. 1998a), presenting as deletions or point mutations in exons 11 or 9. Such mutations are more

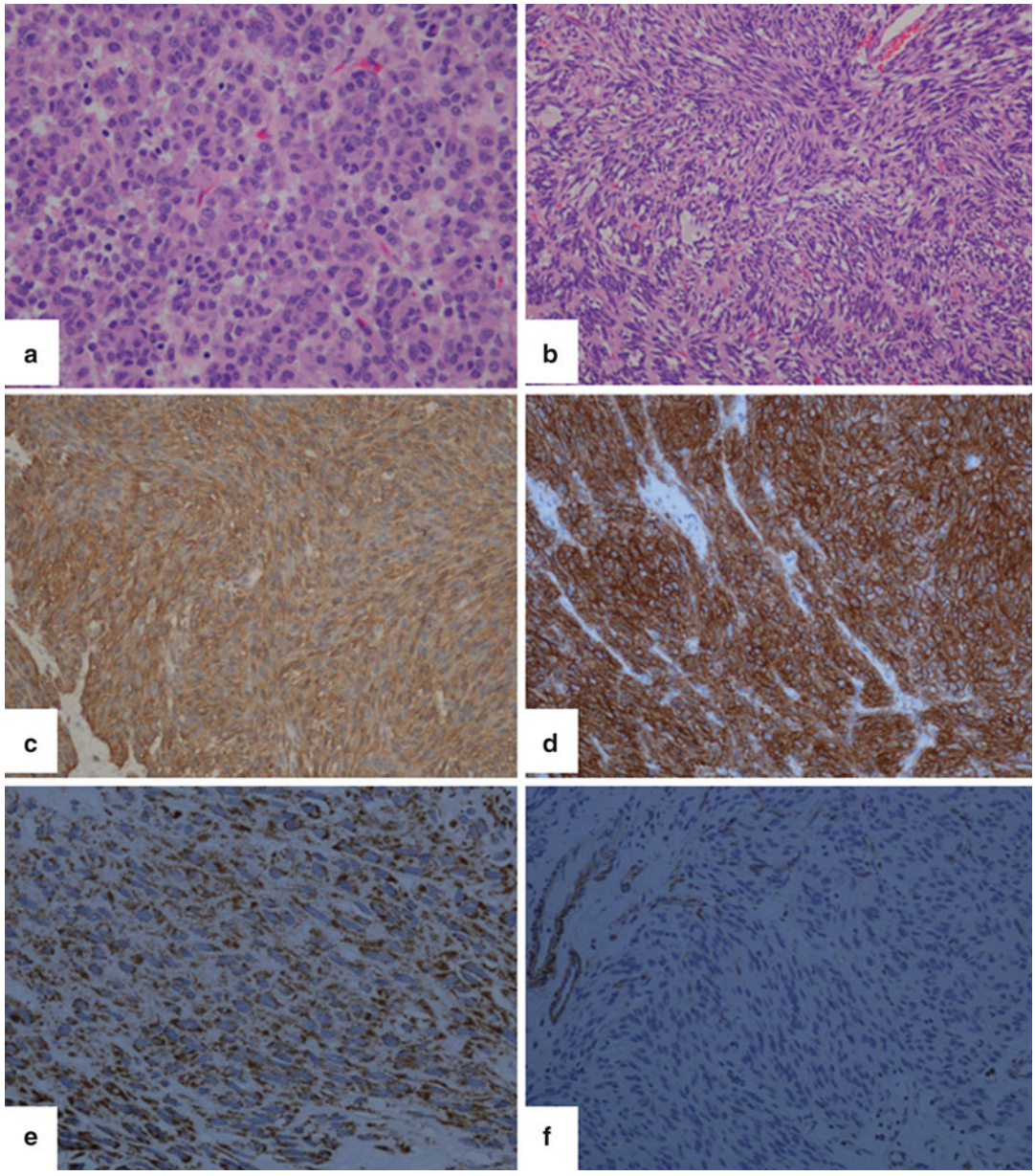

Fig. 27.1 The histology of pediatric GIST shows epithelioid (a H&E ×200) or spindle cell morphology (b H&E ×200). CD117 (C-kit) immunohistochemical stain is essential for confirming the diagnosis of these tumors (c ×200). DOG-1 immunohistochemical stain is another sensitive marker for the diagnosis of GIST (d ×200). Activity of succinate dehydrogenase-B is demonstrated by an immunohistochemical stain as positive (e ×400) or negative (f ×200)

common in spindle cell GIST than in epithelioid types. A smaller number of GIST, however, have mutations in the platelet-derived growth factor receptor alpha gene (*PDGFR-A*) (Heinrich et al. 2003). These tumors frequently have epithelioid morphology and exhibit good prognosis (Lasota et al. 2004). Immunohistochemical overexpression of CD117 also occurs in many of these

PDGFRA mutant tumors (Miettinen et al. 2005), suggesting a different mechanism of KIT protein expression.

Pediatric GIST differ from those in adults because *KIT* or *PDGFRA* mutations are found in less than 15% of pediatric cases (Agaram et al. 2008). The presence of tyrosine kinase mutations makes only a small number of these tumors susceptible to molecular therapy that targets activated tyrosine kinase receptors. Imatinib mesylate, a tyrosine kinase inhibitor, has proved clinically useful in the treatment of recurrent or metastatic GISTs in adults and in a few pediatric cases.

Syndromic Associations

Although GIST tumors mostly occur in adults aged over 30 years, cases in pediatric age groups have also been reported where they can be syndromic or non-syndromic. Recognized predisposing syndromes contribute to only a minority of cases. In a recent review of predisposition to GIST, Agarwal and Robson (2009) identified familial gastrointestinal stromal tumor syndrome, Carney triad, Carney-Stratakis syndrome and neurofibromatosis (NF) type I as syndromes that predispose individuals to develop GIST. The occurrence of GIST in association with neuroblastoma has also been previously described (Changho et al. 2011).

While the gastrointestinal involvement of neurofibromatosis was well known for several decades, Schaldenbrand and Appelman (1984) described poorly differentiated stromal tumors in small intestine in two patients with neurofibromatosis that were distinctly different from neurofibroma. The GIST in NF type I tend to exhibit c-kit expression by immunohistochemistry but lack the typical mutations in *KIT* and *PDGFRA* genes (Fuller and Williams 1991). A familial occurrence of GIST was also described by Nishda et al. (1998) in seven members of a family. Subsequently, multiple reports of familial gastrointestional stromal tumor syndrome have been published. Unlike the GIST seen in association with NF type I, the GIST of the familial gastrointestional stromal tumor syndrome have germline mutations of either *KIT* or *PDGFRA* (Hirota et al. 1998b; Chompret et al. 2004). The stromal tumors in the familial syndrome tend to be multiple, more common in small intestine and exhibit c-kit expression by immunohistochemistry.

The Carney triad comprises of gastric stromal sarcoma, paraganglioma and pulmonary chordoma that Carney (1983, 1999) reported to be more frequent in young women and had an indolent course. The GIST in Carney triad are usually multiple and located in stomach with or without metastases to lymph nodes, peritoneum and liver. Mutations in *KIT* or *PDGFRA* genes are not found. Carney and Stratakis (2002) described a familial dyad of paragangliona and gastric stromal sarcoma. The GIST in Carney-Stratakis syndrome are usually multiple, located in stomach, and the patients tend to be young and show autosomal dominant inheritance. Pasini et al. (2008) have shown that the GIST associated with Carney-Stratakis syndrome do not exhibit mutations in *KIT* or *PDGFRA* genes but have a reduced succinate dehydrogenase activity (SDH) and may harbor mutations in *SDHB*, *SDHC*, or *SDHD* genes.

Classification

Noting the genetic and biologic differences in the adult and pediatric GIST, Gill et al. (2010) proposed classifying pediatric GIST tumors into type 1 and 2 tumors. According to this proposal, type 1 tumors are frequently seen in young adults. These tumors do not show predilection to any gender or site, frequently harbor mutations of *KIT* and *PDGFRA* genes, are not associated with the Carney triad or Carney-Stratakis syndrome, and do not show lack of SDH activity. SDH activity can reliably be demonstrated by immunohistiochemical stain for SDH-B (Fig. 27.1e). The histology commonly exhibits spindle tumor cells.

Type 2 tumors are frequently seen in children or in association with Carney triad or Carney-Stratakis syndrome and show predilection to younger females. They are multifocal, invariably involve the stomach, do not typically harbor

mutations of *KIT* and *PDGFRA* genes, and show lack of SDH activity (Fig. 27.1f). These SDH-deficient tumors have an estimated frequency of 7.5% among gastric GIST tumors and commonly exhibit epithelioid histology. SDH-B immunohistochemistry is a useful tool in the diagnosis of these tumors. They often have a chronic clinical course similar to other pediatric GIST tumors, with prolonged survival even among patients with peritoneal or liver metastasis (Miettinen et al. 2011).

Prognostic Factors

GIST tumors, in general, may be classified as benign or malignant based on the tumor size and the mitotic count. Gross features suggesting malignancy include obvious invasion of adjacent organs and presence of metastasis. Vascular invasion and atypical mitotic figures are other good predictors of malignancy (Trupiano et al. 2002). The management and prognosis of these tumors in adults has traditionally been classified through a risk stratification scheme into low, intermediate or high risk (Wong et al. 2003). In contrast to tumors in adults, the majority of pediatric GISTs appear to follow a benign course. The exception is that non-syndromic cases with epithelioid morphology in females tend to be high grade. Pediatric GIST have a somewhat unpredictable but slow course of disease despite the presence of metastasis to the lymph nodes, peritoneal cavity and liver (Prakash et al. 2005). Metastasis to local regional lymph nodes is more common in pediatric GIST than adults. Thus, it seems that the conventional criteria for assessing risk of malignancy, such as tumor size, mitotic activity, proliferation index and anatomic location, are not reliable in pediatric GIST. Some pediatric patients develop metastasis despite being classified as low risk.

Treatment

Complete surgical resection is the mainstay of therapy of pediatric GIST. For localized and non-progessive metastatic tumors, resection is followed by follow-up and clinical observation at specific intervals. Tyrosine kinase inhibitor therapy, such as imatinib or sunitinib, is reserved for patients with tumor progression. The absence of oncogenic kinase mutations implies potential unresponsiveness to tyrosine kinase inhibitors since adult tumors negative for *KIT* mutations do not respond as well (Murray et al. 2008). Imatinib mesylate blocks the activated *KIT* gene by attaching to its ATP-binding site. Thus, type 1 tumors respond well to Imatinib whereas type 2 tumors do not. Not unexpectedly, type 1 tumors that do not harbor *KIT* mutations, such as in tumors that have wild type KIT and those with mutations in exon 9 (but not exon 11) respond poorly to Imatinib. The type 1 tumors that have mutations of *PDGFRA* are a mixture of responders and non-responders to Imatinib. The *PDGFRA* mutation D842V renders the GIST resistant to Imatinib (Gramza et al. 2009). The second generation kinase-inhibitor, Sunitinib, inhibits both *KIT* and *PDGFR* and is used in patients with resistance or intolerance to Imatinib.

Type 2 tumors frequently carry a wild type *KIT* mutation status and therefore are not as responsive to Imatinib. Agaram et al. (2008) reported that second generation kinase inhibitors such as Nilotinib, Sorafenib, Dasatinib and Sunitinib lead to apoptosis of these tumor cells at relatively lower drug concentrations and thus may be used in treatment. In the same study, the authors found the gene expression of the pediatric GIST (type 2) to differ from the adult wild type GIST with overexpression of BAALC, FGF4, PLAG1, IGF1R and NEFL genes. Janeway et al. (2010) found that the *IGF1R* gene is overexpressed in the pediatric (type 2) but not in adult GIST; and it was suggested that targeting IGF1R is a potential therapy of type 2 GIST (Belinsky et al. 2008).

Despite the multifocality and early metastases frequently seen in type 2 tumors, the biology of these tumors suggests an indolent course with long-term survival but with risk of disease recurrence and repeat surgical procedures. The molecular genetics, clinicopathological presentation and biology of the type 2 tumors indicate that there is a need for developing better therapeutic agents that target the involved genes.

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Pediatric Pituitary Adenomas: Germline Genetic Defects

28

Margaret F. Keil and Constantine A. Stratakis

Contents

Abstract

In childhood and adolescence pituitary tumors occur infrequently and are rarely malignant. However, these tumors may result in significant morbidity due to the essential role of the pituitary in the maintenance of homeostasis and the regulation of growth and puberty. Approximately 2–6% of surgically treated pituitary tumors occur in children and are primarily of two types, craniophayrngiomas and adenomas. The majority of pituitary tumors are sporadic; however, in children these tumors may be part of a genetic condition predisposing to pituitary and other tumors. The investigation of pituitary tumors in the context of genetic syndromes, such as MEN-1, Carney complex, familial isolated pituitary adenoma, and McCune Albright syndrome, has advanced our knowledge of the molecular basis of pituitary tumors.

Introduction

Although pituitary adenomas in children are rare, accurate data regarding their incidence and prevalence is lacking. Results of autopsy studies (primarily adults) indicate that pituitary adenomas develop in approximately 17–25% of the population(Asa and Ezzat 2002), which is consistent with results of studies of radiological imaging that report pituitary tumors in approximately 20% of the general population, with no gender predilection. Recently reported cross-sectional studies report a prevalence of one in 1064–1289 clinically relevant pituitary

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adenoma in well-defined populations(Daly et al. 2006). Approximately 3.5–8.5% of all pituitary tumors are diagnosed prior to the age of 20 years and they account for approximately 3% of all diagnosed intracranial tumors in childhood (Melmed 2011). There are two predominant types of tumors that occur within the pituitary fossa, craniopharyngiomas and adenomas. Typically pituitary tumors in children and adolescents are histologically benign; however, significant morbidity may result due to mass effect and/or interference with normal pituitary hormone function (Asa and Ezzat 2002). Sporadic lesions comprise the majority of pituitary tumors. However, in children more frequently than adults, pituitary tumors may be a manifestation of a genetic condition, such as multiple endocrine neoplasia type 1 (MEN1), Carney complex, familial isolated pituitary adenoma (FIPA), and McCune-Albright syndrome. These genetic syndromes have provided a model to help advance our knowledge of the molecular basis of pituitary tumorigenesis. In this chapter, we review recent findings on the diagnosis, evaluation, treatment, and molecular genetics of the two most common tumors of the pituitary gland in childhood, craniopharyngiomas and pituitary adenoma.

Pituitary Development

The pituitary gland has an essential role in the maintenance of homeostasis, normal growth, and reproductive function. The pituitary gland forms around the middle of the fourth embryonic week from an invagination of the oral ectoderm (stomodeum) to the rudimentary primordium (Rathke's pouch). Cell fate studies document a placodal origin of the anterior pituitary in all vertebrates. The pouch has elongated and constricts at the attachment to the oral epithelium around the 5th week of development; the adenohypophysis (pars anterior, pars intermedia, and pars tuberalis) develop from the ectoderm of the stomodeum. The neurohypophysis develops from the neuroectoderm (infundibulum)(Faglia and Spada 2001). The anterior and posterior pituitary lobes develop concurrently and continue to interact closely despite the different embryologic origin of the two tissues.

The adenohypophysis contains six different cell types that are characterized by their hormone secretion: corticotrophs secrete corticotropin (adrenocorticotropic hormone (ACTH)), somatotrophs secrete growth hormone (GH), thyrotrophs produce thyrotropin (thyroid-stimulating hormone or (TSH)), gonadotrophs secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and lactotrophs produce prolactin (PRL). The posterior pituitary lobe contains axonal terminals from the magnocellular hypothalamic neurons that are surrounded by pituitocytes (astroglia). Peptide hormones (oxytocin and vasopressin) are synthesized by the magnocellular neurons and transported to the axonal terminals in the posterior lobe from where they are secreted to the general circulation. The hypothalamus secretes releasing hormones (RH) that regulate the function of the anterior pituitary through modulation of cell proliferation, hormone synthesis, and secretion: corticotropin-releasing hormone (CRH) controls ACTH, GHRH and somatostatin (SMS) regulate GH secretion, thyrotropin-RH (TRH) for TSH, and gonadotropin RH (GnRH) for LH and FSH. In addition dopamine inhibits PRL secretion and TRH stimulates it; therefore a putative PRL-RH or releasing factor (PRF) has long been postulated to exist (Zhu et al. 2007).

Craniopharyngiomas

Craniopharyngiomas comprise the majority (80–90%) of neoplasms found in the pituitary fossa of children: up to 15% of all intracranial tumors in childhood are craniopharyngiomas. These tumors have a bimodal age-specific incidence: they occur most frequently at age 5–14 years and rarely in the fifth decade of life (Bunin et al. 1998) and incidence does not vary with race or gender. Craniopharyngiomas arise from squamous rest cells left as remnants from the Rathke's pouch; these cells are located between the adeno- and neurohypophysis and this is where most craniopharyngiomas first form. By the time most craniopharyngiomas (70% of the total) give symptoms they are extended in both the intrasellar and

suprasellar regions; 30% of the tumors may be either intra- or suprasellar in location (Jagannathan et al. 2007). Histology of craniopharyngiomas identifies primarily two categories, adamantinomatous, with cyst formation (ACF, typical pediatric form) and squamous-papillary (adults), although transitional forms have been reported.

Craniopharyngiomas typically present with endocrine dysfunction, diabetes insipidus, vision problems, and intense headaches or vomiting or other symptoms related to increased intracranial pressure. By histology, these tumors are benign; however, craniopharyngiomas can behave aggressively through papillae that invade surrounding bony structures and tissues. In addition, they can have cystic components that may enlarge and compress adjacent structures (Bunin et al. 1998). The molecular mechanisms underlying craniopharyngiomas have not been well characterized, some studies suggest it is a monoclonal tumor. In addition, cytogenetic abnormalities have been identified in up to 50% of tumors, most commonly gains in 1q, 12q, and 17q (Rienstein et al. 2003). Recently, β-catenin gene mutations were found in up to 20% of the rare adamantinomatous craniopharyngiomas, and Gaston-Massuet et al. (2011) reported that craniopharyngiomas arise from activation of β-catenin in pituitary progenitors during embryogenesis. However, the more common papillary craniopharyngiomas to this date have no common genetic abnormality (Sekine et al. 2002).

At the time of diagnosis of a craniopharyngioma, endocrine dysfunction is found in about 80% of the patients. GH deficiency is the most frequent endocrine finding (75%), followed by gonadotropin deficiency (40%), corticotropin and thyrotropin deficiency (Rienstein et al. 2003). Although craniopharyngiomas are frequently large at presentation, pituitary stalk disruption is not typically seen and hyperprolactinemia secondary to pituitary stalk compression is noted only in approximately 20% of patients. Diabetes insipidus (DI) is frequently a presenting symptom (in about 9–17% of the patients). The treatment of choice for craniopharyngiomas is surgical resection. Because the recurrence rate is higher than in all other pituitary tumors adjunctive radiotherapy is often indicated, except for small, entirely intrasellar lesions. Morbidity may be significant and is associated with treatment and also dependent on the size, location, and invasiveness of the tumor, the experience of the surgeon and the route of surgical approach. Craniopharyngiomas are generally radiosensitive and stereotactic radiosurgery has been used with success; since up to 60% of craniopharyngiomas are both solid and cystic, adjuvant treatments such as cyst aspiration or stereotactic Ommaya reservoir (for intracavity brachytherapy with bleomycin, radioactive phosphorus, or alpha-emitting 90Yt) are used to avoid surgery in situations where the solid part of the tumor is small or surgery is not possible or not indicated in younger patients (Sanford 1994).

Pituitary Adenomas

Among functional pituitary tumors in early childhood, ACTH-producing adenomas are probably the most common, although overall these tumors are still considerably rare. To date, no genetic defects have been consistently associated with childhood corticotropinomas, which only rarely occur in the familial setting, and then, most commonly in the context of multiple endocrine neoplasia type 1 (MEN 1)(Marx et al. 1999). GH- and/or PRL-producing are the second most frequently found functional pituitary tumors in early childhood; these tumors in children almost always occur in the familial setting or in the context of known genetic defects: *GNAS*, *menin*, *PRKAR1A*, *AIP* and p27 (*CDKN1B*) mutations; somato- and/or mammotropinomas become significantly more frequent than corticotropinomas in late childhood, adolescence and adulthood (Stratakis et al. 2010). Hypothalamic and pituitary factors are involved in pituitary adenoma development and cell growth. In addition, other factors and genetic events appear to influence pituitary-cell clonal expansion and oncogene activation, which is necessary to propagate tumor growth (Fig. 28.1; Melmed 2011; Xekouki et al. 2010).

Corticotropinomas are the most common pituitary adenomas in prepubertal children; their

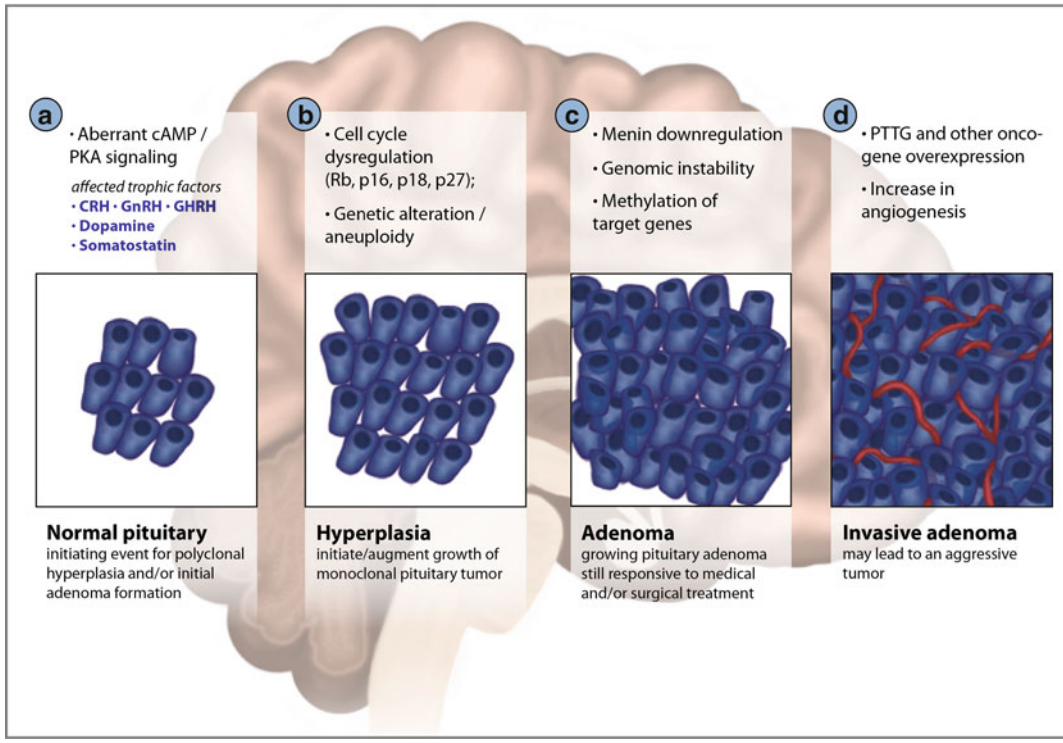


Fig. 28.1 Human molecular genetics of pituitary tumorigenesis. Possible pathways of pituitary tumorigenesis due to: (**a**) Aberrant cAMP signalling (initiating event for the polyclonal hyperplasia an/or initial formation of adenoma (i.e. *GNAS* and *PRKAR1A*); (**b**) Cell-cycle dysregulation and aneuploidy (i.e. events or genes that initiate or augment growth of a monoclonal pituitary adenoma); (**c**) *Menin* downregulation, methylation of specific target genes, aneuploidy and/or disruption of genomic integrity; (**d**) PTTG and/or other growth factor or oncogene overexpression, increased angiogenesis

frequency decreases during puberty and in adolescence, when prolactinomas become more prevalent. The cumulative incidence of ACTH-producing tumors (also known as Cushing disease) in children does not exceed a tenth of the annual incidence of 2–5 new cases of Cushing syndrome per million people per year. The most characteristic clinical presentation of Cushing disease is significant weight gain concomitant with failure to gain height. Other common symptoms include headaches, hypertension, glucose intolerance, and delayed pubertal development and amenorrhea despite often-significant virilization and hirsuitism. Compared to adults and older adolescents, children and younger adolescents do not typically report problems with sleep disruption, muscle weakness, or problems with memory or cognition.

Corticotroph adenomas are significantly smaller than other types of pituitary tumors (usually 3 mm or less). Rarely, corticotropinomas can be exophytic, growing into the subarachnoid space, or they may invade the cavernous sinus or wall; there are also case reports of tumors that originate in the posterior lobe (Magiakou et al. 1994). Most recently our group suggested a 3-day inpatient evaluation of a child suspected of having Cushing syndrome for confirmation of the diagnosis and investigation of a corticotropinoma (Batista et al. 2007). First-line treatment for Cushing disease in childhood is always surgical; transsphenoidal adenomectomy or hemihypophysectomy in situations where the surgical exploration is negative has been shown to be nearly 90% curative with an expert care facility.

Radiation or gamma-knife therapy is reserved for these patients in whom surgical intervention failed (Magiakou et al. 1994). Bilateral adrenalectomy may be considered for inoperable or recurrent cases; however it is associated with a significant risk of development of Nelson's syndrome.

Somatotropinomas comprise approximately 5–15% of pediatric pituitary adenomas in children and adolescents before the age of 20 years. Excess GH production results from an adenoma, usually macroadenoma or, rarely, somatotroph hyperplasia, which occurs in certain genetic conditions such as McCune-Albright syndrome or Carney complex. GH excess due to dysregulation of GHRH signaling as a result of a local mass effect may occur with optic glioma seen in neurofibromatosis type-1 (NF-1) or from an ectopic GHRH-producing tumor (almost unheard of in children). These tumors may also stain for prolactin and thyrotropin, which is usually of no clinical significance.

Clinical presentation in children and adolescents varies depending on whether the epiphyseal growth plate is open. Prior to epiphyseal fusion, significant acceleration of growth velocity is noted, a condition also known as 'gigantism'; as epiphyseal fusion is completed, the clinical symptoms become more similar to those in acromegalic adults (coarse facial features, broadened nose, large hands and feet, obesity, organomegaly, sweating, nausea). Since somatotropinomas are often macroadenomas, headaches and visual disturbances are frequently reported (Lim et al. 2004). First-line of treatment for childhood gigantism or acromegaly is transsphenoidal surgery; however, unlike Cushing disease, GH-producing tumors are often large and locally invasive. With small, well-circumscribed tumors transspheniodal surgery may be curative, while with larger and locally invasive tumors surgery may be beneficial to decompress tumors but persistent or recurrent disease is common and adjuvant therapy is needed. Radiotherapy, either primary or post-surgical, has slow onset of treatment effect and high treatment related morbidity of panhypopituitarism (Lim et al. 2004).

Pharmacologic agents are often indicated both before and after surgery and have been shown to be effective at shrinking tumor size and improving biochemical abnormalities. Long-acting somatostatin analogs have been shown to be effective at normalizing IGF-1 levels in most patients (Lim et al. 2004). Suppression of insulin secretion is a side effect of treatment with long-acting somatostatin analogs, and therefore may increase the risk for development of glucose intolerance. Pegvisomant, a GH receptor antagonist, has been shown to be effective therapy for normalization of IGF-1 levels with no detrimental effects on glucose metabolism and recent studies report that combination therapy with pegvisomant and long-acting somatostatin analog offers an additional benefit since tumor suppression is combined with GH receptor blockade (Lim et al. 2004). A study of the long-term efficacy and safety of combination therapy (long-acting somatostatin analog plus twice weekly pegvisomant) reported that IGF-1 levels normalized for all 32 patients; however, transient elevation in liver enzymes was observed in 11 patients, with a higher risk for patients diagnosed with diabetes mellitus. There is limited data on pegvisomant treatment in children, mostly case studies, which report successful outcomes.

Prolactinomas are the most common pituitary adenomas in older children, accounting for approximately 50% of pituitary adenomas, with the majority occurring in adolescence with a female preponderance (Jagannathan et al. 2007). Prolactinomas arise from acidophilic cells from the same embryonic lineage as somatotropes and thyrotropes. Prolactinomas may be seen in several inherited syndromes, including MEN 1, Carney complex, and familial isolated pituitary adenomas (Ciccarelli et al. 2005). A pituitary adenoma may be the first clinical manifestation of MEN 1, with the youngest reported case in a 5-year old boy with a pituitary somatomammotroph macroadenoma (Stratakis et al. 2000). Clinical presentation varies depending on the age and gender of the child, although growth arrest is typically seen in children and adolescents before ephiphyseal fusion is completed. Females may present with pubertal delay, amenorrhea, and other symptoms of hypogonadism. In males, macroprolactinomas are more frequent; accordingly, males with prolactinomas also have a higher incidence of neurological

and ophthalmological abnormalities (i.e., cranial nerve compression, headaches, vision loss), growth or pubertal arrest and other pituitary dysfunctions. Contrary to common belief, gynecomastia is not a finding in hyperprolactinemia. Since various factors such as neurogenic (emotional stress, nipple stimulation, chest wall lesions), pharmacological (phenothiazine, metoclopramide, centrally acting antihypertensive) or mechanical processes (craniopharyngiomas, Rathke cleft cyst, non-functioning adenoma, and infiltrative processes) can lead to loss of dopaminergic suppression of pituitary lactotrophs with hyperprolactinemia as a result, the differential diagnosis of hyperprolactinemia in children and adolescents is rather large (Lafferty and Chrousos 1999).

Pharmacological management with dopamine agonists (e.g. bromocriptine, pergolide, or cabergoline) is typically the first line of treatment for prolactinomas. The goals of treatment include the normalization of prolactin levels and pituitary function and the reduction of tumor size. Dopamine agonists are effective in reducing tumor size and controlling prolactin levels in approximately 80–90% of patients with microadenomas and about 70% of macroadenomas. Studies report that cabergoline, a selective D2 receptor agonist, is more effective and often better tolerated than bromocriptine. In addition, cabergoline has been shown to be effective in treatment of tumors resistant to other dopamine agonists (Schlechte 2003). In some cases treatment with dopaminergic agents can be withdrawn and prolactin levels will remain within normal limits.

Surgical intervention for prolactinomas is reserved for emergency situations such as acute threat to vision, hydrocephalus, or cerebral spinal fluid leak, or for rare tumors that grow despite exposure to increasing doses of dopamine agonists. Compliance is often a problem in long-term management of prolactimonas, since cessation of medical treatment leads to recurrence of hyperprolactinemia and tumor re-growth. At the initiation of therapy, commonly reported side effects of dopamine agonist treatment include nausea, dry mouth, dyspepsia, or dizziness. Treatment doses of 2.5–10 mg daily (bromocriptine) or 0.25–2 mg weekly (cabergoline) have not been associated with long-term adverse effects. However, recent reports of cardiac valve regurgitation in patients with Parkinson's disease treated with pergolide and cabergoline raised concern about the safety of long-term treatment with dopamine agonists. The safety of cabergoline was evaluated in a study of 1,200 patients with Parkinson's disease (controlled and uncontrolled studies) at doses of up to 11.5 mg/day, which exceed the maximum recommended dose for treatment of hyperprolactinemic disorders. The risk of cardiac valvular disease appeared to be higher in patients treated with at least 3 mg per day of cabergoline, a dose that is 10–20 times higher than the standard regimen for macroprolactinomas. Since the risk of long-term, low-dose treatment is unknown, discussion of potential risks of therapy with the patient and decision about the need for echocardiogram is advisable (Schlechte 2003).

Non-Functioning Adenomas

Non-functioning pituitary tumors in childhood and adolescence are rare; these tumors represent only 4–6% of pediatric cases while in series of adult patients, hormonally silent tumors account for approximately 33–50% of the total number of pituitary lesions (Lafferty and Chrousos 1999). Most silent adenomas arise from gonadotroph cells and often are macroadenomas at diagnosis; they occasionally grow and may present with headaches and visual disturbances, as well as deficient growth and/or pubertal delay (Lafferty and Chrousos 1999). Large adenomas may obstruct the foramen of Monro and cause hydrocephalus, while pituitary adenomas and sellar tumors that impinge on the optic apparatus and/or cavernous sinus can result in cranial nerve palsies, cavernous sinus syndromes, and/or additional visual disturbances. Non-functioning pituitary adenomas may present with GH deficiency (up to 75%), LH/FSH deficiency (~40%), or ACTH and TSH deficiency (~25%) (Lafferty and Chrousos 1999; Jagannathan et al. 2007).

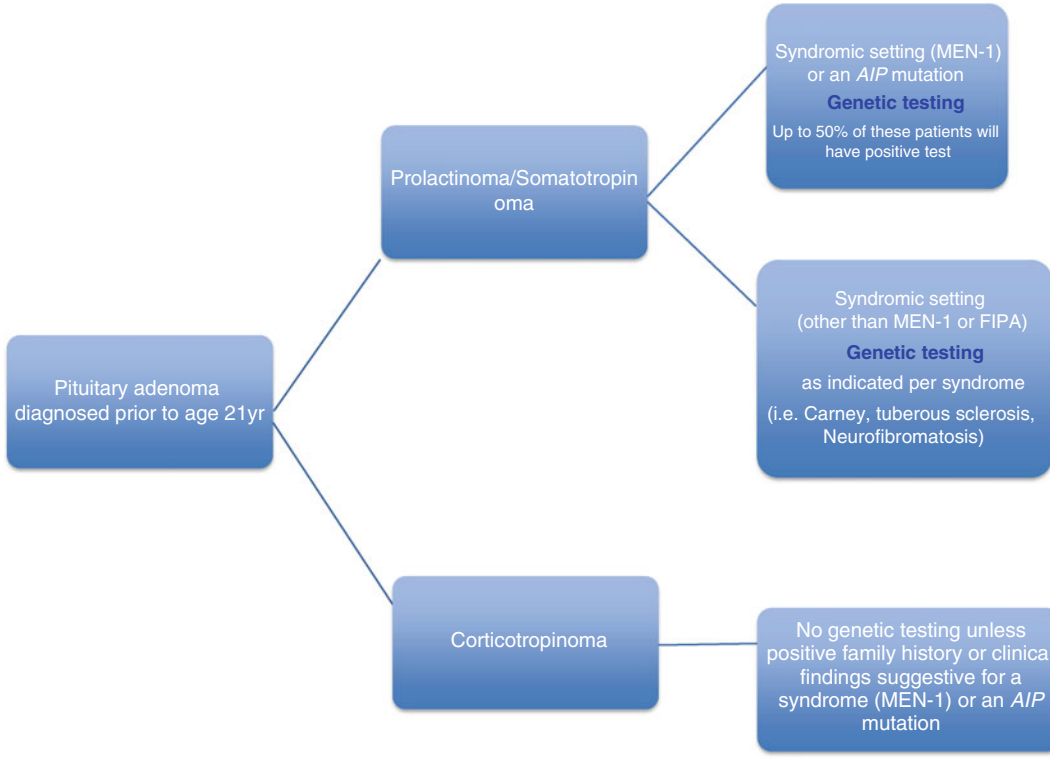
Compression of the pituitary stalk by pituitary adenoma has been reported but secondary hyperprolactinemia is typically seen in less than 20% of patients. DI is also rare (9–17%) but is more commonly seen in patients with Rathke's cleft cysts (Jagannathan et al. 2007). Recommendation for surgical excision of a hormonally silent intrasellar tumor or cyst depends on the tumor size, location, and potential for invasiveness (Freda et al. 2011).

Molecular Genetics of Pituitary Tumors

Genetic conditions associated with pituitary tumors include MEN 1, Carney complex, familial isolated pituitary adenomas (FIPA), and McCune-Albright syndrome. MEN1 is caused by germ line mutations in *menin*. Recently, a mutation in the *CDKN1B* gene (also known as p27 and KIP1) was reported to be associated with a MEN 1-like syndrome known as MEN 4 (MENX in rodents) in a rat disease model and human kindred. Genetic defects in one of the regulatory subunits of protein kinase A (PKA) (regulatory subunit type 1 alpha, *PRKAR1A*) causes Carney complex (Horvath et al. 2008). Vierimaa (2006) reported that inactivation mutations of the gene encoding aryl hydrocarbon receptor-interacting protein (AIP) were found in patients with pituitary tumors (predominantly acromegaly) in both sporadic and familial settings (Vierimaa et al. 2006). Somatic mutations on the adenylate cyclase-stimulating G alpha protein (GNAS complex locus, GNAS) are found in McCune-Albright syndrome (Stratakis et al. 2010). Familial growth hormone secreting pituitary adenomas may occur as an isolated autosomal dominant disorder (familial somatotropinoma) or as part of MEN 1 and Carney complex (Stratakis et al. 2010). McCune-Albright syndrome (MAS) is a genetic, but not inherited, disorder. A recent study of the prevalence of germline mutations in *MEN1*, *AIP*, *PRKAR1A*, *CDKN1B*, *and CDKN2C* reported that AIP or MEN1 mutations are frequent in pediatric patients with either GH- or PRL-secreting pituitary adenomas, however are rarely found in corticotropinomas (Stratakis et al. 2010). A proposed algorithm for genetic testing for pituitary adenomas in paediatrics is listed in Fig. 28.2 (Xekouki et al. 2010).

Carney complex(CNC), first described by Carney in the mid-1980s, is a rare autosomal dominant disorder that includes myxomas, lentigines, endocrine overactivity, and a variety of other tumors such as schwannomas and pituitary adenomas. In approximately 60% of the patients who meet diagnostic criteria, an inactivating mutation in the gene encoding *PRKAR1A* has been identified and a second, as yet uncharacterized locus at 2p16 has been implicated in some families (Carney et al. 1985; Stratakis et al. 2010). We recently reported that GH-producing tumors were identified in a cohort of adult patients (mean age 35.8 years) with clinical acromegaly (Boikos and Stratakis 2006). Acromegaly in CNC is characterized by a slow progressive course and aggressive pituitary tumors are not common. Of note, in many of these patients, clinically significant acromegaly did not present until after surgical treatment of their Cushing syndrome (72% of these patients were diagnosed with CS due to primary pigmented nodular adrenocortical disease). This change in clinical phenotype in patients with concurrent Cushing syndrome and acromegaly is not surprising given the known relationship between cortisol and growth hormone metabolism, but as phenomenon deserves further investigation in patients affected with CNC or similar conditions, such as McCune Albright syndrome (Boikos and Stratakis 2006).

For patients with CNC who have elevated GH and/or IGF-1, it is important to identify clinically significant acromegaly as defined by generally applied criteria (2004). Most CNC patients will have some abnormality of GH secretion due to the underlying pituitary hyperplasia, but almost all of them will have negative imaging studies (Boikos and Stratakis 2006). It is common practice to treat CNC patients with elevated IGF-1 levels with somatostatin analogues with the goal of normalizing IGF-1(Melmed 2011). For CNC patients with abnormal response to oral glucose



Adapted from: Xekouki P, Azevedo M, Stratakis, CA. Anterior pituitary adenomas: inherited syndromes, novel genes, and molecular pathways. 2010. Expert Rev Endocrinol Metab, 5, 697-709.

Fig. 28.2 Recommended algorithm for genetic testing in children with pituitary adenomas. *MEN-1* Multiple endocrine neoplasia type 1, *AIP* Aryl hydrocarbon-interacting protein

tolerance tests but normal IGF-1 levels and normal pituitary imaging, evaluations should be performed annually to assess for changes that may require treatment.

McCune Albright syndrome (MAS) is characterized by polyostotic fibrous dysplasia, café-au-lait pigmented lesions, endocrine abnormalities (precocious puberty, thyrotoxicosis, pituitary gigantism, and Cushings syndrome) and rarely by other tumors. MAS is caused by mosaicism for activating mutations of the *GNAS* gene. *GNAS* maps to chromosome 20q13 and encodes the ubiquitously expressed Gs-α subunit of the G protein. The phenotype of MAS, including hypersomatotropinemia, is due to the cellular response to the activation of adenyl cyclase signaling pathways. As mentioned above, *GNAS* mutations were also identified in sporadic GH-producing tumors. As seen with patients affected by CNC or carriers of *PRKAR1A* mutations, GH excess in MAS is frequently observed (approximately 20% of the patients) but pituitary tumors are not typically detectable by MRI (Stratakis et al. 2010).

Typical histological findings in pituitary glands of MAS patients are GH- and PRL-producing cell hyperplasia (Horvath et al. 2008); similar to what one sees in CNC pituitaries. Hypersomatotropinemia in MAS can be associated with significant morbidity due to exacerbation of polyostotic fibrous dysplasia in the presence of elevated GH levels. Treatment of GH- producing tumors in MAS with cabergoline has consistently shown an inadequate response, while long-acting octreotide has demonstrated an intermediate response. Recently, GH-receptor antagonists have been proposed as effective medical intervention for patients with inoperable MAS pituitary tumors or

hypersomatotropinemia without a visible tumor (Chanson et al. 2007).

MEN 1 is a genetic disorder inherited in an autosomal dominant manner and characterized by a predisposition to peptic ulcer disease and primary endocrine hyperactivity involving the pituitary, parathyroid, and pancreas. The disorder is due to inactivating mutations in the *menin* gene, which was identified in 1997. *Menin* is a tumor suppressor, which has been localized to chromosome 11q13. Several studies have reported that menin interacts with various proteins involved with transcriptional regulation, genome stability, cell division and proliferation (Marx et al. 1999). Pituitary adenomas occur in approximately 30–40% of patients with *menin* mutations (Asa and Ezzat 2002). The most common pituitary tumors are those secreting PRL (~60%) and GH (~20%), while ACTH-secreting and non-functional adenomas represent less than 15% of MEN 1-associated pituitary adenomas (Jagannathan et al. 2007; Marx et al. 1999). Data from studies in recently developed mouse models report similar frequency (~37%) of PRL-producing and other pituitary tumors in heterozygote mice with one *menin* allele inactivated (Bertolino et al. 2003). Although no genotype-phenotype correlation has been noted in *menin* mutation carriers, in familial MEN 1 the frequency of pituitary disease is significantly higher than in sporadic MEN 1 cases (Marx et al. 1999). In addition, in MEN 1 patients with pituitary adenoma and acromegaly, an increased female-to-male ratio has been reported for both familial and sporadic cases (Asa and Ezzat 2002).

Familial isolated pituitary adenoma (FIPA) is a clinical condition that refers to kindreds with two or more pituitary adenomas that are genetically negative for mutations in *menin* or *PRKAR1A*. Homogeneous mutations refer to similar pituitary tumor type occurring within the same family and heterogeneous mutations refer to families with two or more different tumor types (Beckers and Daly 2007). All pituitary tumor phenotypes have been reported in FIPA kindreds, and typically at least one prolactin- or GH-secreting adenoma is noted in each family. Recently, a genome-wide and DNA mapping study identified inactivating mutations in the gene that encodes aryl hydrocarbon receptor-interacting protein (AIP) gene on chromosome 11q13.3. In this series, combinations of somatotropinomas, mixed GH- and PRL-secreting adenomas, and prolactinomas were noted. Lack of functional AIP was shown by loss of heterozygosity in the tumor FIPA specimens. AIP mutations were reported in 15% of FIPA families and half of those with isolated familial somatotropinoma, which is a well-described clinical syndrome related only to patients with acrogigantism. Typically tumors in patients with AIP mutations are larger and diagnosed at a younger age than patients without AIP mutations or in sporadic tumors (Daly et al. 2010).A recent study of clinical characteristics and therapeutic response in 96 patients with Germ-line AIP mutations and pituitary adenomas, reported that somatotropinomas were most frequent presentation (almost 80%) and more than half of these tumors were co-secretors of GH and PRL, while prolactinomas accounted for 13.5, 7.3% nonsecreting (all tumors were macroadenomas), and a single *AIP-mut* TSH-secreting tumor. The predisposition for aggressive tumors in children and adolescents (often in a familial setting) highlights the importance of early detection to improve treatment outcomes (Daly et al. 2010).

In conclusion, early identification of pituitary tumors in children is necessary to avoid serious adverse effects on both physiological and cognitive outcomes as a result of pituitary hormone dysregulation during the critical periods of growth in childhood and adolescence. Treatment of rare disorders, such as pediatric pituitary tumors, requires a multidisciplinary team with expertise in the diagnosis, treatment, and long-term management to facilitate early diagnosis and treatment and reduce morbidity. The family of a child diagnosed with a pituitary tumor as part of a genetic syndrome should be offered genetic counselling and surveillance of family members as appropriate. As ongoing studies identify gene and protein expressions, mutations, and candidate genes important for the development and function of the anterior pituitary gland, this information will facilitate earlier diagnosis and provide opportunities to develop therapeutic targets.

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Pediatric Ependymomas: Role of Methylation

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Mariana Bohns Michalowski and Dominique Plantaz

Contents

Abstract

During the last 10 years, a new mechanism of tumor development has been described: the hypermethylation of tumor suppressor genes. These epigenetic modifications were rarely studied in childhood cancer and large series of patients didn't exist before 2003. Ependymomas (EP) represent the third most frequent type of central nervous system (CNS) tumor of childhood. No prognostic biological markers are available, and differentiation from other tumors may be difficult. The objective of describing a methylation profile in these cancers is to try to find a relationship between genes methylation and clinical evolution and to define new biological markers. The actual results from tumoral series indicate that hypermethylation of tumor suppressor genes may be important in the development and evolution of childhood cancers. Thus, these epigenetic alterations could be used as a marker of the disease and genes regulating methylation should be considered as possible novel therapeutic targets.

Introduction

Ependymomas, glial neoplasms that develop from ependymal cells lining the cerebral ventricles and the central canal of the spinal cord, are the third most frequent type of tumor of the central nervous system (CNS) in childhood, after astrocytomas and medulloblastomas.

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Most pediatric ependymomas develop in the ventricles, and they are difficult to differentiate from choroid plexus tumors. Ninety percent are intracranial and the majority is located in the posterior fossa. Histological WHO classification (Kleihues et al. 2002) defines as ependymomas the tumors that microscopically present the ependymal rosette or a perivascular pseudorosette, but they vary from a well-differentiated tumor with no anaplasia and little polymorphism to a highly cellular lesion with significant mitotic activity, anaplasia and necrosis. Although these tumors are frequently well circumscribed with calcification, hemorrhagic and cystic zones, they are also locally invasive.

The single most important prognostic factor is the extent of tumor resection. Tumors that were completely resected and those that have a residual disease of less than 1.5 cm^2 in postoperatory IRM have a better prognosis than those that were partially resected. Survival varies from 66 to 75% after a complete tumor resection and from 0 to 11% after a partial tumor resection. The younger children (<4 years of age) have frequently posterior fossa tumors that are more invasive and, consequently, more difficult to make a complete resection. The younger age is described as a marker of bad prognosis. Until today, no biological marker was identified as a diagnostic or prognostic factor in these tumors (Strother et al. 2002).

The treatment is based on surgery as described above. Radiation therapy seems to help to improve overall survival rates, but has serious undesirable late effects, mainly to the cognitive development of young children (Grill et al. 2003; Merchant et al. 2004). Although different types of polychemotherapy have been tried, no regimen has shown an unquestionable improve in survival (Strother et al 2002).

Furthermore, the pathogenesis and the genetic abnormalities of ependymomas are poorly characterized, and biological markers of prognostic or diagnostic value are not yet available. Multiple genomic imbalances have been described in cytogenetic and comparative genomic hybridization analyses, including partial or total loss of chromosomes (1p, 4q, 6q, 9, 10, 11, 13, 16, 17, 19q, 20q, and 22q). In 40% of childhood ependymomas, however, no chromosomal imbalances can be detected.

The most frequent alterations are loss of 22q and 6q. These anomalies are present in ~23 and ~15%, respectively, of all childhood ependymomas analyzed (Ransom et al. 1992; Grill et al. 2002; Jeuken et al. 2002; Dyer et al. 2002). In addition to point mutations or gene deletions, transcriptional repression by hypermethylation of promoter sequences is an alternative mechanism of inactivation of tumor-suppressor genes in cancer. Thus, tumors can present a mutation in one allele of a gene with the other allele hypermethylated, leading to its functional inactivation. This silencing can be partially relieved by demethylation of the promoter region.

Cytogenetics and Ependymoma

Recently, many different research groups are studying genetic anomalies in ependymomas aiming to find a specific biological marker. Multiple genomic anomalies have been described as partial or complete chromosomal losses (1p, 4q, 6q, 9, 10, 11, 13, 16, 17, 19q, 20q, and 22q), but 40% of childhood ependymomas have no chromosomal alteration (Reardon et al. 1999; Ward et al. 2001). The most frequent chromosomal anomalies are the partial loss of the long arm of the chromosome 22 and 6. These alterations are present in 23 and 15% of these tumors, respectively (Zheng et al. 2000; Grill et al. 2002). These findings suggest the presence of tumor suppressor genes in theses chromosomes. Another genetic anomaly that was described is the gain of 1q in 20% of ependymomas (Ward et al. 2001). All these studies also demonstrated that genetic alterations are different in children ependymomas when compared to adults ependymomas and that intracranial tumors are genetically different from spinal tumors (Jeuken et al. 2002). We will start describing what is actually known about cytogenetic alterations in chromosome 22 and 6 in ependymomas before explaining epigenetic alterations and research in this matter.

Chromosome 22

Ependymomas are more frequent in Neurofibromatosis 2 patients (NF2), a hereditary disease characterized by the development of bilateral vestibular schwannomas and meningiomas (Martuza and Eldrige 1988; Rubio et al. 1994). NF2 gene, that is located on chromosome 22, is mutated in a high percentage of adult meningiomas and schwannomas. NF2 could be an important gene in childhood ependymomas genesis. Ebert et al. (1999) observed a frequent deletion of chromosome 22 in spinal ependymomas related to a NF2 gene mutation. On the other hand, in intracranial ependymoma the 22q deletion was not related to this mutation. This author suggests that another tumor suppressor gene located in this region (INI1/hSNF5/SMARCB1) could be responsible for carcinogenesis in these tumors. This observation has been confirmed by others (Carter et al. 2002).

Results about INI1 and ependymomas were highly attended since this gene is located in chromosome 22q, and mutated in pediatric rhabdoid tumors (Versteege et al. 1998). Kraus et al. (2001) studied a series of 53 ependymomas and found no homozygous mutation or deletion in INI1. They concluded that INI1 has no role in ependymoma pathogenesis but they also described once more the high frequency of 22q losses, mainly in spinal tumors (Kraus et al. 2001).

Chromosome 6

Huang et al. in 2003 showed that the most frequent LOH in ependymomas was located on 6q (30.3% of tumors presented a partial or complete deletion of the long arm of chromosome 6) (Huang et al. 2003). Before him, Reardon et al. (1999) showed that deletions and variable rearrangements in 6q were more frequent than those observed in chromosome 22. These deletions and rearrangements on 6q had been previously described in some sort of gliomas, primitive neuroectodermal tumors (PNET) and acute leukemia. Besides that, punctual deletions on 6q22 and 6q23-q24 were correlated to malignant progression of endocrine tumors of the pancreas. In a study by Huang et al (2003) the locus that was most frequently deleted was 6q24-25.3, but these anomalies were also detected at 6q15-16 and 6q21-22.1. This region presents many genes that have already been cloned as, for example, IGF2R, MAP3K5, and ZAC. Independently of the deletion target gene in this region, it seems to play an important role in ependymoma pathogenesis.

Epigenetics and Ependymoma

When we started studying epigenetic alteration in children neoplasias, there was no published study on DNA methylation and ependymomas. Alonso et al. (2003) were the first to suggest that hypermethylation could play an important role in the development of many different cerebral tumors including seven ependymomas. Further, they confirmed this observation in a series of 27 ependymomas (Alonso et al. 2004) and found more than 20% methylation in MGMT, TIMP3, THBS1 and TP73. NF2 and Caspase 8 was less than 10% methylated and RB1 and p16INK4a were methylated in 4 and 18%, respectively. In this series of tumors, 14 patients were adults and 13 were children. No significant difference was found between the two groups. Later, some groups showed a relationship between tumor localization, patient age and methylation profile. As shown in Fig. 29.1, Rousseau et al (2003) observed a significant difference in CDKN2A promoter gene methylation between adult and pediatric tumors ($p = 0.043$ CDKN2A) (Fig. 29.1) (Rousseau et al. 2003).

Hamilton et al. (2005) published the methylation status of 9 different genes (RASSF1A, CASP8, MGMT, RB1, p14 (ARF), p15 (INK4B), p16 (INK4A), TP73 and TIMP3) in a small serie of 20 ependymomas where 11 were from children. RASSF1A were frequently methylated (85%); CASP8, MGMT and TP73 a little bit less methylated and no methylation was detected to p14, p15, p16, Rb1 and TIMP3. There was a trend toward an association between CASP8 methylation and histology.

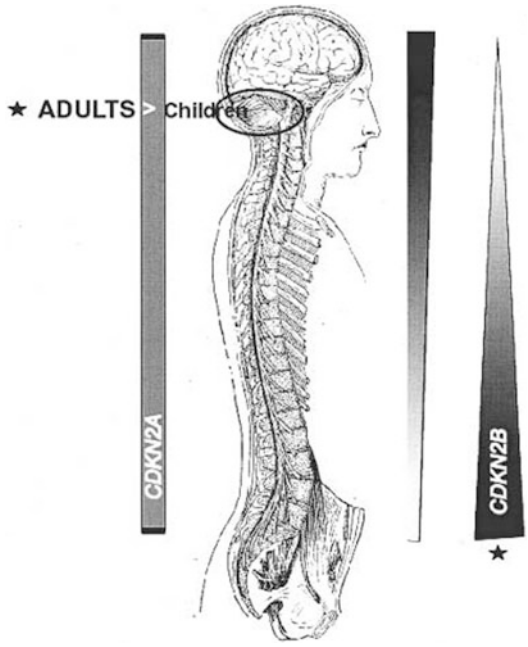

Fig. 29.1 Differences in ependymoma methylation profile. There was no craniocaudal difference in CDKN2A methylation, but adult cerebral tumors were more frequently methylated than pediatric tumors. *$p < 0.05$ (X^2) Rousseau et al. 2003

From 2002 until 2006, we analyzed a total of 19 genes in childhood ependymomas: 9 well-characterized tumor suppressor genes involved in cell-cycle regulation (p15 or p15INK4a, p16 or p16INK4a, p14 or p14ARF, APC, RB1, RASSF1A, BLU, FHIT, RARB), one DNA repair gene (MGMT), two genes related to metastasis and invasion (DAPK, ECAD), three tumor necrosis factor-related apoptosis- inducing ligand (TRAIL) pathway genes (CASP8 and the receptors TNFRSF10C and TNFRSF10D), and one more, the negative regulator CASP8 (FLIP). These genes were chosen because they were previously described as methylated in oligodendrogliomas and ependymomas, as well as in other adult cancers. A more specific study on genes located on chromosome 22 (INI1, TIMP3, NF2) was also performed, because, as described above, loss of this chromosome is the most frequently described abnormality in ependymomas, suggesting that one or more genes located in this region may be related to genesis of this tumor (Michalowski et al. 2006). Hypermethylation of RASSF1A and hypermethylation of the genes implicated in the TRAIL pathway were found to be very frequent abnormalities in both ependymomas and benign plexus choroid tumors. Here we will discuss the implications of this and related findings for the diagnosis and prognosis of ependymomas.

To our knowledge, this was the largest study of hypermethylation of tumor suppressor genes in intracranial childhood ependymoma, and the first study to compare an ependymoma group with a benign tumor group in this context. Choroid plexus papillomas are rare benign intracranial neoplasms of the choroid plexus, a neuroepithelial-lined papillary projection of the ventricular ependyma. In the pediatric age group, papillomas are not easily differentiated from ependymomas, because they have similar localizations. The former occurs most commonly in the lateral ventricle (67%), followed by the fourth ventricle (19%), with a lesser number presenting primarily in the third ventricle (14%). Several studies have now reported methylation profiles in different tumor types as proof of principle for cancer risk assessment, early cancer diagnosis and prognosis, and response to chemotherapy.

The RASSF1A gene located at 3p21.3 was the most frequently hypermethylated in the ependymoma series (56%). We observed a comparable percentage of methylation of this gene in benign tumors (66%). The TRAIL pathway genes (CASP8, TFRSF10C, and TFRSF10D) were also frequently methylated in ependymomas (30, 9.5, and 36.4%, respectively) and in choroid plexus papillomas (50, 50, and 16.7%, respectively). In the literature, the study by Alonso et al. described only one methylation of the CASP8 gene amongst the 27 ependymomas tested. The differences seen are probably due to the heterogeneous populations studied and histological differences (ependymomas of WHO grade II versus III; frozen tissue as opposed to paraffin-embedded tumors). It should be noted that the genes that were methylated in both groups of tumors are mainly associated with apoptosis. This finding may be explained by the regulatory role of these genes in cellular replication and cellular death control in the development

of both benign and malignant tumors. There was no methylation of genes associated with invasion and metastases in benign tumors. Genetic hypermethylation in benign tumors or normal tissues has rarely been described.

FHIT was methylated in 22% of the ependymomas studied and in none of the choroid plexus papillomas. As can be seen from the literature, FHIT is inactivated in 60% of human tumors (range 20–100%, depending on tumor type); this figure makes FHIT the most commonly altered gene in human cancer. Several recent studies have provided support for the tumor-suppressor activity of FHIT. The exact molecular mechanism or functional pathway of FHIT action is still unknown, but it may play a role in the early stages of cancer development.

The RARB and BLU genes were methylated in 14.8 and 13.6%, respectively, of all ependymomas tested and in none of the choroid plexus papillomas. These methylations were described mainly in adult cancers such as breast cancer and gliomas, which implicates RASSF1A and BLU promoter methylation in the pathogenesis of adult gliomas. Loss of expression of the p16 gene, often associated with aberrant methylation, was observed not only in astrocytic tumors but also in oligodendroglial tumors. Note that only 3 out of 27 childhood ependymomas showed a hypermethylation of this gene (11%). This result is comparable to some data available in the literature, but discordant with other data that describe no methylation of the gene p16 in posterior fossa ependymomas grade II WHO in children. In the latter study by Rousseau et al. (2003) p 14 and p 15 were methylated in ~10 and ~20% of the pediatric posterior fossa ependymomas. These differences may be due to histological differences (different pathology center, paraffin versus frozen tissue) or simply because series of childhood intracranial ependymomas are still too small and limited to allow a complete view of the complex epigenetic anomalies.

Because chromosome 22 has been described as the one most often deleted in childhood ependymomas, we supposed that this region contains tumor suppressor genes involved in ependymomas formation. The INI1 gene, located on chromosome segment 22q11.2, has been implicated in transcriptional activation and repression. It is mutated in early childhood rhabdoid tumors and in various malignancies of the central nervous system. The NF2 gene, located at 22q12, is frequently inactivated in sporadic cord forms and several studies have investigated the possibility that the NF2 gene may be the candidate tumor suppressor in ependymomas. The hypermethylation of this gene has been observed in 7% of ependymomas. We also tested TIMP3, which is an angiogenesis inhibitor that has been found to be methylated in 33% of ependymomas. No hypermethylation was detected in these three genes, all of which are located on chromosome 22. In our series, only 5 of the 27 ependymomas showed a 22q deletion. DAPK, MGMT, and APC were rarely methylated in our series and p14, p15, FLIP, and RB1 were never methylated. We observed some differences between our results and the results obtained previously for adult ependymomas, mainly for MGMT, DAPK, and p14 genes.

This may suggest that childhood ependymomas and tumors with different localization represent different entities with different methylation profiles. We did not find a statistically significant difference between gene methylation in ependymomas and choroid plexus papillomas, although some genes were never methylated in the benign tumor group. This is probably due to the small number (seven) of tumors studied in the benign tumor group (Michalowski et al. 2006). In addition, we were unable to evaluate whether methylation and deletion are coexistent, as proposed by Knudson, because only a small sample of our tumors presented a 22q deletion and no tumor showed methylation of 22q genes. This analysis also suggests that a low percentage of ependymomas are hypermethylated at the MGMT promoter. We could not establish a relationship between this methylation and response to chemotherapy because the only patient who was MGMT hypermethylated presented a very rapid tumor progression. Clinical trials with MGMT modulating agents may have a role in the treatment of these tumors and, although a relationship between hypermethylation and clinical presentation and evolution was not established, the epigenetic

alterations could be used in the future for differential diagnosis and detection of tumor regrowth (e.g., by a methylation-based molecular diagnostic assay in the cerebrospinal fluid).

In conclusion, recent studies tried to define a methylation profile in childhood intracranial ependymomas. We observed that the methylation pattern seems not to be randomly assigned in ependymomas, and may represent a mechanism of tumor development and evolution. Tracing the methylation profile offers the advantages of early detection, differential diagnosis, and detection of tumor regrowth by a methylation-based molecular diagnostic assay. In future studies, it will be of interest to test different genes, located in commonly deleted regions, in larger and comparative series.

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Pediatric Intramedullary Cavernoma: Surgical Treatment

30

Erwin Cornips and Mariel Ter Laak-Poort

Contents

Abstract

The natural history of pediatric intramedullary cavernomas (ImCs) is essentially unknown, however, they tend to present at a mean age between 9.1 and 13.2 years with an acute neurological deficit, often followed by rapid deterioration due to hematomyelia. Imaging of the entire neuraxis is warranted, as those affected by multiple lesions and the familial form of the disease (12–40%) tend to suffer a more aggressive behaviour and earlier age of clinical onset. The cumulative literature is inconclusive with regard to indications for surgical treatment, thus supporting the concept of highly individualized decision making. However, considering the lifetime risks in children, surgical treatment to prevent future hemorrhage should strongly be considered, at least for those lesions that are surgically accessible. Except for the very young child, most authors would favor the use of intraoperative neuromonitoring during resection using either a posterior midline, dorsal root entry zone, or lateral myelotomy approach. With adequate surgical treatment either in the acute phase or after clinical recuperation, prognosis may be surprisingly good. Incompletely resected lesions and/or syndromal cases tend to recur, requiring further treatment. Definite answers await more cases with longer follow-up especially after

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subtotal resection, however, as long as the child remains in a stable condition, careful clinical and radiological follow-up may be an alternative to reoperation.

Introduction

Cavernomas are well-circumscribed, mulberry-like vascular lesions composed of thin-walled sinusoidal channels. They have neither a large arterial supply, nor a significant venous drainage. They may be found throughout the body, including the central nervous system (CNS), but only rarely occur within the spinal cord (5%) with diameters ranging from just a few millimeters to several centimeters (Cornips et al. 2010; Khalatbari et al. 2011; Nagib and O'Fallon 2002). The absence of intervening neural tissue and the presence of surrounding gliosis make cavernomas, even in eloquent areas such as the spinal cord, more amenable to resection. Appropriate management of intramedullary cavernomas (ImCs), however, requires a thorough knowledge of their clinical characteristics, including their natural history, as well as the macroscopic and microscopic anatomy of the spine and spinal cord, including those corridors that allow a complete and safe removal (Mithka et al. 2011). Moreover, it appears that ImCs – like their cerebral counterparts - behave differently in children as compared to adults (Acciarri et al. 2009; Cornips et al. 2010; Nagib and O'Fallon 2002; Noudel et al. 2008; Santoro et al. 2007; Xia et al. 2009). Confronted with an ImC in a child, due to the rarity of the condition, the attending neurosurgeon until recently did not find any guidelines in the literature. Consequently, he had to decide if and when to operate on a case-by-case basis, based on the child's clinical presentation, MR findings, and his or her personal preference. Fortunately, substantial progress has been made in recent years (Acciarri et al. 2009; Cornips et al. 2010; Gross et al. 2010; Liang et al. 2011; Nagib and O'Fallon 2002; Noudel et al. 2008; Toldo et al. 2009; Xia et al. 2009) as we will now discuss.

Epidemiology and Pathogenesis

ImCs account for only 5% of all central nervous system cavernomas,and for 5–12% of all spinal cord vascular lesions (Mithka et al. 2011), however, their true incidence in the general population is essentially unknown (Liang et al. 2011). As many as 12–40% of patients and especially children and young adults with ImCs also have intracranial lesions (familial and syndromal cases) (Gross et al. 2010; Mithka et al. 2011), justifying imaging of the entire neuraxis in all (Cornips et al. 2010). ImCs are especially rare in children, in whom they represent less than 1% of all intramedullary lesions, occurring equally in the cervical and thoracic regions. As such, less than 30 symptomatic pediatric cases have been described in the English literature to date (Cornips et al. 2010; Khalatbari et al. 2011). Remarkably, these predominantly occurred in boys (66%) (Cornips et al. 2010), as compared to a more equal gender distribution in adults according to a recent meta-analysis of 352 patients (173 males, 175 females) (Gross et al. 2010).

The pathogenesis of cavernomas is incompletely understood, as both congenital and de novo formation have been documented in the brain as well as in the spinal cord. Genetic loci of chromosomes 7q, 7p, and 3q, representing the expression of proteins CCM1 (KRIT1), CCM2 (MGC4607, malcavernin), and CCM3 (programmed cell death 10), respectively, are associated with the development of familial cavernomas, the so-called inherited cerebral cavernous malformation disorder. This disorder (as opposed to the more common sporadic form) is transmitted in an autosomal dominant fashion with incomplete penetrance and variable expression (Acciarri et al. 2009; Cornips et al. 2010; Khalatbari et al. 2011; Toldo et al. 2009). These proteins supposedly play a role in signalling pathways that contribute to vascular development. Affected patients may have cavernomas at different sites besides brain and spinal cord, including retina, liver, skin, and vertebrae (Toldo et al. 2009). Other genetic syndromes such as Klippel-Trenaunay-Weber syndrome have been associated with the development of ImCs (Mithka et al. 2011).

Natural History and Clinical Presentation

The natural history of ImCs is unknown because their incidence in the population in general and the pediatric population in particular is not well documented (Cornips et al. 2010; Kharkar et al. 2007; Mithka et al. 2011). The potential that hemorrhage (intralesional or more diffuse (hematomyelia)) may cause significant morbidity, however, is likely higher for ImCs than for their cerebral counterparts, because of the confined parenchymatous space in which they are located (Cornips et al. 2010). The bleeding risk is estimated at 1.4–4.5% per lesion per year for ImCs (Liang et al. 2011; Nagib and O'Fallon 2002; Noudel et al. 2008), as compared to 0.7–1.3% for cerebral cavernomas, and 2.7% for brainstem cavernomas. Nevertheless, in a recent study of ten patients with a conservatively managed symptomatic ImC the authors did not observe a significant, permanent neurological decline during a mean follow-up of 80 months (Kharkar et al. 2007). It is important, however, to realize that all but one patient in this study were adults, who may have a different clinical course (as outlined below), and that the study design was retrospective and therefore non-randomized. As such, these conservatively managed patients had mainly presented with paresthesias whereas those patients who were surgically managed had mainly presented with pain and paresis. The authors carefully conclude that they may have identified a distinct subgroup of patients with symptomatic ImC that may have a much lower risk of rehemorrhage.

The clinical presentation of ImCs in general is quite variable, often including both sensory and motor symptoms, resulting in central pain and paresis (Cornips et al. 2010). This variability may be understood by the dynamic nature of these lesions, as intra- and perilesional hemorrhage, thrombosis, gliosis, organization, hyalinization, wall thickening, calcification, cyst formation, and even involution of the caverns may all contribute to the observed changes over time (Gross et al. 2010; Khalatbari et al. 2011; Santoro et al. 2007). The following patterns have been reported: first, a pattern of discrete and acute episodes of neurological deterioration over months or even years, separated by various degrees of recovery, likely caused by episodic intralesional hemorrhage, thrombosis, or both. Second, a slow, progressive decline in neurological function, likely caused by repeated small hemorrhages that cause progressive hyalinization of the vascular walls and/or thrombosis leading to progressive enlargement of the lesion. Third, the acute onset of symptoms followed by rapid neurological decline, most likely caused by hemorrhage within the spinal cord parenchyma (hematomyelia). Fourth, the acute onset of mild symptoms followed by a gradually progressive decline in neurological function over weeks to months, caused by hemorrhage within the lesion followed by thrombosis or changes in the surrounding microcirculation. Fifth, the acute onset of back pain, with or without neurological deficits, caused by subarachnoid hemorrhage from a cavernoma located on the surface of the spinal cord (Mithka et al. 2011).

The clinical presentation of ImCs in children, however, for some reason seems to be less variable, as almost all reported cases presented with an acute, more or less severe neurological deficit, often followed by rapid deterioration due to hematomyelia (type 3) (Cornips et al. 2010; Santoro et al. 2007). The mean age children present with hematomyelia reportedly varies between 9.1 and 13.2 years (Cornips et al. 2010), which makes some authors suggest that hormonal factors may influence the onset of cavernomas in puberty (Noudel et al. 2008). Children affected by multiple lesions and the familial form of the disease tend to suffer a more aggressive behaviour with an earlier age of clinical onset (Acciarri et al. 2009).

Indications and Timing for Surgery

Indications for surgical treatment of ImCs in the general population include symptomatic, surgically accessible lesions in patients who are medically fit. Surgically inaccessible, incidental, or mildy symptomatic lesions may be followed up by serial clinical examination and MR imaging, including those lesions observed in patients with hereditary forms of the disease. Resection may

be considered in case they progressively enlarge, or in case the patient experiences one or more symptomatic hemorrhages (Mithka et al. 2011). Thus, for accessible symptomatic ImCs surgical treatment is recommended, whereas for accessible asymptomatic ImCs surgical treatment is recommended by some (Nagib and O'Fallon 2002) based on a presumed relatively high incidence of spontaneous hemorrhage and relatively low risk of surgery, but not recommended by others (Jallo et al. 2006; Kharkar et al. 2007) who would prefer regular clinical and radiological follow-up. Moreover, for deep symptomatic ImCs surgical treatment would be recommended at least by some authors in spite of the significantly higher surgical risks involved (Nagib and O'Fallon 2002), whereas for deep asymptomatic ImCs all would agree with regular clinical and radiological follow-up. Finally, for multiple ImCs, only the symptomatic lesion is usually targeted (Khalatbari et al. 2011).

Clearly, the cumulative literature is inconclusive with regard to indications for surgical treatment of ImCs, thus supporting the concept of highly individualized decision making. However, when the affected individual is a child, and the lesion surgically accessible, we share the opinion of many others that surgical treatment to prevent future hemorrhage should strongly be considered, because of the more frequent acute, severe neurological deficits, and lifetime risks in children as compared to adults (Acciarri et al. 2009; Cornips et al. 2010; Khalatbari et al. 2011; Noudel et al. 2008; Xia et al. 2009). There simply are no data to guarantee safe, conservative therapy over a child's lifetime, yet there are data from several recent studies that allow to estimate the surgical risks (Acciarri et al. 2009; Cornips et al. 2010; Khalatbari et al. 2011; Nagib and O'Fallon 2002; Noudel et al. 2008; Xia et al. 2009).

Timing for surgical treatment in a patient with a symptomatic ImC remains controversial. Some authors argue that symptomatic hemorrhage should prompt urgent resection in order to prevent recurrent hemorrhage and subsequent neurological deterioration. Hemorrhage outside the boundaries of the cavernoma arguably increases the surgical accessibility of the lesion, and facilitates resection in the acute setting (Mithka et al. 2011). Other authors argue that immediate operation in case of severe cord swelling could be harmful for an already stressed spinal cord. As long as the neurological condition of the patient is not declining, postponing surgery for 4–6 weeks or even up to 3 months will be helpful for resolving the hematoma, diminishing cord swelling and, as a result, creating a discrete border (gliotic plane) on the lesion itself (Choi et al. 2011; Cornips et al. 2010; Liang et al. 2011).

Most authors would agree that progressive neurological decline, that may indicate a steep pressure rise inside the spinal cord and imminent infarction, should prompt urgent resection. On the other hand, patients with mild/non-progressive symptoms may be followed up clinically to allow potential recovery (often necessitating physical rehabilitation) before surgery (Cornips et al. 2010; Mithka et al. 2011). Importantly, symptomatic yet otherwise neurologically intact patients with surgically accessible lesions should fully understand the potential complications of surgery, including transient or even permanent neurological injury (Mithka et al. 2011). The surgical management of spinal intradural-extramedullary and spinal epidural cavernous malformations, both very rare in children (Acciarri et al. 2009; Sarikaya-Seiwert et al. 2010), is beyond the scope of this chapter.

Surgical Approach and Technique

Resection is the only definitive treatment for symptomatic ImCs, but entails the risks of a general anesthetic, infection, hemorrhage, pain, instability, spinal cord tethering, cerebrospinal fluid leakage, and of course transient or even permanent neurological injury due to the high density of eloquent tissue within the spinal cord (Mithka et al. 2011). These deficits depend on the location of the myelotomy, the depth and size of the cavernoma, the skills of and instruments used by the attending neurosurgeon, as

well as the preoperative neurological status of the patient. Thus, the surgeon must carefully weigh the natural history of ImCs in a particular patient against the risks of surgery as previously outlined. Despite these risks, surgery remains the definitive treatment for symptomatic lesions, preferably using an en bloc resection technique to minimize the chance of leaving any residual that may lead to further hemorrhage (Santoro et al. 2007).

The surgical approach should be carefully chosen depending on the exact location of the cavernoma inside the spinal cord. An anterior approach may significantly compromise spinal cord vascularisation (to the point of an eventual infarction) and should therefore be avoided. The most common and safest approach to ImCs is through a posterior midline myelotomy, well suited for posterior or centrally located lesions. Alternatively, an approach through the dorsal root entry zone may be used for lesions situated posterolaterally in the spinal cord that do not reach the posterior or lateral surfaces. Finally, an approach between the ventral and dorsal nerve roots may be used for lesions situated laterally or anterolaterally in the spinal cord that do not reach the surface. The latter approach typically requires a more extensive bony removal (drilling one or more ipsilateral facet joints and pedicles) and gentle rotation of the spinal cord after cutting the dentate ligaments (Fig. 30.1a–c, used with permission from Barrow Neurological Institute).

The vertebral level should be carefully localised, using preoperative MRI (preferably in prone position), intraoperative X-ray (lateral projection), or other measures, especially when the cavernoma is situated in the thoracic spinal cord. The laminae and spinous processes are removed en bloc using a high-speed drill with low-profile (pediatric) footplate, and kept under traction to prevent shortening of the ligaments by the time they are replaced. This laminoplastic technique may reduce the incidence of postoperative kyphoscoliosis. After bony removal, intraoperative ultrasound may be very useful to exactly localise the lesion prior to myelotomy, especially for those lesions that are not immediately apparent at the (posterior) surface of the spinal cord (Fig. 30.2). In this regard, the surgeon should

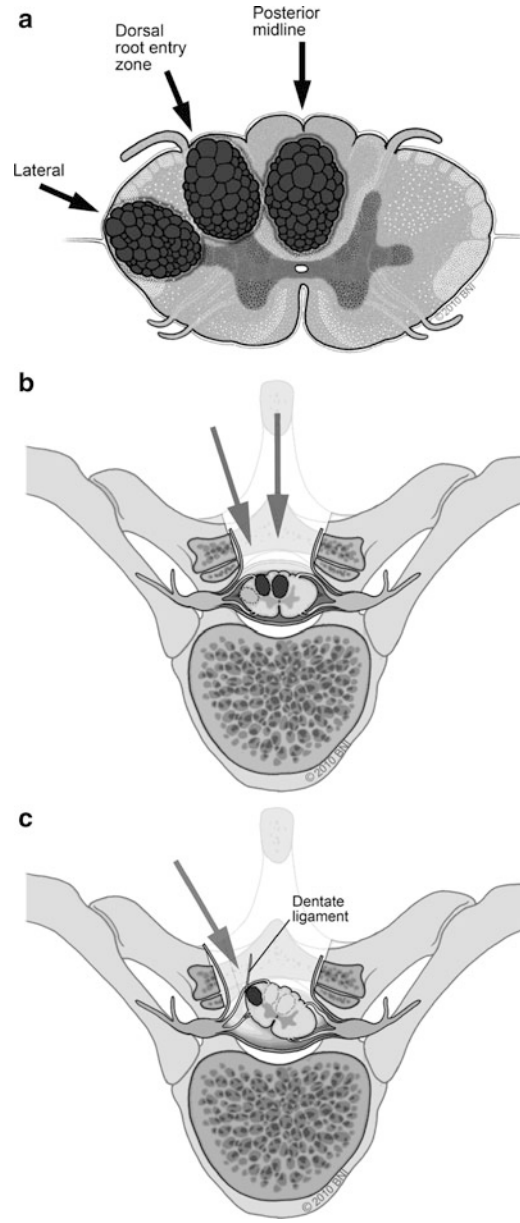


Fig. 30.1 Axial illustration of the thoracic spinal cord, demonstrating three possible myelotomy sites to approach an ImC (**a**). A myelotomy at the posterior midline or at the dorsal root entry zone requires standard laminectomy (**b**), whereas a lateral myelotomy between the ventral and dorsal nerve roots requires additional ipsilateral bone removal (**c**) (Reprinted with permission from Barrow Neurological Institute)

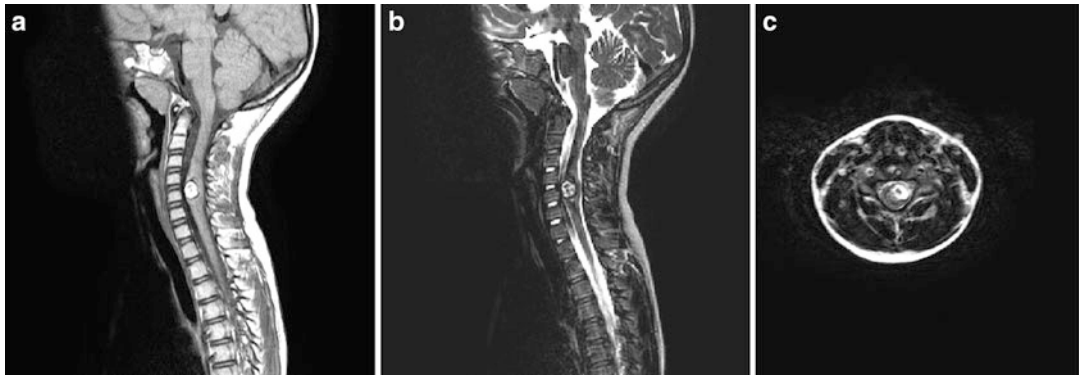
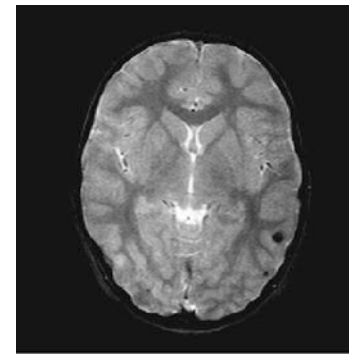
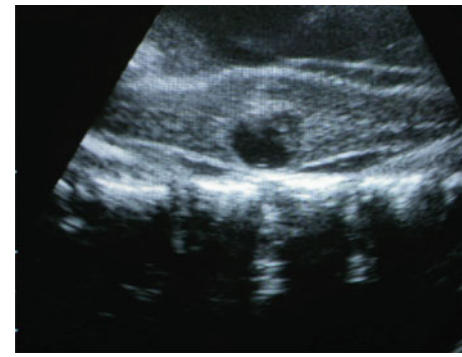


Fig. 30.2 C4-C5 left anterolateral intramedullary cavernoma with signs of recent hemorrhage (sagittal T1 (**a**), sagittal T2 (**b**), and axial T2 (**c**) images). The lesion was removed after substantial clinical recuperation 3 months later, using an approach between the dorsal and ventral nerve roots

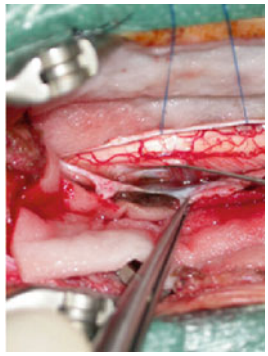
realize that preoperative MR imaging is imperfect in defining whether a lesion is superficial or deep, with as many as 17.6% false positives (Gross et al. 2010). Moreover, there may be substantial movement of the thecal sac and spinal cord in relation to the vertebral column in different body positions. Subsequently, the spinal cord is sharply incised over the entire length of the lesion using an ophthalmologist knife. Once the cavernoma is reached, a plane between the lesion and the surrounding yellow-brown, hemosiderin-stained tissue (that should not be resected) is sought, and the lesion carefully resected using microsurgical techniques, avoiding any unnecessary retraction. Similar to their cerebral counterparts, any associated venous anomaly inside the spinal cord close to the cavernoma should be preserved, as it may be draining normal cord tissue (Vishteh et al. 1997). The significance of these so-called cryptic venous malformations, however, has been questioned by others (Nagib and O'Fallon 2002). Once the lesion has been removed, the operative bed should be carefully inspected to exclude an eventual remnant, that may lead to a clinical recurrence. In fact, the incidence of rebleeding may be as high as 17.6% (Vishteh et al. 1997), and some authors would even advocate the use of ultrasonography to rule out any remnant before closure (Santoro et al. 2007).



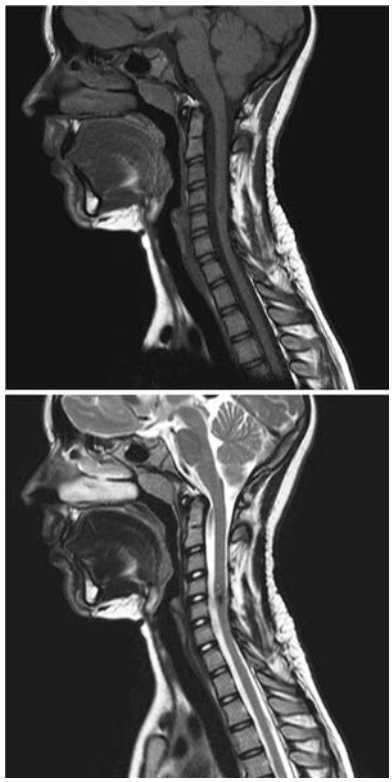
Cerebral T2 FFE image showing several intracranial cavernomas



Intraoperative ultrasound used to exactly localise the lesion



Intraoperative image showing slight bluish discoloration where the lesion reaches the lateral cord surface



Sagittal T1 and T2 images 5 years postoperatively. The child has a moderate residual left hemiparesis

Intraoperative Neuromonitoring

Although some authors would question the value of time-consuming intraoperative neuromonitoring, especially when dealing with ImCs that are always surrounded by a thin layer of hemosiderin laden gliosis that protects the adjacent spinal cord tissue (Noudel et al. 2008), most authors would favor intraoperative neuromonitoring (Nagib and O'Fallon 2002; Liang et al. 2011) that has been established as an essential adjunct to spinal cord surgery in recent years (Sala et al. 2002). Intraoperative SSEPs and MEPs provide information on intraoperative injury to the spinal sensory and motor pathways respectively. This information combined with other signals may help the surgeon determine whether a specific maneuver is potentially harmful to the spinal cord in general and motor tract function in particular. The recording of muscle-MEPs with transcranial electrical stimulation provides the most reliable tool for detection and especially prevention of intraoperative injury to the spinal motor pathways. Combining these muscle-MEPs with D-wave recording makes the technique even more sensitive and reliable (Kothbauer 2007).Ideally, muscle MEPs and D-waves should be monitored in every intramedullary tumor resection. However, if D-waves are not available, combined SSEP and MEP monitoring should be performed because together they cover both somatosensory and motor pathways and provide a higher sensitivity, positive predictive value, and negative predictive value than either modality on its own (Mithka et al. 2011). Finally, intraoperative neuromonitoring also serves the neuroanesthesiologist, reminding him or her to keep mean arterial blood pressure at a sufficiently high level, which is of utmost importance when exploring the delicate substance of the spinal cord.

Postoperative Outcome and Long-Term Follow-up

The outcome after surgical intervention depends on depth and size of the lesion, location of the myelotomy, preoperative neurological status (probably the single most important clinical factor related to outcome) (Steiger et al. 2010), and last but not least on institutional and surgeon experience. With appropriate patient selection, careful surgical planning, intraoperative neuromonitoring,

and state-of-the-art anesthesiological and surgical technique, the outcome may be surprisingly good, with long-term neurological improvement rates as high as 46–58% in the general population (Mithka et al. 2011).

The immediate outcome after surgical intervention may be transient worsening of sensorimotor symptoms in approximately one-third of patients (Gross et al. 2010). However, in a recent series including 81 surgically treated patients (of whom 9 below 18 years old), such worsening was observed in only five patients (6%), including four patients who were operated before the acquisition of intraoperative neuromonitoring. The large majority of patients remained in unchanged clinical condition (n = 75, 93%), and one patient experienced an immediate improvement (1%) (Liang et al. 2011). The long-term outcome after surgical intervention according to a recent meta-analysis of 352 adult patients would be 61% improvement, 27% unchanged, and 12% worsening as compared to their preoperative status (Gross et al. 2010).

Although surgical intervention dramatically reduces continuing neurological decline due to rehemorrhage, subtotally resected lesions and/or syndromal cases may recur, requiring further treatment (Cornips et al. 2010). Little if anything is found in the literature regarding the difficult dilemma between follow-up or reoperation in case of residual cavernoma, especially in the setting of multiple cavernomas in children who have a long life expectancy, and are otherwise doing fine (Cornips et al. 2010). It is generally believed subtotally resected lesions and/or syndromal cases tend to recur (Nagib and O'Fallon 2002). In this regard, it is important to realize that especially early postoperative MR images may be misleading and therefore misinterpreted, as blood products and low-intensity signals of postoperative scar tissue may be misdiagnosed for residual cavernoma. Therefore, comparison with MR images at least 6 months later is strongly recommended (Gross et al. 2010; Noudel et al. 2008).

Long-term complications beside symptomatic rehemorrhage include spinal cord tethering and spinal deformity. Spinal cord tethering may cause severe headaches, neck pain, arm or leg pain, refractory to medical treatment, and/or progressive neurological deficit, which are all related to the level of spinal cord tethering, and may necessitate surgical detethering (Cornips et al. 2010). Spinal deformity may develop in 16–100% of children, especially younger children (<13 years old) subjected to multilevel laminectomies, and may complicate functional recovery years after surgical treatment (Yao et al. 2007). Whether it may be prevented by replacement laminoplasty techniques, or may warrant early stabilization, remains debated (Cornips et al. 2010; Nagib and O'Fallon 2002; Noudel et al. 2008; Yao et al. 2007). Clearly, future research should focus on a better understanding of the short- and long-term risks and benefits of surgical intervention (Mithka et al. 2011).

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Pediatric Patients with Bone Malignancy: Therapeutic Options

31

Lukas M. Nystrom and Jose A. Morcuende

Contents

Abstract

Primary bone malignancy, namely osteosarcoma and Ewing sarcoma, is predominantly encountered in the pediatric population. After histological confirmation of diagnosis done through a carefully performed biopsy these malignant tumors are generally treated with neoadjuvant chemotherapy and wide surgical resection, followed by adjuvant chemotherapy. Reconstructive options after resection depend on tumor size and location as well as patient age and goals. Limb ablation, either by amputation or rotationplasty, is commonly a less appealing option although the literature supports excellent long-term outcomes and durability. As a result of improved neoadjuvant regimens limb sparing surgery has become the mainstay of surgical treatment for the vast majority of bone tumors. Limb sparing options primarily include allograft reconstruction, allograft prosthetic composite reconstruction, and megaprosthesis reconstruction with or without expansion capabilities. Each of these options has features that make them attractive as well as potential complications. The choice of reconstruction should be made after in-depth discussion of the benefits, alternatives and potential complications on a patient by patient basis.

Introduction

Optimal treatment of pediatric bone malignancy relies on accurate diagnosis by a carefully performed biopsy. Biopsy by the surgeon

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performing the resection is recommended whenever possible (Mankin et al. 1996a). Confirmation of diagnosis is followed by a multidisciplinary team approach to administer neoadjuvant chemotherapy. Following the neoadjuvant regimen the surgeon's goal is to obtain a wide resection of the tumor (Rougraff et al. 1994). Wide resection can be accomplished with limb ablation or limb sparing techniques. In general, limb sparing surgical resection has become the treatment of choice. This is made possible by improved chemotherapy regimens that are thought to manage micrometastases as well as decrease the actual tumor size allowing for improved ability to perform a wide resection. Recent studies show equivalent survival and recurrence between amputation and limb salvage groups (Rougraff et al. 1994).

Limb sparing reconstructive options include allograft reconstruction, allograft prosthetic composite reconstruction and prosthetic reconstruction, and others. Because of advances in the medical treatment and accurate staging of malignant bone tumors there is a higher likelihood that patients will survive their disease; therefore there has been, an ever increasing focus on improved functional outcome rather than solely survival (Hillmann et al. 1999). Rougraff et al. (1994) showed in their comparative series of 227 patients who underwent either limb salvage, above-the-knee amputation or hip disarticulation that those undergoing limb salvage had significantly higher MSTS and Knee Society scores. Which reconstructive option is best for an individual patient is a patient by patient decision and must include considerations of tumor grade, anatomic factors, patient goals, and patient age. An additional consideration is that these bone tumors are frequently found at the physes of long bones, with the knee being the most common location (Kumta et al. 2002). Therefore, the surgeon must take into account that any limb salvage technique requiring physeal resection will result in a limb length discrepancy (DiCaprio and Friedlaender 2003). Complications rates are high for limb salvaging techniques and must be carefully discussed with the patient and family prior to surgery to ensure reasonable expectations. The aim of this chapter is to highlight several of the major options when faced with the need to resect a malignant tumor of bone in the pediatric patient.

Biopsy

The histologic diagnosis of pediatric bone malignancy relies on a carefully performed biopsy to obtain tissue representative of the tumor. We recommend this be performed by a surgeon experienced in musculoskeletal oncology, preferably the surgeon performing the eventual resection. Although a seemingly "minor" outpatient procedure, a poorly performed biopsy can lead to wound complications, unplanned surgeries, change in the resection plan and potentially patient survival (Mankin et al. 1996b). These authors reported on the experience of the members of the Musculoskeletal Tumor noted that for biopsies diagnostic of osteosarcoma 18.2% of those biopsies performed at a referring center had complications which altered the treatment course compared with only 3.8% of those performed at the treatment center.

The principles that should be adhered to are: longitudinal incisions performed in the area of an eventual extensile approach for tumor resection, careful hemostasis, dissection through single compartment (avoiding tissue planes), and insurance of diagnostic tissue prior to completing the procedure. For these reasons, in addition to other logistical considerations, we recommend that whenever possible the biopsy be performed at the treatment center that will be definitively managing the patient.

Limb Ablation Surgery

Amputation

Amputation, or disarticulation, was once considered the only acceptable method to eradicate malignancy. With improved chemotherapeutic regimens this is no longer the case (Sluga et al. 1999; Rougraff et al. 1994; Simon et al. 1986). However, there are certain patient and tumor factors

that that make amputation a desirable, or sometimes the only, option. A prerequisite for limb salvage is the ability to maintain the neurovascular bundle without compromising local control of the malignancy (Kropej et al. 1991). If this cannot be reliably accomplished, amputation may be the more prudent choice. Despite the shift to limb sparing surgery, amputation is known to have excellent patient outcomes. A multi-institutional study demonstrated that when comparing limb-salvage with amputation that amputation has a significantly lower reoperation rate with equivalent emotional acceptance and quality-of-life (Rougraff et al. 1994).

Rotationplasty

Van Nes rotationplasty is an option in the patient requiring a large resection, primarily of the distal femur, although various rotationplasties are feasible depending on the resection required (Hanlon and Krajbich 1999). The benefit of rotationplasty is that one can resect a large portion of distal femur or proximal tibia and still reconstruct a viable below "knee" amputation. Reports in the literature have generally noted favorable results with this procedure. Hanlon reported on 14 patients treated with rotationplasty for osteosarcoma followed for a mean of 8 years (Hanlon and Krajbich 1999). All patients had good or excellent results according to the Enneking functional evaluation and all but one had no pain, with the other having only intermittent pain controlled by non-narcotic means. Thirteen of the fourteen patients had excellent emotional acceptance of the procedure, with the other having good acceptance. All patients reported that they would choose to have the procedure again.

The reported complication rate with this procedure is relatively low when considering the patient population and surgical alternatives (Hanlon and Krajbich 1999; Gottsauner-Wolf et al. 1991; Kotz 1997). Hanlon noted that three of 14 patients in his series required reoperations for surgical complications. However, a larger series noted that 30 of 70 patients had some type of early or late complication (Gottsauner-Wolf et al. 1991); with 16 of the early complications requiring operative intervention to manage their complication. The main complications described in all series are vascular occlusions, nerve complications, non-union, infection and development of thigh length inequality.

Despite the good results reported in the literature, the primary disadvantage of this technique is thought to be the cosmetic and potential psychological implications of this operation. For this reason this procedure is infrequently performed in the United States. The psychological disturbance often cited as a potential deterrent to this procedure has been somewhat discredited by the literature (Hopyan et al. 2006). In properly selected patients this operation is very well-tolerated and has good results with relatively low incidence of immediate and long-term complications.

Allograft Reconstruction

Standards in tissue banking have increased dramatically over the past several decades, allowing for a wide range of allograft options. Allografts are available for intercalary (metaphyseal-diaphyseal) as well as osteoarticular reconstruction. Advantages of allograft reconstruction include the ability to tailor the reconstruction nearly exactly to the resection, the potential for biologic healing to host bone, and the soft tissue attachments available for repair when performing an osteoarticular reconstruction. Disadvantages of allograft reconstruction techniques include risk of infection, fracture, non-union and late joint complications including subchondral collapse or degenerative joint disease.

The appeal of allograft reconstruction is increased with the ability to perform a resection preserving the articular surface and ligamentous attachments (Fig. 31.1), thus performing an intercalary reconstruction. Long-term retrieval studies have demonstrate histologic evidence of healing at the host-allograft interface, although it does occur slowly over a period of approximately one year and the type of union achieved renders the bone susceptible to fracture through that area in the

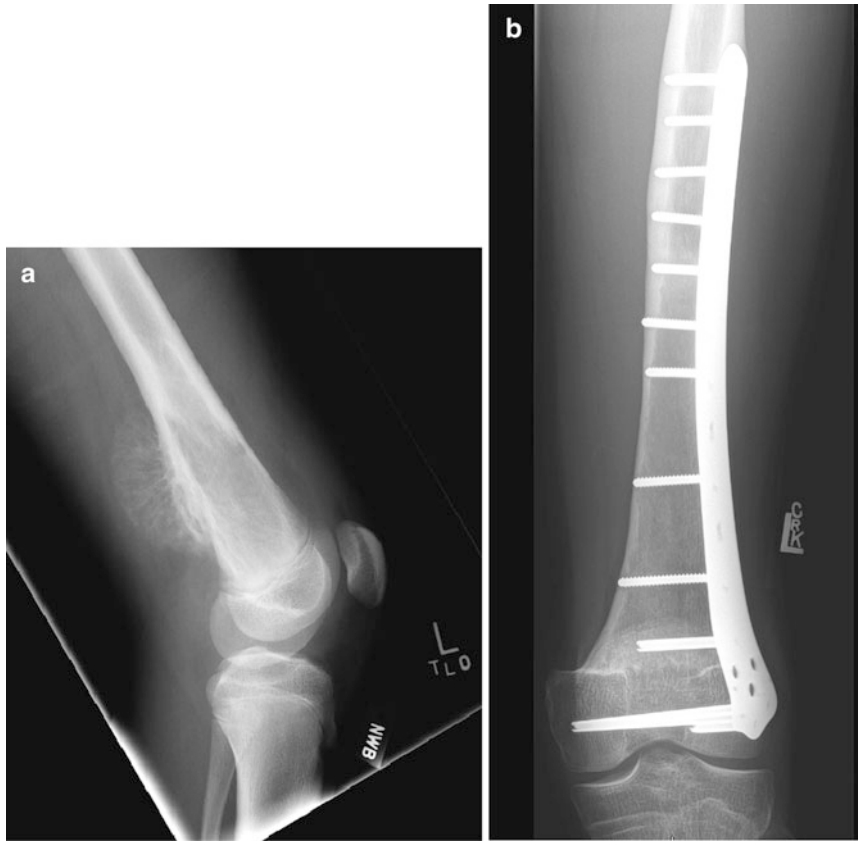

Fig. 31.1 (**a**) Lateral radiograph of a young boy with low-grade osteosarcoma of the distal femur. (**b**) AP radiograph 3 years post-operatively showing healing at both the proximal and distal osteosynthesis sites

future (Enneking and Campanacci 2001). Fracture rates, according to a series of 274 allograft reconstructions, are reported to be ~16% (Berrey et al. 1990). These fractures happened late, an average of 29 months post-operatively, in this series.

Long term outcomes of allograft reconstructions are generally thought to be good with 75% of patients retaining their graft according to a large series of over 800 patients with greater than 20 years of follow-up (Mankin et al. 1996a). However, when focusing on osteoarticular reconstructions around the knee a lower survival rate of 40% at an average of 10 years has been reported (Campanacci et al. 2010). In the long-term follow-up reported by Campanacci the most common modes of failure were fracture of the graft and joint collapse requiring conversion to allograft-prosthetic composite. In patients treated with osteoarticular allograft reconstruction there is also a concern for the development of degenerative joint disease at the reconstructed joint. According to the series reported by Mankin et al. (1996a) 16% of patients with reconstructions around the knee and 20% with proximal femur reconstructions required eventual joint arthroplasty at an average of 6 years after their reconstruction. These findings were corroborated by the retrieval analysis performed by Enneking who noted that in osteoarticular allograft retrievals there were no surviving chondrocytes in the allografts and that once the subchondral surface was revascularized the structural integrity of the joint was also lost (Enneking and Campanacci 2001). The observations of subchondral collapse, and degenerative joint disease, in the osteoarticular allograft reconstruction has been the main impetus for development of the allograft-prosthetic composite reconstruction technique.

Allograft Prosthetic Reconstruction

In effort to incur all of the advantages associated with allograft reconstruction, while avoiding the disadvantages of loss of articular integrity, the technique of allograft-prosthetic composite reconstruction has become popular. This involves a wide osteoarticular resection of the tumor and reconstruction of the bulk of the resection with allograft and the articular surface with arthroplasty components (Fig. 31.2). This technique becomes especially advantageous when reconstructing anatomic regions in which the resected soft-tissue envelope renders that joint unstable and/or non-functional, such as the proximal humerus or proximal tibia. When resecting a tumor of the proximal humerus, whether intra-articular or extra-articular, a portion of the rotator cuff and capsule must be sacrificed potentially rendering the joint unstable and non-functional. In the proximal tibia the primary concern is the extensor mechanism. Because the allograft used should have the periarticular soft-tissues left intact, this allows for a soft tissue reconstruction of the periarticular structures.

Abdeen et al. (2009) reported a series of 36 allograft-prosthetic composite reconstructions of the proximal humerus and found an overall 88% survivorship of the implant at 10 years with revision as the endpoint. Further this group noted that the functional results in patients where the deltoid was able to be preserved were superior to those reported in the literature for osteoarticular allograft and endoprosthetic reconstructions. This series reported functional results with average forward flexion and abduction of ~70°; this is in comparison to results in the ranges of 30–40° reported with other techniques. Overall the functional outcome was found to correlate with the amount of deltoid resected and whether an extra-articular resection was necessary. However, regardless of the functional outcome all patients reported no or only mild pain and there was only one dislocation reported in the series (Abdeen et al. 2009).

Similar concerns exist in the proximal tibial resection due to the sacrifice of the extensor mechanism insertion. Whereas it is possible to secure native patellar tendon to an implant there is currently no clinical data to support ingrowth

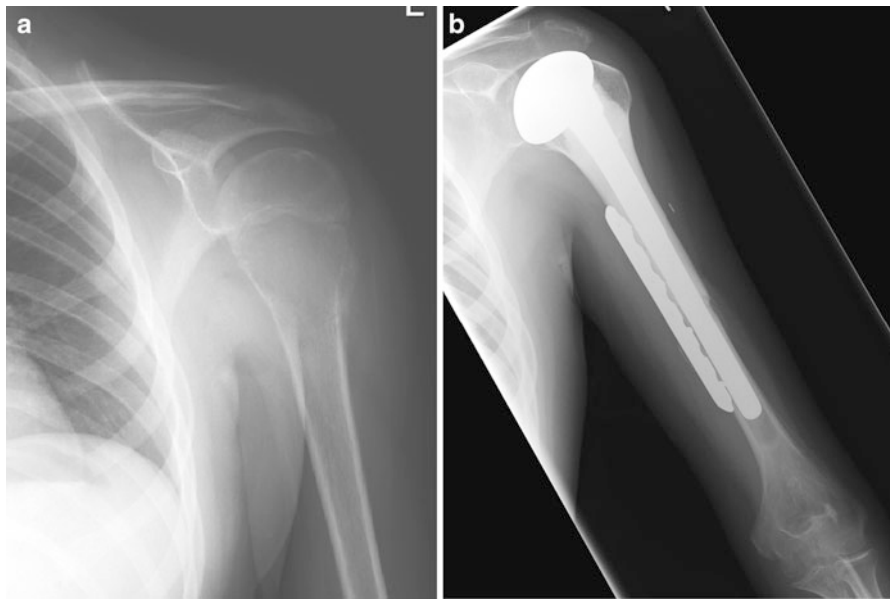

Fig. 31.2 (**a**) Pre-operative AP of the left shoulder showing an osteosarcoma of the proximal humerus. (**b**) Post-operative AP of the left humerus showing the allograft-prosthetic composite reconstruction with plate fixation at the osteosynthesis site

of this tissue into a prosthesis. There has, however, been encouraging evidence in a dog model showing tendon ingrowth to porous metal (tantalum) that may have future clinical implications (Reach et al. 2007). Allograft-prosthetic composite reconstruction in this area offers the advantage of a resurfaced joint combined with soft-tissue reconstruction of the extensor mechanism. Gilbert and colleagues reported a series of 12 allograft-prosthetic reconstructions of the proximal tibia with an average follow-up of ~4 years (Gilbert et al. 2009). They noted no failures in their group and extensor lags of only 5–15°.

Prosthetic Reconstruction

Prosthetic reconstruction of the skeletally immature limb can be done with essentially two different modalities: modular prosthetic components and growing or expandable components. When choosing a prosthetic reconstruction in a pediatric patient careful planning must occur in order to have equal, or near equal, leg lengths at the time of skeletal maturity. Each type of prosthesis has avenues to accomplish this but are associated with different types of potential complications for which the patient surgeon should be aware of and prepared to deal with.

Modular components have the advantage over expandable components in that they offer increased prosthetic construct strength at maximal lengthening. The disadvantages are that each lengthening requires an operation that involves a rather large exposure and all of the risks associated with surgically reopening a wound with an underlying prosthesis. In the patient requiring multiple lengthenings this can result in excessive scar formation and higher risk of infection (Balke et al. 2009). This option has not gained great favor in the very young patient due to the need for multiple surgeries and subsequent difficulties with participation in rehabilitation and the accompanying poorer results (Tunn et al. 2004).

Expandable components offer the advantage of either minimally invasive or non-invasive expansion procedures (Eckardt et al. 2000). The disadvantages of these devices, however, are the potential for failure of the expansion mechanism and failure of the prosthesis at maximal lengthening. The modular component may be more desirable in an older patient where lengthening requirements will be minimal, whereas the expandable component may be desirable in a younger patient who will require multiple lengthening procedures.

The expandable endoprosthesis, first described by Lewis (1986), functions through an expanding drive mechanism which is incorporated into the housing of the prosthesis. This mechanism was originally accessed through a very minimal surgical approach; however, newer prostheses are capable of non-invasive expansion in-situ (Gitelis et al. 2003).

Principles of Expandable Prostheses

When considering implanting an expanding endoprosthesis it is important to calculate estimated remaining growth. It is important to note that chemotherapeutic and radiation treatment protocols have been shown to affect physeal growth, which further complicates these calculations (Glasser et al. 1991). A child at or near skeletal maturity does not need an expandable device. Depending on their remaining growth the surgeon can consider some amount of acute lengthening with implantation of a conventional or expanding endoprosthesis with or without a contralateral epiphysiodesis. Calculation of remaining growth is instructive when counseling the patient and family members on surgical options and approximate number of lengthening procedures required.

Pre-operative planning will give an indication to the extent of the tumor and size of resection required. Magnetic resonance imaging (MRI) can best define the extent of the tumor (Fig. 31.3a). These should be done both prior to, and following neoadjuvant chemotherapy sessions. Estimated resection and imaging data must be communicated to the manufacturer as these devices are custom made for each patient.

The amount of bone resected will limit the lengthening capability of the prosthesis; as the telescoping portion can be only as long as the prosthetic implanted. The amount of acute lengthening

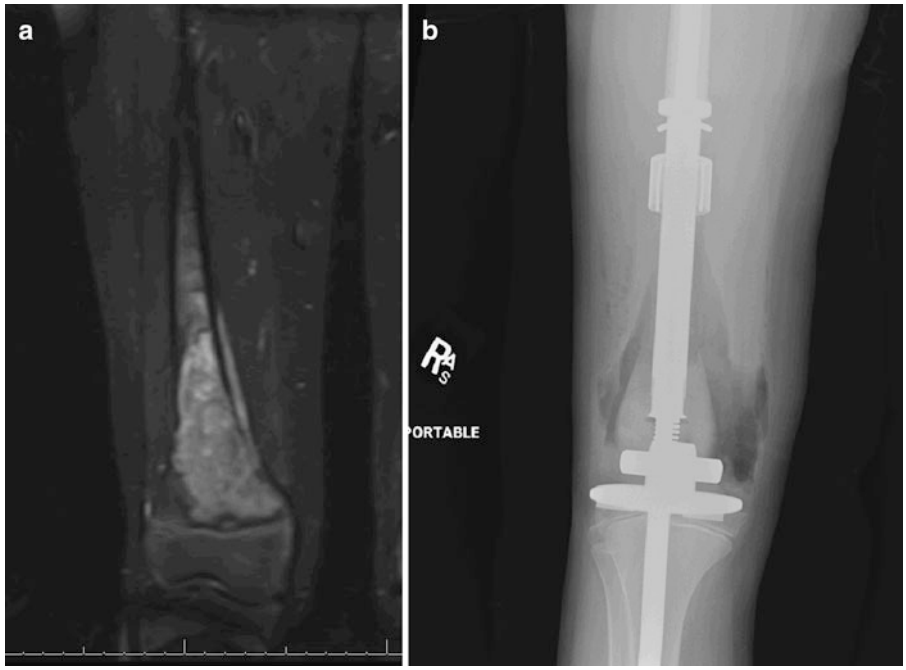

Fig. 31.3 (**a**) Coronal STIR MRI demonstrating Ewing's sarcoma of the distal femur. (**b**) Post-operative AP view of the knee demonstrating the implanted expandable endoprosthesis

(i.e., Lengthening done at the index procedure) must be conservative to avoid neurovascular complications. In general it is recommended that this number not exceed 1–2 cm (Schindler et al. 1998); however, current evidence is insufficient to recommend an amount of acute lengthening that is truly "safe."

Lengthenings

At each surgical follow-up visit radiographs of the prosthesis are obtained to evaluate for potential complications. Orthoroentgenograms are regularly obtained to calculate the limb length discrepancy. When a discrepancy exists of 1–2 cm, lengthening is undertaken. The lengthening procedure is done under radiographic guidance, typically in the fluoroscopy suite. Anesthesia is not required, and light intravenous sedation and/or analgesia are optional. In addition to negating the risk of the anesthetic to the patient, and additional advantage of performing awake lengthening is the ability to monitor pain and neurovascular examination (Gitelis et al. 2003).

Conversion

Due to the diminished strength of the expanded endoprosthesis has been recommended by some authors that children undergo conversion to a conventional fully-constrained hinge prosthesis upon reaching skeletal maturity (Baumgart et al. 2005). However, there is no consensus on this point as some investigators feel that a fully expanded implant is strong enough to withhold stresses at full lengthening (Schindler et al. 1998). In general there is increased concern for failure with full expansion of a telescoping prosthesis compared with a modular lengthened prosthesis.

Outcomes

Several small series reporting the results of expandable endoprosthesis have been reported in the literature. Kenan (1999) reported on 54 children with osteosarcoma or Ewing's sarcoma who were treated with the Lewis Expandable Adjustable Prosthesis (LEAP) (Kenan 1999). Of the 34 patients available for 2–12 year follow-up,

Table 31.1 Summary of outcome data available of expanding endoprostheses for treatment of pediatric malignancy (Nystrom and Morcuende 2010)

Series	N	Avg F/U (yrs)	# lengthen/pt	cm length/exp	AMP	REV	AL	INF	FX	HW	NV
Kenan (1999)	34	2–12	4.0	1.5	5	24	16	4	NA	3	NA
Schiller et al. (1995)[a]	6	6.3	7.8	0.6	0	7	1	3	1	NA	2
Neel et al. (2003)	14	1.8	4.3	0.9	1	8	3	0	3	2	0
Eckardt et al. (1993)	7	3.1	1.6	1.7	1	6	0	0	0	7	1
Eckardt et al. (2000)	19	8.8	2.0	1.0	3	NA	5	0	2	6	4
Schindler et al. (1997)[b]	14	8.7	4.3	1.2	2	10	6	1	1	1	1
Gitelis et al. (2003)	14	2.1	4.1	0.9	0	5	1	1	0	5	0

NA = data not available from review of manuscript
N number of patients, *AMP* amputations, *REV* revisions, *AL* aseptic loosening, *INF* infections, *FX* fractures, *HW* hardware failures, *NV* neurovascular complications
[a]Only included survivors who reached skeletal maturity
[b]Excluded amputations from final analysis

24 patients had required revision procedures at some point, with all revisions successful. Twelve patients reached skeletal maturity without leg length discrepancy.

A series published by Eckardt et al. (2000) reported on 32 patients (19 survivors) treated with four different prosthetic styles. In this series they demonstrated a disappointing rate of device failure of 25%. However; the failures were largely salvageable and of the 19 surviving patients, limb salvage was successful in 16. Additionally, of the nine patients who reached skeletal maturity at the time of the report, six had equal leg lengths.

The Repiphysis prosthesis (Wright Medical Technology, Arlington, TN) is the only expandable endoprosthesis that we are aware of that is available for use in the United States (Fig. 31.3b). The only study, to our knowledge, examining the outcome of the Repiphysis prosthesis was reported by Gitelis et al. (2003). The 14 patients in this series were reported at greater than 2 years follow-up. There was a 100% limb-salvage rate in this series, although there were five necessary revision surgeries all related to failure or fracture of the implanted components. Patients had an average MSTS score of 83.5% at the time of most recent follow-up. A summary of the available literature is compiled in Table 31.1 (Nystrom and Morcuende 2010).

Complications

Aseptic loosening is the most frequently encountered complication in this particular limb-salvage procedure. This is thought to be largely due to the long lever arm created at the bone-implant interface which is necessitated by the extensive resection. Supporting this mechanical concept of device failure, Schindler noted that in their series the patients who developed aseptic loosening had an average of 57.8% of their femur length resected, compared with 46.2% in those who did not develop this complication (Schindler et al. 1998).

Mechanical failure is another frequently reported which most frequently involves failure of the expansion mechanism, but there are also reports regarding fatigue fracture of the prosthesis (Eckardt et al. 2000; Gitelis et al. 2003). As experience with different expansion mechanisms has increased, this mode of failure seems to be reported with less frequency in the literature.

The available series reviewed here indicate a variable incidence of deep infection (Nystrom and Morcuende 2010). These numbers are difficult to interpret given the inherent small numbers included in these studies. Deep infection with this procedure is a disastrous complication and many reports describe necessary treatment with limb ablation depending on the severity of the infection.

Less frequently and variably reported complications include fracture, neurologic compromise and post-operative stiffness.

In conclusion, the decision for definitive surgical management of the pediatric bone malignancy is multifactorial involving patient and tumor specific factors. All treatments have advantages and disadvantages. Despite a higher complication rate, limb salvage techniques have largely become the method of choice due to improved function without compromising survival. Although it is tempting to try and develop a treatment algorithm which can be applied to all patients, decisions are best made on a case-by-case basis involving discussion with the patient and family.

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Late and Acute Effects of Pediatric Cancer Therapy on the Oral Cavity

32

Christopher Rowland, Cesar A. Migliorati, and Sue C. Kaste

Contents

Abstract

Pediatric cancer therapy has advanced to become curative for many types of cancer. The overall survival of patients treated for childhood cancer is now in the range of 90%. The May 2009 issue of the *Journal of Clinical Oncology* reported improved survival from about 30% in 1960 to 80% in 2004. An epidemiologic study (Mariotto et al. 2009) estimated that in the United States alone, there are more than 300,000 survivors of childhood cancer. However, this success has not come without a price. Pediatric cancer therapy is given during a time of growth, and late effects in the oral cavity can alter the growth and development of teeth and bones and affect overall health for the duration of a patient's life. The severity of these clinical and anatomical complications depends on tumor diagnosis, therapy exposure (chemotherapy, radiation, hematopoietic stem cell transplantation or a combination of several therapies), patient age and developmental status at the time of therapy and the resulting toxicity. In this chapter we will discuss some of the most important oral complications in pediatric oncology, with a focus on late effects of cancer and its treatment. Early complications will also be briefly described. Although the emphasis of this book is neurologic malignancy, oral complications occur in association with such malignancies because of the type of cancer therapy used. The prevention and management of these complications will be

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discussed, as well as the need for collaboration between dental providers and the oncology team for the improvement of outcomes.

Introduction

Cancer therapies comprise chemotherapy, radiation, surgery and hematopoietic stem cell transplantation, used alone or in combination. Each can cause acute oral complications that can resolve after therapy or persist for many years (Brennan et al. 2010). In pediatrics, some of the complications affect the development of head and neck bones, cervical vertebral bodies, and oral cavity structures like the teeth and jaws (Kaste et al. 2009).

Chemotherapy can alter tissue development and integrity and can impair the function of oral tissues and salivary glands. This typically occurs during cancer treatment and usually resolves after therapy is discontinued (Majorana et al. 2000). Radiation therapy can cause the same effects, but part of the damage is permanent (Raney et al. 1999). Surgery physically alters craniofacial function and the physical appearance of the patient. High-dose chemotherapy and irradiation of the head and neck area can cause serious ulcerative lesions (oral mucositis). This acute toxicity of the oral soft tissues can be so severe that it requires modification or discontinuation of cancer treatment, thereby potentially affecting prognosis (Sonis 2009). Unlike the acute effects of therapy described above, late oral cavity effects in the pediatric population are usually related to altered growth and development of craniofacial structures and teeth (Kaste et al. 2009; Raney et al. 1999; van der Pas-van Voskuilen et al. 2009). In addition, salivary gland dysfunction, leading to a decrease in salivary secretion, increases the risk of caries and periodontal disease (Hong et al. 2010). The main factors that determine the severity of oral cavity effects are the patient's age, the toxicity of high-dose chemotherapy, the dosing and duration of radiation therapy and the anatomic areas of the head and neck that are irradiated. Severe late effects after hematopoietic stem cell transplantation include graft versus host disease, increased risk of secondary malignancies of the oral cavity, and adverse psychosocial complications (Bhatia et al. 2007; Curtis et al. 1997; Ferry et al. 2007; Leahey et al. 1999).

The oral cavities of pediatric patients can be devastated by cancer therapy due to high cell turnover. It has been reported that more than half of patients treated for rhabdomyosarcoma with multiagent chemotherapy developed dental toxicities (Kaste et al. 1995). Additional evidence has shown that 70% of patients who received chemotherapy for nephroblastoma were found to be at risk of dental sequelae (Marec-Berard et al. 2005). In another study, it was reported that 70% of children treated with chemotherapy, irradiation of the head and neck or hematopoietic stem cell transplantation (HSCT) for neuroblastoma developed dental abnormalities (Kaste et al. 1998). Other authors have found that among a group of children who underwent head and neck radiation therapy for rhabdomyosarcoma, 11/30 (~30%) experienced facial growth deficiencies and 7/30 (~20%) experienced dental abnormalities (Paulino et al. 2000). A study of long-term survivors of childhood cancer reported that patients with central nervous system tumors, neuroblastoma, and soft tissue sarcomas had the highest risk of oral cavity abnormalities (Kaste et al. 2009). Therefore, oral cavity and head and neck complications affect children treated for cancer. The severity and duration of these complications depend on the toxicity of the therapy used, the age of the children and the developmental stage of the skeleton, head and neck, and oral cavity structures. Below we will discuss pediatric oral cavity considerations before, during, and after cancer therapy, with an emphasis on late complications.

Oral Considerations Before Cancer Therapy

The oral cavity and oral structures should be evaluated before the start of cancer therapy so that the dentist can diagnose and treat existing oral/dental disease and implement interventions

Table 32.1 Checklist of pre-therapy oral considerations

Oral examination by dental team
Radiographic evaluation
Oral hygiene instructions (brushing, flossing, fluoride, etc.)
Prescription of prophylactic agents (fluoride, mouth rinse, saliva substitutes, etc.)
Nutritional counseling (sugar intake, balanced meals, etc.)
Review of oral complications associated with planned cancer therapy
Optimization of oral health (caries control, dental extractions, treatment of infections)
Orthodontic appliance risk assessment

to prevent or minimize oral complications. Collaboration between the dental and oncology teams is important in facilitating this preliminary evaluation. Depending on the type of cancer therapy that is going to be used and the expected toxicity, a proper dental care can be optimized for each patient. For example, the decision as to whether or not to extract teeth can be made by weighing expected toxicity and the risk of having to perform an emergency extraction of a decayed tooth during the myelosuppressive phase of cancer therapy. Thus, restorable decayed teeth could be immediately treated and teeth that are infected and non-restorable could be extracted before the patient becomes immunosuppressed or radiation therapy involving the oral cavity begins. In addition, oral hygiene education of both parents and children with focus on the importance of maintaining oral health during and after therapy can significantly improve treatment outcomes (Table 32.1).

Common effects of cancer therapy observed during treatment include oral mucositis, infections and bleeding. Early detection and control of oral disease is important to reduce the risk of acute dental and periodontal disease activation during myelosuppressive periods, improve the outcome of cancer therapy, and improve patient quality of life. When patients are treated with radiation therapy to the head and neck, the oral tissues should be protected and shielded. For example, the use of lead shields and collars can shield almost all oral structures from the radiation beam in patients with nasopharyngeal carcinoma. New radiation protocols using intensity-modulated radiation therapy protect and spare vital structures like the salivary glands from the radiation beam. Preventing radiation damage of the oral tissues can reduce oral sequelae (Peterson et al. 2010).

When oral complications like mucositis and xerostomia emerge during radiation therapy, oral intervention cannot always be completed. Ideally, patients should be screened before the start of radiation therapy. Potential or active lesions such as dental caries, abscesses, and ulcerations can be diagnosed and treated. Teeth that cannot be saved can be extracted. Plaque that accumulates on the tooth surface is composed of bacteria and its byproducts and is a source of infection that should be professionally removed.

During high-dose radiation therapy involving the craniofacial structures, normal blood flow and tissue oxygenation are compromised. Thus, the third molars (already developed in teenagers and young adults) and severely decayed teeth, which could become an additional source of plaque accumulation and infection during or after therapy, should be extracted before therapy starts. Such surgical procedures are contraindicated after radiation therapy has compromised tissue healing and imposed the risk of osteoradionecrosis.

There is an additional risk of oral complications if orthodontic appliances are not removed before cancer therapy. They can be a source of dental plaque build-up, making oral hygiene difficult. Therefore, the removal of orthodontic appliances should be considered, especially if treatment is expected to be myelosuppressive. Ideally, appliances should be removed a week before pre-transplant conditioning or initiation of radiation.

Oral hygiene procedures, specific oral hygiene products, and fluoride use should be discussed with patients and parents. There is increasing evidence that the use of high-dose fluoride in association with calcium phosphate increases the protection of tooth structures against demineralization (Papas et al. 2008). Implementation of an oral hygiene protocol will help to protect against the deleterious effects of impaired salivary gland function and reduced salivary secretion (Jensen et al. 2010).

If cancer therapy must be started urgently, pre-treatment evaluation of the oral cavity and optimization of oral health may not be possible. Recommended oral hygiene procedures should be made available to parents and oncology staff members and implemented as soon as possible. Although hygiene recommendations alone are not ideal for oral health prognosis, good hygiene will reduce the oral bacterial load and enhance tooth resistance against demineralization. Routine dental care can continue as the patient's health status improves after the completion of cancer therapy.

Oral Considerations During Cancer Therapy

The oral care team plays an important role in the prevention and management of oral complications (McGuire et al. 2006). It is important that patients maintain good oral hygiene throughout cancer therapy to reduce the risk of oral complications, including dental caries, periodontal disease and infection. During active therapy, the dental team has limited interaction with the patient, and physicians and nurses are more likely to note changes in the oral cavity. Routine patient evaluations should include attention to oral hygiene and evaluation of the oral cavity for complications. The medical staff can also encourage the patient and parents to maintain daily hygiene procedures. High-dose chemotherapy and radiation can lead to the development of acute oral complications such as dental or periodontal infection, bleeding, dryness and mucositis. When these complications are diagnosed, the oral care team should be advised so that the complications can be addressed promptly (Table 32.2).

Prevention and management of oral mucositis can be a challenge. Oral mucositis can be so severe as to limit the administration of cancer treatment, thus increasing the risk of an adverse treatment outcome and potentially death (Sonis 2005). Oral mucositis is frequently accompanied by bleeding and offers oral bacteria an entryway into the systemic circulation, thus placing immunosuppressed patients at risk of life-threatening infection. Oral mucositis can also be intensely painful and distressing. Patients may have difficulty eating, swallowing and performing oral hygiene. When severe oral toxicity is expected (as in hematopoietic stem cell transplantation), patient-controlled analgesia (PCA) with morphine can be initiated (Keefe et al. 2007). After 2–3 weeks of therapy (radiation or chemotherapy), patients may experience dryness of the mouth. This uncomfortable symptom can also influence oral health and eating habits. Thus, minimizing the risk of oral complications and diagnosing them early is of great importance (Table 32.3).

Table 32.2 Observation of the oral cavity by medical staff: What to look for

Ability to swallow, eat solids and/or drink liquids
Mouth pain, oral discomfort, bleeding
Color and consistency of saliva
Oral mucosa: soreness, redness, ulcers, lesions and dryness
Scalloped tongue
Signs of infection (e.g., herpes simplex, candidiasis)

Table 32.3 Evaluation of the oral cavity during cancer therapy

Examination by dental team every 3–6 months
Radiographic evaluation (as recommended by the ADA)
Monitor salivary function
Emphasize oral hygiene instructions (brushing, flossing, etc.)
Monitor therapeutic agents and prescribe other therapies as needed
Review nutritional counseling
Discuss dental complications of therapy if noted
Correct dental caries and infections

ADA American Dental Association

Oral Considerations After Cancer Therapy

After completion of cancer treatment, children may experience problems with teeth, gingival tissue, salivary glands, and bone. Chemotherapy and radiation can cause long-term effects in the oral

cavity, but the risk of acute oral complications from high-dose chemotherapy decreases with bone marrow recovery. Pediatric patients who undergo HSCT receive immunosuppressive therapy for prolonged periods, increasing the risk of opportunistic infections in the oral cavity. Additional complications after high-dose chemotherapy include chronic salivary gland dysfunction and dryness, graft-versus-host disease and second primary tumors of the oral cavity (Demarosi et al. 2005).

Age is an important factor in the development of oral complications; the younger the patient, the greater the risk. The detrimental effect of therapy is caused by inhibition of the normal development of the oral structures, which include teeth, bone, temporomandibular joint, muscles of mastication, salivary glands, and oral mucosa (Holtta et al. 2005a, b).

Complications Affecting the Teeth

Odontogenic complications may affect different parts of the tooth structure (Fig. 32.1). The crown is composed of the enamel (outer surface) and dentin (internal portion). The root is composed of cementum (outer surface) and dentin (internal portion). The root canal is the space that contains the nerve and blood vessels within the tooth structure. When therapy is administered during the development of dentition, developmental arrest or defects in all three structures (crown, root, root canal) may result. This interruption in development can lead to partial development or complete absence of the tooth (Table 32.4).

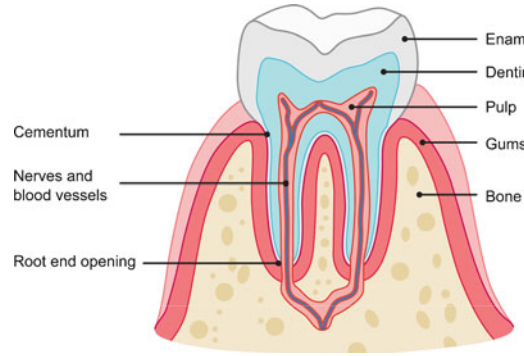

Fig. 32.1 Structural diagram of a tooth

Normal tooth development involves the formation of a primary set of teeth that exfoliate between the ages of 6 and 12 years of age and are replaced by the permanent teeth. When the primary teeth are affected by cancer therapy, the permanent teeth may still develop normally. Panorex films show the developing dentition in Fig. 32.2 and Fig. 32.3. Figure 32.2 is a panorex of the normal developing dentition of a 6 year old. Figure 32.3 is a normal panorex of a teenage patient. During development of the enamel or dentin, oncotherapeutic exposures can adversely affect crown development, causing hypocalcification of the tooth structure, defects in the surface, and anatomical malformation. Clinically, these effects appear as discoloration, malformation,

Table 32.4 Late effects in the oral cavity after therapy

Disease	Dental sequelae	%	Source
Rhabdomyosarcoma	Root stunting	54	Kaste et al. (1995)
	Microdontia	23	
	Hypodontia	50	
	Multiple issues	36	
	Cosmetic or functional	23	
Nephroblastoma	Root stunting	44	Marec-Berard et al. (2005)
	Enamel hypoplasia	22	
	Microdontia	18	
	Hypodontia	7	
Neuroblastoma	Caries		Hutton et al. (2010)
	Microdontia (below 3.5 years old)		

Adapted from Lopes et al. (2006)

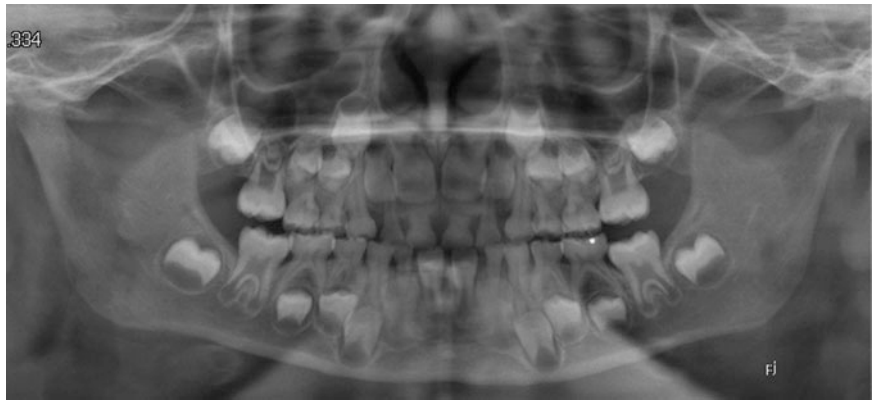

Fig. 32.2 Panorex of a 6 year old showing normal mixed dentition. The patient underwent placement of a small restoration in left mandibular second primary molar

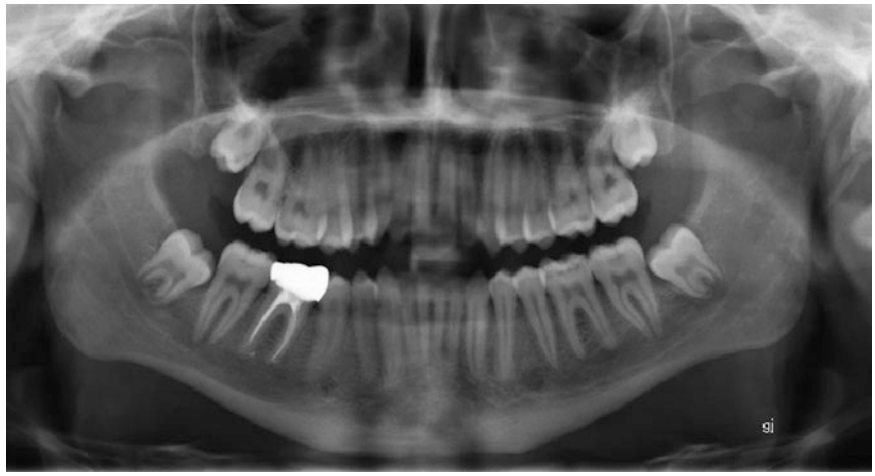

Fig. 32.3 Normal panorex of a teenager. Patient has undergone root canal therapy and placement of a crown right mandibular first molar

and abnormal size of the tooth (Holtta et al. 2002). When the tooth structure is compromised, formation of dental caries is facilitated. For example, patients less than 3.5 years of age who undergo HSCT for lymphoma and solid tumors, particularly neuroblastoma, have teeth that are microdontic (small) and show a significant number of caries (Hutton et al. 2010) Caries can be exacerbated by xerostomia if the salivary glands and secretion of saliva are affected in patients treated with high-dose chemotherapy and radiation therapy (Fig. 32.4).

When a tooth erupts into the oral cavity, problems can be identified and treated upon visual evaluation of the crown structure. Radiographs are the most effective tools to assess the development of root structure and pulpal tissue. Developmental defects of the root can be devastating and can go unnoticed in the absence of radiographic evaluation. Some of these defects include root stunting, "thistle tube-like" malformation and dilacerations (Fig. 32.5). Root stunting is the most destructive effect, as it disrupts dental eruption patterns and can cause future loss of the tooth (Duggal 2003; Vaughan et al. 2005). Root stunting also interferes with orthodontic treatment, as the underdeveloped root structures

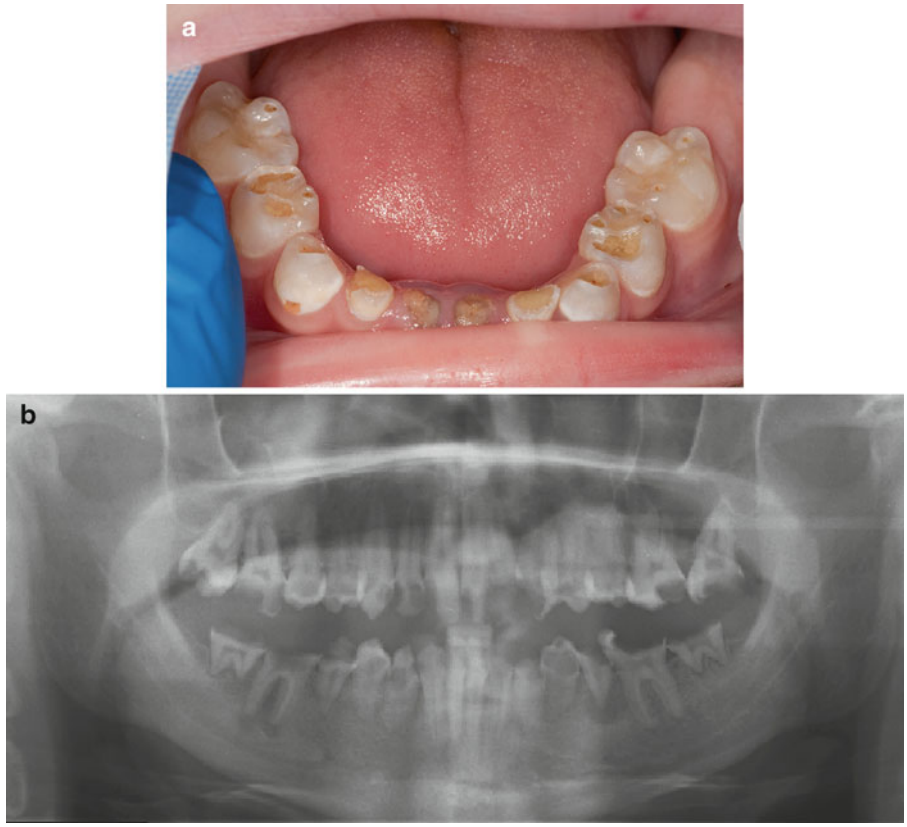

Fig. 32.4 Clinical (**a**) and radiographic (**b**) aspects of severe tooth decay associated with radiation therapy and xerostomia

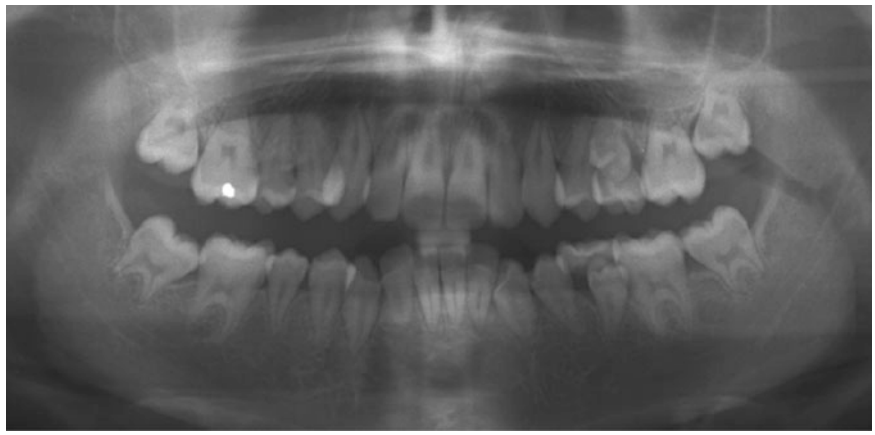

Fig. 32.5 Panoramic radiograph showing root stunting (arrested tooth development)

may be inadequate for secure fixation of an orthodontic appliance.

The pulpal tissue and the root canal can be adversely affected in a number of ways. The canal can be obliterated and enlarged, and the dental pulp can become necrotic. If the dental pulp becomes necrotic the existing obliteration of the pulp chamber will make the necessary root canal therapy impossible. As a consequence, the tooth will have to be extracted. Taurodontism is an abnormality commonly noted after therapy of young patients (Lopes et al. 2006). This abnormality is a benign elongation of the body of the root canal chamber. However, necrosis of the pulp can be a rare complication.

Complications of Bones of the Head and Neck

The bone structure of the oral cavity is composed of the maxilla and mandible. These structures are best evaluated through imaging such as panoramic radiographs and cephalometric studies. The panoramic view will reveal bone density, bone height, and general development from a frontal view, whereas the cephalometric film shows growth and deficits of the mandible and maxilla (Fig. 32.4).

The maxilla and mandible can be affected independently during radiation therapy, depending on the anatomic distribution and scatter of the radiation beam. In children, for example, some retardation of growth may result from doses as low as 10 Gy, depending upon the age at irradiation and the conditions of exposure. Other skeletal changes have been observed after therapeutic irradiation in childhood at doses exceeding 20 Gy. However, the susceptibility of these tissues to subsequent trauma months or years later may be increased, but precise dose-response data for such long-term effects are fragmentary (International Commission on Radiological Protection 1984). The area primarily affected by radiation therapy is that included in the radiation ports. Shielding of vital areas in the head and neck prior to radiation can help to prevent future complications. The treatment of rhabdomyosarcomas of the head and neck with multiagent chemotherapy can result in defects that require orthodontic and structural correction in 23% of patients (Kaste et al. 1995). Radiation therapy to the head and neck can cause deficient mandibular growth leading to retrognathia, while irradiation of the sinus cavity can cause maxillary hypoplasia. Because the opposing mandible will develop normally, bone development will show a discrepancy that may necessitate future orthodontic therapy and, in severe cases, orthognathic surgery to correct jaw positioning and occlusion. If altered jaw formation is noted, referral to an orthodontist for evaluation is recommended (Estilo et al. 2003).

Irradiated bone can pose a problem when tooth extraction is needed. Patients who have received more than 50 Gy of radiation to the head and neck are at risk of osteoradionecrosis (ORN). When invasive dental procedures like dental extractions or surgery involving bone manipulation are needed in areas within the radiation fields, the use of hyperbaric oxygen therapy (HBO) could reduce the risk of ONR. The usual HBO protocol is 20 hyperbaric chamber treatments of 90 min each before oral surgery and ten treatments afterward. However, it must be considered that this protocol is still in search of scientific support (Peterson et al. 2010). The overall risk of ORN of the jawbones is low but may persist indefinitely. The mandible is at higher risk because of its limited blood supply.

Osteonecrosis of the jaw is observed in cancer patients with skeletal metastasis of solid tumors (breast, prostate, and lung) and multiple myeloma as well as in patients with osteoporosis who take bisphosphonates. Bisphosphonates do not appear to affect the pediatric population in this manner; no pediatric cases have been reported to date (Brown et al. 2008).

Like other joint structures, the temporomandibular joint may be affected by radiation. Some of the complications are trismus, joint adhesion, and joint necrosis. The main functional complication is trismus, or limited opening of the jaw. Trismus hinders oral hygiene, compromises the patient's ability to eat and masticate properly, and interferes with dental treatment. The muscles of mastication can also

be affected, with the development of scar tissue. Physical therapy can improve mouth opening and the range of motion of the jaw. All of these developmental complications affect the overall health and quality of life of the patient. Maintaining good oral and maxillofacial function is important for eating, swallowing, speech, and good oral hygiene.

Complications Associated with Saliva and Salivary Glands

Salivary gland function and saliva production can be severely affected by cancer therapy (Jensen et al. 2010). Chemotherapy typically affects the glands during treatment, and dryness of the oral cavity can persist for a short time after completion of treatment. Progressive improvement may lead to complete normalization of gland function and saliva production. However, xerostomia is a serious problem for patients after irradiation of the head and neck that involves the salivary glands. The dose of radiation and the extent of glandular involvement determine whether normal function and saliva production can be restored. Among its multiple functions, saliva is a natural cleansing agent of the oral mucosal tissues and teeth. Salivary buffering capacity maintains a neutral ph, protecting teeth from the acidity of sugars (Sreebny 2000). When salivary glands are functionally impaired and saliva production is decreased or absent, an acidic oral environment that is detrimental to the teeth can develop. Patients with dry mouth after radiation therapy are at very high risk of caries, which can be rampant (Fig. 32.4) and affect every tooth in the mouth (Purdell-Lewis et al. 1988). This condition is very difficult to treat and usually leads to complete tooth loss and the need for dentures. Thus, attention should be paid to the salivary glands and saliva production before cancer therapy begins, especially if radiation therapy of the head and neck that involves the salivary glands is necessary. During the pre-treatment evaluation, patients must be educated about the importance of maintaining good oral hygiene during cancer treatment and for life. Preventive measures include good oral hygiene even during therapy, the use of saliva substitutes, alcohol-free mouth rinses, and non-irritating toothpastes with high fluoride content. There is, however, no substitute for good brushing techniques and regular follow-up at the dental office. The patient must understand that more frequent visits to the dentist are needed during therapy. The dentist has the important role of early detection and treatment of dental and other oral problems and monitoring their progression.

In addition to good oral hygiene and periodic follow-up visits to the dentist, other issues must also be considered. Dental visits should be more frequent than that for general pediatric populations. Access to dental care maybe limited for patients with low income, less education, and no medical insurance (Casillas et al. 2010). Patients who have completed cancer therapy are likely to be in these categories. Further, in some cases, dentists may decline to treat cancer patients due to lack of adequate training. Thus, lack of access to dental care may lead to the long-term failure of a patient's oral and dental health after cancer therapy (Kaste et al. 2009).

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Air Embolism in a Pediatric Patient: A Very Rare Complication of Endoscopic Retrograde Cholangiopancreatography

33

Marta Di Pisa, Giuseppe Chiaramonte, Ilaria Tarantino, and Mario Traina

Contents

Abstract

There are ample data in the literature regarding the occurrence of venous air embolism during an endoscopic procedure, in which the gas, insufflated into a body cavity, enters the vascular system through a traumatic or surgical lesion. In the last years, almost three fatal events have already been described during endoscopic retrograde cholangiopancreatography (ERCP), following liver biopsy and sphincterotomy, but very few records exist on this complication in paediatric patients. For this reason, it's imperative to recognize it in time, because it can be fatal or can have severe neurologic consequences.

Introduction

Vascular air embolism (VAE) is the entrainment of air (or exogenously delivered gas) from the operative field or other communications with the surroundings into the venous or arterial vasculature, producing cardiopulmonary dysfunction and other systemic effects (Bisceglia et al. 2009). Its incidence is actually undervalued for two main reasons: first, because of the sensitivity of detection methods used during the procedure, and second, because, sometimes, it can have a subclinical onset; also, very few data exist in the paediatric population and for all of these reasons it could not be promptly recognized and could have fatal consequences. To have clear knowledge about this

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complication is particularly important to treat it on time and to avoid unfortunate consequences.

The first case report on a fatal air embolism, which occurred in a 4-month-old infant undergoing gastroscopy after a Kasai procedure and confirmed on autopsy, is attributed to Lowdon and Tidmore (1988). Recently, other two cases of VAE were reported in a 2 years old baby girl, who received home infusion therapy via central venous access (Laskey et al. 2002) and the other one in a neonatal patient with acute respiratory distress syndrome, in which a massive entrainment of air occurred into the extracorporeal membrane oxygenation circuit (Timpa et al. 2011).

In the past, the conditions under which air embolism was encountered were limited only to neurosurgical procedures, conducted in the "sitting position", with an incidence of VAE in children (Bithal et al. 2004) of 9–33%, without significant differences between adults and children. In recent years, we have seen that it also occurs in interventional radiology and endoscopy suite (Di Pisa et al. 2011) or laparoscopic surgical centre (Bisceglia et al. 2009).

The morbidity and mortality of VAE are related to the volume of air, rate of entrainment, the patient's underlying cardiorespiratory status, and the patient's position; from these factors, the basic factor determining the morbidity and mortality is primarily related to the volume of air entrainment within the right ventricle. Lethal volumes of air entrained as an acute bolus have been concluded to be ~0.5–0.75 ml/kg in rabbits and 7.5–15.0 ml/kg in dogs (Toung et al. 2001); it is not known in humans, although mortality has been reported with injection of as little as 100–300 ml. If entrainment of air is slow, the heart may be able to withstand large quantities of air despite entrainment over a prolonged period of time or if the volume is limited, it is rapidly reabsorbed or excreted by the lungs. Otherwise, there may be complete outflow obstruction from the right ventricle as failure from the inability to decompress the tension of the ventricular wall, causing cardiovascular collapse (Mohammedi et al. 2002). Air entrainment into the pulmonary circulation may lead to pulmonary vasoconstriction, release of inflammatory mediators, bronchoconstriction, and an increase in ventilation/perfusion mismatch (Romberg 2009).

From the Point of View of the Endoscopist and Anesthesiologist

Hepatic portal venous air embolism is the rarest complication of gastrointestinal endoscopy, resulting from penetration of gas into the portal veins, and may occur especially during endoscopic retrograde cholangiopancreatography (Fig. 33.1) (Bisceglia et al. 2009) and endoscopic biliary sphincterotomy (Cotton et al. 1991). Also, it was described during procedures following liver biopsy (Siddiqui et al. 2005) or during lung biopsy (Mokart et al. 2011) and after percutaneous CT guided transthoracic needle biopsy (Hiraki et al. 2007).

During endoscopy, one proposed mechanism of air embolism is mechanical irritation of the bile duct wall from endoscopic instrumentation, (for example, secondary to bile duct stones extractions, clearing of a metallic stent, deployment or removal of a biliary prosthesis) that may cause intramural dissection and injury to surrounding veins (Rabe et al. 2006). Consequently,

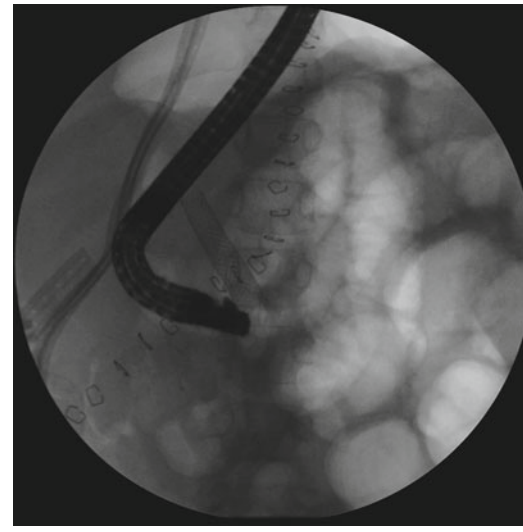

Fig. 33.1 A metal stent positioned into the biliary duct during ERCP

a biliovenous shunt is created which serves as a portal of entry of air into the venous; high pressures generated in the gastrointestinal tract, during endoscopic procedures, and sphincterotomy play a crucial role (Barthet et al. 1994).

Air is introduced into the gastrointestinal tract through the endoscope at relatively high pressures of up to 43 kPa and at a high flow of up to 2,000 ml/min, and it may inadvertently enter the venous circulation (Rabe et al. 2006). In the presence of portal venous air, it is mandatory to quickly interrupt the procedure; otherwise, continuing the procedure in an attempt to correct iatrogenic lesions may result in increased air delivery and further decompensation. Immediate decompression of the lumen with subsequent nasogastric tube suction and placement of the patient in a left lateral decubitus and trendelenburg position are the first manoeuvres needed to be done to safeguard infant patients (Cha et al. 2010).

Over the last years many case reports have been published on air embolism in patients who underwent endoscopic procedures. Few of them are related to pediatric patients. Anesthesiologists should be aware of this potential and life threatening complication, especially when they are dealing with pediatric patients, because even small amount of air passing in the venous vessels can reach the heart and cause fatal pulmonary air embolism (Benítez et al. 2003). Metal stents and venous shunts are also additional risk factors (Rabe et al. 2006).

Cases of paradoxical air embolism are also reported following endoscopic procedures (Kim et al. 2010). Furthermore, in children who are younger than 1 year of age, the risk of paradoxical air embolism is higher because the percentage of children with patent foramen ovale can be as high as 70% (Calvert et al. 2011). General anaesthesia with endotracheal tube placement is the preferred technique of anaesthesia in children undergoing ERCP. The minimum standard of monitoring is EKG, SpO2, Capnography, non Invasive Blood Pressure or Mechanical Ventilation parameters. All the equipment and drugs for pediatric advanced cardiac life support should be available for ready use. In an anesthetized patient massive air pulmonary embolism creates a big drop in cardiac output with the signs of a very poor peripheral perfusion (cyanosis, mottled skin, reduced capillary refill,). On the monitor, a rapid severe drop of etCO2 and SpO2 is visualized (Navarro et al. 2011), with consequent increasing of the central venous pressure (if a central line is in place), brady or tachi-arythmias, peri-arrest or cardiac arrest rhythms, severe hypotension, circulatory arrest (Pandia et al. 2011).

Differential diagnosis can be a challenge because symptoms are common with anaphylaxis, tension pneumothorax, hypovolemic shock, cardiac tamponade and severe aorto-caval compression due to bowel overinflation. The transesophageal or transthoracic echocardiography (TEE) plays a central role in diagnosis because it allows seeing directly the air bubbles inside the heart chambers and the failure of the right ventricle (Park et al. 2010).

Once the massive air embolism is suspected the patient should be ventilated on 100% oxygen and the procedure should be immediately stopped. Positioning the patient on trendelenburg, and left tilt may help to trap air bubbles in the right ventricle (Beloiartsev and Theilen 2011). In arrested patients CPR and advanced cardiac life support should be started promptly; cardiac massage may be beneficial reducing large bubbles into smaller, which are more easily and rapidly reabsorbed. Aspiration of air from the right atrium can also be useful as an option of treatment but requires central access (Shaikh and Ummunisa 2009). Once the patient is resuscitated, he/she should be transferred to ICU and investigated and treated for any organ damage due to cardiac arrest or paradoxycal air embolism.

Clinical Presentation and Diagnosis

Clinical manifestations may be of interest to the cardiovascular, pulmonary, and neurologic system, depending on the rate and entrained volume of VAE. The most common cardiovascular symptom is tachyarrhythmia, and electrocardiographically the main sign is change on ST – T tract (Cooney et al. 2011); also, decreases of blood

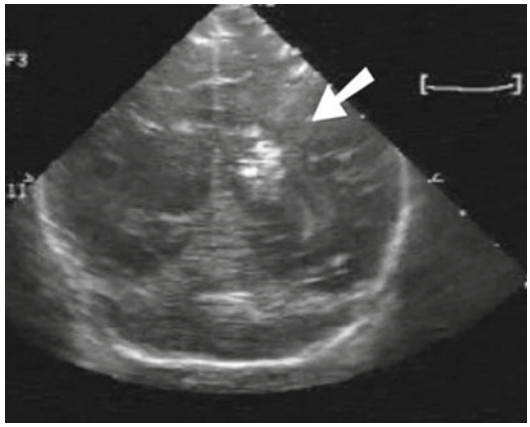

Fig. 33.2 TTE illustrates a thrombus into the left atrium

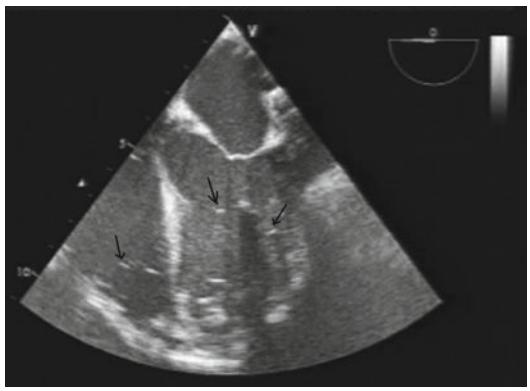

Fig. 33.3 Some echo dense shadows, seen at the TTE, consistent with air

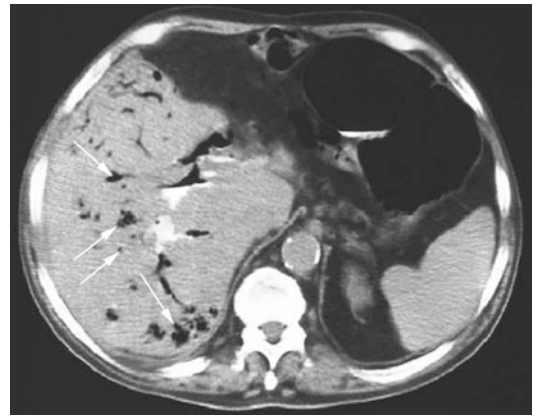

Fig. 33.4 CT shows some air content within the hepatic parenchyma

pressure in consequence of the cardiac output falters and increase in pulmonary artery pressure and in the central venous pressure measurements as a secondary effect of right heart failure are observed determining, as a final effect, hypotension until the shock (Finsterer et al. 2010).

Pulmonary clinical signs in awake patients are acute dyspnea, wheezing, and tachypnea. Both cardiovascular and pulmonary systems have effects on neurological structures, as a consequence of cardiac output, leading to cerebral hypoperfusion and edema, which means focal deficits until coma (Klein and Zarka 2000). Also, direct cerebral air embolism may arise via a persistent patent foramen ovale, which is present in ~20% of the adult population (Marek et al. 2007). In a sedated infant patient, warning signs are: a sudden and unexplained decrease in the end tidal carbon dioxide, hypoxia, bradycardia, hypotension, and arrhythmia.

The most sensitive diagnostic test to demonstrate presence of venous air is transesophageal or transthoracic echocardiography (TEE) (Figs. 33.2 and 33.3), which shows any amount of air in the cardiac chambers and in the circulation (Glenski et al. 1986). The major deterrents to TEE are that it is invasive, is expensive, and requires expertise and constant vigilance that may limit its use by a noncardiac anesthesiologist. In case of large volumes of air and if is still present in the heart, VAE can be diagnosed with a chest radiograph (Cooney et al. 2011). Finally, CT (Fig. 33.4) is highly sensitive for the detection of cerebral intraparenchymal gas if obtained early (Tan et al. 2008).

In conclusion, venous air embolism is a very rare complication of many gastroenterology procedures including ERCP, and because of its rarity, is not easy to recognize it. Unfortunately, if not diagnosed and treated quickly, it can rapidly lead to a fatal cardiac arrest. Prevention of air embolism during endoscopy requires awareness of the possibility of this silent complication, particularly in procedures in which vessels can be damaged. A prompt diagnosis and emergency measures immediately taken prevent permanent neurological damage, and lethal consequences. Attention to limitation of the pressure of insufflated air is necessary and carbon dioxide should be considered the insufflating gas of choice, even if not particularly practical because it is really adsorbed if there is an embolus.

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