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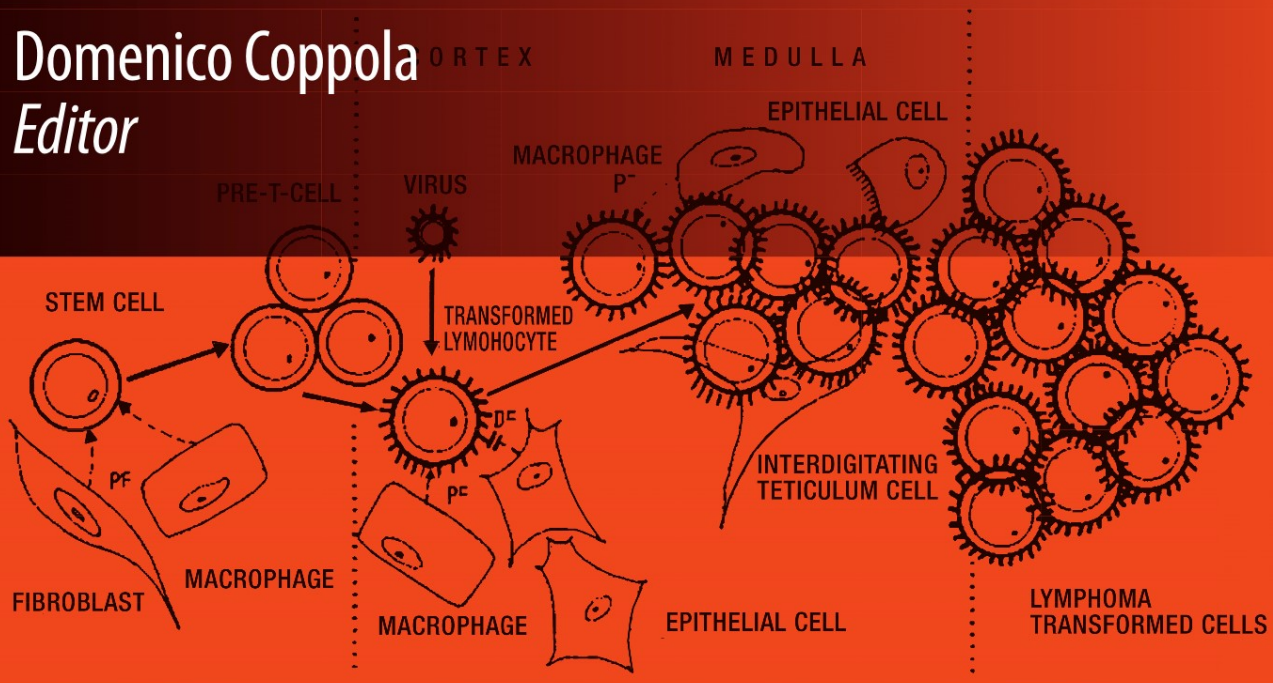

Mechanisms of Oncogenesis

An Update on Tumorigenesis

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MECHANISMS OF ONCOGENESIS

Cancer Growth and Progression

Volume 12

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Preface

This volume is a continuation of the encyclopedic work entitled "Cancer Growth and Progression," that was originally edited by Professor Hans E. Kaiser. As a tribute to the memory of Dr. Kaiser and his contributions to the field, this new edition is following his example of providing the most current information about cancer literature and comprehensive coverage of all aspects of tumor growth and progression.

This particular tome is divided into two sections: the first addressing general causes of oncogenesis such as environmental factors and tumor mechanisms; and the second addressing the oncogenic mechanisms of tumor in specific organs. This book is written and compiled by world renowned leaders in their respective fields and focuses on the most recent developments in basic oncogenesis. This definitive text will provide the practitioner, whether in research, academia or clinical practice, with a fount of easily accessible information on the most common tumors.

Contents

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Chapter 1

Cytokines and Stressors: Implications for Cancer Immunotherapy

Alexander W. Kusnecov and Hymie Anisman

Contents

1.1 Introduction

Stressful events have been implicated as being fundamental in provoking some pathologies and exacerbating the course or symptoms of others. In this regard, it has been suggested that certain pathological states, such as depressive and cardiovascular illness, may be related to antecedent stressors (or emotional states), although the biological outcomes leading to such effects remain to be fully deduced [1–3]. The view has likewise been expressed that life-stressors (or depressive illness that might be associated with stressful events) may contribute to the exacerbation of neoplastic disease [4–6], although the available data in humans supporting this position has been limited, and the validity of this perspective has been challenged [7–11]. Yet, treatment of depression in cancer patients has been found to increase survival times suggesting that psychological attributes, possibly involving stressor-related processes, may influence the course of cancer progression [6, 12].

Studies in animals indicated that stressors exacerbated the growth of some types of transplanted or carcinogen-induced tumors, but it was clear that such an outcome was dependent on characteristics of the tumor system being examined (e.g., syngeneic vs. nonsyngeneic, hormone-dependent *vs*. hormone-independent, fast vs. slow growing). Moreover, at least some of the effects of the treatments were independent of immune functioning, as the stressor-elicited tumor augmentation was evident in syngeneic tumors that presumably had escaped immune surveillance [13]. Of course, this does not belie the possibility that stressor effects on immune functioning may have influenced some types of tumors. Likewise, it is certainly possible that stressors may have impacted tumor growth by affecting the activity of cytokines (signaling molecules of the immune system), which influenced hormonal and other processes, and thus tumor growth.

Given the complexity of the processes associated with cancer development and progression, it is not surprising that the impact of stressors on these processes is not well understood. The difficulty in appraising the stress-cancer relationship is compounded by the fact that stressors may have diverse effects on various biological systems (e.g., autonomic nervous system,

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central nervous system, neuroendocrine and immune processes), and these effects are dependent on a variety of characteristics related to the nature of the stressor, including the type of stressor employed, its controllability, predictability and chronicity, as well as various attributes of the organism, including age, sex, species and strain.

Although any number of processes may contribute to the effects of stressors on tumor development, one promising avenue of research in this respect concerns the analysis of stressor effects on cytokine functioning. To be sure, cytokines have been used increasingly in the treatment of certain types of cancer. In the present review, we report on the effects of stressors on the activity of various cytokines, as well as the effects of cytokines on neuroendocrine and central neurotransmitter processes. Inasmuch as cytokines may also contribute to the provocation of depressive illness, we suggest that psychological state and tumor growth, by virtue of the related neurochemical changes, may be intertwined. In this review we will briefly describe (a) the effects of stressors on neuroendocrine and cytokine processes, (b) the influence of cytokines on neuroendocrine and central neurotransmitter functioning, and finally (c) the impact of cytokine immunotherapy on cancer progression, and the effects evident with respect to psychological processes, particularly those affected by stressors (especially depressive states).

It should be stated at this point that the study of stress has typically been approached from a negative perspective. That is, the driving hypothesis is that stressors are thought to produce disruptive physiological changes that might contribute to pathology, whether physical or psychological. However, in the review of data below, it should be considered that all changes taking place in response to a given environmental and/or psychological challenge represent normal adjustments in function that may or may not return to pre-stress levels, but nonetheless represent engagement of resources that evolved to cope with the stressor. The challenge of stress research is to determine the conditions under which the consequences of stressor exposure result in biological functioning that as a result of the "stress experience" no longer operates to serve the interests of health and survival. Many of these conditions, including the intensity, chronicity and frequency of stressor exposure, as well as various organismic variable, including sex and genetics, have already been alluded to above.

1.2 Stressor Effects on Neurochemical Processes

Stressors have repeatedly been shown to instigate a cascade of central and peripheral neurochemical changes that are thought to facilitate the organism's ability to contend with the challenge. Among other things, these varied neuroendocrine and neurotransmitter changes may have permissive or suppressive actions, enhance the effects of other neuroendocrine factors, blunt the physiological and psychological impact of stressors, and preclude excessive physiological activation [14].

1.2.1 Hypothalamic-Pituitary-Adrenal Effects of Stressors

Ordinarily, acute stressors increase the activity of the prototypical neuroendocrine system comprising hypothalamic-pituitary-adrenal functioning. In response to stressors, the paraventricular nucleus of the hypothalamus is activated, giving rise to the release of corticotropin releasing hormone (CRH) from the median eminence, which then stimulates ACTH release from the anterior pituitary gland. The ACTH thus enters circulation, provoking glucocorticoid secretion from the adrenal gland [15–17]. In the case of some stressor-related pathologies, such as depression, elevated circulating cortisol levels may be evident [18], although levels of morning cortisol may be reduced among individuals that experienced traumatic events that resulted in the development of posttraumatic stress disorder (PTSD) [19].

Typically the HPA response to stressors occurs rapidly and normalization of corticoid levels occurs fairly soon after stressor termination [14]. Interestingly, however, with the passage of time following acute stressors, or in response to chronic stressors, long lasting phenotypic variations of neurosecretory neurons occur so that increased arginine vasopressin (AVP) is present within corticotropin releasing hormone (CRH) terminals located within the external zone of the median eminence [20–22]. When released concurrently, AVP and CRH synergistically stimulate pituitary ACTH release, so that exaggerated

responses are elicited by later stressor exposure, particularly when this involves a novel insult [22]. Thus, in assessing the impact of stressors on pathological states, whether these involve psychological or biological processes, it is important to consider that stressors have proactive effects in addition to their immediate impact. Indeed, in assessing the development of PTSD, it was shown that the neuroendocrine response to trauma is influenced by previous traumatic experiences [23] and adverse early-life events likewise influenced the adult neuroendocrine response associated with a subsequent laboratory stressor [24].

1.2.2 Central Neurochemical Effects of Stressors

In addition to the HPA hormonal effects of stressors, a variety of challenges influence the activity of neurotransmitters in numerous brain regions including hypothalamic nuclei and in limbic sites. For instance, stressors promote CRH mRNA expression and increase in vivo CRH release at the central amygdala (CeA) [25–26], possibly through 5-HT and NE processes [27]. In addition to these CRH variations, stressors markedly influence the utilization of monoamines, norepinephrine (NE), dopamine (DA) and serotonin (5-HT), and the magnitude of the effects observed are dependent on several experiential and organismic factors (e.g., age, strain) [28]. The NE and 5-HT variations are notable in specific hypothalamic nuclei (e.g., paraventricular nucleus of the hypothalamus and various mesolimbic sites), as are the DA alterations (e.g., arcuate nucleus, mPFC, ventral tegmentum, nucleus accumbens shell) [28–29], and even mild stimuli (tailpinch, novelty, social defeat) and psychosocial stressors increased in vivo mesolimbic DA release [30–32]. However, if the stressor is sufficiently severe, then the increased amine utilization may exceed synthesis and levels of the transmitter decline [28]. It ought to be underscored at this juncture, however, that not all stressors necessarily induce identical outcomes. Specifically, like neurogenic (physical) stressors, psychogenic insults (e.g., psychological threats such as learned fear cues, predator odor) influence NE and amygdala CRH release [33]. However, it seems that the effects of certain stressors, particularly "prewired" predator-related challenges, induce monoamine and neuroendocrine alterations distinguishable from those elicited by learned stressors [34–35]. It is equally possible that differential neurochemical changes in humans may likewise be related to the nature of the stressor experienced.

While stressors encountered by humans can be either acute or chronic, relatively severe stressors may involve anticipatory periods (e.g., anticipation of surgery) or rumination over stressors already encountered (e.g., loss of a loved one), and in this sense these stressors have a chronic component. Thus, it especially important to consider the impact of chronic strain on neurochemical processes that may influence pathology. Of course, in this context it is essential to consider that individuals differ widely in their appraisal of stressors and in the way they cope with varied insults. Thus, in human studies that attempt to link stressors and pathology, it will be necessary to consider the contribution of these and other psychosocial factors that may influence illness vulnerability and progression.

Studies in animals have indicated that the course of stressor-provoked neurochemical changes varies with the severity, chronicity and predictability of the stressor experience. For instance, the reductions of monoamine levels associated with relatively intense acute stressors may be absent following exposure to a repeated or chronic stressor [28, 36], likely owing to a compensatory enhancement of NE and 5-HT synthesis [36–37] and moderation of DA utilization [28]. Chronic stressors also promote down regulation of ß-NE receptor activity and the NE sensitive cAMP response [38], and chronic challenge may decrease 5-HT_{1B} receptor expression [39]. It is important to underscore, however, that while the increased amine turnover may be of adaptive significance over the short-term, if neuronal changes are sustained, then the wear and tear on physiological systems may become excessive (allostatic load), ultimately rendering the animal vulnerable to a variety of pathological outcomes [40]. As will be seen shortly, it also appears that the effects of stressors on immunity may be fundamentally linked to stressor chronicity, in that the immunoenhancement elicited by acute stressors turns to immunosuppression after protracted insults [41].

As in the case of the CRH changes described earlier, it appears that the increased amine utilization elicited by an acute stressor persists for a brief period,

but can readily be re-induced upon reexposure to the same stressor (sensitization) or to a different stressor (cross sensitization) [28, 42–45]. In addition to the sensitized monoamine utilization seen upon reexposure to an acutely experienced stressor, it appears that chronic stressors also promote sensitization of mechanisms associated with amine synthesis [46]. The amine changes elicited by stressor reexposure or stressor-related cues can be attenuated by anxiolytics (diazepam) [47–48] and by 5-HT_{1A} receptor manipulations [48]. The important point for the present purposes is that both acute and chronic stressors may have long-term neurochemical repercussions, including neurotransmitters within brain regions thought to be important in contending with stressors and in mediating mood states.

1.3 Cytokine Contribution to the Stress Response and Mood States

Traditionally stressors have been considered to be of a "processive" nature, comprising either neurogenic or psychogenic insults that involve the appraisal or processing of information. Yet, the view has been taken that this definition may be too narrow and that systemic insults, such as bacterial or viral infection, ought to be considered as being stressors [49], despite the fact that they do not engender the appraisal in the same way that psychogenic and neurogenic insults do.

1.3.1 Neurochemical Consequences of Cytokine Treatment

Beyond their other functions, cytokines may contribute to communication processes between the immune system and the CNS. Despite the fact that cytokines are large, hydrophilic polypeptides that do not readily cross the blood brain barrier (BBB) [50–51], entry into the brain can occur at circumventricular organs [52], which lack an efficient BBB, or through saturable carrier mediated transport mechanisms [50], ultimately reaching various brain nuclei through volume diffusion [53]. As well, systemic administration of proinflammatory cytokines, such as IL-1β and TNFα appear to non-selectively stimulate cells of large blood vessels and small capillaries, and can disrupt the BBB [54–55]. Once present in the brain, cytokines can bind to specific cytokine receptors [56–57], and may promote activation of intracellular second messengers [58]. In addition to direct actions within the brain, increased CNS activity can be provoked indirectly through stimulation of afferent vagal fibers [52, 59–60] or receptors located at circumventricular and other vascular regions [51, 61]. As well, it seems likely that neuronal activity within limbic sites, such as the CeA, can be provoked through stimulation of the parabrachial nucleus and paraventricular thalamus [62]. Irrespective of how the brain changes come about, it seems that cytokine factors may come to promote neurochemical and behavioral changes akin to those characterizing mood disorders.

There are ample data indicating that, like processive stressors, challenges with viral and bacterial products, as well as cytokines, influence central monoamine activity [63–64] and profoundly increase the release of stress-reactive hormones, including ACTH and corticosterone [64–65]. Given the similarity between the effects of processive and systemic insults, we argued that the brain may be interpreting systemic challenges as if they were stressors [63], and as in the case of stressors, cytokine challenges were found to promote the sensitization of neuropeptide functioning so that the response to later psychogenic stressors was increased [22]. Furthermore, it was reported that treatment with an interleukin antagonist attenuated the ACTH, corticosterone and hypothalamic monoamine changes induced by a psychogenic stressor [66], indicating that cytokines act as part of the stressor-sensitive HPA loop. Of course, as already alluded to, this does not imply that processive and systemic stressors have identical effects. In this regard, it was suggested [49] that these insults impact HPA functioning through different neural circuits. It will be recalled, as indicated earlier, that processive stressors profoundly influence central amygdala and prefrontal cortical neuronal functioning and may contribute to the affective and cognitive processes related to mood and anxiety disorders. Limbic neurochemical changes are likewise influenced by cytokine challenges [52, 63], but likely do not involve appraisal processes such as those associated with processive stressors. Yet, systemic and processive stressors may act synergistically, and cytokines may provoke the sensitization of neurochemical systems,

thereby leading to exaggerated stress responses upon subsequent encounters with processive challenges [22, 63].

1.3.2 Behavioral Effects of Cytokines

Immune activation or administration of IL-1β and TNFα, induce an array of behavioral symptoms often referred to as "sickness behaviors" [67]. Although peripheral factors contribute to these effects, it appears that they may be mediated, at least in part, by central mechanisms [59, 68]. For instance, systemically administered cytokines elicit soporific effects, anorexia fever, fatigue, reduced motor activity, curled body posture [59, 69] sleep [70], and reduced sexual behavior [71], and several of these behavioral outcomes are provoked by central cytokine administration [59]. It has further been reported that cytokines disrupt operant responding for food reward [72], and disturb exploration and social interaction [59, 68]. These behavioral changes are thought to be adaptively significant as they act to minimize energy expenditure and sustain body temperature [52, 69]. In this vein, it is significant that the expression of the sickness behaviors are contextually dependent in that the behavioral signs are suppressed under conditions where this would be advantageous to the organism, e.g., in a threatening environment [73–74].

As indicated earlier, cytokines affect central neurochemical functioning and affect hormonal processes. Moreover, the nature of the changes observed are reminiscent of those associated with stressors, and are also those that have frequently been implicated as factors associated with depressive illness [28]. Indeed, the position has been advanced that activation of the inflammatory immune response may play a provocative role in the evolution of depressive illness [75]. Consistent with this view, increased cytokine activity may elicit anhedonia (i.e., a diminution in the rewarding value of otherwise positive stimuli), a symptom that is a characteristic feature of depression [76–80], and it appears that the effects of cytokines are exacerbated in previously stressed animals [81].

While cytokines clearly influence peripheral processes, it was suggested that at least some of the effects of cytokines are mediated by central mechanisms as they can be induced by direct administration into brain, and the actions of systemically administered cytokines can be attenuated by central antagonist administration [59, 82]. To be sure, it is difficult to dissociate the sickness from the motivational attributes of the cytokine treatment. After all, an animal feeling sick may be less motivated to respond in response to otherwise hedonic stimuli. Yet, it does seem that a prime characteristic of sickness (anorexia) elicited by IL-1β can be distinguished from the motivational (anhedonic) effects of this treatment. In particular, while anorexia induced by the treatment is fairly short lasting (1–2 days) the anhedonia (e.g., reflected by operant responding for sucrose on a schedule of reinforcement where a progressively greater number of responses is required for a fixed amount of sucrose, thus providing an index of the motivation to work for reward) is much longer lasting (∼4 days). Furthermore, while chronic antidepressant (fluoxetine) treatment attenuated the responding for sucrose reward, this treatment did not influence the anorexia [80].

1.3.3 Cytokines and Mood States

Consistent with the view that the inflammatory immune response might be related to depression, it has been reported that the immune profile of severely depressive patients was reminiscent of that ordinarily associated with an acute phase reaction. Specifically, increased concentrations of soluble IL-2 receptors (sIL-2R), IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-6, sIL-6R, and IFNγ were repeatedly observed among melancholic patients [75]. As well, depression was associated with increased levels of complement proteins, C3 and C4, as well as positive acute phase proteins, haptoglobin, α1-antitrypsin, β1 and β2 macroglobulin, coupled with reduced levels of negative acute phase proteins. The elevated levels of IL-1β, IL-6 and α1-acid glycoprotein normalized with antidepressant medication; however, the upregulated production of sIL-2R, IL-6, IL-10, sIL-6R and IL-1Ra in severe depression, and the elevated IL-1β seen in patients suffering from chronic low grade depression (dysthymia) [83], were not attenuated with antidepressant medication [75, 84–85]. Thus, the possibility exists that

these factors may be trait markers of the illness but do not play a provocative role in depressive illness. Of course, the possibility cannot be dismissed that normalization of these cytokines occurs with alleviation of depressive symptoms but this requires more sustained treatment or might occur over time following symptom remission.

In considering the data suggesting that cytokines affect central neurochemical processes and hence mood states, it is important to underscore that although numerous reports have examined the effects of IL-1, TNFα and IL-6, far fewer studies examined the effects of IL-2. Indeed, those studies that assessed the effects of IL-2 generally indicated that this cytokine had either weak or no effect on HPA hormonal activity [86–87], and had limited effects on central neurotransmitter functioning [88]. The central effects of other cytokines, such as IFNγ, have received still less attention. Nevertheless, studies in animals have indicated that IL-2 may induce anhedonic-like effects [86], and may affect cognitive processes [88–89]. Moreover, as will be discussed later, cytokines appear to be rather potent depressogenic agents.

1.4 Immunological Consequences of Stressor Exposure

The fact that stressors, or the behavior associated with stressors, produce changes in immune function is well documented [89]. In fact, much of the evidence has been so extensively reviewed [90–98], that little can be added that might shed new light on this phenomenon. What is perhaps more important is to address the question of whether the information that has been gathered to date can be used to aid clinical practice, especially in the field of oncology, where efforts to increase the aggressiveness of cytotoxic immune mechanisms against cancer cells may be compromised by suppressive feedback regulation by the CNS. Indeed, the neuroendocrine and autonomic nervous system responses that are elicited by immune challenges, and cytokines in particular, are believed to provide a feedback regulatory influence on ongoing immune processes. Much of this research has focused on the HPA axis, with adrenocorticoid hormones, such as glucocorticoids, serving to inhibit or attenuate the magnitude of immune responses. This may prevent the appearance of autoimmune dysregulation, as well as septic or bacteraemic shock. Whether this benefit takes place at the cost of effective immune surveillance or elimination of tumor cells, is not presently certain, since few studies systematically addressed this question. Nonetheless, there is growing evidence that administration of cytokines as part of cancer immunotherapy can produce significant deleterious effects on behavior, and these, in turn, may be associated with neuroendocrine and neuromodulatory changes that could negatively impact on therapeutic outcome.

Prediction of what types of immune parameters and how they may be affected by a specific stressor regimen is not fully understood. Studies in humans have largely relied on blood measures of immune function and in this regard, mainly in vitro measures of mitogen-induced proliferation or natural killer (NK) cell activity. Both suppression and enhancement of responsiveness have been noted, which only serve to complicate the question of prediction. More recently, there have been studies in humans that have addressed in vivo outcomes of immune function. Among the more dramatic demonstrations of this is the impact of chronic life stressors, such as caregiving (e.g., for a partner with a neurodegenerative disorder such as Alzheimer's), on reductions and/or delay of wound healing [99], possibly through an impact on reparative immune mechanisms (eg., IL-1 production) [100]. These particular findings are clinically relevant since post-operative recovery of cancer patients may be influenced by psychological factors that either preceded or followed surgical intervention. Other studies in humans have addressed antibody production in response to immunizations [101]. Here the data provide some support of the animal literature, in that humoral immune responses can be modulated by stressors, although a comprehensive review of the human literature concluded that this is by no means dramatic, nor readily predictable [101]. Much of this uncertainty is likely due to methodological compromises inherent in doing human studies of normal antibody reactivity in humans – especially, where this involves a merger with established guidelines for carrying out community vaccinations. For example, control of antigen dose and even the composition of the injected vaccine (in the case of influenza studies) can vary, as is the difficulty of determining the relationship between stressful episodes and measures of antibody.

1.5 Stress and Cytokine Production

One aspect of immune function that may be especially sensitive to stressor effects is that of cytokine production. The ability of lymphocytes and macrophages to alter synthesis and rates of cytokine production as a function of neuroendocrine impact represents an important influence on the cascade of events that culminate in the effector phase of the immune response. Hence, regulation of T-helper cell cytokines may influence antibody production and the antigen specificity of antibody subtypes. Moreover, the amplification and lytic ability of cytotoxic T cells and natural killer cells can be influenced by a variety of T cell and macrophage-derived cytokines, including interferon-γ (IFNγ) and tumor necrosis factor-α (TNF-α).

1.5.1 Stressors Influence Th1 and Th2 Derived Cytokines

In recent years there has also been an emphasis on examining the balance between Th1 and Th2 cytokine production. Shifts in this balance are considered to reflect a bias towards either proinflammatory processes through activation of macrophages (if shifted towards Th1) or humoral immune responsiveness (if shifted towards Th2). Excessive and prolonged skewing in either direction can promote various types of infectious and autoimmune pathology. Investigation of the effects of stressors on cytokine production has focused on the following Th1 cell cytokines: IL-2, IFNγ, and TNF-α; while IL-4, IL-6 and IL-10 *inter alia* – the main Th2 cell cytokines – have received the most attention (it should be noted, however, that cytokines from either Th cell subtype are also produced by other types of immune cells, as discussed further below). Early in an immune response to pathogen, predominance of Th1 cell function is typically desired to drive increased phagocytic functions (through activation of macrophages and stimulation of opsonizing antibodies). However, eventual down-regulation by Th2 cell cytokines, in particular, by IL-10, can serve to shift the immune response away from the development of an unnecessarily protracted and potentially damaging impact on tissue function. Therefore, if stressors modify the production of Th1 and Th2 cytokines, this may induce critical imbalances in their mutual counter-regulatory functional relationship, and that may result in pathology.

1.5.1.1 Impact on Interferon-γ

Assessment of the effects of stressors on cytokine production has focused on in vitro determinations after stimulation with mitogens (eg., phytohemagglutin [PHA] or concanavalin A [Con A]) and recall antigens that were used to prime animals or humans in vivo. Splenic lymphocytes from rats exposed to acutely applied stressors (e.g., electric shock or conditioned fear) showed suppressed IFNγ production following mitogenic stimulation [102–104]. Similarly, in mice, exposure to a 24 h session of restraint suppressed Con-A stimulated spleen cell IFNγ production [105], while repeated daily restraint produced the same effect in response to tetanus toxin, herpes simplex virus, influenza virus, tumor antigens, ovalbumin, and CD3 crosslinking with monoclonal antibody [106–109]. Suppression of INFγ production is not restricted to the spleen, but has also been demonstrated among cells isolated from regional lymph nodes [102–110].

As in the case of INFγ, there have been multiple reports of suppressed IL-2 production following various psychogenic stressors in rats [104–111] and mice [108, 110, 112], although failure of suppression in rat spleen cells stimulated with Con A has been noted [113]. In general, however, it is evident that exposure of laboratory rodents to a number of commonly used experimental stressors exerts a suppressive influence on Th1 cytokine production, when this is assessed by in vitro restimulation methods. However, less work is available on measures of Th1 cytokine production in vivo, which may help to determine whether the implications of these studies to susceptibility to Th1 dependent diseases can be complicated by stress. Yet, it has also been demonstrated that infectious disease processes can be promoted by stressors, indirectly validating the significance of the in vitro findings [114–115].

1.5.1.2 Impact on Interleukin-4

Interestingly, the production of IL-4, a Th2 cytokine important in regulating B cell activation and differentiation, but most prominent in promoting IgE

antibody responses, was reduced in response to a brief psychosocial stressor or academic examinations [125, 116], although in another study utilizing a social stressor (public speaking task), no changes of mitogen-induced IL-4 production were noted [127]. The discrepancy between these studies may have been due to the source of T cell activation, since Buske-Kirschbaum et al. [125] utilized the T cell superantigen, Toxic-Shock Syndrome Toxin (TSST-1) as the activating agent. Nonetheless, it is clear that further research is needed to address important interactions between stress and immunological mechanisms underlying allergic reactions, which in some cases represent a significant life-risk.

With respect to Th2 cell cytokine production induced in vitro, it has been found that IL-10 production in response to tetanus toxin and influenza virus is suppressed following prolonged restraint [106]. In contrast, restraint exposure was without effect on IL-4 production in response to T cell mitogenic stimulation [105, 108], but suppressed splenic IL-4 production induced by herpes simplex virus [112]. At first glance, the case could be made that under infectious circumstances, Th2 cell cytokine production is more likely to be inhibited by stressors in order to extend the course of Th1 mediated immune reactivity that drive inflammatory and phagocytic functions relevant to the elimination of pathogen. While suppression of IFNγ by stressors may be inconsistent with this view, reduced production of nitric oxide (NO) by macrophages stimulated with IFNγ may promote disinhibition of T cell functions, since NO has been shown to be activated by a conditioned stressor and to suppress T cell proliferation [117].

It seems that it may still be premature to make definitive conclusions concerning the impact of stressors on Th1 and Th2 cytokine functioning. It remains to be determined whether stressors differentially influence cytokine activity within different immune compartments (e.g., spleen vs. blood), and whether the effects observed are unique to certain types of stressor regimens, or even to certain types of stressors. What the available data do make clear, however, is that stressors are able to modulate cytokine activity. As more studies are conducted that vary stressor parameters, alternative interpretations may arise that will hopefully provide a uniform picture of how stressors affect Th1 and Th2 cytokine function. This is particularly pertinent since the extrapolation of animal data to human studies requires conceptual agreement that is sometimes lacking. While both human and animal data agree that stressors impact the immune system, closer examination of how cytokine production by human immunocytes is affected by stressors reveals a divergence from the animal literature. Admittedly, human studies are restricted largely to the analysis of the peripheral blood compartment of the immune system, and the obvious issues of control over subject variables such as genetic background, experiential history and age are inherently difficult to address. Nonetheless, important observations have been made with potential clinical relevance.

1.5.1.3 Impact on Interleukin-10 and Interleukin-2

Just as stressors have been found to either enhance or reduce immune functioning in humans, stressor exposure appears to be associated with both suppression and enhancement of T cell cytokine production in response to mitogens, as well as antigens. Of particular interest are studies examining the relationship of IL-10 to that of the Th1 cytokines IL-2 and IFNγ. This relationship is particularly important in that elevations of IL-10 serve to increase inhibitory feedback effects on Th1 cell activity, thereby limiting the magnitude of proinflammatory processes. Among elderly subjects vaccinated against influenza, increased levels of perceived distress as well as social activity were associated with greater IL-10 production in response to influenza antigen restimulation in vitro [118]. Interestingly, younger caregivers experiencing high levels of stress showed elevated levels of IL-10^+ T cells, without any changes in IL-2^+ or IFNγ^+ T cells [119]. This same category of subjects (viz., caregivers) had previously been shown to display attenuated responses to influenza vaccine, and reduced IL-2 production [120], which may be consistent with higher levels of IL-10 producing T cells. Similarly, in response to the distress of academic examinations, participants showed elevated IL-10 production [121–124], which was associated with reduced production of IFNγ in some studies [121, 123], but increased production of IFNγ in others [122, 124]. The latter finding is consistent with exposure of human subjects to experimentally-induced psychosocial stressors [125–127]. Moreover, the increased IFNγ

production is seen in participants with atopic dermatitis or multiple sclerosis [125, 127], suggesting that increased Th1 responses may exacerbate ongoing clinical conditions. To complement the increased production of IFNγ, it has been noted that another Th1 cytokine, IL-2, is also increased in response to examination stress, along with an associated down-regulation of IL-2 receptor expression, suggesting increased ligand-receptor interactions [128]. Overall, the reported studies suggest that elevated production of IL-10 might introduce the risk of greater inhibition of Th1 cytokine production. This may impair the initial phase of immunological responsiveness during which memory formation and effector function needs to be optimal against potential pathogens. However, the observation that IL-2 and IFNγ are also increased in response to stressor exposure suggests possible changes in sensitivity to IL-10 and/or changes in the percentage number of Th1 subsets. Moreover, the observed increases in IL-10 may be compensatory responses to the elevated Th1 cytokines. Much of this is speculative at present, and therefore, additional data are needed to fully examine this set of complex relationships.

1.5.1.4 Impact on Interleukin-5

The effects of stress on the production of Th2 cytokines other than IL-10 have also been investigated. The stress of academic examinations was found to increase the production of IL-5 in sputum-derived cells obtained from students with mild asthma, although IFNγ production was unaffected [129]. Interleukin-5 has been shown to play an important role in recruitment and/or activation of eosinophils, granulocytic leukocytes that are concentrated around the epithelium of mucosal areas such as the gut and respiratory systems, and hence important in protection against microbial infection. In fact, the stress-induced increase of IL-5 production in asthmatic individuals was associated with eosinophilia [130]; once again suggesting that increased production of certain cytokines can result in increased inflammation and life-threatening allergic reactions in susceptible individuals. Indeed, in subjects suffering from atopic dermatitis, stressor experience was shown to promote peripheral blood eosinophilia [125].

1.5.1.5 Impact on Interleukin-6

The cellular origins of cytokines tend to be quite variable, with IL-2 and IL-4 originating mainly from T cells, as opposed to other cytokines such as IL-5 and IL-10, which in addition to arising from T cells, can be produced by cells of the innate immune system, such as eosinophils, monocytes and macrophages. Moreover, many cytokines have been identified in the central nervous system, where astroglial cells tend to be the main cellular source of production. Aside from T cell-derived cytokines, therefore, it should come as no surprise that alterations in the production of cytokines emanating from innate compartments of the immune system have been observed following stressor experiences. In particular, considerable attention has been directed to IL-6, which appears to be measurable under induced and spontaneous conditions (without antigenic/mitogenic stimulation). One of the major functions of this cytokine is promotion of cell growth and differentiation, in particular among activated B cells [130], although fibroblast and neuronal growth is also influenced by IL-6 [130–131]. Consequently, stressor-induced alterations in the production and/or release of IL-6 may impact humoral immune functions, as well as the functional status of immune cells and the CNS.

The production of IL-6 among spleen cells stimulated with PHA was enhanced by exposure to a conditioned stressor, but only in lactating female rats [132], suggesting an important role for IL-6 during critical periods of physiological change. Moreover, while the IL-6 measured in this study may have been derived from T cells and macrophages present in culture, the enhanced production is consistent with evidence in mice that social disruption increases the in vitro IL-6 response of macrophages to LPS [133]. However, in vitro enhancement following stressor exposure is not universal, as viral stimulation of IL-6 production is reduced following persistent restraint [110, 112], although this may be regionally determined, since lymph nodes (and not splenic cells) displayed augmented IL-6 output in response to influenza virus [96]. Human studies have similarly reported bi-directional effects on mitogen or antigen induced IL-6 production. For example, highly stressed parents of young cancer patients showed suppressed IL-6 production [134], although the distress of exams, public speaking or exercise has been shown to augment in vitro IL-6 production in response to mitogens [121, 124,

135]. To some degree, the latter differences may be accounted for by the chronicity (and possibly intensity) of the stressor experience, since intuitively, at least, it is expected that significant qualitative differences exist in the psychological impact of coping with a child that has cancer as opposed to sitting for an exam or performing a public speaking task. As such, it is essential to examine more closely the differential impact of chronic versus acute stressor exposure, as well as the nature or severity of the stressor experiences.

Of considerable interest in view of the current emphasis on using cytokines to predict various neuropathological, psychiatric and cardiovascular disorders, is the impact of stressors on basal levels of circulating plasma cytokines. At issue in these studies is the cellular source of the cytokines that have been measured. One cytokine that has been closely monitored is plasma IL-6. Since the initial studies by LeMay et al. [136] and Zhou et al. [137] there has been a growing recognition that elevations of plasma IL-6 follow exposure to a variety of psychogenic and neurogenic stressors, such as electric shock, restraint, social-disruption, novel environments, conditioned fear stimuli, and handling [137–140]. In human studies, exposure to stressors results in similar findings [141–142]. The reliability of stressor-induced plasma IL-6 elevations has all the hallmarks of an endocrine index of distress, such as corticosterone, with which IL-6 may have a close functional relationship, in that stressor-induced corticosterone responses may rely on IL-6 production [143]. The cellular source of IL-6 in plasma within a mere hour of stressor exposure is unlikely to be an immunocyte, since splenic mRNA for IL-6 is not detectable after stressor exposure [139]. However, liver parenchymal cells respond with increased mRNA for IL-6 after exposure to restraint [139], suggesting that these may partly contribute to circulating levels of IL-6 following stressor exposure.

1.5.1.6 Impact on Interleukin-1 and Tumor Necrosis Factor-α

The determination of cytokine responses in vitro may carry the problem of biological meaningfulness. This is typically addressed through in vivo studies, although in some cases the range of stimuli used in vivo deviate significantly from those used in vitro. For example, replicating the in vitro effects of Con A or PHA is difficult, since commonly used T cell antigens (e.g., KLH) do not readily induce measureable cytokine responses in vivo. However, the macrophage activating stimulus, LPS, has proven to be a reliable in vitro and in vivo stimulus. Indeed, injections of LPS produce robust elevations of plasma cytokines in rats and mice, and it has been shown in mice that an acute episode of restraint stress can inhibit the magnitude of the IL-1 response to systemically administered LPS [144]. Interestingly, after several days of social disruption, mice challenged with LPS responded with greater amounts of IL-1 and TNFα in lymphoid regions (e.g., spleen and lung) and brain [145]. This finding is surprising in that repeated stressor exposure might be expected to reduce the proinflammatory response to LPS. However, such was not the case, and reinforces the notion that different types of stressors interacting with different forms of immunological stimulation may reveal unexpected patterns of responding that heretofore had not been expected. Indeed, stressor-induced augmentation of immune responses has previously been demonstrated in both rats and mice challenged with T cell antigens [146–148], albeit following acute stressor exposure. Alternatively, chronic stressor exposure has been shown to suppress T cell mediated immune responses in vivo [149]. Hence, the observation that LPS challenge after repeated – as opposed to acute – exposure to social disruption results in augmented cytokine reactivity [145], suggests that innate immune mechanisms, such as those involving macrophages, may be primed to react in a more exaggerated manner after chronic stressor exposure. Similar observations have recently been made in rats exposed to a single session of inescapable tailshock and subsequently shown to display a heightened IL-1β and TNFα response to LPS challenge [150]. Interestingly, the latter study also found that brain IL-1β increases were also sensitized by the prior neurogenic stressor exposure [150].

Proinflammatory cytokines, such as IL-1, have also been measured in significantly higher concentrations after stressor exposure [138, 151]. Some of these effects can actually be prevented by prior activation of the immune system, since it was shown that restraint failed to increase plasma levels of IL-1 and IL-6 only if animals had been pretreated with LPS more than a week earlier [137]. While the mechanism for this immunologically-induced “desensitization” to a psychological stressor remains to be determined, there

are opposite phenomena with respect to the neurobiological and behavioral effects of cytokines, such as TNF-α and IL-1β which was discussed earlier. For example, pretreatment with TNF-α or IL-1 has been shown to sensitize animals to greater neuroendocrine and behavioral responses to additional cytokine and/or psychogenic stressor exposure.

The foregoing discussion of the effects of stressors on cytokine production by lymphocytes and macrophages, as well as the elevation of plasma cytokines (most notably IL-6), highlights the importance of CNS-mediated effects on immunological processes. As discussed earlier, certain cytokines, such as IL-1, TNFα and IL-6, target neuroendocrine pathways in the brain, ultimately causing elevated peripheral levels of noradrenergic and glucocorticoid activity. Whether this efferent output from the brain is in response to psychogenic or systemic stimulation, the consequences may be similar, namely modification of peripheral cytokine production. This is important to keep in mind, considering that immunotherapy with cytokines is in principle similar to experimental studies that established the impact of cytokines on CNS function. Therefore, the patient that receives large doses of IL-2 or IFNα may respond to these cytokines both at the immunological level and that of the CNS. Activation of the latter can result in neuroendocrine responses that may conceivably impact ongoing immune processes. Moreover, neurobehavioral changes may render the individual more sensitive to psychogenic stressors, ultimately leading to the question as to whether exogenous treatment with cytokines for therapeutic reasons, may actually result in serious dysregulation of endocrine functions that militate against optimal therapeutic outcome. These questions have not been fully addressed, but as will be indicated shortly, there is evidence that cytokine immunotherapy results in serious behavioral consequences.

1.6 Cytokines and Depression

Studies in animals, as indicated earlier, have revealed that treatment with IL-2 as well as proinflammatory cytokines gives rise to central neurochemical changes that have been linked to depressive illness. Likewise, in animals these cytokines promote neurovegetative and behavioral changes reminiscent of those that characterize depression in humans [63]. Paralleling these findings, severe depression in humans has been associated with increased circulating levels of various cytokines and their soluble receptors, as well as increased mitogen-stimulated cytokine production [75]. The relationship between cytokines and depression was further reinforced by the finding that the administration of an endotoxin to humans induced changes of mood, particularly the induction of mild depression [152].

Another line of evidence comes from studies that evaluated the effects of immunotherapy (IL-2, IFNγ and TNFα in humans undergoing treatment for various forms of cancer or for hepatitis C). These studies indicated that high doses of these cytokines induced neuropsychiatric symptoms, including depression and/or anxiety, often of sufficient severity to require discontinuation of therapy [153–170]. Moreover, it seemed that many of the diverse effects of the cytokine treatments were dissociable (e.g., sickness vs. mood changes). Specifically, it appeared that the somatic and neurovegetative symptoms (such as anorexia, fatigue and pain) emerged during the initial 2 weeks of IFNγ treatment, whereas mood-related symptoms (depression, anxiety) and cognitive disturbances tended to appear later [156].

The processes through which cytokines induce depressive symptoms remain to be elucidated. It will be recalled that cytokines affect CRH and monoamine activity within several limbic sites, and it was suggested that these neurochemical alterations give rise to the affective disturbances [78]. Thus, it is particularly significant that the depressive symptoms provoked by IFNγ were attenuated by treatment with the selective 5-HT reuptake inhibitor, paroxetine [171]. Moreover, it appeared that the antidepressant markedly influenced mood-related symptoms, whereas fatigue and anorexia were hardly attenuated by the antidepressant treatment. In effect, these data are consistent with a causal role for cytokines in depressive illness, and also indicate that the cytokine-provoked mood and sickness effects can be dissociated from one another, such that antidepressants act principally on mood-related characteristics. Further, these data raise the possibility that the efficacy of immunotherapeutic treatments, by virtue of the diminution of side effects, can be augmented by appropriate antidepressant pretreatment. At the same time, it ought to be considered that the effects of cytokine therapy were assessed in a fairly atypical

population, namely patients with severe illness that certainly would have been associated with considerable distress. In effect, the actions of the cytokines may have reflected the additive or synergistic actions of the treatment superimposed on the backdrop of a stressor. In this regard, there are indeed indications that stressors and cytokines may synergistically affect neurochemical functioning [63].

1.7 Conclusion

Admittedly, we have circumvented the issue of whether stressors exacerbate cancer progression. Simply put, the available data are frequently confusing or contradictory, and insufficient prospective information is available to assess this relationship adequately. There are certainly ample data indicating that stressors impact immune functioning, although the nature of the effects observed vary with a host of conditions, including the nature of the stressor, its severity and chronicity, previous stressor experiences, and the immune compartment being examined. Importantly, the way a stressor influences these processes, as it affects others, is likely related to the way the individual appraises or interprets challenges, and the coping method endorsed to deal with such insults. In the case of cytokine changes an array of different factors influence the impact of stressors, and these effects seem to vary with the specific cytokines being appraised, and no doubt will vary across individuals, depending on a constellation of organismic and experiential variables. Clearly, whether the various immune and cytokine changes provoked by stressors affect tumor progression will be difficult to decipher.

Paradoxically, while a link has often been proposed between stress and cancer progression, cytokines which have multiple stressor like effects and may induce depression, have been shown to attenuate the course of some cancers. Thus, it might reasonably have been expected that stressors, like cytokines, would actually have an attenuating effect on neoplastic disease. A resolution of this mismatch may not be readily forthcoming. However, we provisionally offer the suggestion that to understand the relationship between stressors and cancer, it is essential to distinguish between acute, subchronic, and chronic unpredictable stressors. While mild and acute stressors may instigate adaptive neurochemical systems that militate against adverse outcomes, including tumorigenic actions, stressors experienced on a chronic, unpredictable basis may lead to excessive utilization of those adaptive resources that ordinarily act against pathology. It will be recalled that with sustained stressors the wear and tear on neurochemical systems may become excessive (termed allostatic load), precluding adaptive responses being mounted [40], hence rendering the organism more vulnerable to pathology. Further, it is proposed that among individuals who are, for whatever reason, particularly vulnerable to stressor effects, subchronic insults may be sufficient to exacerbate the development of pathology. We suggest that such outcomes will be most pronounced among those individuals that had previously encountered traumatic events or protracted stressors that sensitized those neurotransmitter, neuroendocrine, or cytokine systems that otherwise would serve in an adaptive capacity.

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Chapter 2

The Role of Oncogene Activation in Tumor Progression

Michael J. Gray and Gary E. Gallick

Contents

2.1 Introduction

Normal cells become tumorigenic after multiple genetic and epigenetic alterations. This process alters complex signaling networks within these cells as well as interactions between these cells and the extracellular matrix. Cell growth and proliferation is a tightly regulated process in normal cells. Several cytokines and growth factors are capable of inducing proliferation, motility, and survival. Among the most widely studied mitogens are epidermal growth factor (EGF) and hepatocyte growth factor (HGF). These cytokines and/or growth factors activate specific transmembrane receptors that undergo phosphorylation events and in turn activate intercellular secondary messengers such as the Ras and Src oncogenes. Targets of these secondary messengers are numerous but include proteins that are involved in gene regulation, cell cycle components, and survival pathways.

The number of identified oncogenes involved in the malignant transformation process is abundant and growing. Oncogenes generally fall into one of three classes, those that initiate a signaling event from external stimulation such as tyrosine kinases receptors at the cell surface, those that are components of intracellular signal transduction cascades, and those that control gene expression. While it is beyond the scope of this review to focus upon the role of all oncogenes linked by one or more studies to malignant transformation, an examination of key discoveries to date will illustrate members of several types of oncogenes, the mechanisms of their activation, and roles in tumorigenic growth and tumor progression.

2.2 The Epidermal Growth Factor Receptor Family

The epidermal growth factor receptor (EGFR) mediates numerous essential processes in normal cells

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including proliferation, survival, differentiation, adhesion and migration. Four EGFR family members (also known as the erbB family) exist in vertebrates: EGFR/ErbB-1, HER2/ErbB-2/neu-2, HER3/ErbB-3, and HER4/ErbB-4. The EGFR family contains an extracellular ligand binding domain, a trans-membrane domain, and a cytoplasmic domain with tyrosine kinase activity that is required for signal transduction [1]. At least eight ligands are capable of activating these receptors including EGF and transforming growth factor α (TGF-α) [2–5]. Unstimulated EGFR exists as a monomer but upon ligand binding forms either homo- or heterodimers with other EGFR family members [6]. Dimerization of the receptor results in activation of the receptors intrinsic kinase ability and tyrosine autophosphorylation and/or transphosphorylation occurs [1]. The ability of the EGFR family to heterodimerize allows for diversification of signal pathways [7, 8]. The phosphorylated residues of EGFR recruit and activate both transducer and adapter proteins that coordinate and activate downstream signal events. Numerous studies have shown the requirement for the cytoplasmic kinase domain of EGFR in normal and neoplastic signal transductions.

2.3 Mechanisms of EGFR Dysregulation

Dysregulation of the EGFR occurs by a variety of mechanisms including overexpression of ligand, EGFR, or both. In patients with lung carcinomas overall survival was greatly reduced in those groups that expressed high levels of EGF or TGF-α compared to those that did not [9, 10]. Some neoplastic tissues express both EGFR and one or more ligands capable of activating the receptor allowing autocrine activation of the receptor [11]. Overexpression of EGFR has been shown to inhibit receptor turnover presumably by overwhelming limiting factors involved in EGFR degradation [12, 14]. Mechanisms other than overexpression of EGFR or its ligands can also affect EGFR signaling. Mutations resulting in constitutive activation of the kinase domain have been identified in numerous cancers including brain, lung, stomach and breast, but have yet to be documented in nonmalignant tissues.

An alternative mechanism that results in increased activation of EGFR dependent signal transduction pathways occurs from EGFR heteodimerization with the EGFR family member HER2/ErbB2 as well as with other heterologous receptor systems. Overexpression of HER2 is associated with breast cancer and coexpression with EGFR is linked to poor patient prognosis [15, 16]. HER2 does not bind EGF or other EGF related ligands; rather it acts as a coreceptor by increasing the ligand affinity of its binding partner in the heterodimeric complex. EGFR/HER2 heterodimers result in elevated basal levels of activated receptors and increased activation of EGFR signaling networks [17–19]. Coexpression of EGFR and HER2 promotes cellular transformation and contributes to hormone independence of some cancers in vitro and overexpression of EGFR/HER2 correlates with poor patient prognosis [20–23]. Heterodimerization of EGFR and HER2 also results in increased receptor stabilization in part by down-regulating degradation and recycling pathways [8, 6]. In addition, EGFR-HER2 heterodimers differentially activate additional signal transduction pathways from those of EGFR homodimers [6].

Cross-talk between EGFR and other heterologous receptors such as the thrombin, endothelion-1, and LPA receptors can also influence EGFR activation [24–26]. These receptors, known as G-protein-coupled receptors (GPCRs) activate numerous downstream events that can in turn activate EGFR directly or indirectly. These mechanisms include the activation of Src which can phorsphorylate EGFR at sites not targeted via EGFR autophosphorylation [27, 24, 28]. GPCRs can also activate metalloproteinases that target membrane-anchored pro-ligands that are activated by cleavage which can then bind and activate EGFR [29, 30].

2.4 The EGFR Receptor Signal Pathway

Activation of EGFR results in a series of complex downstream signal transduction events. Auto- and transphosphorylation of the kinase domain of EGFR allows the recruitment and activation of Src homology-2 domain (SH2) containing adapters and transducers including SHP-2, GRB2, phosphatidylinositol 3-kinase (PI3K), Akt, phospholipase C-γ (PLC-γ), and members of the STAT transcription family [31–37]. A significant pathway activated via GRB2 is the Ras/Raf/mitogen activated kinase (MAPK) cascade [38, 39]. In addition to promoting cellular

proliferation, EGFR activation of PI3K also plays an important role in tumorigenesis and cell cycle progression [36, 37]. The cell cycle inhibitor p27 has been shown to be down-regulated via the PI3K/Akt pathway [40]. In addition, the angiogenesis promoting peptide vascular endothelial growth factor (VEGF) is transcriptionally upregulated by EGFR activation of PI3K [41–43].

2.5 EGFR-Targeted Therapy in Human Cancers

It is well established that disruption of normal EGFR activity occurs in numerous human tumors including breast, head and neck, gastric, ovarian, renal, pancreatic, bladder, colon, and non-small cell lung cancer (NSCLC) [44]. EGFR activation contributes to tumor progression by promoting angiogenesis, cell survival, proliferation, and invasion/metastasis [45–49]. Increased EGFR activation in malignant tissues can also result in resistance to standard therapies and poor patient prognosis [50–57].

Therapies directed towards the specific downregulation of EGFR on malignant tissues are being developed [58, 59]. A number of monoclonal antibodies designed to prevent EGFR activation by blocking ligand binding are being employed (e.g. IMC-225 and ABX-EGF). IMC-225, also currently in clinical trials, inhibits cell growth and survival in vitro and in vivo, inhibits angiogenesis, induces apoptosis in some cell lines, and reduces metastatic capability [60, 48, 61, 62]. Bispecific antibodies that target both EGFR and epitopes on immune surveillance cells are also being studied in the attempt to initiate immune system recognition and removal of malignant tissues (MDX-447). Additional antibody derived therapies employing the conjugation of toxins to antibodies specific for EGFR have also shown some success (scFv-14e1-ETA-Fusion toxin) [63].

Targeting the EGFR tyrosine kinase domain is also being extensively pursued as a therapeutic approach using chemical inhibitors. These EGFR tyrosine kinase inhibitors (EGFR-TKIs) include ZD1839 (Iressa) and OSI-774, and prevent receptor activation by blocking ATP from reacting with the kinase domain. ZDS1839 has been shown to increase apoptosis, reduce cell proliferation, induce cell arrest, inhibit angiogenesis, and reduce invasion/metastasis [64–67]. ZD1839 is undergoing evaluation in phase III clinical trials on non-small cell lung carcinoma (NSCLC) in combination with other therapeutic agents but results have been somewhat disappointing after encouraging results from phase I and II trials [68–70]. Phase I and II clinical trials using OSI-774 in mono and combinatorial therapy have provided some encouraging results and phase III trials in pancreatic and lung cancers is currently being undertaken [71, 72]. Other anti-EGFR therapies include nucleotide antisense technology to inhibit translation of EGFR and/or its ligands, but of the current therapies IC-225 and the EGFR-TKIs ZD1839 and OSI-774 are the furthest developed tumoricidial agents.

2.6 Role of the EGFR Family Member ErbB2/HER2 in Breast Cancer

Abberant expression of the HER2 (also know as c-neu) gene occurs in a variety of human cancers but perhaps has been most studied for its role in breast carcinoma. Amplification of the HER2 gene in breast cancer is estimated at 20–30% [73]. HER2 expression/amplification is used as an independent prognostic factor towards patients' survival rates. HER2 dysregulation is associated with an aggressive disease and poor prognosis [74]. Overexpression of HER2 enhances proliferation, metastasis, and induction of angiogenesis and anti-apoptosis promoting factors [75, 76]. Activation of HER2 requires heterodimerization with other EGFR members for transphosphorylation as HER2 has no distinct activating ligand [77]. Interestingly homo- and heterodimers of the HER family have differential degrees of mitogenic stimulation. Heterodimers of HER2-HER3, the dominant HER complex found in carcinoma cells, are the most potent mitogenic combination [78–79]. The neuregulins (NRG, also known as neu differentiation factor (NDF), and heregulin) are the activation ligands of both HER3 and HER4. HER2 increases the affinity of its dimerization partner for its ligand in addition to increasing receptor stabilization, thus allowing enhanced activation of downstream pathways [8, 6].

The signaling pathway of HER2 is still being elucidated but, HER2 activation induces downstream

effector molecules involved in cell proliferation and metastasis [80–82]. These effectors include the cell proliferation ERK/MAPK pathway and members of the ETS transcription factors that regulate the expression of matrix remodeling proteins [83–85]. While these findings suggest that the aberrant activation of effectors pathways by HER2 overexpression may contribute to the development of metastatic breast cancer, the molecular pathway is still incompletely understood. This is complicated in part due to the ability of the EGFR/HER receptors to form some 9 potential dimer combinations allowing considerable signal diversification [78, 86]. Further investigation into HER2 biology will not only help decipher its role in breast cancer progression but also assist in the development of therapeutic agents.

Because gene amplification in cancer cells results in HER2 protein levels that may be 100 fold greater than in normal tissues, it has received rigorous examination as a potential therapeutic target [87, 88]. Therapies directed towards blocking HER2 expression and function are the two primary focuses. These therapies include the introduction of antisense oligonucleotides into cells directed towards HER2 or blocking the kinase activity of the HER2 receptor by chemical inhibitors [89, 90]. The greatest progress in HER2-targeted therapies has been made using monoclonal antibodies directed towards the extracellular domain of the HER2 receptor. Studies have suggested that these monoclonal antibodies may function by blocking requirements of cancer cells including angiogenesis, proliferation, and survival [91–94]. Currently the recombinant human antibodies (rHuMAb-HER2) Herceptin and tratuzumab have shown encouraging results in phase II and II clinical trials and are currently being used clinically in the treatment of patients with metastatic breast cancer [95, 96].

2.7 The c-Met Receptor

The tyrosine kinase receptor c-Met was first identified by chemically induced transformation of human osteogenic sarcoma (HOS) cell line [97]. In this transformed HOS cell line, the c-Met protooncogene on chromosome 7 underwent a translocation that resulted in a fusion protein that contained a constitutively active Met kinase domain [98]. Isolation of the cDNA of this oncogenic protein led to the identification of the full length c-Met receptor [97]. The transmembrane protein encoded by the c-Met gene is unusual compared to other kinase receptors in that the inactive single chain precursor undergoes intracellular proteolytic processing yielding a disulfide-linked heterodimer. The cytoplasmic c-terminal domain of the c-Met receptor contains a multifunctional site that has been shown to interact with a variety of signaling molecules [99–100]. The structural and biosynthetic properties define the Met receptor tyrosine kinase family which consists of three related members, c-Met, Ron, and c-Sea [101–103].

The ligand of the c-Met receptor is hepatocyte growth factor (HGF), also known as scatter factor (SF) [104, 105]. HGF/SF was initially identified as a mitogen for hepatocytes and a scattering/motility factor for epithelial cells [106–109]. Additional studies have shown that other cell types are biological targets of HGF/SF including endothelium, myoblast, hematopoietic and some neuronal tissues. The c-Met-HGF/SF signaling pathway effects a wide range of biological processes, including angiogenesis, embryonic development, organ regeneration, wound healing, cellular motility, proliferation, and invasion [110–118, 106, 119, 120]. Null mutations, of either HGF/SF or c-Met, result in embryonic lethality [116–117].

While regulating a wide range of essential physiological processes, the dysregulation of c-Met receptor kinase activity result in tumor development and progression [121]. c-Met-HGF/SF signaling can increase tumorigenicity in part by escalating cell invasiveness and metastatic capability [122]. Accumulation of high levels of HGF/SF in tumors is correlated with poor patient prognosis and a highly aggressive and invasive phenotype [123–125]. Overexpression of c-Met has been shown to occur in carcinomas of the colon, pancreas, ovary, and thyroid in addition to other types of cancers [98, 126–127]. Expression of both HGF/SF and the c-Met receptor is seen in gliomas, osteosarcomas, pancreas, lung, and breast cancers among others creating an autocrine loop and increased tumorigenicity [128–133]. Point mutations of the c-Met receptor resulting in constitutive activation occur in hereditary and sporadic papillary renal carcinomas, hepatocellular and gastric carcinoma and squamous cell carcinomas of the head and neck [134–138]. Mutations in the c-Met receptor are also a contributing factor in primary cancers progression towards metastasis [122, 139].

Reduction of c-Met expression or activation in malignant tissues reduces or inhibits tumor growth, invasive, and metastatic potential [140–144].

2.8 The c-MET-HGF/SF Signal Pathway: Role in Malignant Progression

Activation of the c-Met receptor initiates a wide variety of signal pathways in the target cell. The c-Met receptor is a cell surface glycoprotein composed of an extracellular α-subunit and a transmembrane β-subunit. Binding of HGF/SF to c-Met, causes an increase in the tyrosine kinase activity of the β-subunit resulting in receptor autophosphorylation [145–147]. C-Met signaling is controlled through a multifunctional docking site comprised of two tandem arranged phosphotyrosines [146]. Mutational analysis has shown an absolute requirement of the multifunctional docking element for both physiological and pathophysiological properties of the c-Met receptor. Upon autophosphorylation this docking site binds and activates numerous SH2-containing adapters including GAB1, SHC, GRB2, and the signal transducers phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ (PLC-γ), Src, Akt, SHP2 phosphatase, and STAT3 [99, 148, 100, 126, 149–156] (Fig. 2.1), Interestingly, promotion of invasive growth by activated wild-type c-Met in neoplastic tissues is dependent upon physical association with the α6β4 integrin. The integrin serves as an additional docking platform for HGF/SF dependent transducers including PI3K and SHC that act synergistically with activators binding the c-Met catalytic domain [157].

Considerable data exist upon the individual roles of these signaling molecules and adapters in C-Met-mediated tumor progression. GRB2 is essential for

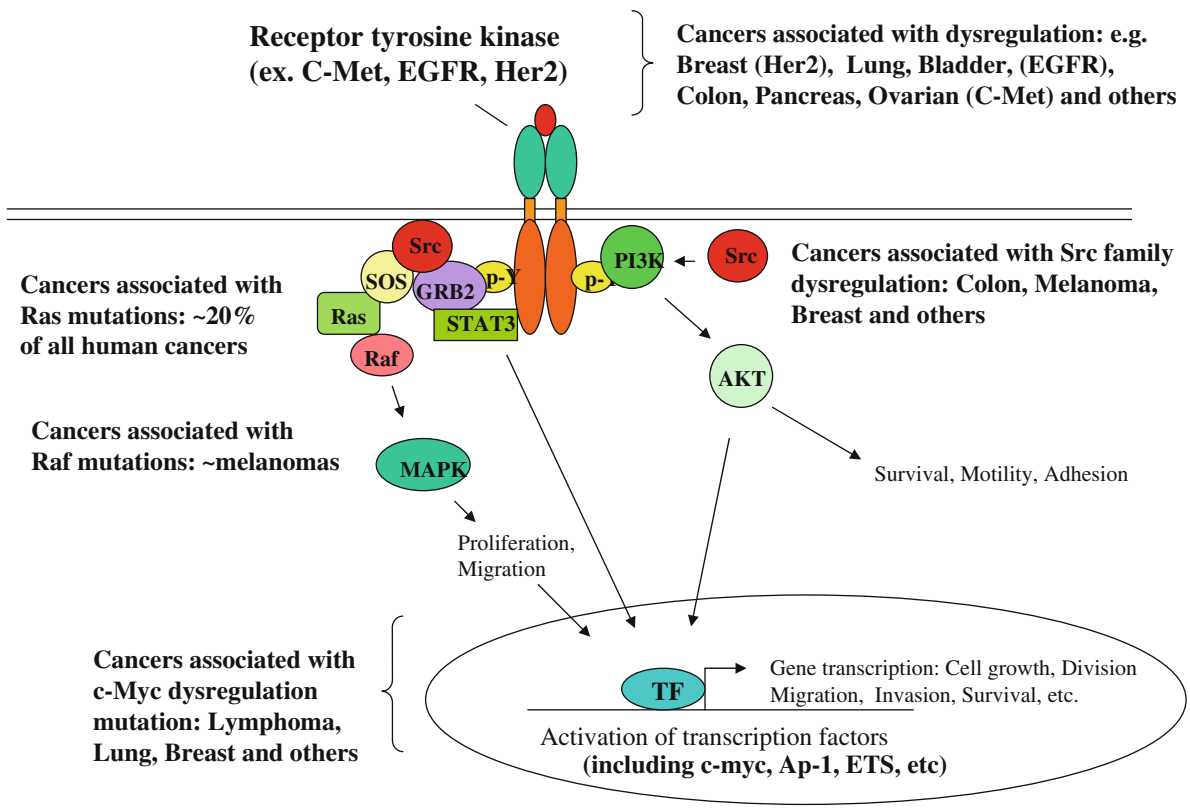

Fig. 2.1 Abbreviated signaling pathway mediated by protein tyrosine kinase receptors. Overexpression of receptors often contributes to malignant progression, and leads to activation of "downstream" proto-oncogenes. As discussed In the text, activation of these downstream signaling molecules may occur independently of receptor activation. Some of most frequent tumors in which activation occurs are shown in the Figure; however, many other tumors have these alterations.

HGF induced c-Met function including motility and invasion [158, 147, 159]. GRB2 links SOS to the activated c-Met receptor and results in the initiation of Ras signaling leading to MAPK activation [147]. In addition to controlling cell proliferation MAPK increases the invasiveness and metastatic capability of c-Met hyper-activation via upregulation of the serine protease urokinase (uPA) and it's receptor (uPAR) [160, 161]. Protease urokinase degrades components of the extracellular matrix (ECM) while activating additional proteases including plasmin and metalloproteinases (MMPs) that further degrade both the ECM and basement membrane [162]. PI3K is involved in the mitogenic effect of c-Met-HGF/SF activation and in conjunction with AKT inhibits apoptosis and enhances DNA repair [163, 164, 153, 156]. C-Met activated Src results in increased motility and anchorage independent growth [152]. Src has been shown to interact with paxillin and focal adhesion kinase (FAK), two kinases that participate in cytoskeletal rearrangements [165]. Currently, small molecule inhibitors of c-Met are in development and may prove to be important tools in the treatment of tumors with aberrant c-Met expression or activation.

2.9 The Ras Oncogene

The three members of the Ras family small GTP-binding proteins, H-, N-, and K-Ras, are important regulators of essential cellular processes including proliferation, differentiation, survival and apoptosis. While the Ras genes share a high degree of homology they are not entirely functionally redundant as only K-Ras is essential for development [166–168].

Ras acts as a membrane bound molecular switch, which cycles between a GTP-bound active and a GDP-bound inactive state. The cycle is regulated via guanine-nucleotide exchange factors (GEFs) that promote the active GTP-bound Ras, and GTPase activating proteins (GAPs), which increase the low-intrinsitic GTPase activity of Ras. Ras activation occurs following extracellular stimulation and receptor phosphorylation events that recruit GEFs to the plasma membrane where they bind via adapter proteins and induce the active Ras-GTP complex. The GTP-bound Ras activates several critical effectors including the serine-threonine kinase Raf-1. Raf-1 activates the extracellular-regulated kinase pathway (Erk, also known as MAPK) which regulates proliferation and differentiation [169, 170]. Other Ras activated effectors include phosphatidylinositol 3-kinase (PI3K), which is involved in survival, proliferation and metabolism, and the nucleotide exchange factors for Ral GTPase RalGDS [171–173].

2.10 Mechanisms of Ras Dysregulation

Ras signaling in normal tissues is transient due to the gradual intrinsic guanine triphosphatease (GTPase) activity of Ras itself and by cytoplasmic GTPases that rapidly convert Ras into the GTP-bound inactive state. Dysregulation of Ras function typically occurs from overexpression, mutation, or loss of GTPases that target Ras. Ras overexpression has been documented in a variety of human cancers including neuroblastoma, colon, lung, breast, bladder, head and neck, and stomach [174–188]. Interestingly, the correlation of elevated Ras expression to patient prognosis is extremely variable. Head and neck cancers and neuroblastomas with elevated Ras have a favorable patient prognosis while gastric, colorectal, and lung do not [176, 181, 184, 186]. This disparity may be due in part to the stage of disease when Ras dysregulation occurs. Ras overexpression is postulated to be an early event in head and neck and neuroblastoma cancers, while in colorectal, lung, and gastric cancers a late stage event. Ras overexpression in late stage disease may accompany other genetic alterations that promote a more aggressive disease.

Ras mutations found in human cancers that induce malignant transformation have been reviewed extensively. Ras mutations are almost exclusively found in amino acids 12, 13, 59, and 61 [189]. These mutations alter the GTPase activity of Ras by preventing GAPS from promoting the hydrolysis of GTP-bound Ras resulting in constitutive Ras activation. Hyperactive Ras can also result from the loss of GAP expression or activity. Loss of the GTPase tumor suppressor gene product NF1 results in elevated Ras activation and neurofibromatosis type 1 cancers. These cancers include benign and malignant tumors of neural crest origin including melanomas [190, 191]. Enhanced Ras activation can also occur in response to increased stimulation by upstream mediators. These upstream mediators are

numerous but include kinase receptors such as c-Met, EGFR, and platelet derived growth factor receptor (PDGFR).

2.11 Ras Mediated Signaling Cascade: Implications in Malignant Progression

Increases in Ras activation result in the activation of downstream effectors that can promote oncogenesis and malignant progression. The most heavily studied Ras effector is the serine-threonine kinase Raf-1. Activated Raf phosphorylates and activates the mitogen-activated protein kinases 1 and 2 (MEK1 and MEK2), which in turn activate the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 (extracellular signal regulated kinases 1 and 2). Targets for the ERK proteins are numerous but include the Ets family of transcription factors [192, 193]. Genes activated by the Ets family include components of the AP-1 transcription factors which induce division by activating the cellular machinery that drives proliferation such as the D-type cyclins [194–196]. Ets also regulates genes that encode genes that are involved in invasion and metastasis including matrix metalloproteinase MMP-1 and MMP-9. The products of these genes participate in the degradation and remodeling of the extracellular matrix [197–198].

Additional effectors activated by Ras include phosphatidylinositol 3-kinase (PI3K). PI3K regulates a number of important cellular processes such as proliferation, cytoskeletal rearrangements, transformation, and survival. Akt/PKB promotion of cell survival results from the induction of survival signals and downregulating of apoptotic inducing signals [47, 199]. Targets of AKT/PKB survival signals include both transcription factors and regulatory proteins [200, 47, 201–203]. Transcription factors activated by AKT/PKB include CREB, NFκB, and the Forkhead family member FKHRL1 [204]. Phosphorylation of FKHRL1 by AKT/PKB results in reduced transcription of the apoptosis-inducing factor Fas ligand [205]. Additional AKT/PKB survival signals include the desensitization of TNF-related apoptosis-inducing ligand (TRAIL) and downregulation of glycogen synthase-3 (GSK-3) which suppresses proliferation and survival, and inactivation of the pro-apopotic Bcl-2 family member BAD [205–210].

2.12 Ras in Human Cancers and Targeted Ras Therapies

Approximately 20% of all human cancers contain Ras point mutations resulting in activated Ras. Some specific cancers have a high prevalence of Ras mutations (90% of pancreatic cancers, 50% of colon carcinomas, and 30% of lung carcinomas) [189, 166]. Due to the large numbers of tumors having Ras mutations the development of Ras specific inhibitors that target specific components of the signal transduction pathway is being pursued. One therapeutic strategy employs farnesyltransferase inhibitors (FTIs), which block the covalent attachment of a farnesyl isoprenoid group to the carboxy-terminal of Ras and are a requirement for membrane localization and activation. Despite preclinical evidence that these inhibitors (R115777 and SCH66336) may be effective in interrupting Ras mediated pathways, clinical results have been disappointing. Potential discrepancies between preclinical and clinical results may be due to the inability of the FTIs to effectively block the function of all isoforms of endogenous Ras. This is supported by the lack of toxicity from the FTIs in normal tissues, where Ras proteins are required for essential cellular functions. Additionally, FTIs may not exclusively target the farnesyl isoprenoid of Ras, but also other proteins similarly modified.

Other Ras specific therapies utilize kinase inhibitors that target components of the Ras pathway. These inhibitors target upstream activators of Ras such as growth factor receptors (EGFR receptors as discussed earlier), and downstream components including the RAF/MEK kinase pathway (BAY 43-9006 and CI-1040/PD184352 respectively). CI-1040/PD184352 has been shown to effectively inhibit proliferation, survival, and metastasis of some tumor cell lines in some preclinical models and is currently undergoing evaluation in clinical trials. The Raf inhibitor BAY 43-9006, which also targets the BRAF kinase, has been shown to reduce some proliferative pathways in patients and analysis of this drug in phase II and III trials will be significant. Additional therapies that may reduce Ras activation target the upstream

activators of Ras including EGFR and are discussed previously.

2.13 The Src Kinase Family

Src was initially discovered as an oncogenic protein of the Rous sarcoma retrovirus [211]. The transforming gene of the Rous sarcoma virus, v-src, is a mutated and activated form of a normal cellular gene, c-src, which encodes a protein with intrinsic tyrosine kinase activity. Following its initial discovery, eight closely related members have been identified which comprise the Src family kinases (SFKs); Src, Fyn, Yes, Lck, Hck, Fgr, Lyn, and Blk. All Src family members are composed of unique protein domains including an amino terminus membrane-targeting known as the SH4 domain (Src homology 4), a poorly conserved unique domain, an SH3 domain, SH2 domain, tyrosine kinase domain, and a regulatory region [212]. Src is maintained in an inactive state by intramolecular interactions between the SH2 and SH3 domains controlled via phosphorylation of Y-527 that restricts accessibility of the kinase domain for ATP and substrates. Activation of SFKs occurs by two primary mechanisms, dephosphorylation of Y-527 or by phosphotyrosine proteins that bind to the SH2 domain and prevent the inactive conformation. These mechanisms allow the activation of the intrinsic protein kinase activity and phosphorylation of critical tyrosine residues in the activation domain [213, 212].

The SFKs are membrane-associated non-receptor tyrosine kinases that regulate critical signal transduction pathways. While most of the SFKs are expressed in cells of hematopoietic origin, Src, Yes and Fyn show a ubiquitous pattern of expression and are highly expressed in platelets, neurons, and some epithelial tissues [214]. The Src kinases are activated in response to specific cellular signals and induce proliferation, survival, motility, and invasion. These kinases also participate in the regulation of cytokine receptors, receptor protein kinases, g-protein linked receptors and integrins [214]. While the Src kinase families are critical in normal cellular responses, overexpression and/or hyper-activation of specific family members occurs in some human cancers. C-Src c-Lck, and c-Yes dysregulation occurs in cancers of epithelial origin including colorectal, head and neck, and breast [215–221]. Other family members are postulated to have a role in cancers of hematopoietic origin including leukemia and lymphomas [222, 223].

2.14 Src Dysregulation

The dysregulation of Src family members has been extensively studied in human colorectal cancers and to a lesser extent in breast cancer. Src protein levels and kinase activity are frequently elevated in colon carcinoma relative to normal colonic muscosa [224, 215]. Studies also suggest that the progression of primary tumors to metastasis in colon cancers correlates with an increase in Src kinase activity and protein expression [217, 225]. The most common mechanism leading to aberrant Src expression or activity is through increased expression or activation of growth factors receptors that recruit and activate Src, or through improper regulation and/or loss of non-receptor factors that regulate Src kinase activity. The epidermal growth factor receptor (EGFR), Her2/Neu, and c-Met receptors are all commonly overexpressed or overactivated in a variety of human tumors including breast and colorectal cancers [226, 44, 227]. These receptors activate Src and elevated Src activity has been shown to increase metastatic and tumorigenic potential of some cancers [148, 228, 229]. Dysregulation of Src activity can also occur though aberrant expression or activity of proteins that regulate Src. The Csk family is comprised of critical negative regulators of Src and reduced expression of Csk with a subsequent increase in Src activation occurs in some cancers [230–232]. Csks overexpression in animal models results in reduced Src activation and suppression of metastasis of colon cancer cells [233]. Several potential positive regulators of Src activation have also been identified including PTP1, PTP-α, PTPO1, SHP-1 and SHP-2. Reduced expression of SHP-1 is capable of function as a positive regulator of Src function and elevated expression of PTP1B, with accompanying elevated Src activity, occurs in some breast cancer cell lines [234, 235]. Interestingly, mutations of the Src catalytic region resulting in a constitutively active isoforms has been reported but similar studies have not substantiated these results, suggesting that mutations of Src resulting in activation are a rare event and not a major contributor to Src's role in malignant tissues.

2.15 The Src Signaling Pathway–Implications in Malignant Progression

Downstream targets of Src family members are numerous and regulate many of the same functions essential to development, including proliferation, apoptosis, angiogenesis, and invasion/migration [214]. Src participation in cell proliferation is in part controlled via positive and negative regulators of the cell cycle. V-Src suppresses the cyclin kinase inhibitor p27 and induces expression and activation of cyclins which prevents a quiescent state and promotes proliferation [236]. Additional studies have shown that c-Src is required for mitogenic functions including DNA synthesis induced by the PDGF, EGF and CSF (colony stimulating factor) tyrosine kinase receptors [237, 238]. Src is also specifically required at the G1 and G2 to M phase transition in cell division [239, 240]. Transcription factors that undergo activation by Src include c-myc and members of the STAT family that regulate genes central to cell proliferation [241, 242]. STAT3 and STAT5 are have been shown to be constitutively activated in squamous cell carcinomas of the head and neck (SCCHN) where EGFR is commonly overexpressed and hyperactivation of Src occurs [242]. While these studies suggest a direct role of Src in inducing cellular proliferation, other reports utilizing mutated PDGF receptors which fail to activate Src yet DNA synthesis still occurs, contradict these conclusions [243]. Possible explanations for these opposing results may be that basal levels of active Src are sufficient for cell proliferation or that Src activation does not require direct interactions with the PDGF receptor but occurs via other PDGF dependent mediators.

Src signaling has also been implicated in cell survival [244]. V-Src mediated anti-apoptotic pathways are dependent upon activation of the PI3K and AKT/PKB pathway [245]. Prior studies have shown that Akt/PKB promotes cell survival by phosphorylation of BAD, Caspase-9, and FKHR1 [47, 246, 205]. Akt/PKB phosphorylation of BAD prevents its interaction with the Bcl-2 and Bcl-xL, both apoptotic-inducing proteins, thus promoting cell survival [247]. Phosphorylation of caspase-9 by AKT inhibits the pro-apoptotic protein's degradation of critical cellular enzymes, while phosphorylation of the transcription factor FKHR1 reduces expression of several apoptosis inducing genes including the Fas ligand [246, 205].

The angiogenesis promoting vascular edothelial growth factor (VEGF) has been shown to stimulate Src activation in endothelial cells and activated Src associates with the VEGF receptor KDR/Flt1 [248, 249]. VEGF expression is also important in the neovascularization of growing tumor cells and both constitutive and inducible expression of VEGF by colon carcinoma cells has been shown to be Src dependent [250].

Src also contributes to an increase in invasiness and metastasis through regulation of proteins that are involved in cytoskeletal rearrangements. In cells with kinase-defective Src aberrant focal adhesions occur and their migration/invasive capabilities are reduced [251]. In addition downregulation of Src activation via the Src regulator protein Csk resulted in reduced FAK phosphorylation and aberrant cellular adhesion [252]. Src activation by VEGF results in activation of the focal adhesion kinase (FAK) that is involved in cytoskeletal rearrangements and migration/invasion. Enhanced FAK expression, like that of Src, commonly occurs in some cancers and is postulated to facilitate invasion and metastasis [253]. Src activation may also contribute to the invasiness of certain cancers including colon carcinoma through upregulation of matrix proteases that facilitate the degradation of the extracellular matrix [254]. Overexpression of the Src inhibitor Csk resulted in reduced expression of matrix metalloprotease MMP-2, further supporting a role for Src in metastasis and invasion [233]. An addition to degradation of the extracellular matrix is another important step in the progression of a metastatic phenotype is the disruption of cadherin-mediated cell-to-cell contacts. Studies in colon cancer have indicated that members of the Src kinase family can disrupt these cell-to-cell junctions [255]. Src expression results in a large decrease in cell-to-cell adhesion accompanied by cadherin phosphorylation and loss of cadherin/catenin association [221]. Another requirement of metastasis is the ability to detach from the primary tumor and surrounding matrix. Loss of cellular adhesion results in a form of programmed cell death termed anoikis. Activation of Src in colon carcinoma contributes to anoikis resistance and overexpression of activated Src enhanced this resistance [256].

2.16 The c-Myc Oncogene

C-myc, is the cellular homolog of v-myc oncogene, which was initially identified as a retroviral transforming factor in chickens [257, 258]. Following the initial discovery of c-myc four other closely related proteins have been identified: B-myc, L-myc, N-myc, and S-myc. These proteins comprise the myc family but only c-myc, N-myc and L-myc have transforming potential. Altered expression of c-myc occurs in a variety of human cancers including Burkitt's lymphoma, lung carcinoma, and breast carcinoma [259–261]. N-myc expression is altered in neuroblastoma and retinoblastoma [262–264]. Elevated expression of L-myc occurs in small cell lung carcinomas [265].

The c-myc, L-myc and N-myc proteins have a role in proliferation, growth, apoptosis, and terminal differentation [266–268]. Myc is rapidly induced in response to a variety of mitogenic stimuli and is an immediate early type response, requiring no gene expression or protein synthesis. Initial characterization suggested the myc proteins may function in gene regulation but failure of isolated full-length c-myc to bind DNA contradicted these conclusions [269]. It was the identification of Max, the heterodimerization partner of c-myc, which showed that myc could function as a sequence specific transcription factor capable of binding DNA and activating a variety of genes in mammalian and yeast cells [270–273]. Additional support for the requirement of the Max:Myc complex in c-myc mediated gene transcription was derived by showing that myc's transforming potential is negated by disruption of the heterodimer complex and deletion of either c-myc or Max results in embryonic lethality [274–276, 268, 277].

Early interpretations of the role of Max in myc regulation suggest that Max, which lacks a transactivation domain [273], targets the heterodimer complex to sequence specific DNA regions where c-myc activates transcription of downstream genes. It was also proposed that Max homodimers may compete with available DNA binding sites and act as a myc repressor, but actual in vivo repression eludes detection [278, 272, 273, 279]. Recently the identification of additional Max binding partners, the Mad family proteins, which when coupled to Max antagonize myc transactivation has shown that complex and cooperative regulation patterns are mediated by the myc:Mad:Max proteins [280, 281]. Numerous excellent reviews on the functional relationship of the myc:Mad:Max network have been published in recent years which discuss in detail the molecular interaction of these proteins [269, 282].

2.17 Mechanisms of c-Myc Dysregulation

In human cancers loss of normal myc regulation occurs though several mechanisms. In lymphoid cancers such as Burkitt's lymphoma, the c-myc proto-oncogene, which is located on chromosome 8q24, undergoes translocation in B-cells to one of the three immunogloblin genes located on chromosome 2, 14, or 22 and results in improper activation of the myc gene [259, 260, 283]. The c-myc gene is also amplified in a variety of human cancers including lung and breast, and elevated expression of the c-myc gene occurs in approximately 30% of both breast and colon carcinomas [261, 284–287]. Aberrant c-myc expression can occur via loss of regulatory mechanisms that control myc transcription. The adenomatous polyposis coli (APC) gene is frequently mutated in some human cancers including colon carcinoma [288, 289]. APC negatively regulates β-catenin, which is a coactivator for the transcription factor Tcf [290–292]. Tcf directly activates c-myc expression and loss of functional APC results in constitutive transcriptional activation by a beta-catenin-Tcf complex and increased expression of myc [292, 293]. In bladder cancer mutation of beta-catenin results in overexpression of c-myc and the cell cycle regulatory protein cyclin-D [294]. Occurrences of point mutations resulting in altered isoforms of the c-myc protein have also been reported [295, 296]. In Burkitt's lymphoma these sites occur at regions surrounding phosphorylation sites that are postulated to control negative regulation of c-myc activity and degradation of the c-myc protein [297–299].

2.18 The Role of c-myc in Oncogenesis and Malignant Transformation

Array analysis has identified a myriad of genes that are targets of c-myc regulation including the cyclin/cyclin-dependent kinases (CDK) and CDK inhibitors

[300–303]. The cyclins/CDKs and CDK inhibitors are essential cell cycle regulatory proteins whose dysregulation often occurs in the development of cancer [304, 300]. Cyclin D2 expression has been shown to be directly regulated by the myc/mad/max network [305]. Over-expression of cyclins A and E and CDK activation occurs with enhanced myc activity [306–308]. Activation of the D and E cyclin/CDK complexes is required for the synthesis (S) phase of the cell cycle [300]. Myc activation also downregulates the CDK inhibitor p27 (kip) and p21, which facilitates activation of the cyclin/CDK complexes and entry into the G1-S phase of the cell cycle [309–313]. Targets of the cyclin/CDKs include the retinoblastoma (RB) tumor suppressor gene [314, 315]. The RB protein, also a critical regulator of the G1-S stage of the cell cycle, represses transcriptional regulation of the E2F transcription factor. Inactivation of RB via cyclin/CDK phosphorylation events releases E2F and promotes the G1-S phase [316, 317]. Myc also interferes with RB function by enhancing expression of the RB repressing protein Id2 thus further ensuring RB inactivation and E2F activity [318].

Myc target genes also include those that are associated with cell growth and metabolism and are a separate function from that of cell cycle progression [319, 320]. These genes included those that are involved in protein and nucleotide synthesis, translational regulation, and protein folding, turnover and transport [301, 321]. In B cells and fibroblasts overexpression of myc results in enlarged cells and augmented growth independent of myc control of cell cycle regulation [322–324]. These processes, while being independent of cell cycle regulation by myc, further support the considerable role that myc participates in cell growth and division.

2.19 Conclusion

While considerable data exists, upon the role of oncogenes in the development of cancer and malignant transformation, our understanding is still incomplete. This is due in part because cancers themselves are characterized by several oncogenic events, each of which contributes to the maligiant phenotype. It is both these multiple genetic events and signaling pathway redundancy that present one of the biggest challenges in designing antineoplastic therapies. To date only the inhibitor STI-571, which targets the frequently mutated BCR-ABL oncogene in chronic myloid leukemia (CML), has shown great success as an inhibitor of a proto-oncogene. Other therapeutic agents have had mixed results in clinical trials, but combining therapies that target different oncogenes may prove effective. Ultimately it will be the molecular understanding of the collective roles of these oncogenes in each indivdual cancer and utilizing these observations in designing specific antineoplastic agents that will provide clinical impact.

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Chapter 3

Carcinogenic Effects of Ionising Radiation

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Contents

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3.1 Introduction

Within less than a decade after the discovery of the X-ray by Roentgen, in 1895, cancer was recognized to be a late complication of radiation injury. For decades thereafter, however, it was assumed that cancer would result only from doses large enough to cause severe damage and disorganization of tissue [1].

The possibility that there might be no threshold for carcinogenic effects of radiation was not widely considered until 1957, when the rates of leukaemia in A-bomb survivors, radiologists, and patients treated with radiation for ankylosing spondylitis were postulated by some observers to have increased as a linear, non-threshold function of the dose [2]. This hypothesis was greeted with scepticism at the time, however, and the precise shape of the dose-response curve for leukaemia has remained controversial ever since. Nevertheless, certain other forms of cancer have more recently been observed to exhibit dose–response relationships that are not inconsistent with linear, non-threshold functions [3]. Consequently, although the risks of carcinogenic effects from low-level irradiation are still uncertain, the threshold theory has come to be abandoned for purposes of radiation protection [4–7].

The evolution of our knowledge of radiation carcinogenesis – and, in turn, the development of principles to guide in formulating policies for purposes of radiation protection – provide lessons of strategic importance in assessing the health hazards of environmental agents in general.

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3.2 Historical Highlights

3.2.1 Early Radiologists

The first cancer attributed to radiation arose in a radiologist at the site of long-standing radiation injury on the skin of his hand [8]. It was soon followed by scores of similar cases, owing to the practice among pioneer radiologists of exposing their hands repeatedly in focusing their primitive fluoroscopic equipment. By 1910, 94 such cases of skin cancer had been reported among radiologists, X-ray technicians, and radium handlers in Europe and America [9]. The course of injury in such victims commonly began with reddening and blistering of the exposed skin, followed within a few weeks by epilation, and subsequently by atrophy of the epidermis, development of keratoses, and ultimately malignant growth. In many cases, the cancers were multiple and occurred on both hands. The tumours characteristically developed after a latent period of years or decades and were superimposed on progressive radiation dermatitis. Such cancers are no longer an occupational disease among radiologists, but they continue to occur among other workers who are exposed to radiation without adequate safeguards [10].

Leukaemia was another occupational malignancy noted in pioneer radiologists, the first cluster of cases being reported as early as 1911 [11]. The induction of the disease has since been confirmed in other irradiated populations, in which over 200 "radiation-induced" cases were reported between 1911 and 1959 [12]. Although the incidence of the disease was several times higher than normal in radiologists who entered practice in the U.S. during the first decades of the twentieth century [13], the excess has nearly disappeared in recent cohorts, owing to improved safety standards [14, 15].

3.2.2 Radium Dial Painters

As early as 1929, the frequency of osteosarcomas and carcinomas of the cranial sinuses was observed to be elevated in radium dial painters [16]. The induction of these cancers in dial painters resulted from their practice of pointing their fine-tipped brushes between their lips, resulting in their gradual ingestion of toxic quantities of radium and mesothorium [16, 17].

3.2.3 Underground Hard-Rock Miners

Cancer of the lung has been known for hundreds of years to be an occupational disability of pitchblende miners in Czechoslovakia, but not until the twentieth century was the disease linked to the inhalation of radon in the mines [1]. The incidence of the disease has since been found to be increased similarly in miners of uranium, fluorospar, and other radioactive ores, as a result of their occupational exposure to high concentrations of radon [17, 18].

3.2.4 Medically Irradiated Patients

Benign and malignant tumours of many, but not all, types have been reported to arise as late complications of radiation therapy [17]. Noteworthy examples include: (1) leukaemia and certain other cancers (e.g., bone, lung, pharynx, stomach, and pancreas) at irradiated sites in patients given X-ray therapy to the spine for ankylosing spondylitis [19]; (2) carcinoma of the breast in women given X-ray therapy to the breast for acute post-partum mastitis and other benign diseases [20]; (3) leukaemia and gastrointestinal cancer in women treated for menorrhagia by ovarian irradiation [21, 22]; (4) thyroid tumours, leukaemias, osteochondromas, salivary gland tumours, and other neoplasms at irradiated sites in patients given X-ray therapy to the mediastinum in infancy for enlargement of the thymus or other non-neoplastic conditions [23]; (5) solid tumors (chiefly sarcomas) arising at sites of previous irradiation in patients treated with X-rays for various lesions [24]; (6) skeletal tumours in patients treated with radium-224 for ankylosing spondylitis or tuberculous osteitis [25]; (7) tumours of the skin, thyroid, and brain in patients given X-ray therapy to the scalp in childhood for treatment of tinea capitis [26]; (8) leukemia in patients treated with phosphorus-32 for polycythemia vera [27]; (9) leukaemia in patients treated with iodine-131 for thyrotoxicosis [28]; (10) cancers of the urinary bladder, rectum, endometrium, ovary, small intestine, bone, and connective tissue in women treated with radiation for carcinoma of the cervix [29]; and (11) leukaemia and cancers of the thyroid, bone, connective tissue, and other sites in persons treated with radiation in childhood for Hodgkin's disease, Wilm's tumour, retinoblastoma, neuroblastoma, or other malignancies [30].

Exposure to radiation for diagnostic purposes also has been shown to cause cancers in some groups of patients; e.g., (1) breast cancers in women who had received repeated fluoroscopic examinations of the chest during the treatment of pulmonary tuberculosis; (2) leukaemia in children who had been exposed prenatally in the radiographic examination of their mothers; (3) patients who had been injected with thorium oxide (thorotrast) for angiographic examination; and (4) patients who had been examined radiographically for various other conditions [7, 17, 31].

3.2.5 Marshall Islanders Exposed to Radioactive Fallout

Natives of the Marshall Islands who were exposed accidentally to radioactive fallout from a nuclear weapons test in 1954 have shown an increased incidence of thyroid cancer. In almost 80% of those who were heavily irradiated when less than 10 years of age, thyroid nodules appeared between 8 and 16 years after exposure. In several such persons, whose thyroid glands are estimated to have received 7–14 Gy from internally deposited radioiodine and 1.75 Gy from external γ-rays, the tumours were preceded by overt hypothyroidism [32].

3.2.6 Experimental Radiation Carcinogenesis

Within only a few years after the first radiation-induced cancers in humans were reported, tumors were induced by irradiation in laboratory animals [1, 33]. Since then, neoplasms of many types have been induced experimentally in animals of various species [31, 33, 34].

In view of the diversity of benign and malignant growths that have been observed to be increased in frequency in irradiated human and animal populations, ionising radiation has come to be regarded as a "universal" carcinogen. This inference should not be interpreted, however, to mean that radiation is capable of inducing every type of cancer or of increasing the incidence of every induced cancer equally by a given dose. On the contrary, from the wealth of data that are now available, it can be concluded that: (1) neoplasms of most, but not necessarily all, types can be induced by irradiation under appropriate conditions in animals of suitable susceptibility; (2) the relation between dose and incidence varies, depending on the type of tumour in question, the dose, dose rate and linear energy transfer (LET) of the radiation, the sex, age, genetic background, and physiological state of the exposed subjects, and other variables; (3) low-LET radiations, such as X-rays and γ-rays, are generally less tumorigenic for a given dose than high-LET radiations, such as alpha particles, and their tumorigenic effectiveness decreases with decreasing dose rate, in contrast to that of high-LET radiations, which tends to be relatively independent of the duration of exposure; (4) for no type of neoplasm do the existing data suffice to define the dose-response relationship unambiguously at doses in the range of only a few mSv; (5) irradiation acts to increase the incidence of neoplasms through a variety of mechanisms, some of which involve direct effects on the cells that undergo transformation, and others which are mediated through effects on neighboring cells ("bystander" effects) or effects on more remote organs and tissues; (6) the various effects in question include the activation of oncogenes, the inactivation or loss of tumour-suppressor genes, and alterations in hormone levels, other growth factors, immunological responses, and other homeostatic mechanisms; (7) the process of radiation-induced neoplasia characteristically evolves through a sequence of steps, including initiation, promotion, and progression, completion of which may occupy a considerable fraction of the normal life span; (8) the degree to which the risk of cancer may be increased by a given dose of radiation will depend on the extent to which the process is influenced by other factors before, during, or after irradiation [3, 33, 35].

3.3 Sources, and Levels of Ionising Radiation in the Environment

Ionising radiation exists in two forms: (1) electromagnetic waves of extremely short wavelength (e.g., X-rays and γ-rays) and (2) accelerated atomic particles (e.g., electrons, protons, neutrons, alpha particles). In both forms, ionising radiation causes its biological effects though energetic and disruptive interactions with atoms and molecules in its path, as noted below. A given dose of ionising radiation is therefore customarily expressed in terms of the amount of energy that is

Table 3.1 Quantities and dose units of ionising radiation

Quantity being measured	Definition	Dose unit[a]
Absorbed dose	Energy deposited in tissue	Gray (Gy)
Equivalent dose	Absorbed dose weighted for the ion density (potency) of the radiation	Sievert (Sv)
Effective dose	Equivalent dose weighted for the sensitivity of the exposed organ(s)	Sievert (Sv)
Collective effective dose	Effective dose applied to a population	Person-Sv
Committed effective dose	Cumulative dose to be received from a given intake of radioactivity	Sievert (Sv)
Radioactivity	One disintegration per second	Becquerel (Bq)

[a]The units listed are those of the International System [4] and have largely replaced the earlier units; namely, the rad (1 rad = 100 ergs/gm = 0.01 Gy), the rem (1 rem = 0.01 Sv), and the curie (1 Ci = 3.7×10^{10} disintegrations per second = 3.7×10^{10}Bq).

deposited in the cells or tissues in which it is absorbed (Table 3.1).

Human populations are exposed to ionising radiation from various natural and man-made sources. Sources of natural background radiation include: (1) cosmic rays, which impinge on the earth from outer space; (2) terrestrial radiations, which emanate from radium and other radioactive elements in the earth's crust; (3) internal radiations, which are emitted by potassium-40, carbon-14, and other radionuclides that are normally present in the body; and (4) radon and its daughter elements which are present in inhaled air (Table 3.2).

The dose that is received from cosmic rays can differ appreciably from the value tabulated, depending on one's elevation; i.e., it can be twice as high in mountainous regions as at sea level and up to two orders of magnitude higher at jet aircraft altitudes. Likewise, the dose from terrestrial radiation varies widely from one area to another, depending on local variations in the radioactivity of the soil. Larger than the dose from these and all other natural sources combined, however, is the dose that is typically received by the bronchial epithelium from the inhalation of radon and its daughter elements, the concentration of which in indoor air can vary by an order of magnitude or more [31, 36].

Table 3.2 Average doses of radiation received annually by a resident of the US[a]

Source	Dose[b](mSv)	(%)
Natural		
Radon[c]	2.0	55
Cosmic	0.27	8
Terrestrial	0.28	8
Internal	0.30	11
Total natural	2.94	82
Artificial		
X-ray diagnosis	0.39	11
Nuclear medicine	0.14	4
Consumer products	0.10	3
Occupational	<0.01	<0.03
Nuclear fuel cycle	<0.01	<0.03
Miscellaneous	<0.01	<0.03
Total artificial	0.63	18
Total natural and artificial	3.57	100

[a]Adapted from National Academy of Sciences [31]
[b]Average effective dose to soft tissues
[c]Average effective dose to bronchial epithelium alone

Of the diverse man-made sources of radiation to which the general population is commonly exposed (Table 3.2), the largest is the use of radiation in medical diagnosis. Much smaller contributions come from other sources, including radioactive minerals in phosphate fertilizers, building materials, and crushed rock, radioactive fallout from atomic weapons, nuclear power production, and radiation-emitting components of various consumer products (color TV sets, smoke detectors, luminescent clock dials, airport security baggage inspection systems, etc.).

In various occupations, workers receive additional doses of ionising radiation, depending on their job assignments and working conditions. The average annual effective dose received occupationally by monitored radiation workers in the U.S. is smaller than that received from natural background, and in any given year less than 1% of such workers receives a dose that approaches the maximum permissible yearly occupational exposure limit [50 mSv (5 rem)] [37].

3.4 Carcinogenic Effects on Specific Tissues

3.4.1 Skin

Cancer of the skin, which occurred as a late complication of radiation dermatitis in scores of pioneer radiation workers [1], as noted above, has since been observed to be induced by radiation in patients treated with X-rays to the scalp in childhood for tinea capitis [38], patients treated with X-rays to the chest in infancy for enlargement of the thymus [23], patients treated to various parts of the body for other conditions [39], Czechoslovakian uranium miners [40], and atomic bomb survivors [41]. In addition to basal cell carcinomas, which predominate at lower doses, the induced neoplasms include squamous cell carcinomas and smaller numbers of fibrosarcomas, melanomas, and sweat gland tumours [17,38]. The existing data do not suffice to define the dose-incidence precisely for any of these neoplasms, but it has become evident that cutaneous basal cell carcinomas can be induced with little or no antecedent clinical evidence of radiation damage [38].

In laboratory mice and rats, in which the induction of skin tumours has been studied extensively, the incidence of such tumours has been found to rise steeply in the dose range above 20 Sv [42]. At doses below 5 Sv, however, the incidence is too low to be investigated readily; in fact, 0.5 Gy of beta radiation delivered thrice weekly to the skin throughout life was found to induce no tumours in any of 50 exposed rats [43]. The *c-myc* oncogene has been observed to be amplified in the progression of radiation-induced skin tumours in the rat [44].

3.4.2 Hematopoietic and Lymphoid Tissues

All major forms of leukaemia except the chronic lymphatic form have been observed to be induced by irradiation of the whole body or a major part of the active bone marrow in humans. The radiation-induced excess appears within 2–5 years after irradiation, is dose-dependent, and persists for 15 years or longer, depending on the type of leukaemia and age at irradiation [7, 17].

In a-bomb survivors, patients treated with spinal irradiation for ankylosing spondylitis, and women treated with pelvic irradiation for menorrhagia, the combined excess of all forms of leukaemia (other than the chronic lymphatic form) averaged over the first 25 years after irradiation has approximated 1–2 cases per 10,000 persons per year per Sv to the bone marrow. Furthermore, the overall excess in the a-bomb survivors is consistent with a linear-quadratic dose–incidence relationship [3, 7, 45]; however, the different types of leukaemia appear to differ in their dose–incidence relationships and time distributions, and in none of the populations do the data suffice to define the shape of the dose-response curve precisely. Children who were exposed *in utero* during the radiographic examination of their mothers have shown a similarly increased frequency of leukaemia [46]; however, no such excess has been evident in Japanese children who were exposed prenatally to atomic-bomb radiation, possibly because of the limited numbers involved [47]. The earlier suggestion that the cluster of cases in children residing in the vicinity of the Sellafield nuclear plant may have resulted from the occupational irradiation of their fathers [48] has since been discounted on the basis of further evidence [7, 49].

For other types of hematologic malignancies, the data are variable. The risk of multiple myeloma

appeared until recently to have been increased by irradiation in some populations, but the additional evidence that is now available argues against this interpretation [7]. For Hodgkin's disease and non-Hodgkin's lymphomas, likewise, the data show no clear evidence of a causal association with radiation [7].

All species of laboratory animals studied to date appear to be susceptible in varying degrees to the induction of hematologic malignancies, but the effects of radiation on the frequency of a given neoplasm vary with the growth in question, the conditions of irradiation, host factors (species, strain, sex, age at exposure), and other variables [50]. In short, the experimental data indicate that: (1) many, but not all, types of hematologic growths can be induced by ionising radiation; (2) the dose-incidence curve for low-LET radiation typically rises less steeply at low dose rates than at high dose rates; (3) the dose-incidence curve for high-LET radiation typically rises more steeply than the curve for low-LET radiation and is less dependent on the dose rate; (4) at high dose rates, the dose-incidence curve typically passes through a maximum in the intermediate-to-high dose range, above which it declines with increasing dose; (5) the data do not suffice to define the shape of the dose-incidence curve in the mSv dose range; (6) the precise pathogenetic mechanisms of the various neoplasms remain to be elucidated, but specific chromosomal aberrations and mutations have been implicated in some instances [51, 52]; (7) uncertainties about the dose-incidence relationships and relevant causative mechanisms complicate extrapolation from the animal data to man [3, 50].

3.4.3 Thyroid Gland

Epidemiological data show the thyroid gland to be highly susceptible to radiation carcinogenesis during childhood. A dose-dependent excess of thyroid tumours has been observed in a-bomb survivors, patients given radiotherapy to the neck in infancy for thymic enlargement and other non-neoplastic conditions, patients treated with radiation to the scalp for tinea capitis in childhood, Marshall islanders exposed to radioactive fallout from a nuclear weapons test in 1954, persons exposed during childhood to radionuclides downwind from the Nevada test site, persons in eastern Europe exposed during childhood to radionuclides released from the Chernobyl accident, and others treated with external thyroid irradiation [7, 53–56].

The induced neoplasms are chiefly adenomas and adeno-carcinomas of the papillary type, many of which have exhibited distinct rearrangements of the *ret* oncogene [57]. The tumors are typically preceded by a latent period of 10 years or longer and carry a low risk of mortality. Susceptibility is 2–4 times higher in females than in males and is similarly higher in children than in adults [7, 56]. Susceptibility also appears to be increased in those of Jewish ethnicity [56].

In those exposed to X-rays during childhood, the excess of thyroid tumours has been observed after a dose as low as 65 mSv, and the dose-incidence data are consistent with a linear-nonthreshold relationship, corresponding to an excess of approximately 4 cancers per 10,000 person-yr-Sv [49, 55]. Little or no excess of tumours has been observed in persons treated with iodine-131 for hyperthyroidism, but the patients in question were treated mainly as adults, and the doses they received are large enough (60–100 Gy) to have caused substantial cell killing [7]. The data from Chernobyl [58, 59] suffice to indicate that exposure to radioiodine in childhood can cause thyroid cancer, but the magnitude of the risk per unit dose from radioiodine remains uncertain.

In laboratory animals, tumours of the thyroid gland have been induced by internal, as well as external, irradiation. With external irradiation, the dose-incidence curve rises with increasing dose up to about 15 Gy, above which it passes through a maximum and decreases with further increase in the dose, owing presumably to excessive damage to the follicular epithelium [50]. Protracted irradiation by internally deposited iodine-131 has appeared to be several times less tumorigenic to the thyroid than acute X-irradiation in some experiments but not in others [50]. The carcinogenic effects of a given dose of radiation can be enhanced by any drug, dietary factor, or condition that elicits hormone-induced hyperplasia of the follicular epithelium [3].

3.4.4 Other Endocrine Glands

Adenomas of the parathyroid glands have been reported to be increased in frequency in atomic-bomb survivors [60] and some other irradiated populations

[61]. Analysis of the dose-incidence relationship in these populations is complicated by the small numbers of cases that have been observed and the possibility that they may be confounded in some instances by the presence of the multiglandular endocrine neoplasia syndrome [17].

Adenomas and adenocarcinomas of the adrenal cortex, pancreatic islets, parathyroid gland, and anterior pituitary have also been observed to occur with increased frequency in irradiated mice and rats [34, 62].

3.4.5 Breast

The female breast has been found to be highly susceptible to radiation carcinogenesis, through studies of women: (1) exposed to atomic-bomb radiation [63], (2) treated with radiation to the breast for acute postpartum mastitis or other non-neoplastic diseases [20, 64], (3) subjected to repeated fluoroscopic examinations of the chest during treatment for pulmonary tuberculosis with artificial pneumothorax [20], and (4) employed as radium dial painters [65].

In the above groups, an increased frequency of breast cancer became evident within 5–20 years after irradiation, depending on the dose and age at exposure, and it has persisted for the duration of follow-up. The excess is larger in women irradiated during childhood or adolescence than in women irradiated at older ages, and susceptibility has been observed to decline markedly after the menopause. Although the excess became evident within 5–9 years after irradiation in the older age group, it did not appear until 15–20 years after irradiation in women exposed during adolescence or until 35 years later in those who were exposed at less than 10 years of age, implying that expression of the carcinogenic changes depends on promotion by age-related hormonal stimulation of breast tissue [66]. The risk of cancer appears typically to have increased linearly with the dose up to about 3–5 Gy, above which it has turned downward; however, no simple unified model adequately describes the excess in all groups. In women who were irradiated acutely or in fractionated high-dose-rate exposures, the dose-incidence curves are remarkably similar, implying that successive high-dose-rate exposures are highly additive in their carcinogenic effects on the breast. Conversely, in women who received protracted low-dose-rate exposures, in the treatment of cutaneous hemangiomas, the risks per unit dose have been several times lower [20], implying that the carcinogenic effects of successive doses on the breast are substantially less than fully additive if the radiation is absorbed gradually enough.

A high susceptibility of the mammary gland to radiation carcinogenesis is also evident in female laboratory animals of certain genetic backgrounds. In female rats and mice of some strains, the incidence of mammary gland tumours can be elevated detectably by doses as low as 100 mGy of X- or γ-radiation or 2.5 mGy of fast neutrons [3]. The shape of the dose-response curve varies, however, depending on the type of neoplasm in question, the dose rate and LET of the radiation, the age and genetic background of the exposed animals, and other variables [3]. The tumours evolve through a succession of stages (initiation, promotion, and progression), the completion of which is strongly dependent on appropriate hormonal stimulation [3]. Although the roles of specific oncogenes or tumour-suppressor genes remain to be elucidated, the frequency with which radiation initiates tumour formation greatly exceeds the rate with which radiation is known to induce mutations at any given genetic locus, implying that epigenetic factors may be involved [67]. That radiation-induced genomic instability is also likely to be involved in the process is suggested by the observation that neoplastic transformation, delayed chromatid instability, and delayed point mutations in the p53 tumor-suppressor gene are induced by irradiation at a far higher frequency in mammary cells from mice of the cancer-susceptible BALB/c strain than in mammary cells from mice of the cancer-resistant C57BL/6 strain [68].

3.4.6 Respiratory Tract

A dose-dependent increase in the incidence of lung cancer has been observed in a-bomb survivors [63], patients treated with spinal irradiation for ankylosing spondylitis [19], underground hardrock miners [18], and other irradiated populations [17]. The induced neoplasms include squamous-cell carcinomas, small-cell anaplastic carcinomas, and smaller numbers of cancers of other types [17].

The appearance of the tumours has typically been preceded by a latent period of 10 years or more,

depending on age at exposure. In a-bomb survivors, no excess of lung tumours was evident until after 10 years in those more than 50 years old at the time of irradiation, after 15 years in those 35–49 years old at exposure, and after 25 years in those 20–34 years old at exposure. Adjusted for age and duration of follow-up, the excess of lung cancer in a-bomb survivors, irradiated spondylitics, and underground miners approximates 2–3 cases per 10,000 persons per year per Sv, and although the data do not suffice to define the shape of the dose-response curve precisely, they are consistent with a linear-nonthreshold relationship [7, 18]. In underground miners, however, in whom the effects were due primarily to the high-LET radiations emitted by radon, the dose-incidence curve rises more steeply than in those populations that were exposed primarily to low-LET radiations [31, 69]. It is noteworthy, moreover, that the rates of lung cancer per unit dose in populations exposed to radon in indoor air appear to be comparable to the rates observed in underground miners [70]. Conversely, no significant excess of lung cancer has been evident in women who received repeated fluoroscopic examinations of the chest during the treatment of pulmonary tuberculosis, suggesting that the carcinogenic effectiveness of low-LET radiation for the lung may be greatly reduced if the dose is accumulated sufficiently slowly [7].

In populations exposed primarily to high-LET radiation, the carcinogenic effects of cigarette smoking generally appear to have been multiplicative with those of radiation [7, 31, 69], whereas in populations exposed primarily to low-LET radiation, the effects of cigarette smoking generally appear to have been additive, or only slightly more than additive, with those of radiation [17, 31]. Noteworthy in this connection, is evidence that the mutations of the p53 gene in lung cancers induced by radon appear to differ significantly from those in lung cancers induced by tobacco smoke [71].

In laboratory animals of various species and strains, carcinogenic effects of radiation on the lung have been studied extensively [3, 72, 73]. The tumours have been observed to include benign and malignant growths arising at all levels of the respiratory tract, depending on the distribution of the radiation dose. Susceptibility to a given neoplasm varies among the different types of cells in the respiratory tract, as well as with the genetic background of the exposed animals, so that no one dose–response relationship fits all patterns of response. In general, high-LET radiation is appreciably more tumorigenic to the lung than is low-LET radiation, and its tumorigenic effectiveness varies relatively little with changes in the dose and dose rate, in contrast to the tumorigenic effectiveness of low-LET radiation, which tends to decrease with decreasing dose and dose rate [72]. After a given dose, irrespective of the LET of the radiation, the yield of tumours may be increased by the application of proliferative stimuli [74]. Although the relevant molecular mechanisms of tumorigenesis remain to be elucidated fully, evidence suggests that activation of the *ras* gene may be involved early in the induction of proliferative lesions by plutonium [73] but that neither the *rb* gene nor the *p53* gene play a major role in the induction of lung tumours. In dogs, moreover, the expression of the epidermal growth factor receptor gene has been found to be elevated in a significant percentage of plutonium-induced proliferative foci and lung tumours [75].

3.4.7 Gastrointestinal Tract

Cancers of the esophagus, stomach, colon, rectum, liver, pancreas, and salivary glands occur with increased frequency after irradiation, depending on the dose and conditions of exposure [7, 17]. Small increases in the frequency of tumours of the pharynx, hypopharynx, and larynx also have been observed in some irradiated populations, but such findings have been inconsistent, and no significant excess has been noted in a-bomb survivors or other populations exposed to doses in the range below 1 Sv [17, 31]; thus, the susceptibility of the latter tissues to the carcinogenic effects of radiation remains to be established.

Carcinoma of the *esophagus* has been observed to occur at twice the expected frequency in patients treated with spinal irradiation for ankylosing spondylitis [76], and to be increased in frequency for a time in a-bomb survivors as well [63]. Other irradiated populations, however, have not consistently shown a significant increase in the frequency of the disease [7, 17].

In rodents of several species, carcinomas of the *esophagus* and *forestomach* have been induced by experimental irradiation. In general, however, an excess of such tumours has been detected only after a relatively large dose (>5 Sv), and the yield per unit dose has been larger with fast neutrons than with X- or γ-rays [77].

G*astric carcinoma* has occurred with increased frequency in a-bomb survivors [63], in whom the data are consistent with a linear dose-response relationship, corresponding to a lifetime risk of 110 fatal cases per 10,000 persons per Sv [4]. Patients treated with radiation for cervical cancer also have shown an excess of the disease [28]. In other irradiated populations, however, elevated risks of the disease have been noted only inconsistently [7, 17].

Carcinoma of the *glandular stomach* also has been induced by irradiation in laboratory rodents [77]. In general, however, such tumors have been rare and have been detectable only after a relatively large dose, and the excess per unit dose has been larger with neutrons than with X- or γ-rays [77].

Carcinomas of the *colon* have occurred with increased frequency in a-bomb survivors, in whom the data are consistent with a linear dose-response relationship [63], corresponding to a lifetime risk of 8.5 fatal cases per 10,000 persons per Sv [4]. An elevated risk of the disease has been observed also in women treated with abdominal irradiation for benign pelvic disorders [17]. In other irradiated populations, however, it has been seen only inconstantly [7, 17].

In laboratory animals, intensive irradiation has been shown to induce adenocarcinomas of the *colon* [33]. The observed neoplasms include polypoid tumours of the large bowel in rats and dogs resulting from localized irradiation by neutron beams or by dietary polonium-210 or cerium-144 [78].

Cancer of the *rectum* has occurred with increased frequency in women treated with radiation for carcinoma of the cervix [29, 79]. No significant excess of the disease has been documented, however, in women of other irradiated populations, in whom the doses to the rectum have been much lower [17].

Primary cancers of the *liver* have occurred with increased frequency in a-bomb survivors [6] and in patients injected with thorotrast for angiographic examination [80–83]. In the a-bomb survivors the excess is statistically significant only at doses in the range of 1 Sv, and the magnitude of the risk at lower doses is uncertain. The types of liver cancer associated with thorotrast have typically been cholangiocarcinomas and smaller numbers of angiosarcomas and hepatocellular carcinomas, while those types associated with low-LET irradiation in the a-bomb survivors have been primarily hepatocellular carcinomas [7].

An excess of *liver* tumours has also been produced in laboratory animals by external irradiation and by the intravenous injection of colloidal radionuclides [77]. The induced tumours have occurred mainly at high dose levels and have included neoplasms of virtually all histologic types, depending on the exposure conditions, species, and strain in question.

Carcinomas of the *gall bladder* have occurred with increased frequency in patients injected with thorotrast for angiographic examination [80–84]. A significant excess of such tumors has not been observed consistently in other irradiated populations [17].

Tumours of the *salivary glands* have appeared with increased frequency in patients treated with radiation of the head and neck in childhood for various benign conditions; in such persons, the excess has corresponded to a risk of 0.6–2.5 cases of benign and malignant tumours per million exposed children per year per Gy [17, 85]. A dose-dependent increase has also been observed in a-bomb survivors [86], in whom the excess over a 20-year follow-up period has amounted to about 0.5–4 tumours per million persons per year per Gy [17].

Salivary gland tumours have rarely been observed in irradiated animal populations [87, 88], indicating that the susceptibility of these glands in the species investigated is relatively low.

Carcinoma of the *pancreas* has appeared to be increased in frequency in some irradiated population groups, but the excess has been of equivocal significance and has been noted only inconsistently [17, 49]. At present, therefore, there is no clear evidence of a causal association between pancreatic cancer and previous irradiation [17, 49].

3.4.8 Skeleton

An excess of benign and malignant bone tumours has been observed in radium dial painters [89–91], patients treated for ankylosing spondylitis by intravenous injection of radium-224 [25], patients injected with thorotrast for angiographic examination [78], and patients treated with therapeutic X-radiation for various conditions [7, 17]. The induced tumours have been evident only at doses above several Gy, however, with the result that no clear evidence of an excess has been observed in the a-bomb survivors or other populations exposed at lower dose levels [7, 17]. In patients

injected with radium-224, the data are consistent with a linear dose–response relationship over the range between 10 Gy and 100 Gy, but the shape of the dose-response curve at lower doses is highly uncertain [7].

In patients injected with radium-224 or treated with X-rays, in whom the radiation was received over a relatively brief period, the resulting excess of tumours was evident within 4 years, reached a maximum at 6–8 years, and declined thereafter [31]. In radium dial painters, however, who continue to accumulate dose from internally deposited radium-226 throughout life, osteosarcomas have appeared as late as 52 years after the onset of exposure [89, 97]. Susceptibility to the induction of osteosarcomas has been observed to be higher in children than in adults, and also to vary from one part of the skeleton to another, being highest at sites where spontaneous bone tumours arise most frequently (e.g., near the knee) and lowest in the vertebrae [92].

In laboratory animals, likewise, comparable carcinogenic effects of radiation on the *skeleton* have been observed. In mice, rats, and dogs, high-LET radiation from internally-deposited radium or plutonium has been found to be many times more tumorigenic to bone than low-LET radiation from internally-deposited strontium-90 or from external γ-radiation [93]. High-LET radiation has also proven to be more effective per unit dose at low dose rates than at high dose rates, whereas the opposite has been true of low-LET radiation [94]. Even with high-LET radiation, however, the average latent period for tumour induction has been observed to vary inversely with the dose rate and thus to exceed the mean life span for the species in question when the dose rate is sufficiently low [94]. In some instances, the tumours have exhibited the activation of certain oncogenes [95], the inactivation or loss of certain tumour-suppressor genes [95–96], or the presence of oncogenic viruses [97].

3.4.9 Genital Organs

A dose-dependent excess of carcinoma of the *ovary* has been observed in a-bomb survivors [63], but no clear excess has been evident in other irradiated populations [17]. On the basis of the existing data, the lifetime risk of the disease has been estimated to approximate10 fatal cases per 10,000 women per Sv [4].

In mice, tumours of the ovary are induced at high frequency by a dose that is large enough to sterilize both ovaries. The neoplasms include tumours of granulosa cells, lutein cells, theca cells, and other stromal elements, and their induction is attributable to the disturbance of hormonal regulation resulting from radiation-induced sterilization [50, 77]. In the mouse, susceptibility declines markedly with age at the time of irradiation [77].

Cancers of the uterus have not consistently been increased in frequency in irradiated women, and their induction by irradiation remains to be established [17, 31].

Cancers of the testis, penis, scrotum, and prostate . The existing epidemiological data reveal no clear evidence of a causal connection between radiation and cancers of the male genital organs [7, 17]. Interstitial tumors of the testis have been induced by intensive irradiation of the scrotum in rats of certain strains, an effect tentatively attributed by some observers to radiation-induced hormonal disturbances [77].

3.4.10 Kidney and Urinary Bladder

Cancers of the kidney and urinary bladder are increased in frequency in a-bomb survivors [63], patients treated with radiation for ankylosing spondylitis [19], uterine bleeding [21], or other diseases [29], and patients injected with thorotrast for retrograde pyelography [80]. The tumors are characteristically preceded by a latent period of 25–30 years, depending on the conditions of irradiation and age at exposure. The bladder appears to be more susceptible than the kidney [17], and the lifetime risk of bladder cancers for members of the general population is estimated to approximate 30 fatal cases per 10,000 persons per Sv [4].

In rats and mice, tumors of the kidney are readily induced by irradiation [77]. The tumors include benign and malignant growths, and their incidence for a given dose depends on physiological variables as well as on the conditions of irradiation [77]. Susceptibility is greatly increased in “Eker” rats, which are heterozygous for a mutated Tsc2 gene [98]. In these animals, the induction of renal tumors is postulated to constitute a 2-step process, the first step being the inheritance of the Tsc2 gene, and the second step being the loss or mutation of the remaining wild-type allele [98].

3.4.11 Central Nervous System

Brain tumors have been observed to be increased in frequency in patients given radiation therapy to the scalp in childhood for tinea capitis [26, 54], patients treated with radiation to the head and neck in childhood for tonsillitis, adenoiditis, and other non-neoplastic conditions [17], and children exposed to diagnostic X-radiation in utero [46]. Most of the onserved tumors have been benign, and of these, neurilemmomas aappear to be the type of neoplasm at highest risk. The data do not suffice, however, to define the dose–incidence relationship [7, 17].

Brain tumors have been induced in primates by intensive thermal neutron- or proton-irradiation [99].

3.4.12 Cancers, All Sites Combined

In a-bomb survivors, the total incidence of solid cancers during the first 50 years after irradiation appears to have increased as a linear-nonthreshold function of the dose over the range 0–2 Sv (Fig. 3.1). A significant elevation of the risk is detectable at doses below 0.1 Sv, and the upper confidence limit on any possible threshold has been computed to be 0.06 Sv [100]. As a consequence of the increased mortality from cancer and other diseases, the mean survival time of the population has been correspondingly reduced [101].

In laboratory animals, likewise, the oncogenic effects of whole-body irradiation on all organs combined cause the lifespan to be reduced by an amount that appears to increase linearly with the dose [3, 102].

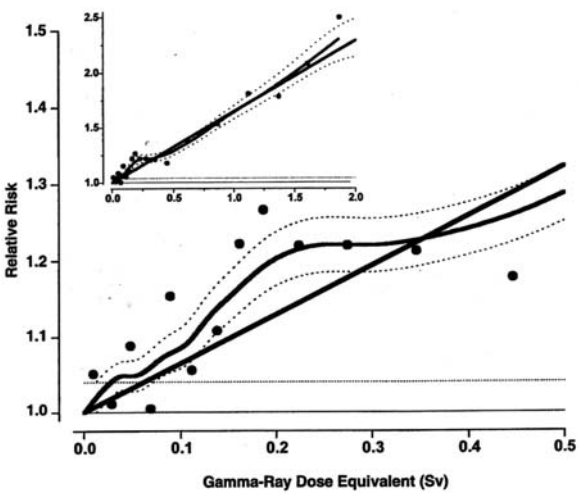

Fig. 3.1 Dose-response relationship for relative risk of cancer, all types combined, excluding leukemia, in a-bomb survivors, 1958–1994 (from 100). The data represent age-specific incidence rates in irradiated survivors relative to those in non-irradiated survivors, averaged over the follow-up period and over sex, and for exposure at age 30. The *straight line* represents the linear risk estimate computed over the 0–2 Sv dose range, and the *dashed curves* represent ±1 standard error for the *smoothed curve*

3.5 Mechanisms and Dose–Incidence Relationships

3.5.1 Effects of Radiation at the Cellular and Subcellular Levels

As ionising radiation penetrates living cells, it collides randomly with atoms and molecules in its path, giving rise to *ions* and *free radicals*, which break chemical bonds and cause other molecular alterations that may injure the cells. The spatial distribution of such events along the path of the radiation depends on the energy, mass, and charge of the radiation; e.g., X rays and gamma rays are sparsely ionising, in comparison with charged particles, which typically are more densely ionising; e.g., an alpha particle typically gives up all of its energy in traversing only a few cells [103]. The physico-chemical changes result almost instantaneously, but the evolution and expression of any ensuing biological effects may take minutes, days, or years, depending on the types of effects in question.

Any molecule in the cell may be altered by radiation, but *DNA* is the most critical biological target because of the limited redundancy of the genetic information it contains. DNA can be damaged directly by an impinging radiation, and it can also be damaged indirectly by radiation-induced effects on the surrounding cytoplasm or through the release of reactive oxygen species, cytokines, and other factors from neighboring cells (so-called “bystander” effects) [104]. A dose of radiation that is large enough to kill the average dividing cell {2 Sv (200 rem)} suffices to cause hundreds of lesions in its DNA molecules [105]. Most such lesions are reparable, but those produced by a densely ionising radiation (e.g., a proton or an alpha particle) are generally large scale in nature and less reparable than

those produced by a sparsely ionising radiation (e.g., an X-ray or a gamma ray) [3, 7, 103]. For this reason, densely ionising [high-linear energy transfer (LET)] radiations are typically more potent than are sparsely ionising (low-LET) radiations for most forms of injury [3, 4, 7].

Damage to DNA that remains unrepaired or is misrepaired may be expressed as a *mutation*. The frequency of mutations typically increases as a linear-nonthreshold function of the dose, approximating 10^{-5} to 10^{-6} per locus per Sv (100 rem), which is interpreted to indicate that that traversal of the DNA by a single ionising particle may suffice to cause a mutation [3, 7, 31]. With high-LET radiation, the curve rises more steeply than with low-LET radiation, and the yield of mutations per unit dose is relatively independent of the dose rate [3]. With low-LET radiation, in contrast, the yield of mutations per unit dose typically decreases with decreasing dose rate down to a minimum in the range of 0.1–1.0 cGy per minute, below which it rises again with further reduction of the dose rate [106]. The fact that the mutagenic effectiveness of low-LET radiation is reduced to a minimum at a dose rate in the range of 0.1–1.0 cGy per minute, is interpreted to signify that dose rates in this range are optimal for the error-free repair of DNA damage, and that the adaptive response needed for the purpose is elicited progressively less effectively as the dose rate is reduced below this level [106].

Pre-exposure to a small "conditioning" dose of low-LET radiation has been observed to reduce the yield of mutations produced by a larger "test" dose administered a short time later in some types of cells, owing to the induction of an adaptive repair system [107]. An appreciable dose appears to be required, however, to elicit such an adaptive response; hence the extent to which the response can be expected to protect humans against the mutagenic effects of low-level radiation remains to be determined [3, 107].

In view of the evidence that genomic instability is an important characteristic of cancer cells [35], it is noteworthy that the mutation rate in various experimental systems has been observed to remain elevated for many cell generations following irradiation, indicating the induction of transmissible genomic instability in surviving cells and their progeny [3, 10, 108, 109].

Radiation damage to the genetic apparatus may also cause the breakage of *chromosome*s. Although cells normally possess the ability to rejoin the severed ends of a broken chromosome, when two or more breaks occur close enough together in space and time, the broken ends from different break points may be joined together erroneously, giving rise to translocations, inversions, dicentrics, and other changes in chromosome number and structure [110]. The frequency of such "two-break" aberrations typically increases as a linear-quadratic function of the dose with low-LET radiation and as a steeper, linear function of the dose with high-LET radiation; the dose-response relationship has been characterized well enough so that the frequency of aberrations in blood lymphocytes can serve as a useful biological dosimeter [111, 112]. In human lymphocytes cultured soon after irradiation in vitro the frequency of dicentric and ring aberrations approximates 0.1 per cell per Sv [113], from which it may be inferred that the dose required to double the frequency of such aberrations is roughly 0.05 Sv, or about 50 times the dose ordinarily received each year from natural background radiation. Under certain experimental conditions, and in some but not all cells, prior exposure to a small "conditioning" dose of radiation has been observed to elicit an adaptive response that reduces the frequency of chromosome aberrations produced by a larger "test" dose administered a short time later [107]; however, as with protection against the induction of mutations, an appreciable dose appears to be required to elicit the response, so that the extent to which it can be expected to protect humans against the effects of low-level irradiation remains to be determined [3, 107].

Among the earliest reactions to irradiation is the inhibition of cell division, which appears promptly after exposure, varying both in degree and duration with the dose. Although the inhibition of mitosis is characteristically transitory, radiation damage to genes and chromosomes may be lethal to dividing cells, which are highly radiosensitive as a class [114]. Measured in terms of proliferative capacity, the survival of dividing cells tends to decrease exponentially with increasing dose, 1–2 Sv (100–200 rem) generally sufficing to reduce the surviving population by about 50%.

3.5.2 In Vitro Neoplastic Transformation

Clonogenic cells that survive irradiation in vitro exhibit a dose-dependent increase in the probability of *neoplastic transformation*. The dose-response curve for in vitro transformation is complex, however, and

it varies, depending on the dose rate and LET of the radiation, the genetic background of the exposed cells, the culture conditions, and other variables [3]. Human cells are relatively resistant to transformation unless previously immortalized [115, 116]; consequently the bulk of our data on in vitro transformation come from experiments with rodent cells. From the existing data, it appears that: (1) the percentage of cells transformed increases with the dose, often reaching a plateau in the range of 10^{-3} at doses of 3–5 Gy; (2) high-LET radiation has a greater transforming effectiveness than low-LET radiation; (3) the transforming effectives of low-LET radiation generally decreases with decreasing dose rate, in contrast to that of high-LET radiation, which tends to remain constant, or even to increase, with decreasing dose rate; (4) cells are most sensitive to radiation-induced transformation in the G_2/M stage of the division cycle; (5) depending on the cell system employed, transforming effects may be detectable at doses as low as 100 mGy of γ-rays, 10 mGy of neutrons, or after the passage of an average of only one alpha particle per cell nucleus; (6) transformation of a given cell may be enhanced by, or mediated through, radiation-induced effects on neighboring cells ("bystander effects"); (7) prior exposure to a small, "conditioning" dose of radiation may reduce the susceptibility of the cell to a second, larger, "test" dose administered a short time later; (8) transformation evolves through a multi-step process; (9) the initiating step in transformation typically occurs at a frequency far exceeding the rate of radiation-induced mutations at any given genetic locus in rodent cells, suggesting that epigenetic events may be involved; (10) the initiating step is characteristically followed by the progressive accumulation of genetic changes in subsequent cell generations, indicating that the induction of genomic instability is involved; (11) the rate of transformation by ionizing radiation may be modified by various other physical and chemical agents applied before or after irradiation [3].

3.5.3 Carcinogenesis In Vivo

As noted above, many but not all, types of neoplasms have been induced by irradiation in laboratory animals and human populations, with dose-response relationships that vary markedly, depending on the neoplasm in question. In laboratory animals, moreover, neoplasms of some types have actually decreased in frequency with increasing dose over the range of doses tested [3, 34].

For those types of neoplasms that exhibit a dose-dependent increase in frequency, the dose-response curves typically pass through a maximum at intermediate dose levels and decrease with further increase in the dose [3, 34, 50]. The decrease in their frequency at higher doses is attributed to cell killing or other forms of damage that interfere with the expression of the carcinogenic changes [34, 50]. Conversely, cell killing can play a key role in the induction of some other types of neoplasms – such as ovarian tumours and thymic lymphomas in the mouse, hair follicle tumours in the rat, and osteosarcomas in the dog – by eliciting growth stimuli that promote the formation of these neoplasms [50].

As yet, however, the relevant mechanisms of oncogenesis are not known in sufficient detail to explain the diversity of dose-incidence patterns that have been observed. In relatively few instances, moreover, has the dose–incidence relationship been characterized at doses below 0.5 Sv, or has the influence of the dose rate and quality (LET) of the radiation for tumorigenesis been defined in detail. Pending further information, therefore, the carcinogenic risks from low-level radiation must remain uncertain [3, 7]. Nevertheless, since the oncogenic effects of radiation are postulated to be initiated by the activation of oncogenes, inactivation of tumour-suppressor genes, or other appropriate genetic damage to the occasional cell [35], the dose-incidence relationships for low-level radiation carcinogenesis in vivo presumably resemble the corresponding dose–effect relationships for the induction of mutations and chromosome aberrations, mentioned above [3].

Given existing data on the molecular mechanisms of leukaemia and other neoplasms, therefore, it is noteworthy that the dose-response curves for leukaemia and for solid cancers (all types combined) in a-bomb survivors closely resemble the curves for chromosome aberrations and mutations, respectively [117]. Furthermore, the age-distribution of solid cancers (all types combined) in a-bomb survivors, coupled with the corresponding dose-response data, are consistent with the hypothesis that cancer results from the gradual accumulation of mutations in the body's stem cells throughout life, and that irradiation can add any one of the requisite mutations and thereby advance the process significantly in any given cell [118].

Influence of dose rate. In experimental animals, the dose-incidence curves for oncogenic effects of

low-LET radiation generally decrease in slope with decreasing dose rate, owing to repair of some of the incipient damage apace with its gradual accumulation [3]. As a result, the overall age-specific mortality from radiation-induced neoplasms – and the associated reduction in life-expectancy – are typically several times lower if a given dose of low-LET radiation is received gradually in small increments over a period of weeks than if it is absorbed in a single, brief exposure [3]. With high-LET radiation, on the other hand, the dose-response curve may rise even more steeply with prolongation of the exposure [3, 31, 119].

Age-related changes in susceptibility may also modify the dose-response relationship when the period of irradiation is greatly protracted. In the induction of ovarian tumours in the mouse, for example, the effect of aging and the effect of protraction both act to reduce the ultimate yield of tumours per unit dose when the period of irradiation is greatly prolonged [7].

Influence of host factors. Susceptibility to the induction of neoplasms of any given type also varies markedly among laboratory animals of different species and strains [35] and among humans of differing genetic constitutions [3, 7, 35, 120]. Susceptibility is heightened, for example in children with familial retinoblastoma and in those with the nevoid basal cell carcinoma syndrome, ataxia telangiectasia, or certain other inherited disorders [35, 120]. In children with familial retinoblastoma, the heightened susceptibility has been interpreted as evidence of a two-step mutational etiology of the induced cancers, the first mutation being inherited and the second caused by irradiation [120].

As noted above, age also influences susceptibility to radiation carcinogenesis, the effects of age varying with the type of neoplasm in question [33, 34]. For example, susceptibility to the induction of thyroid tumours and susceptibility to induction of tumours of the female breast both decline markedly with increasing age at exposure in human populations [7]. Conversely, however, the total incidence of cancer per unit dose tends to increase with age at exposure in adults, in parallel with the age-dependent increase in the underlying spontaneous baseline cancer incidence in the general population [118].

Another factor that may conceivably modify the dose–response relationship for radiation carcinogenesis at low dose levels is the potential influence of an adaptive response to radiation; i.e., under certain experimental conditions, as noted above, exposure to a small "conditioning" dose of radiation has been shown to stimulate DNA repair and to reduce the yield of mutations and/or chromosome aberrations that is induced by a subsequent "test" dose [49, 107]. As yet, however, it remains to be determined whether such an adaptive response can be elicited by low-level irradiation and, if so, the extent to which it might be expected to affect susceptibility to the carcinogenic effects of radiation [3, 107].

3.6 Modifying Effects of Other Physical and Chemical Agents

The carcinogenic effects of radiation may be enhanced or inhibited by various other physical and chemical agents. In endocrine glands and their target organs, for example, radiation carcinogenesis can be promoted by appropriate hormonal stimulation [3, 7]. Similarly, as noted above, the combined effects of radiation and cigarette smoking on the risks of lung cancer may be additive or more than additive, depending on the conditions of exposure [7]. Additional examples of interactions between the effects of radiation and those of other agents include: (1) the synergistic effects of ultraviolet radiation and X-radiation in the induction of squamous cell carcinomas of the skin in persons irradiated for tinea capitis in childhood [38]; (2) the synergistic effects of X-radiation and asbestos in the induction of mesotheliomas in rats [121]; and (3) the enhanced risks of second cancers in patients treated with combinations of radiation and various chemotherapeutic agents, as compared with radiation alone [7].

Depending on the conditions of exposure, additive, synergistic, or mutually antagonistic interactions have been observed among different agents in combination with radiation [7]. The existence of such variations is not unexpected in view of the multi-causal, multistage nature of carcinogenesis, the differences among diverse agents in their modes of action, and the different ways in which homeostatic processes may affect neoplasia.

3.7 Assessment of the Carcinogenic Risks of Low-Level Irradiation

Assessment of the carcinogenic risks of low-level irradiation in human populations is complicated by the

following factors: (1) the existing data on the quantitative relationship between irradiation and the risk of cancer come primarily from observations on the effects of relatively large doses, with the result that estimates of the risks at lower dose levels must be based on models, the reliability of which is uncertain; (2) contributing to the uncertainty of the models currently in use are questions about the extent to which they may fail to allow appropriately for the possibility that adaptive responses to radiation may modify the dose–incidence relationship at low dose levels; (3) the cancers induced by radiation do not appear until years or decades after exposure and are indistinguishable individually from those induced by other causes, so that their causal connection to irradiation can be inferred only on statistical grounds, based on an increase in their frequency above that expected; (4) the frequency of cancer at any one site is so low that few study populations have been large enough and/or exposed to high enough doses of radiation to yield highly quantitative dose-incidence data; (5) the average latent period between irradiation and the appearance of the induced cancer is so long that the follow-up of exposed persons and the evaluation of their doses is severely hampered; (6) none of the irradiated populations studied thus far has been followed long enough to disclose the total, cumulative lifetime effects of radiation on its cancer incidence; (7) many of the existing dose-incidence data have been derived from the study of patients treated with radiation for medical purposes, in whom the effects of radiation may be confounded by effects of other forms of treatment or of the underlying disease itself; (8) some of the existing data are based on effects of internally deposited radionuclides, interpretation of which is complicated by unknown variations of the radiation dose in space and time; and (9) the natural incidence of cancer varies so widely from one organ to another and under the influence of so many variables (e.g., genetic background, age, sex, geographic location, diet, socio-economic factors, etc.) that dose-incidence data derived from one population may not be strictly applicable to another [3, 7].

The above complications notwithstanding, the weight of existing epidemiological, experimental, and theoretical evidence suggests that lesions which are precursors to cancer (i.e., mutations and chromosome aberrations) and some forms of cancer themselves can be expected to increase in frequency as linear-nonthreshold functions of the radiation dose at low-to-intermediate levels of exposure [3]. Therefore, although the data do not exclude other dose–effect relationships, including those with thresholds, the linear-nonthreshold model, adjusted to allow for expected dose-rate- and LET-dependent differences in the tumorigenic effectiveness of radiation, is the model that is presently used in assessing the risks of radiation-induced cancer for purposes of radiation protection [3, 4, 6]. The linear-nonthreshold model has also come to be used as a basis for risk assessment in compensation cases involving the occurrence of cancers in previously irradiated persons [122, 123].

The use of this type of model has yielded a range of risk estimates for cancers of different sites (e.g., Table 3.3).

Table 3.3 Estimated lifetime risks of cancer attributable to 0.1 Sv low-dose-rate irradiation[a]

Type or site of cancer	Excess cancer deaths per 100,000 (No.)	(%)[b]
Colon	95	5
Lung	85	3
Bone marrow (leukaemia)	50	10
Stomach	50	8
Breast	45	2
Urinary bladder	25	4
Esophagus	10	3
Liver	15	3
Gonads	15	3
Thyroid	5	5
Bone	3	3
Skin	2	2
Remainder	100	2
Total	500	2

[a]Modified from [4, 124]

[b]Percentage by which the spontaneous, "background" risk would be increased

The estimates imply that less than 3% of all cancers in the general population can be attributed to natural background irradiation [7, 31]. Since a percentage that small would not be detectable with existing epidemiological methods, the estimate is not inconsistent with the fact that studies have thus far failed to find referable differences in cancer rates among populations residing in areas of widely differing natural background radiation levels [3, 7]. It is noteworthy, however, that although the percentage of all cancers attributable to natural background radiation is relatively small, the data imply that up to 20% of lung cancers may result from inhalation of the radon in indoor air [3, 18].

Furthermore, because the average radiation dose to the general population from medical sources in developed countries now exceeds that from all natural sources other than inhaled radon, growing attention is being given to limitation of the doses delivered in medical and dental practice [4, 6]. Methods used for the purpose include: (1) reduction of the number of radiographs per patient, with avoidance of unnecessary exposures; (2) reduction of the duration and intensity of exposure per radiograph; (3) use of other imaging techniques in preference to radiography and fluoroscopy whenever possible; (4) reduction of the field size to a minimum; (5) shielding of tissues outside the field to be examined, especially the gonads; (6) proper training and supervision of staff engaged in radiological examinations; (7) proper calibration and operation of radiological apparatus; (8) achievement of an appropriate balance between risk and benefit in the use of radiographic procedures for mass screening of asymptomatic populations, as in the development of guidelines for X-ray mammography in mass screening of women for the early detection of breast cancer [125].

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Chapter 4

Chemical Carcinogenesis Role of Chloroform – Further Studies

Elizabeth K. Weisburger

Contents

4.1 Introduction

Since the previous version of this chapter [1], there have been numerous studies on chloroform ($CHCl_3$ (CAS No. 67-66-3)) in order to explain the mechanism of its action. $CHCl_3$ had detrimental or beneficial effects in various animal studies, depending on the solvent, the species, and the sex of the animals, in addition to other factors. The presence of very small levels of $CHCl_3$ in chlorine-treated water remains a matter for much discussion among environmental groups, the US EPA and the community of toxicologists [2]. The International Agency for Research on Cancer (IARC) considers that there is inadequate evidence for the carcinogenicity of chlorinated drinking water in either animals or humans [3]. A report that weathering of organic matter leads to accumulation of halogenated organic compounds indicates that such occurrences should be considered in risk assessment [4]. However, risk from ordinary use of chlorinated water is extremely small, and the margin of safety is considerable [5]. The health benefits of chlorinated water, given with the small amount of $CHCl_3$ outweigh the risks from using untreated water.

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4.2 Short-Term Studies

Various short-term trials have continued investigation of the species and sex associated effects of $CHC1_3$. Administration by gavage in corn oil had a more harmful effect than did giving the compound in the drinking water. Gavage in oil led to cytolethality and regenerative cell proliferation in the liver and kidneys. Mice were more susceptible than rats, but the final result depended on the dosing vehicle, length of treatment and the level of $CHCl_3$ [6–14]. The dosing vehicle has less influence on the toxicity in rats than it did in mice [15–17].

The results in animals were reinforced by microsomal or hepatocyte tests which confirmed that $CHCl_3$ was metabolized to a greater extent, presumably to toxic intermediates as phosgene, by susceptible animals [18–20]. Reduction in glutathione content and its related enzymes was also noted.

As a volatile liquid, the usual route of exposure to $CHCl_3$ is by inhalation; there is a Threshold Limit Value (TLV) of 10 ppm for this compound [21]. Thus inhalation studies would appear more relevant to human exposure. One such study at 90 ppm for 6 h/day for 4 consecutive days with F-344 rats and several strains of mice indicated clearly that rats were not affected [22, 23]. Although B6C3F1 and Sv/129 mice had severe hepatic and renal necrosis, CYP2E1 knockout mice or mice given a P-450 inhibitor did

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not have pathologic changes, indicating that CYP2E1 is involved in cytotoxic-related metabolic conversion [23]. Further investigation demonstrated that inhalation exposure of susceptible BDFl mice increased the labeling index, a measure of cell proliferation, in target tissues [13]. This index was used as a marker for determining inhalation toxicity of $CHCl_3$ in both rats [14, 24] and mice [9, 25] and to examine differences between susceptible and nonsusceptible rat strains [14]. The technique was also employed to estimate risk from continued exposure [25–27].

Results from a 13-week inhalation study at several levels of $CHCl_3$, led to a conclusion that 5 ppm was a no observed adverse effect level (NOAEL) for nephrotoxicity in male mice; the NOAEL for hepatic proliferation in female mice was 10 ppm [26, 24]. In male F344 rats the NOAEL and lowest observed adverse effect level (LOAEL) for liver toxicity were 0.25 mmol/kg and 0.5 mmol/kg, respectively, after administration of $CHCl_3$ in an aqueous emulsion [28]. However, compensatory cell proliferation and tissue damage may not be the only factors in toxicity and tumor induction. Intense cell regeneration was observed in organs where $CHCl_3$ did not induce tumors. Thus the purely epigenetic role ascribed to $CHCl_3$ may be under question [29].

4.3 Carcinogenicity Studies

In one long-term test, $CHCl_3$ was given in the drinking water at levels of 0, 200, 400, 900 and 1,800 mg/l for 2 years to male Osborne-Mendel rats and female B6C3F1 mice. In rats, the high dose levels led to renal tumors [30]. Re-examination of the tissue slides led to the conclusion that the two highest dose levels for 6 months or longer gave a 95–100% incidence of kidney tumors, but at the 400 mg, or lower level, no tumors were seen [31].

The key events were sustained cellular toxicity and chronic regenerative hyperplasia with resultant changes in the convoluted tubules. In contrast $CHCl_3$ in the drinking water did not increase the liver tumor incidence in female mice, even at the highest dosage, equivalent to 263 mg/kg for 104 weeks [30].

Another lifetime test in Wistar rats afforded a different picture. A dose level of 24 mmol $CHCl_3$ in drinking water led to increased hepatic adenofibrosis in both sexes, while females had a significant increase in neoplastic liver nodules. However, test females had no mammary tumors vs. a 49% incidence in controls [32].

An inhalation study with male and female F-344 rats exposed to $CHCl_3$ at 90 ppm, 5 days a week for 2 years, failed to induce cancer [23]. A follow-up study showed lack of a direct genotoxic activity, only marginal cell proliferation in the kidneys of male rats and lower tissue-specific susceptibility in female rats [24]. In contrast, male but not female BDFl mice developed kidney tumors in this inhalation study, while female mice had an increase in liver tumors [23]. Additional investigation demonstrated that male mice had histologic changes and regenerative cell proliferation, in the kidneys [26]. However, the need to investigate other mechanisms, such as glutathione conjugation and reductive metabolism, was also emphasized [33].

4.4 Mechanistic and Interactive Effects

It has been mentioned that CYP2E1 appeared to be involved in the metabolic activation of $CHCl_3$ [23]. On the other hand, deprivation of food, which also induces CYP2El, had little or no effect on blood levels or toxicity when rats were exposed by inhalation. Metabolism of $CHCl_3$ was increased threefold [34]. Oral administration caused more severe hepatic damage than did intraperitoneal injection. The result was attributed to the first pass metabolism unique to oral administration [34]. Mice exposed to $CHCl_3$ in a closed recirculating chamber had significant decreases in body temperature and enzyme activity. Blood/air and tissue/air partitions increased with falling temperatures. The data were used to determine a physiologically based pharmacokinetic model (PB-PK) for $CHCl_3$ metabolism [35]. However, PB-PK dose measures did not reconcile the rat and mouse kidney tumor response data, although it performed well for the liver tumor data [36]. Further study of the P450s involved in $CHCl_3$ activation was carried out in rats pretreated with inducers of CYP2E1 and CYP2B1/2. These treatments potentiated hepatic damage. Histologic, innnunoinhibition and immunoblot analyses led to the conclusion that both CYP2E1 and CYP2B1/2 contribute to the hepatic damage but they do so quite differently [37].

In contrast, other tests showed that in DBA/2N mice there was a decided increase in CYP2A5 expression in the liver, but no change or decrease in levels of CYPlA, 2B, 2C, 2E1 and 3A4. No explanation for these differences in enzyme induction has been suggested [38].

$CHCl_3$ interacts with or is affected by administration of various other compounds. Its hepatotoxicity and lethality were increased by concurrent administration of a series of alcohols, from methanol to decanol [39]. Most are P-450 enzyme inducers, but in some cases enzyme inducers had little or no effect [40]. Similar results were noted with methyl isobutyl ketone and some of its metabolites [41], or with 2-hexanone [42]. Such compounds induce CYP2E1 and CYP2B1 which increase metabolic activation [43] Combinations of $CHCl_3$, carbon tetrachloride or trichloroethylene had synergistic toxic actions, as indicated by plasma enzyme activities [44]. Various other compounds have inhibited the effects of $CHCl_3$, even, $CHCl_3$ itself. Giving it in drinking water, at levels from 120 to 1,800 ppm, to female B6C3F1 mice for a month protected them against hepatotoxicity and enhanced cell proliferation when they received $CHC1_3$ in corn oil [45]. Dimethyl sulfoxide, even when given 10 h after an oral dose of $CHC1_3$, protected male SD rats against hepatic injury and tubular necrosis of the kidney [46]. Methoxsalen, an inhibitor of P-450, prevented the toxic action of $CHC1_3$ in mice by decreasing metabolic activation [47], while pyrazole acted similarly for rats [48]. However, in Mongolian gerbils, pretreatment with the enzyme inducers phenobarbital, chlordecone, mirex or 3-methylcholanthrene decreased the toxicity of $CHCl_3$ [49].

Unexpectedly, $CHC1_3$ has emerged as an inhibitor of some other carcinogens. In male F-344 rats given standard doses of 1,2-dimethylhydrazine to induce colon tumors, $CHC1_3$ at 900 or 1,800 mg/l of drinking water, led to a significant decrease in tumors; 36% in controls vs 13% in $CHCl_3$ treated rats [50]. Likewise, $CHCl_3$ in drinking water reduced the incidence of liver tumors in mice given an initiating dose of ethylnitrosourea or diethylnitrosamine, indicating lack of a promoting effect [11]. Further, when male F-344 rats were initiated by partial hepatectomy and diethylnitrosamine, followed by phenobarbital and $CHCl_3$ in drinking water, the $CHCl_3$ treatment had a tumor-inhibiting action. In this case, $CHCl_3$ affected the activating enzymes [51].

4.5 Risk

In evaluating the risk from exposure to $CHCl_3$ in water, the greater toxicity of other trihalomethanes or other compounds formed during chlorination is often overlooked [3, 52]. Of special interest is MX or 3 chloro-4-(dichloromethy1)-5-hydroxy-2 (5H)-furanone which is a very potent mutagen [3]. The method used by the US EPA to estimate risk is often criticized because it is a linearized multistage model, whereas animal studies indicate a non-linear dose-response relationship [53, 54]. As an example, the EPA method yields a virtually safe dose (VSD) of 0.000008 ppm for inhaled $CHCl_3$, while animal data indicate 0.01 ppm is a VSD, even with an uncertainty or safety factor of 1,000 [26]. The use of a more realistic model would still allow for protection of public health without wasting resources to attain unrealistic goals [55].

Estimating the risks from $CHCl_3$ exposure appears to be a continuing activity within many groups [56, 57]. The result is that risk of cancer from the usual exposures to chlorinated water is quite small and would not lead to increases in liver cancer [5, 58]. Risk to health from not using chlorinated water would be greater.

4.6 Conclusion

Many studies have shown that metabolic activation of $CHCl_3$ with consequent cell injury and regeneration appears to be involved in its harmful effects. Route of administration, species, strain and sex influence the final outcome. The presence of tiny amounts of $CHCl_3$ in chlorinated drinking water remains a matter of controversy, even though other more harmful compounds are also formed. Animal studies at below 400 ppm of $CHCl_3$ in water showed no effect; this level is many times higher than levels in treated water. Risk analyses indicate the actual risk from $CHCl_3$ in water remains extremely small.

4.7 Addendum

Since the major portion of this chapter was written, additional studies on chloroform have been reported. Most have been directed at the processes involved in

the hepatic and renal toxicity of chloroform. One relatively long-term study of 26 weeks was done with mice carrying the rasH2 transgene [59]. Contrary to expectations, chloroform led to no significant increase in neoplastic lesions in these mice. This mouse model may not be suitable for detection of nongenotoxic carcinogens.

Short term in vivo or in vitro tests have led to further discoveries on the mechanistic aspects of the effects of chloroform. The known metabolite, phosgene, interacts with proteins, especially lysine residues and other cellular constitutents [60], but some evidence for involvement of a trichloromethyl radical has emerged also [61].

Chloroform toxicity, leading to cell death, involved glutathione depletion and oxidative stress with protein nitration [62, 63]. The extent and rapidity of tissue repair reduced the hepatotoxicity of chloroform [64, 65].

However, dietary restriction, even for 1 day, changed hepatic metabolism and increased the hepatoxicity of chloroform in rats [66].

The toxicity of chloroform in SW mice was diminished by an initial subchronic dose which then protected the mice from a subsequent lethal dose through increased exhalation and tissue regeneration [65, 67]. Administration of COX-2 inhibitors also decreased the hepatoxicity of chloroform [68].

However, combined exposure via inhalation and the drinking water increased the degree of renal carcinogenesis [69]. The renal toxicity of chloroform was not dependent on hepatic P450 enzymes, as shown in a mouse model null for P450 reductases, where chloroform induced renal lesions [70, 71]

In utero or lactational exposure of Wistar rats to chloroform via the drinking water of the dams did not lead to changes typical of type 2 diabetes. However, this treatment did cause impaired postnatal growth [72].

The risk of chloroform to humans has been reviewed [73] and considered by several groups, using pharmacokinetic and pharmacodynamic (PBPK/PD) parameters. Reference doses (RfDs) for liver and kidney, respectively, have been calculated as 0.4 mg/kg/day and 3 mg/kg/day [74, 75].

Since the usual levels of chloroform in drinking water are in the order of micrograms/liter, these RfDs are considerably higher than the exposures usually attained from drinking water.

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Chapter 5

Use of Organ Explant and Cell Culture in Cancer Research

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Contents

5.1 Introduction to Tissue Culture and References

Cancer is a multi-factorial disease. The primary cause of cancer is genetic instability in the cellular fidelity of DNA replication. When DNA is not copied correctly, the resulting mutations may lead to cancer. The causes of these mutations can be single or multi-factorial. Many things are known to cause these mutations. The major factors or agents include: viruses (e.g. sv40, ebv, etc [1]), physical factors (e.g. asbestos, radiation, [2–3]), oncogene activation (e.g. ras, c-met, her2-neu, v-abl), as well as familial inherited mutations [4–8]. There is a delicate inter-relationship between the genetics and the environment that leads to cancer. Metabolic pathways (e.g. p450 system, debrisoquine pathways) are also critical in this interaction between genes and the environment [9–10]. The lesions that evolve may go through a series of steps or cascades (e.g. Vogelstein and Kinzler model [11–13]), or they may arise seemingly spontaneously. There can be inherited familial genetic syndromes [7–8], known or unknown environmental exposure to toxicants/carcinogens [14], or, most likely, combinations of these two mechanisms that cause or predispose individuals to cancer.

The fundamental defect, however, is within the DNA of cells. Errors in the copying of DNA that are propagated in dividing cells may induce damage and lead to a phenotypic lesion. These mutations, if they are in the DNA synthesis/repair system (e.g. p53), cell cycle control pathways (e.g. cdc7 and cdk genes), or growth factor ligand/receptor pathways (e.g. c-Met), can be significant in their carcinogenesis potential. Since cancer is a disease that starts in the DNA of a single cell or cloned cells, it is logical and appropriate that the study of cancer has developed through in vitro models. Isolated monolayer or suspension cells from carcinomas were among the first cells to be grown (reviewed in Chapter 9 of Chapter 15). Seemingly immortalized cells like those of the HeLa cell line have served researchers for generations [15]. Cells maintained as a cohesive group or as a tissue have been used to study metabolism and morphology, and whole organs have been maintained in vitro for organotypic culture. There are numerous review articles, textbooks, and chapters that define, explain, and document the use

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D. Coppola (ed.), *Mechanisms of Oncogenesis*, Cancer Growth and Progression 12,
DOI 10.1007/978-90-481-3725-1_5,

Table 5.1 Tissue culture review references

References	Description
Modern Cell Biology, Alan R. Liss, Inc., New York, NY, 1989	Epithelial cell culture
Methods in Enzymology, Volume 58; Academic Press, Boston, MA, 1979	Methods for cell culture
Culture of Animal Cells. Alan R. Liss, Inc., New York, NY, 1983	Introduction and methods
Methods in Cell Biology, New York Academic Press, 1981	General methods

of cell, tissue, and organ culture in cancer research. The Table 5.1 lists many of the standard and complete references on the field and discipline of tissue culture. In the context of this review, "tissue culture" includes cell, monolayer, organ explant, and organotypic cell culture.

Our previous chapter [16] described advances in the field of in vitro cancer research, particularly the use of human tissues in vitro to study cancer. We also documented the relationship and comparison between animal models and human systems. Figure 5.1 from our earlier chapter summarizes the original work. The fundamental premise of the original chapter was that one could understand carcinogenesis in humans by studying cells, tissues, and organs in vitro. The original work described how one could take a well-characterized animal model of cancer and isolate the cells, tissues, or organs that developed into cancer in that animal, and maintain the cells in vitro. The genetics, phenotype, and metabolism of the animal could then be studied in vitro for similarities and differences. Human cells obtained through donation, autopsy, and surgery could be similarly studied. Human in vivo studies are not ethically possible. However, if there is a consistency between the whole animal and the animal cells or tissues, and the observations, measurements, and characterizations at the genetic and molecular level between the animal cells and human cells are equivalent, then one can draw the conclusion that the carcinogenic process under study would, in the human, be carcinogenic as well.

5.2 Standard Definitions and Uses of Tissue Culture

In the original chapter we concentrated on how one could study cancer in vitro from human cells, tissues, and organs. That concept and method of tissue culture is now well characterized and accepted in the field of cancer research. This edition of the chapter will focus instead on the quantification, imaging, and analysis of cancer. It will also explain how one can use in vitro models, not only to describe the carcinogenic process qualitatively, but also to concentrate on the important aspect of quantification. There is no need to rewrite the methods and techniques of cell, tissue and organ culture when so many excellent reviews and books already exist.

Cell culture is the growth of dispersed, disaggregated, single cells of a unique cell type, which do not necessarily retain the histologic structural relationships of the cells and tissues from which they were removed. It can be a monolayer, mixed population, and can be

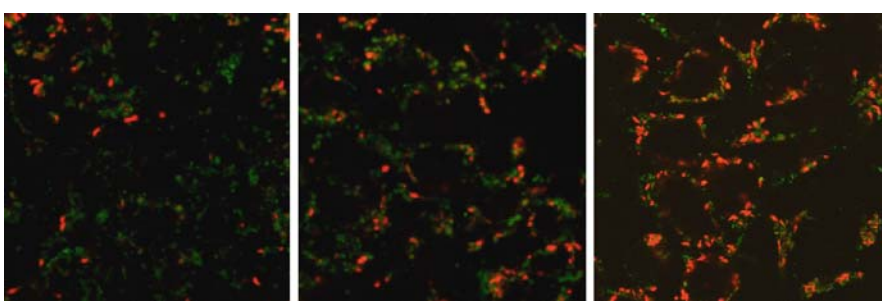

Fig. 5.1 Use of tissue culture to study human carcinogenesis. Cells and explants are cultured from tumor tissue to characterize their morphologic and biologic properties (e.g. metastatic potential, susceptibility to chemotherapeutic drugs). Normal cells and explants are cultured in protocols which expose these cells to carcinogens in order to induce transformation in vitro. The transformed tissues can then be assayed the same way as the tumor tissues are studied

in either a two or three-dimensional culture, depending on the substrate. Removal and isolation of the cells is achieved by enzymatic, chemical, mechanical, or physical separation of the cells. Such cells are initially isolated and identified as a primary cell culture. Later, in their in vitro life span, they may appear immortal, and are then called "cell lines" (human tumors have been shown to possess the ability to give rise to immortal cell lines [17]). Cell lines have proved to be an invaluable resource in the field of cancer research. *Organ culture* is the three-dimensional, multi-cellular, multi-tissue, in vitro growth of sections or pieces – explants – of organs that retain at least some of the histologic structural integrity of the tissue from which it was taken. Explant organ culture therefore contains the multiple cell types comprising the tissue from which the explants are resected. For the purposes of this chapter, both organ culture and cell culture, along with the in vitro studies of cellular and molecular pathophysiology will be referred to by the more general term of tissue culture. Table 5.2 lists some of the important review articles describing the methods of tissue culture. The important references in the field of imaging, including molecular imaging and cellular based imaging, are listed in Table 5.3.

Carcinoma cells possess specific morphologic criteria that enable them to be identified as malignant [18–19]. They are characterized by an angularity in the nuclear and cytoplasmic organelles and cytosol material, lack of uniformity in cellular characteristics and structures (e.g. lumens, nuclear membranes, membrane thickness), and accentuations of normal phenotypic features (e.g. clearing, amount of cytoplasm, clumping, size of cell). Tissue culture has provided several other criteria by which to distinguish such cells. Cancer tissues tend to have decreased adhesion between cells, and cells isolated from the tumors are often described as being anchorage independent [20]. Cancer cells do not exhibit contact inhibition restrictions, and tend to grow differently on the cultured

Table 5.2 Tissue culture methods references

References	Subject
Cancer Res 59(7 Suppl):1757–1763s; discussion 1763–1764s, 1999	Tissue culture, genome/environment/3D tissue structure interactions
Cancer Res 53(10 Suppl):2446–2448s, 1993	In vitro carcinogenesis methods
Cancer Res 53(10 Suppl):2455–2456s, 1993	Nutrition and carcinogenesis in vitro
Cancer Res 61(3):799–807, 2001	Nutrition and carcinogenesis in vitro
Cancer Res 35(10):2619–2630, 1975	Differential sensitivity of cells to anticancer agents
In vitro 19(4):317–325, 1983	Environment/tissue interactions
Cancer 48:1490–1496, 1981	Tissue culture methods for pancreatic cancer models
J Natl Cancer Inst 66:849–858, 1981	Environment/tissue interactions

Table 5.3 Imaging references

References	Subject
Laser Capture Microscopy. *In* Abelson JN, Simon MI (series Ed.) *Methods in Enzymology*, Academic Press, Boston, MA, 2002	Laser capture methods
Handbook of Fluorescent Probes and Research Products, Ninth Edition. Molecular Probes, Inc., Eugene, OR, 2002	Reagents, methods, probes for fluorescence
Methods in Cell Biology, Academic Press, Boston, MA, 1993	Confocal applications
Confocal Microscopy: Methods and Protocols. *In* Walker JM (series Ed.) *Methods in Molecular Biology*, Humana Press, Totawa, NJ, 1999	Confocal applications
Handbook of Biological Confocal Microscopy, Second Edition. Plenum Press, New York, NY, 1989	Confocal applications
Fluorescence Microscopy of Living Cells in Culture: Part A. and Part B. *In* Wilson L (series Ed.) *Methods in Cell Biology*, Academic Press, Boston, MA, 1989	Microscopy applications
Am J Physiol Cell Physiol 283:C905–C916, 2002	Multi-photon microscopy intra vital imaging
Genes and Devolopment 17:545–580, 2003	Molecular imaging
TRENDS in Cell Biology 13(2):101–106, 2003	Fluorescent imaging

vessels as plaques or clusters of raised colonies. Cancer cells or tissues grown in culture often release specific marker substances, such as mucins [21] or tumor angiogenesis factor (TAF) [22]. However, while such criteria aid in distinguishing cancer cells from healthy tissues, they do not provide an adequate basis for the classification of cancers. Thus far, cancer classification has been based primarily on morphology and empirical parameters, such as tumor histology and patient history, as well as the expression of the aforementioned markers, which are often unreliable. Such classifications are far from accurate, the major limitation being that many morphologically similar tumors exhibit dramatically different clinical outcomes and responses to treatment. The recent development of DNA microarray technology [23–26] and the subsequent development of gene-expression profiling [27–29] have provided many exciting possibilities for the future of cancer classification and prediction of patient survival [30–31].

5.3 Gene-Expression Profiling

Studies of leukaemia have demonstrated that gene-expression profiling can be used to determine tumor classification in the absence of any previous knowledge [32]. Other studies involving diffuse large-B-cell lymphoma (DLBCL) patients have indicated that gene-expression profiling can be applied to predict clinical outcome [33]. The use of gene-expression profiling to determine classification and projected clinical outcome has important implications for the future of cancer treatment. The more accurate classification afforded by such profiling allows for more specific treatments, and, as in the case of breast cancer, can be used to prevent unnecessary treatment with adjuvant therapy, which often is accompanied by severe toxic side effects. Currently, the determination of metastases or the likelihood of its occurrence (an essential parameter used in considering the administration of adjuvant treatment) is based, in breast cancer patients, upon the detection of lymph node metastases at the time of surgery [34]. Authors of a study on gene-expression profiling in breast cancer patients were able to determine a group of 70 differentially expressed genes in lymph-node-negative patients, which they termed a "poor prognosis signature," that closely corresponded with the disease outcome. Another significant finding was that this poor prognosis signature already exists in primary breast tumors at the time of surgery, and it has been demonstrated that gene-expression profiling is a far more accurate predictor of outcome for breast cancer than any other currently used criteria [35]. Studies of melanomas, medulloblastoma, and other cancers have further demonstrated gene-expression profiling to be a useful tool in developing a better understanding of the molecular processes resulting in cancer, as well as the identification of novel potential targets for therapy [30, 32, 33, 36–46].

5.4 Laser Capture Microdissection

When performing gene-expression profiling on biopsied tumors, the subpopulation of cells desiredfor analysis often constitutes only a microscopic 5% of the total tissue volume. Including any of the remaining 95% of the tissue in analysis can contaminate the whole sample and greatly skew results [47]. Previously, to avoid such contamination, the desired cells were harvested in culture. However, cultured cells often lose a number of desired inherent properties when removed and grown separately from the host tissue [47]. Today, these drawbacks of gene-expression profiling combined with cell culture can be avoided using laser capture microdissection (LCM).

LCM has quickly become known as "a fast and dependable method of capturing and reserving specific cells from tissue, under direct microscopic visualization" [47–79]. Cells are transferred from a fixed or frozen tissue sample to a thin polymer film, using a laser beam with a diameter of 7.5, 30, or 60 μm [50]. Genetic components extracted from these cells can then be used for gene-expression profiling of individual patients, microarrays, cDNA libraries, and other techniques of genetic mutational analysis [47, 51–56]. LCM has also been successfully applied in the removal of intact proteins from specific cell populations derived from tissue sections (fixed, stained, or frozen) on glass microscope slides, "under direct microscopic visualization" [47, 57, 58]. Proteins removed in such a manner have been employed in two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), a technique that has previously required an amount of protein analogous to millions of cells.

5.5 Molecular Imaging

Molecular imaging is a rapidly developing biomedical field that can be defined as the visual representation, characterization and quantification of biological processes at the cellular and sub-cellular levels within intact living cells and organisms. The extensive development of this field in recent years has been achieved largely as a result of the many recent advances in molecular and cellular biology.

New imaging technologies, initially studied on tissue cultures and organ explants, now provide researchers the ability to monitor in vivo tumor development, specific molecules, and the efficacy of treatment on individual, narrowly-defined populations of cells. Technologies previously used in clinical diagnostics of other ailments, such as magnetic resonance imaging [59–60] and x-ray computed tomography (CT), have found their way into the laboratory, providing valuable new insights and possibilities in the arena of cancer research.

Molecular imaging techniques can be divided into several fields; radionucleotide imaging (e.g. positron emission tomography–PET), magnetic resonance imaging (MRI), computed imaging (CT), ultrasound (US) and optical imaging. These differ in several aspects, such as spatial and temporal resolution, depth of penetration, and the respective detection threshold of the specific probes used in each technology [61]. Optical imaging techniques now utilize specific molecular fluorescent probes, which can be used at different resolutions and depth penetration ranging from micrometers (intravital microscopy – IVM) to centimeters (fluorescent molecular tomography – FMT) [62]. A major obstacle of optical imaging is the auto-fluorescence obtained from the different tissues. The availability of certain treatments, such as Sudan Black quenching [63–64], together with the recent development of smart detectors combined with confocal and multi-photon microscopy enables the researchers to overcome the problem of auto-fluorescence of certain tissues and distinguish between their signal and the specific signal of the fluorescent probe used [65]. An important benefit of molecular imaging assays is their quantitative nature, as well as the ability to extract three-dimensional information regarding the spatial distribution of the tumor or phenomenon within a particular cell, organ, or throughout the entire body. Optical imaging is widely used in cancer research, both at the cellular as well as at the whole animal levels [66].

In recent years, fluorescent proteins such as Green Fluorescent Protein (GFP) have served as an important tool, both for tumor detection as well as the monitoring of tumor development. Tumor growth is monitored by following cells that over-express GFP [e.g. 67, 68–70]. In these studies, cancer cells expressing GFP were injected into mice, and tumor development was followed using the GFP signal. These studies employed different optical imaging techniques, and were carried out by either illumination with blue light [68] or intravital fluorescence microscopy [67, 69]. Chimeric fluorescent proteins were used to tag oncogenes and other proteins that are involved in cancer development and metastasis [71]. These tagged proteins have been used for both sub-cellular localization and molecular real-time interaction studies [10]. Optical molecular imaging of fluorescent-tagged proteins enables the study of the expression of these proteins with high spatial and temporal resolution. Protein interactions and activity are studied by using fluorescence resonance energy transfer (FRET) microscopy [72] using specifically designed combinations of fluorescent molecules that will pass the emission signal to an adjoining protein if close or co-localized and induce fluorescence (e.g. blue laser light will excite FITC to emit green light that will excite rhodamine to emit red light). These techniques enable the study of the effects of treatments in real time at the cellular and whole organ levels. Molecular imaging of living animals (intravital) in cancer research offers several advantages over cell and tissue culture studies. First, bio-distribution of molecular probes and related biological processes can be studied with high temporal and spatial resolution, in the context of the intact living object. This enables the visualization of the functions and interactions of particular proteins within the live animal. Additionally, it eliminates the need to euthanize the animals, permitting repetitive imaging of the same animal over time, and allows the use of different imaging strategies on the same animal. The basic problems associated with optical imaging of a living animal (e.g. low penetration, which limits the imaging of internal organs and high auto-fluorescent background) still remain the main obstacle in the use of optical imaging in the lab and in the clinic. Nevertheless, the constant improvement of instruments and probes used for optical molecular imaging is providing novel insights

into molecular mechanisms of tumorigenicity by real time mapping of the location and the interaction of key players in cancer development and metastasis.

5.5.1 In Vitro Mouse Models

Thus far, many of the groundbreaking advancements in imaging have been achieved in miniature on mouse and other animal models [73]. Transgenic and knock-out mice are now the standard for cancer research, and mice, as well as hamsters, are also a favorite for explant research [66, 74–75]. The high-resolution imaging of mice with scaled-down versions of equipment clinically-available for humans has provided us with invaluable insights into the physiological and molecular processes underlying cancer development and metastasis, and, consequently, new possibilities for treatment of the disease.

New imaging equipment in miniature has made possible the three-dimensional reconstruction of organs and tissues. With the advent of injectable "smart" reporter probes, scientists now have the capability to monitor tumors in vivo, thus providing valuable insights about host-tumor interactions, tumorigenesis, and therapy efficacy. In vivo imaging in mice allows the non-invasive visualization of primary metastatic tumors. Such imaging also provides the ability to monitor the physiological events of cancer. Ultimately, this non-invasive imaging of cancer in mice holds promise for translation to clinical application in humans, allowing earlier detection and phenotyping of tumors, and resulting in treatments custom-tailored to specific patients.

5.5.2 Magnetic Resonance Imaging

A wide array of imaging technologies is now available for cancer research. One of the most important of these technologies, high-resolution magnetic resonance imaging (MRI) provides information on the physico-chemical state of tissues, flow, diffusion, motion, and molecular targets [73]. Capable of producing detailed anatomical images, it is inarguably one of the most useful techniques available in the screening of transgenic mice for tumors and other anatomical abnormalities. MRI is used to visualize tumor size, location, metastatic burden, and phenotype, and can also be used to quantify vascular parameters such as capillary permeability, flow, and vascular volume, even deep within tumors [59, 76–82]. Due to these impressive capabilities, MRI is slowly replacing more invasive (and usually terminal) histological procedures that involve meticulous analysis of micro-vessel density. MRI is also used in the detection of the expression of receptors, transgene products, and tumor-specific markers [62]. Recently, magnetic nanosensors, which detect certain DNA or mRNA sequences, have been used in conjunction with MRI for the rapid analysis of non-purified tumor samples, as well as for the determination of tumor phenotype in vivo [83]. With this promising combination of technologies, specific populations of cells can be magnetically labelled and followed using MRI, and could thus be useful in stem-cell-based therapies requiring long-term in vivo tracking of specific cell populations. Magnetic resonance spectroscopy (MR) is used to image metabolic activity in tumors and detect the expression of specific molecules, proteins, and tumor-specific markers.

5.5.3 Fluorescence Imaging

Fluorescence imaging is one of the least expensive and most rapid methods of imaging a specific cell or molecule in mice, and is capable of monitoring both normal and carcinoma cells. Fluorescence reflectance imaging (FRI) detects molecular events in surface-based tumors, while fluorescence-mediated tomography (FMT) is used for the quantitative imaging of deep tumors. FMT requires the use of targeted, or "smart," fluorophore reporters; with this technology, a method of tomographic reconstruction for in vivo imaging of fluorescent probes has been developed [84]. In the lab, FMT is used to quantify protein expression or localization in vivo in the absence of radioactive labelling. This technique holds promise for translation to clinical use [85]; patients could be injected with fluorescently labelled affinity molecules, and tumor reactivity to these probes could then be used to identify breast, prostate, or colon cancers in their earliest forms. Fluorescence correlation spectroscopy (FCS) is used at the single molecule level to investigate molecular dynamics. The one significant drawback of fluorescence imaging is the need for tumor-bearing regions to be surgically exposed.

5.5.4 Intravital Microscopy

Perhaps the most important and useful of today's imaging options is that of intravital microscopy (IVM), through confocal or multiphoton imaging [61, 86–87]. Such intravital microscopy is useful for investigating tumor pathophysiology, and has provided many useful insights into the various aspects of tumor biology, including molecular, functional, genetic, and cellular, as well as host-tumor interactions and tumor response to therapy [88]. Confocal microscopes enable the visualization of organelles and cell processes, such as receptor-ligand interactions and co-localization of proteins with organelles. The great strides made in increasing depth capabilities and microscope miniaturization in multiphoton laser-scanning microscopy have resulted in the recent advances in the study of living, and even behaving, non-anaesthetized animals [89–90]. Nonlinear optical microscopy (NLOM) relies on the nonlinear interactions of laser light with specific molecules in a biological sample, resulting in the emission of fluorescent light. The most commonly used method is two-photon excitation (2PE) of fluorescence, utilized in two-photon-excited fluorescence microscopy (2PM), and involves the near-simultaneous absorption of two (usually near-infrared, or NRI) photons [91]. Other multiphoton processes receiving recent attention are three-photon excitation and second harmonic generation (SHG) [92]. The key advantage of multiphoton microscopy over single-photon is that multiphoton processes provide high-resolution images from deep within intact living tissue [93]. The use of fiber optics has enabled the miniaturization of two-photon microscopy, allowing for increased flexibility and mobility. Miniaturized optics, fiber delivery, and fiber-tip resonant scanning have made possible experiments on behaving animals [89]. Other technologies, such as stimulated-emission depletion (STED) utilize multiphoton processes. While it does not require multiphoton excitation, STED is made more effective by its use, and thus creates excellent resolution comparable to that of near-field microscopy, but with the advantages conferred by a far-field technology [94–95]. Multiphoton microscopy has proven to be particularly useful in in vivo tumor characterization, allowing the investigation of previously inaccessible internal regions of tumors.

While IVM is most commonly used in determining tumor size, architecture, and vasculature, it has recently been utilized to explore gene expression and function in tumors, as well as promoter and enzyme activity in vivo. Other findings have given new insights into the molecular origins of cancer, identified a number of genes involved in oncogenesis, and have emphasized the important influence of host-tumor interactions on angiogenesis, growth, and metastasis [96–97]. These, and other discoveries have resulted not only in a better understanding of the steps involved in cancer development and metastasis, but also in the creation of new methods of detection and treatment of cancer, including improvements in immunotherapy, stressing the importance of scheduling and optimal dosage to maximize efficacy of therapy.

Traditionally, tumor response to therapy (in animal models) has been evaluated in terms of tumor size (either a reduction or stabilization thereof) or survival time [88]. With the advent of IVM, it is possible to simultaneously monitor multiple parameters, allowing for a better understanding of tumor response to therapies, and even the physiological determinants of drug delivery to tumors [88]. IVM requires the use of an animal model, a molecular probe, a microscope equipped with a digital camera detection system, an image acquisition system, and computer analysis of data [88]. Using multiphoton laser-scanning microscopes (MPLSMs) and confocal laser-scanning microscopes (CLSM), the depth of imaging can be further increased [98, 99]. Any molecule that can be detected by optical microscopy can also be tracked by IVM in vivo [88], and, thus, combined with the ability to continuously and non-invasively monitor molecular and cellular processes, IVM provides a valuable method by which to monitor gene expression and regulation in living animals [88]. Ultimately, it is hoped that IVM will yield new options for the detection and treatment of cancer in humans, as well as provide valuable insights into the function and expression of genes in a live, intact host.

5.5.5 Other Imaging Methods

There are a number of useful imaging technologies available other than those described in the preceding sections, and these should not be neglected in the discussion of molecular imaging. This group of "others"

includes x-ray computed tomography (CT), ultrasound, optical imaging, and nuclear imaging. CT allows the researcher to obtain three-dimensional images, and is most useful in lung and bone-tumor imaging. Ultrasound, most often used in vascular and interventional imaging, generates images based on acoustic echoes and is useful in the rapid screening of pathologies. Optical imaging often includes the utilization of a charge-coupled device (CCD) camera for optical detection. A number of imaging technologies require the additional use of molecular probes which recognize tumor-specific markers and reporter probes that mark specific biological processes. The recent development of "smart probes" that are activated and detected only when they interact with the specific target has further improved the accuracy and capabilities of these technologies. Single photon emission tomography (SPECT) requires the use of reporter probes or contrast agents to image antibodies, peptides, and other probes. Through radio-labelling of DNA, SPECT allows the monitoring of therapeutic gene-delivery vectors and antisense oligonucleotides [100–101]. The efficacy of such gene-therapy vectors [102–103], as well as the expression of extra cellular receptors [104–105], can be further monitored through positron emission tomography (PET), which also requires reporter probes or contrast agents. Another available imaging method is that of bioluminescence imaging (BLI), which is used for monitoring gene expression and cell tracking (but which is unlikely to be useful in a clinical setting due to its inability to monitor cells other than those of transgenically modified tumors).

5.5.6 Met-HGF/SF: A System Studied Through Molecular Imaging

One system that was extensively studied in the field of cancer research, using molecular imaging methods, is the Met-HGF/SF system. Met is a proto-oncogene that belongs to the tyrosine kinase growth factor receptor family. It is expressed in a wide variety of tissues, but mostly on the surface of epithelial cells. The intracellular domain of Met contains the tyrosine kinase domain, and its extracellular domain binds its ligand, Hepatocyte Growth Factor/Scatter Factor (HGF/SF), which is normally produced by mesenchymal cells. Binding of HGF/SF to Met leads to autophosphorylation of several tyrosine residues in the tyrosine kinase domain of the receptor and at its docking site [106]. The multi-substrate docking site of Met recruits adapter signalling molecules such as Grb2, Shc, Gab1, Src and Crk/CRKL as well as signalling transducers such as phosphotidylinositol-3-OH kinase (PI3K), the signal transducer and activator of transcription 3 (Stat3), phospholipase C-γ (PLC-γ) and Src [107]. These interactions occur either directly or indirectly via other adapter proteins and signal transducers [reviewed in 108]. It was shown that Met, and its activation, are important for Met-HGF/SF-mediated cell migration and transformation [109–110] as well as differentiation [111]. Recently, a protein was identified, that is up-regulated in cells upon Met activation, named Mimp (Met-induced mitochondrial protein). Mimp encodes a 33 kDa protein, and exhibits both sequence and a structural homology to the family of mitochondrial carrier proteins. It is expressed in a wide range of tissues with an expression pattern similar to that of Met [112]. Using a Mimp tagged to GFP,

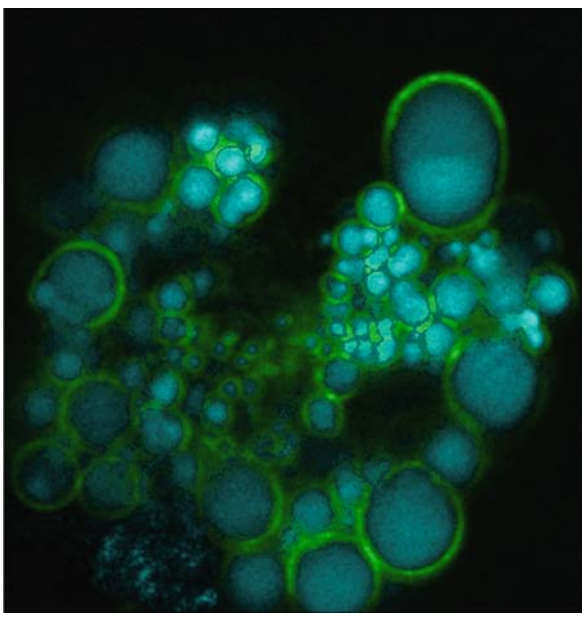

Fig. 5.2 CLSM analysis of 293-T cells transiently co-transfected with GFP-Mimp and ECFP-mitochondrial marker (BD Biosciences Clontech, CA). GFP-Mimp localizes to the mitochondrial membrane while ECFP-mitochondrial marker stains the mitochondrial lumen

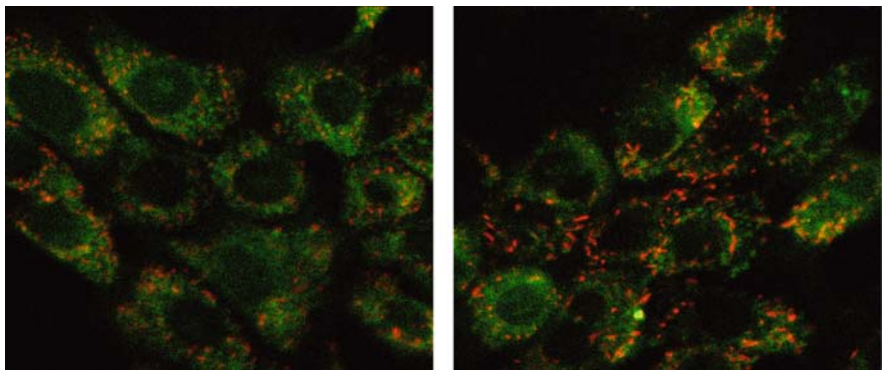

Fig. 5.3 CLSM analysis of cells expressing Mimp-GFP and stained with JC-1 dye. The ratio between the *green* and the *red* fluorescence of each stained cell indicate the mitochondrial membrane potential of the cell population

together with a mitochondrial marker localized Mimp to the mitochondria as can be seen in Fig. 5.2 [112].

The fluorescent dye JC-1, which is a reliable indicator of the mitochondrial membrane potential changes in live cells, was used in cells expressing Mimp-GFP. HGF/SF treatment led to a significant mitochondrial membrane depolarization (Fig. 5.3) [112].

The use of optical molecular imaging in the study of the Met-HGF/SF system has provided important answers to many questions concerning both the location and the interaction of key members in cancer development and metastasis.

5.6 Quantitative Analysis

As described in this text, pathologists and cell biologists have traditionally used microscopy to make histological assessments of tissue phenotype based on morphology and, more recently, protein expression with the aid of immunohistochemistry and immunofluorescence. The advantage of this methodology is that through training and experience, one can make highly sophisticated histological interpretations. The disadvantage is that this sophistication varies in an operator dependant fashion [113–114], and is not easily transferred or communicated. This is an impediment to clinical care as the selection and evaluation of treatments hinges on accurate and precise diagnosis. It is, likewise, an impediment to advances in research, since the study of factors influencing biological events requires precise measurement of those events.

5.6.1 Advantages of Quantitative Analysis

These challenges may be mitigated through quantitative analysis of digital images. The digital image is a quantitative data set comprised of the light intensity at each pixel expressed as a numeric value (typically on a scale of 0–255 or 0–1,024 as a result of technicalities related to binary data storage). This data set may be processed computationally in any number of ways, several of which are further described here. Such analysis provides several advantages over the subjective interpretation of pathologists:

1. Computational methods are reproducible. The same algorithm run on the same images should give exactly the same results time after time.
2. Computational methods are portable. Different operators in different locations can perform exactly the same analysis by running the same computational algorithm on their images. When run on different image sets, the results are directly comparable because they were derived in exactly the same way (although variation in tissue preparation and image acquisition may introduce persistent variability).
3. Computational methods provide data that is scaled continuously. Human interpretation of micrographic images has typically been at best semi-quantitative. For example, Her-2 expression may be reported as “strong”, “weak”, “+4” or “+2”. Computational methods can report results as continuous variables over any desired range, providing much finer granularity (e.g. resolving power) in the data.

However, these advantages of computational pathology are only beginning to be exploited. The computer must be given very specific instructions for performing image analysis, and we have just begun to translate the expertise of clinicians and scientists into a computational language. Indeed, this process itself is of benefit to the scientist, as it requires clear and detailed definitions of the terms to be quantified, such as "atypia", "budding", or "cytoplasmic clearing."

5.6.2 Methods of Quantitative Analysis

Fundamental methods of image analysis are well described in several texts [115–116]. Quantitative algorithms have been developed for a variety of computer vision applications in industry, agriculture, and science [117–120]. Quantification of imaged cells and tissues may include the following:

1. Measurement of protein expression level. Using immunofluorescence, protein expression may be measured as a function of fluorescent intensity within any given region of interest. Protein levels may be compared between different regions of a biopsy specimen, between different types of cells, or between different regions within a cell (such as nuclear vs. cytoplasmic protein levels). For example, we have shown that measures of the expression level of the protein Met, as quantified by immunofluorescence, correlate with prognosis in breast cancer particularly when expressed as the ratio of protein levels in normal tissue compared to tumor tissue within a given patient [121].
2. Quantification of morphological features. The shape and behaviour of cells may be quantified in various ways, including cell scattering and branching. The following steps must be taken to perform this type of analysis:

    a. Threshold intensity must be selected that distinguishes background regions from regions occupied by biological material of interest. For immunohistochemical or H&E stained samples, the background will be bright (white) and the tissue will be darker. The opposite is true of images of immunofluorescently labelled tissue. This process of selecting a threshold intensity separating tissue from background, often termed "segmentation", may be done empirically, or computationally utilizing methods described elsewhere (reviewed in [116]). As an example, one strategy involves selecting the midpoint of the intensity scale (say, 128 on a 0–255 scale) as a first estimate, and then calculating the mean intensities of all pixels above and below this initial estimate. The midpoint between the two means is then used as the next estimate, and the procedure is repeated until the estimate no longer changes [122]. In our analyses of immunofluorescence images, we found that the resulting threshold value was too low, so we modified this algorithm to use the mean of the pixels brighter than the final estimate as threshold.
    b. Structures of interest within the image must be localized by determining the inter-relationship of the bright pixels. The simplest method for doing this is the "blob" algorithm whereby contiguous bright pixels are grouped together. However, this approach is too simplistic for tissue and cell culture analysis, because biological material is highly heterogeneous and the various methods for staining this material usually does not produce a uniformly bright region suitable for detection in this matter. To account for this, we have introduced a modified blob algorithm that divides the image up into many small regions (a grid), and groups together contiguous bright regions based on their average intensity [123]. By varying the size of the grid squares and the cut off value for the number of bright pixels that must be contained by each square, tissue features of varying degrees of heterogeneity may be detected.
    c. Characteristics of identified structures must be quantified. For example, in quantifying cell scattering we used the grid-based blob algorithm described above to identify clusters of cells in cell culture images and then enumerated the number of cell clusters within each image as well as the size of those clusters both in terms of pixels and cells (see Fig. 5.4). These measures were shown to correlate with subjective scattering scores assigned by human interpreters of the images.

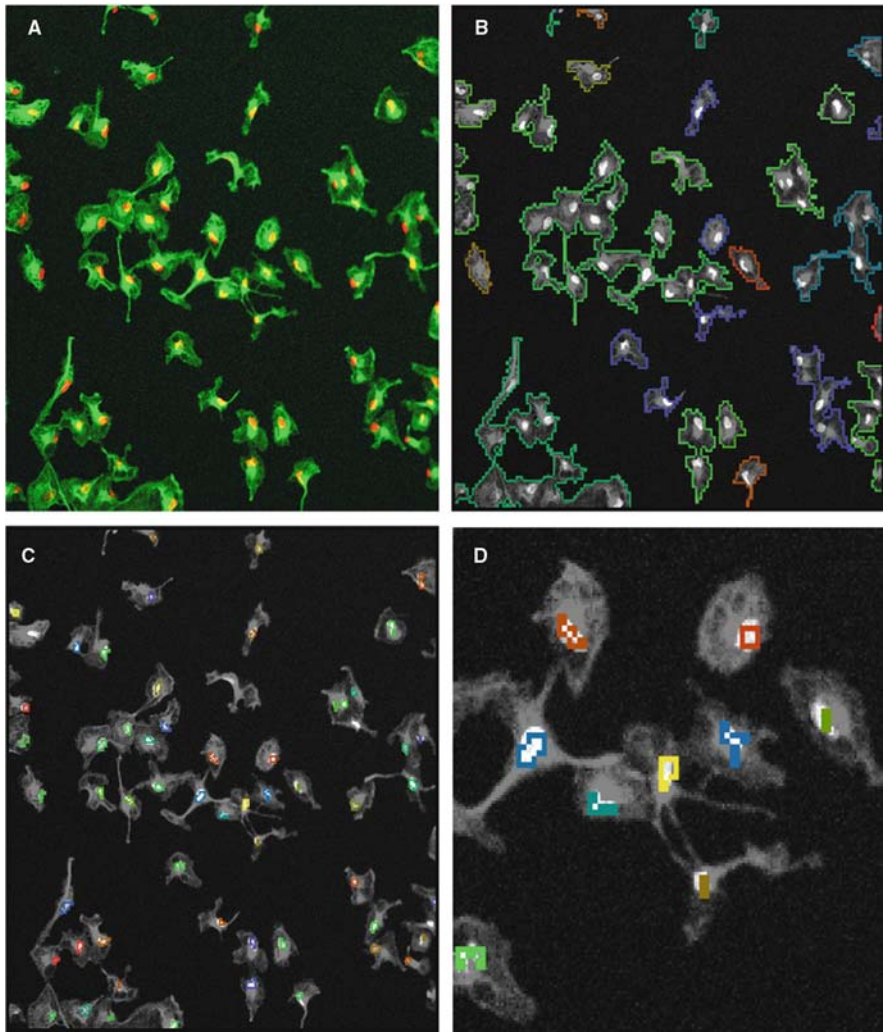

Fig. 5.4 Examples of quantitative scattering analysis. The grid based blob analysis was used to locate cells in the image and quantify the number of cell clusters as well as the size of each cluster in terms of pixels and cells. (**a)** Original image of cells growing in a culture flask. (**b**) The cells have been localized by applying the grid based blob analysis to the green image channel (cytoplasmic marker), and each cluster of cells annotated with a random pseudocolor for visualization purposes. (**c**) The same algorithm applied to the red image channel (nuclear marker) to localize nuclei. The nuclei were located to allow the computer to count the number of cells in each cluster. (**d**) Zoomed image showing the nuclear annotation

Likewise, cell branching may be quantified by relating the circumference of the cell to its area. A perfectly spherical cell has the lowest surface area: volume ratio (it is for this reason that lipid drops in aqueous solution form spherical droplets, since this arrangement minimizes hydrophobic interactions and thereby represents the free energy minimum.) Similarly, in two dimensions, a section through a spherical cell yields a circular structure with the smallest possible circumference: area ratio. As the cell becomes more branched, the resulting sections through the cell become more tortuous, and the circumference increases relative to the area. The ratio of circumference to area, then, may be used as a quantitative measure of cell branching. For convenience, our laboratory typically performs a mathematical transformation of this ratio based on the geometric relationship between area and circumference such that the minimum branching coefficient in 1 (the grid based blob algorithm leads to some rounding error such that coefficients slightly less than 1 may result). This approach to quantifying cell branching is illustrated schematically in Fig. 5.5a, b, and illustrative images from cell culture with corresponding computed quantitative data are shown in Fig. 5.5 c, d.

One nagging question may arise in the mind of the reader in light of this discussion: Do we envision a day when researchers and pathologists will be replaced by computers, at least within the context of the interpretation of histology? The answer is no. Computer algorithms can only apply the expertise of human operators in a rigorous fashion. Human expertise will always, in our view, be required to validate and advance

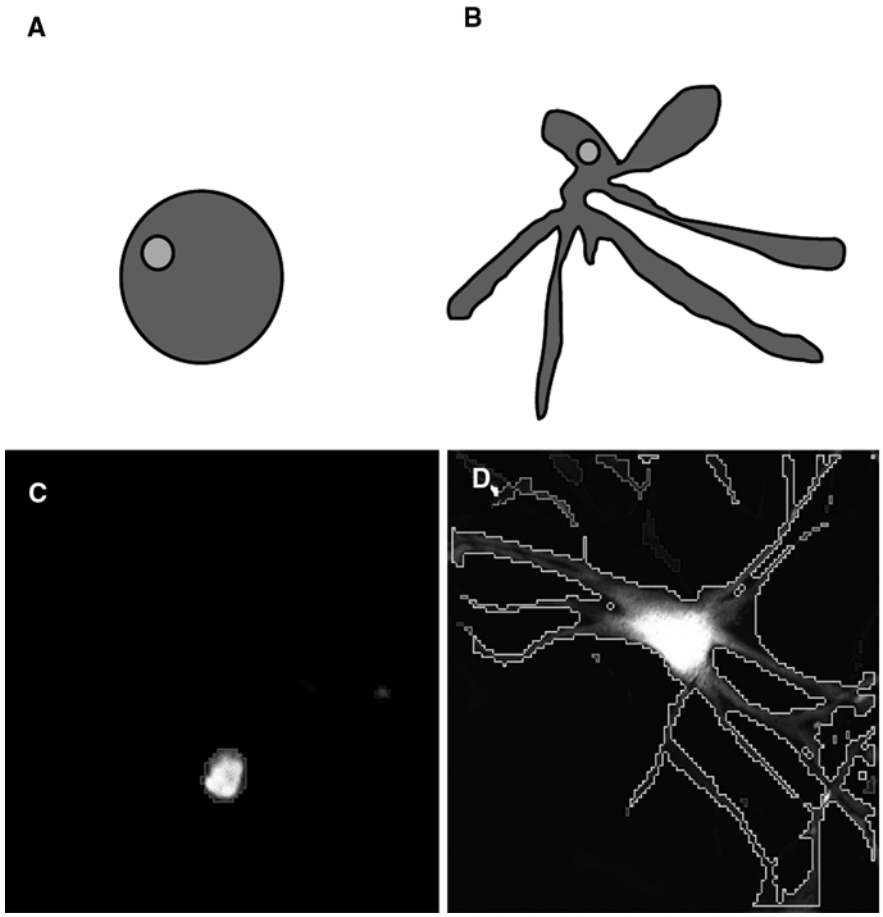

Fig. 5.5 (**a**) and (**b**) Schematic representation of basis of branching algorithm. Both "cells" have similar cytoplasmic area, but the cell in **a** (unbranched) has a much smaller circumference than the cell in **b** (branched). By relating the circumference to the area of the cell, a branching coefficient may be calculated. (**c**) and (**d**) The algorithm was applied to cells growing in culture. The computed branching coefficient for the cell in C was about 1, while that of the cell shown in **d** was 5.4

the work of computational pathology. Computers are well suited to rigorous and reproducible application of rules (something that humans are less well suited to), while humans are well suited to creativity, adaptation, and recognition of exceptions (something that computers are less well suited to). As such, the relationship is synergistic and not competitive, Deep Blue notwithstanding.[1]

[1] When the computer Deep Blue defeated chess Grand Master Gary Kasparov, it was touted in the popular press as a triumph of computer over human. But in reality, it was just the sort of synergy discussed here: the – designed and programmed computer.

5.7 Conclusion

Cancer is a multi-factorial disease, influenced by a combination of genetic and environmental factors. Imaging of the molecular, cellular and organism changes that document the sequence and steps in this process is one of the key reasons tissue culture has been so important in unraveling the process of carcinogenesis.

At the molecular level, cancer is characterized by multiple alterations in genes that play key regulatory roles in various cellular functions resulting in deregulated growth and metastatic spread of the cells that produce the late stage characteristic and specificphenotypic changes. The various disciplines within tissue culture have proven to be an invaluable resource for the in vitro study of carcinogenesis and its many dynamic causes. The developments of gene-expression profiling and laser capture microdissection have further aided

in this study and have provided a number of groundbreaking insights into the development and causation of cancer.

However, the in vitro study of cancer does have its restrictions, largely in its limited potential for translation to clinical use. The recent development of molecular imaging technologies in miniature, such as magnetic resonance imaging, x-ray computed tomography, fluorescent imaging, and, most importantly, intravital microscopy, has helped to overcome these limitations by presenting access to a whole new realm of study in vivo on mouse models. The benefit of such in vivo studies on mice is the ability to study therapy efficacy and molecular interactions in real time in the living animal. Advances in the quantitative analysis of imaging, combined with the development of new technologies hold great promise for not only a better understanding of the many factors underlying the disease, but also for the development of new technologies and therapies to be used in a clinical setting on humans. These technologies have been developed using in vitro models and are now ready to apply to in vivo studies. There still remains much to be learned about the causes of cancer and possibilities for its treatment. The various methods of in vitro study using cell culture have provided researchers with a solid foundation of knowledge, and, along with in vivo research, facilitates the continual building upon that foundation. As greater advances are made in the laboratory, it is hoped that even greater achievements will be made in the clinical application of data acquired through laboratory studies: new methods of detection and treatment, and even, perhaps one day, a cure.

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Chapter 6

Chromosomal Abnormalities in Selected Hematopoietic Malignancies Detected by Conventional and Molecular Cytogenetics: Diagnostic and Prognostic Significance

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Contents

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6.1 Introduction

Theodor Boveri first proposed the somatic mutation theory of cancer in 1914. He proposed that cancer develops from a single cell that acquires a genetic alteration. The hypothesis of the clonal origin of neoplasms, however, could neither be confirmed nor be refuted because the tools for testing his hypothesis were not yet available at the time. With advances in the techniques for obtaining analyzable metaphases over the ensuing years, supporting evidence accumulated. In 1960, Nowell and Hungerford reported the first recurrent chromosomal abnormality associated with a single cancer type, chronic myelocytic (or myeloid) leukemia, or CML [1]. The marker chromosome was named the Philadelphia (Ph) chromosome in honor of the city where it was discovered. Subsequently, through banding techniques, the marker was determined to be the derivative chromosome 22, resulting from the translocation, t(9;22)(q34;q11.2) [2]. Only with further advances in various banding techniques in the 1970s could many more specific chromosomal rearrangements be identified. Some of the technological advances that took place over the last 50 years are described below, as we present specific recurrent chromosomal abnormalities in selected hematopoietic malignancies that were delineated by advances in conventional and molecular cytogenetic technologies.

6.2 Technological Advances in Cancer Cytogenetics

Cancer cytogenetics is the study of chromosomes in cancer tissues. Chromosomal analysis can be performed on a variety of tissue types. For the

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evaluation of chromosomes in hematological disorders, bone marrow is usually utilized. Unlike other tissues, such as nerve and muscle, hematopoietic cells have the capacity for self-renewal. Cell division in the bone marrow is a physiological process which obviates the need for stimulation by mitogens. Mitogens, such as phytohemagglutinin (PHA), pokeweed mitogen, lipopolysaccharide and Epstein-Barr virus, may be needed to stimulate non-dividing cells in diseases with a low mitotic rate. It is known that the role of hematopoiesis in the fetal stage is initiated in the bone marrow. Many cells normally form in the bone marrow, including erythrocytes, basophils, neutrophils, eosinophils, monocytes and megakaryocytes. Replacement of blood cells entails mitotic cell divisions and maturation via differentiation. Bone marrow is the tissue of choice for the cytogenetic study of most hematologic conditions since it more accurately reflects what occurs in vivo. Blood can be used to study the cytogenetics of malignant conditions only if dividing leukemic blast cells are present.

Bone marrow can be subjected to a one- or a two-day unstimulated culture, as well as a direct preparation [3]. Certain chromosomal abnormalities are detected more readily under certain conditions. For example, the t(15;17) characteristic of acute promyelocytic leukemia (APL or AML-M3 subtype) is rarely seen in direct preparations while this abnormality is often revealed in short-term cultures [4, 5], although not all laboratories report the same experience.

6.2.1 Harvesting of Cells for Cancer Cytogenetics

Once cultured, the cells are harvested utilizing a modification of a peripheral blood technique first described by Moorhead et al. [6]. Mitotic arrest is usually achieved employing Colcemid to disrupt the mitotic spindle. A hypotonic solution is added to cause the swelling of the cells to spread the chromosomes. A fixative (usually a mixture of three parts of methanol plus one part of acetic acid) is added for cell fixation. Fixed cells are dropped onto cleaned slides and air-dried. While suspension cultures are harvested manually, in situ cultures can be harvested by automated systems (e.g., Tecan Miniprep^{TM} Harvestor, Tecan-US, or Genial Systems MultiPrep Genie^{TM}, Rainbow Scientific, Inc) [7, 8]. Slide preparation is best performed under controlled temperature and humidity conditions in an environmentally controlled chamber. Examples of commercially available chambers include The Thermotron^{TM} [9] and environmental control chambers designed by Percival Scientific, Inc. (Perry, Iowa). Once prepared, slides are then aged and subjected to the appropriate banding techniques.

6.2.2 Conventional Cytogenetic Banding Techniques in Cancer Cytogenetics

Karyotyping is the arrangement of the chromosomes in a defined systematic manner. Initially, karyotyping was accomplished by exploiting the differences in size and shape of the chromosomes in a cell that had been stained with a dye such as Giemsa. The first "banding" technique reported was Q-banding, using either quinacrine mustard or quinacrine dihydrochloride [10]. However, this technique suffered from an inability of the fluorescent slides to become part of the permanent record due to the effect of fluorescence quenching. Subsequently, G-banding pattern was achieved by using trypsin which allowed the chromosomes to be visualized under a brightfield microscope as a continuous series of light and dark bands. Other staining techniques such as C, R, NOR, etc. are rarely, if ever, used for bone marrow cytogenetics and further discussion will not be pursued.

6.2.3 Molecular Cytogenetic Techniques in Cancer Cytogenetics

Conventional cytogenetic analysis is performed primarily using G-banding. With the advent of molecular technology, molecular cytogenetic techniques have increasingly been used to enhance the information obtained by conventional G-banded methods. Fluorescence or fluorescent in situ hybridization (FISH) can be performed on many sample types using a variety of probes that include: centromere-specific alpha-satellite probes to assess chromosome copy number; subtelomere probes to detect deletion or subtle translocations of the telomeric regions of chromosomes; chromosome painting probes utilizing a

mixture of probes (probe cocktails) to delineate chromosomal structural rearrangements such as translocations; locus-specific or unique sequence probes to focus on specific loci/regions of interest on chromosomes. A combination of molecular probes is often used in cases where a complex chromosomal rearrangement is encountered. The utility of FISH is especially evident when the mitotic index is low and the quality of G-banding is suboptimal. Other specific clinical applications of FISH include the following:

- Rapid FISH for detecting aneuploidies, especially in interphase cells for suspected constitutional as well as acquired monosomies or trisomies in cancer
- The detection of single-cell trisomies for determinating clonality or mosaicism
- Clinical applications in sex-mismatched bone marrow transplants
- The detection of minimal residual disease
- Rapid analysis of bone marrow smears for specific chromosomal abnormalities such as trisomies or suspected unique genetic lesions
- Marker chromosome identification
- Microdeletion evaluations
- Extraction of information in suboptimal specimens with low mitotic index, suboptimal banding, and terminally differentiated cells
- Assessment of chromosome copy number in archival formalin-fixed, paraffin-embedded pathology specimens (e.g., Amplification study of genes such as HER-2/neu in breast cancer, or NMYC in neuroblastomas)
- Sequential flow cytometry and FISH for the study of formalin-fixed paraffin-embedded cancer tissues
- Assessment of chromosome/gene copy number in interphase cells found in cytological specimens, such as buccal smears, cervical smears, semen smears, and extracellular fluids
- Evaluation of bladder cancer recurrence using bladder washes
- Evaluation of lung and prostate cancer using FISH probe kits
- FISH as an adjunct to G-banding to characterize cell lines derived from primary tumors
- FISH for localizing DNA sequences onto metaphase chromosomes and mapping viral integration sites
- FISH as an indispensable tool used in the Human Genome Project

The principles and applications of FISH have been discussed extensively elsewhere [11–29].

Although both in situ hybridization (ISH) and FISH have been performed manually for many years, automated instrumentation is now available which can improve laboratory throughput (e.g., Vysis HYBrite^{TM} and the Vysis VP2000^{TM}) [30].

The use of other FISH-based techniques, such as spectral karyotyping (SKY), chromosome comparative genomic hybridization (CGH) and microarray CGH enhance significantly the utility of cancer cytogenetics. Further detailed elaboration of these newer, still evolving, molecular cytogenetic techniques, however, is beyond the scope of this discussion.

6.3 Numerical and Structural Chromosomal Abnormalities

Cytogenetic abnormalities may be numerical or structural. Numerical chromosomal abnormalities include gains or losses of individual chromosomes (e.g., trisomies and monosomies), or gains and losses of entire sets of chromosomes (e.g., triploidy and haploidy). Structural chromosomal abnormalities include chromosome translocations, paracentric and pericentric inversions, direct (tandem) and reverse duplications, terminal and interstitial deletions, isochromosomes, ring chromosomes and marker or unknown chromosomes. Various chromosomal abnormalities have been summarized by An International System for Human Cytogenetic Nomenclature [31, 32]. In addition, an abnormality is considered clonal if two or more cells are found with a gain of a chromosome, or with a structural chromosomal abnormality; or if three or more cells are found with a loss of a chromosome [33]. Although a loss of a chromosome in one cell is usually considered random due to technical factors (such as over-spreading), each nonclonal abnormality should be carefully evaluated to rule out the presence of a small, but bonafide, clone [14, 19, 20]. Examples of nonclonal abnormalities which should be noted are the presence of the Philadelphia translocation in a cell of a patient with CML [1, 2] and the presence of a t(15;17) in a cell of a patient with APL [34, 35]. The identification of one cell with a normal karyotype is considered sufficient evidence to support the presence of a normal cell line in an abnormal case. Interpretation by an

American Board of Medical Genetics (ABMG) board-certified clinical cytogeneticist is, thus, as important for hematopoietic malignancies as in other areas of cytogenetic diagnosis. Furthermore, it is a College of American Pathologists (CAP) requirement for a CLIA-accredited clinical laboratory.

6.4 The Classification of Hematopoietic Malignancies

The above-mentioned technological advances made possible continuing improvement in the cytogenetic analyses of hematopoietic malignancies. A brief discussion on the classification of hematopoietic malignancies will be presented in order to describe the chromosomal abnormalities associated with some of these diseases.

Hematologic malignancies are cancers of the hematopoietic system. Leukemia is cancer of the white blood cells. Depending on the clinical course, leukemia may be classified as chronic or acute, and may be defined by the source of the leukemic cell population, as either myelocytic (myeloid) or lymphocytic (lymphoid) leukemia. Among the chronic leukemias are chronic myelocytic leukemia (CML) and chronic lymphocytic leukemia (CLL). Among the acute leukemias are acute myelocytic leukemia (AML) and acute lymphoblastic leukemia (ALL).

In the past, the acute leukemias have been classified by the French-American-British (FAB) Cooperative Group [36, 37, 5]. The FAB classification has relied on morphological, cytochemical and immunological characteristics and most subsets are characterized by specified chromosomal rearrangements which frequently correlate with other clinical features and outcomes. Eight subgroups (M0 to M7) are defined for AML and three for ALL. This classification also includes the myelodysplastic syndromes (MDS) and the myeloproliferative diseases (MPD) [38]. However, the earlier FAB system is being replaced by a newer World Health Organization (WHO) classification of hematologic malignancies which has been developed to address the changing needs of the field.

The European Association of Pathologists and the Society for Hematopathology have been developing a new WHO classification of hematologic malignancies since 1995 [39]. This classification, based on the work of 10 committees of pathologists and a Clinical Advisory Committee of international hematologists and oncologists, includes myeloid, lymphoid, histiocytic, and mast cell neoplasms.

The newer WHO system modifies and incorporates the Revised European-American Lymphoma (REAL) classification [40] and extends the principles underlying that schema to the classification of myeloid diseases. Like the REAL system, the WHO system attempts to classify neoplastic lymphohematopoietic diseases into discrete entities based on their unique histopathological and genetic features.

According to the new WHO system, the lymphohematopoietic neoplasms are divided into clinically relevant and biologically discrete entities, including morphology, immunophenotype, clinical history and cytogenetic abnormalities [21]. With respect to cytogenetics, within the category of acute myeloid leukemias, for example, four main groups are recognized: (1) AML with recurrent cytogenetic abnormalities, such as t(8;21), t(15;17), inv(16), and 11q23 abnormalities; (2) AML with myelodysplasia-related features; (3) therapy-related AML and MDS, and (4) AML not otherwise categorized, including FAB subtypes M0 and M7 [41, 42, 21]. The delineation of specific cytogenetic diseases found in numerous studies showed that cytogenetics could successfully predict response to therapy [43–47] and therefore could be used to make tailored treatment decisions [43, 48].

The successful establishment of the WHO classification should facilitate and ultimately lead to progress in the understanding and treatment of hematologic malignancies. Details of the WHO classification can best be obtained by consulting Harris et al. [39, 41] and Jaffe et al. [42]

6.5 Cytogenetic Abnormalities in Selected Hematopoietic Malignancies

6.5.1 The Philadelphia Chromosome in Chronic Myelocytic Leukemia

The t(9;22)(q34;q11.2) is found mostly in CML, but is also reported in ALL and rarely in AML. The chromosomal rearrangement alters the order of the genetic loci

on these two target chromosomes; it fuses the Abelson (ABL) oncogene on 9q34 to the breakpoint cluster region (BCR) locus on chromosome 22, and results in a hybrid gene. The chimeric BCR/ABL gene encodes a constitutively activated protein tyrosine kinase, which leads to the activation of multiple signaling pathways with profound effects on cell cycle, adhesion, apoptosis and eventual myeloid cell transformation. Huntly et al. [49] noted that in murine transgenic and retroviral transduction models, expression of BCR/ABL has been shown to be both sufficient for initiation and necessary for maintenance of a leukemic phenotype. Figure 6.1 depicts the Philadelphia translocation in CML.

Besides its utility in diagnosis, another utility of cytogenetic analysis is that it can provide insight into clinical course and subsequently treatment. For example, the finding of the Philadelphia chromosome as the sole abnormality in the chronic phase of the disease is associated with a good prognosis. However, changes in the karyotype such as additional chromosomal abnormalities or the emergence of a new subclone (usually +8, +19, i(17q), or an extra Ph chromosome) during the course of the disease signifies disease progression into the acute phase and is considered a poor prognostic sign. Figure 6.2 is an example of such a change.

The t(9;22) has been found to be present in approximately 3% of pediatric and 25% of adult patients with ALL . In children, the Philadelphia chromosome confers an unfavorable prognosis, especially when it is associated with either a high leukocyte count, slow early response to initial therapy, or certain secondary chromosomal aberrations [50–51].

Until recently, treatments for CML consisted of either allogeneic stem cell transplantation or an alpha-interferon-based regimen. However, both options are associated with considerable drawbacks. Although potentially curative, stem cell transplantation is associated with considerable morbidity and mortality, while alpha-interferon-based regimens adequately control chronic-phase disease but result in few long-term survivors. Recently, treatment with the protein tyrosine kinase inhibitor imatinib mesylate (Gleevec/Glivec, formerly known as STI571; Novartis, Basel, Switzerland) has resulted in excellent hematologic and cytogenetic responses in all phases of CML [21]. A comparison with historical controls shows improved survival in the later stages of the disease for patients treated with imatinib, and it is hoped that the excellent response rates obtained in chronic-phase patients will also translate into improved survival.

In the era of kinase inhibition, the cytogenetic and molecular analysis of genetic abnormalities in CML takes on increasing importance, in part because the treatment itself now relies on the presence of the

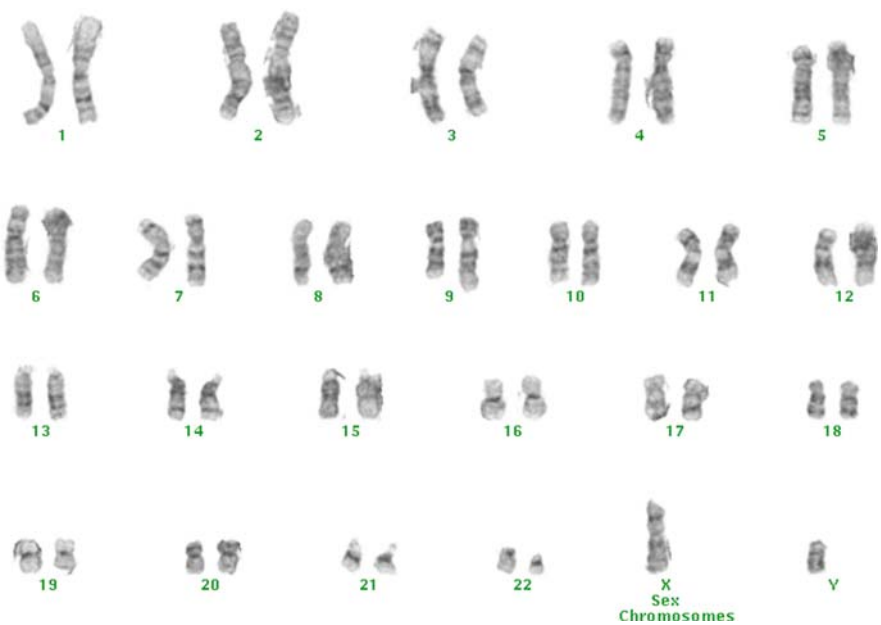

Fig. 6.1 A G-banded karyogram showing the Philadelphia translocation, t(9;22)(q34;q11.2), from a patient with CML

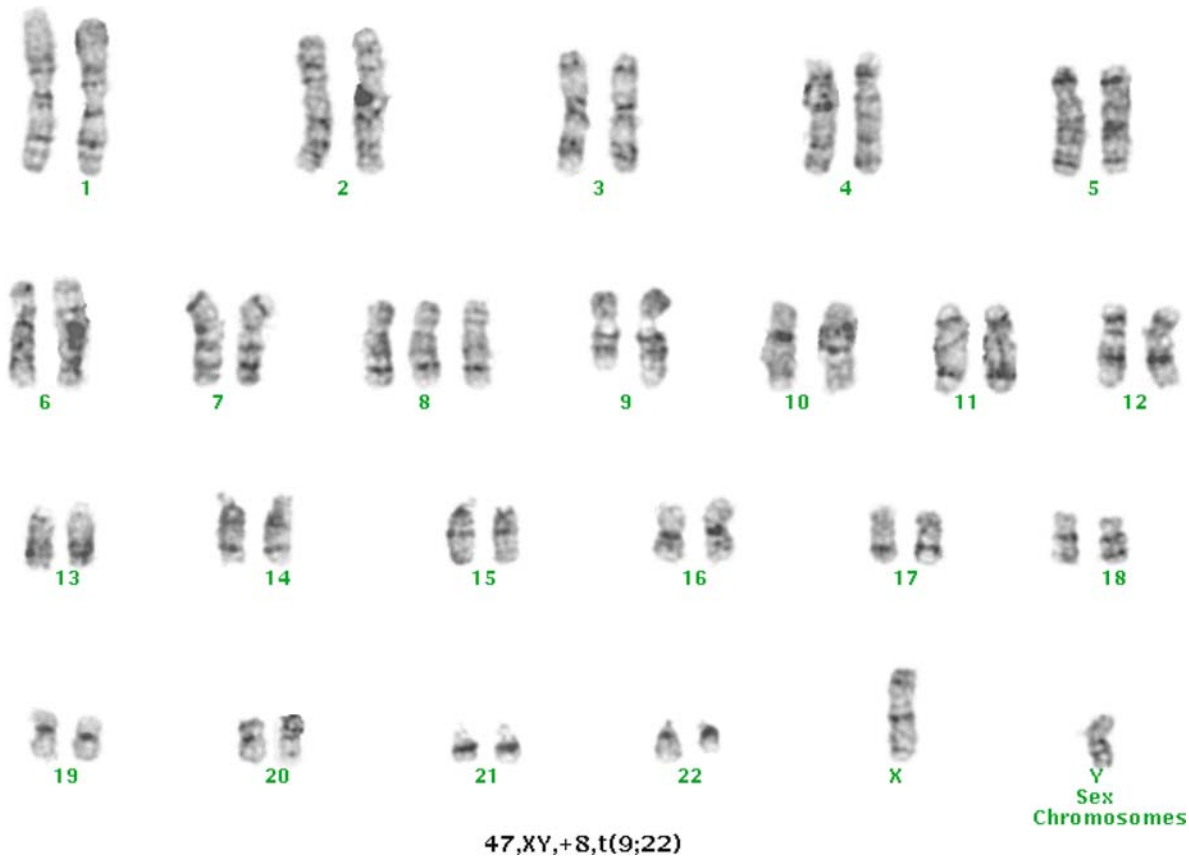

Fig. 6.2 A G-banded karyogram showing the presence of trisomy 8 (+8) in addition to the Philadelphia translocation, t(9;22)(q34;q11.2)

constitutively active BCR-ABL fusion gene. Prior to the use of imatinib, a major cytogenetic response – less than or equal to 35% of cells analyzed expressing the BCR-ABL translocation – was a therapeutic goal, because patients attaining a major cytogenetic response with interferon alpha treatment were known to have increased survival [52]. Patients deemed to be at high risk for transplant-related mortality, by virtue of age, co-morbidity or lack of an HLA matched related donor, were maintained on interferon as long as a response was maintained [53]. Nevertheless, because interferon is rarely curative, patients at lower risk for transplant-related morbidity or mortality were offered allogeneic hematopoietic cell transplantation if at all possible [54, 53]. Contrariwise, patients who had received transplantation were expected to attain a complete cytogenetic response [55]. Patients failing to do so, or patients who lost a complete response after having attained one, were again considered for further therapy [56].

The relevance of a major cytogenetic response to imatinib has not been demonstrated in a prospective trial. Rather, the focus of treatment has been on molecular responses determined by quantitative polymerase chain reaction (PCR). A major molecular response has been defined as a three-$\log_{10}$ decrease in the ratio of BCR-ABL mRNA transcripts to some control transcript, often the normal BCR transcript [57]. In any case, the appropriate therapeutic maneuver to consider if a major molecular response is not obtained has not been determined in prospective randomized controlled trials. Options are to use a second-generation kinase inhibitor [58, 59] or to offer hematopoietic cell transplantation [60]. While the place of cytogenetic monitoring using the latter modality is clear, the role of either cytogenetic or molecular

monitoring after use of a second-generation kinase inhibitor has not been established. Presumably, though, either molecular response, cytogenetic response or both will be important in this challenging clinical setting.

Even if imatinib treatment is successful, it appears that it will be necessary to continue cytogenetic monitoring. Various molecular responses to long-term imatinib treatment have now been described, including, mutation of the BCR-ABL kinase domain and additional molecular or cytogenetic abnormalities [61]. These molecular and cytogenetic abnormalities have often [62], although not always [63], been associated with progression of disease or transformation to a more aggressive lymphohematopoietic neoplasm. Cytogenetic monitoring may detect these events in an early stage, and may therefore impact on the treatment decision or on the outcome of treatment, although these suppositions must be verified in adequate prospective studies. In any case, additional molecular and/or cytogenetic changes can certainly be used to follow the course of evolving disease.

6.5.2 Chromosomal Abnormalities in Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia found in the United States and Europe. The disease occurs almost exclusively in middle-aged and elderly patients. An overwhelming majority of CLL cases are of B-cell origin, with T-cell CLL accounting for only about 5% of all cases [38].

In CLL, the neoplastic cells do not usually proliferate readily in vitro and require stimulation by the appropriate mitogens to achieve a sufficient number of mitoses or dividing cells for analysis. The mitogens of choice are phytohemagglutinin (PHA), concanavalin A (Con-A), T-cell growth factor (TGF), and pokeweed mitogen (PWM) in T-cell malignancies. For B-cell malignancies, mitogens often employed in cytogenetic laboratories include Epstein-Barr virus (EBV), lipopolysaccharide (LPS), which is derived from E. coli, dextran sulfate (DXS) and pokeweed mitogen (PWM). EBV and LPS are especially favored in terms of efficacy in retrieving mitoses with chromosomal abnormalities. An alternative is to perform interphase cytogenetics.

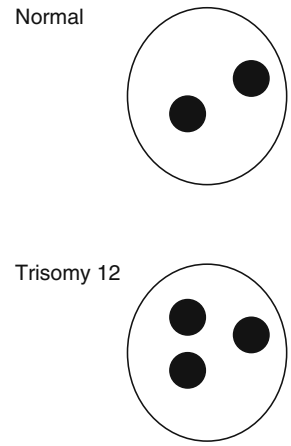

Fig. 6.3 FISH enumeration of chromosome 12 copy number using a CEP 12 SpectrumOrange probe. Two orange/red signals are seen in a normal interphase nucleus. Three orange/red signals are seen in a trisomic 12 (+12) cell

One of the most frequently found chromosomal abnormality in CLL is trisomy 12 (Fig. 6.3), occurring in ~20% of all patients and associated with a poor prognosis [64]. This numerical chromosomal abnormality is especially amenable to molecular cytogenetic analysis. FISH can be performed on routinely prepared as well as previously Wright-stained peripheral blood smears [65].

Other chromosomes frequently involved in abnormalities include chromosome 11, chromosome 13, chromosome 14, chromosome 1, chromosome 3, chromosome 6 and chromosome 17. It has been hypothesized that trisomy 12 may be the primary or earliest karyotypic change in most patients and that other chromosomal changes in addition to trisomy 12 later arise as a result of clonal evolution, dedifferentiation, or treatment.

While risk-adapted therapy of CLL based on cytogenetics is not yet a standard of care, recent data has shown differential survival in various cytogenetic subgroups [66]. In this analysis, del(13q) as a sole abnormality had a better prognosis than normal cytogenetics, as reflected by a delayed time until initiation of therapy. On the other hand, deletions of 17p13 and 11q22–23 were associated with decreased survival. Deletion of chromosome 17p13

is associated with mutated p53, which itself is associated with poor survival after purine analog based therapy [67]. Given the data that 17p13 deletion is associated with poorer survival, and that p53 mutations are associated with both 17p13 deletion and poorer survival after treatment with purine analogues, Byrd and colleagues, in a recent analysis based on CALGB protocol 9,712, examined the relationship of chromosomal abnormalities and survival after modern purine analogue based immunochemotherapy [68]. This paper examined outcomes from a randomized phase II trial of concurrent or sequential treatment of previously untreated CLL with fludarabine and rituximab. A major finding was that both progression-free and overall survival were decreased in patients with poor risk cytogenetics by Döhner's criteria (17p- or 11q-). Interestingly, patients with del(11)(q22.3) had high complete response rates using NCI working group criteria, whereas the CR rate of patients with del(17p) was 0%. Based on these data, it might be reasonable to not use fludarabine-rituximab immunochemotherapy in patients with poor risk cytogenetics, particularly those with del(17p). Alternative treatments to be considered could be alemtuzumab-based therapy or, in appropriately selected patients, allogeneic hematopoietic cell transplantation after either a myeloablative or non-myeloablative preparative regimen. As yet, no peer-reviewed full reports of these strategies are available. Therefore, such treatment should ideally be conducted at the setting of an investigational protocol.

6.5.3 T(8;14) in Burkitt Lymphoma/Leukemia

Burkitt lymphoma is a form of diffuse lymphoblastic non-Hodgkin lymphoma which is endemic in Africa and the West Indies; it is found only sporadically outside of those areas. The tumor shows a close relationship with the Epstein-Barr virus (EBV) in endemic areas. The t(8;14)(q24;q32), or its variant translocations in a minority of cases, t(2;8)(p12;q24) and t(8;22)(q24;q11), is observed in Burkitt lymphoma or its leukemic presentation. Through this translocation, the MYC oncogene (which maps to 8q24) is juxtaposed to the immunoglobulin (Ig) heavy-chain locus

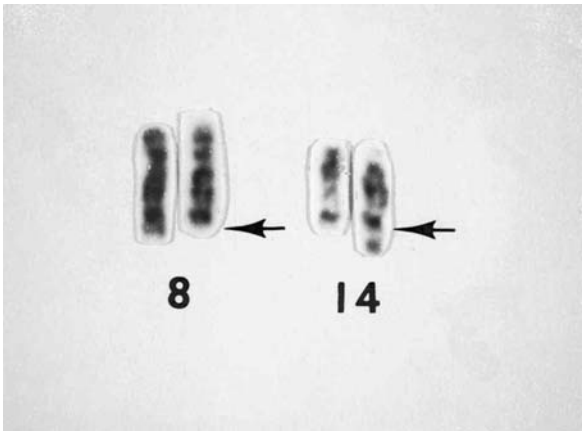

Fig. 6.4 T(8;14)(q24;q32) in Burkitt lymphoma and in ALL

(which maps to 14q32) and consequently becomes deregulated in B cells. Prior to the development of more refined cytogenetic techniques, the "marker' often seen in African Burkitt lymphoma was only identified as a "14q+" structural chromosomal abnormality. It was identified later as a recurring translocation, t(8;14)(q24;q32), as shown in Fig. 6.4.

In African cases this translocation is usually the sole abnormality. In non-endemic areas, however, additional chromosomal abnormalities are usually present.

FISH with either IgH or dual fusion IgH-CMYC fish probes is a good confirmatory test when the subtle t(8;14) is not readily visible , or when a 14q+ is present and the translocation partner has not been visualized. Also the involvement in the bone marrow may be limited and FISH can screen a large number of interphase nuclei. A general discussion of the cytogenetics of lymploid neoplasias can be found in Raimondi [69].

Identification of Burkitt lymphoma/leukemia is usually made on routine histology, where the tissue has a characteristic "starry sky" pattern. In doubtful cases, however, the diagnosis is important to make. Burkitt lymphoma, in either the lympadenopathic or leukemic form, is one of the most rapidly growing tumors of the lymphohematopoietic system, with a doubling time close to 1 day [70]. Unique chemotherapeutic regimens based on high dose methotrexate without prolonged maintenance treatment have been found to be effective [71]. Missing the diagnosis both exposes patients to non-beneficial chemotherapy and deprives them of a chance for cure with the first chemotherapeutic regimen.

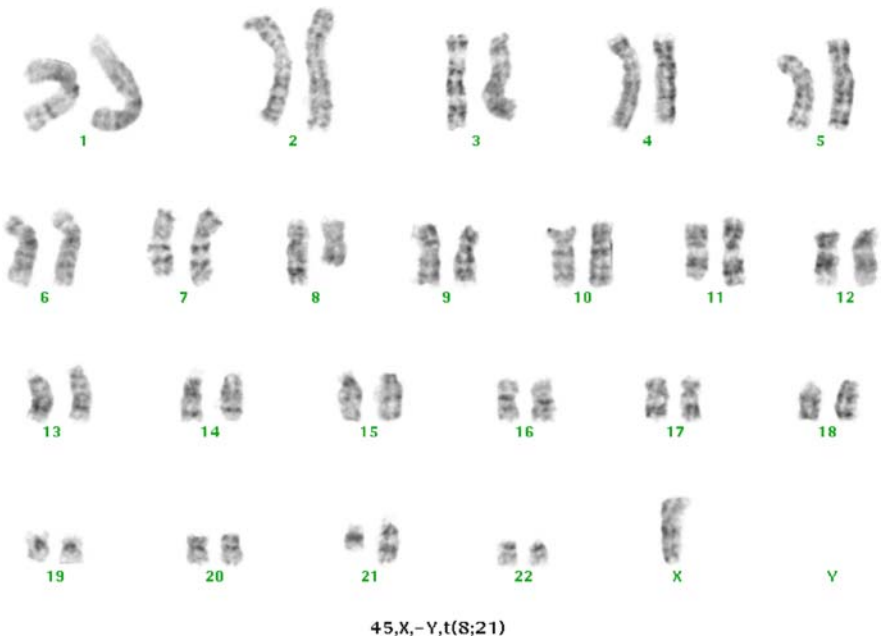

Fig. 6.5 A G-banded karyogram showing the 8;21 translocation. This karyogram is also missing a Y chromosome

6.5.4 Recurrent Cytogenetic Abnormalities in Acute Myeloid Leukemia (AML)

Recurring chromosomal abnormalities in acute myeloid leukemia (AML) include t(8;21)(q22;q22), t(15;17)(q22;q21), inv(16)(p13.3q22), and 11q23 rearrangements. A brief discussion of each ensues.

6.5.4.1 AML with t(8;21)(q22;q22)

The t(8;21) is a recurrent structural chromosomal rearrangement mostly found in AML M2 subtype of the French-American-British (FAB) classification but also in other AML subtypes. The t(8;21) juxtaposes the Acute Myeloid Leukemia 1 (AML1, also called RUNX 1) gene locus on 21q22 with the Eight Twenty One (ETO) gene locus on 8q22. The 8q22 breakpoint clusters are within the putative zinc finger DNA binding gene ETO. The breakpoints in 21q22 are clustered within the AML1 gene. The t(8;21) event produces a fusion of the two genes on the derivative 8 chromosome that results in the novel chimeric gene AML1/ETO. Probes are now available for the detection of the AML1/ETO fusion which is a good complement to conventional cytogenetics as submicroscopic rearrangements are occasionally present. Figure 6.5 shows a G-banded karyogram of the 8;21 translocation. This male patient is also missing a Y chromosome in this metaphase cell.

The clinical utility in the diagnosis of Core Binding Factor (CBF) leukemias [t(8;21), inv(16) and t(16;16)] is sufficient that these diseases have been separated out as a unique pathophysiological entity in the WHO system [39]. Traditionally, these cytogenetic abnormalities have been associated with "good risk" AML. In the MRC 10 trial, good risk cytogenetics had a ~65% overall survival, as opposed to 41% and 14% for intermediate risk and poor risk cytogenetics respectively [72]. This has been interpreted in practice as being a relative contraindication to high dose chemotherapy with allogeneic hematopoietic cell transplantation while in first remission from CBF+ AML (119). CBF+ AMLs are particularly sensitive to high dose cytarabine-based regimens [73].

6.5.4.2 AML with t(15;17)(q22;q12) and Variants

This translocation involves the PML gene on 15q and the Retinoic Acid Receptor Alpha (RARA) gene on 17q resulting in the formation of the PML/RARA fusion gene. The PML/RARA fusion

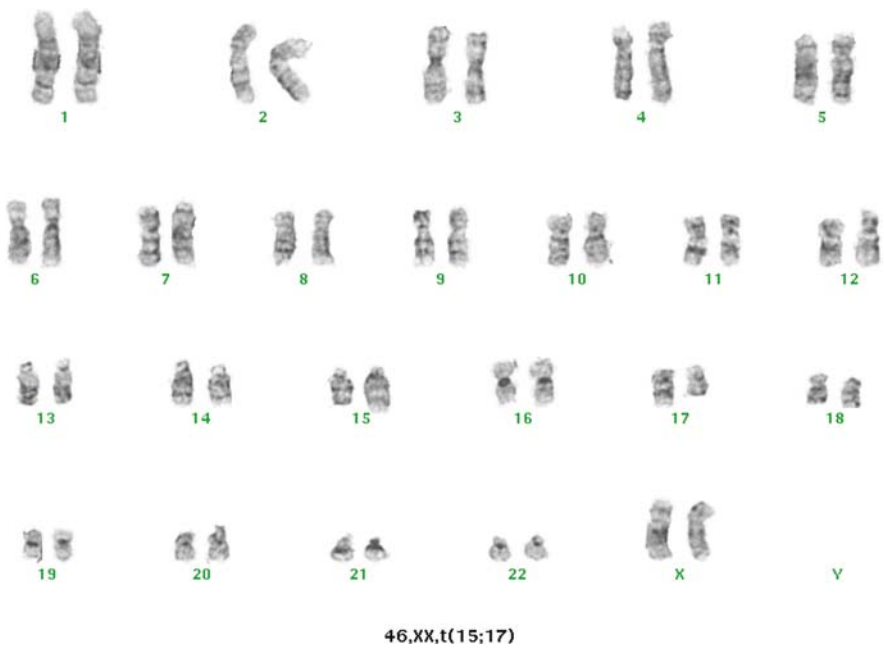

Fig. 6.6 A G-banded karyogram showing the 15;17 translocation

disrupts the retinoic acid receptor resulting in deregulated retinoid signaling. Current treatments include high-dose retinoic acid (all-trans-retinoic acid or ATRA) in combination with chemotherapy.

The translocation is detectable by conventional cytogenetics, RT-PCR and FISH. Detection of the fusion is one of the most sensitive predictor of relapse. It was one of the first examples of targeted therapy in hematopoietic diseases. Figure 6.6 shows a G-banded karyogram of the 15;17 translocation.

As with CML, APL has a unique treatment whose effectiveness is based on the cytogenetic lesion. While APL previously had a very poor outcome with standard anthracycline-cytarabine based therapy, it is now the AML subtype with the best prognosis, thanks to treatment with all-trans retinoic acid (ATRA, tretinoin). Standard treatment for M3 AML now consists of ATRA and anthracycline chemotherapy [74], and the role of any cytotoxic chemotherapy continues to be questioned as efforts are made to reduce or eliminate this component of the regimen [75]. As opposed to cytotoxic chemotherapy, ATRA appears to work as a differentiation agent [76]. In fact, the so-called "ATRA syndrome" or leukocytosis, shortness of breath, and peripheral and pulmonary edema has more recently been termed the "APL differentiation syndrome", as it has been seen with other differentiation-inducing agents used for this disease [77]. Also of considerable practical and theoretic interest is the fact that APL with certain variant translocations, wherein PML is fused to a gene other than RARA, such as PLZF, treatment with tretinoin may be ineffective [78].

6.5.4.3 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)

The inv(16) or t(16;16), are strongly associated with AML $M4_{eo}$ subtype. The inv(16)/t(16;16) has been shown to fuse the CBFB gene on 16q22 with the MYH11 gene on 16p13.1 giving rise to a chimeric protein. FISH is an important tool for the confirmation of these abnormalities as they are subtle and difficult to visualize by conventional cytogenetics, especially when the chromosome preparation is not optimal. Figure 6.7 shows a representative karyogram of a metaphase cell with t(16;16).

6.5.4.4 AML with 11q23 (MLL) Abnormalities

As alluded to earlier, this group of rearrangements involving the disruption of the *MLL* gene is one of the most common cytogenetic abnormalities observed in hematopoietic malignancies. In AML, numerous variant translocations have been reported involving

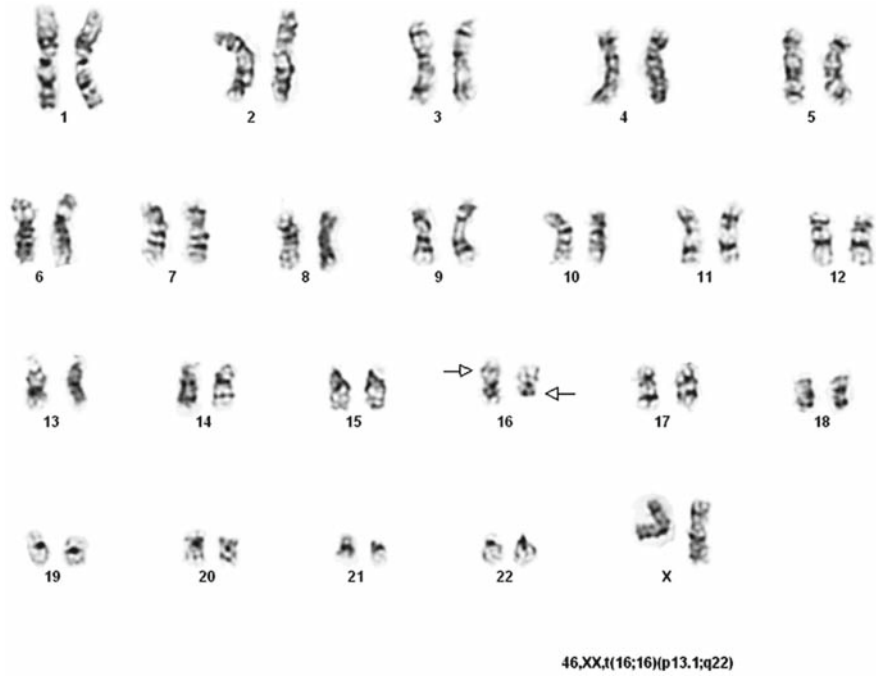

Fig. 6.7 A G-banded karyogram showing t(16;16)(p13.1;q22)

MLL, with the most common being, t(9;11)(p22;q23), t(10;11) and t(11;19)(q23;p13.1), and usually predict a poor outcome. Thus, patients with an unfavorable cytogenetic feature such as 11q23 are often assigned to the most intensive treatment arm.

The detection of recurrent 11q23 chromosomal abnormalities using conventional and molecular cytogenetic techniques is discussed below in the ALL section and in a recent review [21].

6.5.5 Recurrent Cytogenetic Abnormalities in Acute Lymphoblastic Leukemia (ALL)

The most frequent genetic subtypes of B-precursor ALL in children include: hyperdiploidy (~30%), t(12;21)(p13;q22)/TEL-AML1 (25%), t(1;19)(q23;p13.3)/E2A-PBX1 (6%), t(9;22)(q34;q11.2) (4%), 11q23/MLL rearrangements (5% of all B-precursor and ~90% of infant ALL) (Table 6.1). In addition, B-cell leukemia/lymphoma represents 5% of all B lineage ALL. Most of these genetic subtypes of ALL are also found in adults, but they are rare, with the possible exception of t(9;22) which is identified in 25% of all cases.

Table 6.1 Main recurrent cytogenetic/genetic lesions with clinical impact on outcome in ALL

Prognosis	Cytogenetic subtype	Children (%)	Adult (%)
Favorable	Hyperdiploid (mn 51+) +4, +10, +17 or DNA Index > 1.16	25	9
	t(12;21)	25	3
Unfavorable	t(9;22)	4	25
	11q23 (Infants ~90%)	5	8
	Hypodiploid (mn < 44) or DNA Index <0.80	1	1
	t(1;19)[a]	6	4

Abbreviations: mn = modal number of chromosomes.
[a]The outcome has improved with intensive chemotherapy.

6.5.6 Structural Changes in ALL

6.5.6.1 t(12;21)(p13.3;q22)

The most frequent cytogenetic lesion found in 25% of children with B-lineage ALL is the cryptic t(12;21) which is not observed by conventional cytogenetics banding methods, but easily detected by FISH or

RT-PCR methods. The t(12;21)(p13.3;q22) results in the fusion of *ETV6* to *AML1* (renamed *RUNX1* and *CBFA2*), the most common fusion partner of *ETV6* [79–81]. However, *ETV6-CBFA2* is rarely observed in infants with ALL, in pediatric patients with hyperdiploid leukemic cells, or in pediatric patients with T-cell ALL. This genetic abnormality is observed mainly in children 3–5 years of age and occurs in only 1.5–4.4% of adult patients with ALL.

Patients with the *ETV6-CBFA2* fusion have an excellent outcome [79, 81]. There is controversy whether ultimate event-free survival (EFS) is actually superior to that of other patients with B-precursor ALL, or whether the EFS is similar but the timing of relapse is significantly later for patients with the *ETV6-CBFA2* fusion compared to other patients with B-precursor ALL [82–83]. Recent evidence indicates that in a few cases the *ETV6-CBFA2* rearrangement may be acquired in utero, but ALL does not develop until years later. Thus, an additional cooperating mutation(s) may be required for leukemogenesis [84].

ETV6 is rearranged in half of the patients with 12p13 translocations and either lymphoid or myeloid leukemia [85]. *ETV6* has multiple fusion partners: ~40 chromosome bands are involved in translocations with *ETV6*, and ~20 partner genes have been cloned. Likewise, *CBFA2* is also involved resulting in >40 different chromosomal aberrations associated with hematologic disorders. Figure 6.8 illustrates the detection of the TEL/AML1 gene fusion as a result of t(12;21).

6.5.6.2 CBFA2 (AML1/RUNX1) Amplification

In addition to detecting the t(12;21) and determining variation patterns in the signals of both genes, FISH using the *ETV6-CBFA2* probe can confirm *CBFA2* amplification, which occurs in approximately 1–2% of older pediatric patients or adolescents with B-lineage and a low leukocyte count, t(12;21)-negative ALL [86–87]. Patients with amplification of *CBFA2* have been associated with an unfavorable outcome in a small number of patients [88]. Future international collaborations and larger collections of such cases will enable us to refine the clinical and survival associations.

The intrachromosomal amplification of chromosome 21 includes *CBFA2* gene (iAMP21) and are characterized by complex genomic alterations with a common region of amplification (CRA) and a common region of deletion (CRD) in 100% and 70% of iAMP patients, respectively [89].

6.5.6.3 11q23/MLL Gene Rearrangement and t(4;11) in ALL

The q23 region of chromosome 11 is a relatively common site of structural rearrangements in pediatric patients with hematologic neoplasms. In infants with ALL, the incidence of 11q23 abnormalities ranges from 70 to 90%, whereas in children with ALL is from 4.5 to 5.7% [90–91]. Children who have ALL with 11q23 abnormalities are usually young and have high leukocyte counts, organomegaly, and central nervous system (CNS) involvement. The leukemic cells have an early pre-B immunophenotype, CD10 –negative,

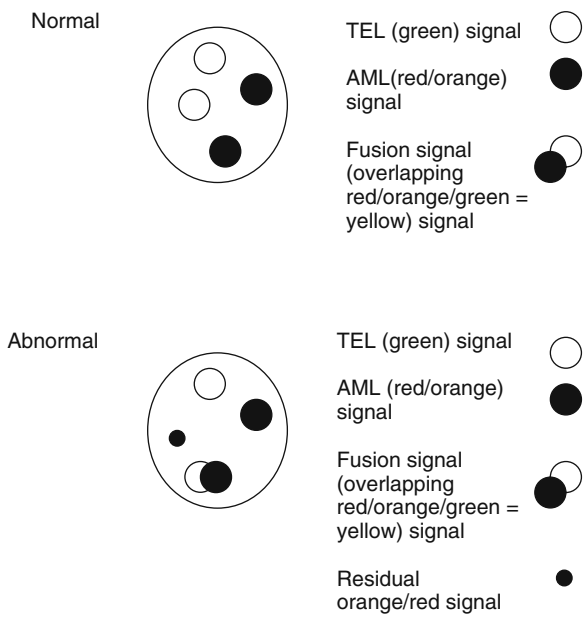

Fig. 6.8 Detection of the TEL/AML1 gene fusion that occurs as a result of a t(12;21)(p13;q22) using the Vysis LSI TEL/AML ES Dual Color Translocation Probe. In a normal interphase nucleus lacking the TEL/AML1 fusion gene, two green (TEL) and two orange/red (AML1) signals can be seen. In an abnormal cell containing the TEL/AML1 fusion, the expected signal pattern is one green (native TEL), one large orange/red (native AML1), one smaller orange/red signal (residual AML1), and one fused orange/red/green (yellow) signal

with myeloid-related antigens. ALL with 11q23 abnormalities, except deletions or inversions, is associated with a poor prognosis [92]. In a large international collaborative study, infants with ALL and 11q23 fared substantially worse than patients with 11q23 who were 1 year or older [93]. Furthermore, a recent study on infants with ALL also showed that the individual *MLL* rearrangements (69% of cases) t(4;11) (30%), t(11;19) (17%), t(9;11) (8%) and other 11q23 (13%) did not have different effects on prognosis [94].

Some *MLL* gene rearrangements can not be detected by conventional cytogenetic methods. The commercially available dual-color *MLL* probe allows FISH evaluation of derivatives of a translocation involving *MLL* in metaphase chromosomes and the splitting of signals in interphase nuclei (Fig. 6.9) [95]. In rare instances, FISH based on this probe detects not only the reciprocal translocation but also a deletion of at least 190 kb from the 3' region of *MLL* gene [96].

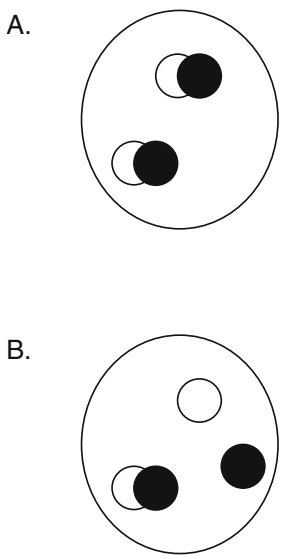

Fig. 6.9 Schematic representation of the Vysis LSI MLL Dual Color, Break Apart Rearrangement Probe for the detection of 11q23 structural chromosomal abnormalities. This probe consists of a centromeric (proximal) portion labeled in green and a telomeric (distal) portion labeled in orange/red. The signal pattern observed in a cell lacking the MLL rearrangement is expected to show a two orange/red/green (yellow) fusion signal pattern. In a cell possessing a MLL translocation, the expected pattern is one orange/red/green (yellow) fusion signal, one orange/red signal, and one green signal. (**a**) Normal cell with no MLL rearrangement, showing two orange/red/green (yellow) fusion signals (*top*). (**b**) Abnormal cell with a MLL rearrangement, showing one orange/red, one green and one orange/red/green (yellow) fusion signal (*bottom*)

About 80 alternative partner chromosome sites have been identified (some only in a small number of patients), and about 50 novel genes involved in the translocations have been cloned [97–98]. Like the t(4;11)(q21;q23), other recurrent 11q23 translocations, such as t(9;11)(p22;q23), t(6;11)(q27;q23), t(10;11)(p variable;q23), and t(11;19)(q23;p13.1), are typically found in acute myelomonocytic and monocytic leukemias. However, the t(9;11)(p22;q23) and t(10;11) can also be found in rare cases of ALL. Figure 6.10 shows an ideogram (Fig. 6.10a) and a representative karyogram (Fig. 6.10b) with the 4;11 translocation (reprinted with permission from Experimental and Molecular Pathology).

The gene on 11q23 that is most often rearranged in acute leukemias is *MLL* (myeloid/lymphoid leukemia or mixed lineage leukemia; also called *ALL1*, *HRX*, and *HTRX*) [99–100]. *MLL* is a homolog of the Drosophila *trithorax* gene, whose function is required for proper expression of homeotic genes and regulation of chromatin structure. Nearly all 11q23 translocations produce a fusion protein possessing the NH_2-terminus of MLL fused to the COOH-terminus of the fusion partner [101].

Studies of gene expression profiles in leukemic cells have shown the receptor tyrosine kinase FLT3 to be highly expressed in *MLL*-rearranged ALL as compared with other leukemias [102]. Further assessment showed that approximately 20% of *MLL*-rearranged ALL samples has activating mutations of FLT3 in the activation loop region [103]. Thus, the presence of FLT3 mutations in *MLL*-rearranged ALL supports activation of FLT3 or other kinases as cooperating events in this disease. Clinical trials designed to assess the efficacy of FLT3 inhibitors in *MLL*-rearranged ALL are in development [104].

6.5.6.4 T(1;19)(q23;p13.3)

In childhood ALL, the t(1;19) is the most frequent translocation detected by conventional cytogenetic methods. This translocation, with a primarily postnatal origin, is found in 6% of all cases of childhood ALL and in approximately 25% of cases of pre-B cytoplasmic immunoglobulin–positive (cIg^+) ALL [105–106]. It occurs in either a balanced form (25% of cases) or an unbalanced form (75% of cases) as der(19)t(1;19)(q23;p13.3). The t(1;19) was initially

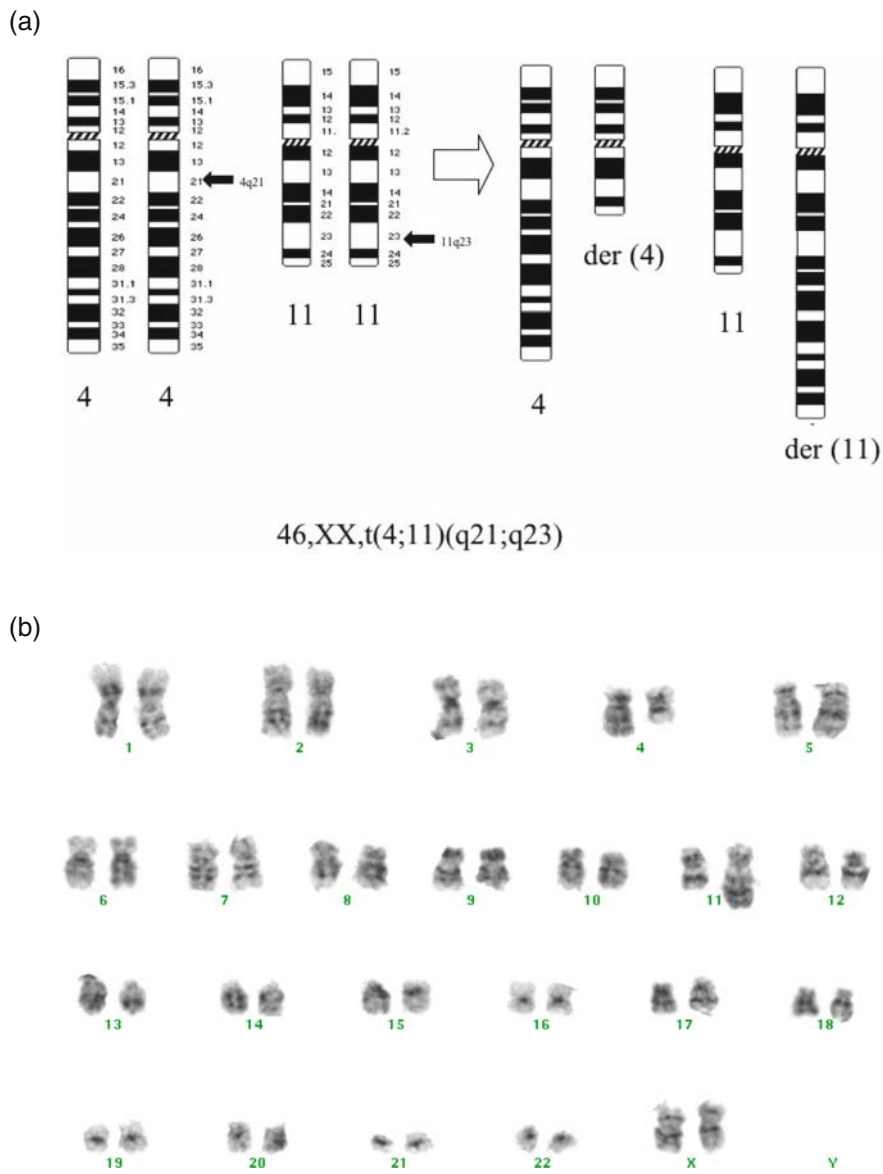

Fig. 6.10 (**a**) is an ideogram showing the breakpoints of the 4;11 translocation; (**b**) shows a representative karyogram with a 4;11 translocation. Reprinted from Hayne et al. [169], with permission from Experimental and Molecular Pathology

associated with inferior outcome in the context of antimetabolite-based therapy but subsequent studies have shown that the poorer prognosis can be largely overcome by more intensive chemotherapy [107]. The t(1;19) leads to the fusion of *TCF3* (*E2A)*, which is on chromosome 19 and encodes a helix-loop-helix (HLH) protein, with *PBX1*, a homeobox-containing gene on chromosome 1 [108–109]. The resulting hybrid gene, *TCF3-PBX1*, is a potent oncogene and can be detected by RT-PCR and/or FISH [110–112]. In about 5–10% the t(1;19) detected by conventional cytogenetics does not involve *TCF3* or *PBX1* but involves other genes [113–115].

6.5.6.5 t(17;19)(q22;p13.3) and inv(19)(p13.3q13.4)

Other rare, nonrandom chromosomal translocations affecting 19p13.3 also involve the *TCF3* gene. The t(17;19)(q22;p13.3) is found in approximately 1% of patients with B-lineage leukemia, most of whom do not respond to therapy [116]. The majority of t(17;19) generate a fusion gene consisting of *TCF3* and the hepatic leukemia factor gene (*HLF*) on chromosome 17 [117]. In a few cases in which the t(17;19)(q22;p13.3) is present, neither *TCF3* nor *HLF* rearrangements have been noted. Thus, this translocation, like the t(1;19),

may be heterogeneous at the molecular level [118]. In addition, a rare cryptic inversion of chromosome 19, inv(19)(p13.3q13.4), fuses *TCF3* to the *FB1* gene on 19q13.4 [119].

6.5.7 Numerical Chromosomal Abnormalities in ALL

ALL can be classified into subtypes based on the modal number (MN) of chromosomes. Recognition of ploidy as a distinctive cytogenetic feature in ALL has improved the ability to predict clinical outcome and devise risk-specific therapy.

6.5.7.1 Near-Tetraploidy and Near-Triploidy in ALL

Near-tetraploidy (MN range, ≥82) occurs in less than 1% of reported cases of childhood ALL. Near-triploidy (MN range, 69–81) is extremely rare (0.3%) in childhood ALL. A strong association of near-triploidy and near-tetraploidy and the cryptic t(12;21) has been noted [120].

6.5.7.2 Hyperdiploidy (>50 Chromosomes)

High-hyperdiploidy (with a MN range of 51–68, or DNA index >1.16) occurs in 25% of pediatric ALL. Favorable presenting features commonly associated with this subgroup include an early pre-B immunophenotype, low leukocyte counts, and age between 2 and 10 years. The patients whose blast cells have trisomy of chromosomes 4, 10, and 17 among the extra chromosomes have a superior prognosis [121–122] and are presently used for favorable low-risk group stratification. Sometimes only normal metaphase chromosomes are found by conventional cytogenetics and flow cytometry indicates a higher-than-normal DNA index (i.e., hyperdiploidy). This discrepancy suggests that dividing hyperdiploid blast cells have a short life-span which may be explained by stringent survival requirements and a marked propensity to undergo apoptosis of these blasts [123].

Recent studies have shown that hyperdiploidy resulting from nondisjunction of chromosomes in childhood B-cell precursor ALL occurs early during leukemogenesis and probably arises prenatally, although ALL was not diagnosed clinically until 2–3 years after birth [124]. This result extends earlier observations on the origins of specific chromosomal translocations in children with ALL suggesting that a genetic lesion is necessary, but not sufficient, in the leukemogenic process, and that additional genetic or epigenetic aberrations are needed for overt leukemia [125].

Recent studies have shown a distinct expression signature for each of the known genetic subtypes of childhood ALL using the Affymetrix microarray system [126–127]. These studies showed the majority of the class-discriminating genes for high hyperdiploid ALL were on chromosomes X and 21, which are tri- or tetrasomic more often. Also, the gene dosage effect for trisomic chromosomes had an average increase of 2-fold. Such systems are currently being validated by international collaborations and may have an impact on the future classification of leukemias.

6.5.7.3 Hyperdiploidy (47–50 Chromosomes)

Low-hyperdiploidy, defined as 47–50 chromosomes, occurs in 10–5% of cases of childhood ALL and was initially recognized because it confers a prognosis that is intermediate to those assigned by other ploidy groups. Gains of almost every chromosome have been observed in leukemic cells with this ploidy designation. The analysis of 86 cases revealed that +21 was the most common numeric abnormality (39%); less common were +X (21%), +8 (9%), and +10 (8%) [128]. The chromosomal arms most often affected by structural abnormalities were 1q (15%), 6q (14%), 12p (21%), and 19p (10%). Non-Down patients with trisomy 21 have a good prognosis, which may account for a strong association between +21 and the cryptic t(12;21)(p13.3;q22) [129].

6.5.7.4 Hypodiploidy and Near-Haploidy

Hypodiploidy (≤45 chromosomes), representing a heterogeneous subgroup of patients, is found in ~8% of cases of childhood ALL. Hypodiploidy has been further divided as near-haploid (23–29 chromosomes),

low hypodiploidy (33–39 chromosomes) and high hypodiploidy (42–45 chromosomes) [130]. There is a significant trend for progressively worse outcome with decreasing chromosome number; near haploidy have the worst outcome.

Most hypodiploid cases (80%) have an MN of 45, and the chromosomal deficiency arises from an unbalanced translocation, the loss of a whole chromosome (predominantly –X), or the formation of dicentric chromosomes [131]. Approximately 50% ALL cases with high hypodiploidy contain the *ETV6-CBFA2* fusion gene, which may explain in part its association with a more favorable outcome when compared to cases with less than 44 chromosomes [131].

Low hypodiploidy is extremely rare (approximately 0.8% of patients with ALL) and is associated with poor prognosis [130, 132, 131] (see Table 6.1). The overall incidence of near-haploidy is low, about 0.5% of all ALL cases and is associated with a poor prognosis (median survival, 10 months from the time of diagnosis), despite the presence of relatively favorable presenting features [132, 131]. The near haploid and low hypodiploid groups are also characterized by the presence of a doubled hyperdiploid population and if undetected by conventional cytogenetics may be misinterpreted as a hyperdiploid clone. In these rare cases, DNA index analysis may aid proper risk assignment [130–131].

6.5.8 Selected Recurrent Cytogenetic Abnormalities in Myelodysplastic Syndrome (MDS)

Myelodysplastic syndrome (MDS) is a heterogeneous group of neoplastic disorders arising from clonal hematopoietic progenitor cells whose degree of differentiation varies [133–134]. MDS include primary idiopathic MDS and secondary or therapy-related MDS (t-MDS) that develop after prior exposure to chemotherapy or radiation. Primary MDS arises largely in older individuals, and the incidence increases with age. The deaths of patients with MDS usually result from cytopenia and transformation to acute leukemia which occurs in 10–15% of cases. Unlike MDS in adults, with the exception of refractory anemia (RA) and MDS with Down syndrome, MDS in children runs an aggressive clinical course with a short survival period and a higher rate of progression to acute myeloid leukemia (AML). Cytogenetic analysis, in combination with evaluation of clinical features, provides information about the predicted median survival estimate and the likelihood of progression to AML [135]. An abnormal clone is found in 50–60% of patients at the time of MDS diagnosis; additional chromosome abnormalities may appear during the course of the disease and are associated with clinical progression and early death [135, 136]. These abnormalities may appear alone or in complex rearrangements, and most are distinct from abnormalities seen in patients with AML. In contrast, cytogenetic abnormalities are observed in as many as 90% of cases with t-MDS [137].

6.5.8.1 Cytogenetic Features and Classification Criteria

An international prognostic scoring system (IPSS) for primary MDS was developed after multivariate analysis of 816 patients with primary MDS who primarily received supportive care [138]. The most significant independent variables identified for determining survival and AML evolution were the percentage of marrow blast cells, number of cytopenias, and subgroups based on particular cytogenetic features. Patients with a del(5q), a del(20q), or a –Y and patients with a normal marrow karyotype had a relatively good prognosis; approximately 70% of adult patients with MDS meet this criteria. The 16% of patients with complex abnormalities (i.e., at least three aberrations) or chromosome 7 anomalies (i.e., –7 or del(7q)) had a relatively poor prognosis. The remaining patients (14%) with other abnormalities had an intermediate prognosis. The median length of survival after diagnosis was 5.7 years for patients with a good prognosis, 1.2–3.5 years for those with an intermediate prognosis, and 4.0 months for those with a poor prognosis [138].

In several series, the survival times correlated well with the prognostic subgroups for adult patients with MDS; the strong correlation confirmed the validity of the IPSS, which was partly based on cytogenetic features [139, 135]. However, other investigators did not show the IPSS to be superior to existing prognostic systems. In the retrospective analysis of children by Sasaki et al. (141), the cytogenetic pattern was the only prognostic variable in the IPSS that was found useful

for predicting outcome of children with MDS. The 4-year survival probability was 83.8% for children with a good prognosis, 48.9% for those with an intermediate prognosis, and 6.5% for those with a poor prognosis [140].

Recently, the WHO classification system has established 8 histopathologic variants [refractory anemia (RA), RA with ringed sideroblasts, RA with multilineage dysplasia, RA with multilineage dysplasia and ringed sideroblasts, RA with excess blasts: Type 1 (5–9% blasts in the bone marrow and < 5% blasts in the blood), RA with excess blasts: Type 2 (10–19% blasts in the bone marrow and 5–19% blasts in the blood), MDS with isolated del(5q), and MDS unclassified] [141].

Classification criteria for childhood MDS have been adapted from classification systems tailored for MDS in adults [142]. Most pediatric series have included patients with MDS and predisposing constitutional disorders, patients with a mild form of the disease (RA), and patients with more aggressive forms juvenile myelomonocytic leukemia (JMML), refractory anemia with excess blast cells (RAEB), and refractory anemia with excess blast cells in transformation (RAEBT). Two other pediatric conditions were included in the recent WHO classification of myelodysplastic/myeloproliferative disorder (MDS/MPD) [42]. One type is transient myeloproliferative disorder (TMD) or transient abnormal myelopoiesis (TAM), which is seen in neonates with Down syndrome. The other type is characterized by rare but recurrent cytogenetic abnormalities involving 8p11 and 5q31–33 (aberrations of tyrosine kinase genes). In few pediatric and adolescent patients with the diagnosis of MDS, the bone marrow at initial examination have cytogenetic features typical of leukemic cells in patients with primary AML {e.g., t(8;21), inv(16), and t(9;11)}. These patients have better responses to AML-directed therapy and they should be treated as AML regardless of the blast count.

It is recognized that MDS is notable for a predominance of chromosomal deletions, whereas AML is characterized by balanced translocations. The monosomy 5 (–5) or del(5q) has a variable prognosis according the subtype of MDS and the presence or absence of other chromosomal abnormalities. Two critical minimal regions of deletion on 5q are at q31 and q33 [143]. Likewise, two critical minimal regions of deletion on 7q are at q22 or q32–33 [144]. Other critical regions have been identified at 17p [145] and at 20q12 [146]. The large deletions detectable by conventional cytogenetics are likely due to late developments in the pathogenesis of MDS. Despite numerous multidisciplinary studies of these commonly deleted regions, the molecular mechanisms of transformation and the critical genes involved remain elusive.

6.5.8.2 Chromosome 5 Abnormality

A del(5q) is present in 15–25% of adult patients with primary MDS and in as many as 50% of adult patients with t-MDS, especially those patients previously exposed to alkylating agents. The del(5q) may be found as a sole abnormality or as part of a complex karyotype. In adults with RA, a del(5q) as the sole abnormality is the hallmark of the 5q– syndrome usually observed in older female patients with low blast counts, normal or elevated platelet count, who has an indolent course and long survival [147–148]. Monosomy 5 has been considered less likely to be a primary karyotypic abnormality and is not of pathogenic significance in MDS. In pediatric patients with MDS, a –5/del(5q) is rarely seen and they do not have the same clinical features at initial examination as do adults [149].

Recently, studies have shown that lenalidomide, a thalidomide analogue, has a significant therapeutic benefit in patients with MDS who would otherwise not benefit from growth-factor therapy; the benefits of lenalidomide were noted particularly in patients with RA with isolated erythroid abnormalities, MDS with isolated del(5)(q31.1), and patients with a low or more favorable IPSS score [150–151]. Based on these findings, lenalidomide was recently approved by FDA to treat patients with a 5q deletion (December 2005).

6.5.8.3 Chromosome 7 Abnormality

A –7 or del(7q) are associated with primary MDS and AML and with t-MDS and t-AML that occurs in children and adults exposed to mutagenic agents or treated earlier with alkylating agents. Abnormalities of chromosome 7 are the most frequent chromosomal changes in malignant cells of pediatric patients with MDS found in 40–50% of the cases [142, 149]. Monosomy 7 is common in younger children with JMML or constitutional disorders, such as Fanconi anemia, congenital neutropenia, or neurofibromatosis,

all of which increase the risk for a malignant myeloid disorder [152]. Children with –7 and MDS have an outcome similar to that of similar-aged patients with MDS without monosomy 7. However, in pediatric patients with –7 and AML (when compared to those without –7) have a lower response rate to chemotherapy and a higher relapse rate [153].

6.5.8.4 Therapy-Related MDS (t-MDS)

The development of t-MDS or t-AML is one of the most serious late consequences of patients with cytopenia 3–7 years after receiving alkylating agents and/or radiotherapy to treat a primary cancer. As mentioned above, the chromosomal abnormalities most frequently observed in t-MDS include –5/del(5q) and/or –7/del(7q), and complex karyotypes with frequent deletion of 12p, 17p, and 20q; the patients have a dismal outcome [154]. Patients treated with topoisomerase II inhibitors also are at increased risk of developing secondary myeloid malignancies, but with a fewer cases developing t-MDS as the majority progress to AML. Most of these latter cases have translocations involving 11q23 deregulating the *MLL* gene or, less often, the *CBFA2* gene at 21q22 [155–156].

In adult hematology, cytogenetics has been important in MDS as well. As in AML, cytogenetics in MDS carry prognostic importance, and certain abnormalities are known to carry a particularly grim prognosis. Treatment related MDS, or t-MDS, is a side effect of cytotoxic chemotherapy given for other tumors. Most typically, aneuploidies such as –5, –7 and +8 arise 3–4 years after treatment with an alkylating agent. In contrast, balanced translocations, especially of 11q23 and also of 3q26 and 21q22, are seen about 2–3 years after treatment with an agent active against topoisomerase II, such as the epipodophyllotoxins or anthracyclines. All t-MDS carries a poor prognosis, and it is unclear if the chromosomal abnormalities mentioned above are the cause of this or simply reflect the underlying aggressive disease.

Notwithstanding the above, there is one bright spot in the cytogenetics of MDS, which has recently gotten slightly brighter still. The so-called "5q- syndrome" consists of a triad of normal to high peripheral blood platelet numbers, a high mean corpuscular volume and small unilobular megakaryocytes on bone marrow examination in addition to an isolated 5q- karyotype [157]. The deleted area may span the chromosomal region from 5q13 to 5q33, but almost always included 5q31.1. Although the diagnosis of the 5q- syndrome requires 5q- to be the only chromosomal abnormality, the presence of 5q- with or without other abnormalities now carries therapeutic importance in that the immunomodulating agent lenalidomide is now approved for treating transfusion dependent patients with MDS with low or intermediate-1 International Prognostic Scoring system (IPSS) scores and with 5q- with or without other abnormalities. This narrow indication is based on a multi-institutional study [158] that showed a reduced transfusion requirement in 112/148 such patients (76%), with transfusion independence being achieved in 99 patients (67%).

Finally, the IPSS itself is based partly on the importance of cytogenetics in MDS. The IPSS assigns points to a patient's MDS based on peripheral blood cytopenias, marrow blasts and cytogenetics. The sum of the points indicates a score associated with a better or worse prognosis [138]. In this system, good risk cytogenetics include normal, -Y, 5q- and 20q-. Poor risk includes complex cytogenetics ($\geq$ 3 abnormalities), and abnormalities of chromosome 7, whether or not they are isolated. Anything else is intermediate risk. Although the IPSS has been validated on a separate data set from that upon which it was built, it has not been prospectively validated as of yet, and its usefulness in addition to or instead of the WHO classification, for either prognosis or therapeutic decision making, is still not formally proven.

6.5.9 Myeloproliferative Diseases

The WHO classification of myeloproliferative diseases (MPD) includes CML, polycythemia vera (PV), essential thrombocythemia (ET), chronic idiopathic myelofibrosis (CIMF) and the related disorders: chronic eosinophilic leukemia (CEL) and idiopathic hypereosinophilic syndrome (HES) [141]. CML has been discussed in a prior section. Other MPDs are discussed below.

6.5.9.1 MPDs Other than CML

Most cytogenetic studies of patients with PV are normal, with 20–30% of cases having a 20q–, +8 or +9.

Patients with ET also have normal cytogenetic studies. The clonal cytogenetic findings seen in 30–40% patients with CIMF include 13q–, 20q– and 1q+ [159].

At initial examination, a small number of patients, mostly adults, have clinical and hematologic features that suggest the presence of CML, but the Philadelphia (Ph) chromosome is absent. Therefore, the disease is classified as atypical CML/MDS/MPD. A number of translocations or cryptic genetic lesions are found in a small proportion of these patients; it is important to identify the chromosomal alterations, because the formed fusion proteins are potential targets for selected signal transduction therapy [160–161].

Although many translocations, each with many variant partners, disrupting tyrosine kinase genes have been described in reports of single cases, there are two key breakpoint clusters at 5q33 (*PDGFRB*) and 8p11 (*FGFR1*) and an occasional disruption of 4q12 (*PDGFRA*). Rearrangements of the *PDGFRB* transmembrane tyrosine kinase receptor gene may respond well to treatment with imatinib mesylate (Gleevec; Novartis, Basel, Switzerland) [161]. The patients with the 8p11 syndrome or EMS (8p11 myeloproliferative syndrome) have chromosomal abnormalities affecting the p11–12 region of chromosome 8, involving the FGFR1 tyrosine kinase gene [162]. Specific therapy has not yet been identified in patients with the 8p11 syndrome; apparently, imatinib mesylate is inactive against *FGFR1* and therefore may not benefit these patients [163]. The most recently recognized *FIP1L1-PDGFRA* fusion gene in cases of hypereosinophilic syndrome encodes an activated tyrosine kinase that is inhibited by imatinib [164–165]. As in CML, mutations in the *FIP1L1-PDGFRA* kinase domain is known to give rise to resistance to imatinib mesylate and other inhibitors appear to be effective to treat these patients [166].

The recently discovered V617F mutation of tyrosine kinase JAK2 (*JAK2*-V617F), specific to MPD except CML, is present in most patients with PV and in more than half of patients suffering from IMF or ET. Furthermore, this mutation has been identified and in other subsets of patients suffering from AML, other MPDs and MDSs [167–168]. The consequence of this mutation on normal hematopoiesis and the involvement in such a diversity of diseases is being evaluated presently with the prospect of targeted therapies to inhibit the mutation.

Therefore, the success seen in the treatment of *BCR-ABL*$^+$ CML with kinase inhibitors may be extended to patients with translocations that disrupt genes encoding other tyrosine kinases. Although these translocations/genetic lesions are found in a small proportion of patients their identification is important because the formed fusion proteins are potential targets for selected signal transduction therapy. Numerous additional genetic lesions have been recognized in MPD and MDS but the molecular pathogenesis of diseases are beyond the scope of this chapter.

6.6 Conclusion

Cancer cytogenetics has become established as a routine component in the management of patients with hematopoietic malignancies. The detection of specific recurring chromosomal abnormalities is important not only for diagnosis, but also for prognosis and treatment. After the initial diagnosis, usually via G-banding, continuous monitoring of the patient through treatment, remission, relapse and bone marrow transplant is often achieved using a combination of conventional and molecular techniques such as FISH and PCR, in addition to conventional cytogenetics

The historical delineation of the Philadelphia translocation is important not only because it was the first consistent recurring chromosomal abnormality found in a cancer subtype, but also because it serves as a paradigm for the later utilization of cytogenetic information in hematology and oncology. Past experience in the cytogenetic study of leukemias has led to important clinical correlations and insights. For example, when an inversion of chromosome 16 in AML M4 subtype is found, the clinician can reasonably conclude that the prognosis will most likely be good whereas when a rearrangement is found involving the 11q23 breakpoint, the associated prognosis will most likely be poor, although this is more controversial. The detection of cytogenetic rearrangements and accurate characterization of breakpoints in these abnormal clones is thus used to determine subsequent therapeutic options and to predict success in treatment outcome. In addition, the knowledge gained through cytogenetic studies of the structural chromosomal abnormalities may eventually lead to the cloning of additional genes involved in the development and progression of cancer

and may one day shed light on the molecular mechanisms of leukemogenesis in the particular hematopoietic malignancy. Only with a thorough understanding can therapeutic agents be rationally designed so that the growth of malignant cells can be specifically inhibited. Despite certain predictions to the contrary, the advent of molecular biology did not diminish the importance of conventional cytogenetics. Instead, the increased availability of molecular probes has continued to propel the field of cytogenetics into the twenty-first century with powerful adjunct techniques such as FISH and FISH-based techniques such as CGH and SKY. Also, concurrent with these cytogenetic advances, other factors continue to converge to offer significant advances in focused diagnostics and comprehensive care for patients with hematologic malignancies [169]. As more is known about molecular and cytogenetic abnormalities in hematopoietic malignancy, close collaboration between cytogeneticists, specialists in immunophenotyping, molecular pathologists and treating clinicians will be necessary in order to select optimal therapeutic and follow-up regimens for our patients. This close collaboration in the use of advanced diagnostic and prognostic methods has already yielded important insights into optimal treatment strategies for several hematologic malignancies, and has led to rationally designed therapeutics as important investigational agents or as standards of care. We anticipate improved treatment outcomes as this field is advanced and the knowledge gained is further applied to patient care.

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Chapter 7

Mechanisms of Cancer Growth and Progression in Lymphoma

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Contents

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7.1 Introduction

Lymphomas are a group of biologically diverse and clinically distinct malignant tumors of hematopoietic origin. They arise from lymphocytes (T-cells or B-cells), that appear to be arrested at different stages of maturation. Lymphomas are typically initiated by acquired alterations such as chromosomal translocations, deletions and mutations that occur in the genome of the lymphoid cell (or their progenitors), rather than inherited DNA-sequence variations. The transforming events in lymphomagenesis represent a multi-step process involving the progressive accumulation of genetic lesions that ultimately result in the clonal expansion of malignant T- or B-lymphocytes. Current classification of lymphomas, adopted by the World Health Organization [1], includes B-cell neoplasms, T/NK-cell tumors, and Hodgkin lymphoma. About 95% of lymphomas are of B-cell origin; the remainder are T-cell malignancies. Within the category of B-cell derived lymphomas, mature B-cell neoplasms comprise over 90% of non-Hodgkin lymphomas worldwide [2, 3, 1], and will be the focus of this review.

During the past two decades, extensive progress has been made to elucidate the cellular origin of B-cell lymphomas by various approaches including histology, immunophenotyping and molecular techniques. Advances in technology have made possible the study of normal and malignant lymphocytes and identification of major transforming events, including the role of chromosomal translocations, antigen activation of B-cell receptor, and the cellular microenvironment in lymphoma pathogenesis.

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7.2 B Cell Development

Lymphocyte precursors (progenitors) originate in the bone marrow and undergo antigen-independent lineage commitment. Maturation and selection of T-cells occur predominantly in the thymus [4]. B-cell maturation occurs in steps, first in the bone marrow from hematopoietic precursors to immature/transitional B cells, then in the periphery from transitional to fully mature B cells [5, 6].

7.2.1 Early B Cell Maturation: Production of B Cell Receptor (BCR)

The initial stage of B cell differentiation begins in the bone marrow where precursor lymphoblasts undergo antigen independent rearrangement of the immunoglobulin heavy and light chain genes, through the process of V(D)J recombination, leading to the generation of B-cell receptor (BCR) (Fig. 7.1). Proliferation and terminal differentiation of B-cells are regulated by antigen triggering, T-cell interaction, macrophages and local factors, that occur predominantly in secondary lymphoid tissue, including lymph nodes, and spleen [7]. There are many different V, D and J segments in the germ line, and therefore each B-cell generates a distinct pair of genes for its heavy chain variable region and another pair for its light-chain variable region. These encode a distinct antigen receptor with unique specificity, thus generating the repertoire diversity of BCR [8]. BCR is composed of two identical heavy- and two identical light-chain immunoglobulin (Ig) polypeptides that are covalently linked by disulfide bridges. Other components of the BCR are the CD79a and CD79b molecules that contain immunoreceptor tyrosine-based activation motifs. These motifs interact with intracellular signaling molecules after BCR cross-linking/antigen binding, and relay signals that lead to proliferation and/or further differentiation of the activated B-cell [9].

The expression of BCR is critically important for the development and survival of B cells. In the bone marrow, B-cell precursors that fail to express a functional BCR undergo apoptosis [9]. The BCR-dependant survival of B-cells is further emphasized by studies that show that in vivo ablation of surface immunoglobulin on mature B-cells by inducible gene targeting results in rapid cell death [10]. Furthermore, receptor specificity is critical for B-cells throughout their life. In bone marrow, B-cell precursors expressing autoreactive receptors either "edit" their receptors by means of secondary V(D)J rearrangements or undergo apoptosis. Following the process of receptor editing, non-autoreactive B-cells expressing a functional surface antigen receptor leave the bone marrow to become mature, naïve (not yet been exposed to antigen) B-cells [11].

7.2.2 Late B Cell Maturation: Production of High Affinity Immunoglobulin

In the peripheral lymphoid organs, the immature/transitional B-cells that have recently emerged from the bone marrow may follow one of two pathways that are either T-cell dependent or T-cell independent, respectively. These two functionally distinct developmental pathways take place in anatomically distinct regions of the secondary lymphoid organs. In the germinal centers of the secondary lymphoid tissue, Ig genes are modified by somatic hypermutation and class-switch recombination. First, in a T-cell-dependent manner, antigen-driven somatic hypermutation of the immunoglobulin variable (IgV) region leads to introduction of point mutations, deletions or duplications in the IgV gene, resulting in generation of a high-affinity B cell response to cognate antigens. Class switching results in the replacement of the originally expressed heavy-chain constant region gene (initially mu constant region for IgM, and the delta constant region for IgD) by that of another downstream constant-region gene, allowing the expression of IgG, IgA or IgE. Consequently, the process of class-switch recombination results in the expression of an antibody with a different effector function but the same antigen-binding specificity. Therefore, the germinal center is the source of memory B-cells and plasma cells that produce high-affinity antibodies necessary to protect against invading microorganisms. Alternatively, B cell development along a marginal zone T-cell independent pathway provides a first line of "innate-like" defense against specific pathogens [12, 13, 5].

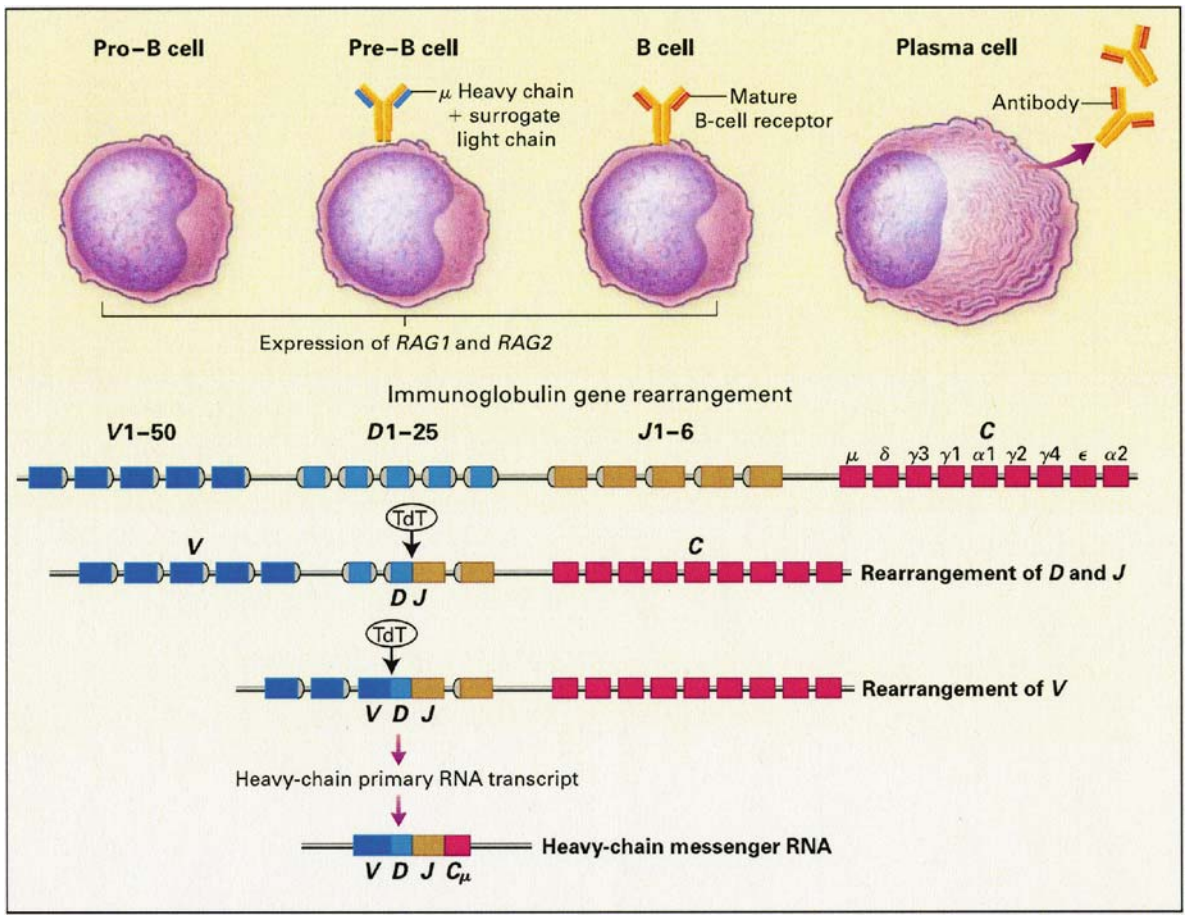

Fig. 7.1 Diversity of antigen receptors. The enormously diverse specificities of the antigen receptors are produced by gene rearrangements during the early developmental stages of the lymphocyte. The events involved in generating a coding sequence for the immunoglobulin heavy chain are shown. Early in B-cell development, pro-B cells mature into pre-B cells, at which stages they express the recombination-activating genes *RAG1* and *RAG2*. The recombinases encoded by these genes mediate the random rearrangement of 1 of 25 diversity (*D*) gene segments next to any 1 of 6 joining (*J*) gene segments. This is followed by the rearrangement of any 1 of 50 variable (*V*) gene segments next to the already rearranged *DJ* segment. Different B cells will rearrange a different segment in each pool, thereby creating one level of diversity. Further diversity is brought about by splicing inaccuracies and by the incorporation of nucleotides mediated by the enzyme terminal deoxyribonucleotidyltransferase (TdT). The heavy-chain primary RNA transcript is processed into messenger RNA (mRNA), with splicing of the rearranged *VDJ* segment next to the constant (*C*) region gene. This mRNA will encode a heavy chain that appears on the surface of the pre-B cell together with the surrogate light chain, which is encoded by genes that do not undergo rearrangement. As the pre-B cell continues to mature, the immunoglobulin light-chain genes undergo rearrangement; the resulting light chain replaces the surrogate light chain, and thereby produces a mature IgM B-cell receptor on the cell surface. The B-cell receptors at this stage also usually include IgD antibodies with the same specificity as the IgM molecule, produced by alternative splicing of the rearranged *VDJ* to either the C_μ or the C_δ gene. The expression of *RAG1* and *RAG2* is then switched off. After encountering an antigen, and in the presence of costimulatory signals, the B cell further differentiates into a plasma cell, which secretes high levels of the specific antibody (or into a memory B cell). The same general principles regarding the rearrangement process apply to the generation of α/ß and γ/δ T-cell receptors. The gene segments in the figure are not drawn to scale (From: Delves and Roitt [74])

7.3 Cellular Origin of B Cell Lymphomas

B-cell neoplasms appear to mimic stages of normal B-cell development, allowing classification based on similarities to their proposed normal B-cell counterpart when assessing a combination of morphology, histology and immunophenotype (Table 7.1). However, some B-cell neoplasms (i.e. hairy cell leukemia) do not

Table 7.1 Characteristics of some matures B-cell lymphomas. Somatic hypermutation (SHM), Diffuse large B-cell lymphoma (DLBCL), Germinal Center (GC), Marginal-zone associated lymphoid tissue (MALT). Adapted from "Table 1" in Shaffer et al. [75], and "Table 2" in Kuppers [63]

Lymphoma	SHM	Ongoing SHM	Chromosomal translocations	Viruses	Putative cell of origin
Mantle cell lymphoma	No (except for a small subset)	No	bcl1-IgH {t(11;14)}	–	Pre-GC B-cell
Follicular lymphoma	Yes	Yes	bcl2-IgH {t(14;18)}	–	GC B-cell
Burkitt lymphoma	Yes	No	myc-IgH {t(8;14)} or myc-IgL {t(2;8) or t(8;22)}	EBV (endemic 95%, sporadic 30%)	GC B-cell
Splenic Marginal zone lymphoma	Yes (in most)			–	GC or post-GC B-cell
MALT lymphoma	Yes	Yes	API-MALT1 bcl10-IgH MALT1-IgH FOXP1-IgH	Indirect roll of H. pylori in gastric MALT lymphoma	GC or post-GC B-cell
Chronic lymphocytic leukemia (CLL)	Yes and no	No	–	–	Antigen-experienced B-cell (pre- or post-GC)
GC B-cell-like DLBCL	Yes	Yes	bcl6-various bcl2-IgH myc-IgH or myc-IgL	–	GC-B-cell
Activated B-cell-like DLBCL	Yes	No	bcl6-various bcl2-IgH myc-IgH or myc-IgL	–	GC-B-cell subset or extra-GC mutated B-cell

obviously correspond to a normal B-cell differentiation stage, while others (i.e. chronic lymphocytic leukemia) appear to be of heterogeneous origin [14].

Distinct stages of B-cell development are characterized by the particular structure of the BCR and expression of surface differentiation markers. Distinct maturation stages often take place in specific histologic structures. A substantial proportion of peripheral blood B-cells (up to 90%) are IgM+ IgD+ CD5+ B-cells that have unmutated IgV, and thus can be assigned to a pre-germinal center stage of development (i.e. naïve B-cells). The memory B-cell compartment generated in the germinal center reaction consists of somatically hypermutated IgV, class-switched B-cells that express surface IgM [15].

Germinal centers of the secondary lymphoid tissues (lymph nodes, spleen and mucosa associated lymphoid tissue) are the main sites where somatic hypermutation of IgV occurs. The GC is surrounded by a mantle zone of naïve B-cells, most of which express CD5. The marginal zone is a B-cell rich zone located between B-cell follicles and the T-cell area in spleen and mucosa associated lymphoid tissue (i.e. Peyer's patches and tonsils) (Fig. 7.2).

7.3.1 Naïve (Circulating) B Cells

At practically every B-cell developmental stage, there is a subtype of lymphoid neoplasm with malignant lymphocytes arrested at a particular stage of maturation (Fig. 7.3). Naïve B-cells are often CD5+ and express surface immunoglobulins IgM and IgD. These small resting lymphocytes circulate in the blood and also occupy primary lymphoid follicles and follicle mantle zones (so-called recirculating B-cells). Among lymphomas derived from mature B-cells, unmutated IgV genes have been found only in mantle cell lymphoma and some chronic lymphocytic leukemias, both of which are CD5+ neoplasms [16, 17]. Most mantle cell lymphomas are thought to be derived from CD5+ (naïve) B-cells of the mantle zone; although, 20–30% carry mutated IgV region genes, indicating that they have passed through the germinal center [18, 19]. Many cases of chronic lymphocytic leukemia (CLL) are believed to correspond to the recirculating CD5+ naïve B-cells, expressing IgM and IgD, with germline configuration of IgV, that are found in peripheral blood, primary follicle, and follicle mantle zones. CLL cases that show IgV gene mutations may correspond to a subset of peripheral blood CD5+ memory B-cells that express surface IgM [20]. Tumors of naïve B-cells are usually histologically low-grade, clinically indolent and often widespread and leukemic, consistent with the recirculating behavior of normal naïve B-cells [14].

7.3.2 Germinal Center B Cells

Upon encountering antigen, naïve B-cells undergo blast transformation, migrate into primary lymphoid follicle and fill the follicular dendritic cell (FDC) meshwork, forming a germinal center (GC), where they ultimately mature into memory B-cells and immunoglobulin-secreting plasma cells (Fig. 7.4). Germinal center blast cells, called centroblasts, switch off expression of BCL2, an anti-apoptotic protein; thus they are susceptible to death by apoptosis [21]. Centroblasts express BCL6 protein, a POZ/zinc-finger transcriptional repressor, as well as the membrane metalloendopeptidase CD10; these markers are not expressed by naïve or memory B-cells, mantle cells or plasma cells, and serve as "markers" for germinal center B-cells [22, 23]. In the germinal center, somatic mutations occur in the immunoglobulin variable (IgV) region gene resulting in altered affinity for antigen and marked intraclonal diversity in a population of cells derived from only a few precursors [24]. In addition, some cells switch from IgM to IgG or IgA, by way of the process of Ig class-switch. Through these mechanisms, the "germinal center reaction" will give rise to the production of high-affinity IgG and IgA antibodies of the late primary or secondary immune response [25]. The BCL6 gene also undergoes somatic mutation in the germinal center [26]. Both IgV gene mutations and BCL6 gene mutations serve as markers of cells that have been through the germinal center. The detection of somatically mutated IgV genes in a lymphoma is an indication that the neoplastic clone originated from GC or post-GC (i.e. memory) B-cells, and the pattern of somatic hypermutation may allow these two possibilities to be distinguished [15]. For example, in follicular lymphoma there is ongoing somatic hypermutation within the tumor clone [27]. Additionally, follicular lymphomas have growth patterns that resemble those

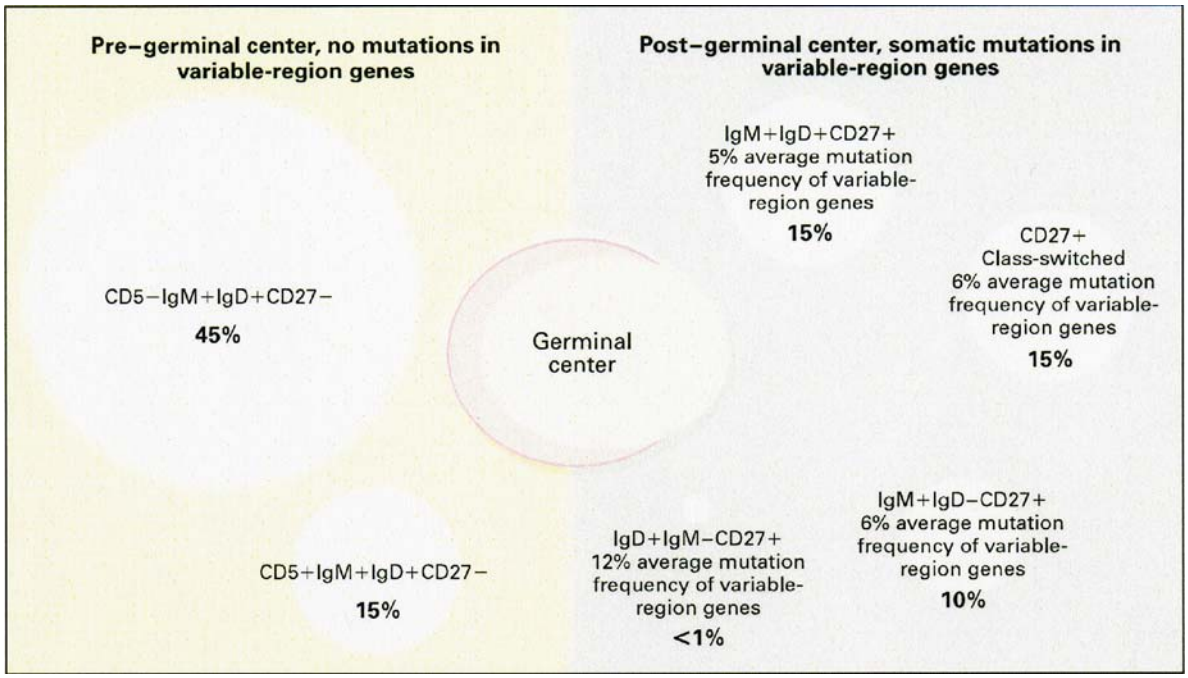

Fig. 7.2 Cellular composition of the peripheral-blood B-cell repertoire in humans. Shown are the phenotypically defined subgroups of B cells, the average frequency of mutations of the heavy-chain variable-region genes, and the percentages of the respective subgroups among all peripheral-blood B cells. Cells with only IgD are not discussed, because they comprise only a very small subgroup in the peripheral blood of some people. Somatically mutated B-cells in the peripheral blood are descendants of pre-germinal-center (naive) B cells that have passed through the germinal center in lymphoid organs where they acquired somatic mutations (From: Kuppers et al. [33])

of the normal germinal center B cells, infiltrated by follicular dendritic cells (FDC) and T cells. Furthermore, the tumor cells express the membrane metalloendopeptidase CD10, which is a hallmark of GC B-cells [23]. Taken together, follicular lymphoma is thus identified as a germinal center B-cell tumor. Burkitt lymphoma cells are BCL6+, express the germinal center surface marker CD10 and have mutated IgV genes, and are thus thought to correspond to a germinal center blast cell [28].

7.3.3 Memory B Cells (Post-germinal Center Cells)

Memory B-cells typically reside in the follicle marginal zones, characteristically lack CD5 and CD10, express surface IgM but not IgD, and have mutated IgV region genes. These post-germinal B-cells retain the ability to selectively traffic back to the tissue in which they undergo antigen stimulation. For example, B-cells that arise in mucosa associated lymphoid tissue (MALT) tend to return there, and those that arise in lymph nodes will home to nodal sites, via integrin homing receptors [29]. Nodal marginal zone lymphoma, extra-nodal marginal zone lymphoma and splenic marginal zone lymphoma are three entities that are thought to drive from post-germinal center marginal zone B-cells [30].

7.3.4 Molecular Profiling Supports Differentiation Stages for Neoplastic B Cells

The relationship between B-cell lymphomas to normal stages of B cell maturation has been further clarified using genomic-scale gene-expression profiling of B cell lymphomas and normal B cell subsets. A unique gene-expression signature distinguishes GC B-cells

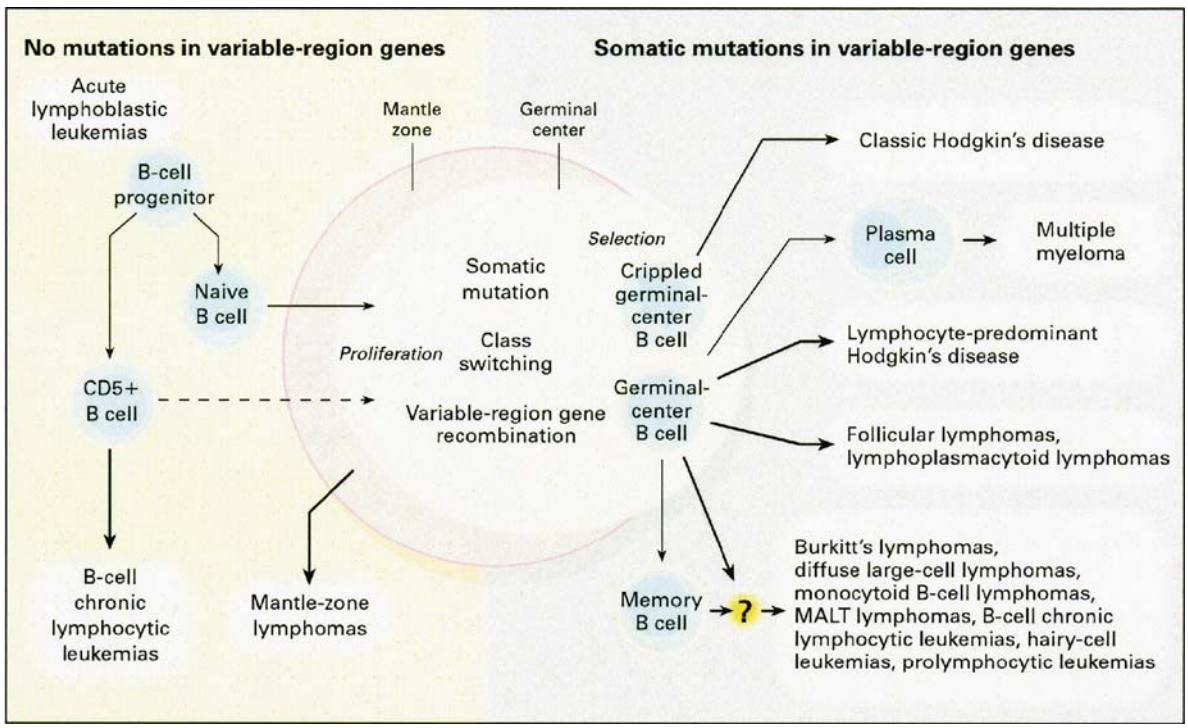

Fig. 7.3 Assignment of human B-Cell lymphomas to their normal B-Cell counterparts. Naive B cells that recognize antigen with their antigen receptors establish germinal centers. In these structures the cells vigorously proliferate. The genomic DNA of these cells may then be subjected to somatic hypermutation, class switching, and perhaps variable-region gene recombination. The extent of variable-region gene recombination is unknown. Acute lymphoblastic leukemia represents a cancer of B-cell progenitors. Germinal-center B cells that acquire affinity-increasing mutations are positively selected and differentiate into memory B cells or plasma cells. The diffuse large-cell lymphomas include centroblastic lymphomas, immunoblastic lymphomas, T-cell-rich B-cell lymphomas, mediastinal sclerosing lymphomas, and large-cell anaplastic lymphomas of B type. Diffuse large-cell lymphomas may represent primary diseases or transformations from low-grade lymphomas. Lymphomas in which intraclonal variable-region gene diversity is absent or is only occasionally present (except in the case of classic Hodgkin's disease) may be derived from progenitors in the germinal center or from post-germinal-center (memory) B cells. Nevertheless, as outlined in the text, important steps in the transformation process take place in the germinal center in lymphomas in which the tumor B cells resemble post-germinal-center B cells. MALT denotes mucosa-associated lymphoid tissue (From: Kuppers et al. [33])

from other stages of B-cell differentiation, including resting naïve and memory B cells, and mitogenically activated peripheral blood B-cells [31]. These studies identified a GC-B cell gene-expression signature that is associated with follicular lymphoma, Burkitt lymphoma and a subset of diffuse large B cell lymphoma (DLBCL). Two molecularly distinct forms of diffuse large B cell lymphoma (DLBCL) with gene expression patterns indicative of different stages of B-cell differentiation were identified. One type expressed genes characteristic of germinal centre B cells (germinal centre B-like DLBCL); the second type expressed genes normally induced during in vitro activation of peripheral blood B cells (activated B-like DLBCL) [31].

7.4 Transforming Events in Lymphomagenesis

7.4.1 Genomic DNA Modification: Point Mutations and Translocations

The fundamental processes that are crucial for B-cell differentiation and survival are found to be involved in the malignant transformation of most B-cell lymphomas. A vast majority of B-cell lymphomas appear to derive from GC or post CG B-cells, suggesting that malignant transformation often occurs, or is initiated in, GC B-cells [32]. The germinal center B-cells

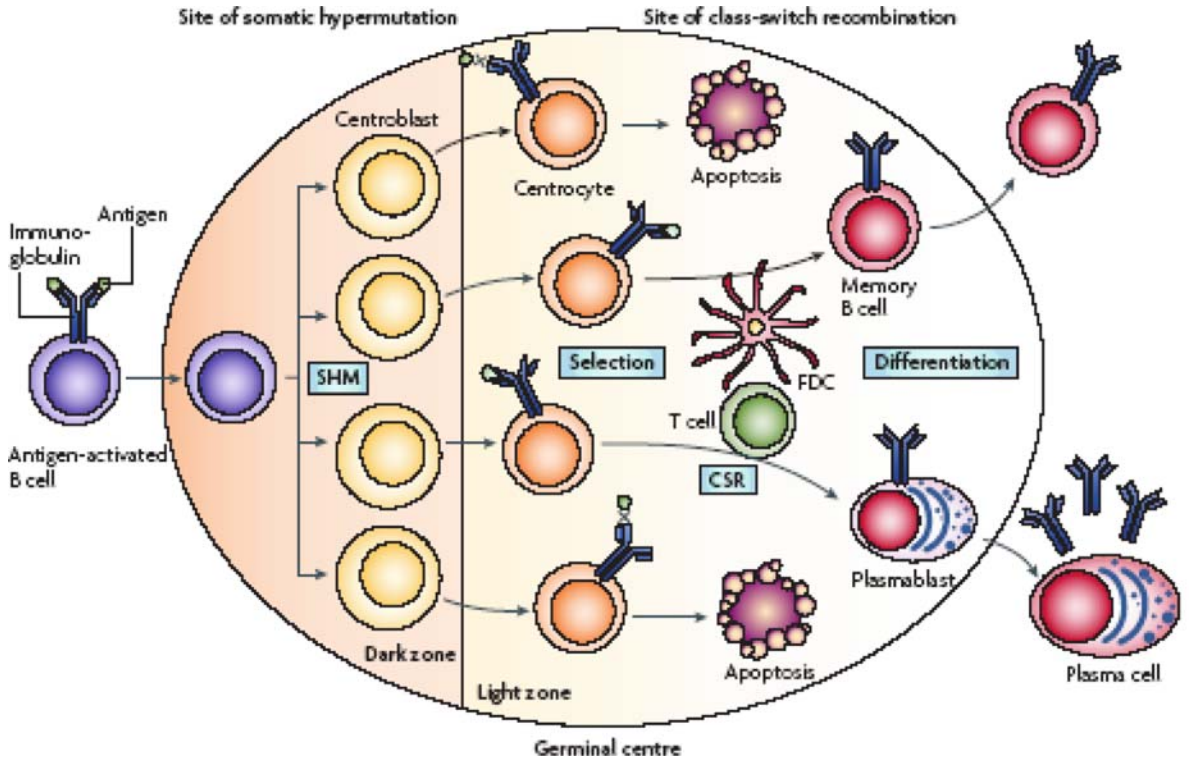

Fig. 7.4 The germinal centre microenvironment. Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion in the dark zone of the germinal centre. During proliferation, the process of somatic hypermutation (SHM) introduces base-pair changes into the V(D)J region of the rearranged genes encoding the immunoglobulin variable region (IgV) of the heavy chain and light chain; some of these base-pair mutations lead to a change in the amino-acid sequence. Centroblasts then differentiate into centrocytes and move to the light zone, where the modified antigen receptor, with help from immune helper cells including T cells and follicular dendritic cells (FDCs), is selected for improved binding to the immunizing antigen. Newly generated centrocytes that produce an unfavourable antibody undergo apoptosis and are removed. A subset of centrocytes undergoes immunoglobulin class-switch recombination (CSR). Cycling of centroblasts and centrocytes between dark and light zones seems to be mediated by a chemokine gradient, presumably established by stromal cells in the respective zones (not shown). Antigen-selected centrocytes eventually differentiate into memory B cells or plasma cells (From: Klein and Dalla-Favera [21])

are programmed to vigorously proliferate and expand, which may by itself increase the risk of acquisition of DNA damage. Also, these GC B-cells undergo molecular processes that modify the genomic DNA (gene rearrangements to produce immunoglobulin diversity, somatic hypermutation, variable region gene recombination and class-switch). Therefore there is abundant opportunity for malignant transformation in the germinal center reaction. [33]. Occasionally, aberrant activity of these processes may result in chromosomal translocations involving the Ig loci and a proto-oncogene. In fact, balanced chromosomal translocations, mostly involving the immunoglobulin (Ig) genes and a variety of partner genes, are a hallmark of many mature B-cell lymphomas [34]. Well-known examples include bcl2/Ig translocation in follicular lymphoma, bcl-1/Ig translocation in mantle cell lymphoma, c-myc/Ig translocation in Burkitt lymphoma, and bcl-6 translocations in diffuse large B-cell lymphoma.

In most instances the translocated partner gene (a proto-oncogene) becomes transcriptionally deregulated and constitutively active, as the oncogene is brought under the control of an active immunoglobulin locus [35]. Some translocations, such as the bcl2/IgH translocation associated with follicular lymphoma, bcl-1 translocations in mantle cell lymphoma, and c-myc translocations in endemic Burkitt lymphoma, have chromosomal breakpoints in the Ig locus that are located at the 5' end of the J (or sometime D) heavy chain gene segments. These translocations

therefore likely happen during V(D)J recombination, a process that takes place predominantly in early B-cell development in the bone marrow (and also occasionally in germinal center B-cells) [36, 37]. V(D)J recombination is a process catalysed by the B-cell specific V(D)J recombinase activating enzymes RAG-1/2 that are expressed in both pre-B-cells and GC B-cells [38].

In other translocations, the breakpoints are found within or adjacent to rearranged V(D)J genes, and these V-region genes are always somatically hypermutated. Indeed, translocations of the c-myc gene into a rearranged Ig gene in a subgroup of Burkitt lymphoma (sporatic Burkitt lymphoma and HIV-associated Burkitt lymphoma) is thought to happen as a by-product of somatic hypermutation [39]. Somatic hypermutation may also occasionally target genes other than IgV region genes and introduce point mutations in oncogenes and/or tumor suppressor genes that are thought to play a role in lymphomagenesis. The genes encoding BCL-6 and FAS (also known as CD95) were found to contain mutations in a considerable fraction of normal GC and memory B-cells (but not naïve B-cells), indicating that these genes are often physiological targets of the somatic hypermutation machinery in normal B-cells [40, 26]. Such mutations may promote lymphomagenesis in certain instances. In deed, point mutations of bcl-6 have been described in a significant fraction of diffuse large B-cell lymphomas and a majority of follicular lymphomas, occurring independently of chromosomal translocations [41, 26]. Also, inactivating mutations of FAS are found in about 20% of post GC lymphomas and could protect lymphoma cells from death induced by FAS-ligand-expressing cells [42]. Thus, the somatic hypermutation process may also promote lymphomagenesis by targeting regulatory and coding sequences of the bcl-6 proto-oncogene and FAS tumor suppressor gene, resulting in either dysregulated expression (BCL-6) or loss of function (FAS) [35].

Finally, translocations characterized by breakpoints in the IgH switch region include c-myc/Ig in sporadic Burkitt lymphoma [43, 44], bcl-3 {t(14;19)} in B-cell chronic lymphocytic leukemia [45], bcl-6 {t(3;14)} in diffuse large B-cell lymphoma [46], and Pax-5 {t(9;14)} in lymphoplasmacytic lymphoma [47]. These chromosomal translocations are probably caused by errors occurring during class-switch recombination in germinal center B-cells.

7.4.2 Transforming Viruses

Certain viruses are capable of promoting the development of lymphoma by transforming the infected cells. A well-known example is Epstein-Barr virus (EBV), a member of the herpes-virus family, that mainly targets B-cells, but can also infect other cells such as epithelial and T-cells. EBV is implicated in the pathogenesis of several types of tumors, including certain hematologic (Burkitt lymphoma, subsets of Hodgkin and T-cell lymphomas, post-transplant lymphomas), epithelial (undifferentiated nasopharyngeal carcinoma, a subset of gastric adenocarcinomas) and mesenchymal (EBV-associated smooth muscle tumor, inflammatory pseudotumor-like follicular dendritic cell tumor) neoplasms [48]. EBV DNA is found in nearly all cases of endemic Burkitt lymphoma, 40% of cases of classic Hodgkin lymphoma, and in the vast majority of post-transplant and primary effusion lymphomas. These lymphomas are usually derived from GC B-cells, pointing to the importance of this microenvironment in the development of EBV-associated B-cell malignancies [49]. Interestingly however, various types of EBV-associated B-cell lymphomas differ markedly, not only in their pathogenesis, but also in the presumed role of EBV in this process. For example, a defining feature of Burkitt lymphoma is the reciprocal translocation between myc and one of the three Ig loci [50, 51]. The role of EBV might be to retain myc-driven proliferation and to evade its apoptotic effects, thereby supporting unrestricted clonal expansion of B-cells that harbor a myc/Ig translocation [52]. Another member of the herpes-virus family, human herpes virus 8 (HHV-8) is associated with all cases of primary effusion lymphoma (PEL) and some other AIDS-related lymphoproliferative disorders [53]. PEL is a rare B-cell lymphoma that develops as serous effusions in pleural, pericardial and peritoneal cavities, without any solid localization [54]. PEL is almost exclusively observed in the context of HIV infection with a particular incidence in men. HHV-8 infected B-lymphocytes in PEL have constitutive nuclear factor (NF)-kappaB activity that is essential for their survival. It has been reported that viral FADD-like interleukin-1-beta-converting enzyme {FLICE/caspase 8}-inhibitory protein (FLIP) activates NF-kappaB more potently than cellular FLIP in B cells, and that it is largely responsible for NF-kappaB

activation in latently infected PEL cells [55]. The lymphoma cells are post-germinal center B-cells, all carry HHV-8 genome, and most carry both HHV-8 and EBV genomes [56].

7.5 Role of Antigen Receptor, Microenvironment and Antigenic Stimulation in B-Cell Lymphomas

Tumor cells in most B-cell (non-Hodgkin) lymphomas express surface Ig. The mutation pattern indicates that the precursors of the tumor clones have been rigorously positively selected for expression of a functional antigen receptor [15]. The notion that BCR supplies important survival signals to B-cell lymphoma cells is supported by the observation that despite frequent oncogenic translocations involving the Ig loci, the vast majority are targeted to nonfunctional alleles [57]. These observations indicate that expression of a functional BCR is essential for survival of the transformed B-cell [10].

7.5.1 Chronic Infection and Lymphoma

Some types of lymphomas are associated with specific microbial infections. Certain infectious agents initiate and/or promote lymphomagenesis by direct lymphocyte transformation. Examples include lymphotrophic oncogenic viruses such as EBV, HHV-8 and HTLV-1 that directly infect a subset of lymphocytes and express viral oncogenes in those cells. Alternatively, chronic antigenic stimulation by pathogens that do not directly infect or transform lymphoid cells and/or autoantigens may trigger a sustained lymphoid proliferation that provides fertile ground for the transformation process [58]. The best characterized example of infection-associated indirect transformation of lymphocytes is gastric mucosa-associated lymphoid tissue (MALT) lymphoma, in which nearly all cases are associated with chronic infection of gastric mucosa by the bacterium H. pylori [59]. Importantly, it is the CD4+ T-helper cells that recognize the bacterium and provide contact-dependent help to promote the survival and proliferation of lymphoma cells [60]. Antigen-driven lymphoproliferation is thought to result from integration of two signals: (1) A prolonged T-cell independent response, generating autoreactive marginal zone B-cells with acquired alterations that confer a clonal advantage to antigen-specific B-cells, ultimately leading to transformation, and (2) A prolonged T-cell dependent response, generating a sustained pool of H. pylori-specific T-cells [12]. The fact that H. pylori eradication by antibiotic treatment often leads to regression of lymphoma highlights the important role of microenvironment in lymphoma progression [59]. Although early transformed B-cells rely on antigenic stimulation for their proliferation and survival, this dependence is not permanent. In fact, constitutive activation of the NF-kappaB pathway by oncogenic activity resulting from recurrent chromosomal translocations {i.e. t(11;18), t(1;14) and t(14;18)} bypasses the requirement for antigen signaling through BCR [58].

7.5.2 Lymphoma and Immunologic Disorders

Certain lymphomas, in particular those arising in the extranodal sites, show distinctive clinicopathologic features that may include association with an underlying immunodeficiency syndrome, autoimmune disease, infection, or other immunologic disorders [61]. In addition to pathogen-derived antigens that cause chronic and sustained stimulation of the immune system, certain auto antigens are also known to stimulate reactive B-cells. In fact, patients with certain autoimmune disorders (i.e. rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, and autoimmune thyroid disease) have an increased risk of developing lymphoid malignancies [62]. The presence of autoantibodies is the hallmark of systemic autoimmune diseases. In these patients, the immune responses to certain autoantigens promote an inflammatory reaction, mediated by both the innate and the adaptive immune systems. The adaptive response is mediated by CD4+ T-cells that recognize antigenic peptides (driven from autoantigens and/or exogeneous antigens), which are bound to HLA class II molecules on antigen-presenting cells. This interaction leads to the production of cytokines by activated T-cells that damage tissue as well as cause activation and proliferation of antibody-producing B-cells. It has been suggested that in some cases, antigen-activation contributes to lymphomagenesis by driving the proliferation of specific B cells and by increasing the frequency of their transformation [63, 64].

7.6 Functional Consequences of Genetic Lesions in Lymphomagenesis

The various genetic lesions that occur in B-cell lymphomas contribute to lymphomagensis by dysregulation of normal B-cell proliferation, survival, differentiation and/or maturation. These oncogenic events (chromosomal translocations, gene amplifications, deletions, point mutations) disrupt B-cell homeostasis by driving the cells through the cell cycle, by preventing apoptosis and by blocking terminal differentiation. These concepts are elaborated on, within the paragraphs to follow. Within the germinal centers, the differentiation of an antigen-activated B-cell into a centroblast is accompanied by dramatic simultaneous upregulation of pro-proliferative and pro-apoptotic genes. Centroblasts up-regulate the expression of genes associated with proliferation, as well as several pro-apoptotic molecules, and lack the expression of anti-apoptotic factors such as BCL-2 [24]. This includes genes involved in proliferation, which are transcriptional targets of c-*myc* [65]. MYC also targets genes involved in apoptosis [66]. Centroblasts undergo clonal expansion and somatic hypermutation, and then differentiate into centrocytes. Few centrocytes that generate an antibody with increased affinity for antigen are positively selected for, and they then re-express BCL-2 protein. Centrocytes that form an unfavorable antibody undergo apoptosis and are removed [24]. BCL-6 is a transcriptional repressor that is specifically expressed by GC B-cells and is essential for germinal center formation [67]. Although BCL-6 is a main effector of a centroblast phenotype, it must be down-regulated for cells to arrest growth, to interact with other cells, and to differentiate into centrocytes and eventually plasma cells and memory B-cells [21].

7.6.1 Blocking Apoptosis

Follicular lymphomas are believed to be tumors of the GC B-cells (centrocytes and centroblasts) in which centrocytes constitutively express BCL-2 protein as a result of the chromosomal translocation t(14;18), and thus fail to undergo apoptosis [14]. The tumor is composed predominantly of centrocytes, which are resting cells, and thus tends to be indolent [14]. Translocations of the bcl-2 gene also occur in 20–30% of cases of diffuse large B-cell lymphomas [68]. Marginal zone lymphomas of the mucosa-associated lymphoid tissue (MALT) harbor the translocation t(11;18), in approximately 50% of cytogenetically abnormal cases, that gives rise to the fusion of an apoptosis inhibitor gene, API2, to a gene on 18q21 named MLT. Hence, the over-expression of the anti-apoptotic protein API2 and a survival advantage for the lymphoma cells results [69].

7.6.2 Enhancing Cell Growth and Proliferation

MYC overexpression, misexpression, and deregulation, caused by translocations and/or mutations are seen in many GC-derived B-cell lymphomas. Burkitt lymphomas [70], as well as some diffuse large B-cell lymphomas [71], harbor translocations of c-myc to one of the Ig loci. This deregulation of MYC plays a critical role in lymphomagenesis by promoting cellular growth and proliferation [66]. Both Burkitt and diffuse large B cell lymphomas are composed of proliferating cells and tend to be clinically aggressive [14]. Although these aggressive lymphomas usually arise de novo, they may occur as a result of transformation of low-grade (indolent) lymphomas. For example, the risk of follicular lymphoma (FL) progression and transformation to a high-grade lymphoma has been reported as being approximately 20% at 8 years. Transformation to DLBCL is observed most frequently (usually centroblastic subtype); rare cases transform to Burkitt or Burkitt-like lymphoma. Acquisition of additional genetic alterations, including c-myc translocations, p53 mutation, deletions of the tumor suppressor genes p15 and p16, and chromosomal 6q23–26 and 17q aberrations, are implicated in progression and transformation to an aggressive lymphoma [72].

7.6.3 Blocking Differentiation

Mutations and translocations of BCL-6 are common genetic lesions in many lymphomas. BCL-6 is a key upstream regulator of terminal B-cell differentiation. Dysregulation of BCL-6 entraps B-cells at the GC stage, thus leading to malignant transformation by simultaneous inhibition of differentiation and enhanced proliferation [73].

7.7 Conclusion

Our current understanding of B-cell lymphoma pathogenesis indicates that tumor initiation and progression is multi-factorial. The transformation process depends on a series of acquired genetic alterations (such as chromosomal translocations), expression of a functional BCR and interaction with the microenvironment (thus the ability to obtain growth and survival signals). The very processes that have a central role in normal B-cell differentiation and maturation, namely the need for intense cellular proliferation within the germinal centers, V(D)J recombination, somatic hypermutation, and IgH class switch recombination, may aberrantly lead to oncogenic alterations, affecting B-cell differentiation, proliferation and apoptosis, and loss of hematopoietic homeostasis.

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Chapter 8

Pediatric Cancer Mechanisms of Cancer Growth and Progression

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Contents

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8.1 Introduction

The multiplication of all cells in normal tissue is limited. The genetic propensity for growth is such that a certain size, once reached, is maintained without further increase. If mutations change the genetic potentialities, or if the environment is altered, such limitation may be lost and a malignant tumor produced. No tumor of the adult grows as rapidly as the normal embryo. Especially in the early stages of development, normal embryonic cells may have some of the characteristics of neoplastic cells.

It is often only from knowledge of the postnatal course of a tumor that proof can be obtained of its malignancy. Neuroblastomas are usually considered malignant, but many are observed in which neuroblasts differentiated postnatally into mature ganglion cells and did not behave as those of a malignant tumor. Neuroblastomatous tissue has been found widely distributed throughout the liver and abdominal cavity at birth and has sometimes been considered to be of metastatic origin. However, since neuroblasts are normally present in the liver and other parts of the embryo, it is more probable that these cells are subject to the same stimulus responsible for the more massive proliferation considered as the primary tumor, but are of local origin rather than disseminated from the principal tumor.

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Neoplasms in the fetus and infant are not entirely the same as those in the adult; the types, incidence, clinical features, behavior, and response to treatment are different [1–13].

8.2 Teratomas

Teratomas arise from foci of plastic pluripotential embryonic tissue that has escaped from the influence of the primary organizer during early embryonic development, this escape being in some way related to disturbances emanating from the invaginating organizing tissues of the primitive streak and so affecting median or paramedian parts in close relationship to these tissues [9]. As it grows, the affected primordium shows no effect of a primary organizer but differentiates in accordance with its own labile determinations, producing a variety of tissues foreign to the part from which they grow. Willis believed that most well-studied teratoid tumors can be distinguished easily as teratomas or abortive fetuses, but he admitted that in rare cases differentiation may be very difficult [9].

8.3 Malignant Tumors

Continued growth without maturation of certain cells that are normally present during embryonic development, but that usually mature after birth, is responsible for many malignant congenital tumors. Wilms tumors are derived from the metanephric blastema that has lost its propensity for differentiation but not for growth. Neuroblastomas of the adrenal gland come from cells that arise in the neural crest and wander into the gland early in embryonic life. Normally these cells differentiate into chromaffin tissue, but occasionally the transformation fails to take place, and a proliferation without differentiation produces a neuroblastoma.

Other tumors that occur predominantly in the first year of life and have certain features of embryonic growth include embryonic sarcomas, yolk sac tumors of the testis, hepatoblastomas, and medulloblastomas of the brain. Some teratomas of the sacrococcygeal and retroperitoneal regions can be included with those having an overgrowth of embryonic components that fail to mature. The cells of many sarcomas resemble immature fibroblasts and are also a result of failure of maturation coupled with an excessive stimulus for growth.

8.4 Incidence

The most common malignancies noted at birth are, in order, neuroblastoma, leukemia, sarcomas, and brain tumors [1, 11, 13–15]. Leukemia-lymphoma, brain tumors, neuroblastoma, and soft tissue sarcomas, in decreasing order of occurrence, are the more common neoplasms noted in older children and adolescents under 15 years of age [16].

8.5 Etiology

Several etiologies have been suggested: viral infections in-utero, maternal drugs and irradiation, congenital malformations, chromosomal defects, and cancer-causing genes [17]. This problem lies at the molecular level in the gene [18–20].

8.6 Environmental Factors

Some drugs associated with congenital malignancies; for example, hydantoin and alcohol are prenatal carcinogens. Neuroblastoma has been described in association with both the fetal hydantoin and fetal alcohol syndromes [21]. Diethylstilbestrol (DES) exposure in-utero may result in vaginal clear cell adenocarcinoma. Some studies [22] suggest a possible increased risk of malignant tumors after in-utero exposure to certain viral agents such as varicella, influenza, rubella, cytomegalovirus, and human immunodeficiency virus. Exposure to therapeutic agents like chemotherapy and ionizing radiation, elevates the risk of a second malignancy especially CNS tumors [23]. The cumulative incidence of secondary brain tumors after irradiation to craniospinal axis as a treatment for ALL is reported to be approximately 20 times greater than that seen in non-cancer control

populations [24–26]. A diagnosis of a genetic disorder also increases the risk of malignancy.

8.7 Mechanisms in Carcinogenesis

Cancer is an uncontrolled growth of cells resulting from an alteration in their DNA That produces a deregulation of their normal growth [18]. The altered cell changes to a malignant cell, becomes independent of normal regulatory control, and multiplies, producing a clone of cancer cells that subsequently develops into a neoplasm. There are several mechanisms, occurring as single or multiple events, by which the DNA of a normal cell becomes transformed into a cancer cell; this process is termed carcinogenesis. The known mechanisms include point mutations in DNA (the replacement of a single correct DNA sequence within a gene by an incorrect one), gene deletions (loss of a large amount of DNA resulting in loss of all or part of the gene), and chromosomal translocations with gene rearrangements (the broken ends of the DNA from two different chromosomes may be joined incorrectly, resulting in parts of each chromosome being exchanged) [18].

8.8 Chromosme Translocations

Lejeune's recognition of trisomy 21 in Down syndrome, predicted the many steps at which altered gene expression can produce abnormal tissue growth (neoplasia) or development (congenital anomalies). The similarities between molecular embryology and molecular oncology are strengthened by anomaly patterns (syndromes) that include cancer predisposition [e.g., Beckwith-Wiedemann syndrome (BWS), neurofibromatosis-1], the dual roles of many proto-oncogene/tumor suppressor genes as developmental genes (e.g., PAX/C-KIT genes in Waardenburg syndrome/Piebald trait), and the stepwise progression from primary mutation, environmental factor, epigenetic change to multifactorial (e.g., HOX genes in human leukemias and limb defects).

The number of chromosomal rearrangements and gene mutations associated with tumors is now so great that almost every cancer patient undergoes some sort of molecular or cytogenetic testing. The same progression from chromosome anomaly to causative breakpoint to cancer/tumor suppressor gene can now be followed for most developmental anomalies, and epigenesis is a central factor in both neoplasia and development [27, 28].

About one-half of the 6,422 Mendelian and multifactorial disorders in the literature involve altered morphogenesis, including over 800 with neoplasia.

Molecular diagnosis of birth defects is as powerful as that for neoplasia. Fluorescent karyotyping and gene expression profiling of single cells [29] is a reality for both fields.

Chromosome analysis is now merging with DNA chips to provide telomere or array analyses, capable of defining subtle deletion/duplication of any chromosome segment by its altered fluorescent pattern of chip representation.

Alveolar rhabdomyosarcoma (RMS) is associated with a recurring chromosomal translocation, t(2;13) in about 50% of cases. This chromosomal rearrangement results in the fusion of a developmentally regulated gene, PAX3, with a member of the fork head family of genes now termed FKHR. The resultant PAX3/FKHR gene is associated in all cases with alveolar RMS, has not been described in any other malignancy, and thus appears to be diagnostic of this tumor. Variations such as t(1;13) may also be found in alveolar RMS when PAX3 located on chromosome 2 is replaced by a related gene, PAX7 on chromosome 1. Both types of gene fusion are etiologic and specific for alveolar RMS [30].

Translocation t(11;22)(q24;q12) is found in at least 85% of cases of Ewing/PNET group of neuroectodermal tumors [31]. This group includes Ewing sarcoma of bone and soft tissue, Askin tumor of the chest wall, peripheral neuroepithelioma, primitive neuroectodermal tumor (PNET), and esthesioneuroblastoma (olfactory neuroblastoma). The translocation results in the fusion of an RNA-binding gene, termed EWS,5' to a known oncogene, FLI-1 (for Friend erythroleukemia virus integration site). A variant t(21;22) occurs when FLI-1 replaces 3' of EWS by ERG, from chromosome 21. Over 95% of Ewing/PNET tumors have demonstrable fusion of EWS with an ETS family oncogene, and this Ewing/PNET and its family are not defined by a single molecular genetic defect.

The intraabdominal desmoplastic small round cell tumor has a t(11;22) but distinct from the Ewing/PNET t(11;22) as 22q12 is fused with 11p13 [32].

8.9 Genomic Imprinting

Genomic imprinting occurs in embryonal rhabdomyosarcoma [33]. There appears to be dysregulated gene expression in the chromosomal region 11p15.5. Both maternal and paternal alleles of the same gene are inappropriately expressed. In Wilms tumor and Wiedemann-Beckwith syndrome, both display abnormal expression of growth-associated growth factor IGF2, secondary to "de-imprinting" or possibly loss of normal allelic suppression secondary to loss of genomic imprinting. P53 mutations appear only in anaplastic Wilms with a known poorer prognosis [34]. The details of genomic imprinting and loss of imprinting are discussed later in this chapter.

8.10 Proto-oncogenes and Oncogenes

Proto-oncogenes encode a variety of cellular proteins involved in a normal cell growth, proliferation and differentiation. These include growth factors, growth factor receptors, intracellular transducers and nuclear transcription factors. They have distinctive spatial and temporal expression patterns in developing organs, and this supports the view that they have important functions in organogenesis. The role of mutations in these genes in the development of many neoplasms is now well recognized. Oncogenes impart to the tumor cell certain properties such as growth advantage, rapid proliferation, and the ability to metastasize [19, 35, 36]. One example is the N-myc oncogene, which is normally expressed in developing organs and tissues and in certain tumor systems (e.g., neuroblastoma, retinoblastoma). The product of the N-myc gene is a nuclear protein, which is produced in increased amounts (or amplified) in dividing embryonic cells and in some tumor cells. The presence of amplification of an oncogene product, e.g., in a neuroblastoma specimen, is used as an indicator of a poor prognosis, since N-myc amplification is associated with an advanced stage of disease and an unfavorable outcome in neuroblastoma patients [35].

8.11 Anti-oncogenes

Anti-oncogenes, which suppress the formation of malignant tumors, have been identified [18, 37] (Table 8.1). One example of an antioncogene is the retinoblastoma gene (Rb). Individuals with the loss of one or both of these two protective genes have an increased susceptibility to retinoblastoma. An important relationship exists between the integrity (homozygosity) of the 13q14 Rb gene and the development of certain childhood cancers [35, 36]. The findings indicate that both copies of the 13q14 Rb gene must be

Table 8.1 Tumor suppressor genes involved in human neoplasms (From: Isaacs [41])

Gene	Chromosomal location	Neoplasms associated with somatic mutations	Neoplasms associated with inherited (germ-line) mutations
WT-1	11p13	Wilms tumor	Wilms tumor
p53	17p13.1	Most human cancers	Li-Fraumeni syndrome: carcinomas of breast and adrenal cortex; sarcomas; leukemias; brain tumors
APC	5q21	Carcinomas of colon, stomach and pancreas	Familial adenomatous polyposis coli; carcinomas of colon
DCC	18q21	Carcinomas colon and stomach	Unknown
VHL	3p25	Renal cell carcinoma	von Hippel-Lindau disease: retinal and cerebellar hemangioblastomas; renal cell carcinomas; angiomas and cysts of many visceral organs
NF-1	17q11	Schwannomas	Neurofibromatosis type 1; neural tumors
NF-2	22q12	Schwannomas and meningiomas	Neurofibromatosis type 2; central (acoustic) schwannomas; meningiomas
Rb	13q14	Retinoblastoma; osteosarcoma; carcinomas of breast, bladder, prostate, and lung	Retinoblastoma; osteosarcoma

altered in some way (by loss, inactivation, mutation, or deletion) before a tumor can develop. If the individual acquires a defective 13q14 gene from either parent, he or she is heterozygous for the altered gene and is a carrier for the gene. However before tumorigenesis results, a second event (or "hit") must occur, i.e., both retinoblastoma genes must be altered [38]. Furthermore, inheritance of a faulty copy of one allele at the 13q14 locus makes the individual susceptible to cancer. When the second allele becomes altered, deleted, or inactivated at the 13q14 locus, tumorigenesis occurs with the development of retinoblastoma at an early age. Second malignancies such as osteosarcoma appear later [36, 39].

An important relationship exits between certain congenital malformations and syndromes and the development of neoplasms [3, 17, 40–42] (Tables 8.2, 8.3, and 8.4). An example is the increased occurrence of neoplasms associated with gonadal dysgenesis in patients having a Y chromosome where gonadoblastoma and germinoma arise in the dysgenetic gonads [2, 17, 43]. Wilms tumor, hepatoblastoma and adrenocortical tumors in patients with hemihypertrophy and Wiedemann-Beckwith syndrome are other examples. Aniridia and malformations of the genitourinary tract have been found in infants and children with Wilms tumor [3, 44]. The list of inherited syndromes and conditions associated with an increased risk of tumors expands considerably each year [45].

Several hereditary syndromes characterized by DNA repair defects have been described [17, 18]. These include ataxia telangiectasia (lymphomas), Bloom syndrome (leukemia), Fanconi anemia (leukemia), and xeroderma pigmentosum (skin carcinomas) [17, 18, 20]. Except for ataxia telangiectasia (X-linked), these are autosomal recessive conditions.

Hereditary conditions such as tuberous sclerosis and forms of neurofibromatosis, which are autosomal dominant, predispose the individual to the development of gliomas and malignant schwannoma [1, 17].

8.12 Controlling Gene Expression

Increasing gene expression occurs by increasing the number of DNA copies of that gene (gene amplification). Human folate resistant cell cultures are seen in aggressive neuroblastomas that amplify NMYC. The new controlling mechanisms offer novel means for understanding and manipulating developmental gene regulation. The relevance of new, non-classical pathways which control gene expression is reinforced by progressive DNA expansions/contractions and myriad gene rearrangements characteristic of most cancers.

Table 8.2 Some syndromes and congenital malformations associated with childhood tumors (From: Isaacs [41])

Malformation or syndrome	Neoplasm
Wiedemann-Beckwith syndrome hemihypertrophy	Wilms tumor
	Adrenocortical adenoma
	Adrenocortical carcinoma
	Hepatoblastoma
	Pancreatoblastoma
Aniridia	Wilms tumor
Genitourinary system anomalies and	Nephroblastomatosis
Perlman syndrome	Wilms tumor
Hirschsprung disease	Neuroblastoma
Poland syndrome	Leukemia
Drash syndrome	Wilms tumor
Tuberous sclerosis	Cardiac rhabdyomyoma
	Angiomyolipoma
	Astrocytoma
Multiple endocrine neoplasia	Thyroid medullary carcinoma
(MEN 2)	Pheochromocytoma
	Submucosal neuromas
Nevoid basal cell carcinoma (Gorlin) syndrome	Basal cell carcinoma
	Medulloblastoma
	Ovarian fibroma
Wiskott-Aldrich syndrome	Lymphoma
Bloom syndrome	Leukemia

Table 8.3 Examples of chromosomal abnormalities associated with childhood tumors (From: Isaacs [42])

Chromosomal defect	Childhood tumor
1p del, 1p32-p36 del, double minutes	Neuroblastoma
11p13 del , trisomy 18	Wilms tumor
13q14 del	Retinoblastoma
	Osteosarcoma
Trisomy 21 (Down syndrome)	Leukemia
Monosomy 7	Leukemia and myelodysplasia syndrome
t(11q23), t(1;22), t(9;11)	Leukemia
t(1:22) (p13;q13)	Leukemia
Gonadal dysgenesis (46,XY; 45X/46,XY)	Gonadoblastoma Germinoma
Klinefelter syndrome (XXY)	Teratoma
t(11;22)(q24;q12) t(21;22)	Ewing sarcoma/ PNET
t(11;22) (p13;q12)	Intra-abdominal desmoplastic small round cell tumor
t(2;13)	Alveolar rhabdomyosarcoma
del 1p	Astrocytoma

Table 8.4 Some syndromes that predispose to solid tumors (From: Isaacs [41])

Syndromes	Chromosomal locus	Gene	Tumor
Neurofibromatosis type 1	17q11	NF1	Rhabdomyosarcoma
Wiedemann-Beckwith	11p15.5	Unknown	Wilms tumor Hepatoblastoma
WAGR Denys-Drash	11p13	WT1	Wilms tumor
Adenomatous polyposis coli	5q21	APC	Hepatoblastoma
Hereditary retinoblastoma	13q14	RB1	Retinoblastoma Osteosarcoma
Li-Fraumeni	unknown	p53	Osteosarcoma Rhabdomyosarcoma

WAGR – Wilms tumor, aniridia, genitourinary defects, retardation

8.13 DNA Methylation

Methylation of DNA contributes to cell differentiation, since the changes in DNA structure and expression will be transmitted to all the daughter cells of a progenitor cell. Methylation of promoters probably plays a major role in cellular differentiation during embryogenesis: cells eliminate the transcription of unwanted genes by methylating their promoters.

Methylation inhibits (and occasionally activates) a very large number of genes, including some proto-oncogenes [46]. In cancers, there is often a decreased expression of DNA methyltransferase which leads to a nonspecific demethylation of 5-methylcytosine. It appears that this demethylation can reactivate the expression of proto-oncogenes which thus act as unregulated growth-promoting agents (e.g., oncogenes, telomerases, etc.) and of genes involved in cell migration, leading to the emergence of progressively more aggressive clones (clonal evolution). Abnormal methylation (hence inactivation) of anti-oncogenes similarly plays a major role in cancer.

8.14 Zinc-Fingers

Transcription factors are those with a zinc-finger motif: zinc molecules cause these proteins to have a tertiary structure resembling fingers. The "fingers" bind the promoters of the genes under their control, so as to activate or inhibit them. Zinc finger proteins illustrate the multiple domains that can exist within transcription factors, allowing them to coordinate multiple different expression pathways. WT-1 (the Wilms tumor-1 gene product), the fly developmental gene hunchback, certain hormone and retinoic acid receptors belong to the zinc-finger class of transcription factors.

8.15 Proteomics

As with DNA chip profiling of gene sequences or transcripts, a particular protein population (proteome) can be profiled by shotgun sequencing techniques, microcapillary or two-dimensional electrophoresis, laser technologies, microarrays and mass spectroscopy [47–49]. This field of study is called proteomics.

The use of proteomic technology links the enormous variability in protein populations and structures to the nucleic acid language of genome and RNA. The laborious work of isolating enzymes and the more modern dilemma of predicting protein function from identified genes can now be simplified by characterizing protein groups: proteins defined by housekeeping, signal-response, cell cycle phase, compartment, or differentiation state can be sequenced en masse, then correlated with their gene structure and expression. Protein–protein interactions can be predicted by looking for encoding sequences within cDNAs that predict shared protein motifs; two-hybrid and multiassay methods for determining all protein interactions in yeast or *Drosophila* have been developed [50].

8.16 Differentiation (Epigenetic Control of Gene Expression)

With exceptions of selective rearrangements in certain genes (e.g., T-cell receptor genes), all cells of an organism have the same genetic formation. Differentiation is characterized by the activation of certain genes, and the inactivation of other genes to address specific developmental needs of cells and tissues (e.g., to specify whether ectoblastic cells will become brain, neural crest or epidermis) [51–53]. HOX genes play a major role in cellular differentiation: their proteins bind promoters of developmental genes and activate or inhibit their transcription; this effect is generally irreversible, but in cancer, a loss of inhibition of transcription of these genes can cause cells to de-differentiate [46]. Differentiation and mitotic activity tend to be inversely proportional (the greater the differentiation of a cell population, the lower its proliferation rate); this is also true in cancer. Oncogenesis can be thought of as a deregulation of basic embrylogic mechanisms, which explains the similarities between molecular embryology and molecular oncology.

The master switch or selector genes control fundamental aspects of development; they do so by activating or repressing batteries of subordinate genes. In humans, the actions of master switch genes are illustrated by the MYOD1/MYF5 genes. Subdivision of embryonic domains by homeotic proteins (PAX, HOX) defines which primitive cells will become a certain tissue (e.g., skeletal muscle). When the gene "Myoblast Differentiation-1" (MYOD1) is expressed in primitive mesenchymal cells, it causes them to differentiate into skeletal myocytes. MYOD1 (the gene product) is a transcription factor that binds multiple promoters specific to skeletal muscular differentiation. Thus, a single gene is sufficient to activate and inactive a whole cascade of subordinate genes. Furthermore, MYOD1 recognizes its own promoter, and therefore activates its own transcription in a positive feedback loop fashion. Once a cell activates the transcription of MYOD1, auto-activation causes this cell and its descendants to express MYOD1 forever. From then on, these cells can take no other differentiation path than that of a myocyte. A second gene is available (called MYF5) that performs similar functions to those of MYOD1.

In oncologic states cellular differentiation inhibits mitotic activity. This is also true for the embryo: MYOD1 expression is inversely proportional to myocyte proliferation. Fibroblast growth factor (FGF) acts as a growth factor (proto-oncogene) in myoblasts; FGF down-regulates differentiation (without inhibiting it totally), by down-regulating the transcription of MYOD1 and MYF5, thus partially inhibiting the stimulation of their downstream cascades.

8.17 Loss of Imprinting and Cancer

Genomic imprinting was discovered in the 1980s, and is now recognized to be very important clinically [54–56]. Imprinting is characterized by the differential activation of alleles according to their parental origin, associated with different patterns of DNA methylation. Mammals are diploid organisms, meaning that all somatic cells possess two copies of the genome. Each autosomal gene is therefore represented by two copies, or alleles, with one copy inherited from each parent at fertilization. For the vast majority of autosomal genes, expression may occur from either allele. However, a small proportion (<1%) of genes are imprinted, meaning that expression occurs from only one allele. Imprinting, therefore, is defined as the parental allele-specific expression of a very limited set of genes. This is an epigenetic phenomenon whereby the DNA of the two alleles of a gene is differentially modified so that only one parental allele, parent-specific for each gene, is normally expressed [57]. The expressed allele is dependent upon its parental origin. For example, the gene encoding insulin-like growth factor II (IGF2) is only expressed from the allele inherited from the father. Similarly, human triploidy with two paternal genomes also produces placental tissue (hydatidiform mole), while triploidy with two maternal genomes biases toward fetal tissues (e.g., ovarian teratomas). This regulation depends upon an epigenetic marking of parental alleles during gametogenesis. Monoallelic expression ensures that the levels of the proteins encoded by imprinted genes, such as important factors of embryonic growth, placental growth or adult metabolism, are assured [58]. Mis-regulation of imprinted gene expression or loss of imprinting (LOI) refers to loss of monoallelic gene regulation and concomitant biallelic expression. LOI can cause activation of the normally silent copy of a growth promoting gene such as IGF2, or silencing of the normally active copy of a growth inhibitory gene such as $p57^{KIP2}$ [59].

If one cell of the morula loses one of its extra chromosomes (i.e., reverts from a trisomic to a disomic state), it will generate mosaic embryos with two cell lines, one with a 1/3 chance that both chromosomes of a pair are derived from one parent (uniparental disomy). If one considers an abnormal allele or deletion on one of the parental chromosomes, then two types of uniparental disomy may be considered; disomy for the same parental chromosome (uniparental isodisomy) or disomy where both parental chromosomes are represented (uniparental heterodisomy).

In BWS, partial moles, complete moles, and many cancers, this equilibrium is altered, leading to LOI and IGF2 over-expression. This has been documented in Wilms tumor, Ewing sarcoma, rhabdomyosarcoma, adrenocortical tumors, hepatoblastoma and hepatocellular carcinoma, and pheochromocytoma; note that the incidence of these tumors is greatly increased in BWS, and that placentas of BWS fetuses can show mole-like changes. IGF2 is also overexpressed in partial and complete moles, choriocarcinoma, leukemias, germ cell tumors, as well as bladder, breast, cervical, esophageal, gastric, colorectal, pulmonary, ovarian, prostatic, renal cell and other carcinomas and tumors.

8.18 Cell Proliferation: Proto-oncogenes and Anti-oncogenes

Proto-oncogenes (growth factor) and anti-oncogenes (tumor suppressors), along with their receptors and signaling molecules, interact to exert a physiologic control of cell proliferation in embryonal and adult tissues [2, 9, 60, 61]. Activation and inactivation of these genes is normally very tightly controlled so as to initiate and arrest cell proliferation at appropriate times and locations. During the 4 weeks following fertilization, the number of embryonic cells double every 2–4 days under the control of these proto-/anti-oncogenes. Regional differences in embryonic growth are secondary to differences in oncogenes expression/repression. Mutations in these genes can result in embryonic defects or neoplasia (i.e., as in Fig. 8.1), but few tumors achieve growth rates of the embryonic cells. Well-illustrated explanations of oncogene/tumor suppressor action and regulation are available in pathology texts [62].

8.19 Intercellular Signaling in Development: Sonic Hedgehog (SHH)

Embryonic cellular signaling pathways can be extremely complex, with core molecules that influence

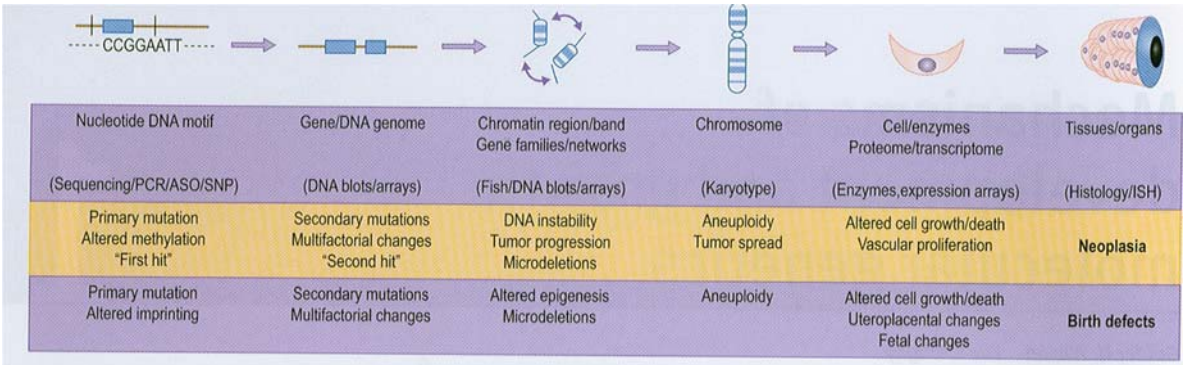

Fig. 8.1 Molecular characterization in development and neoplasia. A developmental pathway from DNA sequence to complex structure is envisioned, with parallel regulatory steps contributing to neoplasia (*middle panel*) or birth defects (From: Wilson and Oligny [133])

the development of multiple organs. A prototype of the SHH pathway (SHH is a member of the hedgehog gene family and is named as a pun from a video game). In the absence of SHH protein, the Patch (PTCH) plasma membrane protein inhibits smoothened (SMO) protein. Binding of SHH with PTCH lifts this inhibition, allowing SMO to activate the SHH cascade, including GL12, GL13, CBP, and SUFU molecules [63, 64]. Mutations involving this cascade can result in a wide range of diseases, including tremors, holoprosencephaly, Greig cephalopolysyndactyly, Pallister-Hall syndrome, Rubinstein-Taybi syndrome, the nevoid basal cell carcinoma syndrome, basal carcinoma, (syndromic and sporadic forms), medulloblastoma, meningiomas, primitive neuroectodermal tumors, breast adenocarcinomas, trichoepitheliomas, squamous cell carcinomas, esophageal carcinomas, fetal rhabdomyomas, and rhabdomosarcomas [63].

8.20 Examples: Sequential Gene Expression in Growth and Neoplasia

Molecular analysis has defined many growth-related molecules through their alteration in tumors [63, 65–71]. The definition of molecular changes that fulfill Knudson's two-hit or two-stage hypothesis has also been reviewed [72]. While Knudson's explanation involved one abnormal RB1 allele from the germline (predisposition or first hit), followed by somatic RB1 gene mutations in susceptible tissue (second hit in retina), epigenetic changes can also be placed on this pathway to neoplasia. This is reflected in the fact that most germline RB1 mutations originate on the paternal chromosome, implying a role for genomic imprinting/DNA methylation. Characterization of the RB1 gene as a cell cycle regulatory element places it within cell proliferation/cell death pathways.

8.20.1 Wilms Tumor

Wilms tumor is a malignant embryonal neoplasm derived from nephrogenic blastemal cells. Several lines of differentiation are commonly seen and often replicate the histology of developing kidneys. Approximately 10% of patients with Wilms tumor have also congenital abnormalities and malformation syndromes. The most common malformations are hemihypertrophy and genitourinary anomalies. The common syndromes associated with Wilms tumor include Beckwith-Wiedemann syndrome, WAGR syndrome (Wilms tumor, aniridia, genitourinary abnormalities and mental retardation) and Denys-Drash syndrome (mesangial sclerosis, pseudohermaphroditism and nephroblastoma) [73–77]. Abnormalities involving Wilms tumor locus, 11p13, are consistently found in the tumors of patients with WAGR and Denys-Drash syndrome. The 11p13 Wilms tumor locus encodes two coordinately regulated zinc-fingered transcripts, WT-1 and WIT-1. These genes are highly expressed in the developing urogenital system [78–80]. The WT-1 Protein binds to several sites on promoters of an

insulin-like growth factor (IGF2) gene as well as to a promoter of the platelet-derived growth factor A (PDGF-A) chain gene [81, 82]. The gene controls mesenchymal-epithelial transition during renal development. Furthermore, WT-1 Expression induces transcription of one of the seven proapoptotic genes, *Bak*, and blocks cellular proliferation and DNA synthesis [83]. Interference with these normal regulatory influences of WT-1 may be an important factor in the genesis of nephroblastoma, which expresses high levels of IGF2 [84] and may also overproduce PDGF [85]. The expression patters of WT-1 and Pax-2 in the metanephros overlap to a considerable extent, however, expression of WT1 peaks as that of Pax-2 is decreasing. It is, therefore, possible that WT-1 represses Pax-2, and Pax-2 expression fails to down regulate in the epithelial component of Wilms tumor and in nephroblastomatosis, the putative precursor of nephroblastoma [86]. Despite the strong association of WT1 mutations with Wilms tumor predisposition, WT-1 is mutated in only a minority of sporadic Wilms tumors [87]. This low prevalence of WT-1 abnormalities in sporadic Wilms tumor led to the recognition of genes other than WT-1 in its pathogenesis. Evidence supporting this is provided by the linkage of familial Beckwith-Wiedemann syndrome to a locus at chromosome 11p15, designated WT-2 [88, 89]. The preferential loss of maternal allele at this locus in cases of sporadic nephroblastoma suggest that genomic imprinting is involved in the pathogenesis of some of these tumors [90]. Approximately 1% of the patients with Wilms tumor have positive family history for the same neoplasm. Most of the pedigrees suggest autosomal dominant transmission with variable penetrance and expressivity. This suggests that genetic loci other than WT-1 and WT-2 are responsible for the pathogenesis of many familial as well as sporadic Wilms tumors [91, 92].

8.20.2 Beckwith-Wiedemann Syndrome

Beckwith-Wiedemann syndrome (BWS) and its related tumors are an excellent example of loss of imprinting. BWS is a clinically heterogeneous disorder, first described four decades ago as a disorder of growth regulation manifesting as somatic overgrowth, congenital malformations and tumor predisposition. The majority of cases are sporadic, however, a small number of pedigrees with autosomal dominant inheritance demonstrated linkage to 11p15.5 [93, 94]. Genomic imprinting in the phenotype was suggested by the preferential loss of maternal alleles in BWS and related tumors [95]. BWS is associated with abnormal transcription and regulation of genes associated with cell cycle and growth control in the imprinted domain on chromosome 11p15.5. The imprinted cluster of genes on chromosome 11p15.5 contains at least 12 imprinted genes. The chromosome 11p15.5 region has been divided into two distinct domains that are thought to be regulated by two imprinting centers separated by a non-imprinted region [96–101]. Domain 1 is telomeric and contains the imprinted genes H19 and IGF2, which are controlled by imprinting center called DMR1 (differentially methylated region 1). DMR1 is normally methylated on the paternal allele and unmethylated on the maternal allele. Regulation of transcription is accomplished by binding of the zinc-finger insulator protein CTCF to its consensus sequence within DMR1. CTCF only binds to unmethylated sequence (maternal allele) and interferes with downstream enhancers interacting with the IGF2 promoters [56]. Domain 2 is centromeric and contains the imprinted genes CDKN1C, KCNQ1, and KCNQ1OT1. Regulation of this domain is controlled by an imprinting center, DMR2 [102] (Fig. 8.2). A brief description of the major imprinted genes associated with BWS is given in the following section.

Imprinted genes associated with BWS.

8.20.2.1 IGF2

This is a potent embryonic growth factor. It is a paternally expressed imprinted gene. In mammals, it controls cell number [103]. Ablation of IGF2 results in newborn animals of approximately 60% of normal weight, while upregulation of IGF2 by twofold yields animals with 131% of wild-type values [104, 105]. Disruption of IGF2 imprinting resulting in biallelic expression and its upregulation can result in overproliferation defects, including BWS [106], expansion of colonic crypts and nephrogenic rests in kidney, as well as in multiple tumors, including Wilms tumor [107–109].

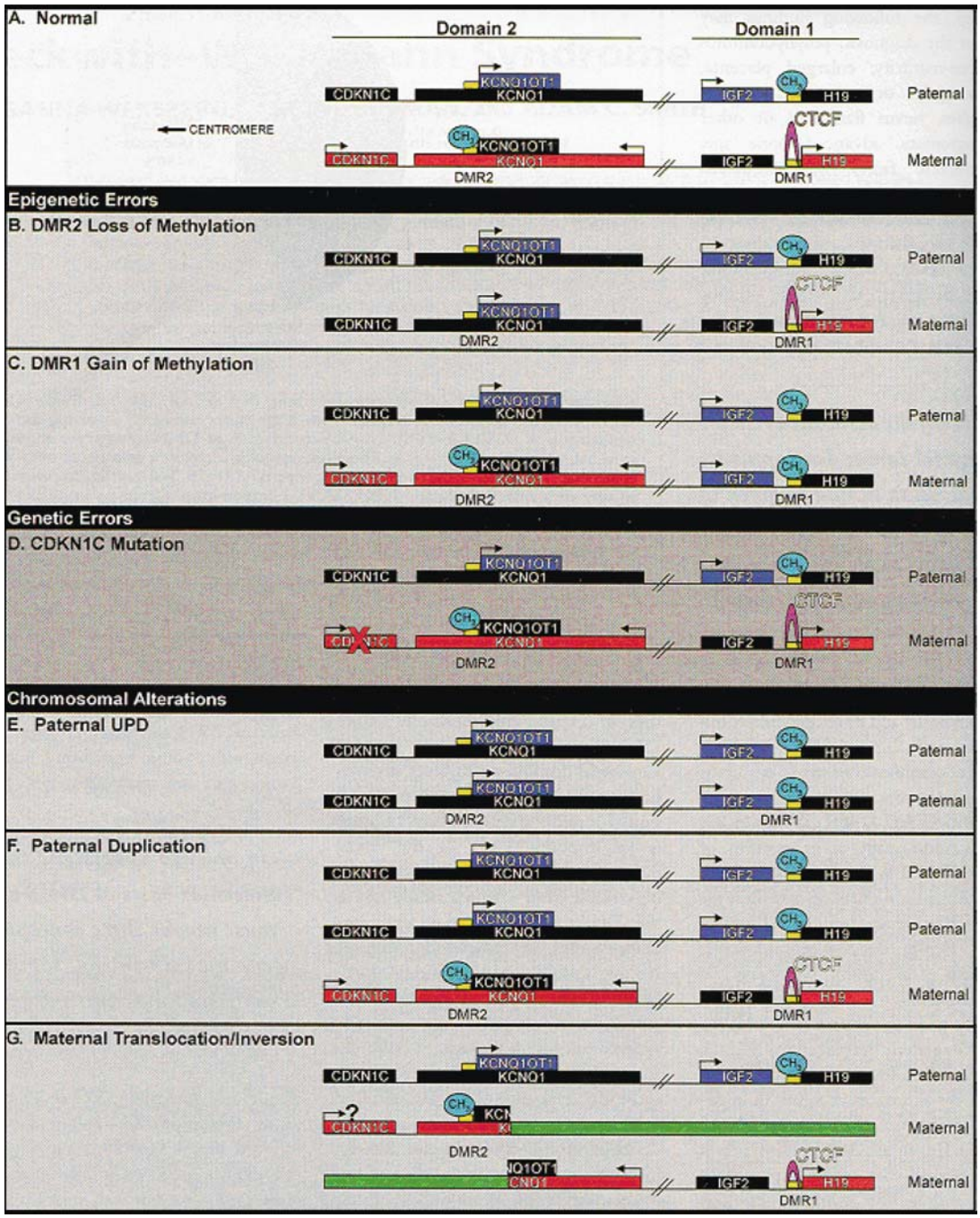

Fig. 8.2 (**a**) Imprinted gene cluster on chromosome 11p15 illustrating selected genes. *Red boxes* represent maternally expressed alleles and *blue boxes* represent paternally expressed alleles. *Arrows* represent the direction of transcription. *Black boxes* denote imprinted alleles that are not expressed. *Yellow boxes* denote the location of differentially methylated imprinting centers 1 and 2 (DMR1 and DMR2). *Light blue circles* with CH_3 represent DNA methylation. Two *diagonal lines* represent an interval of genetic distance not shown. Insulator protein CTCF is shown in *purple*. (**b**) Loss of methylation at DMR2 of BWS patients result in two copies of paternal epigenotype for domain 2. (**c**) Gain of methylation at DMR1 results in H-19 dependent IGF2 biallelic expression with loss of H-19 expression, i.e., two copies of paternal genotype for domain 1. (**d**) Shows mutations in CDKN1C. (**e**) Shows paternal UPD. Patients have two copies of the paternal epigenotypeDomains for 1 and 2. (**f**) Rare paternal duplications (<1%) carry two copies of the paternal genotype and one copy of the maternal genotype. (**g**) Translocations/inversions (<1%) of maternal origin seen in BWS. The epigenotypes are not yet well characterized (From: Weksberg et al. [102])

8.20.2.2 H19

This maternally expressed gene encodes a biologically active non-translated mRNA that may function as a tumor suppressor [110]. Changes in H19 expression or methylation have been reported in cases of BWS [111].

8.20.2.3 CDKN1C

This is a member of the cyclin-dependent kinase inhibitor family, which acts to negatively regulate cell proliferation. This gene is both a tumor suppressor gene and a potential negative regulator of fetal growth. Mutation in this gene have been reported in approximately 5–10% of BWS cases. CDKN1C mutations are found more frequently in cases with omphalocele, cleft palate, and positive family history. However, not all cases of vertical transmission of BWS can currently be ascribed to mutations in CDKN1C [112–114] .

8.20.2.4 KCNQ1

The KCNQ1 gene product forms part of a potassium channel and has also been implicated in at least two cardiac arrhythmia syndromes, Romano-Ward syndrome and Jervell and Lange-Nielsen syndrome. This gene is maternally expressed in most tissues (excluding the heart) and has four alternatively spliced transcripts, two of which are untranslated.

8.20.2.5 KCNQ1OT1

This is an anti-sense transcript which originates intron 10 of KvLQT1. Loss of imprinting occurs in the 5' differentially methylated region (KvDMR) of KCNQ1OT1 in 50–60% of individuals with BWS [115, 116].

8.20.2.6 Other Imprinted Genes

PHLDA2 (also known as IPL, HLDA2, or BWR1C) and SLC22A18 (also known as TSSC5, BWR1A, or ITM) are two identified imprinted genes in the 11p15 region [117, 118]. Both genes show preferential maternal expression in the fetus and are located centromeric to CDKN1C. While neither gene has been directly implicated in BWS, both are hypothesized to have negative growth regulatory functions. PHLDA2 has sequence similarity to PHLDA1 (TDAG51), a gene involved in mediating apoptosis [117], and SLC22A18 mutations have been identified in breast cancer and rhabdomyosarcoma cell lines [119].

8.21 Defects of Differentiation and CNS Tumor Syndromes

Defects of cellular and tissue differentiation may arise at any time in the life of the fetus and infant after initial morphogenesis. Since morphogenesis involves fields, i.e. morphogenetic units consisting of several types of tissues or their precursors, and occur 10–14 days before tissue differentiation, most malformations are histologically normal and few defects disrupt morphogenesis. Teratomas are an important exception. Although genetic factors account for only a minority of childhood CNS tumors, the incidences of particular type of CNS tumors are greatly increased in some tumor syndromes. All of these CNS tumor syndromes share autosomal dominant inheritance.

8.21.1 Neurofibromatosis Type 1 (NF1)

NF1 has an incidence of 1:4,000. The responsible gene is located on chromosome 17q12 [120]. NF1 is frequently associated with optic pathway gliomas in children [121]. Histologically vast majority of these tumors are pilocytic astrocytomas, although diffuse astrocytoma and glioblastomas are also described.

8.21.2 Neurofibromatosis Type 2 (NF2)

This is more commonly associated with adult CNS tumors than with pediatric CNS malignancies. The responsible gene is located on chromosome 22q12.2, encoding a product known as merlin or schwannomin that functions as a tumor suppressor gene. Several types of mutations occur in the gene that lead to the formation of a truncated product. A study by Evans

et al. [122], showed that 18% of patients with NF2 (61/334) presented in the pediatric (0–15 years) age group. Of these, 26 presented with features of vestibular schwannoma, 19 with meningioma and 7 with spinal tumors.

8.21.3 Tuberous Sclerosis (TS)

This disorder complex is characterized by hamartomas and benign neoplastic lesions in the CNS and other organs. There is locus heterogeneity in TS with disease determining genes on chromosomes 9 and 16. The mutant genes occur in small regions of telomeric chromosome bands at 9q34.3, designated as TSC1 (encodes for protein hamartin) and that on chromosome 16 at 16p13.3, designated as TSC2 (encodes for protein tuberin) [123, 124]. Hamartin complexes with tuberin to negatively regulate the cell cycle. Tuberin participates in normal brain development and cardiomyocyte terminal differentiation [125]. The commonest CNS neoplasm in TS is the subependymal giant cell astrocytoma arising from the wall of the lateral ventricles. Other tumors associated with TS include facial angiofibromas, cardiac rhabdomyoma, retinal nodular hamartomas, lymphangiomyomatosis, renal angiomyolipoma, hamartomatous rectal polyps etc.

8.21.4 Von Hippel-Lindau Disease (VHL)

This disease is caused by the mutation of the gene VHL. It is characterized by angiomatosis retinae and hemangioblastomas of the CNS and retina. Hemangioblastomas may also involve the face, adrenals, lungs and liver. Other pathological lesion are renal cell carcinoma, phaeochromocytoma and visceral cysts [6].

8.21.5 Turcot Syndrome

This a heterogenous group of disorder. Both autosomal dominant and recessive inheritance has been described. This syndrome is characterized by the coexistence of colorectal neoplasms with either medulloblastoma (predominantly) or glioblastoma. Some cases are variants of familial adenomatous polyposis (FAP) syndrome, while others are variants of the hereditary nonpolyposis colorectal carcinoma (HNCC) syndrome [126]. At least two defined clinical groupings can be seen within Turcot syndrome. In the first, medulloblastoma is associated with FAP. In these cases, there is mutation in the APC gene, found on chromosome 5q21. The gene encodes a 300 kDa protein that is ubiquitously expressed and modifies the interaction between the beta catenin protein and E-cadherin cell adhesion molecule. In the second clinical group, glioblastoma is seen in patients without FAP, some of whom have HNCC and mutations in DNA mismatch repair genes.

8.21.6 Gorlin Syndrome (Nevoid Basal Cell Carcinoma Syndrome – NBCC)

This is characterized by nevoid basal cell carcinomas and jaw keratocysts, palmar and plantar pits, skeletal abnormalities, ectopic calcifications and ovarian fibromas. The characteristic associated CNS tumor is medulloblastoma, and tends to be of desmoplastic variant histology [127, 128]. The affected gene is PTCH, which lies on chromosome 9q31.

8.21.7 Cowden Syndrome

This is a rare syndrome associated with hamartomatous and neoplastic lesionssuch as dysplastic gangliocytoma of the cerebellum (Lhermitte-Duclos disease), verrucous skin lesions, trichilemmomas, oral mucosa fibromas,, hamartomatous colon polyps, thyroid neoplasms and breast cancer. The affected gene is PTEN/MMAC1, located on chromosome 10q23. The gene product may be involved in cell growth and differentiation [129].

8.21.8 Li-Fraumeni Syndrome (LFS)

This is characterized by the presence of bone or soft tissue sarcomas presenting before the age of 45 years,

with presence of other cancers in the first degree relatives presenting before age 45 years. The pediatric brain tumors associated with LFS are medulloblastoma/PNET, choroid plexus tumor and ependymoma. There are two genetic determinants of LFS. The first is germline mutation of the TP53 gene located at chromosome 17q13. This gene plays a key role in cell cycle control and apoptosis. The second gene is hCHK2 (checkpoint kinase 2), located at chromosome 22q. It has been shown that the gene product of hCHK2 directly phosphorylates p53, indicating its involvement in p53 regulation of DNA damage [130].

8.22 Similarities Between the Embryo and Cancer

As suggested in Fig. 8.1 embryos share many features with cancerous cells [69, 71, 131–133]. Implantation of the embryo and progression of neoplasia both involve invasion of tissues through proteolysis, guidance of cell migration (tumor invasion) by fibroenctins, integrins and other molecules of the extracellular matrix, and secretion of angiotrophic factors to avoid growth-stunting hypoxia.

It has been speculated that cancer cells may modulate their adjacent mesenchyme in ways that favor their metastasis.

Embryos and cancer cells both show rapid cell growth. Blasts double their cell number every 2–4 days in their first 4 weeks, mostly stimulated by proto-oncogenes. Neoplastic growth rates may be less dramatic but have similar signals: regulation cells growth is lost due to mutations activating proto-oncogenes or disrupting tumor suppressors. In carcinogenesis, the disturbed cell growth can result from: overproduction of growth factors; reduced enzymatic degradation of growth factors; mutated oncogene receptors that resist inactivation or become autonomous without need for ligand (the oncogene protein); mutations in other components of signal transduction cascades, as when increased expression of MYC or CDK-4 proteins in the P53-RB cascade stimulates DNA synthesis.

Other common characteristics included extended cell longevity/immortality through activation of telomerase; this enzyme replicates DNA sequences that are unique to ends (telomeres) of chromosomes. The telomeric clusters tend to shorten with each cell division, encoding a timetable for senescence that causes cells to die after a programmed number of cycles. Immortalized cells like those of neoplastic or germline tissues, activate telomerase, which preserves their telomere length and avoids programmed cell death. Cancer cells become like embryonic cells or the stem cells of adult tissues; they outlive and overgrow neighboring somatic cells that cannot replicate their telomeres [131, 132].

Neoplastic cells and embryos are also protected from immunologic responses that would hasten their elimination. The mechanisms responsible for this tolerance are largely unknown, but fibrin may act as an "insulator". Hypercoagulability states, common in paraneoplastic syndromes, would benefit intravascular tumor cells Nitabuch's fibrin layer could also act as a barrier at the placental insertion site. The hypercoagulable state of pregnancy has also been postulated to play a role in eluding the immunologic destruction of the embryo.

The use of the blood circulation for cell migration is used by the germ cells of birds and by hemopoietic cells in mammals. Migrating cells bind to "homing molecules" localized on the surface of endothelial cells. These endothelial molecules are specific to each organ, exemplified by homing molecules in liver endothelial cells that are different from those of other tissues. Only cells with receptors for a specific homing molecule will bind endothelium at that particular site, eventually penetrating the vessel through diapedesis (invasion). Homing molecules are exploited advantageously in bone marrow grafting, where they direct intravenously injected marrow cells to repopulate the marrow. Malignant cells also possess homing receptors, explaining the predilection of tumoral metastasis: colonic adenocarcinoma preferentially spread to liver, breast carcinomas spread to bone, liver, and brain. Despite its vascular perfusion (20% of cardiac output), the kidney is rarely a metastatic target; perhaps cancer cells do not possess the appropriate receptors for renovascular homing molecules.

Another parallel concerns the epigenetic mechanisms of DNA methylation and histone modification, regulating differentiation in the embryo and reactivation of silenced genes in cancer [69, 132]. Epigenetic deregulation of developmentally important genes can also affect imprinted genes; for example, altered imprinting in an adrenocortical carcinoma can lead to overexpression of IGF2 and a loss of H19

expression [68]. Proto-oncogenes may lose silencing signals, being "mutated" to become unregulated oncogenes. Tumor suppressor genes can be pathologically methylated, and thereby inactivated, during the clonal evolution of tumor cells to malignancy. DNA methylation or demethylation can aid tumor progression, prompting "de-differentiation" as a reverse embryology, and these epigenetic alterations can be reversed by drugs in the treatment of cancer [131]. Epigenetic modulation is an early event in colorectal carcinoma, occurring at the early adenoma stage of cancer progression. Though the mechanisms for epigenetic alterations in cancer are poorly understood, they enable neoplastic progression by expression of specific cell adhesion molecules, proteases, angiotrophic factors, telomerase, and apoptotic-inhibiting molecules.

It is apparent that the molecular cascades so perfectly well regulated in embryogenesis can be hijacked by cancer cells to favor their growth, invasion and dissemination. Several "developmental cancer syndromes" are known. For example, in Bloom syndrome, the dosage of BLM protein is crucial to somatic changes in that disorder and to genome instability of those patients' intestinal cells [67]. Inactivation of one BLM allele (haploinsufficiency) causes defective DNA repair with production of a cancer syndrome that predisposes to colorectal cancers. Haploinsufficiency of tumor suppressor genes has been demonstrated in other developmental/cancer syndromes, including ATM in ataxia-telangiectasia, PTCH in basal cell nevus syndrome, and PTEN in Ruvalcaba or Cowden syndrome [67]. The SHH cascade is also crucial to both development and neoplasia, as exemplified by the fact that in humans, abnormal cholesterol synthesis yields an abnormal development of forebrain, and the basal cell nevus syndrome associated with the development of large numbers of basal cell carcinomas. The addition of cholesterol to promote SHH action in forebrain, in addition to the basal nevi is paralleled by farnesylation of RAS which regulates cell proliferation by controlling mitogen-activated protein kinase (MAPK) [66].

The link between CAMs, development, and neoplasia is exemplified by aberrant cell adhesion resulting from the COLLAGEN VII mutations that cause the epidermis-dermis fragility in epidermolysis bullosa [65]. When such mutations preserve the anchoring domain of COLLAGEN VII, COLLAGEN VII promotes squamous cell cancer and allows dermal invasion through its interaction with laminins.

New approaches to birth defects and tumors consider molecular mechanisms behind each step of classical embryogenesis, for these are the vulnerabilities that can be exploited for understanding diagnosis, prevention and therapy.

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Chapter 9

Carcinogenetic Pathway of Malignant Melanoma

Kenneth B. Calder and Michael B. Morgan

Contents

9.1 Introduction

Malignant melanoma is a burgeoning public health concern. In recent decades, the incidence of primary cutaneous melanoma has increased throughout most of the world including the United States. The overall age-adjusted incidence of melanoma has increased nearly 200% among Caucasians in the US between 1973 and 2002 (21.9 cases per 100,000 in 2002). This increased incidence of melanoma has been seen in all age groups, with a disproportionate increase in individuals over 55 years. The incidence of melanoma has also increased among African Americans and Hispanics in the US [1].

Along with the increased incidence of melanoma, between 1973 and 2002, the overall mortality rate among Caucasians increased from 2.1 per 100,000 to 2.9 per 100,000. The mortality rate in men has remained stable while the mortality rate among women has declined since 1992. Between the years of 1973 and 2002 the mortality rate among adults younger than age 55 decreased, while an increase in mortality in both men and women older than 55 was noted. Most importantly, 5-year survival rates for melanoma now surpass 90% in American, Australia, and Sweden [1].

Despite considerable debate, the risk factors associated with melanoma include environmental, phenotypic, and genotypic influences. Such factors include skin and hair color [2], numerous freckles [3], tendency to burn with sun exposure [2], blistering burns [4], presence of nevi [5], immunosuppression [6], and genetic mutations, among others. It has been demonstrated that a personal history of melanoma is one of the strongest predictors for melanoma. In a seven-year follow-up study including 4,484 patients diagnosed with primary melanoma, 9% had a second primary lesion [7]. Other studies have demonstrated an increased risk of melanoma in individuals with a history of nonmelanoma skin cancer [8].

Distinct populations have also been associated with an increased risk of melanoma including transplant patients. The risk of melanoma has also been associated with the presence of precursor large congenital nevi and dysplastic nevi [5]. More recently, the risk of melanoma appears to be greater in individuals with more than 50 nevi or a size greater than 6 mm in diameter [9, 10]. Lastly, 8–12% of melanomas occur in a familial setting, with specific genes responsible for these cases [11].

The tumorigenesis of cutaneous melanoma is a complex, multistep, and enigmatic process. With the many risk factors implicated in the development

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of melanoma, the potential mechanisms leading to carcinogenesis are likely to prove as numerous and variable. These statements are supported by the enormous body of research in the medical literature dedicated to all aspects of melanoma. In an attempt to better understand the complex biology of melanoma and elucidate the tumorigenesis of this malignancy, studies have not placed limits on the potential pathways and mechanisms responsible for melanoma progression. The purpose of this chapter is to present and summarize the most recent advances in the understanding of melanoma tumorigenesis, regarding proposed mechanisms of carcinogenesis, genetic mutations, and the clinical implications of current melanoma research.

9.2 The Role of Photocarcinogenesis in Melanoma

The ultraviolet fraction of the electromagnetic spectrum is considered a major etiological factor in both non-melanoma and melanoma skin cancer. Both UVB and UVA irradiation are responsible for mutations in melanocyte genes responsible for the initiation, promotion, and tumor progression [12]. UVB radiation has been demonstrated to cause mutations as a result of the formation of pyrimidine dimmers and non-dimer photoproducts [13]. On the other hand, the role of UVA radiation is not as well understood. UVA radiation is implicated in the promotion of oxidative mutagenic DNA base alterations and DNA breaks [14].

The complex mechanisms involved in the photocarcinogenesis of melanoma are not completely understood. Studies have demonstrated an increased risk of melanoma associated with latitude gradient [15], skin type and tendency to burn [2], chronic sun exposure with increasing age, and childhood sunburns [4]. At the same time, there appears to be a paradoxical relationship between sun exposure and melanoma. In fair-skinned individuals melanomas are most common in areas intermittently exposed to sun (Trunk, arms, and legs), instead of areas like the face that are chronically exposed to ultraviolet radiation (UVR). It has also been shown that indoor workers and those associated with a higher socioeconomic status are at a higher risk for melanoma [16]. Similarly, all the studies evaluating the effectiveness of sunscreen in the prevention of skin cancer have not demonstrated protective effects [17]. Lastly, certain histologic subtypes of melanoma arise in sites that are exposed to minimal UVR, including the palms, soles, and mucosal membranes.

Melanomas involving skin with chronic solar damage are associated with a location on the face, older age, and chronic UVR exposure. Melanomas associated with intermittent sun exposure present in younger fair-skinned individuals, in such locations as the trunk, arms, and legs. A recent study evaluating 126 primary melanomas using array-based comparative genomic hybridization, classified the melanomas into four groups based upon location and amount of sun exposure; 30 melanomas from skin with chronic solar damage, 40 melanomas from skin with no chronic solar damage, 36 melanomas from the palm, soles, and subungual region, and 20 mucosal melanomas. These lesions underwent genome-wide evaluation for differences DNA copy numbers. The results demonstrate frequent BRAF mutations and losses of chromosome 10 in melanomas arising in areas intermittently exposed to sun. BRAF encodes a serine/threonine kinase responsible for the transduction of mitogenic signals from the cell membrane to the nucleus. In contrast, there was a low frequency of BRAF mutations, and frequent increases in the number of copies of the cyclin D1 (CCND1) gene in melanomas that arise in skin with evidence of chronic solar damage. Acral and mucosal melanomas were associated with a higher frequency of losses of the CDKN2A (p16) locus compared to melanomas in sites with or without evidence of chronic solar damage [18].

The tumor suppressor protein p16 is implicated in the photocarcinogenesis of both melanoma and nonmelanoma skin cancer; squamous cell (SCC) and basal cell (BCC). p16 is a cyclin dependent kinase (CDK) inhibitor preventing the phosphorylation of the retinoblastoma gene (Rb) product; acting as a negative cell cycle regulator [19]. In the normal epidermis p16 is expressed only in the granular cell layer [20]. In the carcinogenesis of SCC p16 protein expression showed gradual up-regulation in the progression from actinic keratosis to SCC in situ to invasive SCC to metastatic SCC [21]. On the other hand, p16 gene mutations in melanoma demonstrate a gradual down-regulation with tumor progression, and complete loss of expression in metastatic tumors [22]. Some have associated p16 mutations (C:G to T:A transitions, CC to TT tandem mutations, and C:G to A:T transversions) with UVR in melanoma cell lines, based upon the similarity of the aforementioned mutations to those observed in mammalian genes exposed to UVR [23].

Unlike SCC carcinogenesis, p53 mutations are described as rare and late events in the tumorigenesis of melanoma that occur in the progression to higher grade malignancy. Nonetheless, p53 mutations have been associated with UVR exposure [24]. Sixty percent of melanomas in patients with xeroderma pigmentosum have TP53 gene mutations, implicating UVR as a potential factor in TP53 gene mutations [25]. TP53 gene mutations in melanoma are usually a result of base pairing substitutions, T:A substituting C:G, also supporting the possible role of UVR in these gene mutations [26]. Compared to nonmelanoma skin cancers, there is a lower frequency of UV-induced TP53 gene mutations in melanoma. Therefore, p53 is unlikely to play a major role in UV-related tumorigenesis of melanoma.

Lastly, UVA exposure is associated with the production of photosensitizers and the generation of reactive oxygen species (ROS). In order to reduce the harmful effects of ROS, the skin produces copper-zinc superoxide dismutase (CuZnSOD), manganese SOD (MnSOD), and catalase. Using immunohistochemical techniques, an up-regulation of CuZnSOD, MnSOD, and catalase was demonstrated in human melanomas compared to age-matched nevi and young control skin. This enhanced antioxidant activity may serve to protect melanoma cells from ROS, allowing selective growth of malignant cells [27].

In conclusion, the role of UVR in the tumorigenesis of melanoma is not as well established as the association between UVR exposure and nonmelanoma skin cancer. As mentioned, there are numerous potential environmental risk factors and models of UV-induced photocarcinogenesis associated with melanoma. It is possible that several of these mechanisms, along with specific environmental and biologic factors, are essential in melanoma progression. Determining the role of UVR in melanoma may potentially assist in further clinical and histopathologic classification of this malignancy.

9.3 Tumorigenesis of Melanoma

9.3.1 p16 (INK4A)

The gene CDKN2A is located at 9p21 and encodes two distinct proteins; INK4A (inhibitor of cyclin-dependent kinase 4) also known as p16 and ARF (alternative reading frame) also designated p14. The p16 protein is a member of the INK4 family and is responsible for controlling the G1/S cell cycle transition. p16 prevents the phosphorylation of the retinoblastoma protein (pRB) by binding to CDK4 and CDK6 which inhibits the formation of the CDK4/6/cyclin D complex, responsible for phosphorylating the pRB. In the presence of p16 expression, the pRB is hypophosphorylated and remains complexed to E2F; resulting in G1 cell cycle arrest. Therefore, loss of p16 expression results in a lack of regulatory control of cell cycle progression due to hyperphosphorylation (inactivation) of pRB [28].

The CDKN2A gene, located at chromosome 9p21, has been identified as one of the familial melanoma genes. Loss of heterozygosity or mutations in this gene is associated with the presentation of melanoma in familial melanoma kindred. More specific, germline mutations or deletions that result in the inactivation of p16 have been identified in cultured melanoma cell lines [29]. p16 mutations have been demonstrated in 25–40% of families with an increased risk of melanoma and in 0.2–2% of sporadic melanoma cases [30, 31]. However, in a more recent study utilizing genomic hybridization, loss of p16 expression has been demonstrated in nearly 50% of primary melanomas [32]. Mechanisms implicated in the loss of p16 expression include: hypermethylation of promoter regions [33], homozygous deletions [34], loss of heterozygosity [32], and microsatellite instability [35], among others.

Decreased expression of p16 (protein and mRNA expression) has been associated with the clinical progression of melanoma in both familial and sporadic cases [22, 36, 37]. Based upon the majority of data at this time, p16 expression is not believed to be altered in nevi [37], and is either unaltered of reduced in melanoma in situ [37]. The expression of p16 has also been studied in melanomas with and without association with a contiguous nevus. Loss of p16 expression was demonstrated in 85% of melanomas not associated with a nevus remnant. On the other hand, 24% of melanomas associated with a nevus demonstrated loss of p16 expression [38].

The loss of expression of p16 in melanoma has been associated with increased tumor thickness [39] and Clark level [40], increased proliferation rate [22], and higher mitotic count [39]; however, not all studies support these findings [32, 36]. Others have associated the reduced expression of p16 with risk of disease relapse

and as an independent predictor of decreased disease survival [41]. In summary, the loss of p16 expression in locally advanced melanoma is characteristic of this malignancy, but the role of p16 in the early stages of melanoma presentation/progression, association with UVR exposure, and prognosis are currently debated.

9.3.2 p53 (TP53)

The tumor suppressor gene TP53 is located on the short arm of chromosome 17 and encodes the p53 protein. DNA damage and other genomic alterations are responsible for activating the p53 protein, which is usually quiescent under normal circumstances. p53 is responsible for activating downstream signals to arrest cells in G1, inhibiting the replication of damaged DNA and allowing DNA repair. Also critical, p53 is responsible for inducing apoptosis if the DNA damage is significant. Hence, alterations in p53 function and/or expression allows replication of cells with damaged DNA, leading to the accumulation of genetic alterations that promote malignancy [42].

Numerous studies have evaluated p53 gene alterations in melanoma with varying results. Some studies have reported the absence of TP53 gene alterations in both primary and metastatic melanoma [43], while other studies report an incidence of mutations between 1 and 29% [26, 44, 45]. Interestingly, metastatic melanomas have been associated with higher frequency of TP53 gene mutations, and nevi have been shown to have less frequent mutations in the TP53 gene [46]. The differences in the reported incidence of TP53 gene mutations in melanoma have been attributed to variations in detection techniques, variable selection of tumors in different stages of progression, variation in anatomic site, and genetic heterogeneity [47].

There is also variable expression of the p53 protein in melanomas, ranging from 5 to 86% of primary melanomas [48, 49]. p53 is thought to be expressed late in the tumorigenesis of melanoma, based upon the absence of or low expression of p53 in nevi and the progressive increased expression of p53 in melanomas as they progress from the radial (horizontal) to vertical (invasive) growth phases and to metastatic disease [46, 48].

p53 has been evaluated in different anatomic sites, in an attempt to demonstrate a relationship between UVR exposure and p53 expression. More frequent (32%) and greater expression of p53 was noted in melanomas arising in the head and neck (chronic sun exposed sites), compared with other sites (6%) [41]. However, there is evidence demonstrating that the expression of p53 is independent of anatomic location and sun exposure [45]. Interestingly, all melanomas demonstrating over-expression of p53 do not have TP53 mutations, implying that the activation of p53 expression is dependent upon other cellular signals and mechanisms [26]. Lastly, some studies have demonstrated an association between p53 expression and tumor thickness, Clark level, and high mitotic rate, while other studies have demonstrated no relationship between p53 expression and such clinical parameters [50, 51].

9.3.3 c-KIT

It is well established that the c-kit receptor and its ligand, stem cell factor (SCF), are important in the development of melanocytes. SCF is responsible for mouse melanocyte migration, proliferation, and differentiation [52]. In the skin, fibroblasts, keratinocytes, and endothelial cells are responsible for the production of SCF [53]. SCF binds to c-kit, a receptor tyrosine kinase, causing dimerization of the c-kit receptor leading to autophosphorylation and activation of downstream pathways (MAP2K). SCF/c-kit are essential in the paracrine regulation of melanocytes in both normal human skin and pigmentary disorders [54]. C-kit expression has been demonstrated in several malignancies, and is implicated in the pathogenesis of gastrointestinal stromal tumors.

Similar to melanocytes, melanoma cells also express the c-kit receptor. C-kit expression has been demonstrated in normal melanocytes and benign nevi, with loss of expression described in advanced melanoma. It is thought that the loss of c-kit expression is associated with the change in the radial to vertical growth phase of melanoma [55]. Immunohistochemistry for c-kit was performed on 261 patients with primary melanoma (AJCC Stage I or II), who had undergone completion of surgical resection with adequate margins. Diffuse expression of c-kit was found in 144 (55%) of cases, while 117 cases did not stain positive with the c-kit polyclonal antibody. Of the 144 cases which stained positive for c-kit, 60 cases demonstrated <30% of positive cells, 64 cases

demonstrated 30–60% of positive cells, and 20 cases demonstrated >60% of positive cells. In this study, c-kit expression showed no association with prognostic significance [56]. Another study evaluated array comparative genomic hybridization data from 102 primary melanomas subdivided in four groupings: mucosal melanoma [38], acral skin [28], skin with chronic sun-induced damage (CSD) [18], and skin without CSD [18]. Oncogenic mutations in c-kit were demonstrated in 3/7 tumors with amplifications on 4q12. Mutations and/or copy number increases of c-kit were demonstrated in 39% of mucosal, 36% of acral, and 28% of melanomas on skin with evidence of CSD. Of note, no mutations and/or copy number increases were present in melanomas on skin without CSD. Increased c-kit protein levels in the vertical growth phase were demonstrated in 79% of tumors with mutations and in 53% of tumors with copy number increases [57].

These findings demonstrate increased c-kit expression in the advanced stages of melanoma tumorgenesis, which contrasts with other studies describing a down regulation of c-kit during progression. The authors of the aforementioned study reconcile their findings by explaining that the prior studies, which demonstrate down regulation of c-kit during melanoma progression, utilized melanoma cell lines of the melanoma subtype that occurs on intermittently sun-exposed skin (superficial-spreading type) with frequent BRAF mutations. Therefore, the absence of c-kit mutations or lack of increased copy number is expected in this melanoma subtype, and further supports the findings of Curtin et al. The melanoma subtypes associated with frequent genetic alterations in c-kit (i.e. lentigo-maligna) typically demonstrate a lentiginous growth pattern, whereas melanomas arising in skin without CSD lack c-kit expression and show a pagetoid growth pattern [57].

Imatinib, a tyrosine kinase inhibitor, has been studied in phase II clinical trials in patients with metastatic melanoma. This study demonstrated no evidence of clinical efficacy of high-dose imatinib in patients with metastatic melanoma [58]. These findings raise the question concerning the true role c-kit in melanoma tumorigenesis. As described by Curtin et al., it is possible that only certain subtypes of melanomas; acral, mucosal, and melanomas in the setting of CSD are associated with mutations and increased expression of c-kit. Appreciating the variability in melanoma subtype expression of c-kit, it is possible that the benefits of imatinib therapy may be seen in a specific group of patients with c-kit mutations.

9.3.4 EGFR

Epidermal growth factor receptor (EGFR) is a member of the tyrosine kinase growth factor receptor family. Tyrosine kinase receptors are responsible for communicating extracellular signals to the nucleus and play vital roles in tumor evolution including: growth, differentiation, inhibition of apoptosis, and metastatic progression. Binding of specific ligands to the extracellular domain of EGFR leads to receptor dimerization and activation of intrinsic tyrosine kinase activity, which in turn induces the RAS/RAF/MEK/MAPK and PI3K/Akt signaling pathways. Over expression, and mutations of EGFR have been demonstrated in several malignancies; including cutaneous melanoma.

EGFR over expression has been described in late stage melanoma, and associated with extra copies of chromosome 7. In a recent study of 81 primary melanomas, 70% of the lesions demonstrated aneusomy of chromosome 7. Copy number alterations of the EGFR gene was demonstrated in 79% of the tumors, and amplification of the EGFR gene was associated with polysomy of chromosome 7. An increased copy number of the EGFR gene has been demonstrated in the progression of melanoma, and has been associated with poor prognosis (increased Breslow and ulceration) [59]. In addition, the expression of EGFR has been shown to increase during melanoma progression and predicts a poor prognosis [60]. Other studies have demonstrated minimal or no expression of EGFR in human melanocytes and melanoma [61]. Therefore, the role of EGFR in the tumorigenesis of melanoma is not fully elucidated nor completely accepted. Further studies are needed to evaluate the significance of EGFR gene alterations as it relates to mRNA and protein expression, to determine the potential utility of anti-EGFR therapeutics.

9.3.5 Cyclin D1

Cyclin D1 is encoded by the CCND1 gene located at 11q13, and has been shown in some studies to be

involved in the tumorigenesis of melanoma. Cyclin D1 is an important positive regulator of the G1-S cell cycle transition, via activation of cdk 4/6, which in turn leads to the inactivation (phosphorylation) of the Rb protein. Inactivation of the Rb protein promotes the release of bound E2F, allowing cell cycle progression [62]. Studies implicating cyclin D1 in the pathogenesis of melanoma have demonstrated amplification of the CCND1 gene in 47% of primary lesions and 35% of metastatic melanomas [63]. A second study demonstrated CCND1 gene amplification in 44% of acral lentiginous melanomas [64]. At the same time, there is evidence demonstrating no amplification of CCND1 in metastatic melanomas [65].

Using immunohistochemistry, increased expression of cyclin D1 has been show in both in cutaneous [66] and uveal melanoma [67]. Nevi and normal skin adjacent to melanomas demonstrate absent or weak expression of cyclin D1 [66]. Some studies report no association between increased cyclin D1 expression and clinical outcomes [67], while there is evidence describing a significant association between increased cyclin D1 expression and thinner lesions [68]. Therefore, the role of cyclin D1 in the tumorigenesis, progression, and prognosis of melanoma is debated at this time.

9.3.6 Telomerase

Telomerase is a ribonucleoprotein DNA polymerase responsible for maintaining telomere length on the ends of chromosomes and is not expressed in somatic cells. In malignancy, the expression of telomerase is implicated in the immortalization of cells [69]. The expression of human telomerase RNA (hTERT) has been evaluated in both benign and malignant melanocytic lesions, demonstrating an increase in telomerase RNA levels from benign to malignant lesion and from primary to metastatic melanomas [70]. In a recent study, Fullen et al. showed increasing mean expression of hTERT from nevi to dysplastic nevi and primary melanoma to metastatic melanoma, but failed to demonstrate a diagnostic role for hTERT in distinguishing between a benign and malignant melanocytic neoplasm [71]. There are also studies reporting uniform moderate to high levels of telomerase expression among ordinary nevi, spitz nevi, and melanomas [72].

Telomerase has also been implicated in the regression phenomenon of melanoma. It is thought that telomere attrition (as a result of several rounds of cell division), leads to open DNA ends and end-to-end fusion of chromatids. During this period of DNA restructuring, the rate of apoptosis is high (regression phenomenon) and ultimately leads to an internal natural selection of a more aggressive phenotype. At this point, the tumors cells activate telomerase, which is responsible for re-stabilizing the telomeres allowing continued proliferation [73]. Further studies are needed to determine the role of telomerase in the pathogenesis of melanoma, which may have prognostic and/or therapeutic implications.

9.3.7 RAS

The mitogen-activated protein kinase pathway (MAPK), via Ras/Raf/Mek/Erk, regulates cell proliferation and survival. Errors in this signaling pathway are implicated in the tumorigenesis of melanoma. The Ras GTPases are small G proteins responsible for mediating growth signals from growth factor receptors to the nucleus. The Ras family of proto-oncogenes includes H-Ras, N-Ras, and K-Ras; with N-Ras more frequently associated with melanocytic lesions [74, 75].

Activating mutations in N-Ras have been demonstrated in 56% of congenital nevi [74], 33% of primary melanomas, and 26% of metastatic melanomas [75]. Of note, N-Ras mutations are not frequently associated with dysplastic nevi [74]. Activating N-Ras mutations have also been associated with sun exposure and nodular lesions [76, 77]. H-Ras is more frequently associated with Spitz nevi, which demonstrate amplification of the H-Ras genomic locus on 11p and oncogenic point mutations [78].

In a study of 126 melanomas subdivided on the basis of UV light exposure, all mutations in the Ras gene were of N-Ras and occurred only in samples without BRAF mutations. The majority of melanomas with N-Ras mutations were from skin with no evidence of chronic sun damage. No significant association between N-Ras mutations and melanoma subtypes or tumor thickness was observed [18]. Therefore based upon the presence of Ras and BRAF mutations in

certain subtypes of melanoma, therapies targeting the Ras/RAF/ERK/PI3K pathways could prove beneficial.

9.4 Conclusion

Noting the number of conflicting studies and ongoing debates concerning the tumorigenesis of melanoma, it is evident that much remains unknown. The complexity of this disease is apparent in the numerous environmental and biologic factors implicated in its pathogenesis. Traditionally, genetic alterations in melanoma were approached according to the histologic subtype. As presented herein, distinct genetic alterations in melanomas are observed not only in the different histologic subtypes, but are also based upon location and degree of sun exposure. Ultimately, a number of different pathways, acting both independently and in concert, are most likely responsible for the carcinogenesis of melanoma. In order to treat this malignancy, therapies will most likely need to exploit the unique genetic and biologic characteristics of the specific tumor.

The heterogeneity of this malignancy is further demonstrated by the numerous pathways implicated in melanoma tumorigenesis. Efforts to elucidate the tumorigenesis of melanoma are not in vain, with an increase in the 5-year survival rate of patients with this disease. Further studies are needed to identify the risk factors, pathogenesis, and the prognostic factors associated with melanoma. Most importantly, based upon the studies and advances made in melanoma, progress may be made in understanding pathways involved in other malignancies as well.

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Chapter 10

Ewing Sarcoma: Molecular Characterization and Potential Molecular Therapeutic Targets

Marilyn M. Bui and Paul J. Zhang

Contents

10.1 Introduction

Ewing sarcoma (ES) is the second most common malignant bone tumor (after osterosarcoma) among children and young adults. According to the most recently published surveillance epidemiology and end results data [1], the overall incidence of ES of the bone has remained unchanged, with an average of 2.93 cases/1,000,000 reported annually between 1973 and 2004 among patients who are 1–19 years of age. ES is slightly male predominant (male to female ratio = 1.3:1) and significantly prominent in whites (almost never shown in blacks) [2].

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10.2 Etiology and Pathogenesis

ES is one of the members of the Ewing sarcoma family of tumors (ESFT), which also includes soft tissue Ewing sarcoma and primitive neuroectodermal tumors (PNET). All members of the ESFT share similar histologic, cytogenetic, and molecular characteristics and have been thought to be derived from neural crest cells and represent a spectrum of tumors with neural differentiation. However, the uniformity of the morphological, immunohistochemical, cytogenetic, and molecular profile suggests that ESFT may derive from special mesenchymal stem cells that are capable of multilineage differentiation. Emerging data have provided evidence in support of this hypothesis by demonstrating the related histopathology of human mesenchymal stem cells in an experimental tumor model [3, 4]. There is also data demonstrating that expression of the *EWS-FLI-1* fusion protein triggers an ES initiation program in primary human mesenchymal stem cells [5, 6]; this is consistent with the hypothesis that ES is most likely the result of spontaneous genetic translocations rather than Mendelian inheritance or environmental (toxic) exposure. Human mesenchymal stem cell models expressing the *EWS-FLI-1* fusion oncogene may provide more information on tumorogenesis and progression of ES. Studies have indicated that *EWS-FLI-1* participates in ES pathogenesis by promoting at least two sets of events that synergize in tumor development and progression: cell proliferation and survival. This is accomplished by inducing other candidate genes, such as *PDGFC, IGF1, MYC, CCND-1,* and *NKX2-2,* and escaping from apoptosis and growth inhibition by repressing p21, $p57^{Kip}$, TGFbRII, and IGFBPP3 [7]. Further study of the translocation-driven events and

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the *EWS-FLI-1* targets will be helpful in identifying potential molecular therapeutic targets.

With the use of classic cytogenetic karyotyping, the hallmark of ES was found to be the presence of one of five distinctive balanced translocations, with EWS at chromosome 22 band q12 rearranged to fuse with one of five members of the erythroblast transformation sequence transcription factor gene family (*FLI-1, ERG, ETV1, E1AF,* and *FEV*) at chromosomes 11, 21, 7, 17, and 2. EWS is an RNA-binding protein of unknown function by itself. However, fusion with one of the erythroblast transformation sequence genes results in a hybrid transcript and an oncogenic chimeric protein, which leads to the tumorigenesis of ES. *EWS-FLI-1,* which translocates in *t*(11;22)(q24;q12), is the most common fusion protein and is associated with 85% of cases of ES. Cytogenetic characteristics of ES are summarized in Table 10.1. *FLI-1* is a transcription factor, which alone is not sarcomagenic. There are as many as 18 variants of *EWS-FLI-1* transcripts. All translocations contain the N-terminal domain of *EWS* (exon 1–7) and the *FLI-1* domain (exon 9). The two most common fusions are *EWS* exon 7 to *FLI-1* exon 6 (type 1), which is a less potent transactivator and confers a better prognosis, and *EWS* exon 7 to *FLI-1* exon 5 (type 2) [8].

In addition to the key translocation of *EWS-FLI-1,* ES is associated with many secondary chromosomal changes (in approximately 20–30% of ES). Numerical chromosomal gains include trisomy 8 (45%), as well as chromosomes 2, 5, 7, 9, and 12 (10–15%); translocation includes *t*(1;16)(q12;q11.2); and mutations or deletions include p53 and p16 ink4A [9–12], which are thought to be associated with poor clinical outcome.

Although *EWS-FLI-1* translocation is necessary for tumorogenesis and progression in many cases of ES, it is reasonable to suspect that this hallmark event interplays with other signal transcription pathways, such as *PDGFC, IGF1, MYC, CCND-1,* and *NKX2-2,* for its full oncogenic effect in ES. Telomeres are specialized structures at the ends of human chromosomes and consist of hundreds of hexanucleotide repeats, which protect the chromosomes from DNA degradation, end to end fusions, rearrangements, and chromosome loss. Telemerase is composed of a multisubunit ribonucleoprotein. Its function is to add telomeric DNA to the ends of linear chromosomes using a RNA template. One of the targets of the *EWS-FLI-1* transcript is telomerase, which is expressed at high levels in ES [13]. Loss of p16 INK4A is commonly associated with ES and is associated with telomerase-immortalized human cell models [14].

10.3 Tissue Diagnosis

Hemotoxylin and eosin staining of formalin-fixed and paraffin-embedded tissue sections has shown ES to be morphologically composed of homogenous small, blue, round cells arranged in sheets with or without pseudorosette formation. Intracytoplasmic glycogen can be detected using periodic acid-Schiff stain with and without diastase digestion. However, results are not specific. With the use of immunohistochemical analysis, ES is shown to characteristically express CD99 and *FLI-1* [15]. CD99 is a 32-kDa cell surface glycoprotein encoded by the MIC2 gene. *FLI-1* nuclear reactivity is typically detected in 71% of cases of ES and PNET, which may also have *EWS-FLI-1* fusion. Both markers are very useful in assisting the diagnosis in patients who have typical clinical

Table 10.1 Cytogenetic characteristics of Ewing sarcoma

Translocation	Hybrid transcript and oncogenic chimeric protein	Frequency (%)	References
t(11;22)(q24;q12)	*EWS-FLI-1*	85–90	Martinez-Ramirez [53]; Mitelman [54]
t(21;22)(q12;q12)	*EWS-ERG*	5–10	Delattre [55]; Mitelman [54]
t(7;22)(p22;q12)	*EWS-ETV1*	<1	Delattre [55]; Mitelman [54]
t(17;22)(q12;q12)	*EWS-EIAF*	<1	Delattre [55]
t(2;22)(q33;q12)	*EWS-FEV*	<1	Delattre [55]

presentation and morphology for ES. However, these markers are not specific, and both can be seen in other neoplasms other than ESFT. Therefore, their diagnostic utility is limited when the clinical presentation and morphology are not typical for the disease. In this situation, the most confirmative study is to identify the hallmark genetic translocation or the fusion gene.

Classic cytogenetic karyotyping requires fresh tumor tissue. The tumor cells are grown in cell culture, and metaphase spread is harvested for karyotyping. Karyotyping provides global information, including structural and numerical chromosomal changes and specific translocation and the secondary alteration information. However, it is a time consuming process and can be useless when tumor cells fail to grow in culture.

Fluorescence in situ hybridization (FISH) is a molecular assay that has been widely used in detection of the hallmark translocation in ES and other members of the family. In addition to frozen and fresh tissue [16], FISH assay is suitable for formalin-fixed paraffin-embedded tissue samples and for cytology smears made from limited volumes of fresh tumor samples or from cell cultures prepared for karyotyping. Fusion probes for *EWS-FLI1* [17–19] and break-apart probes for EWS breakpoint have been used. FISH using fusion probes is very sensitive and specific because it also detects the fusion partner for EWS. The recently described dual-color break-apart cocktail [20] DNA probes flanking the EWS breakpoint region on chromosome 22 only detected breaks at the EWS locus. This break-apart strategy disregarded the translocation partner or fusion type, making it easier to interpret than the probe fusion approach [21]. However, this method is less specific for ES because EWS break-apart signals can be seen in other sarcomas with translocation involving EWS.

Reverse transcriptase polymerase chain reaction (RT-PCR) is another commonly used molecular assay used to confirm *EWS-FLI-1* chimeric transcripts in frozen tissue and formalin-fixed, paraffin-embedded tissue. Because all translocations contain the N-terminal domain of EWS (exon 1–7) and the *FLI-1* domain (exon 9), RT-PCR can be performed using an EWS exon 7 forward primer and an *FLI-1* exon 9 reverse primer, which amplifies all forms of *EWS-FLI-1* of various sizes. However, this approach may yield false-negative results when the tumor harbors large fusions and is more vulnerable when the tumor RNA is partially degraded when formalin-fixed, paraffin-embedded tissue samples are used. The common alternative is to perform RT-PCR using an EWS exon 7 forward primer and an *FLI1* exon 6 reverse primer, which detected *EWS-FLI1* in 85% of ES cases [22]. There are also reports of improved detection of ES chimeric gene fusions by using real-time PCR [23, 24].

10.4 Prognostic Factors

ES by definition is a high-grade malignancy. According to the American Joint Committee on Cancer (AJCC), the most important prognostic factor is tumor stage. A metastatic tumor and larger than 8 cm confers poor clinical outcome. Unlike in patients with carcinomas or melanoma, ES has no propensity to spread via lymph nodes. Other clinical prognostic factors include tumor location and the age of the patient. Without metastatic disease at the time of diagnosis, good prognostic factors include nonpelvic location, younger than 15 years of age, and tumor size less than 8 cm. Type 1 *EWS-FLI-1* translocation has been shown as an independent favorable prognostic factor [25].

The role of molecular markers such as p53 and INK4A in ES remains to be determined [12]. A low p27 protein expression level is associated with poor survival in patients with ES [26]. Other potential markers are connexin [26-a], intracellular signaling pathways [26-b], [27] and thymidylate synthase [27-a]. As shown in various other cancers [28–31], telomere length reduction is also a predictor and shown to be an independent significant predictor of ES relapse [32]. Patients with short telomeres had a 5.3-fold greater risk of relapse than those with unchanged or longer telomeres.

10.5 Treatment and Clinical Outcomes

The traditional treatment approach entails surgical resection, radiation therapy, and chemotherapy based on resectable (localized) or unresectable (locally advanced with distance metastasis) disease stage.

Surgery and radiation are for local control of the tumor, and chemotherapy is for systemic control. For localized disease, preoperative neoadjuvant therapy can facilitate the shrinkage of the tumor and eradicate micrometastatic disease. Surgical resection with negative margin is the preferred method for eradication of all known tumors. In addition, modern surgical approaches have given patients improved limb salvage, limb function, and survival. Subsequent adjuvant chemotherapy and radiation will reduce local recurrence. Although ES is known for its radiosensitivity, due to its side effects and morbidities, this method is less preferred than modern surgical approach. Multimodality therapy is the cornerstone of current treatment of ES. The current generation of chemotherapy uses a combination of actinomycin D and doxorubicin, ifosamide, and etoposide [33–35]. However, clinical trials are trying to improve survival by using alternative cycles of vincristine, adriamycin, and cyclophosphamide and ifosfamide and etoposide (Children's Oncology Group AEWS0031 trial) and with granulocyte colony-stimulating factor [36]. The EUROpean Ewing Tumour Working Initiative of National Groups (EURO-EWING) 99 protocol provides six cycles of vincristine, ifofamide, doxorubicin, and etoposide; results from this protocol are also pending.

According to the surveillance, epidemiology, and end result data [1] the proportion of patients with distant metastasis among all ES cases remained 26–28%, whereas localized disease increased slightly from 57 to 67%. The 5-year survival of patients with localized diseases only increased from 44% in 1973–1982 to 68% after 1993. The 5-year survival of patients with metastatic disease increased from 16 to 39%. The corresponding 10-year survival increased from 39 to 63% in patients with localized disease and from 16 to 32% in patients with metastatic disease. The improvements in survival for patients with localized and metastatic disease reflect the improvements in multimodality therapy.

Conventional chemotherapy is generally considered the "shot gun approach," with inevitably undesirable cytotoxic events to healthy cells in the body. In addition, high-dose chemotherapy supported with autologous bone marrow transplantation may cause increased risk of treatment-related hematopoietic malignancy [37]. The poor outcome of patients with metastatic disease warrants the development of novel therapeutic modalities. The desirable novel therapy should be more effective and less toxic than conventional chemotherapy by targeting the specific and biologically relevant molecular aberrations.

10.6 Approaches to Identify Molecular Targets

Gene microarray technology provides us with the ability to analyze the complete gene expression profile of ES and shed light on the key molecules of the regulatory network in the origination and progression of ES and to facilitate the identification of potential novel therapeutic targets. Schaefer et al. [38], by analyzing 27 ESFT specimens using Affymetrix microarrays, identified genes differentially regulated between metastatic and localized tumor, including PDGF, TP53, NOTCH, and WNT1 signaling. Polychemotherapy-induced regression of 20 primary tumors was correlated with expression of genes related to angiogenesis, apoptosis, and p53 pathways and genes encoding ubiquitin, proteasome, and phosphoinositide 3-kinase. A set of 46 marker genes correctly classified these 20 tumors as responders versus nonresponders. It was concluded that expression signatures of initial tumor biopsies can facilitate identification of ESFT patients at high risk to develop tumor metastasis or to suffer from a therapy refractory cancer. Using gene expression profiles, Cheung et al. [39] developed a novel genomic approach to detect subclinical disease in ES.

The study of ES cell lines is a valuable tool to evaluate potential targeted therapies. For example, dasatinib, an oral multitargeted inhibitor of several kinases including BCR-ABL, SAR-family kinases, c-Kit, and PDGFR, in a study by Timeus et al. [40], induced cytostatic and antimigratory activity in ES cell lines, suggesting a possible use of dasatinib in the treatment of ES when combined with other cytotoxic therapies. However, for cell line studies, it is important to evaluate preclinical models that recapitulate the molecular characteristics of their respective clinical histologic types. Neale et al. [41] applied Affymetrix HG-U133Plus 2 profiling to an expanded panel of models in the Pediatric Preclinical Testing Program. Profiling led to exclusion of some tumor cell lines that did not cluster with human or xenograft

samples. The expression profiles of the remaining 87 models were compared with 112 clinical samples and showed appropriate correlation to clinical histologic type. Analysis of copy number alterations using Affymetrix 100 K single nucleotide polymorphism showed that the models have copy number alterations similar to their clinical counterparts. In addition, the copy number-altered genes were shown to be nonrandom and appeared to identify histologic type-specific programs of genetic alterations. Therefore, preclinical models can accurately recapitulate expression profiles and genetic alterations common to childhood cancer and are valuable in drug development.

The potential molecular targets that are identified by gene microarray assay or cell line studies need to be validated on tumor tissue obtained from ES patients. The tumor samples can be fresh, frozen, or fixed tissue (such as in RNA preservatives or formalin fixed paraffin embedded). Formalin fixed paraffin embedded tissue is most available and abundant in routine pathology practice. Using immunohistochemical study to validate molecular markers on this type of tissue has been a popular and productive approach. Tissue microarray (TMA) is a method for assembling formalin fixed paraffin embedded tissue from multiple patients into a single block, which allows simultaneous testing of potential molecular markers in multiple tumor samples. Our group has constructed an ES TMA with corresponding clinical information including therapy and outcome. Using this method we have assessed muscarnic and nicotinic acetylcholine receptor (AchRs) expression in ES [42]. We found that AChRs are overexpresssed in a significant number of ES. The western blot analysis of 3 human ES cell lines confirms the presence of AChRs. We are following this lead to investigate their potential therapeutic implications.

10.7 Other Therapeutic Alternatives

10.7.1 Dendritic Cell Immunotherapy

Dendritic cells (DC) are the most potent antigen-presenting cells that induces the activation and proliferation of CD8+ cytotoxic T cells with the help of CD4+ T cells. Activated CD8+ T cells function as tumor-killing cells by cytolysis and apoptosis. Cytokines, especially interferon-gamma, play important roles in this interaction [43]. DC-based immunotherapy has shown promise in patients with breast, prostate, colon, and renal cell carcinomas [44–46]. Our group has also preliminary data that demonstrated the effectiveness of DC immunotherapy in combination with radiation therapy in a clinical trial in patients with sarcoma (Finkeitain SE, Gabrilovich D, Bui MM, Cheong D, Heydek R, Janssen W, Letson D, Sondak V, Szekely R, Antonia SJ, unpublished observations). However, the effects of this therapy in overall survival and disease-free survival will require longer follow-up. To verify the hypothesis and develop the most effective DC vaccine against ES, Guo et al. [47] evaluated the antitumoral efficacy of DC-ES hybrids (DC-A673) and dendritic cells pulsed with other antigen-loading methods. The results indicated that the hybrids induce stronger antitumor efficacy.

10.7.2 siRNA Targets Against the EWS-FLI1 *Oncogene*

RNA interference (RNAi) is a process of sequence-specific, posttranscriptional gene silencing. The sequence is composed of 21–23 nucleotides (siRNA) and is associated with a complex of proteins named the RNA-induced silencing complex. The RNA-induced silencing complex directs the siRNA to the complementary target sequence and results in cleavage of the target RNA [48, 49]. The *EWS-FLI1* transcript is composed of a specific mRNA sequence, which constitutes a relevant target. Toub et al. [50] demonstrated the efficiency of siRNA targeted toward the *EWS-FLI1* transcript, free or encapsulated, delivered by means of nanotechnology. These siRNA-loaded nanocapsules were then tested in vivo on mouse xenografted *EWS-FLI1* expressing tumor and found to trigger a dose-dependent inhibition of tumor growth after intratumoral injection. Specific inhibition of *EWS-FLI-1* was also observed. siRNA is a promising strategy in the development of therapeutic applications in ES [51, 52].

The above alternative therapeutic modalities are not expected to replace surgery, radiation, or chemotherapy; rather, they are expected to be an addition to

the current treatment modalities. In general, alternative therapies are more specific to biologically relevant targets and would be expected to have far less toxicity than traditional chemotherapy.

10.8 Future Directions

The modern revolution in molecular biology will lead to characterization of important signaling pathways in ES and the identification of candidate targets for novel therapies. The specificity of targeted agents will make it possible that a therapy will be tailored to the specific and biologically relevant molecular targets of individual tumors, fitting in the era of personalized medicine. However, despite substantial progress made in recent years in molecular oncology and pathology, so far there are no clinically validated tests to assess the efficacy of drugs targeting aberrant activated signaling pathways in individual patients with ES. We are developing a highly sensitive, quantitative multiplex microbead suspension array approach to simultaneously measure phosphorylation and activation of multiple signaling proteins in small tumor samples from patients with ES, similar to what was done on lung cancer by our group [27]. This approach may serve as a clinically validated test to guide future therapeutic decisions with regard to utility of specific inhibitors for the individual patients based on the signaling profile of ES cancer cells. We are developing a primary human xenograpft model of ES to assess the efficacy of new therapeutic agents. We are also working in collaboration with other groups (academic, private, non-profit, and pharmaceutical) in clinical trials for future drug development for ES.

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Chapter 11

Molecular Mechanisms of Central Nervous System Metastasis

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Contents

11.1 Introduction

Brain metastasis occurs in up to 20–40% of cancer patients and regardless of the treatment, prognosis is usually poor [1–3]. The development of metastases undoubtedly contributes significantly to both morbidity and mortality in cancer patients, impacting prognosis and quality of life. It is well accepted that the more aggressive the cancer the more likely the potential for metastasis and vice versa. How and why tumors metastasize is still a topic of ongoing debate, although the migratory and invasive properties of the individual primary tumor are directly related to its metastatic potential. Malignant cells must manifest these properties in order to attain a more aggressive, and therefore more invasive phenotype. These abilities permit tumor cells to migrate and invade through the basement membrane associated with the tumor and its vasculature, compromising its integrity and initiating focal destruction of the extracellular matrix architecture. Although invasion into the lymphatics and/or vasculature followed by eventual extravasation is the primary physical modality that facilitates metastases from the primary site to distant anatomic sites, it is at the molecular level that expression of critical proteins is altered, effecting pathways within the cell that allow enhanced migration and invasion [4–8]. The central nervous system is a prime site for distant metastasis, particularly for such tumors as lung, breast, gastrointestinal, renal and melanoma, although most malignant tumors have been shown to have CNS metastatic potential [9–11].

Typically, metastases to the brain are seen as multiple, well demarcated neoplasms with significant necrosis and peritumoral edema and gliosis (Figs. 11.1 and 11.2). At the macroscopic level the pattern of distribution of lesions is often peripheral, a reflection of the embolic nature of the pathogenetic process as well as the smaller caliber of vasculature seen at the junction of gray and white matter.

11.2 The Metastatic Process

This involves several sequential steps, each one being imperative in its contribution to the final result [12–15]. For CNS metastasis these are: (1) separation from the primary neoplasm; (2) invasion through the basement membrane and extracellular matrix; (3) release into lymphatics or vasculature; (4) trapping in the brain capillary bed; (5) local growth; and

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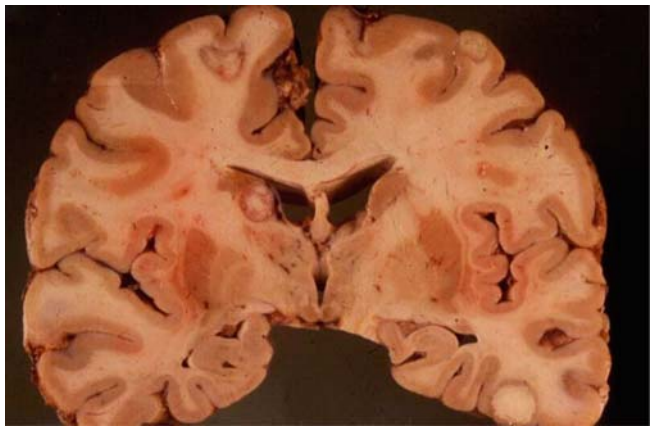

Fig. 11.1 Multiple, well delineated, solid and focally necrotic nodules of metastatic adenocarcinoma (*arrows*) are seen on this coronal section through the cerebral hemispheres

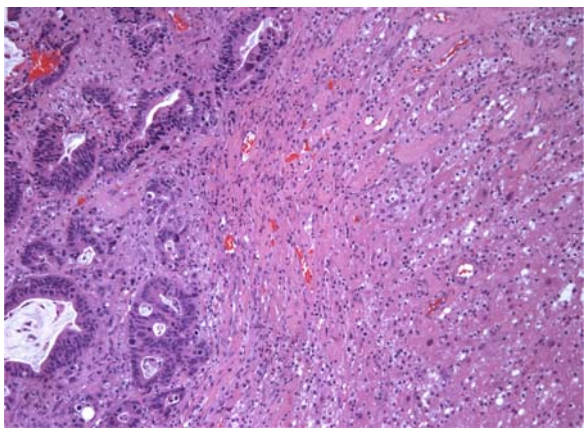

Fig. 11.2 Microscopically, the lesions are frequently well-delineated from adjacent brain which demonstrates reactive gliosis. Formalin fixed, paraffin embedded, Hematoxylin and Eosin stained section – original magnification 200

(6) angiogenesis. Studies show that it is essential that the malignant cells reach the vasculature of the brain and attach there, invade through the endothelial wall, and be able to grow in the brain parenchyma in order for metastasis to form [16–20]. In addition, there are data that suggest that it is not the amount of circulating single cells that correlate with metastasis, rather it is the presence of tumor cell clusters that is more important as a determinant of metastasis [13, 21–24].

11.3 Molecular Mechanisms

Any attempt to answer questions such as why certain cancers notoriously metastasize to the CNS whereas others (such as prostate adenocarcinoma) metastasize widely to other sites but almost never grow in the brain; or what potential targets can be explored for the development of successful therapeutic modalities, we must first examine the molecular mechanism of metastasis. Although the steps outlined in the preceding paragraphs identify the basic steps of the metastatic process, the molecular mechanisms are far more complex and challenging. Many of the molecules and pathways are involved in several different steps of metastasis and often influence multiple pathophysiologic components such as growth, proliferation, angiogenesis, apoptosis and migration. Many of these actions occur concurrently, and frequently are interdependent.

11.4 Hedgehog Signaling Pathway

The first steps in metastasis are separation from the primay tumor and local invasion. It is well recognized that the hedgehog (Hh) signaling pathway, plays a critical role in cell growth and tissue differentiation during embryonic development [25–28]. Hedgehog signaling plays a role in regulating the motility and migration of multiple cell types, including endothelial progenitor cells involved in microvascular remodeling, during would healing, as well as in yolk sac and embryonal vasculogenesis [29–31]. The mammalian Hh ligand, sonic hedgehog (SHh), activates the Hh signaling pathway by binding to their receptor, Patched 1 and 2. This halts Patched suppression of Smoothened (Smo), a membrane protein related to the G-protein receptor family, which upon activation promotes nuclear translocation of a family of transcription factors, Glis, to active Hh genes [26, 32, 33].

There are a variety of human cancers known to be caused by mutations leading to inappropriate SHh pathway signaling, including basal cell carcinoma, gastric carcinoma, medulloblastoma, pancreatic cancer and prostate cancer. Mechanisms include excessive activation due to mutations in the Smo gene and/or loss-of-functions mutations in the Patched gene. Inhibition by using a ligand-blocking antibody or Smo inhibitor, such as KAAD-cyclopamine, inhibits tumor growth [34–37]. Recently it has been reported that SHh signaling is involved not only in the *de novo* vascularization of certain embryonic tissues, but also in angiogenesis [38]. In addition, studies by Hochman et al. have shown that components of the SHh pathway may directly participate in cell migration and angiogenesis. Inhibition of the SHh pathway blocks SHh-induced migration and vascular generation [39]. Young et al. showed that higher concentrations of N-SHh increased cell motility and invasiveness in malignant cells and this enhancement was halted by inhibition of the SHh pathway by KAAD-cyclopramine or anti-SHh antibodies [40]. In addition, interference with SHh signaling decreases the activity of key metalloproteinases (MMP-2 and -9) involved in cell migration and invasion, suggesting these proteins may also be linked to SHh-mediated metastasis [40]. Studies on pancreatic cancer showed reduced metastasis with disruption of the SHh pathway and increased Gli expression has been linked to lymphatic metastasis [41, 42]. These findings, taken together, suggest that SHh activity is with a major molecular player in stepwise progression of metastases.

11.5 Transforming Growth Factor-β

Transforming growth factor (TGF)-β is a pleiotropic cytokine that plays a critical role in regulation of cell growth, differentiation and migration [43–45]. The primary mechanism of TGF-β is signal binding to specific receptors with serine/threonine activity, TGF-β type 1 and 2 receptor (TGF-βR1, TGF-βR2). TGF-βR1 is also referred to as activin receptor-like kinase (ALK) which also has a role in TGF-β signal mediation [46]. Coupling of TGF-β to TGF-βR2 leads to activation of ALK and the Smads receptor [47–50]. Mutations in each of these components can contribute to tumorigenesis [51–53]. In particular, mutations leading to continued activation of ALK5 enhance invasion and angiogenesis through the regulation of matrix metalloproteinases (MMPs) [54–56]. MMP overexpression correlates with invasiveness of certain tumors [57–60]. Also, TGF-β activation of several related pathways (mitogen-activated protein kinases and PI3K/Akt) can enhance malignant cell migration and immunosuppresson, thus altering the tumor micro-environment [61–63]. TGF-β can aid in metastasis by breaking down basement membrane barriers and promoting malignant cell motility [54, 55, 64, 65]. Recent research by Young et al. revealed that TGF-β is more effective in promoting invasion and migration in the presence of SHh. Anti-TGF-β-blocking antibody reduces migration and invasiveness of malignant cells. Blockade of the ALK5 kinase significantly reduces SHh-induced cell motility. Taken together, the results show that SHh promotes motility and invasiveness of malignant cells through TGF-β-mediated by activation of the ALK5-Smad 3 pathway [40].

11.6 Angiopoietin Pathway

There are numerous additional proteins involved in invasion by tumor cells. Angiopoietin 2 (Ang2) is a known angiogenic regulator found on both endothelial cells and tumor cells that plays important roles in angiogenesis and tumor progression and has been significantly associated with tumor metastasis and invasion [66–71]. It has been shown that Ang2 functions via the integrin receptor family, which is crucial for migration and invasion of tumor cells [72–74]. Mutations within specific integrins have been shown to affect cell adhesion, invasion and metastasis [75]. Specifically, Ang2 is a potential substrate for integrins in endothelial cells, fibroblasts and myocytes enhancing cell adhesion and triggering intracellular signaling pathways [76–78]. Recent studies show Ang2 stimulates tumor cell invasion via up-regulation and activation of matrix-metalloprotease 2 (MMP-2) [79]. This mechanism appears to overlap with the TGF-β pathways discussed above. Ang2 interacts specifically with αvβ1 integrin activating focal adhesion kinase (FAK), p130Cas, extracellular signal-regulated protein kinase (ERK)/mitogen – activated protein kinase, and c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase, thereby enhancing MMP-2 expression and secretion [80–84]. MMP-2 then degrades the

extracellular matrix allowing invasion of tumor cells [79]. Ang2 and MMP-2 are found at the invasive edge of tumors, but not in the more central areas [57, 59]. Vascular endothelial growth factor proteins enhance endothelial cell adhesion, migration, and survival via the integrin family receptors [85].

11.7 Matrix Interactions

An important component of the metastatic process is the implantation and subsequent proliferation of metastatic tumor cells within the host organ. This interplay between factors associated with the newly arrived cells and the host microenvironment is a key factor determining the success of the metastatic process. In addition to their ability to physically create a space for themselves, these tumor cells must also generate a significant blood supply for them to grow to a size greater than 2 mm in diameter and become clinically detectable. As noted, MMPs are critical to various steps in the sequence of events leading to metastasis, but particularly as the principle pathway to remodel the extracellular matrix [86–89]. Most invasive human malignancies are associated with up-regulation of the metalloproteinases, especially MMP-2 and MMP-9. Tissue inhibitors of MMPs (TIMPs) are considered the classical regulators of the proteolytic activites of the MMPs, a family of zinc dependent endopeptidases [87–89] and hence tumor inhibitors [90–94]. However, more recently, it has become evident that TIMPs are truly multifunctional proteins with far-reaching effects. They encompass an extensive repertoire of functions that are both MMP dependent and MMP-independent.

These effects are either tumor inhibitory or paradoxically, tumor promoting. TIMP1 is a mitogen for various cell lines [91–97]. Elevated TIMP1 levels is associated with increased invasion and poor prognosis in many malignancies, including non-small cell lung carcinoma [92]; breast cancer [93] and colorectal carcinoma [94]. In concert with the multiplicity of tumors impacted by these mechanisms, an equally diverse range of mechanisms have been implicated including cell proliferation, tumor infiltration and growth, angiogenesis and apoptosis involving a yet unidentified receptor mechanism. These studies have reinforced the concept of MMP independent activity with involvement of signaling pathways.

We examined the effects of overexpression of TIMP-1 in a CNS model of metastasis, focusing primarily on the interaction of TIMP1 in the CNS microenvironment, particularly its impact on the implantation and growth of tumor. Following implantation of lung adenocarcinoma cells transfected to overexpress human TIMP-1, we demonstrated both increased tumor size as well as more aggressive tumor growth patterns with multiplicity of tumors and increased invasion. Utilizing in vivo and in vitro analysis of vascular patterns, we noted increased angiogenesis either in tumors from these cells or when serum-free medium from TIMP-1 overexpressing clones was used. Gene expression profiling of TIMP-1 clone exhibited a 3-fold reduction of thrombospondin-1 (TSP1) expression. TSP-1 is a well documented inhibitor of angiogenesis. An elegant study by Watnick et al. (2003) describes the role of *ras* and *myc* oncogenes to repress TSP-1 leading to increased angiogenesis [95]. These studies indicate that TSP-1 is a crucial inhibitor of angiogenesis, just as VEGF is an activator. These studies further confirm the role of TIMP-1 to promote tumor growth and suggest yet another possible mechanism for its interaction in the host microenvironment.

11.8 VEGF Pathway

The growth and spread of metastases are dependent on the establishment of an adequate blood supply [22, 23, 90, 96–100]. Angiogenesis occurs by sprouting and non-sprouting methods. The prior involving branching of new capillaries from preexisting vessels, and the latter resulting from enlargement, splitting and fusion of preexisting cells produced by proliferation of endothelial cells within the wall of a vessel [101]. Vascular endothelial growth factor (VEGF) is a well known participant in the increased vascular density of tumors and correlates directly with a poor prognosis [102–110]. There are several possible mechanisms to explain this phenomenon: cells that have increased expression of VEGF may be more successful in outgrowing their dormant state after metastasis and the probability of cellular entry into the vasculature may increase with vessel density [97, 111]. However, there is research that shows that for certain sites, induction of angiogenesis is not a prerequisite as the pre-existent vascular

bed may be sufficient for tumor growth as tumors can survive and infiltrate by co-option of pre-existing vessels [107, 112–120].

When VEGF is involved in tumor metastasis it appears that endothelial cells and pericytes in dilated vessels are induced to proliferate and migrate, thereby covering the micronodules protruding into the lumen of these vessels [121]. There is also speculation that this encasement of tumor cells by endothelial cells can provide protection from the immune system, thus increasing the possibility of successful metastasis [121], providing a protected vascular "niche". Tumor cells that do not usually have efficient metastatic potential can successfully travel with VEGF expressing tumor cells, providing an explanation for the observation that metastasis form with little angiogenic potential despite origin from highly vascular tumors [119, 121]. Also, these tumor nodule out-pouchings are frequently found in the vasculature of patients with poor prognosis [107, 122]. Many tumors with a nodular phenotype and intravascular growth often have high VEGF expression [123]. For brain metastasis, in particular, studies show that VEGF is necessary, but not sufficient by itself, for the production and growth [124]. Rapidly enlarging brain metastases have numerous large blood vessels and the expression of VEGF directly correlates with angiogenesis and growth [124]. In one study, tumor cells with a mutant-VEGF had significantly decreased incidence of brain metastasis, though this was not found for all cancers [124]. Additional studies showed potential involvement in neovascularization of brain tumors by platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) [125]. Using similar pathways to VEGF these angiogenic molecules overcome inhibition of angiogenesis at the primary tumor site leading to disintegration of the basement membrane and migration of endothelial cells towards the tumor [126].

11.9 Other Molecular Pathways

In addition to the mechanism of actual growth, many studies have investigated why certain tumors metastasize more often to specific organs. For example, certain tumors have a predilection for neural tissue. In a series of studies by Schackert and Fidler it was demonstrated that certain cell lines metastasize to the meninges, whereas other preferentially formed tumors within the brain parenchyma [18–20]. A primary example of this is the study by Onodera [127] that showed that neural cell adhesion molecule (NCAM) expression was significantly higher in primary colorectal tumors that had brain metastases than those with metastases to other sites [127]. NCAM is involved in the formation of neuronal networks as well as in neuromuscular synapses [120, 128–130]. Another study involving melanoma cells showed that expression p75NTR, a common receptor for the neurotrophin family, correlated to brain metastasis as well as greater survival therein [131]. Mammalian neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic binding factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) [132]. All have been shown to affect certain malignant cell lines and to work through their own specific tyrosine kinase receptor [133–140]. Marchetti has also linked human heparanase to brain metastases [139]. In addition, nuclear factor com 1 was shown to be increased in brain metastatic tumors with significant increases of expression upon activation of receptor tyrosine kinase [141].

Tyrosine kinase receptors in their own right have been shown to aid in metastasis of small cell lung cancers to the brain, as well as other organs [142]. Another player in brain specific metastasis is the polymorphic cell adhesion molecule CD44. CD44 is a family of proteins generated by extensive splicing of CD44 pre-messenger RNA that is present in normal brain and primary brain tumors. A variant of CD44 (CD44v) has been shown to be increased in metastatic brain tumors, but not in normal brain or primary brain tumors. Metastases to the spine are almost always negative for CD44v expression [143, 144].

A 2003 study by Gerlach et al. revealed that malignant tumors with intracerebral metastasis had higher levels of tissue factor pathway inhibitor (TFPI) compared to their benign counterparts, as well as being higher in primary brain tumors [145]. In prostate cancer, higher levels of a splice variant of the Kruppel-like factor 6 (KLF6) suppressor gene was shown to lead to poorer survival due to more rapid metastasis and dissemination to distant sites, including the CNS [146]. Recent study of the notch signaling pathway in experimental brain metastasis showed that activation of this pathway led to more migratory and invasive cell lines implying that it may play a crucial role in brain metastasis [147, 148]. Finally, galectin-3 is increased

in metastatic breast cancer, but not in normal breast tissue [149].

11.10 Endogenous Inhibitors

An interesting adjunct to the topic of metastasis is the idea of endogenous inhibition. The concept of tumor dormancy suggests that there is a prolonged period of latency for micrometastasis prior to their growth and clinical appearance [150, 151]. While fast growing tumors have been shown to have strong angiogenic properties, recent findings suggest that the primary tumor may actually exert anti-angiogenic effects on silent metastasis. Brain metastases have been reported months or years after removal of the primary tumor [152–159]. It is believed that the balance between proliferation and apoptosis at the secondary tumor site is influenced by anti-angiogenic mediators released by the primary tumor and that removal of this primary site may then result in loss of inhibition of the secondary tumors angiogenesis. Two factors have been shown to take part in this phenomenon, angiostatin and endostatin. Angiostatin was the first endogenous anti-angiogenic factor isolated in 1994 [160–161]. It was shown to keep lung metastasis in a dormant stage by inducing an insufficient vascularization and a higher apoptotic rate [150, 160–163]. Endostatin is a carboxyl-terminal fragment of collagen XVIII 1 originally purified from a hemangioendothelioma [164]. Its anti-angiogenic properties are mediate specifically by inhibiting proliferation, migration, and tube formation of endothelial cells [164–168]. Though angiostatin and endostatin are the only two factors whose mechanism is well understood to date, research is being done to elucidate other proteins that may be involved in endogenous inhibition as well as their possible utilization for cancer treatment.

11.11 Conclusions

Investigations into the mechanisms of metastatic disease remain an integral part of neuro-oncology research. Many of the findings are recent and, in the final analysis, the pathogenetic mechanisms that are critical to the spread of cancer in general and the evolution of metastasis within the central nervous system still remain elusive. Despite the advances that have significantly contributed to our understanding of the intricacies of the metastatic process at cellular and subcellular levels, it remains abundantly clear that understanding molecular mechanisms will perhaps provide us the most useful information. Success in deciphering complex interactions in the tumor microenvironment, to the identification of specific therapeutic targets, as well as predictions of prognosis and response, all fall within the realm of these endeavors, and therefore the search for a better understanding must continue if we are to impact this devastating disease.

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Chapter 12

Carcinogenesis of Human Papillomavirus in Head and Neck Squamous Cell Carcinoma

Dahui Qin

Contents

12.1 Introduction

In 2000, head and neck cancer was ranked as the eighth leading cause of cancer death worldwide. Approximately 481,100 new cases developed, and 320,000 persons died of the disease [1]. In 2007, approximately 45,660 new cases developed in the United States and 11,210 patients died of the disease [2]. Head and neck squamous cell carcinoma is the most common head and neck malignant tumor. Carcinogenesis of head and neck squamous cell carcinoma is an important issue. Smoking and drinking are well known in carcinogenesis of head and neck squamous cell carcinoma. In recent years, Human papillomavirus (HPV) has drawn attention for its possible role in the carcinogenesis of head and neck squamous cell carcinoma.

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12.2 Squamous Cell Carcinoma

Squamous cell carcinoma is a malignant epithelial neoplasm arising from squamous epithelial cells [3], stratified squamous epithelial cells to be exact, not including simple squamous epithelial cells. For the purpose of simplicity, most pathologists use the term squamous epithelium for stratified squamous cell epithelium. We will do the same in this chapter. The squamous epithelium can be found on the surface of mucosa and surface of skin. The squamous cell carcinoma of head and neck can arise from both mucosa and skin. The head and neck squamous cell carcinoma originating from skin is the same as other skin squamous cell carcinoma. Very often, the term head and neck squamous cell carcinoma implies an origin from squamous mucosa of head and neck. The squamous mucosa of head and neck can be found at oral cavity, pharynx, larynx, nasal vestibule and other locations. Apart from the locations mentioned above, it is not unusual that other type of epithelia at head and neck may have squamous cell meteplasia. Squamous cell carcinoma can arise from such metaplastic epithelia.

12.3 Squamous Epithelium

The squamous epithelium is a thick epithelium. Its major function is protection. It consists of four layers, including stratum basale, stratum spinosum, stratum granulosum, and stratum corneum [4].

The stratum basale consists of a single layer of cuboidal or low columnar cells, which are often called basal cells. This layer of basal cells rests on the

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basal lamina. The nucleus of basal cell is relatively large and the cytoplasm is relatively more basophilic when compared with other layer of squamous epithelium. The basal cells are able to undergo mitosis and proliferation. The proliferation of basal cells is mainly responsible for the continual renewal of basal cell itself and the layers of cells above the basal layer. Mitosis can normally be seen at the basal layer. Immunohistochemistry stain using Ki-67 shows proliferative activity of basal layer. The stratum spinosum consists of polygonal cells with more cytoplasm. The cytoplasm is somewhat basophilic, but less so when compared to basal cells. The stratum granulosum consists of flattened cells with irregularly shaped coarse granules that are strongly basophilic in H&E staining. The stratum corneum consists of flat cornified cells, which lose their nuclei. In squamous mucosa, the thickness of stratum corneum varies, depending on it anatomic location. All cells of squamous epithelium are originated from basal cells and moved upwards towards the surface. As the cells move towards the surface, the cells become more and more mature. In other words, cells become differentiated and show characteristics of squamous cell epithelium. Squamous cell carcinoma cells may show different differentiation, recapitulating different stages of normal squamous cell maturation. If tumor cells resemble very mature squamous cells, the tumor is called well differentiated. If tumor cells show little squamous cell maturation features, the tumor is called poorly differentiated. In between, the tumor is called moderately differentiated.

12.4 Carcinogeneis of Head and Neck Squamous Cell Carcinoma

Carcinogenesis of head and neck squamous cell carcinoma involves multiple factors. The interaction between extrinsic factors and intrinsic genetic profiles may determine the outcome. It is known that tobacco and alcohol play an important role in etiology. Human papillomavirus (HPV) has been found in head and neck squamous cell carcinoma. Its presence in the head and neck squamous cell carcinoma of non-smoker and non-drinker implies its role in carcinogenesis. The related findings will be the focus of this chapter.

12.4.1 Human Papillomavirus (HPV)

HPV has been grouped in the Papovaviridae family [5]. It is DNA virus with double-stranded circular DNA genome of about 8,000 bp. HPV has no envelope and has pronounced tropism for squamous epithelial cells. The viral DNA and RNA transcripts for early gene expression are found in the basal cell layer, whereas virus replication, including the production of capsid proteins and assembly of infectious virions occurs at more superficial layers of squamous cell epithelium. The infected cells suffer from failure of terminal differentiation, leading to accumulation of cells at stratum spinosum. Cells that are permissive for the production of infective virions commonly display a ballooned cytoplasmic vacuole and darkened irregular-shaped nucleus. Such morphological features are termed koilocytosis. Over 100 different HPV serotypes have been identified [6], of which about 40 are associated with female genital tract HPV infection. HPV infection is associated with uterus cervical squamous cell carcinoma. Based on its correlation with squamous cell carcinoma, HPV is divided into two groups, high risk and low risk. Eleven HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 56, and 58, are consistently classified as high risk. Recently, seven additional types are added to the high risk list: 26, 53, 59, 66, 68, 73, and 82. The genomes of the various types of HPV resemble each other in genomic organizations. There are nine designated open reading frames (genes) that encode seven early proteins (E1, E2, E3, E4, E5, E6, and E7) and two late proteins (L1 and L2).

12.4.2 Role of HPV in Head and Neck Squamous Cell Carcinoma Carcinogenesis

More than 95% of uterus cervical squamous cell carcinoma is associated with HPV infection. Histologically, the squamous cell mucosa at head and neck bears similarities with cervical squamous mucosa. The evidence of HPV in head and neck squamous cell carcinoma carcinogenesis comes from three aspects, including epidemiology, laboratory research, and clinical observation.

12.4.2.1 Epidemiology Analysis

Several studies have compared the presence of HPV DNA in head and neck squamous cell carcinoma and control groups in the matched anatomic sites. Correlation of HPV DNA and head and neck squamous cell carcinoma is observed with Odds ratios between 0.5 and 6.2 [7–11]. One study shows that the Odds ratio after adjusting age, smoking and drinking is 3.7 [8].

12.4.2.2 Laboratory Analysis

Laboratory research data also indicates that HPV play a role in carcinogenesis of head and neck squamous cell carcinoma. In the 1980s, it was discovered that two HPV genes (E6 and E7) are necessary and sufficient for human keratinocytes to become immortal [12]. The expression of E6 and E7 is directed by the E6/E7 promoter in genital HPVs, which is tightly controlled by a complex interplay of positively and negatively acting host transcription factors, whose binding sites are present in the long control region (LCR) [13]. The long control region of genital HPVs contains four E2 binding sites in conserved positions, two of which are very close to the TATA box. Binding of E2 to these two sites has been shown to repress the promoter in a dose dependent regulation [14]. Intergration of HPV virus into genital squamous epithelium often interrupts E2 and hence may increase E6 and E7 expression [15]. Later, it was found that HPV16 can also transform oral epithelial cells [16, 17]. Furthermore, deletion of PDZ binding motif in HPV 16 E6 prevents the virus from inducing immortalization of the infected squamous cells [18]. The observation indicates the HPV 16 E6 may be important oncogene. It also indicates that PDZ motif is an important component of E6 oncogene. PDZ domains are about 90 amino acids long and form a 3-dimensional binding pocket. These sequences were initially discovered in PSD-95, DLG and Zo-1, hence PDZ [18]. HPV E6 and E7 play an important role in uterus cervical carcinogenesis mediated by high-risk HPV types [19]. The E6 protein binds to the tumor suppressor protein p53 [20]. The interaction of these two protein results in rapid degradation of p53 via the ubiquitin pathway [21]. E7 protein can bind to RB1 tumor suppressor protein, which leads to rapid degradation of RB1 via the ubiquitin pathway [22]. Destabilization of pRB causes release of E2F from pRb/E2F complexes. This permits E2F, a transcriptional regulator of cell proliferation genes, to transactivate S-phase-related genes. The functional inactivation of pRB by E7 leads to overexpression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ [23, 24]. The function loss of p53 and RB1 not only interferes with apoptosis, but also increase cellular proliferation. Both processes contribute to the carcinogenesis (Fig. 12.1). However, HPV carcinogenesis may be more complicated than our current understanding. A recent study reports that in animal, deletion of RB1 does not recapitulate all E7-mediated phenotypes [25], which imply that E7-mediated carcinogenesis may involve mechanisms beyond the RB1 pathway.

12.4.2.3 Clinical Analysis

Clinical data provides another line of evidence indicating that HPV may play a role in carcinogenesis of head and neck squamous cell carcinoma. Koilocytosis is a morphological change associated with HPV infection. It is commonly seen in the uterus cervical lesion and associated with cervical carcinoma. Koilocytosis is also identified in the head and neck squamous mucosa [26]. Head and neck squamous cell carcinoma may arise from a precursor lesion, leukoplakia. It has been reported that 31% of leukoplakia biopsy shows positivity for HPV DNA [27–31]. Lind et al reported a 20 case follow up. Among 20 cases, seven develop into squamous cell carcinoma in 10 year of follow up period [32]. Furthermore, HPV DNA has been identified in squamous cell carcinoma in head and neck [33–39]. Two oncogene products (E6 and E7), which are identified in uterus cervical carcinoma, have also been found in head and neck squamous cell carcinoma [40–43]. Women with uterus cervical carcinoma have higher incidence of second primary head and neck squamous cell carcinoma, when compared with a control group of women who have other cancers [44]. A recent case report shows a coupled-husband and wife has synchronous squamous cell carcinoma of head and neck [45]. The tumors in both of them are positive for HPV. The HPV in both tumors belongs to same prototype (HPV 16R). Not only that, the HPV in both tumors shares an uncommon signal variant nucleotide. Such observation implies that this unique HPV is transmitted between this couple and may be associated with

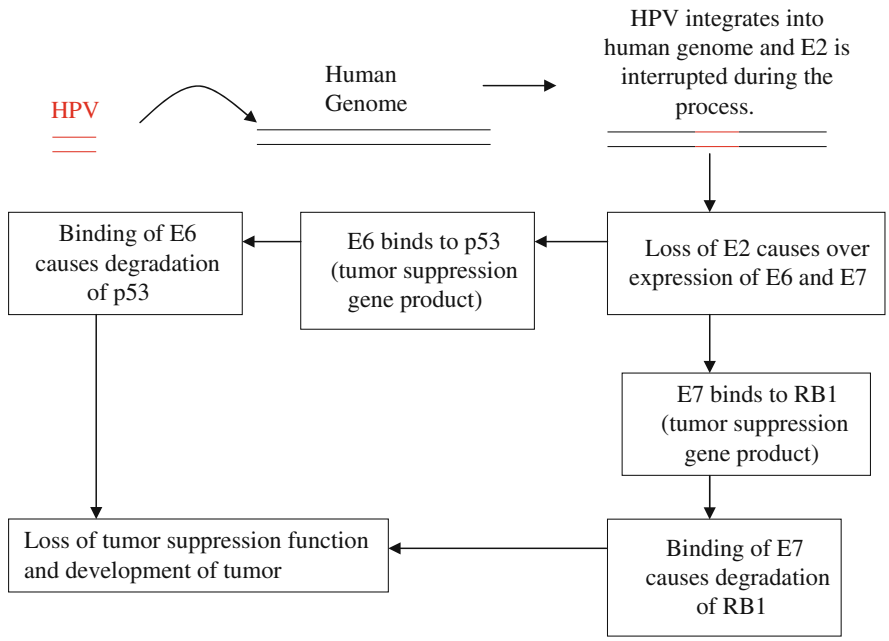

Fig 12.1 Carcinogenesis of HPV. The integration of HPV into human genome interruptes E2 of human genome. The loss of E2 causes over expression of E6 and E7, which eventually results in degredation of RB1 and p53. RB1 and p53 are tumor suppressor factors. Loss of RB1 and p53 will increase the risk of developing carcinoma

carcinogenesis of both tumors in this couple. HPV DNA is not only found in head and neck squamous cell carcinoma, but also changes host cell gene expression. A recent study compares the gene expression profiles of HPV positive and HPV negative head and neck squamous cell carcinoma. The results show that HPV positive and HPV negative head and neck squamous cell carcinoma have different gene expression profiles [46]. This result implies that HPV DNA in head and neck squamous carcinoma not only correlates with squamous cell carcinoma occurrence, but also affects cancer cell gene expression. This also implies that carcinogenetic process induced by HPV may be different from the one induced by other carcinogenetic factors. Several studies show that the HPV associated head and neck squamous cell carcinoma has better survival rate than that of HPV-negative ones. These data also implies that HPV oncogenesis mechanism may be different from other oncogenetic processes in squamous cell carcinoma. However, different observation about the correlation of HPV infection and better prognosis is also mentioned [43].

HPV positive rate found in head and neck squamous cell carcinoma varies among different reports. On average, positive rate is about 22% [34, 47]. One of the factors that cause the variation of positive rate is the anatomic location. Head and neck squamous cell carcinoma can arise from different locations, for example, buccal mucosa, tongue, and tonsil etc. It has been reported that the squamous cell carcinoma at tonsil has a much higher HPV positive rate. Syrjanen has reviewed 422 tonsil squamous cell carcinomas, with 216 positive for HPV DNA [48]. HPV is not only identified in primary tumor, but also identified in metastatic tumor. Five studies show that HPV were found in both primary tumor and their lymph node metastasis [49]. Two most common types of HPV found in oral squamous cell carcinoma are HPV 16 and 18. Among these two, HPV 16 is found far more often than HPV 18 [50–54]. Low risk HPV 6/11 are also found in head and neck squamous cell carcinoma, esp. in verrucous carcinoma, in which a positive rate of 47% has been reported [47]. Low risk HPV 6/11 are more frequently found in recurrent respiratory papillomatosis with a positive rate of about 80% [55–60]. These data indicate that low risk HPV 6/11 are usually associated with benign lesion. In other words, low risk HPV 6/11 might only promote head and neck squamous cell proliferation. It may not stop squamous cell differentiation and maturation. The squamous cells infected by HPV 6/11 may not have the capacity of infiltrating growth and metastastis, which are the features of

malignant squamous cell carcinoma. Although HPV 6/11 has been identified in small portion of head and neck squamous cell carcinoma, its role in carcinogenesis of squamous cell carcinoma may not be significant. Its presence in head and neck squamous cell carcinoma may be a coincidence, whereas the squamous cell carcinoma is caused by other carcinogenetic mechanism. On the other side, high risk HPV, for example, HPV16 and 18 appear to play an important role in squamous cell carcinoma carcinogenesis. The difference of HPV 6/11 and HPV 16/18 may shed the light on the understanding of squamous cell carcinoma carcinogenesis. One of the differences between high risk and low risk HPV is PDZ domain. In high-risk cancer-causing HPV subtypes, the E6 proteins contain a PDZ binding motif, whereas low-risk HPV types lack this motif [18]. However, it is too early to rule out possible role of low risk HPV in carcinogenesis.

It is an intriguing fact that the squamous cell carcinoma at tonsil has high rate of HPV infection. Anatomically, the squamous cell epithelium at tonsil is continuous with adjacent oral and pharyngeal squamous epithelium. Underlying the squamous epithelium, there is abundant lymphoid tissue with germinal centers. The lymphoid tissue is an important part of our immune system. Theoretically, the vicinity of the lymphoid tissue should offer protection to tonsil squamous epithelium from HPV infection. The paradox is that tonsil squamous cell epithelium has significant higher rate of HPV infection. Why does tonsil squamous epithelium, which has such close proximity to oral and pharyngeal squamous epithelium, has significant higher incidence of HPV infection. One of the explanations may lay in its unique anatomic structure. Tonsil squamous mucosa has convoluted surface with numerous crypts. These crypts are easy to trap infective agents. A well-known fact is bacterial tonsilitis, which is a frequent site in oropharyngeal infection. The tonsil, as an immune organ, is designed to increase its exposure to infectious agents so that the tonsil can effectively launch immune responses to the infectious agents. The lymphocytes activated in the immune response will migrate into circulation and to reach other organs of our body. By doing this, our body is better protected from infective agents. However, the infectious agents trapped at the tonsil can be overwhelming sometimes. The tonsil is like a guard of our body. It attracts "enemy fire" and sometimes, takes the casualty itself. Tonsil squamous cell carcinoma not only has higher rate of HPV infection, but also has higher viral load. One report shows that E6 level of tonsil squamous cell carcinoma could be 80,000 times higher than non-tonsil squamous cell carcinoma [43]. This difference is striking. It may provide important clue leading to understanding HPV mediated carcinogenesis. It deserves further exploring as to why tonsil squamous carcinoma has such a higher viral load. Anatomically, tumor consists of tumor cells (malignant squamous cells in case of head and neck squamous cell carcinoma) and stroma. In case of tonsil squamous cell carcinoma, the stroma contains significant amount of lymphoid tissue with numerous germinal centers. It is necessary to know the distribution of HPV in tonsil squamous carcinoma, i.e. if the HPV is in malignant squamous cells or in underlying lymphoid stroma. In the tonsil lymphoid tissue, there are many germinal centers. It is well known that germinal centers contain follicular dendritic cells [61, 62]. The dendrites of follicular dendritic cells form three-dimensional network in germinal center. One of the unique features of this network is to trap antigen-antibody complexes, including antibody-virus complexes [63–67]. Therefore, it is necessary to know if there is any viral load in tonsil squamous cell carcinoma that is located in lymphoid tissue rather than in malignant squamous cells. In situ hybridization data for HPV in tonsil carcinoma should provide the answer for such a question. Although it is not fully understood why the squamous cell carcinoma at tonsil has higher rate of HPV infection, this unique fact provides a window for exploring the role of HPV carcinogenesis in head and neck squamous cell carcinoma. Finding an effective HPV test to monitor tonsil HPV infection and squamous epithelial dysplastic changes associated with HPV infection may help to reduce the incidence of head and neck squamous cell carcinoma.

12.5 HPV Testing Methods

Different methods have been used to monitor HPV infection. Most of these experiences come from monitoring uterus cervical HPV infection.

Most PCR protocols for HPV testing make use of consensus primers targeted to HPV L1 gene [19]. Such design is potentially capable of detecting all HPV types that affect the anogenital region. The primer sets

that amplify shorter regions of the L1 gene can be used to increase the sensitivity of analysis , especially when testing FFPE (formalin-fixed paraffin embedded) tissue because the DNA in FFPE tissue tend to be in smaller fragments. Following amplification using consensus primers, the HPV type can be determined by DNA sequence analysis. The different HPV types have differences in their L1 sequences. The amplified HPV sequence can also be detected using membrane hybridization with type-specific probes. In such an assay, the oligonucleotide probes for individual HPV types are arrayed on a membrane strip. The probe for each HPV type has a fixed position on a membrane strip. The PCR amplified HPV DNA will hybridize to the complement probes on the membrane strip at its unique position. The bound PCR amplified HPV DNA will be detected by a chromogenic reaction and the position of chromogenic reaction product in the array indicates the HPV type.

Another assay uses solution hybridiztion methods. A cocktail of RNA probes for either high risk or low risk HPV types will be used to hybridize. DNA will be extracted from the specimen. The cocktail of RNA probes will be mixed with the DNA and RNA:DNA hybrids will be formed if the specimen has HPV DNA. The RNA:DNA hybrids will be captured by antibodies specific for RNA:DNA hybrids, and the bound RNA:DNA will be detected using a chemiluminescent probe.

In situ hybridization is another assay that can identify HPV DNA in tissue sections. Florescence in situ hybridization (FISH) and chromogenic in situ hybridization (CISH) can be used. The advantage of CISH is that the florescent microscopy is not needed because chromogenic hybridization product can be observed using light microscope. Tissue can be counterstained and better correlation of HPV DNA and morphology can be obtained. Cocktails of probes for high risk HPV are available. The probes for individual HPV type can be developed. New assays have been developed; for example, the assays targeting on E6 and E7 instead of L1 and new algorithm of HPV test has been proposed [68]. The assays that have been used to detect HPV in head and neck are usually those used in uterus cervidal cancer. Therefore, not all HPV types have been tested in head and neck squamous cell carcinoma. Greater variation has been observed in head and neck squamous carcinoma-HPV study, including HPV positive rate and HPV viral load. The most prominent HPV type in head and neck squamous cell carcinoma is HPV 16. However, it may not be a bad idea to keep our minds open at present. For the culprits in head and neck squamous cell carcinoma carcinogenesis, HPV16 is a devil that we know. At the same time, one may wonder what is the devil that we don't know.

12.6 Conclusion

Head and neck squamous cell carcinoma is a relatively common malignant tumor and has tremendous impact on human health. Understanding the carcinogenesis of head and neck squamous cell carcinoma will help to reduce the incidence and help to treat the disease. Apart from smoking and drinking, HPV may be an important factor in carcinogenesis of head and neck squamous cell carcinoma. However, more study is needed in this field.

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Chapter 13

Postmenopausal Hormone Replacement Therapy and Breast Cancer – Clinicopathologic Associations and Molecular Mechanisms

Geza Acs and Michael J. Wagoner

Contents

13.1 Introduction

Breast cancer is the leading malignancy in women with an estimated 178,480 new cases and 40,460 deaths in 2007 in the United States [1]. Although the cause of breast cancer is currently unknown, several molecular pathways have been identified to play a role in breast cancer development and progression. Perhaps the most important pathway involves the estrogen receptor (ER). The causal relationship between ER expression and cellular responsiveness to estrogens and antiestrogens has been extensively studied in cell lines [2], animal models [3] and humans [4], and makes ER one of the most important therapeutic targets in breast cancer [5]. More than two thirds of breast cancers show ER expression at the time of diagnosis [6], and immunohistochemical detection of ER expression is routinely used in making decisions on hormonal (anti-estrogen) therapy for breast cancer [7]. Current anti-estrogen treatment strategies include blocking by selective modulators (e.g. tamoxifen, raloxifene), destabilizing and degradation of ER by selective down-regulators (e.g. fulvestrant) and disruption of estrogen synthesis (i.e. aromatase inhibitors), any of which alone can result in a substantial decrease of tumor growth in about 30–50% of patients with ER positive breast cancer [8].

13.2 Endogenous Sex Steroid Hormones and Breast Carcinogenesis

A large and compelling body of epidemiological and experimental data implicates estrogens in the etiology of human breast cancer [9]. Animal studies repeatedly demonstrated that estrogens can induce and promote mammary tumors in rodents and that removing the animals' ovaries or administering an anti-estrogenic drug has the opposite effect [10]. The most widely accepted risk factors for breast cancer (such as early menarche, late menopause, obesity, etc.) can be thought of as measures of the cumulative dose of estrogen that breast epithelium is exposed to over time [11–13]. Indeed, an association between the risk of breast cancer and persistently elevated blood levels of estrogen has

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been found consistently in many studies [14, 15]. Not only do increased circulating levels of estradiol confer an increased relative risk, women with levels in the highest quartiles of fellow hormones such as estrone sulfate, androstenedione, dehydroepiandrosterone, and testosterone all have similarly increased relative risk of breast carcinoma [15]. In addition to circulating steroid hormones, estrogens are produced locally within the breast tissue as well, the levels of which cannot be measured with simple blood tests. Enzymes such as aromatase and steroid sulfatase can convert precursors into active estrogen metabolites in the local breast environment and since many of these enzymes are cytosolic, the estrogens they produce can act within the cell immediately. This form of hormonal synthesis and action has been termed "intracrine" and it is thought to play as large a role in overall estrogen exposure of breast tissue as do circulating hormone levels [16].

Studies have shown that endogenous estrogens may exert their effects in breast carcinogenesis through either promotion of growth of epithelial cells or via the formation of DNA toxic metabolites [13, 17, 18]. The degradation of estrogens follows a multistep oxidative metabolism process. Phase I metabolism begins with one of several cytochrome P-450 enzymes which catalyzes estrone or estradiol to a hydroxycatechol estrogen. These metabolites are further broken down to estrogen quinones which can constitutively form unstable adducts with adenine and guanine in DNA [19–21]. The reduction of estrogen quinones back to hydroquinones and catechols further produces reactive oxygen species which have been theorized to be responsible for DNA damage associated with estrogens [22]. Phase II detoxification pathways active in breast tissue involve multiple sulfation, methylation, and glutathione pathways designed to protect against the aforementioned DNA toxicity of estrogen metabolites. 4-hydroxyequilenin, a reactive catechol metabolite of equilenin formed from the degradation of equine estrogens present in hormone-replacement-therapy prescriptions have been found to inhibit detoxification enzymes such as glutathione S-transferase P1-1 and catechol O-methyltransferase [23, 24]. It was hypothesized that such inhibition might shift the balance of the overall reaction towards the toxic estrogen metabolites and thus promote DNA damage.

The role of hormones other than estrogen is less clear [25]. While estrogen promotes ductal proliferation and maturation, progesterone, acting through the progesterone receptor (PR), is required for mammary gland side branching and alveolar differentiation [25]. Several studies have shown progesterone to impart protective effects on the breast tissue and unlike estrogens, elevated serum levels of progesterone have not been associated with increased risk of breast cancer in postmenopausal women [26, 27]. In fact, an inverse relationship between serum progesterone levels and breast cancer risk has been demonstrated in premenopausal women [28, 29]. This effect has been best studied in relation to pregnancy associated breast cancer risk reduction. Pregnancy confers a significant risk reduction if full term gestation is reached in the first 30 years of life. Interestingly, this protection does not seem to be conferred in the setting of preterm labor or aborted pregnancies. In contrast, preeclamptic pregnancies confer an increased risk reduction compared to normal pregnancy. Examination of the hormonal influences at play in these findings reveals that progesterone levels are relatively increased in preeclamptic women, while levels of estrogens are decreased [30–32]. Physiologically, the levels of progesterone peek in the last several weeks of pregnancy and are believed to promote the final maturation stages of the breast glandular epithelium in preparation for lactation. Progesterone is also the only hormone to show such a sharp increase in the last weeks of pregnancy. These findings in combination with the lack of benefit of abrupt pregnancy termination and increased benefit in preeclampsia strongly suggest that progesterone is the hormonal agent which confers the protective effects seen with a full term pregnancy.

13.3 Long Term Use of Postmenopausal Combined Hormone Replacement Therapy (HRT) Is Associated with an Increased Risk of Breast Cancer

Menopausal symptoms (i.e. hot flushes, vasomotor changes, mood disturbances, genitourinary symptoms such as atrophic vaginitis, etc.) affect more than 50% of women, and approximately 40% of women suffer from symptoms severe enough to seek medical help [33]. Among the spectrum of therapies prescribed, estrogen treatment, with or without a progestin, is the most effective therapy [34–36]. The trends in formulation have moved from estrogen-only or unopposed

replacement (ERT) to combinations of estrogens and progestins (HRT) due to the protective effects of progestins on the endometrium. In the US, the main replacement preparations contain conjugated equine estrogens (CEE) alone or combined with medroxyprogesterone acetate (MPA) [37]. Non-hormonal therapies have been reported to improve menopausal symptoms to a lesser degree than HRT, but have other beneficial effects [38–42]. HRT alone or combined with physical exercise and supplements, has also been demonstrated to have a positive influence on post-menopausal loss of bone mass and subsequent osteoporosis.

Given the associations between sex steroid hormones and breast cancer, the possibility that ERT and/or HRT may increase the risk of breast cancer has been a concern since their use in the 1950s. Accordingly, many observational and cohort studies examined the influence of HRT on breast cancer risk in the past decades. Although the results were contradictory, some studies have been interpreted as suggesting a small but significant increase in the risk of breast cancer in women using HRT at the time of diagnosis [15, 43–52]. The collaborative reanalysis from 1997 was an attempt to bring together and re-examine the individual data of all 51 relevant studies published thus far. It revealed that breast cancer risk increased by 2.3% per year of hormone use, compared with an increased risk of 2.8% per year of natural delay in the onset of the menopause [49]. The risk of breast cancer, which was not significant until 5 years of use, increased by 35% in women who had used HRT for 11 years on average. Within 5 years after discontinuation of treatment, the elevated risk has returned to baseline [49]. Overall, most studies indicated a greater risk associated with combined HRT than with unopposed estrogen treatment, particularly for hormone receptor-positive carcinoma. Between the mid 1970s and 1980s surveillance data showed that the incidence of ER positive tumors increased by an average of 131% in the population-based tumor registry of Kaiser Permanente, Portland, OR [53], and some investigators suggested that this finding may implicate the involvement of HRT use in the rising incidence of breast cancer [54].

Since randomized placebo-controlled trials are regarded as the highest level of evidence for the investigation of the impact of drugs on disease risk, the results of the Heart and Estrogen/Progestin Replacement (HER) study and both arms of the Women's Health Initiative (WHI) study were highly anticipated [55–57]. The HER study on the secondary prevention of coronary heart disease by continuous treatment with CEE/MPA, observed a statistically non-significant 27% increase in the relative risk of breast cancer after 6.8 years of use [55]. In the combined HRT arm of the WHI study, 5.2 years of treatment with CEE/MPA increased the relative risk of breast cancer by 24%, which reached statistical significance [57]. The authors of the study estimated eight additional breast cancer cases for 10,000 women-years for CEE/MPA treatment, which corresponds to the results of the collaborative re-analysis [57, 58]. Interestingly, a 2006 reanalysis of the WHI CEE/MPA data [59], revealed that breast cancer risk was significantly different in women who used HRT prior to the initiation of the trial compared to those not using hormones before: Among 4,311 prior users, the adjusted hazard ratio (HR) for CEE/MPA versus placebo was 1.96, significantly different from that among 12,297 never users (HR: 1.02). At the same time, the Million Women Study (MWS), a massive observational follow-up study designed to assess the risk of breast cancer among HRT recipients in a population of British women undergoing screening mammography, observed a relative risk of breast cancer in HRT users of up to 2.0 [60]. On the other hand, the arm of the WHI study investigating the effect of CEE alone in hysterectomized women, revealed no increased risk of breast cancer after 6.8 years of treatment [56, 61]. Similarly, in the study of Chen et al. [62] no significantly increased breast cancer risk was seen in women using unopposed estrogens for up to 20 years; however, these authors found a significantly increased risk (relative risk of 1.42) in women using unopposed estrogens for more than 20 years.

Although both the combined arm of the WHI study and the MWS were widely criticized and have not conclusively ruled out various sources of bias [63–68], their results were highly publicized in the media. The use of HRT at menopause has become a matter of intense debate, and its utility and safety has been questioned. Despite ongoing debate [67], the theory that combined HRT increases the risk of being diagnosed with invasive breast cancer appears widely accepted and the International Agency for Research on Cancer (IARC) has recently classified combined HRT as a class I carcinogen in humans [69]. However, many important questions remain unanswered. One of the major limitations to our understanding of the effect

of HRT on breast cancers is due to the fact that most reports are based on epidemiologic studies utilizing data extracted from cancer registries without central pathologic review and confirmation of diagnoses and tumor features. Given the typically small number of cancer cases included even in the largest studies and the known significant disagreement rate in the determination of tumor size, histological type, grade and receptor status even among expert breast pathologists, misclassification of even a few tumors could drastically change the results of such studies.

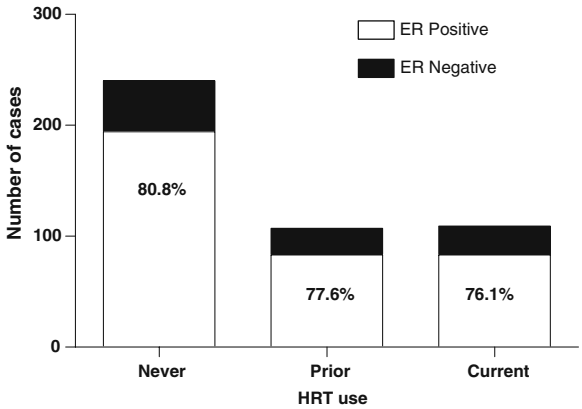

Fig. 13.1 Comparison of the ratio of estrogen receptor (ER) positive and negative cases of 456 breast carcinomas diagnosed in postmenopausal women according to use of combined hormone replacement therapy (HRT)

13.4 Association of HRT Use with Histologic Features and Prognosis of Breast Cancers

The expression of specific receptors by the tumor cells is considered to be a pathophysiological prerequisite for potential hormone effect on breast cancers. The analysis of data from the Nurses' Health Study revealed that postmenopausal women who used HRT had a higher probability of developing ER positive and progesterone receptor (PR) positive tumors [70]. HRT use increased the frequency of ER/PR positive invasive breast cancers 2- to 2.5-fold, whereas the effect on receptor-negative carcinoma was less pronounced [52, 71–73]. Other epidemiologic studies have reported a similar greater risk in current users of continuous combined HRT, concentrated in ER positive disease [48, 50, 72–74]. Indeed, use of HRT was found to be the greatest predictor of ER positive disease [75]. In contrast, other studies, such as the WHI, did not find a difference between HRT users and non-users with regard to hormone receptor status. Similarly, our own review of 456 consecutive breast carcinoma cases diagnosed in postmenopausal women at the Moffitt Cancer Center [76] showed no difference in hormone receptor status between HRT users and non-users (Acs et al., manuscript in preparation) (Fig. 13.1).

Other investigators have focused on the reported increase in the rate of lobular carcinoma compared to ductal (no special type) cancer [52, 77]. Given the usually ER positive nature of lobular carcinomas, it was hypothesized that HRT increases the incidence of lobular more than other types of breast cancer and account for the reported rise in the incidence of the former [52, 78, 79]. Seven epidemiological studies investigated the risk of breast cancer associated with HRT according to histologic type of tumors [71, 73, 77, 80–83]. In these studies, 387 lobular cancer cases and 1,582 ductal cancer cases were current hormone users. The results (summarized in Table 13.1) indicated that while the use of estrogens alone was associated with no or a slightly elevated risk, combination HRT increased the incidence of lobular and "mixed ductal-lobular" cancers to a much greater extent than that of ductal carcinoma. In addition to the fact that these results are based on relatively few cases of lobular cancer, several of these studies are from a small geographic region [71, 77, 80, 82, 84]. None of these studies had central pathologic review of the cases, which significantly limits the reliability of their conclusions. Given the small number of lobular cancer cases included, misclassification of even a few tumors could significantly change the results. It should also be kept in mind that the validity of comparing lobular carcinoma, a relatively well defined, single entity in its classic form, to the rest of the majority of breast cancers (designated "ductal" or "no special type" carcinoma simply based on the lack of diagnostic features of special subtypes of breast cancer) characterized by a wide variety of genetic alterations, phenotypic features and degrees of differentiation, is rather questionable. In addition, the entity of "mixed ductal-lobular" carcinoma is not defined and pathologists use this diagnosis to designate a variety of breast carcinomas ranging from ductal (no special type, NST) carcinomas with focal single cell

Table 13.1 Summary of results of epidemiological studies on HRT use and breast cancer histology

		Lobular carcinoma			Ductal carcinoma		
	Studies (*n*)	User cases (*n*)	RR (95% CI)	*P*	User cases (*n*)	RR (95% CI)	*P*
E only	6	164	1.44 (0.97–2.13)	0.001	795	0.90 (0.69–1.18)	0.083
E+P	6	182	2.82 (1.95–4.07)		629	1.15 (0.86–1.54)	
All hormones	7	387	2.19 (1.61–2.99)		1,582	1.08 (0.84–1.39)	

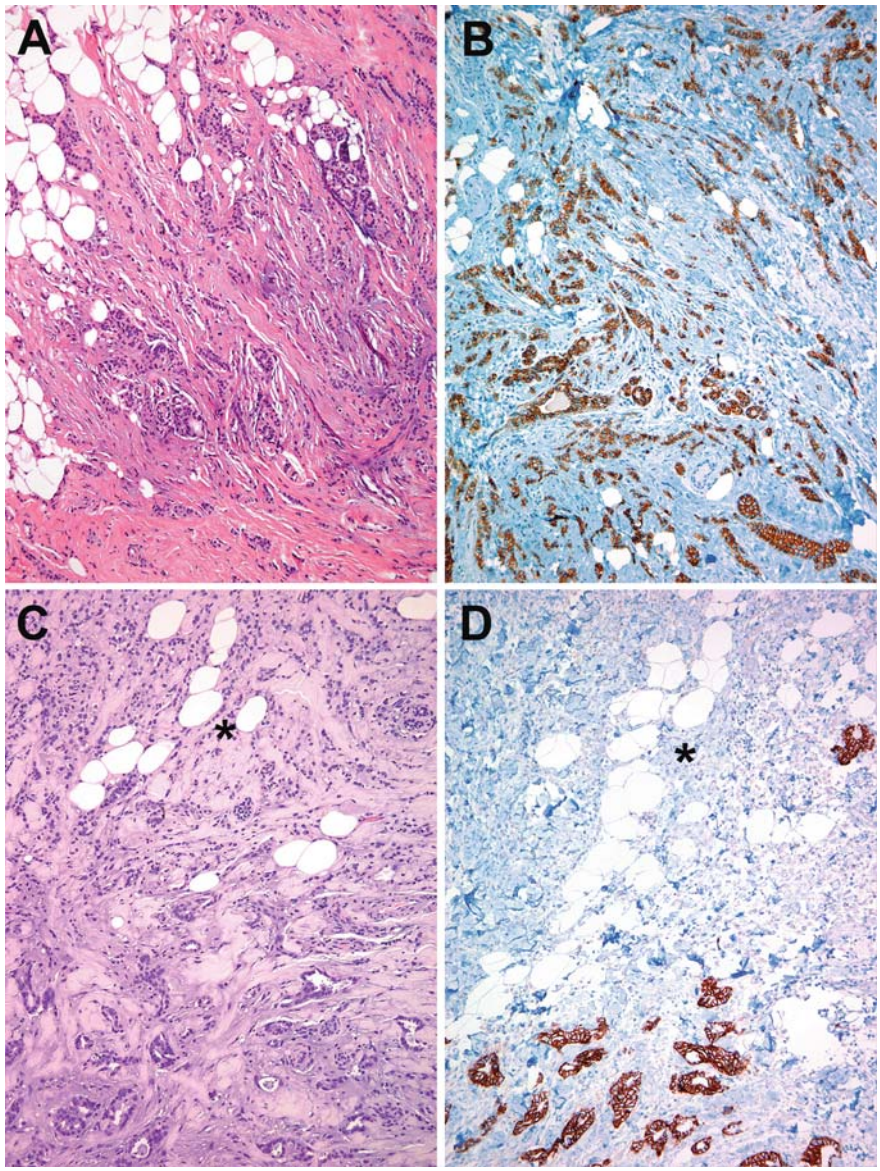

Fig. 13.2 **A**. Invasive carcinoma "with ductal and lobular features". The tumor cells focally show a single file infiltrating pattern reminiscent of lobular carcinoma. Small areas of lobular-like growth pattern can be often seen in otherwise usual ductal (no special type, NST) carcinomas and their presence does not warrant a diagnosis of mixed type carcinoma. **B**. The foci of carcinoma showing lobular-like growth patterns retain strong membrane staining for E-cadherin. **C**. Invasive carcinoma, mixed ductal NST and lobular type. The NST component of the carcinoma is composed of cohesive nests and glands, while the distinct lobular component shows dyscohesive cells growing in a single file infiltrative patter. **D**. E-cadherin immunostaining shows strong membrane reactivity in the NST carcinoma component and complete lack of staining in the lobular component

infiltrating pattern to carcinomas having clearly distinct components of classic lobular and ductal (NST) areas (Fig. 13.2). In fact, the only study using central pathology review [85] indicated that HRT is associated with low tumor grade, rather than a specific histologic type. In this study, the agreement rates for the diagnosis of lobular and "mixed ductal-lobular" carcinomas were only 68 and 18%, respectively, highlighting the

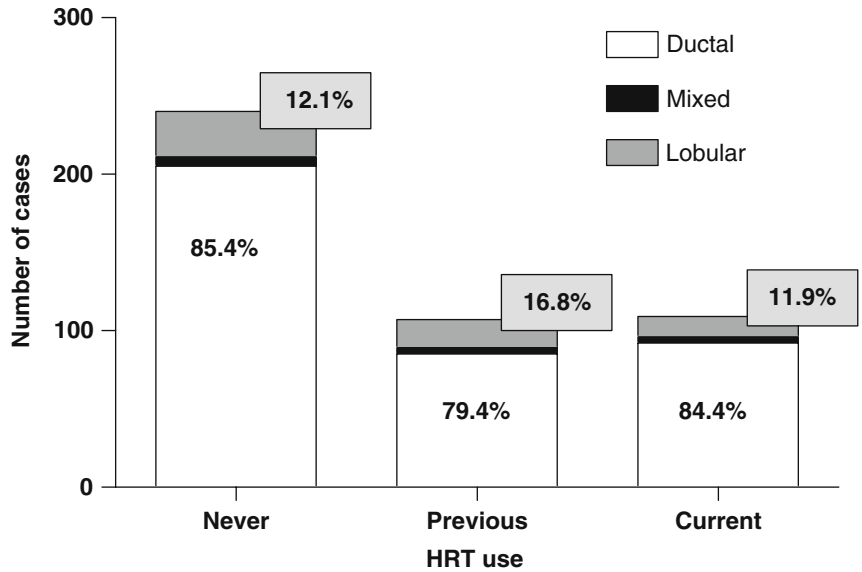

Fig. 13.3 Comparison of histologic type of 456 breast carcinomas diagnosed in postmenopausal women according to use of combined hormone replacement therapy (HRT)

importance of central pathology review. Our recent review of 456 consecutive cases of invasive breast carcinoma diagnosed in postmenopausal women at the Moffitt Cancer Center [76] showed no difference in the rate of lobular carcinoma according to HRT use when strict histologic criteria were applied (Acs et al., manuscript in preparation) (Fig. 13.3). The WHI CEE/MPA trial did not show such a differential risk either: 11.2 and 10.6% of the cancers in the HRT and placebo groups, respectively, were lobular cancers [58].

Although HRT has been shown to be associated with an increased risk of being diagnosed with invasive breast cancer, no increased breast cancer mortality has been demonstrated in HRT users in the reports. In contrast, most studies which investigated mortality have shown improved breast cancer survival in women using HRT [86–96]. Although the improved survival was suggested to be simply due to early detection of tumors by some investigators, studies also indicate that HRT users develop smaller, better differentiated tumors [97–112]. These findings suggest a possible effect of HRT on pre-existing tumors and that surveillance/detection bias is not the only explanation for better survival. Differing with most reports in the literature, the WHI CEE/MPA study found that tumors in the HRT group were larger and more likely to be associated with lymph node metastases than those in the placebo group [58]. The WHI investigators argued that their results are consistent with the stimulation of growth of established breast cancers and concluded that invasive breast cancers diagnosed in women who use HRT may have a worse prognosis. A recent systematic review of 25 studies on the influence of hormone use on prognostic factors of breast cancers concluded that, because of their methodology, the epidemiological studies cannot negate the findings of the WHI [113].

13.5 Initiation of New Breast Cancers Versus Promotion of Pre-existing Tumors

The exact pathogenetic mechanisms of breast cancer initiation and/or promotion are still poorly understood [51, 114–117]. One of the most important questions is whether postmenopausal HRT initiates the growth of new breast cancers or whether the epidemiologic results reflect a hormonal impact on pre-existing tumors. Studies in rodents have demonstrated that estrogens or their catechol metabolites are carcinogens in various tissues, including the mammary gland [13, 17, 118–122]. Although estrogens may theoretically be involved in the initiation of breast cancer, a carcinogenic/mutagenic role of HRT seems improbable. In almost every study reporting an increase in breast cancer risk with HRT, the increase in incidence has been found within a few years. There is general acceptance

that 30–35 tumor doublings are required to achieve a tumor size of 1 cm in diameter, which is more or less the smallest lesion that can be diagnosed in the clinic [65, 123, 124]. Although the doubling time of breast cancers is highly variable, in general a tumor doubles in size every 100 days. Thus, it is estimated to take approximately 7 years for a single malignant cell to grow large enough to become detectable by mammography and 10 years to grow to a clinically detectable 1 cm mass [125]. The rapid finding of an increased risk of breast cancer within a few years of HRT use suggests that the epidemiologic studies are detecting pre-existing tumors [126]. Accordingly, it is currently believed that HRT does not initiate new tumors, but may increase (promote) the likelihood of tumor growth at a later stage of carcinogenesis. Although it was also suggested that by stimulating cell division and thus increasing the rate of spontaneous mutations [10], hormones may contribute to the emergence of frankly malignant tumors from atypical precursor lesions [127], the available studies indicate that HRT use does not further elevate the breast cancer risk associated with proliferative breast disease with or without atypia [128–131] and thus, do not support such a hypothesis. Two recent studies examining the effect of either unopposed CEE or combined CEE/MPA treatment on the risk of benign proliferative breast disease within the WHI trials found that both CEE (average duration of 6.9 years) and CEE/MPA (average duration 5.5 years) treatments conferred an increased risk of benign proliferative breast disease without atypia (HR: 2.34 and 2.00, respectively), but neither had any significant effect on the risk of proliferative breast disease with atypia (atypical hyperplasia, HR: 1.12 and 0.76, respectively) [132, 133].

Recent US statistics have indicated a rapid decrease in breast cancer incidence immediately after the publicity surrounding the reports from the WHI and the resulting drop in HRT use. National data revealed a 7% decrease in breast cancer incidence in 2003, which was greatest in women aged 50–69 years and mostly confined to ER positive tumors [134, 135]. Breast cancer declined 10% in the Northern California Kaiser program in the years 2003 and 2004 [136]. It was suggested that these findings most likely reflect pre-existing cancers just below the detection limit in 2002 that slowed or stopped growing upon HRT removal [54, 68, 135]. An impact of HRT on pre-existing tumors also appears to be supported by statistics derived from the area around Geneva, Switzerland [137]. Beginning in 1997, the peak breast cancer incidence in the Geneva area has moved to a younger group of women (aged 60–64), with the increase occurring only in early stage disease with ER positive tumors in HRT users. However, the drop in incidence appears to be too immediate after cessation of hormone use, it was also seen for advanced stage disease, and other explanations (e.g. a decline in the prevalence of screening mammography [138]) have not been excluded. As MacMahon and Cole [139] noted, the reported increase in breast cancer incidence rates before 2000 probably reflects the increasing use of more effective methods of breast cancer screening leading to earlier diagnosis. As they point out, the cases detected by screening during a fixed time period will increase the number of cases diagnosed and hence the incidence rate of the diagnosed disease over time. At the same time, effective screening also decreases the number of preclinical cases to be found by screening in subsequent years, which could have contributed to the decline in breast cancer incidence after 2003.

13.6 The Effect of HRT on the Proliferation of Breast Cancers

Epidemiological studies suggest that the impact of estrogens on the relative risk of breast cancer is modest, but it is significantly enhanced by the addition of progestins. In contrast to the endometrium, epithelial cell proliferation in the normal breast reaches its peak during the progesterone-dominant luteal phase of the menstrual cycle [140–142]. This observation has been the driving force behind the argument that progestins are the major hormonal mitogens in the breast and that combined HRT stimulates the proliferation of pre-existing breast cancers. However, most experimental studies do not support a major role for an adverse progestational influence on breast cancer. In animal models, estrogen, not progesterone, is the major inducer of proliferation, and evidence indicates that with increasing duration of exposure, progesterone can limit breast epithelial growth as it does in endometrial epithelium [143–145]. In vitro studies of normal breast epithelial cells also revealed that progestins inhibit proliferation [146]. The story with benign human breast tissue

specimens removed after women were treated with estrogen and progestin is more confusing, indicating on one hand that progestins inhibit in vivo estrogen-induced proliferation [143, 144, 147], and on the other hand that markers of epithelial cell proliferation are higher in women being treated with estrogen-progestin [115, 148]. Similar effects of CEE and CEE/MPA were observed in a monkey model [149]. Nevertheless, progestins have also been demonstrated to decrease anti-apoptotic protein expression [150], and apoptosis in breast tissue is also higher in the luteal phase than in the follicular phase [151].

Importantly, there are profound differences between benign and malignant breast tissue concerning the hormone dependent regulation of cell proliferation. In the resting normal mammary tissue ERα and PR are expressed in very few epithelial cells, while ERβ is present in 70% of the cells. The minority (approximately 2%) of epithelial cells which are proliferating, do not contain ER and the mitoses are probably controlled by paracrine interactions of adjacent epithelial cells [152, 153]. In contrast, the transition of benign to malignant mammary tissue is characterized by a switch from paracrine to autocrine regulation of epithelial cell proliferation by sex steroids, i.e., in breast tumors ERα and PR are also expressed in proliferating cells [154–156]. Studies have shown that the mitotic rate of both ER/PR positive and ER/PR negative breast carcinoma is higher in the luteal phase than in the follicular phase of the menstrual cycle [142]. In addition, studies indicate that neither ethinylestradiol, plus norethisterone, nor tibolone had a significant effect on the proliferation of normal breast epithelium [157, 158], even though both tibolone and all types of estrogen/progestin combinations were found to be associated with an increased risk of breast cancer [60, 73, 74, 159, 160]. It is thus questionable whether the effects of different HRT preparations on benign mammary epithelium reflect those on breast carcinoma. In fact, most studies which have investigated the proliferation rate of breast cancers in women using HRT found that tumors detected in these patients showed significantly lower S-phase fraction, mitotic activity and Ki-67 labeling index, compared to tumors in non-users [61, 97–111, 161].

Studies of patients with invasive breast carcinoma have shown that early changes (<3 weeks) in cell proliferation and PR expression occur after antiestrogen therapy in ER positive but not in ER negative tumors [162–165]. These changes are accepted surrogate markers of clinical tumor response. In one study [165], the decrease in proliferation correlated with subsequent reduction in tumor size in patients receiving tamoxifen therapy. Despite its clear clinical importance, there is only a single study reported in the literature investigating the effect of HRT withdrawal on

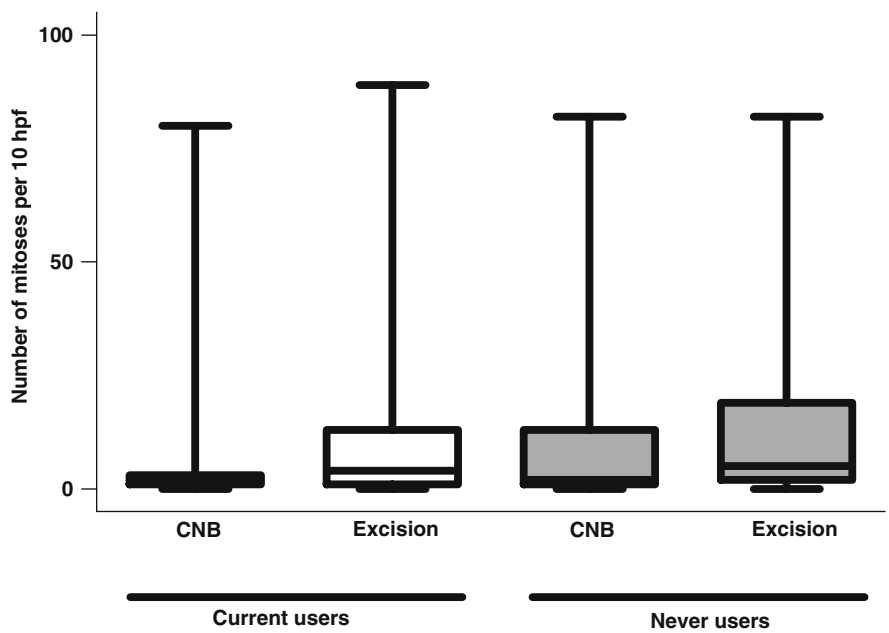

Fig. 13.4 Comparison of mitotic activity (number of mitoses per 10 high power fields) in core needle biopsy (CNB, taken when patients were receiving combined hormone replacement therapy (HRT) in the current user group) and corresponding subsequent excisional biopsy samples (taken at least 3 weeks after cessation of HRT) of breast cancer in current HRT users and never users

the proliferation rate of breast cancers [166]. Although limited by small sample size and methodological flaws, the results suggested a decrease in proliferation after withdrawal of HRT in ER positive, but not in ER negative tumors. On the other hand, similar to prior results, this study also showed that tumors in HRT users had significantly lower proliferative activity compared to cancers in non-users. Our recent results obtained in 404 consecutive postmenopausal breast cancers showed that there was no significant decrease in proliferation after HRT withdrawal in the tumor cells measured by either the number of mitotic figures per 10 high power fields or Ki-67 labeling index (Acs et al., unpublished data) (Fig. 13.4).

13.7 Effect of HRT on Hormone Receptor and Gene Expression Profile in Breast Cancers

It is well recognized that early pregnancy produces changes in the breast that result in resistance to carcinogenesis [167–169]. In rodents a similar effect is accomplished by treatment with estrogen plus a progestin. The refractory phenotype produced is associated with progestin-induced changes in the expression of genes involved in cellular proliferation [124, 170]. The PR is induced by estrogens at the transcriptional level and decreased by progestins at both the transcriptional and translational levels [171]. The PR has two major isoforms, designated PR-A and PR-B receptors [172]. The two PR forms are expressed by a single gene, a consequence of transcription from distinctly different promoters, in a complex system of transcription regulation [173]. The breast tissue of normal women expresses equal amounts of PR-A and PR-B, while breast cancers are associated with increased PR-A levels [174, 175]. PR-A and PR-B have different molecular functions and affect different genes. Therefore, the target tissue response to progestins depends on the differential expression of each receptor and the ratio of their concentrations, as well as the target tissue context of adaptor proteins [176, 177]. In most cells PR-B is the positive regulator of progesterone-responsive genes, and PR-A inhibits PR-B activity. The broad activity of PR-A suggests that this PR isoform regulates inhibition of steroid hormone action wherever it is expressed [178]. In the absence of progestins the PR-A isoforms are dominant and they can exert gene regulation in ER positive breast cell lines even in the absence of their ligand [175]. In the absence of progestins, unliganded PR-A can up-regulate genes associated with aggressive growth, invasion and poor prognosis, including genes that provide resistance to apoptosis, and adversely influence the biology of ER positive tumors [174, 175]. Indeed, ER positive breast cancers with a higher rate of recurrence were shown to be rich in the PR-A isoform [174, 175]. PR-A-rich tumors with a high PR-A/PR-B ratio do poorly and respond less well to tamoxifen [174]. In the presence of progestins, however, PR-B is a stronger regulator of gene transcription. In monkeys the breast levels of PR-A were unchanged after 3 years of treatment with CEE alone [179]. In contrast, treatment with CEE/MPA produced a decline in PR-A levels with a 10-fold beneficial change in the PR-A/PR-B ratio. It thus seems possible that exposure of an ER positive tumor to estrogen-progestin treatment can prevent an unfavorable PR-A/PR-B ratio, promoting the beneficial actions of PR-B.

Early molecular biology studies also provide support for a favorable effect of estrogen-progestin exposure in breast cancers. Estrogen regulated genes can be separated into two groups [180, 181]: The first group is associated with poor prognosis with high expression of cell proliferation and anti-apoptosis related genes. The other, good prognosis group is associated with better differentiated tumors with better survival and response to tamoxifen. In vitro studies using microarray analysis have profiled the gene networks regulated by estrogen [182]. Genes that are up-regulated by estrogen are down-regulated by estrogen-progestin treatment [183]. There is only a single study reported in the literature that examined the gene expression profile of breast cancers in HRT users [183]. This study showed that HRT use altered the gene expression profile only in ER positive cancers. Comparison of HRT users and non-users, 276 genes were found to be activated by HRT exposure. Among the genes regulated, many were involved in either DNA repair or cell cycle regulation. Interestingly, the pattern of changes in the HRT treated tumors was very similar to those observed in breast cancer cells treated with tamoxifen [183]. All women in this cluster were free of recurrence 5 years after diagnosis. In a cohort of 131 breast cancer patients, the women whose tumors exhibited the gene expression

profile associated with combined HRT exposure preferentially benefited from tamoxifen treatment [183].

Additional supporting data for a potential beneficial effect of combined estrogen-progestin exposure can also be found in two recent studies. A retrospective cohort study in the Southern California Kaiser program showed a reduction in breast cancer case mortality that was significant only among women with breast cancer who were users of estrogen-progestin and not among users of estrogen alone [184]. A large study of 374,465 women screened in six US mammography centers reported that an increase in lower grade, lower stage, ER positive cancers was found only in current users of estrogen-progestin [105]. The molecular and clinical data thus suggest a potential beneficial effect of estrogen-progestin treatment on the biology of breast cancers, likely based on changing the PR-A/PR-B ratio and activity.

13.8 Chemo-/Hormonal Prevention

Numerous studies have shown that tamoxifen use in high risk patient populations can significantly reduce the development of ER+ breast carcinoma [185–187]. Furthermore, these studies have shown that the improvements in risk profiles increase with time after the 5 year course of Tamoxifen has been completed. Recently, investigators have examined the use of prepubertal hormonal exposure simulating pregnancy in order to reduce the risk of breast carcinoma in mouse models [188]. Specifically, mice implanted with mammary tissue harboring deleted p53 and exposed to short term (2–4 weeks) hormonal therapy received both significant short term and long term protective effect from the treatment compared to control mice. Additionally, the mice received similar protective effects from estrogen alone or estrogen plus progesterone. Investigation as to the cellular mechanisms of such conferred protection revealed a 53–85% reduction in the proliferative potential of the mammary cells of treated mice compared to control animals. This protective effect was also observed in other model systems [188]. The results of such animal studies certainly raise the intriguing question whether this model might translate into a “hormonal vaccine” in humans. Numerous studies have demonstrated the protective effects of early pregnancy on breast tissue [167–169, 189, 190]. While the exact cellular mechanisms of this protection are not fully understood, the effect likely involves the attenuation of the proliferative potential of mammary cells, up-regulation of tumor suppressor genes, and maturation of terminal duct lobular units to a more stable form [168, 169, 191].

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Chapter 14

Carcinogenesis of Lung Cancer

Jeffrey Aufman and Farah Khalil

Contents

14.1 Incidence and Epidemiology

There are an estimated 1.2 million deaths per year world wide from lung cancer, making it the most common cause of mortality worldwide [1]. More people die from lung cancer in the United States, estimated 162,000 in 2007, than colon, breast and prostate cancer combined, estimated 124,000 deaths [2]. The health-related economic loss associated with cigarette smoking is approximately $157 billion [3]. The incidence rate in men has declined since 1984 and increased in women since 1990. Lung cancer usually occurs between the ages of forty and seventy years, with a peak in the fifties or sixties. Fifty nine percent of patients diagnosed with lung cancer die within one year and only 15% survive after five years [1]. Before the use of tobacco around the 1920s primary lung cancer was considered very rare in making up less then 1% of all malignancies in the US [4]. With the advent of smoking, lung cancer has increased to be one of the more common causes of mortality. Despite decades of warnings, cigarette smoking remains the most common risk factor for development of pulmonary carcinoma [5]. In spite of recent advances in the diagnosis, staging and treatment few advances have been made in overall patient survival over the past decade [6]. Although abstinence from smoking is the only known way to lower cancer risks, attempts at detecting markers for early lung cancer are being studied. Early detection of cancer helps improve the chances of survival in patients and produce fewer complications. Studies for detecting lung cancer include higher resolution imaging, and markers for early lung cancer. This chapter will describe the molecular abnormalities so far found in adenocarcinoma of the lung and its possible origins.

14.2 Cancer Risk in Smoking

The first scientific study associating cigarette smoke with an increase risk of death was conducted in 1938 [7] but it was not until 1950 that epidemiological studies clearly demonstrated that smoking was associated with lung cancer and death [8]. In 1964 the Surgeon General of the United States after reviewing the large collection of scientific data definitively concluded that smoking is the major cause of lung cancer [2]. This was confirmed, again in 2004, after review of massive law suites data.

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It is well known now, that both the number of cigarettes smoked per day as well as the life time duration of smoking, increases the relative risk of lung cancer. This is referred to as pack history. The risks of lung cancer in a smoker compared to a nonsmoker is 10–30 fold, light to heavy smoking respectively, however, even non smokers have a 1% risk of developing lung cancer [2]. People who smoke over 15 cigarettes a day are considered heavy smokers. When a heavy smoker reduces his intake by 50%, it decreases his relative risk by 27% after 18 years. When a smoker quits smoking, the relative risk decreases by 20%, starting in just 5 years and decreases to 90% in 15 years, compared to a smoker who does not quit [9]. Cigars, pipes, second hand smoke exposure, marijuana and cocaine all are associated with increased risk of lung cancer but less than tobacco cigarettes [9].

Other chemical causes of lung cancer include asbestos, radon, arsenic, bis-choromethyl ether, chromium, formaldehyde, ionizing radiation, nickel, polycyclic aromatic hydrocarbons, hard metal dust and vinyl chloride and work synergistically with tobacco smoke to increase the risk of cancer [2].

Other factors like race and gender can cause increased risks. African Americans and native Hawaiians are more susceptible when fewer than 30 cigarettes are smoked per day. However, no difference is observed between races if more than 30 cigarettes are smoked per day [9].

Women are at a twofold greater risk for cancer than men for similar number of cigarettes smoked. This may be due to different smoking habits and genetic factors. Current brands with low tar and nicotine cigarettes preferred by women have lead to smoking more cigarettes per day and deeper inhalation to sustain a personal threshold of nicotine [4]. The deeper inhalation causes carcinogenic particles to reach more peripheral portions of the lung and higher burning temperatures that cause increased levels of nitrosamine carcinogens [10]. Unfiltered cigarettes have larger tar particles that settle in the more proximal portions of the trachea and bronchi causing squamous cell carcinoma compared to filtered cigarettes that create smaller particles which diffuse to the more peripheral alveoli causing adenocarcinomas [5]. These two factors have caused a reverse in the incidence of squamous cell carcinoma to adenocarcinomas from past decades. There are polymorphic differences in the genes that detoxify and activate carcinogens between men and women and this may also cause a difference in the incidence of cancer.

Less understood risk factors for lung cancer are familial risk, inflammation, dietary factors and other lung diseases. Epidemiological studies have shown that first-degree relatives of patients with lung cancer have an increased risk of developing lung cancer. The studies suggest, that certain people have higher risks from certain environmental carcinogens because of genetic and acquired susceptibility factors [11].

Chronic inflammation is also associated with lung cancer. Patients who have high C reactive protein levels greater than 3 mg/dl, without a known malignancy had increased risk of developing lung cancer [12]. The antithesis of this was seen with decreased risk in patients with chronic obstructive pulmonary disease (COPD) taking inhaled corticosteroids greater than 1,200 mcg/day [13].

Dietary factors like low serum concentrations of antioxidant compounds, especially vitamins A and E, are associated with development of lung cancer. Trials supplementing retinoids, beta carotene and alpha tocopherol have shown not to reduce the incidence of lung cancer and interestingly some trials have even shown an increase in lung cancer [14]. Cruciferous vegetables, such as broccoli and cabbage, rich in isothiocyanates may have protective effect against lung cancer. GSTM1 and GSTT are genes that encode enzymes responsible for eliminating isothiocyanates and if there is an increase in transcriptions of the genes, it may lead to increased cancer. Diets high in phytoestrogens are also associated with a decrease in the incidence of lung cancer in both smokers and non smokers and is consistent with other studies that estrogens may protect against lung cancer [15].

Other lung diseases like pulmonary fibrosis due to asbestos exposure and COPD (emphysema and chronic bronchitis) have a two to four fold increase in frequency of primary lung cancer [16].

14.3 Etiology and Pathogenesis

It is now known how some of the toxins from tobacco smoke cause cancer. Lung cancer can be divided into two major histopathological groups, non small cell lung cancer NSCLC (adenocarcinoma, squamous cell carcinoma, bronchioloalveolar carcinoma and large

cell carcinoma) comprising 80% of carcinomas and small cell lung cancer SCLC (neuroendocrine) comprising 20% of carcinomas of the lung [17, 18].

Tobacco smoke has a vapor phase and a particulate phase with both phases containing more than 100 mutagens and carcinogens [19]. Specific chemicals in tobacco smoke include polycyclic aromatic hydrocarbons (PAH), N-nitrosamines, aromatic amines, ethylene oxide 1, 3 butadiene and others. It is thought that tobacco specific nitrosamines (TSN) and PAH are the compounds that most commonly cause lung cancer [10]. PAHs are metabolically activated by the genes CYP1A1 and CYP1B1 and CYP3A4 which make enzymes that conjugate glutathione-S-transferases to the toxins for excretion [20].

TSN's form three different classes of DNA adducts, or in other words, cancer causing chemicals that are covalently bonded to DNA. The first class of DNA adducts is methylation of different nucleotides that cause mutations, the second is inactivation of different repair enzymes that repair mutations (tumor suppressors) and the third is bulky adducts that interfere with replication or repair [21]. ERCC1 and RRM1 are DNA nucleotide excision repair genes and a decrease in these gene proteins correlate with a poor prognosis and no benefit from platinum based chemotherapy [22] There are polymorphisms of alleles at loci of carcinogen activating and detoxifying enzymes such as cytochrome P450, glutathione S transferase, p53 and DNA repair proteins that cause variability in susceptibility to lung cancer in different individuals [23]. One study has shown that high levels of toxins covalently bonded to the chromosomes (adducts) are associated with an increased risk of lung cancer [10].

14.4 Histology of Lung Adenocarcinoma, Pneumocyte Hyperplasia and Emphysema

Adenocarcinoma is the most common subtype of lung carcinoma and makes up 40% of all primary lung carcinomas. Adenocarcinoma is more common in women than men and most arise in the peripheral areas of the lungs and are subpleural [24]. Adenocarcinomas are subcategorized into adenocarcinoma no special type (NST), acinar, papillary, bronchioloalveolar, solid and mixed types.

Adenocarcinoma of the lung, like cancer at other sites, arises by a stepwise accumulation of genetic abnormalities that transforms benign bronchial epithelium to neoplastic tissue. Atypical alveolar hyperplasia (AAH) and bronchioloalveolar carcinoma (BAC) are both postulated to be precursors of invasive adenocarcinomas, analogous to dysplasia and carcinoma in situ of squamous cell carcinoma. Atypical alveolar hyperplasia and invasive adenocarcinoma share many molecular abnormalities and have clonal populations, which support the possibility that alveolar hyperplasia leads to adenocarcinoma [5]. Emphysema is a destructive disease of the alveoli walls that produces enlarged alveoli and may also be a precursor to lung adenocarcinoma [16]. Not all precursor lesions progress to invasion and research is underway to determine which precursor lesions will progress to cancer development.

Atypical alveolar hyperplasia has cells that are cuboidal and uniform with scant cytoplasm and the nuclei are mildly atypical. AAH morphologically looks very similar to BAC, showing identical dysplastic nuclei, making differentiating the two lesions rather difficult in some cases. It is separated from BAC by the size, which is usually less than 5 mm, milder cytological atypical and has a less monotonous cell population unlike BAC which is more monotonous [5].

Bronchioloalveolar (BAC) carcinoma is rare and occurs more often in women and nonsmokers. BAC can present as solitary or multiple nodules that appears like pneumonia on radiological imaging. BAC grows in a lepidic pattern with moderate to large atypical cells lining the alveolar spaces (Fig. 14.1). It maintains the alveolar architecture and has no signs of invasion, like desmoplasia or frank destruction of tissue [5].

Bronchioloalveolar carcinoma is divided into type I and type II. In Type I BAC the cells are mucinous and resemble goblet cells of the intestine. These produce abundant mucin that may cause bronchorrhea and can even fill up the alveoli. Type I BAC is usually multifocal and the cells grow along the walls in a non-continuous fashion.

Type II BAC's are more often solitary and the cells resemble alveolar type II pneumocytes and clara cells. The cytoplasm may have PAS positive apical granules and the nuclei often have intranuclear pseudoinclusions [5].

Adenocarcinoma of no special type is the most common form of adenocarcinoma and has low power features of glands or tubules (Fig. 14.2) and/or solid

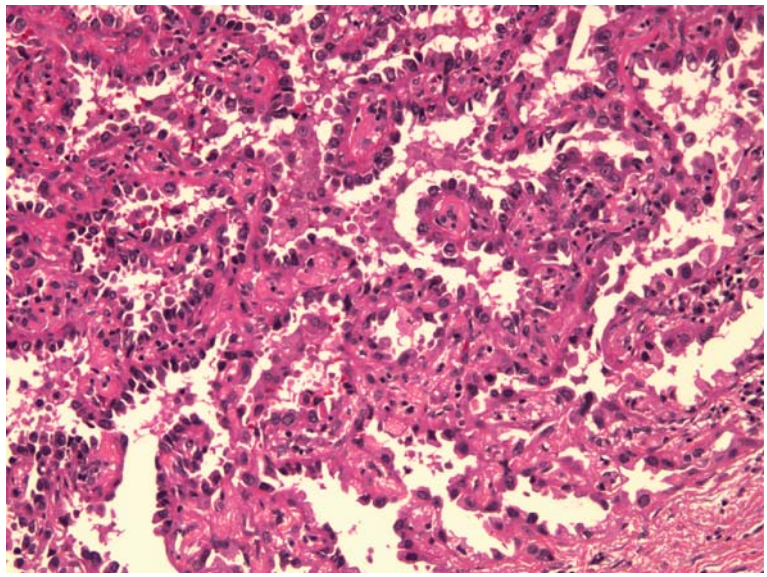

Fig. 14.1 Bronchioloalveolar carcinoma of the lung 20× magnification

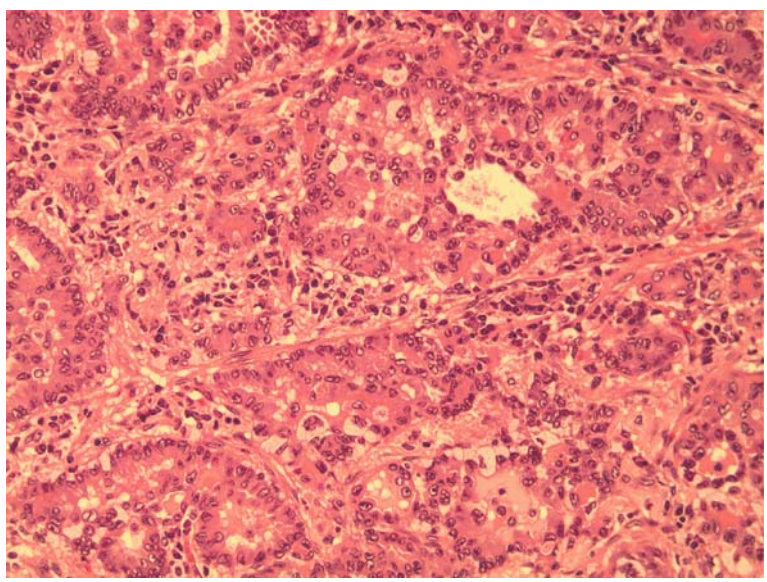

Fig. 14.2 Pulmonary adenocarcinoma 20× magnification

growth pattern. The cells at high power have very bland well differentiated to anaplastic forms. Eighty percent of these tumors have mucin in their cytoplasm. Peripheral tumors are easily treated with surgery due to accessibility. Since the tumors are located at the surface of the lungs they may involve the pleura and then disseminate in the pleural space. Lymphatic, vascular and lymph nodal invasion is common even in small peripheral tumors [5].

It is very important and usually difficult to be able to morphologically distinguish poorly differentiated metastatic adenocarcinoma from primary lung poorly differentiated adenocarcinoma. Immunoperoxidase stains are helpful in making this distinction in most instances. Lung adenocarcinoma is immunoreactive for CK 7 and TTF-1 in a majority of cases, except in mucinous BAC and adenocarcinomas that are very poorly differentiated. CDX-2 reactivity in metastatic lesion from colorectal adenocarcinomas is helpful in differentiating it from lung primary adenocarcinomas which are usually nonreactive. TTF-1 reactivity does not entirely rule in primary lung adenocarcinoma since

thyroid carcinomas and neuroendocrine carcinomas from other sites can be reactive for TTF-1. Pulmonary adenocarcinoma may also express general carcinoma markers including CEA, B72.3 related antigen, CD15 and MOC31 [5].

Emphysema shows abnormal, permanent enlarged airspaces, distal to the terminal bronchiole of the lung caused by destruction of the alveolar walls without fibrosis. Centriacinar emphysema occurs predominantly in heavy smokers with chronic bronchitis. The etiology of emphysema hinges around the fact that chronic inflammation disrupts the protease antiprotease ratio. Alveolar wall destruction results from activated neutrophils and macrophages releasing their proteases (neutrophil elastase, proteinase 3, and cathepsin G). Nicotine and reactive oxygen species found in smoke attract and activate neutrophils and macrophages in the alveoli. Smoking also increases elastase and metalloproteinase's in macrophages and neutrophils. Tobacco smoke contains numerous free radicals and decreases the normal amount of superoxide dismutase, a normal antioxidant that neutralizes endogenous free radicals, allowing increase free radicals to cause tissue damage. Tissue damage is therefore caused by decrease amounts of protective antiproteases caused by free radicals of cigarette smoke and increase amounts of proteases caused by activation of macrophages and neutrophils [1]. It is interesting that the carcinogens in tobacco smoke that cause cancer also cause direct tissue damage as in emphysema and patients with COPD have a 2–4% increase in the risk of developing carcinoma [16].

14.5 Cytology of Lung Adenocarcinoma

Cytologic evaluation of lung masses is performed using sputum, bronchial washes, bronchial brushes, bronchial lavage, capillary wedge, fine needle aspirations and pleural effusions specimens [25]. The ability to detect cancer in these specimens varies due to the number of cells collected by the different techniques. Screening asymptomatic smokers for cancer by sputum cytology has not shown to decrease mortality and thus it is used only in symptomatic patients [25].

Type II pneumocyte hyperplasia is seen in pneumonia, sepsis, embolus, chemotherapy, radiation, oxygen toxicity, and smoking. The cells seen are sometimes single and more commonly found in three-dimensional clusters. The cells have large nuclei with coarse chromatin and prominent nucleoli. Type II pneumocyte hyperplasia that is florid can often look similar to adenocarcinoma [25].

Majority of adenocarcinomas occur in the periphery of the lung and are often associated with desmoplastic reaction and pleural puckering. The malignant features of adenocarcinoma include cells with fine foamy to vacuolated cytoplasm, and secretory vacuoles that tend to have cohesive sheets with three dimensional clusters and acini. The nuclei are eccentric, irregular and vesicular with prominent nucleoli. Tumor diathesis background is usually seen in squamous cell carcinomas and not in adenocarcinomas [25]. Mucinous bronchioloalveolar carcinoma has well differentiated mucinous cells that are difficult to recognize as malignant. Features that help recognize malignant cells of a well-differentiated mucinous cell carcinoma are abundant cells in sheets or three dimensional groups of monotonous cells. The nucleus is enlarged and has irregular contours and nucleoli [25]. Mucinous bronchioloalveolar carcinoma cytologically resembles papillary thyroid carcinoma when it demonstrates psammoma bodies, intranuclear pseudoinclusions, nuclear grooves and clear nuclei [26].

14.6 Molecular Pathways

Over the past two decades, progress has been made in understanding the molecular pathogenesis of cancer. Identification of genes that help suppress tumor growth and the identification of genes that activate tumor cell growth are common in many different types of cancer including lung cancer. Lung cancer cells often show deletions at multiple chromosome sites and several tumor suppressor genes are located at these regions which can cause loss of heterozygosity. There are a small number of tumor suppressor genes inactivated (loss of heterozygosity) in lung cancer, which include p53, RB, p16, FHIT and PPP2R1B. Loss of heterozygosity can occur by chromosome breakage, deletions, nucleotide changes, amplification and hypermethylation. These causes have been identified in lung cancer, pre-neoplastic tissue and even in normal lung epithelium of nonsmokers [18]. In non small cell carcinomas

especially squamous cell carcinoma a stepwise progression occurs from hyperplasia, metaplasia, dysplasia, carcinoma in situ and finally to invasive tumors. Other cancers like adenocarcinoma and neuroendocrine follow similar progression. It is now thought that the precursor cell of adenocarcinoma is the bronchioloalveolar stem cell with the K-Ras, Pten, phosphoinositide 3 kinase and cyclin dependent kinase pathways implicated in the proliferation of these stem cells [27].

14.6.1 Chromosomal Changes

Chromosome analysis of lung tumor cells has shown multiple nonrandom breaks of chromosomes 1, 3, 7, 15, 17 [28]. These breaks can cause loss of heterozygosity in tumor suppressor genes which cause tumor development. Lung cancer cells often show deletions at multiple chromosomal regions and deletion mapping have found more than 30 regions in 21 different chromosomes as candidates for tumor suppressor loci [29].

NSCLC often has extensive chromosomal abnormalities which include structural changes, and numerical changes in chromosomes often near triploid [6].

Numerical changes include losses of chromosome 9, 13, Y (in males). Trisomy 7 has also been observed in early changes of NSCLC and premalignant lung tissue. Chromosome imbalances include gains of chromosome arms 1q, 3q, 5p and 8q and losses of 3p, 8p, 9p, 12q and 1p. Losses of 5p, and 13q are also prominent changes found in NSCLC's [30].

Genetic analysis of lung adenocarcinomas lesions show a stepwise loss of heterozygosity (LOH) first in chromosome, 3p21.3 (site of RASSF1A a member of the Ras association Domain family, and FUS1) 3p14.2 (FHIT, a fragile histidine triad gene) followed by 9p21 (p16), 8p, 17p13 (p53), and finally 5q which are all tumor suppressor genes [31]. The loss of heterozygosity is one of the earliest molecular change found in 50% of adenocarcinomas and greater than 90% of squamous cell carcinomas and SCLC of slightly abnormal and even normal appearing epithelium of smokers [18]. In the future surgical margins may be examined by molecular analysis to identify patients most likely to benefit from adjuvant therapy [32].

Microsatellites are repeated sequence motifs of 1 to 6 base pairs found in the regulatory regions of genes and influences gene expression and transcriptional activity. Microsatellite alterations are found in 22% of NSCLC and 35% of SCLC's and correlate with a younger age of incidence and more advanced stage at diagnosis [31].

Normal somatic cells have decreasing amounts of telomerase that eventually leads to cell death unlike tumor cells and germ cells that have increased telomerase that extend the telomeres and cell immortality. How telomerase is re-expressed in lung cancer is unknown but this may be used as a marker and a target for therapy. Telomerase activity and telomere replications are increased in 80% of NSCLC and almost 100% of SCLC. It is associated with advanced stage in primary NSCLC's [31].

14.6.2 Tumor Suppressor Oncogenes

The p53 gene is located on chromosome 17. The p53 protein binds to DNA which stimulates another gene to produce a protein called p21 and GADD45. P21 binds with cdk2 a cell division-stimulating protein and inhibits the cell from passing through to the next stage of cell division. P21 controls G1/S cell cycle advancement, and GADD45, controls the G2/M cycle both stop the replication of DNA if it is damaged [24]. BAX, PERP and other proteins can activate p53 and cause apoptosis. MDM2 a proteasome degrades p53 and keeps the levels low by auto-regulatory feedback found that MDM2 is over expressed in 25% of NSCLC's [18]. Mutant p53 can not bind DNA or is ineffective so no p21 or GADD45 is made to stop cell division therefore the cell divides uncontrollably creating cancer. The p53 and Rb genes are the most common tumor suppressor genes affected in lung carcinoma and are inactivated by mutations, chromosomal deletions, gamma radiation, UV radiation, and carcinogens like tobacco smoke. Tobacco smoke often causes a p53 mutation, with G to T transversions, and hypermethylation of the promoter regions of p53 gene. Benzoapyrene a tobacco smoke metabolite damages three specific loci on the p53 gene. These three loci are known to be abnormal in approximately 50% of primary lung cancer and 75% of SCLC [31]. Recently two functional and structural

homologues of the p53 gene, p73 and p51 were identified but their genetic alterations seem to be rare in lung cancer [29]. P53 mutations can be found in lung tumors of nonsmokers but at a significantly lower rate than that of smokers [33].

The Retinoblastoma gene is found on chromosome 13 which encodes a protein that also stops the cell cycle. The Rb protein prevents cells from entering S phase of the cell cycle. The Rb protein binds to a transcription factor E2F. E2F is not able to bind to the promoters of proto-oncogenes c-myc and c-fos. Transcription of c-myc and c-fos is needed for mitosis so blocking the transcription factor needed to turn on these genes prevents cell division. In summary, if there is a mutation in the Rb gene, no suppressor protein is made to bind with EF2, and EF2 is free to start transcription of genes c-myc and c-fos that creates uncontrolled mitosis and tumorgenesis. Mutations in the RB protein have been found in 15–30% of NSCLC and 90% of SCLC. Mutations of the RB and $P16^{INK4A}$ are rarely found in the same lung tumor however, $P16^{INK4A}$, cyclin D1, and cdk4 are usually seen in NSCLC whereas RB gene inactivation is usually seen in SCLCs. Other members of the RB family, p107 and pR2/p130 are found mutated rarely in NSCLCs and SCLCs [18].

The RAS genes encode a GTPase protein that signals growth and survival after the membrane bound RAS tyrosine kinase receptors are activated. The GTP is hydrolyzed to GDP and starts the RAS signaling. If there are RAS mutations, sometimes GTP hydrolysis is decreased or non functional and there is an increase or constant signaling that causes uncontrolled cell growth [18]. The RAS genes (HRAS, KRAS, and NRAS) are transformed into oncogenes by a point mutation with a G-T transversion caused by bulky DNA adducts present in tobacco smoke like benzopyrene diethlyoxide and nitrosamides [34]. The mutations of the RAS gene are highly associated with smoking and found in 20–30% of lung adenocarcinomas and in 25–40% of atypical alveolar hyperplasia [5]. These point mutations are most commonly found at codon 12 [34] followed by codons 13 and 61 and affect the KRAS gene 90% of the time. Patients with tumors that have K-ras, N-ras or H-ras mutations have a decreased survival [28, 34].

BCL-2 is an anti-apoptotic protein and counteracts BAX, a pro-apoptotic protein that acts on p53 that stops the cell cycle. The proto-oncogene BCl-2 is over expressed in 10% of adenocarcinomas, 25–30% of squamous cell carcinomas and 75–95% of SCLC's. BCL-2 expression in NSCLC is believed to be a favorable prognostic factor, while BCL-2 expression does not influence survival in SCLC's. High BCL-2 and low BAX expression are frequently found in SCLC's that are p53 deficient and are very sensitive to chemotherapy [18].

MYC protein is a basic helix-loop-helix transcription factor that regulates the expression of genes involved in DNA synthesis, RNA metabolism and cell cycle regulation [18]. Over expression of MYC is found in 5–10% of non-small cell lung carcinomas but up to 36% of SCLC and may indicate chemotherapy resistant tumors. Activation of MYC genes c-myc, L-myc, and N–myc occur by amplification or loss of transcriptional control and cause an uncontrolled cell growth and tumor genesis.

Notch-3 is involved in differentiation and neoplasia and influences differentiation of lung cancer cells. Notch-3 is found to be over expressed in NSCLC after chromosome 19p translocation [18].

The $p16^{INK4A}$ is a major step in controlling the G1/S cell cycle advancement. $P16^{INK4A}$ binds to cyclin-dependent protein kinase 4 (CDK4) which inhibits CDK4 to interact with cyclin D1. The cyclin D1 associated CDK4 phosphorylates RB, releasing the cell from RB mediated cell cycle arrest. $P16^{INK4A}$, cyclin D1-CDK4-RB pathway is usually altered, mutated or hypermethylated in 30–50% of NSCLC and rarely in SCLC [18]. P16 methylation is very rare in adenocarcinoma and occurs in the atypical alveolar hyperplasia stage [32]. Promoter methylation of p16 is associated with recurrence after resection [32]. CDK4 and cyclin D1 over expression have been found in NSCLC and is correlated with a poor prognosis [18].

$p14^{ARF}$ interacts with MDM2 and prevents p53-MDM2 interaction that causes degradation of p53. This mutated $p14^{ARF}$ allows mutated DNA to not repair itself and replicate (Fong et al. 2003) [18]. $P14^{ARF}$ mutations are found in 20% of NSCLC's.

The FHIT gene (family histidine triad) encodes a diadenosine 5′,5‴-P1,P3-triphosphate hydrolase involved in purine metabolism. The gene encompasses the common fragile site FRA3B on chromosome 3p14.2, where carcinogen-induced damage can lead to translocations and aberrant transcripts of this gene. The FHIT gene shows LOH in 40% of NSCLC's and 80%

of SCLC even though the FHIT protein is found in only 50% of all lung cancers. Other candidate tumor suppressor genes in the 3p region include RASSF1, SEMA3B, FUS1 and RARB [18].

Another pathway tumor suppressor genes are inactivated is by hypermethylation of the promoter regions resulting in transcriptional inactivation of one allele with the other allele lost by mutation [18]. Hypermethylation has been shown to lead to the silencing of mRNA expression [29]. This occurs in NSCLC and SCLC but can also be detected in early preneoplastic lesions of smokers. Methylated promoter rejoins of genes TIMP-3, P16, p14, CDH13 (H-cadherin) DAPK, GSTP1 and the genes of the chromosome 3p region have been reported [18]. Hypermethylated spots on chromosomal regions 4q, 10q and 17p are present in both NSCLC an SCLC, but so far no candidate tumor suppressor genes have been found in these regions (Fong et al. 2003) [18].

14.6.3 Proliferation Markers

Lung cancer cells can express receptors for growth promoting and inhibitory factors and are often associated with poor prognosis [18].

Gastrin-releasing or other bombesin like peptides (GRP/BN) can stimulate the growth of both normal and malignant lung cells. Increase in GRP levels occurs in fetal lung development and differentiation [28, 31]. Most SCLC and NSCLC express gastrin-releasing or other bombesin like peptides (GRP/BN) receptors and GRPBN peptides although no mutations or amplifications of these genes have been found the mechanism of this growth stimulatory pathway remains unknown. High levels of GRP-like activity have also been recorded in bronchial lavages of smokers compared with nonsmokers [28]. The GRP receptor is located on the X chromosome but does not undergo X inactivation so that women have two genes but men have only have one gene. This may be one reason why women are more susceptible to lung adenocarcinomas from smoking [31].

Another signaling loop uses receptors for tyrosine kinase like the receptors erb-b1 and erb-b2 known as Her-2/neu. Erb-b2 is over expressed in 30% of NSCLC mostly adenocarcinomas and not SCLC. Erb-b1 or epidermal growth factor receptor is over expressed along with EGF hormone or TGF alpha in 13% of NSCLC's. HER2 mutations are associated with resistance to of the EGFR tyrosine kinase [35].

Hepatocyte growth factor (HGF) induces differentiation of lung epithelial cells and is found in NSCLC but not SCLC [31, 36].

EGFR regulates proliferation, apoptosis, angiogenesis, and tumor invasion. EGFR is frequently over expressed in 10% NSCLC's. EGFR mutations are increased in women and nonsmokers and 80% of these mutations involve in frame deletions within exon 19 or the L858R mutant within exon 21 [37]. EGFR mutations can transform fibroblast and lung epithelial cells into atypical alveolar hyperplasia, then into bronchioloalveolar carcinoma and finally into adenocarcinomas in transgenic mice [38]. Clinical trials of the EGFR tyrosine kinase inhibitor, erlotinib a monoclonal antibody against EGFR, are used for treatment of previously untreated advanced lung adenocarcinoma.

AKR1B10 is a gene that produces aldo-keto reductase family 1, member B10 (AKR1B10) was examined by immunohistochemical analysis of 101 non-small cell lung carcinomas (NSCLC) and its over expression was observed in 27 of 32 (84.4%) SCC's and 19 of 65 (29.2%) adenocarcinomas [36].

IGF-1 and IGF-2 are tyrosine kinase receptors that bypass EGFR to activate downstream signaling pathways like KRAS [39]. IGF-1 and IGF-2 also induce self regulation in NSCLC and SCLC. C KIT receptor and hormone are highly expressed and cause growth in SCLC but much less in NSCLC and is an important negative prognostic factor [31].

VGRF induces neovascularization and is secreted by tumor cells and stromal tumor cells. VEGF expression is associated with loss of p53 function. VGEF is expressed in 50% of NSCLCs and is associated with increase microvascular density in tumors and poor prognosis. IL-8 is also a strong angiogenic factor and is part of the CXC chemokine family and is expressed in 50% of NSCLCs.

PD-ECFG are expressed in 30–40% of adenocarcinomas. Metalloproteinases (MMP) and their inhibitors are major causes of metastasis and promotion of tumor related angiogenesis. MMP expression in NSCLC and SCLC is not well studied and undetermined prognostic significance.

E-cadherin-catenin is important for maintaining normal tissue architecture. Lung cancers have reduced expression of laminins and integrins which is associated with disrupted tumor cells and extracellular matrix. This disrupted tumor cells and extracellular

matrix leads to fragmentation of the basement membrane and invasion into the surrounding stroma. E-cadherin-catenin complex loss is seen in lung cancer invasion and metastasis and is associated with poor prognosis.

Cyfra 21-1 is a serum marker uniquely found in high levels in NSCLC's like adenocarcinoma and squamous cell carcinoma and low levels were found in patients with non-malignant pulmonary diseases and patients with small cell lung cancer [40].

Retinoic acid induces early cancer cells to stop proliferating but advanced cancer cells lose responsiveness to retinoids. In a M.D. Anderson trail, 13-cis retinoic acid was used to reduce the development of a new primary cancer in people previously treated for head and neck cancer. Most head and neck caners and lung cancer are caused by cigarette smoke and if their first cancer is controlled, they have a very high risk of developing a second primary cancer of the upper aerodigestive tract. In the M.D. Anderson trail none of the patients who received retinoic acid developed lung cancer, whereas four of the patients receiving placebo did 3. In contrast to the rate of new primary cancers, the rate of head and neck primary cancer relapse were not affected by the treatment of retinoic acid [28].

14.7 Conclusion

Fifty percent of all newly diagnosed lung cancers in the US occur in patient's who had stopped smoking in the last 5 years. If we can detect some of these genetic alterations in preneoplastic respiratory epithelial lesions before cancer develops, early intervention and chemoprevention in such high risk individuals could greatly increase survival rates. Patients with increase baseline risk may be appropriate candidates for screening with procedures such as bronchoscopy to obtain epithelial cells to detect amplifications, mutations, or deletions of genes involved in signal transduction, regulation of gene expression or cellular proliferation and may result in clinically useful detection of early cancer. Interventional approaches that interfere with tumor growth and invasion may keep cells from undergoing malignant transformation [28].

Never smokers are people who have smoked less than 100 cigarettes in their lifetime. In Asia 60–80% of women with lung cancer never smoked [41]. In the US 19% of lung cancer in women occurs in never smokers, compared to 53% in the rest of the world [42]. Adenocarcinoma is more associated with never smokers, light smokers and former smokers while squamous cell carcinoma and small cell lung cancer are associated with heavy smokers [41]. The molecular biology is different between adencocarcinoma caused by smoking and never smoking people. Adenocarcinoma of never smokers more commonly shows mutations of the EGFR receptor (kinase domain) and adenocarcinoma of smokers more commonly shows K-Ras mutations [43]. Lung adenocarcinoma in never-smokers has a very distinct immunohistochemical expression profile of EGFR-related biomarkers as compared to lung adenocarcinoma in smokers. Differences are also seen in the p53, chromosomal abnormalities and methlyation of p16 [44]. Survival of nonsmokers with adenocarcinoma is greatly influenced by CEA level than that of smokers [45]. High levels of EGFR and Ki-67 are observed in smokers, while never-smokers are characterized by high levels of pAKT and p27 [46].

In summary, early detection of adenocarcinoma in smokers may help in increasing the survival rate. The different chromosomal, molecular tumor suppressors, oncogene and proliferation markers may be the key to identifying early lung adenocarcinomas in\break smokers.

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Chapter 15

Genesis of Barrett's Neoplasia: Current Concepts

Domenico Coppola, Nelly A. Nasir, and Leslie Turner

Contents

15.1 Introduction

Barrett's esophagus (BE) is the result of chronic gastro-esophageal reflux, and is characterized by the replacement of the normal stratified squamous epithelium in the lower esophagus with metaplastic columnar epithelium of various types [1]. BE predisposes to the development of adenocarcinoma of the esophagus, a condition that has dramatically increased in frequency (>300%) over the past 30 years [2]. In addition, this type of tumor has a dismal 5-years survival rate of 14–22% [3].

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15.2 Etiology and Pathogenesis

In general, patients with BE acquire columnar metaplasia in the lower esophagus as a consequence of gastroesophageal reflux [3]. Therefore, any condition increasing the reflux of acid from the stomach to the esophagus represents a risk factor. These conditions include a hiatal hernia, the presence of duodenogastric reflux, delayed esophageal acid clearance time, and decreased resting pressure of the lower esophageal sphincter [4, 5]. In a minority of cases, other etiologic factors may be involved such as bile reflux following gastrectomy [6], esophageal injury (lye ingestion) [7], and possibly congenital rest of gastric epithelium (especially in cases of infantile BE) [8]. The last possibility is plausible since, during fetal life, the esophagus is lined by mucin-secreting cells.

The exact mechanism by which squamous epithelium is replaced by metaplastic mucosa is not certain. However, it seems that initially, following erosion of the squamous mucosa by the acid-peptic action of the gastric content, undifferentiated progenitor cells migrate into the denuded areas. These cells are multipotential stem cells that, in the presence of persistent gastroesophageal reflux, selectively differentiate into columnar mucin-secreting epithelium [9]. Alternatively, metaplasia could occur simply by upward migration of the columnar epithelium from the stomach to re-epithelialize the ulcerated mucosa.

15.3 Cancer Risk in Barrett's Esophagus

BE predisposes to the development of adenocarcinoma. It is estimated that of the patients with

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symptomatic gastroesophageal reflux who seek medical attention and undergo endoscopy, approximately 10–20% will have BE [10–14]. Of these, 7–15% already will have adenocarcinoma at the time of their first endoscopy [15]. It has also been shown that 18% of all patients undergoing upper gastrointestinal endoscopy for any reason are found to have BE [16]. The incidence of BE has been increasing in recent years and, consequently, adenocarcinoma arising in BE is the most rapidly increasing cancer in the last two decades [15, 16]. It is estimated that patients with BE have a 30- to 125-fold increased risk of developing adenocarcinoma [15, 16]. Patients who develop adenocarcinoma are usually elderly white men with metaplastic or dysplastic epithelium [17]. This is also true for patients with very short segment BE, which suggests that even small areas of metaplastic epithelium increase the cancer risk [18]. It has been speculated that cancer in BE arises through a multistep sequence of events initiated by gastroesophageal reflux that induces metaplasia and eventually progresses to dysplasia and carcinoma. It has also been observed that the presence of ulceration, in a biopsy of a patient with BE and higher grade dysplasia is most likely associated with invasive carcinoma [18].

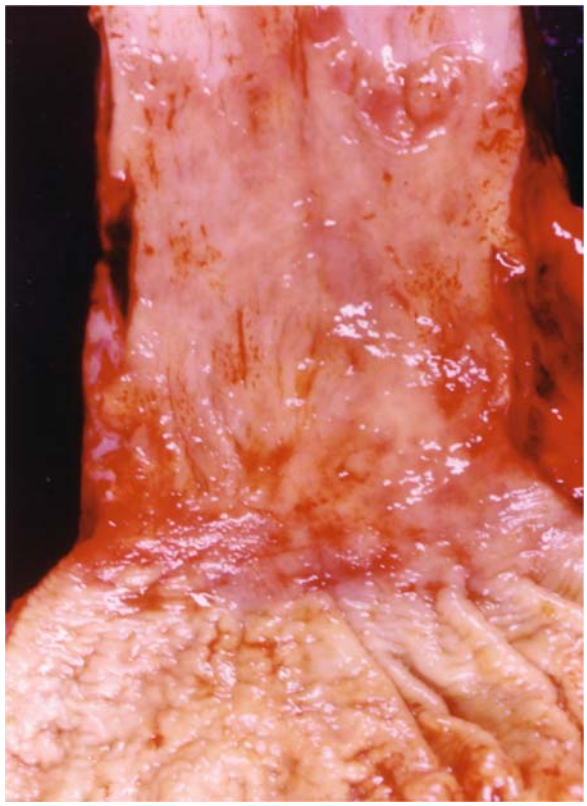

Fig. 15.1 Esophago-gastrectomy specimen showing the salmon-colored Barrett's mucosa replacing the pearly white esophageal squamous mucosa at the esophago-gastric junction

15.4 Pathology of Barrett's Esophagus and of Barrett's Esophagus Associated Neoplasia

Grossly, Barrett's mucosa is usually represented by a well-defined area of salmon-pink, velvety mucosa similar to the adjacent gastric mucosa. It has irregular margins and may contain islands of residual squamous, pearly white esophageal mucosa, or it may be ulcerated (Fig. 15.1). It is usually limited to the lower third of the esophagus, but in severe cases, it may extend to the middle and upper esophagus. The endoscopic diagnosis of BE may be challenging, especially if the gastroesophageal junction is difficult to identify [10, 19].

Histologically, problems in diagnosing BE may arise if the precise site of the biopsy is not known or if one does not realize that the metaplasia in BE can exhibit different patterns. Barrett's epithelium may be of the gastric fundic type, gastric cardiac type, or specialized (intestinal type) (Fig. 15.2) [11]. The first two types of epithelium are histologically indistinguishable from their normal counterpart in the stomach and could represent hiatal hernia. However, this is not a diagnostic problem since it is now accepted that dysplasia and carcinoma arise almost exclusively from the specialized (intestinal type) Barrett's metaplasia [12]. Therefore, it is believed that a diagnosis of BE should be made only if goblet cells are present (Fig. 15.2, arrows) [13, 20]. These are barrel-shaped cells with a distended, acidic mucin-filled cytoplasm, which can be easily identified using either an Alcian blue pH 2.5 stain or an Alcian blue PAS stain. If this rule is followed, then knowing the exact landmark of the biopsy is not so critical since any intestinalized epithelium carries an increased risk of cancer regardless of its precise location.

If gastroesophageal reflux persists in patients with BE, dysplasia can develop [19]. Dysplasia is the

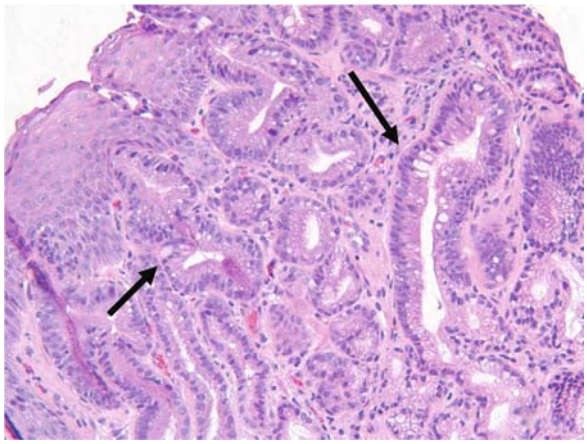

Fig. 15.2 Barrett's esophagus. Transition of esophageal squamous mucosa into a columnar epithelium containing scattered goblet cells (intestinal metaplasia)

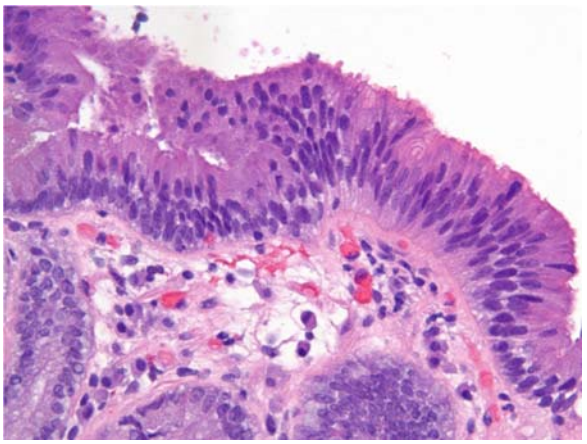

Fig. 15.3 Barrett's esophagus with low grade dysplasia. Barrett's mucosa showing glands with mild architectural distortion and gland lined by cells containing elongated (*cigar shaped*) and stratified nuclei which still retain nuclear polarity

development of neoplastic epithelium, which is confined within the superficial layer of epithelium by an intact basement membrane [20]. When neoplastic cells bridge the basement membrane, an invasive carcinoma is born. Dysplasia in BE has been graded following criteria similar to those used by the Inflammatory Dysplasia Morphology Study Group [21]. Barrett's metaplasia can be negative, indeterminate, or positive for dysplasia. It is indeterminate if features of dysplasia are present but do not extend to the surface epithelium or if these changes are associated with severe-grade inflammation, thus raising the possibility of reactive atypia [22].

Low-grade dysplasia (Fig. 15.3) is characterized by preservation of the glandular architecture, stratified cigar-shaped nuclei (which do not reach the cell surface), nuclear hyperchromasia, a moderate increase in mitotic activity, a decrease in goblet cells, and the presence of dystrophic goblet cells (mucin lies on the basal side of the nucleus). These changes are extending to the surface epithelium. High-grade dysplasia (Fig. 15.4) is characterized by marked distortion of the crypt architecture with cribiform pattern (back-to-back glands). The nuclear stratification involves the cellular surface; there is nuclear anisocytosis and pleomorphism, prominent nucleoli and loss of nuclear polarity. The mitotic figures are numerous. Areas of intestinal metaplasia are often intermingled with areas of dysplasia and adenocarcinoma that may not be endoscopically or grossly visible; therefore, small areas of dysplasia or carcinoma may be missed [23, 24].

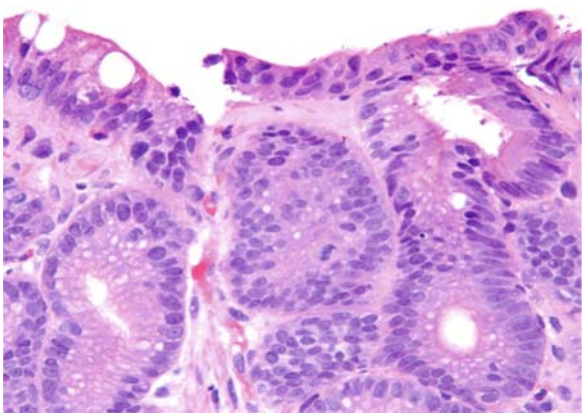

Fig. 15.4 Barrett's esophagus with high grade dysplasia. Intestinalized mucosa showing distorted and crowded glands containing enlarged cells. The cellular nuclei are polygonal, hyperchromatic, and exhibit anisocytosis and prominent nucleoli. Most importantly for the diagnosis, there is loss of nuclear polarity

Intramucosal adenocarcinoma is present when highly dysplastic cells invade through the basement membrane into the lamina propria, or into the muscularis mucosa but not beyond (Fig. 15.5).

In invasive adenocarcinoma, tumor cells infiltrate beyond the muscularis mucosa often eliciting desmoplastic reaction (Fig. 15.6). In most institutions, four-quadrant biopsies are performed, beginning at the top of the gastric folds and proceeding every 2 cm throughout the entire length of the columnar lined esophagus, in addition to biopsies of any endoscopic suspicious

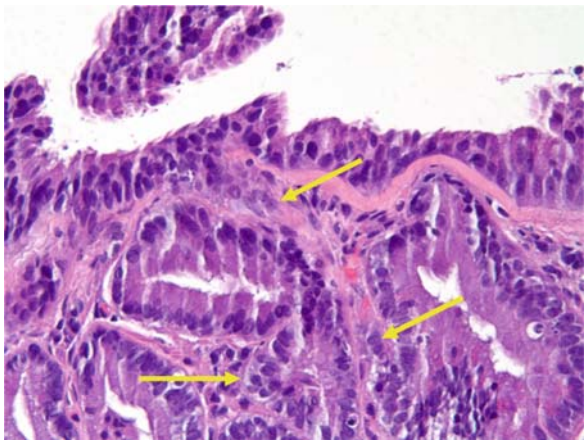

Fig. 15.5 Barrett's esophagus with intramucosal carcinoma. Barrett's mucosa with high grade dysplasia and glands exhibiting early syncytial pattern of growth. There are cellular clusters or single cells infiltrating the lamina propria. An attempt to desmoplastic reaction around these infiltrating cells is also noted (*arrow*)

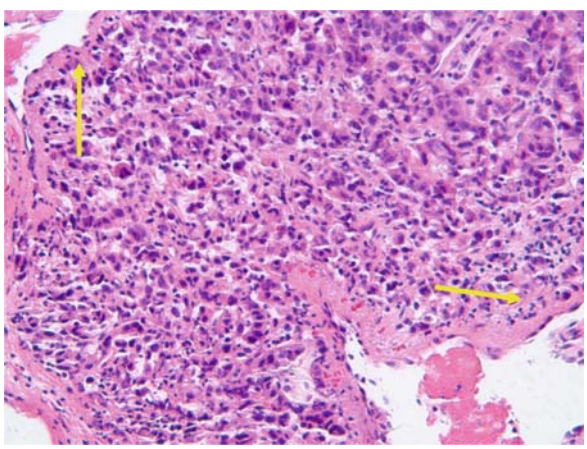

Fig. 15.6 Invasive adenocarcinoma arising in Barrett's esophagus. A poorly differentiated adenocarcinoma infiltrating the esophageal mucosa and surrounding vascular structures. Some of the tumor cells are seen within the vascular wall (*arrow*)

area [23, 25]. The use of this protocol has provided good correlation between endoscopic and pathologic diagnosis [26].

Three problems are associated with the application of the grading system: (1) the assessment of degree of dysplasia, which is subjective, (2) the lack of correlation between the degree of dysplasia and subsequent biologic behavior of the lesion, and (3) inter- and intra-observer variation, especially when assessing intermediate grades of dysplasia (indefinite or low grade).

15.5 Differential Diagnosis of Barrett's Esophagus

The main differential diagnosis of BE is gastric cardia with intestinal metaplasia. This is especially important, and more difficult to recognize, when attempting to discriminate between reactive cardia and dysplastic Barrett's epithelium. It is important to ascertain a correct diagnosis when at all possible since the natural history is different between these two conditions [27, 28]. Short segment BE has been found to have a higher prevalence of dysplasia when compared to chronic carditis with intestinal metaplasia (11.3% vs. 1.3%) and a higher incidence of dysplasia (4.6% vs. 1.5% per year) [29]. Due to this apparent lower risk of neoplasia, patients with chronic carditis with intestinal metaplasia do not currently undergo routine endoscopic surveillance [30]. Srivastava et al. reported several morphologic features that are in favor of a diagnosis of BE over intestinal metaplasia of the cardia in biopsies of the gastroesophageal junction. The reported morphologic features include: crypt disarray and atrophy, incomplete intestinal metaplasia and diffuse intestinal metaplasia, multilayered epithelium, squamous epithelium overlying columnar crypts with intestinal metaplasia, hybrid glands, and esophageal glands or ducts. In their study group, the coexistence of 4 or more of these features was 95% sensitive and 95% specific for Barrett's esophagus. In addition, three of the features (squamous epithelium overlying columnar crypts with intestinal metaplasia, hybrid glands and esophageal glands/ducts) were seen exclusively in BE [31].

Gastric heterotopia (inlet patch) can also be present in the esophagus and may be confused with Barrett's epithelium since Barrett's epithelium may be of the gastric fundic type, gastric cardia type, or specialized (intestinal) type [11]. As mentioned previously, however, it is now accepted that dysplasia and carcinoma arise almost exclusively from the specialized (intestinal type) Barrett's metaplasia [12]. Since BE is diagnosed only in the presence of goblet cells, which are not present in gastric heterotopia, this is usually

not a difficult differential. Borhan-Manesh and Farnum report an incidence of 10% for heterotopic gastric mucosa in the esophagus. These patches of heterotopic gastric mucosa were located just below the upper esophageal sphincter [32]. Pancreatic acinar metaplasia in the esophagus has been reported to occur in up to 24% of the evaluated patients. In one study, no association was found between pancreatic acinar metaplasia and any other clinical or histologic abnormalities in the esophagus or stomach, raising the possibility that this finding is actually congenital in nature [33].

15.6 Cytology and Barrett's-Associated Neoplasia

Some studies have reported the application of cytologic methods in the diagnosis of BE. Studies using brushing cytology have shown good correlation with routine histologic examination in identifying the metaplastic epithelium and carcinoma [34].

Balloon cytology has been used to evaluate the degree of dysplasia in BE. This technique was found to have 66% sensitivity and 100% specificity when using histology as the "gold standard." However, balloon cytology has poor sensitivity in detecting low-grade dysplasia [35, 36]. Prospective studies are underway in several institutions, including ours, to further assess the value of this technique that has a potential cost advantage.

15.7 Molecular Pathways of Neoplastic Progression

Not all patients with BE will progress to adenocarcinoma. Some live for years without developing dysplasia, and they eventually die of unrelated disease. Others demonstrate a rapid progression to dysplasia and carcinoma and will die of esophageal adenocarcinoma, if it is not diagnosed early and treated appropriately. Recent attempts have been made to identify molecular markers to predict which patients with BE will progress to carcinoma. The availability of such markers would allow closer patient follow-up and earlier intervention, preventing the late diagnosis of BE associated adenocarcinoma when the tumor is already disseminated.

15.8 Cell Cycle and DNA Ploidy

Cyclin D1 regulates the transition from G1 to the S phase of the cell cycle via the formation of cyclin D1-cyclin dependent kinase (cdk) complexes which phosphorylate the retinoblastoma (Rb) protein. This change in Rb phosphorylation enhances transcription of growth promoting genes. A study of 307 biopsies from Barrett's patients has shown that positivity for cyclin D1, but not p53, has a statistically significant increased risk of progression to adenocarcinoma [37].

Cyclin B1 is involved in the G2M phase transition of the cell cycle. It is synthesized early in G2 phase and it is believed to promote chromosome condensation, disruption of the nuclear membrane, and mitotic spindle formation. Altered cyclin B1 expression may induce deregulation of the cell cycle, and uncontrolled cell growth. Overexpression of cyclin B1 has been demonstrated early in the transition of BE into low grade dysplasia [38]. Similarly, $CDKN_2$/p16 gene becomes mutated and is detected early, in association to allelic loss of 9p21 chromosome, in diploid cells, just before turning to aneuploid during the neoplastic progression [39].

To date, the most reliable marker of tumor progression in BE associated neoplasia has been DNA ploidy. It has been reported that dysplasia arising in BE is commonly associated with aneuploidy [26, 40]. Reid et al. [34] observed that 9 of 13 patients with aneuploidy and increased G_2/tetraploid cell population developed high-grade dysplasia or carcinoma within 34 months. Forty-nine patients without these abnormalities did not progress to dysplasia. However, these results have not been confirmed [41]. The difficulty in endoscopically differentiating between metaplastic and dysplastic mucosa renders reproducible sampling for flow cytometry, and appropriate correlation with follow-up biopsies, problematic.

15.9 Proliferation Markers and Adhesion Molecules

Proliferation markers including oncogenes, cell receptors, and nuclear proliferation antigens have been reported in patients with BE associated neoplasia.

Al-Kasspooles et al. [42] described epidermal growth factor receptor (EGF-R) gene amplification

and protein overexpression in 31% of 13 human esophageal adenocarcinomas. Alterations have also been described for Src-specific activity, which is 3- to 4-fold higher in BE and 6-fold higher in adenocarcinomas compared to the control tissues [43].

Among the adhesion molecules, beta-catenin is an oncoprotein that mediates cell-cell adhesion via the transmembrane E-cadherin-catenin complex, and it may contribute to carcinogenesis when the APC/beta catenin/Tcf signal transduction pathway is disrupted. It has been shown that E-cadherin is significantly lower in patients with BE, compared to those with normal esophageal epithelium [44]. The nuclear accumulation of beta-catenin is a common and early event during neoplastic progression in BE [45], occurring before the mutated in colon cancer gene (MCC) loss of heterozygosity [46]. In addition, it has been observed that abnormal beta-catenin expression is present in 61% of 70 BE associated adenocarcinomas [47].

Tselepis et al. have reported the upregulation of tumor necrosis factor - α in the progression of BE, with secondary NF-κB independent transcription of beta-catenin and of c-myc [48].

Following the discovery of increased expression of claudin 3, 4 and 7 in gastric adenocarcinoma and dysplasia, as compared to normal gastric mucosa, Montgomery et al. have extendend their investigation to BE specimens. Their findings show that the increase in claudin proteins is an early event during the progression of Barrett's neoplasia [49]. Claudins are involved in the formation of intercellular tight junctions which are important for the maintenance of intercellular cell adhesion and of tissue osmotic homeostasis.

Other molecular alterations identified during the progression from Barrett's metaplasia to carcinoma include the sequential accumulation of acidic fibroblast growth factor [50], and of a novel acidic isoform of proliferating cell nuclear antigen (csPCNA). This protein has been shown to be cancer specific [51]. Finally, Ray et al have described the increase of Rab-11, a small GTP-binding protein, in BE with low grade dysplasia [52].

15.10 Tumor Suppressor Genes

p53 and the retinoblastoma (Rb) genes are important tumor suppressor genes involved in the regulation of cell proliferation and apoptosis. Phosphorylation of Rb induces its molecular conformational changes with release of the transcription factor E2F and translation of genes involved in cell cycle progression. In a study of 56 patients with adenocarcinoma arising in BE, we found progressive loss of Rb protein expression as the intestinal metaplasia progressed to dysplasia and carcinoma [53]. p53, increased in response to DNA damage, stimulates transcription of genes that mediate cell cycle arrest (p21), DNA repair (GADD45), and apoptosis (Bax). Nuclear accumulation of abnormal p53 tumor suppressor protein has been described in approximately 33–50% of adenocarcinomas arising in BE [54, 55]. When genetic sequencing is performed, p53 abnormalities in Barrett's cancer are found in up to 90% of cases.

Independent investigators have detected increased frequency of p53 mutations that parallel increasing degree of dysplasia [56–59]. Chatelain and Flejou reported strong expression of p53 by immunohistochemistry in high grade dysplasia and superficial adenocarcinoma. They detected no significant p53 expression in non-dysplastic metaplastic mucosa and in low grade dysplasia [59]. It has been shown that p53 gene mutations, with protein accumulation, can be detected in routinely processed biopsy samples [60]. In addition, Weston et al. have demonstrated that p53 localization in areas of low grade dysplasia represents a risk factor for progression to a higher degree of dysplasia. The authors suggested that these patients may benefit from a proactive secondary prevention-intervention program, rather than routine close observation [61]. However, mutated p53 has also been reported in BE without dysplasia [57, 58], and some investigators concluded that neither p53 mutation nor p53 protein accumulation had independent prognostic value in patients with BE associated adenocarcinoma [62]. Recent data seem to support the value of p53 as a predictor of BE progression to dysplasia or carcinoma [59, 63, 64]. On the other hand, p63, a p53-related protein, plays no role in BE associated carcinogenesis [65, 66].

15.11 Death-Inducing Signaling Molecules

Many factors involved in the apoptotic pathway of BE associated neoplasia have been studied to gain more

insight to the process of carcinogenesis. Fas/APO-1, a cell receptor that induces apoptosis when activated, is reduced on the cell surface of esophageal adenocarcinoma cells, but it is retained within their cytoplasm as a mechanism to evade Fas-mediated apoptosis [53, 67].

The Bcl-2 family of apoptotic regulators includes pro-apoptotic members (Bax, Bid, Bad, and Bak) and anti-apoptotic members (Bcl-2, and Bcl-xl). Proapoptotic Bcl-2 proteins increase the mitochondrial membrane permeability, allowing the leakage of cytochrome C from mitochondria into cytoplasm, with subsequent caspase activation and apoptosis. In Barrett's epithelium, Bcl-2 protein is highly expressed in low-grade dysplasia, protecting the cells from apoptosis, but it is decreased in high-grade dysplasia and adenocarcinoma [68]. In the same study the authors show high expression of the antiapototic protein Bcl-xl in BE associated dysplasia and carcinoma, but not in intestinal metaplasia. Other investigators have shown a significant reduction of Bcl-2 expression by immunohistochemistry, during the progression of Barrett's mucosa to adenocarcinoma. In this study, Bcl-2 expression correlated with improved patient survival [69].

Van Der Woude et al. concluded that a shift to an "antiapoptotic phenotype" occurs during the progression of Barrett's metaplasia to dysplasia and to adenocarcinoma, due to increased Bcl-x and decreased Bax expression [68].Others have demonstrated that CpG island hypermethylation (and consequent decrease protein expression) of the proapoptotic death associated protein kinase (DAPK) is an early change during BE neoplasia progression [70]. Finally, Li Y. et al. found a negative correlation between metallothionein (MT) expression and apoptotic index in BE associated neoplasia. The authors postulated that MT may function as a zinc-donor for DNA replication and repair in BE [71].

15.12 Angiogenic Markers

It is currently believed that angiogenesis is associated with early stages of tumor invasion, and experimental studies have shown that angiogenesis may occur during the transition from hyperplasia to neoplasia [72].

Hypervascularization/neovascularization has been observed around dysplastic colon adenomas [73], and in a background of mammary ductal carcinoma in situ [74]. Similarly, the increased expression of angiogenenic markers (VEGF, CD31) has been reported early during the progression of Barrett's neoplasia, but did not predict the progression of BE to adenocarcinoma or the survival of patients with BE associated adenocarcinoma [75, 76]. While VEGF expression and high angiogenesis score correlated with lymph node metastatsis [76], mean microvessel count, using CD31 stain, did not correlate with the tumors clinical pathologic features [77]. Others have reported the significant upregulation of VEGF and basic fibroblast growth factor messenger RNA expression in esophageal and gastroesophageal juction adenocarcinomas, suggesting a role of these factors in the development of esophageal cancer [78].

The use of non steroidal, anti-inflammatory drugs has shown to reduce the risk of gastrointestinal cancer by blocking the cyclooxygenase enzyme COX2 [79]. Cyclooxygenase-2 (COX-2) downregulates the expression of angiogenic factors VEGF and basic fibroblast growth factor, inhibiting angiogenesis [80]. Several investigators have observed an increase of COX 2 protein expression, possibly as a response to bile salts exposure, early during the BE associated neoplastic transformation [81, 82]. Others have reported that high COX-2 protein expression is associated with reduced survival of patients undergoing surgery for esophageal adenocarcinomas [83]. Finally, increased inducible nitric oxide synthase (iNOS) has been observed in 76% of Barrett's tissues in one study, however, the meaning of this finding is controversial [84].

15.13 Other Molecular Markers

Microsatellite instability (MSI) is the lost of control of the DNA fidelity, as a result of structural defects of proteins (including MLH1, MSH2, PMS2, MSH6) involved in the identification and correction of nucleotide matching defects, occurring during DNA replication. MSI has been documented in several hereditary and non hereditary malignancies. Kulke et al. have reported the absence of high levels of MSI in 80 cases of BE associated adenocarcinomas. Low levels MSI were identified in only 16% of the tumors,

and immunohisto-chemical expression of MLH1 and MSH2 was retained in all cases. The presence of low MSI was not associated with clinicopathologic features of the tumors [85].

HER-2 protein overexpression or gene amplification has been observed with variable prevalence in BE associated cancer, and it may represent a biomarker of progression from intestinal metaplasia to dysplasia, to carcinoma [86]. Using image-based three-dimensional fluorescent in situ hybridization in thick sections, Rauser et al. uncovered HER-2 low level copy gains in BE associated cancer, not detected by standard FISH. It has been proposed that this finding defines a biologically distinct subpopulation of BE associated cancer patients with unfavorable outcome [87].

A cDNA microarray analysis of BE, BE with dysplasia and BE with adenocarcinoma revealed a 2–16 fold overexpression of L-type Amino Acid Transporter-1 (LAT-1) mRNA in 7 of 8 tumor samples studied. This finding was confirmed in 28 of 28 esophageal adenocarcinoma using RT-PCR. LAT-1 is a sodium-independent, high affinity transporter of large branched chain and aromatic neutral amino acids. LAT-1 is also involved in the transport of the amino acid related chemotherapeutic agent melphalan and, theoretically, LAT-1 positive esophageal adenocarcinomas may be sensitive to therapeutic doses of this drug [88].

In another study, Lin J et al. studied the expression of melanoma-associated antigens (MAGEs) family A members, including MAGE-A10, in BE associated adenocarcinomas. These are tumor-specific antigens recognized by cytotoxic T lymphocytes and may provide potential targets for immunotherapy [89].

We should also mention studies on the expression of Glutatione S-transferase π (GST) in BE. GST are a superfamily of enzymes protecting cells from the cytotoxic effects of free radicals, and from DNA damage caused by tobacco carcinogens, chemotherapeutic drugs, and others. Brabender et al. have shown that down-regulation of GSTPI expression is an early event in the development of BE and of BE associated adenocarcinoma, and it is a marker of disease progression [90].

15.14 "Omics" of Barrett's Neoplasia

The recent advances of cDNA microarray technology with associated bioinformatics tools have allowed the interrogation of thousands of genes at once. This technology has improved the accuracy in tumor diagnosis and classification, and in predicting tumor progression and tumor response to therapy.

Several investigators have reported on the modulation of global gene expression profiling during Barrett's carcinogenesis and tumor progression [91–93]. For example, Wang et al have shown that the gene profile of BE samples is similar to that of BE associated adenocarcinoma and not to the gene profile of normal esophagus. CXCL3, TNFRSF12A, and FN14 are among the 12 differentially expressed genes between BE and esophageal adenocarcinoma [93]. This finding is in agreement with the concept that BE is biologically premalignant. In another study, filtered genes from a previous cDNA microarray study of BE associated adenocarcinoma were used to select a panel of 23 genes potentially capable to discriminate between premalignant and malignant BE changes. Using quantitative real time PCR (qRT-PCR) and bioinformatics (logistic regression analysis and linear discriminant analysis) these authors demonstrated that the combination of only 3 informative genes (BFT, TSPAN, and TP) was able to reliably discriminate between BE and BE associated adenocarcinoma [94].

It has been reported that human BE associated adenocarcinoma cells respond to acid exposure by downregulating apoptosis related genes and upregulating cell proliferation related genes [95]. Similarly, Cheng et al., using a rat model, have shown that esophageal epithelium exposed to an excess of duodenal and gastric reflux, develops BE, dysplasia, and eventually carcinoma [96, 97].

We have previously shown that the histologic progression from BE to adenocarcinoma is associated with early loss of genes regulating differentiation that begins before histologic changes, and a late gain in genes involved in remodeling and invasion [98]. Using a oligonucleotide microarray ("MitoChip") for rapid sequencing of the entire mitochondrial genome, Sui et al. have shown somatic mutations of mitochondrial DNA (mtDNA) in 39% of 23 samples of BE with dysplasia, but in only 13% of 15 samples of BE without dysplasia. The somatic mtDNA alterations were observed in preneoplastic lesions even in the absence of histopathologic evidence of dysplasia, suggesting that the mitochondrial genome is susceptible at the earliest stages of multistep cancer progression. The authors proposed the use of mtDNA analysis as a biomarker for early diagnosis of BE associated dysplasia [99].

MicroRNAs (miRNAs) are a newly discovered class of small non-coding RNA molecules that may function as either oncogenes or tumor suppressor genes. Feber et al. found a subset of 13 miRNAs capable of discriminating between adenocarcinoma, squamous cell carcinoma, and normal squamous epithelium of the esophagus [100].

Finally, the recent advances in mass spectrometry and bioinformatics have improved the discrimination of cancer specific peptides. The usage of high resolution, two dimensional, polyacrylamide gel electrophoresis allows the separation of up to 2,000 proteins at a wide range of pH gradients. This methodology maximizes protein separation, allowing the excision and identification of the selected proteins by matrix-assisted laser desorption/ionization, time of flight and tandem mass spectrometry (MALDI TOF MS) [101]. Using this approach a series of novel proteins differentially expressed in the progression from Barrett's metaplasia to esophageal adenocarcinoma were identified [102–104]. Peng et al. in their comparison of esophageal tumors to normal esophageal samples, identified a group of upregulated proteins (ErbB3, Dr5, Cyclin D1, and members of the zinc finger proteins) and a group of downregulated proteins (Lgi1 and Klf6). Of these ErbB3, Dr5, Znf146, and Lgi1 have not been previously described in BE [104].

15.15 Conclusion

To overcome the limitations of the pathologic criteria for detecting and evaluating BE-associated neoplasia, attempts have been made to identify molecular markers that can predict neoplastic progression in BE. It is possible that the future routine use of brush cytology in the diagnosis of BE will allow sampling of larger areas of diseased mucosa, thus increasing sensitivity and specificity in detecting dysplasia and/or carcinoma. The use of the more advanced molecular tests is promising, and may detect alterations that precede morphological changes.

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Chapter 16

Genesis of Pancreatic Ductal Neoplasia

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Contents

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16.1 Introduction

The epithelial components of the pancreas consist of acinar cells, ducts and islet cells. While the acini compose the majority of the pancreatic parenchyma, the majority of pancreatic carcinomas are of ductal type, rather than islet or acinar cell type. Indeed, when referring to pancreatic carcinoma, it is understood that one is referring to ductal adenocarcinoma of the pancreas.

The estimated incidence of pancreatic carcinoma in the United States for 2008 is 37,680, while the estimated number of deaths for 2008 is 34,290 [1]. Pancreatic cancer is the tenth most frequent cancer in men, accounting for approximately 3% of cancers in men, and accounts for less than 3% of cancers in women, and is not in the top ten for women, yet it ranks fourth as the overall leading cause of death in both sexes [2]. The 5-year overall relative survival rate for all stages is 5.1% [1]. Pancreatic cancer is such a lethal disease because it typically presents at a late stage, only 7% of patients present with carcinoma confined to the pancreas, 52% present with distant metastases, 26% are diagnosed after the carcinoma has spread to regional lymph nodes, and in the remaining 15%, the stage is unknown. Patients with localized disease have the best survival, with a 5-year survival rate of 20%. Those presenting with distant metastases have a 5-year survival rate of 1.8% [1].

Better understanding of the cellular and molecular processes which lead to the development of pancreatic carcinoma are vital both to improving the outcome for patients who present with either early or late stage disease, and also for early detection. The last two decades have seen an explosion in the knowledge of pancreatic

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cancer progression, resulting in the development of a progression model.

16.2 Epidemiology and Etiology

Pancreatic carcinoma presents primarily in older patients, with a median age of 72. The majority of cases present between the ages of 65–85 [1]. It occurs slightly more often in men than women. Black males are at the greatest risk, having a 50% increased risk of developing pancreatic carcinoma compared to whites [3], and it is slightly more common in people of Jewish descent.

The etiology of pancreatic carcinoma is unknown. Smoking, obesity, diet, alcohol, diabetes mellitus and chronic pancreatitis have all been reported to be associated with an increased risk [4]. Cigarette smoking is the most consistently identified risk factor, with an increased the risk of 2–3 fold [5]. The association with smokeless tobacco is not as clear, with one study reporting an increased risk between that of nonsmokers and smokers [6] and another study finding no increase [7]. Other risk factors include obesity, long standing diabetes, and chronic pancreatitis [4, 8–11]. The association with diet has been more controversial [12]. An association with eating red meats has been reported, but what appears to be consistent is that the method of cooking meats and eating pork and red meats is associated with a significant risk [13]. There is also an association resulting in long-standing diabetes and chronic pancreatitis.

16.3 Hereditary Pancreatic Carcinoma

Significant insight into the understanding of pancreatic carcinogenesis has been gained from studying the familial syndromes with which it is associated. Pancreatic carcinoma aggregates in families, accounting for 5–10% of all cases. A family history of pancreatic carcinoma is an indicator of pancreatic cancer risk [14], increasing the risk on average by 1.5–13 fold [15]. Syndromes with specific genetic alterations have been identified, but not all cases of familial pancreatic carcinoma are attributable to known syndromes; these cases are referred to as familial pancreatic cancer [16, 17] and show an autosomal dominant pattern of inheritance most likely due to a rare allele [15, 18]. The specific syndromes and germline mutations associated with pancreatic cancer in families include ataxia-telangiectasia syndrome (p53) [19, 20], hereditary nonpolyposis colorectal cancer Lynch II variant (hLMH1, hMSH2) [21], familial Atypical Multiple Mole Melanoma Syndrome (p16) [22], Peutz-Jegher Syndrome (STK11/LKB1) [23], Hereditary Breast and Ovarian Cancer (BRCA2) [24], and Hereditary Pancreatitis (PRSS1) [25, 26]. Recently, germline mutations to BRCA1 have also been found to contribute to the development of pancreatic cancer [27]. Identification of these genes contributed to understanding the carcinogenesis of pancreatic carcinoma, as the same genes that are mutated in pancreatic carcinoma are mutated in sporadic cases [28].

16.4 Pancreatic Ductal Adenocarcinoma Precursor Lesions

The morphology and nomenclature for ductal pancreatic cancer precursor lesions was standardized by the Pancreas Cancer Think Tank, sponsored by the National Cancer Institute, which met in Park City, Utah, in 1999 [29]. Up until this meeting, the terminology utilized to report on ductal precursor lesions included lesions, metaplasia, hyperplasia, dysplasia and neoplasia. The result was that data from different researchers evaluating the molecular progression of pancreatic carcinoma could not be compared. The terminology pancreatic intraepithelial neoplasia (PanIN), which applies to lesions arising in the smaller caliber pancreatic ducts, was unanimously adapted based on growing evidence that the spectrum of these lesions reflect a tumor progression model with neoplastic potential. The authors standardized the morphologic criteria for grade 1A, 1B, 2 and 3 PanIN, (Figs. 16.1–16.4) based the grading system on the degree of architectural complexity and cytological atypia.

PanINs harbor many of the same genetic alterations found in invasive ductal adenocarcinoma of the pancreas. There is a clear accumulation of genetic alterations associated with the histological progression from PanIN 1 to PanIN 3 and invasive carcinoma.

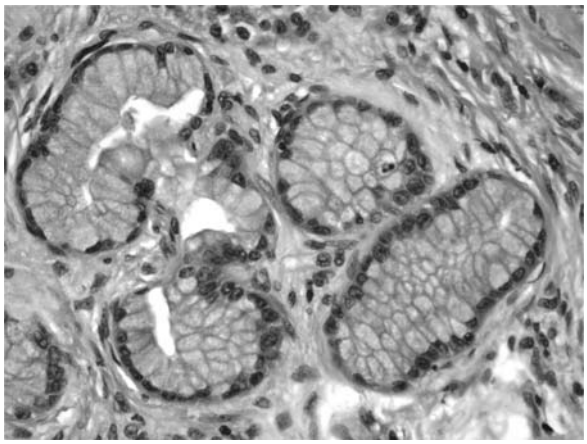

Fig. 16.1 Grades of dysplasia in PanIN 1a. The normal ductal epithelium in this small caliber duct is replaced by tall, columnar, mucinous epithelium

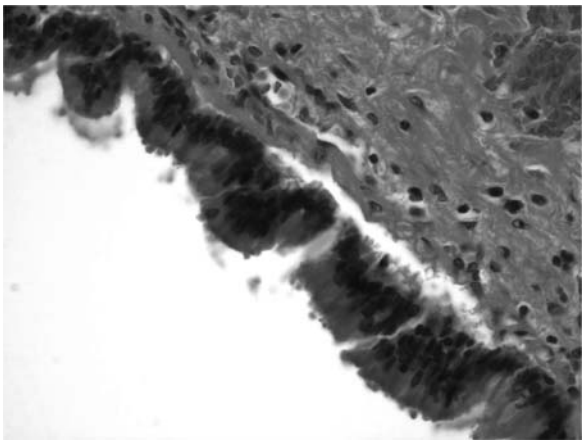

Fig. 16.3 Grades of dysplasia in PanIN 2. The nuclei are enlarged, hyperchromatic and elongated. They are crowded and overlapping but remain oriented to the basal layer

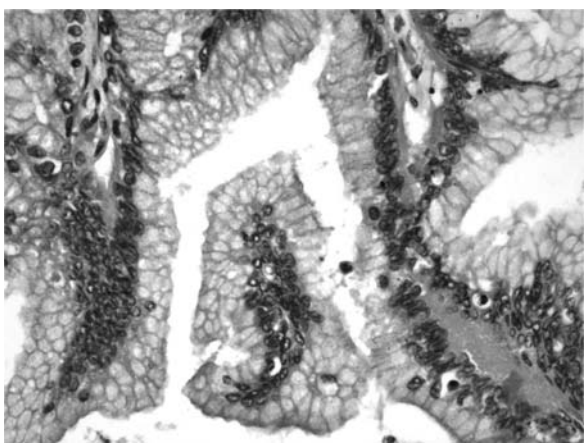

Fig. 16.2 Grades of dysplasia in PanIN 1b. The lining epithelium becomes architecturally complex, and is thrown into small papillae. The nuclei remain basally located, with minimal atypia

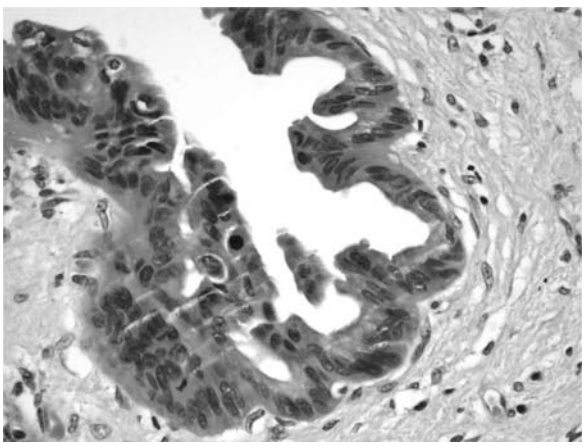

Fig. 16.4 Grades of dysplasia in PanIN 3. The nuclei are enlarged with vesicular nuclei and prominent nucleoli. There is significant mitotic activity, and the mitoses extend to the surface. The nuclei show loss of polarity, extending to the luminal surface

A progression model for pancreatic carcinoma has been developed based on these observations [30].

Intraductal papillary mucinous neoplasms and mucinous cystic neoplasms are the other forms of pre-invasive pancreatic neoplasia, both of which present as visible masses on imaging studies [31]. Intraductal papillary mucinous neoplasms (IPMN) are grossly visible neoplasms arising in the main pancreatic duct or one of its branches, characterized morphologically by a mucinous epithelium replacing the normal ductal epithelium that may also exhibit a papillary growth pattern. These lesions are distinct from PanINs in that they arise in the main duct system and present as a visible mass on imaging studies. Mucinous cystic neoplasms (MCN) are also grossly visible lesions. In contrast to IPMN, they do not communicate with the pancreatic ductal system, and in contrast to both PanIN and IPMN, the cysts are surrounded by an ovarian type stroma. Just as in PanIN, both IPMN and MCN show a progression of the degree of atypia of the duct lining epithelium, graded as low-grade, moderate grade and high grade.

Since PanIN are still considered to be the most common of these three precursor lesions, and most of the studies and progress made in understanding progression has focused on these lesions, the remainder of the discussion will be limited to PanIN.

16.5 Genetic and Molecular Alterations in Pancreatic Carcinoma

16.5.1 Telomeres

One of the earliest initiating events in pancreatic carcinogenesis is telomere dysfunction and shortening, which leads to chromosomal instability. Utilizing an in-situ hybridization technique assessing telomere length on a tissue microarray of PanINs, van Heek et al. [32] found telomere shortening in 96% of all PanINs examined, with 91% of all PanIN 1A harboring this abnormality. The reduction in telomere length was found in any PanIN lesion, whether it was associated with invasive adenocarcinoma or chronic pancreatitis. Ducts with atrophy or inflammation retained telomere length. This finding indicates that all PanIN lesions are truly neoplastic. Telomere shortening is the most common early genetic event occurring in PanIN 1A, with its reported frequency of 96% exceeding even that of K-ras oncogene activation (50%). The genesis of telomere shortening has not yet been elucidated.

16.5.2 Tumor Suppressor Genes

p16/CDKN2A is the most frequently inactivated tumor suppressor gene in pancreatic carcinoma [33], occurring in 90% of pancreatic carcinomas [34]. The loss occurs by homozygous deletion, intragenic mutation with loss of the second allele, and epigenetic silencing by promoter methylation. It is first identified in PanIN 2 lesions. The p16/CDKN2A gene encodes for the $p16^{INK4A}$ protein, part of the cyclin –dependent kinase (CDK) inhibitor family, regulating the cell cycle through the p16/Rb pathway. This protein normally inhibits cell cycle progression through the G1-S checkpoint. Loss of p16 is seen in 30% PanIN 1, 55% PanIN 2 and 71% of PanIN [35].

Inactivation of p53 is identified in 50–75% of pancreatic carcinomas. The method of inactivation typically involves intragenic mutation in one allele coupled with loss of the second allele [36]. The functions of p53 in the cell include G1/S cell cycle checkpoint, maintenance of G2/M arrest and induction of apoptosis. p53 mutations lead to dysregulation of apoptosis and cell cycle control in pancreatic cancer. Mutations in p53 occur late, showing up in PanIN 3 [37].

The DPC4/MADH4/SMAD 4 gene is inactivated in 55% of all pancreatic carcinomas and occurs late in pancreatic carcinogenesis [38]. This gene codes for Madh4 protein, a mediator in the TGFα pathway, which is a tumor suppressive pathway. A number of receptors in the TGFα pathway, including TGFα receptor types 1 and 2 and the activin receptors types 1B and 2 exert their effects through Madh4. Emphasizing the importance of this pathway in pancreatic carcinogenesis, mutations in the genes encoding for these receptors, TGFαR1 and 2, ACVRB/ALK4, and ACVR2, have been found in pancreatic and carcinomas [39–41] in low frequencies.

Mutations in the STK11/LKB1 gene occur in both familial and sporadic cases of pancreatic carcinoma. Mutations in this gene are the cause of the autosomal-dominant inherited disorder Peutz-Jegher syndrome. Patients with this disorder have a relative risk for pancreatic cancer of 132 [42]. In a Peutz-Jegher syndrome patient with both intestinal polyps and pancreatic cancer, sequencing analysis revealed loss of the STK11/LKB1 wild-type allele in the pancreatic cancer. As part of this same study, 127 sporadic cases of pancreatic and biliary tract cancers were evaluated for mutations in STK11/LKB1. Homozygous deletions or somatic sequence mutations coupled with loss of heterozygosity, were demonstrated in 4–6% of these cancers [23].

Other tumor suppressors targeted less frequently include mitogen activated protein kinase-4 (MKK4) [43], EP300 which codes for p300 which is a histone acetyltransferase regulating transcription [44] and FBXW7, a cyclin E regulator [45].

16.5.3 Oncogenes

K-RAS gene mutations are the most frequently identified mutations in pancreatic cancer, found in approximately 90% of all pancreatic carcinomas [46].

Most mutations are single point mutation in codon 12, with mutations in codon 13 and 61 occurring infrequently. Kras mutations play two roles in pancreatic carcinogenesis: initiation and maintenance. Kras gene mutations occur early and are an initiating event [47]. Constitutively active KRAS signaling, induced by the mutation in the KRAS protein, is needed for pancreatic cancer maintenance.

Mutations in the BRAF gene have been identified in one-third of pancreatic cancers lacking a mutation in KRAS [45]. Mutations in both Kras and BRAF are mutually exclusive, but both affect the RAF-MAP signaling pathway, suggesting that this is a very important pathway.

16.5.4 BRCA 2 and Fanconi Anemia Pathway Genes: Caretaker Genes

Germline mutations in BRCA2 are a source of familial cases of pancreatic carcinoma [48] and are identified in up to 7% of pancreatic carcinomas [24], even in patients with apparently sporadic disease. Mutations in BRCA2 also appear to be a late event in pancreatic carcinogenesis, similar to p53 and DPC4, presenting in PanIN 3 [49].

Recent studies have linked BCA2 to some cases of Fanconi anemia, and also identified some Fanconi anemia genes (FANC) as being mutated in pancreatic cancer. Biallelic mutations in BRCA2 gene are responsible for a subset of Fanconi anemia cases [50]. Other Fanconi genes were surveyed in 22 pancreatic cancer xenografts and 11 pancreatic cancer cell lines. A germline nonsense mutation was identified in FANCC in one cell line and somatic frameshift mutations were identified in FANCG in one xenograft. Both of these were accompanied by loss of a second allele [51]. The rate of mutations in FANCC and FANCG is estimated to be about 3% [52].

16.5.5 Gene Overexpression

Her2neu overexpression occurs in approximately 70% of pancreatic adenocarcinomas. It occurs early, being identified in 82% of PanIN 1A lesions [53].

16.6 Epigenetic Changes

Epigenetic changes, characterized by both hypo and hypermethylation of genes, play an important role in pancreatic carcinogenesis [54] by either silencing or activating a number of genes. Aberrant hypermethylation of the promoter region of the p16 gene is responsible for inactivation of this tumor suppressor gene in the 15–20% of cases lacking bi-allelic mutational silencing [33]. Examples of other genes that are frequently hypermethylated include SPARC(55) and ppENK [56]. Aberrant methylation of p16 and ppENK were detected in PanIN and in IPMN at various frequencies. As the neoplasms progressed, the frequency of aberrant methylation increased [57, 58].

Aberrant hypomethylation of genes can cause aberrant gene activation (54). An example of one such gene, identified as being overexpressed in pancreatic carcinoma using gene expression profiling is S100A4 [59]. Other genes identified as being hypomethylated in pancreatic cancer include claudin4, lipocalin2, 14-3-3σ, trefoil factor2, mesothelin and PSCA [60]. Of note in this list is 14-3-3σ, which is abnormally methylated in 85 and 97% of pancreatic cancer cell lines and xenografts, respectively, making it the most frequently hypomethylated gene in pancreatic cancer.

16.7 Alterations in Core Signaling Pathways

A comprehensive genetic analysis of nearly all of the protein-coding genes in the human genome performed in 24 pancreatic cancers defined a core set of 12 cellular signaling pathways and processes that were altered in 67–100% of the pancreatic carcinomas examined. The specific component that may be altered in a specific tumor could vary widely. While individual studies have identified specific gene mutations, this process provides an understanding of the significance of certain pathways to the development of pancreatic cancer [61]. Table 16.1 lists the regulatory pathway and processes. Specific mutations or genetic alterations relating to a number of these pathways have been touched upon in the preceding discussion. Two pathways, the hedgehog signaling pathways and Wnt/Notch

Table 16.1 Regulatory processes or pathways altered in pancreatic carcinoma, identified using global genomic analysis

Regulatory process or pathway	Fraction of tumors with genetic alteration of at least one of the genes (%)
Apoptosis	100
DNA damage control	83
Regulation of G_1/S phase transition	100
Hedgehog signaling	100
Homophilic cell adhesion	79
Integrin signaling	67
c-Jun N-terminal kinase signaling	96
KRAS signaling	100
Regulation of invasion	92
Small GTPase-dependent signaling (other than KRAS)	79
TGF-β signaling	100
Wnt/Notch signaling	100

signaling pathways merit further discussion since these are relatively recently identified alterations in pancreatic carcinoma, and both pathways play a role in normal pancreatic development.

16.7.1 Developmental Signaling Pathways

Hedgehog signaling has a critical role in pancreatic development and results in malignant transformation when mutated. Misregulation of hedgehog signaling plays a role in both initiation and maintenance of pancreatic cancer. Hedgehog exerts its effects at the cellular level through two receptors, Smoothened (Smo) and Patched (PTCH). Mutational inactivation of PTCH or overexpression of Smo lead to activation of the Hegehog pathway. Immunohistochemical studies demonstrating overexpression of sonic hedgehog and Smo in PanINs and adenocarcinomas, but not in normal ducts, confirm that hedgehog is both an early and late mediator of pancreatic carcinogenesis [62]. This pathway is required for cell proliferation and suppression of apoptosis in pancreatic cancer.

Notch pathway receptors (Notch 1–4), ligands (Jagged 1 and 2), and transcriptional targets (Hes1), are overexpressed in both invasive adenocarcinomas and PanINs. Activation of Notch in PanINs is dependent on the ligand, particularly Jagged1, identified in microarray analysis Overexpression of Notch occurs in acinar-ductal metaplasia (see section on Pancreatic Cancer Cell of Origin) and in PanIN 1 lesions in the mouse model. Overexpression of TGFα leads to upregulation of Notch [63]. Augmented EGF, particularly TGFα activity, may be an initiating event, with resulting Notch pathway activation in exocrine tissue [63].

Activation of the Wnt pathway occurs via activating mutations in b-catenin or loss of function mutations in APC. Wnt pathways mutations are rare in pancreatic ductal adenocarcinoma, but are observed more frequently in nonductal tumors. As one would expect based on these findings, Wnt is a rare finding in PanIN lesions [37]

16.8 Altered Protein Expression

In addition to genetic mutations, alterations in protein expression occur due to upregulation.

16.8.1 Cyclooxygenase 2 (COX-2)

COX-2 levels are upregulated in pancreatic cancer. COX 2 inhibitors delay progression of PanIN to adenocarcinoma in the mouse model and slow the growth of pancreatic cancer cell lines [64–66]. COX 2 overexpression appears to occur early in PanIN, being identified in PanIN and in all of the subsequent grades.

16.8.2 Matrix Metalloproteinase 7 (MMP-7, Matrilysin)

MMP-7 is a member of the MMP family of zinc dependent extracellular proteases. This protein is observed in invasive adenocarcinoma and in greater than 70% of PanIN lesions. It is associated with resistance to apoptosis, and plays a role in cancer invasion and metastasis [67–69].

16.8.3 Mucins

MUC1 and MUC 4 are associated with pancreatic cancer. MUC1 is expressed at the early stage of gestation in the fetal pancreas. In normal pancreas, expression of MUC 1 is confined to the apical portion of intralobular ductules. During pancreatic carcinogenesis, it is expressed in PanIN, and its expression increases with the progression to invasive cancer. MUC 4 is not expressed in normal pancreatic development or the normal pancreas, but is expressed in PanIN and invasive cancer. Both of these mucins appear to exert their effects by disturbing cell–cell or cell–matrix interactions [70].

16.8.4 Cell Cycle Regulation Proteins

PAnIN lesions show abnormalities in cell cycle regulation demonstrated by utilization of immunohistochemistry for proliferation antigens such as ki-67 and for cell-cycle proteins. Immunohistochemical labeling for Ki-67 shows increasing expression from PanIN 1A to 3. The highest percentage of Ki-67 positive cells is identified in invasive carcinoma [71]. Topoisomerase IIα, needed for the relaxation of the DNA supercoil, is expressed in PanIN in concordance with the expression of Ki-67 [37]. Cyclin D1, a co-factor in the phosphorylation and inactivation of the Rb gene, is overexpressed in invasive pancreatic adenocarcinomas. Overexpression of cyclin D1 within PanIN occurs in a third of PanIN 2 and half of PanIN 3 lesions, placing it as an intermediate event [37].

$P21^{WAF/CIP1}$ is a cyclin dependent kinase inhibitor whose role is to prevent phosphorylation of the Rb gene. Immunohistochemical expression of $P21^{WAF/CIP1}$ shows expression of this protein at PanIN 1A level, preceding even cyclin D1 [72].

16.9 Gene Expression Profiling

A large number of studies have been published using a variety of platforms to evaluate gene expression abnormalities in pancreatic carcinoma. Novel genes identified include prostate stem cell antigen (PSCA) and mesothelin [73]. Genes that are overexpressed in pancreatic carcinoma compared to normal ducts include topoisomerase IIα, fascin, heat shock protein 47 and pleckstrin. Two members of the S100 family of genes, namely S100P and S100A6, showed significant upregulation in one study. S100P was found to be specific for pancreatic cancer [74].

16.10 miRNA Analysis

MicroRNAs (miRNAs) regulate cellular differentiation, apoptosis, and cell growth, thereby functioning as tumor suppressors or oncogenes. Recent studies have shown that miRNAs are deregulated in pancreatic cancer. One study identified eight miRNAs as being significantly upregulated in most pancreatic cancer tissues and cell lines. These include miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95 [75]. Another study found miR-221, -376a and -301 overexpressed by tumor cells and not stroma or normal acini or ducts [76]. Upregulation of these miRNAs must play a role in the control of various pathways involved in pancreatic carcinogenesis.

MicroRNAs are an important component of the p53 transcriptional pathway. A direct role for one miRNA, miR-34a, has been found in pancreatic cancer. This miRNA is directly transactivated by p53. It is commonly deleted in human cancers, including pancreatic cancers. It appears to modulate and fine-tune the gene expression program initiated by p53 [77]. The abnormalities in this particular miRNA illustrate the important role of miRNAs in normal cellular function and how they contribute to pancreatic carcinogenensis. Further studies of other specific miRNAs may lead to better understanding of the function of miRNAs in pancreatic cancer.

16.11 Progression Pathway in PanINs

A pancreatic adenocarcinoma progression model has been developed incorporating the molecular alterations with the stage of neoplasia at which they first occur [78]. Changes occurring prior to the development of PanIN lesions, in the precursor cells include upregulation of Notch signaling pathways. The earliest event

is telomere shortening. Other early events, occurring in PanIN 1 lesions, include KRAS mutations, Her2neu overexpression, p21 (WAF1/CIP1) up regulation, and overexpression of PSCA, mucin 5, fascin and MMP 7. Mutations occurring in the intermediate phase, in PanIN 2, include p16 inactivation, epigenetic alterations and cyclin D1 activation. Late phase alterations occurring in PanIN 3 include p53, BRCA 2 and DPC 4 mutations and upregulation of 14-3-3σ. Proliferation markers Ki-67 and topoisomeraseIIα are expressed late, also. Mesothelin is upregulated in the process of or after invasion by the neoplastic cells [37, 79].

16.12 Mouse Models of Pancreatic Cancer

The importance of the genetic alterations in KRAS, P53 and $p16^{INK4a}$ in the molecular pathogenesis of pancreatic ductal adenocarcinoma is underscored by studies in mouse models of pancreatic cancer [80–82]. In recent years, at least 12 different genetically engineered mouse models of pancreatic neoplasia have been generated [81]. The successes and failures of these various models to faithfully recapitulate the evolution from PanIN1 to invasive ductal adenocarcinoma that occurs in humans has been essential in the effort to define the minimal number of essential genetic lesions needed to produce pancreatic cancer.

Some of the first attempts to make a mouse model of pancreatic ductal adenocarcinoma employed transgenic expression of TGFα or mutated KRAS ($KRAS^{G12D}$) driven by the rat elastase gene promoter [81]. While the elastase promoter is active in the pancreas, it directs expression primarily in the acinar cell lineage. These mouse models are characterized by prominent acinar to ductal metaplasia. The mice developed primarily acinar cell carcinomas and a small number of undifferentiated carcinomas. Characteristic PanIN lesions were not a prominent feature of pancreatic carcinogenesis in these mice. Whether or not acinar to ductal metaplasia is a prominent feature of human pancreatic ductal adenocarcinoma remains controversial, and will be discussed in the next section on cellular precursors of pancreatic carcinoma. In any case, the disease that results in these mice does not resemble the human disease.

Mice have also been engineered that have targeted disruption of $p16^{INK4a}$. These mice do not develop PanIN or ductal adenocarcinoma, indicating that this genetic change is not likely to be an initiating event in ductal carcinogenesis. Since targeting the expression of oncogenes to the acinar lineage did not yield a true mouse model of pancreatic ductal adenocarcinoma, efforts were made to use promoter elements from genes that are expressed in the ductal cell lineage. To this end a transgenic mouse model was made in which expression of mutated KRAS ($KRAS^{G12V}$) was driven by the Cytokeratin-19 promoter. This promoter is active in mature differentiated ductal epithelium. Remarkably, these mice did not develop pancreatic tumors of any kind or ductal neoplasia. This result may indicate that the presence of KRAS alone in the ductal epithelium is insufficient for tumorigenesis and that other mutations are required. Alternatively, expression of KRAS late in the terminally differentiated duct when Cytokeratin-19 is being expressed is not a permissive time to initiate neoplasia. An alternative strategy has been employed in which conditional mouse mutants are generated in a tissue specific manner. In the Pdx1-cre; LSL-$Kras^{G12D}$ model, an endogenous KRAS allele is modified to contain an activating mutation at codon 12. Constitutive expression of this allele is prevented by insertion of a segment of DNA that stops transcription and translation of the mutant allele. This DNA segment is flanked by LoxP recombination sites for Cre recombinase. A separate line of mice was generated that express Cre recombinase in pancreatic ductal epithelial progenitor cells using the Pdx-1 promoter. Pdx-1 is a transcription factor that is expressed at a very early stage during the development of the pancreas. When the Pdx1-Cre mice and the LoxP-Stop-LoxP-$Kras^{G12D}$ mice are crossed, the resulting line of mice expresses the mutant Kras allele only in the pancreatic epithelium. With this approach the mutant Kras is expressed at physiological levels. The mice develop PanIN1, PanIN2 and PanIN3 with 100% penetrance. Over a period of several months, these mice go on to develop invasive ductal adenocarcinomas that are well to moderately differentiated, with focal areas of anaplastic tumor. Metastatic spread of the tumors to lymph nodes and liver is also seen. The median survival of this mouse model is 16 months. When the Pdx1-cre; LSL-$Kras^{G12D}$ mouse is interbred with a mouse line that is deficient in $p16^{INK4a/Arf}$ or a mouse that carries the gain of function P53 mutation

($Trp53^{R172H}$), the development of invasive pancreatic ductal adenocarcinoma is dramatically accelerated. Invasive cancer appears within 4 weeks. In the absence of $p16^{INK4a/Arf}$ the median survival is 10 weeks and in the presence of $Trp53^{R172H}$ the median survival is 5 months. Therefore, these mouse models have confirmed that mutation of KRAS and loss of p16 or p53 are sufficient to produce a disease in mice that is molecularly and histopathologically indistinguishable from human ductal adenocarcinoma [81].

16.13 Pancreatic Cancer Cell of Origin

The studies in the mouse models have not only confirmed that the proposed molecular progression pathway is accurate, but also introduced new questions about the cell of origin of pancreatic cancer. One mouse model which targeted the mature ductal epithelium did not produce mPanIN or neoplasia, suggesting that the mature ductal cell is not the cell of origin for pancreatic ductal adenocarcinoma [83]. Other options include pancreatic cancer stem cells, centroacinar cells (CAC) and acinar cells. A highly tumorigenic subpopulation expressing the CD 44, CD 24 and epithelial specific antigen (ESA) was identified in human pancreatic adenocarcinomas [84]. These cells had a 100-fold increased tumorigenic potential compared to nontumorigenic cancer cells. They also had the stem cell properties of self renewal, the ability to produce differentiated progeny, and increased expression of sonic hedgehog. The centroacinar cell has the potential of being resident stem or progenitor cells based on the fact that they are the only cell in the mature exocrine pancreas with retained Notch activation, as assessed by Hes1 expression. Since Notch signaling is known to repress differentiation in the developing pancreas, this suggests that centroacinar cells may retain a precursor-like transcriptional program [63, 85]. While centroacinar cells appear to retain a precursor like transcriptional program, acinar cells appear to be facultative progenitor cells, taking on a progenitor role when the pancreas is damaged and regenerating.

Three lines of evidence support this theory. The first is that acinar cells dedifferentiate in caerulein induced chemical pancreatitis, expressing Pdx1 and Hes1, markers of progenitor cells [86]. The second is the presence of acinar-ductal metaplasia, induced by growth factors, specifically TGFα [63]. That acinar cells differentiate into ductal cells has been confirmed by lineage tracing experiments in reporter mice [87]. Finally, in a mouse model which targeted $KRAS^{G12D}$ allele to elastase expressing acinar cells, mPanIN and adenocarcinoma arose from acinar-ductal metaplasia. Acinar to ductal metaplasia is illustrated in Fig. 16.5.

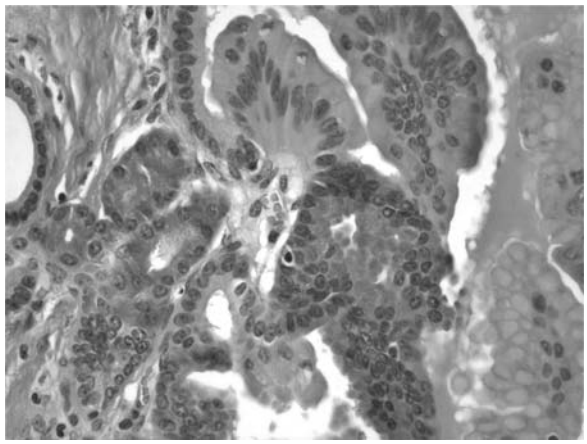

Fig. 16.5 Acinar-ductal metaplasia in human pancreas. This image is taken from a pancreatic resection specimen. This duct is lined by mucinous epithelium. Evident within the duct are also acinar cells. A transition from the acinar cells to the ductal mucinous cells is evident

16.14 Conclusion

PanIN has been established as the precursor lesion for invasive pancreatic carcinoma. A progression model with sequential accumulation of mutations and overexpression of various pathways has been elucidated through numerous studies. This model has been validated in the mouse model of pancreatic carcinogenesis. Future studies in the mouse model will lead to further understanding of the mechanisms involved in the development of pancreatic cancer, and identify new targets both for therapy and prevention.

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Chapter 17

Recent Advances in the Pathogenesis of Pancreatic Endocrine Neoplasms

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17.1 Introduction

The pancreas is thought by some to be the least understood organ after the brain [1, 2]. While the pancreas gets its name from the Greek *pankreas* meaning "all flesh," it is actually a complex lobulated organ with endocrine and exocrine functions. The exocrine pancreas, which produces digestive enzymes, accounts for 80–85% of the pancreatic volume and is composed mainly of ductal and acinar cells admixed with pancreatic parenchyma [3]. The endocrine pancreas is composed of endocrine cells that secrete polypeptide hormones and accounts for 2% of the pancreatic volume. The endocrine cells are found in the islets of Langerhans, which are scattered in the pancreatic parenchyma, and close to pancreatic ducts [1, 4, 5]. The pancreatic islet cell population consist of 10% alpha (α) cells, 70% beta (β) cells, 15% pancreatic polypeptide (PP) cells, and 5% delta (δ) cells [6]. Thus, the pancreas controls nutrient resorption and glucose metabolism via the functional activity of ductal, acinar, and endocrine cell types.

Tumors may develop in either the exocrine or endocrine pancreas. Endocrine pancreatic cancers, also known as Pancreatic Endocrine Neoplasms (PENs), are diverse rare tumors with a wide range of presenting symptoms that are thought to arise from the endocrine cells of the pancreas [7, 8]. PENs account for approximately 2% of all pancreatic neoplasms and are rare in comparison with pancreatic exocrine tumors [8]. Approximately 2,500 new PENs are diagnosed yearly [9, 10]. The incidence of PENs is less than 1 per 100,000 person-years in population studies, although at autopsy the incidence ranges from 0.8 to 10% as these tumors often go unnoticed [11].

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PENs have a better prognosis than exocrine pancreatic tumors. The 5-year survival rate is between 35 and 60% [12]. Currently, no environmental risk factors have been significantly associated with the development of PENs. A recent study found no association with first-hand tobacco exposure or alcohol use [11]. Further case-control and cohort studies are needed to investigate whether life-style factors are associated with the development of these rare tumors. The gene mutations of exocrine adenocarcinomas (TP53, p16INK4A, SMAD4, and K-ras) [13] are found only rarely in association with PENs. The molecular pathogenesis and histogenesis of PENs needs further elucidation. The purpose of this chapter is to summarize recent literature in the pathogenesis of PENs.

Table 17.2 WHO classification of pancreatic endocrine tumors

1. Well-differentiated endocrine tumor
1.1. Benign behavior
Confined to the pancreas, <2 cm in diameter, ≤2 mitoses per 10 HPF, ≤2% Ki-67-positive cells, no angioinvasion, or
perineural invasion
1.2. Uncertain behavior
Confined to the pancreas and one or more of the following
features: ≥2 cm in diameter, >2 mitoses per 10 HPF, >2%
Ki-67-positive cells, angioinvasion, perineural invasion
2. Well-differentiated endocrine carcinoma
Low-grade malignant
Gross local invasion and/or metastases
3. Poorly differentiated carcinoma
High-grade malignant
>10 mitoses per HPF

HPF, high-power field.

17.2 Categorization and Classification

PENs are categorized as functional or nonfunctional. Functional tumors secrete polypeptide hormones such as insulin, gastrin, glucagon, somatostatin, vasoactive intestinal peptide, adrenal corticotrophic hormone, parathyroid hormone-related peptide, growth hormone, calcitonin, melanocyte-stimulating hormone, vasopressin, and norephinephrine and elicit a clinically recognizable, hormone-related syndrome. Nonfunctional tumors secrete biologically inactive hormones and do not produce hormone-associated symptoms (Table 17.1) [14]. Approximately half of all PENs are nonfunctional [10].

In an attempt to define these diverse neoplasms, several classification systems have been proposed, of which the World Health Organization system is the most commonly used (Table 17.2).

Classifying PENs according to size, proliferative activity, angioinvasion, organ invasion, metastases, hormone activity, and clinical syndromes has been shown to be useful in predicting the clinical behavior of these tumors [10, 15]. However, a better understanding of the pathogenesis of these tumors will result in better tumor characterization and improved diagnostic, therapeutic, and prognositic modalities and may lead to a potential cure for patients with these unpredictable neoplasms [16].

Current genetic and molecular techniques have found that the biology of PENs is complex, without a well-recognized pathway being strongly influential. These tumors appear to develop in stages with multiple sequential mutations required to progress to malignancy (Fig. 17.1) [17]. Myriads of factors interact in complex ways to influence development, differentiation, secretion, and the interaction of tumor with its environment [18].

Table 17.1 Phenotyping and clinical features of the different types of PENs (Heitz 2004)

Tumor type	Hormone	Syndrome	Frequency (%)
Functioning tymors			
Insulinoma	Insulin	Hypoglycemia	27.2
Gastrinoma	Gastrin	Zollinger Ellison syndrome	12.5
Glucagonoma	Glucagon	Glucagonoma syndrome	8.0
Vipoma	Vasoactive intestinal peptide	Verner-Morrison syndrome	6.4
Somatostatinoma	Somatostatin	Somatostatinoma syndrome	3.8
ACTH producing Tumor	ACTH	Cushing syndrome	2.4
Serotonin producing Tumor	Serotonin	Carcinoid syndrome	<1
Non-Functioning tumors	Different hormones, slightly elevated	No syndrome	39.7

The table is reproduced from the WHO monograph on Pathology and Genetics, "Tumors of Endocrine Organs, 3rd edition", 2004.

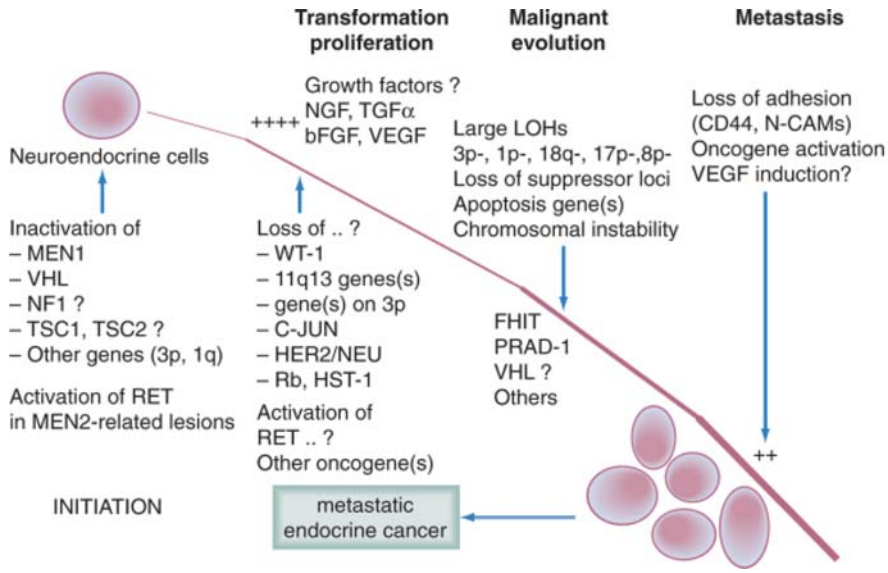


Fig. 17.1 [17] Diagram summaryizing the of major events involved in pancreatic endocrine tumor initiation, progression, and the pathogenic mechanisms involved in metastasis. bFGF, basic fibroblast growth factor; FHIT, fragile histidine triad; MEN1, multiple endocrine neoplasia type 1; NF1, neurofibromatosis type 1 (neurofibromin); NGF, nerve growth factor; PRAD-1, parathyroid adenoma–related protein; TGF, transforming growth factor; TSC1 and TSC2, tuberous sclerosis genes; VEGF, vasculoendothelial growth factor; VHL, von Hippel-Lindau genes

17.3 Composition and Development of the Normal Pancreas

A better understanding of normal pancreatic development will facilitate the understanding of the development of pancreatic neoplasia. The pancreas originates from the forgut endoderm [19]. During the seventh week of gestation, the dorsal and ventral foregut outpouchings rotate and fuse to form a single gland. The majority of the gland, including the body, the tail, the superior/anterior aspect of the head, and the accessory duct of Santorini, is derived from the dorsal outpouching of the foregut. The posterior/inferior part of the head of the pancreas is derived from the ventral outpouching of the foregut and drains into the papilla of Vater through the duct of Wirsung [3].

17.4 Formation and Regulation of Endocrine Cell Mass

Differentiation of the pancreas into a complex tissue structure combining duct, acinar, and islet cells is controlled by multiple physiologic, environmental, and hormonal mechanisms. The regulation of β-cell mass studied in diabetes best describes the current knowledge of endocrine cell formation and regulation.

Four physiological processes determine β-cell mass: neogenesis, replication, hypertrophy, and apoptosis [1, 20], abnormalities of which could be associated with tumorigenesis.

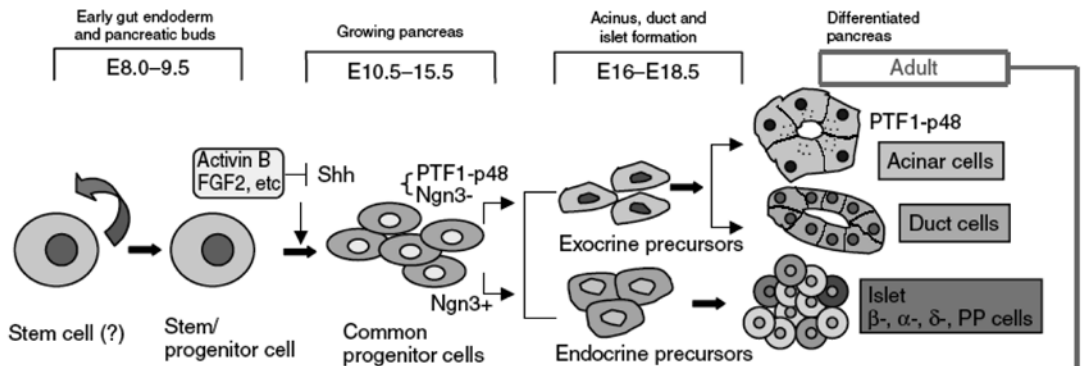
17.4.1 Beta-Cell Neogenesis

Neogenesis is the formation of new cells from pluripotent precursors. This mechanism appears to be the most significant mechanism to increase β-cell mass in humans. Neogenesis appears to occur in waves. The first wave of neogenesis occurs in the embryo. A second wave occurs during weaning. Thereafter, neogenesis occurs at a slow rate throughout adult life and may vary with diet [1, 21]. Neogenesis is demonstrated morphologically as endocrine cells budding from pancreatic ducts and/or clusters of β-cells scattered within the exocrine pancreas [1, 20, 22, 23]. The cell from which neogenesis derives has neither been definitively identified in embryogenesis nor in adult tissues. The neogenesis of β-cells is generally believed to originate

from stem/progenitor cells that reside among the duct cells from which they migrate to form new islets, differentiate, and regenerate both during organ formation and in regeneration of the adult pancreas [20, 24–28]. However, multiple studies suggest other possible locations of stem cells ranging from islets and/or acini to extrapancreatic cells [20, 24].

The inability to find islet stem/progenitor cells is due to the lack of specific cell markers [24]. Evidence exists that there may be more than one kind of stem cell. Studies have identified possible stem cell candidates expressing varied progenitor markers. These include glucose transporter 2, insulin, somatostatin (SST), nestin, pancreatic duodenal homeobox 1 (PDX-1), and islet neogenesis-associated protein [24, 29–31]. There are multiple transcription factors and other molecules that are potential candidates for islet progenitor markers (Fig. 17.2) [24].

Other mechanisms implicated in the neogenesis of β-cells include transdifferentiation of adult ductal, acinar, or even extra-pancreatic cells such as liver [32–35] or intestine [36]. Multipotential adult tissues (differentiated or stem cells) have been shown to cross lineage boundaries. For example, under proper stimulation, cells within the pancreas have been shown to give rise to hepatocytes [24, 37, 38]. Adenovirus-mediated uptake of PDX-1 into liver cells in vivo induced a β-cell phenotype that produced insulin and controlled the hyperglycemia of streptozocin-induced diabetes in rats [39]. Another study showed overexpression of an activated form of PDX-1-induced liver cells to become pancreatic cells in vitro and in vivo [33]. Hepatic stem/progenitor cells (also known as oval cells) can differentiate into insulin-producing cells [32]. There are several possible mechanisms of transdifferentiation. It is likely that a common progenitor cell from the



Transcription factors :

PDX-1, PBX 1	++	++	+	+				
Hlxb9	+	+/–	+	+				
PTF1-p48	+	+	+	–				
Isl-1, Nkx2.2, Nkx6.1	+	+	+	+				
Pax 4, Pax 6	+	+	+	+				
Ngn3	+	+	+	–				
Beta2/NeuroD	+	+	+	+				

			Adult pancreas			Adult pancreatic regeneration		
Other molecules:			Acinar cells	Duct cells	Islet	Acinar cells	Duct cells	Islet
Activins/Act RII	n.d.	+	–	+	+(α,δ)	–	+++	+(α,δ)
Nestin	–	+	–	+	+	–	+	+
PGP9.5	n.d.	+	–	–	+	–	++	+
PYY	+	+	–	–	+(α.δ)	–	+	+(α,δ)
TH	+	+	–	–	+(β)	–	+	+(β)
Glu-2	n.d.	+	–	–	+	–	–	++
Vimentin, Bcl-2, CK20	n.d.	+	–	+	–	–	+	+

Fig. 17.2 [24] A schematic model for the process of pancreatic differentiation. The identified transcription factors and other molecules that are candidates for islet progenitor markers are shown. + + + : high expression; + + : relative high expression; + : moderate expression; +/– : very low expression; – : no expression. n.d. : not determined

endoderm or adjacent area of the liver, intestine, and pancreas can be stimulated to dedifferentiate or transdifferentiate. Furthermore, mature tissues harbor a few pluripotent stem cells that have lineage plasticity.

Deregulated transdifferentiation or additional genetic events could lead to oncogenesis of islet cells. Evidence for this theory exists in the studies of hamsters treated with N-nitroso-bis (2-oxopropyl) amine, a carcinogen causing pancreatic ductal carcinomas. In this scenario, ductal structures begin to appear around and within islets, first exhibiting hyperplasia, then dysplasia, and finally malignant gland structures [2, 4, 40].

17.4.2 Beta-Cell Replication

Replication is the formation of cells from the mitotic division of preexisting mature cells. The term β-cell hyperplasia refers to an increase in β-cell numbers secondary to increased replication, increased neogenesis, or decreased apoptosis. Studies in rats have shown high rates of β-cell replication in the neonate, which slowly decreases to a rate of 2–3% new cells per day in the adult rat. This scenario is also likely in humans [1, 41], although the significance of replication as a means of maintaining β-cell mass in humans is controversial [42, 43].

17.4.3 Beta-Cell Hypertrophy

Hypertrophy is defined as an increase in the size of existing cells. Increasing β-cell mass via hypertrophy may be more important for cells that can no longer replicate [1]. Hyperglycemia has been implicated as a stimulus for β-cell hypertrophy in rat models [44, 45]. However, contradictory studies also exist [42].

17.4.4 Apoptosis in Beta Cells

Apoptosis is programmed cell death. The rate of β-cell apoptosis varies inversely with insulin requirements in the normal pancreas and occurs at slower rates with increasing age [20]. Apoptosis is a key process in islet plasticity. In a study by Jamal et al. [46], adult human islets cultured in vitro in specific medium were shown

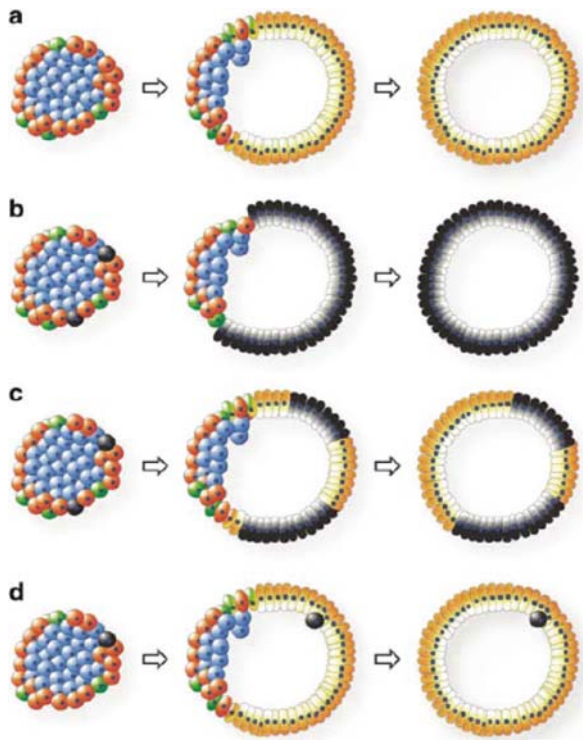


Fig. 17.3 [46] Proposed mechanisms for the phenotypic switch from a solid islet to a regeneration-competent DEC. (**a**) Direct transformation of islet mantle cells to duct-like cells, with concomitant loss of centrally located β-cells. (**b**) Activation and proliferation of an intra-islet progenitor cell that comes to form all cells of the cystic structure. (**c**) Cells of the newly formed duct-like structure are derived from both direct transformation of mantle endocrine cells and putative intra-islet progenitor cells. (**d**) The cystic duct-like structure forms solely through direct transformation of mantle endocrine cells, while an intra-islet progenitor cell remains associated with the duct-like structure

to change into ductlike epithelial cystic structures. The structures are formed by the islet cells located in the islet periphery. β-Cells, normally located in the islet core, undergo selective apoptosis forming the lumina of the duct-like epithelial structures (Figs. 17.3 and 17.4) [46].

Ductlike epithelial cystic structures can be transformed back to islet-like structures, which are morphologically and functionally similar to isolated islets (Fig. 17.5) [46]. The results of this study suggest that adult human islets possess morphogenetic plasticity. Of note, islet-to-duct transformation has been suggested to play a role in the development of pancreatic adenocarcinomas. The observations made in this study may contribute to better understanding of islet neogenesis and pancreatic carcinogenesis.

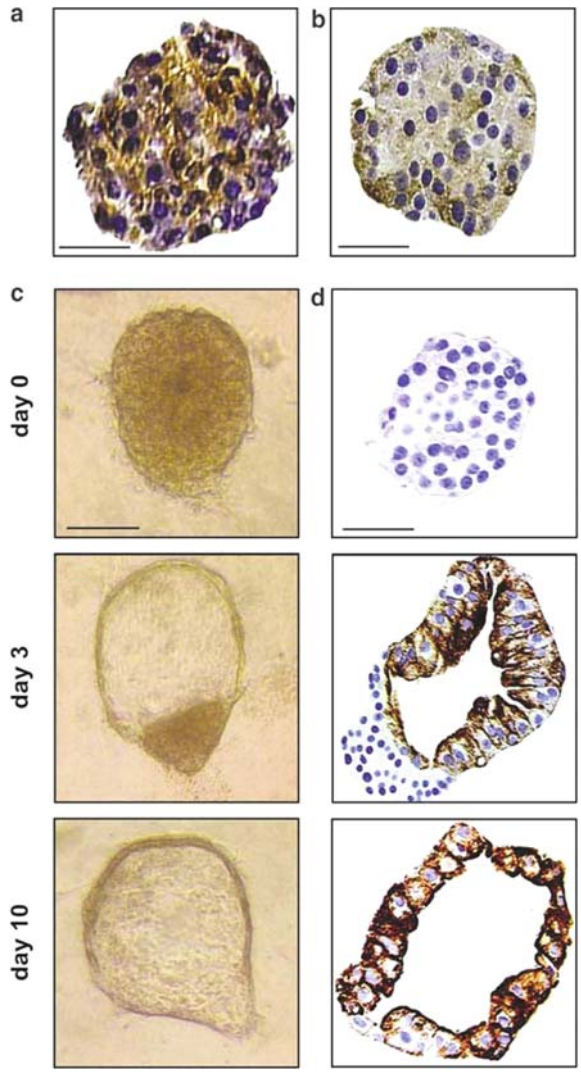


Fig. 17.4 [46] Islet-to-DEC transformation. Freshly isolated adult human islets expressed (**a**) insulin and (**b**) glucagon + somatostatin + pancreatic polypeptide. (**c**) Inverted microscopy and (**d**) CK-19 immunoreactivity demonstrated a typical islet-to-DEC transformation, starting from a freshly isolated islet (day 0), through a transitional structure (day 3), to a fully formed DEC (day 10, scale bars 100 mm)

17.5 Regulators of Pancreatic Endocrine Cell Mass

There are numerous hormones and growth factors that interact in the complex islet cell environment and that are affected by mediators from the exocrine pancreas and systemic circulation. Feedback mechanisms, adjusting to levels of islet hormones, regulate islet cell growth and morphology [1]. Once again, β-cells have been studied the most extensively in an effort to understand diabetes. However, generalizations of principles learned in these studies will increase understanding of the mediators in PENs.

Glucose seems to be the most important regulator of β-cells [1, 31, 47]. Hyperglycemia can induce either adaptation with an increase in β-cell mass or can result in failure to compensate with resultant diabetes [44]. Hyperglycemia-induced increase in β-cell mass may be secondary to an increased proportion of β-cells entering the cell cycle [48]. Also, hyperglycemia causes pancreatic duodenal homeobox 1, a transcription factor that activates the insulin gene, to migrate from the cytoplasm to the nucleus to increase insulin gene transcription [49]. However, hyperglycemia has also been shown to impair insulin secretion, β-cell replication, islet neogenesis, and increased β-cell apoptosis [50–52]. The effect of insulin on β-cell mass is not fully understood. Some studies suggest that it stimulates β-cell growth, while others show growth only in the presence of hyperglycemia [53–56].

c-Myc is a transcription factor of the basic helix-loop-helix leucine zipper. It is an extensively studied protooncogene, involved normally in cell cycle progression. c-Myc promotes cell growth and proliferation in several tissues, but induces apoptosis in others [57]. c-Myc is a key target gene of the Wnt/β-catenin pathway [58]. It is activated during pancreatic development [59]. Pelengaris et al. [205] noted that although Myc activation initially promotes both proliferation and apoptosis in pancreatic β-cells, apoptosis predominates, giving rise to islet involution and diabetes. The upregulation of cell cycle inhibitors, such as p21 that inhibits c-Myc induced proliferation and apoptosis, play a role in β-cell hypertrophy.

Activation of the *phosphatidylinositol 3-OH-kinase/protein kinase B (P13K-Akt/PKB)* pathway by *insulin growth factor-1 (IGF-1)* or *islet neogenesis-associated protein (INGAP)* upregulates β-cell mass. This pathway activates downstream messengers and transcription factors such as pancreatoduodenal homeobox gene-1 (PDX-1), neurogenin 3 (NGN-3), islet-1 (ISL-1), NeuroD/Beta2, and NK homeobox gene 2.2 (NKX-2.2), known to act during pancreatic embryogenesis [46].

PDX-1 is critical for pancreatic development [60]. It is also a key regulator of several factors that

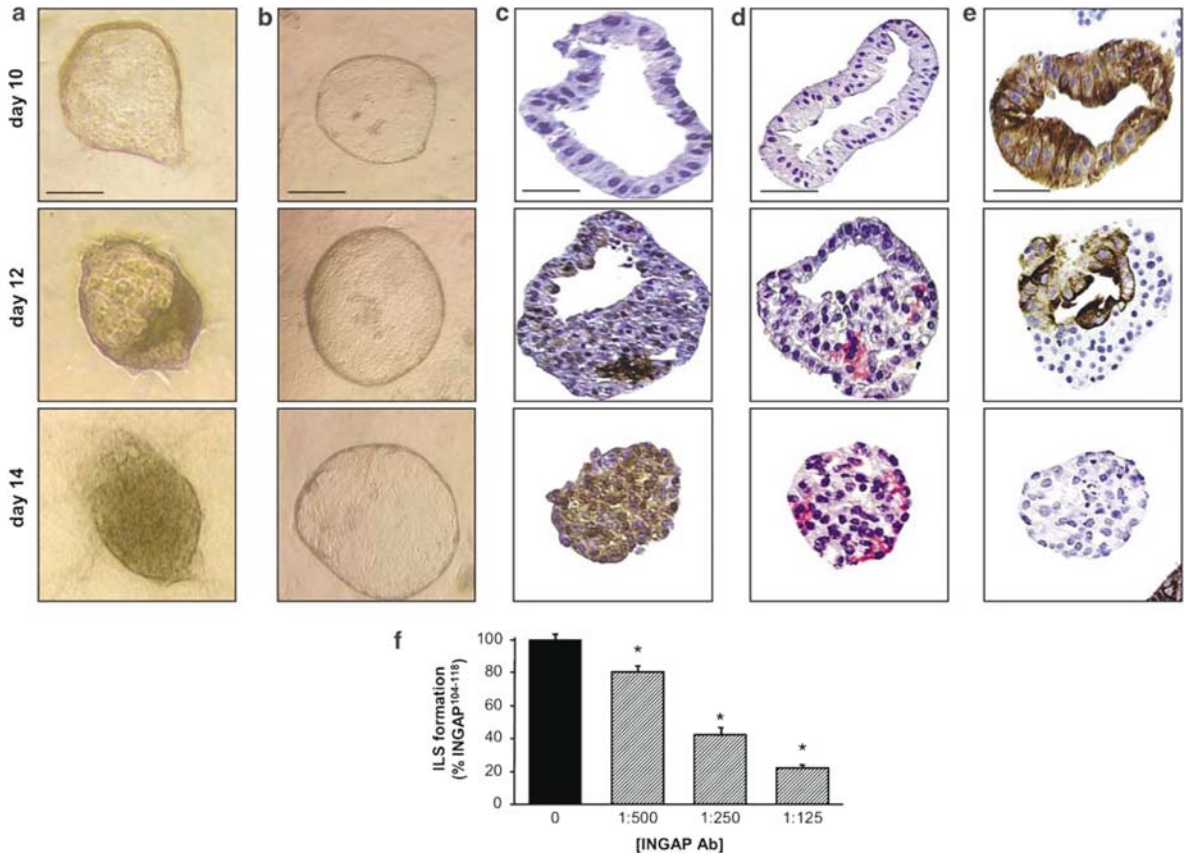


Fig. 17.5 [46] DECs can be transformed back to ILS that are morphologically similar to isolated islets. (**a**) INGAP104–118 induced the formation of neoislets, starting from a DEC (day 10), through a transitional structure (day 12), into an ILS (day 14). (**b**) Neither control medium, GLP-1 nor exendin-4, resulted in any phenotypic transformation (representative photomicrographs; scale bars 100 mm). Immunodetection of (**c**) insulin and (**d**) glucagon + somatostatin + pancreatic polypeptide established the reemergence of normal islet architecture during DEC-to-ILS progression (days 10, 12 and 14), while (**e**) CK-19 immunoreactivity was lost (scale bars 100 mm). (**f**) The effect of INGAP104–118 was inhibited in a dose-responsive manner by a rabbit polyclonal a-INGAP101–121 antibody (mean ± S.E.M.; $^{*}P<0.05$ versus INGAP104–118)

differentiate and maintain islet cell phenotype and function including insulin [61], glucokinase [62], and glucose transporter-2 (GLUT-2) [63]. PDX-1 has been associated with islet progenitors with both the development [64], and regeneration of the adult pancreas [29, 65].

NGN-3 is expressed in putative islet progenitors of pancreatic epithelium prior to their endocrine differentiation. NGN-3 expression initiates signaling events that result in the development and maintenance of progenitor cells in various tissue types. During pancreatic morphogenesis, NGN-3 helps to induce transcription factors such as ISL-1, NeuroD/Beta2, and NKX-2.2, that are involved in the development of mature islets [66, 67].

The intracellular signaling molecules and transcription factors above have been proposed to play a prominent role in the phenotypic differentiation of the developing pancreas. However, their exact role remains to be defined further. Hormones and growth factors affecting islet cell mass are summarized in Table 17.3 [1].

The following general observations have been made regarding the hormones and growth factors involved in β-cell mass regulation [1]:

1. β-Cell mass is not static but increases in response to increased insulin demand.
2. The same mechanisms that operate during pancreatic development in the embryo regulate β-cell mass in the adult [68–70].

Table 17.3 Hormones and growth factors affecting islet cell mass [1]

	Apoptosis	Hypertrophy	Neogenesis	Replication	Other effects	Mechanism	References
HGF	↓	↑		↑ X2.5	↑ Islet size and number, ↑ insulin production and secretion, ↑ Reg expression	Bcl-xL and BAG-1 overexpression	[249]
PL	↑	↑		↑			[250, 251]
hPRL				↑			[250]
hGH				↑			[250]
PTHrP		↔		↑	↑ X2 Islet size and number, ↑ insulin release, no changes in apoptotic rates		[252]
IGF-1				↑	Synergistic effect with glucose	Activation of P13K pathway	[202]
IGF-2	↑			↑	Increased insulin secretion, abnormal islet morphology with α-cells in islet core		[253]
INGAP			↑		Mediator of islet plasticity, reverses diabetes in mice	Activation of P13K pathway	[46]
Reg I	↑	↑		↑	Promotes acinar-to-islet cell transdifferentiation (β); reverses diabetes in mice and rats		[254, 255]
NeuroD/ βcellulin			↑				[35]
GLP-1	↓		↑	↑	Promotes transdifferentiation of acinar and ductal cells to islet cells		[256]

3. Some of the mechanisms of β-cell regulation may contribute to the pathogenesis of pancreatic endocrine neoplasia.

The precise effects of these hormones and mediators on different islet cell types as well as their specific roles in the genesis of pancreatic endocrine neoplasms remain to be further elucidated.

17.6 Pathways of Pathogenesis in Multiple Endocrine Neoplasia Syndromes

Further understanding of pathogenesis of PENs originates from elucidation of the genetic causes of multiple endocrine neoplasia syndromes, where endocrine neoplasms are associated with characteristic genetic abnormalities (Table 17.4) [71].

The multiple endocrine neoplasia syndromes associated with PENs include multiple endocrine neoplasia type 1 (MEN1 gene), von Hippel-Lindau disease (vHL gene), neurofibromatosis (NF-1 gene), and tuberous sclerosis (TSC1 and TSC2 genes). Understanding the development of PENs as a part of these syndromes contributes to better understanding of the genesis of sporadic PENs.

17.7 Multiple Endocrine Neoplasia Type 1

Multiple endocrine neoplasia type 1 (MEN-1) was identified as a clinical and familial syndrome by Moldawer and colleagues [72] and Wermer [73] in 1954 and presents with manifestations of parathyroid, pancreatic islet, or pituitary neoplasia or a combination of these [74]. Other rarer tumors include bronchial and thymic carcinoid tumors, adrenocortical tumors, and cutaneous lesions (lipomas, and collagenomas) [75]. While hyperparathyroidism is the most common endocrine manifestation of MEN-1,

Table 17.4 Genetic syndromes associated with an increased incidence of PENs [71]

Syndrome	Location of gene mutation and gene product	PEN's seen/frequency
Multiple endocrine neoplasia-type 1 (MEN-1)	11q13 (encodes 610 amino acid protein, MENIN)	80–100% develop PEN: (nonfunctional>gastrinoma> insulinoma)
von Hippel-Lindau disease	3q25 (encodes 213 amino acid protein)	12–17% develop PENs (almost always nonfunctional)
Von Recklinghausen's disease {neurofibromatosis 1 (NF-1)}	17q11.2 (encodes 2485 amino acid protein, neurofibromin)	Duodenal somatostatinomas
Tuberous sclerosis	9q34 (TSC1) (encodes 1164 amino acid protein, hamartin) 16p13 (TSC2) (encodes 1807 amino acid protein, tuberin)	Uncommonly develop PEN (nonfunctional and functional)

neoplasia of the pancreatic neuroendocrine cells is the second most common endocrine manifestation and eventually occurs in about 60% or more of MEN-1 patients. The most common enteropancreatic tumors are gastrinomas [76, 77], which often occur in the wall of the duodenum and in peri-pancreatic lymph nodes. Gastrinomas and insulinomas are the most common functional PENs associated with MEN-1. Nonfunctioning tumors are also common [76]. Clustering of subvariants of MEN-1 such as insulinomas [78, 79] and aggressive gastrinomas [80] within small MEN-1 families suggest specific MEN-1 mutations may correlate with specific clinical variants. Pancreatic neuroendocrine tumors in MEN-1 are invariably multifocal and may be widely dispersed in the pancreas and duodenum [76]. Consequently, the role of surgical management is controversial [81, 82]. PENs in MEN-1 may also present as multiple clinically silent enteropancreatic macroadenomas, discussed later, which may be found at surgery or at autopsy in almost 100% of MEN-1 patients older than 40 years [74, 78, 83]. Approximately 80% of MEN1 cases are familial, whereas 20% appear to be associated with mutations based on negative familial history [84].

17.7.1 MEN1 Gene

The *MEN1 gene* is a tumor suppressor gene [85–87] mapped to chromosome 11q13 that encodes a protein termed menin. Menin interacts with other proteins including junD, a member of the activator protein-1 (AP1) transcription factor family, although the importance of the menin-jun D interaction in the development of MEN-1 is unknown [88]. Other proteins that potentially interact with menin include SMAD1, SMAD3, SMAD5, PEM, NM23, nuclear factor kB, runx2, and several others, the importance of which is also unknown [89–95]. The precise physiologic role of menin has not been elucidated, and it is not clear why its absence results in endocrine tumor pathogenesis.

17.7.2 MEN-1 Tumorigenesis: A Two-Step Inactivation

The tumorigenesis in MEN-1 patients is thought to be a two-step inactivation of the MEN1 gene. Both copies of the MEN1 gene must be inactivated in order for tumorigenesis to occur. A "two-hit" hypothesis has been proposed whereby germline inactivation of one allele is followed by somatic inactivation of the second allele in a predisposed cell, leading to clonal proliferation [96]. Alfred Knudson [86, 87] developed the two-hit model for tumorigenesis to account for the epidemiologic observations in hereditary retinoblastoma in which tumors occurred earlier and in multiple sites compared to sporadic cases. Thus, current theory would suggest that, in every cell of MEN-1 patients, an obligatory germline mutation is present. Thus, multiple cells are susceptible for somatic mutations at the second allele, allowing for early development of multiple kinds of tumors [74].

17.7.3 First Step

Virtually all first hits at the MEN1 gene are small mutations involving one to several bases [97, 98].

Most MEN1 gene mutations occur in the locus of exon 2 [99]. However, hundreds of unique germline and somatic mutations, broadly distributed across the MEN1 open reading frame, have been found [74, 98, 100]. Most of the first-hit mutations predict premature truncation of the menin protein, while other mutations predict missense mutations or replacement of one to three amino acids, all with resultant inactivation or absence of menin.

17.7.4 Second Step

The second step in MEN-1 tumorigenesis occurs after the first hit, always occurs in somatic tissue, and usually occurs postnatally. Second-hit mutations are usually large chromosomal or subchromosomal rearrangements with a resultant deletion that includes the remaining normal MEN1 gene.

17.7.5 Loss of Heterozygosity at 11q13

Loss of heterozygosity (LOH) is used mainly to show loss of the normal copy of the MEN1 gene. In MEN-1, LOH at 11q13 was found in almost 100% of gastrinomas and other pancreatic islet tumors, as well as non-pancreatic endocrine neoplasia [101, 102]. Some sporadic endocrine tumors of the type found in MEN-1 show frequent LOH at 11q13. An underlying mutation at 11q13 has been traced to the MEN1 gene in approximately half of sporadic MEN-1 like tumors with 11q13 LOH. Somatic mutations of the MEN1 gene occur in approximately 20% of sporadic, solitary pancreatic endocrine tumors [84, 90, 103]. Therefore, MEN1 gene mutations are among the most common mutations in sporadic pancreatic endocrine tumors. The frequency of MEN1 gene mutations approximates 25% in gastrinomas [104–106], 10–20% in insulinomas [104, 107], and 50% in VIPomas [104, 107].

17.7.6 Events Following Inactivation of MEN1 Gene

After MEN1 gene inactivation, other unknown genes or undetected mutations in MEN1 gene may contribute to MEN-1 tumor development. Studies suggest that the tumorigenic pathway of MEN-1 overlaps and interacts with other homeostatic cell pathways, as illustrated in Fig. 17.1 above [108–111]. Early histologic effects in the tumorigenesis of MEN-1 have been subtle and ill-defined. Multifocal microadenoma is the term given the mono or oligoclonal islet lesion in MEN-1, which may represent a hyperplastic precursor stage to subsequent tumor development (Fig. 17.6) [112].

Evidence for microadenomas includes hyperplastic foci of gastrin cells seen by light microscopy in the duodenum of gastrinoma specimens from MEN-1 but not from sporadic gastrinomas [113]. Furthermore, in the heterozygous knockout of the MEN1 gene in mice, giant hyperplastic islets precede the development of insulinoma, suggesting that subtle islet hyperplasia may be an unrecognized precursor lesion in MEN-1 of humans despite the presence of one normal MEN1 allele. One could speculate that hyperplasia is an expression of MEN1 heterozygosity [114, 115]. Further studies are needed to link these findings with an as yet undiscovered genetic basis of tumor development.

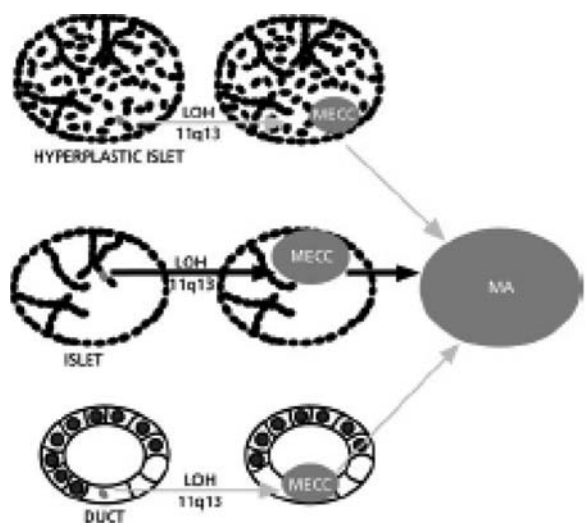


Fig. 17.6 [112] Proposed development of pancreatic microadenomas in MEN1. Monohormonal endocrine cell clusters (MECCs) develop most frequently within normal islets (*middle*) but also in ducts (*bottom*) and hyperplastic islets (*top*) through 11q13 LOH. MECCs progress to microadenomas (MA). The development of MECCs and their progression to microadenomas cause disruption of the normal islet structure. The pathogenetic mechanism leading to islet cell hyperplasia is unknown

17.8 Von Hippel-Lindau Syndrome

Von Hippel-Lindau syndrome (vHL) is an autosomal dominant syndrome characterized by hemangioblastomas of the central nervous system, renal cell carcinomas, retinal angiomas, visceral cysts, pheochromocytoma, and islet cell tumors (in 10–20% of patients) [116–119]. The *vHL gene*, mapped to chromosome 3p25.3 [120, 121], is a tumor suppressor gene that has an inhibitory effect on transcription elongation and facilitates the proteasome-mediated degradation of the hypoxia-inducible factor 1 (HIF-1) protein [74, 122]. The alpha subunit of HIF-1 is highly sensitive to tissue oxygen levels. In the presence of normal oxygen levels, it is bound by the vHL protein complex and covalently linked to ubiquitin in order to be targeted for degradation. In the absence of the vHL protein, HIF-1 alpha levels increase, leading to overproduction of hypoxia-associated cytokines, including erythropoietin, vascular endothelial growth factor (VEGF), and platelet-derived growth factor [8, 123–128]. These cytokines have been implicated in tumor growth. However, the precise mechanism of tumorigenesis is unknown. Other factors contributing to tumor pathogenesis may include matrix metalloproteinases such as MMP1 [129, 130]. VEGF inhibition, with resultant inhibition of angiogenesis in a PEN, may be a useful therapeutic strategy and is currently under investigation [131, 132].

The majority of patients present with a germline mutation of the gene from the affected parent and a normal copy of the gene from the unaffected parent. Tumor develops when both alleles are inactivated, usually as the result of a deletion [8]. Several studies support the role of the vHL gene in vHL-associated PEN tumorigenesis. In one study 12.3% of 155 patients with vHL went on to develop PENs [133]. Other studies found that PENs in patients with vHL were composed of clear cells, like renal carcinoma cells in vHL [101, 134]. LOH of the vHL gene was found in 100% of PENs (6 of 6 tumors) analyzed by PCR-single strand conformational polymorphism and fluorescent in situ hybridization. All the tumors in this study were nonfunctional [101]. These findings support a role for vHL gene mutation in the formation of vHL-associated PENs. In patients with sporadic PENs, no mutations specific to the vHL gene were found, although allelic loss on chromosome 3p was found in 33% of 43 patients with sporadic PENs [135].

17.9 Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF-1) is a neurocutaneous syndrome with characteristic features of neurofibromas, Lisch nodules on the iris, and dermal café-au-lait spots, as well as a variety of endocrine neoplasms, including somatostatin-producing carcinoid tumors of the duodenal wall, pheochromocytoma, hyperparathyroidism, hypothalamic or optic nerve tumors [74], and rarely somatostatinoma of the pancreas [8, 136]. NF-1 is caused by a mutation of the *NF-1 gene*, a tumor suppressor gene located on chromosome 17q11 that encodes the protein neurofibromin. Mutations cause the premature truncation of neurofibromin. The precise role of the NF-1 gene in the development of PENs still remains to be elucidated.

17.10 Tuberous Sclerosis

Tuberous sclerosis is a rare autosomal dominant syndrome associated with the development of hamartomas and benign tumors in multiple organs, including skin, brain, and kidney. Two gene mutations have been described: *TSC1on 9q34 encoding hamartin* [137] and *TSC2 on 16p13.3 encoding tuberin* (with identification and characterization of the tuberous sclerosis gene on chromosome 16 [138]). Together, these proteins function as a tumor suppressor complex and control the activity of mammalian target of rapamycin (mTOR) [74]. A complex of hamartin and tuberin is thought to regulate cell-cycle progression, possibly through upregulation of the mTOR cell-signaling pathway [139, 140]. mTOR is an intracellular protein that is key in the control of cell growth, protein synthesis, and autophagy [132] and is involved in the regulation of β-catenin stability and activity [8, 141]. 1–5% of patients with tuberous sclerosis can develop PENs that demonstrate LOH on 16p13.3 or lack of tuberin immuoreactivity [8, 16, 141, 142]. Based on these findings, the RADIANT (RAD In Advanced Neuroendocrine Tumors) trial, a phase II study, is

under development to evaluate everolimus, an mTOR inhibitor, in patients with advanced PENs who have failed cytotoxic chemotherapy [132].

17.11 Findings of Molecular Genetic Analyses

The majority of PENs are sporadic and unassociated with germline mutations. The genetic aberrations implicated in sporadic PENs are poorly understood. Oncogenes and tumor suppressor genes that are mutated in common human malignancies (p53, APC, Rb, K-ras) do not appear to be associated with PENs [48, 143, 144]. Within genetic syndromes and in sporadic development of PENs, genomic studies have facilitated attempts to identify genomic patterns that characterize individual PENs. Investigation of the molecular processes underlying development and progression of insulinoma has unraveled a variety of molecules, genes, and pathways that seem to play a role in insulinoma tumorigenesis (Fig. 17.7, Table 17.5) [145]. These molecular and genetic studies investigated insulinoma development using primary tumors, transgenic mouse models, or tumor-derived cell lines. Several of these genes and markers have been studied in other PENs, and current findings are discussed below.

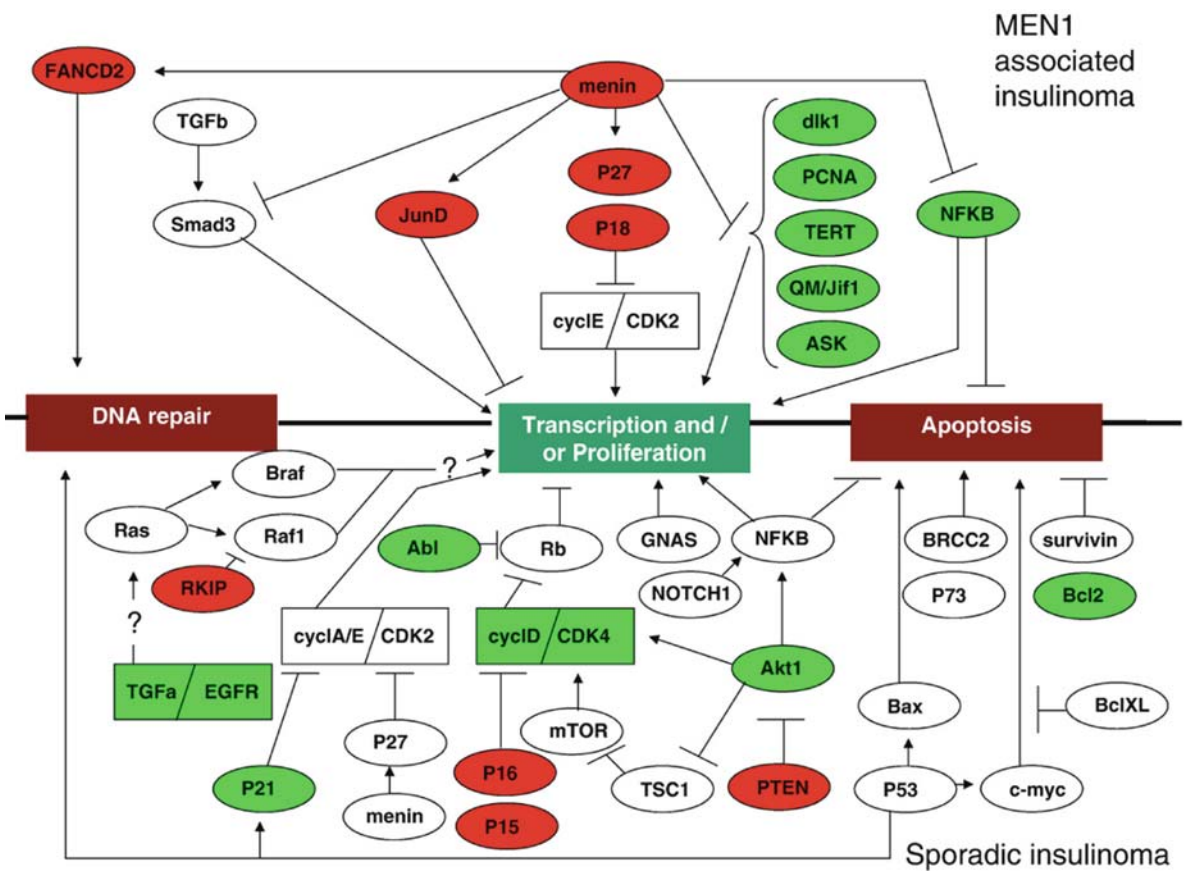


Fig. 17.7 [145] Schematic representation of the signaling molecules and pathways involved in insulinoma tumorigenesis. The upper part of the figure represents the proteins playing a role in MEN-1-associated insulinomas and the lower part those suggested to be involved in sporadic insulinoma tumorigenesis. Proteins with a loss of function are highlighted in red and those with a gain of function are highlighted in green. Proteins, whose role in insulinoma tumorigenesis has been suggested but is not yet clear, are represented in white

Table 17.5 Candidate genes for insulinoma tumorigenesis [145]

Gene symbol	Gene name	Chromosome localization	Function	Reference(s)	Array CGH result
Cell cycle progression					
K-Ras	C-K-Ras 2 protein	12p12	Control of cell cycle progression	[201]	Gain UB and M
ASK	S-phase kinase	7q21	S-phase entry	[257]	Gain M
P27	Cyclin-dependent kinase inhibitor 1B	12p13	Inhibitor of cell cycle progression	[258, 259, 187]	Gain UB and M
P16	Cyclin-dependent kinase inhibitor 2A	9p21	Inhibitor of cell cycle progression	[260, 261, 190]	No changes
P15	Cyclin-dependent kinase inhibitor 2B	9p21	Inhibitor of cell cycle progression	[190]	No changes
P18	Cyclin-dependent kinase inhibitor 2C	1p32	Inhibitor of cell cycle progression	[258, 259]	No changes
Proliferation/ transcription					
Dlk1/pref1	Delta like 1	14q32	Proliferation regulation	[262]	Gain UB and M
CCND1	Cyclin D1	11q13	Proliferation regulation	[193, 194]	No changes
Akt1	v-akt murine thymoma viral oncogene homolog 1	14q32	Proliferation regulation	[263]	Gain UB and M
GNAS	Guanine nucleotide binding protein α subunit	20q13	Proliferation regulation	[128]	No changes
PCNA	Proliferating cell nuclear antigen	20p12	Replication	[262]	Gain UB and M
TGFα	Transforming growth factor alpha	2p13	Growth factor	[263, 264]	No changes
EGFR	Epidermal growth factor receptor	7p11	Growth factor receptor	[263, 264]	Gain M
ABL	v-Abl Abelson murine leukaemia viral oncogene	9q34	Tyrosine kinase, proto-oncogene	[265, 266]	Gain B, UB, and M
TSC1	Tuberous sclerosis 1 protein	9q34	Anti-proliferative	[267]	Gain B, UB, and M
JunD	JunD proto-oncogene variant	19p13	Growth suppressor	[268]	No changes
QM/Jif1	Jun-interacting factor 1	Xq28	Jun binding gene tumor suppressor	[262]	Loss B, UB, and M
Rb	Retinoblastoma	13q14	Tumor suppressor gene	[213, 216, 269]	No changes
P21	Cyclin dependent kinase inhibitor 1A	6p21	Anti-proliferative kinase	[187]	Loss M

Table 17.5 (continued)

Gene symbol	Gene name	Chromosome localization	Function	Reference(s)	Array CGH result
PTEN	Phosphatase and tensin homolog	10q23	Anti-proliferative	[168, 270]	Loss UB and M
RKIP	Raf-1 kinase inhibitory protein	12q24	Raf-1 inhibition	[271]	Loss UB, loss and gain M
MEN1	Multiple endocrine neoplasia type 1	11q13	Growth suppressor	[107, 265, 104, 272, 273, 115]	No changes
Repair DNA damage					
TP53	Tumor protein p53	17p13	Repair DNA damage	[274, 201, 275]	Gain and Loss UB and M
FANCD2	Fanconi anemia complementation group D2 isoform	3p25	Repair DNA damage	[276]	Loss M
Apoptosis					
c-Myc	Cellular myelocytomatosis oncogene	8q24	Pro-apoptosis	[201, 277]	Loss M
BRCC2	Breast cancer cell 2	11q24	Pro-apoptosis	[278, 279]	Loss UB and M
TP73	Tumor protein p73	1p36	Pro-apoptosis	[157]	Loss UB and M
Bcl2	B-cell lymphoma protein 2 beta isoform	18q21	Anti-apoptosis	[277, 280]	Gain M
BIRC5	Baculoviral IAP repeat-containing protein 5	17q25	Anti-apoptosis	[281]	Gain M
NOTCH1	Notch1 preprotein	9q34	Cell growth, Anti-apoptosis	[282]	Gain B, UB, and M
NFKB	Nuclear factor kappa-B	4q24	Cell growth, anti-apoptosis	[93]	Gain M
Chromosomal instability					
TERT	Telomerase Reverse transcriptase	5p15	Telomere maintenance	[283]	Gain UB and M

17.11.1 Comparative Genomic Hybridization

The current status and recent advances in the assessment of the molecular basis of tumorigenesis of PENs from 1981 to 2004 was elegantly reviewed by Zikusoka et al. [16]. These investigators compared 6 studies using comparative genomic hybridization to detect gains and losses in chromosomes in PENs (Fig. 17.8) [16]. The most frequent gains were on chromosomes 7 and 20. The most frequent losses were on chromosomes 2, 6q, 21q, and Y. Chromosomal aberrations associated most frequently with metastasis included gains of chromosomes 7, 14q, 4, and Xq, as well as losses of chromosomes 6p, 3p, 6q, and 21q. Other chromosomal aberrations noted include gains of chromosomes 19, 5, 14p, 12q, 17, 20q, 15, 18, 9q, and 17p, as well as losses of chromosomes 1p, 6, 11q, 3q, 11p, and Xq [146–151]. Nonfunctional PENs contained more genetic aberrations than functional tumors [146], metastases had a higher average number of chromosomal aberrations than matched primaries [147], and 11q losses and 7q gains were commonalities between all 5 studies, pointing to their importance in PEN development.

17.11.2 Specific Chromosomal Aberrations

Many genetic alterations have been described in PENs [16, 145] as summarized in Table 17.6 [14, 145]. LOH

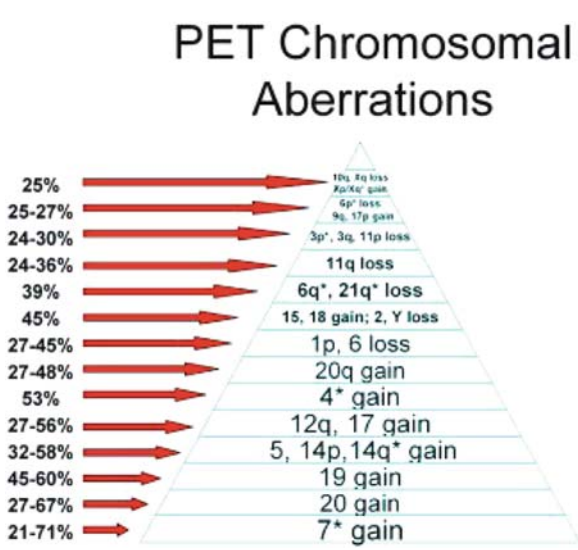


Fig. 17.8 [16] In this pancreatic endocrine tumor (PET) chromosomal aberration pyramid, chromosomal losses or gains from comparative genomic hybridization studies are listed from the most frequent (*bottom*) to the least frequent (*top*). An asterisk identifies the chromosomal aberrations that were associated most frequently with metastatic tissue, suggesting a possible role in tumor progression toward metastatic behavior. Adapted by Zikusoka, et al. [16] from Speel et al. [146]; Stumpf et al. [147]; Zhao et al. [148]; Tonnies et al. [149]; Terris et al. [150]; and Kytola et al. [151]

analysis is a powerful molecular tool and is used to identify tumor suppressor gene loci that are involved in the formation and progression of neoplasms. An LOH frequency greater than 35% at a specific chromosomal locus exceeds the rates of random genomic instability and strongly suggests a relevant tumor suppressor gene at that locus [152, 153].

A number of chromosomal aberrations have been identified in PENs. *Most common abnormalities are found in chromosomes 1, 3 and 6* as summarized in Table 17.7. Loss of heterozygosity at chromosome 1 was found in 34% of PETs and was found to be more common in tumors with hepatic metastasis [154, 155]. LOH for chromosome 1 has also been reported in several other tumors, including colorectal carcinoma, neuroblastoma, breast cancer, hepatocellular carcinoma, and melanoma [156]. In these tumors, the most frequently involved region is 1p36, the recently identified location of p73 [157], a possible tumor suppressor gene. One of two regions of LOH on chromosome 1 associated with malignant PENs includes a region of 1p between D1S1597 and p*ter*, the region of 1p36 lost in other tumor types. Chen et al. [158] identified chromosome 1 LOH on 1q31–32 and 1q21–23 in almost half of the gastrinomas studied and found

Table 17.6 Genetic alterations described to occur in PENs [14, 145]

Chromosomal locus	CGH	LOH	Gene	Mutation	Reference
1p36–	21/102 (21%)	10/29 (34%)			[154]
1q32–	16/102 (16%)	8/29 (28%)			[171]
3p23–	19/102 (19%)	23/31 (74%)			[161]
3p25–26–	19/102 (19%)	31/73 (42%)	vHL	1/75 (1%)	[171, 216, 284, 176]
6q22–	29/102 (28%)	43/69 (62%)			[171, 164]
9p–	0/102 (0%)	12/37 (32%)	CDKN2A/p16	1/44 (2%)	[165, 285]
9q+	29/102 (28%)				[167]
10q23–	14/102 (14%)	8/16 (50%)	PTEN	1/31 (3%)	[168]
11p14–	28/102 (27%)				[147]
11q13–	31/102 (30%)	75/111 (67%)	MEN1	33/155(21%)	[284, 176, 286, 107, 287]
11q22–23–	31/102 (30%)	20/37 (54%)	SDHD	0/20 (0%)	[172]
12p12+	23/102 (23%)		K-ras	1/39 (3%)	[165]
15q–	6/102 (6%)		SMAD3	0/18% (0%)	[175]
17p13–	2/102 (2%)	15/40 (38%)	TP53	1/40 (3%)	[171, 176]
17p+	32/102 (31%)				[167]
18q21–	6/102 (6%)	23/68 (34%)	DPC4	0/41 (0%)	[284, 288]
22q12.1–	4/102 (4%)	9/12 (75%)			[180]
Xq–	14/46 (30%)	11/23 (48%)			[184]
Y–	14/56 (25%)	5/14 (36%)			[184]

Table 17.7 Chromosomal aberrations in metastatic and non-metastatic pancraetic endocrine neeoplasms

Chromosome aberration	Total PENs analyzed	PENS with chromosomal aberration	PENs with chromosomal aberration (%)	Metastatic PENs				Non-metastatic PENS			Reference Number
				Site of metastasis	Analyzed	With aberration	Percentage	Analyzed	With aberration	Percentage	
1 LOH	26	10	38	LN, LIV	17	8	47	9	2	22	[162]
1LOH	29	10	34	LIV	16	7	44	13	3	23	[154]
1 LOH	32	13	41	LN, LIV	20	9	45	12	4	33	[171]
1q	27	11	41	LIV	12	8	67	15	3	20	[158]
3p26–3q 13LOH	4	2	50	LIV	2	2	100	2	0	0	[159]
3p14.2–3p21	21	13	62	LIV &/or LN	6	5	83	15	8	53	[160]
3p25.3-3p23	79	45	57	LIV &/or LN	41	17	41.5	38	28	73.7	[161]
3q LOH	16	4	25	LIV	12	4	33	4	0	0	[155]
6p LOH	44	12	27	LIV, LN, PPT	18	8	44	22	3	14	[146]
6q LOH	44	17	39	LIV, LN, PPT	18	11	61	22	5	23	[146]
6q	93	55	59	LN	36	26	72	55	27	49	[164]
9p	12	11	92	LIV, LN	6	5	83	6	6	100	[166]
11q LOH	44	16	36		22	6	27	18	8	44	[146]
22q LOH	23	22	96	LIV, LN, LUNG	20	20	100	3	2	67	[180]
X LOH	17	7	41	LIV, LN, 0, P	8	6	60	9	1	5	[152]
X LOH	25	10	40	LIV	11	8	73	14	2	14	[184]
Y LOH	15	5	33	LIV	8	4	50	7	1	15	[184]
TOTALS	551	263	**48%**	15 LIV, 10 LN	273	154	**56%**	264	103	**39%**	

LIV: Liver
LN: Lymph Node
PPT: Peripancreatic Tissue
O: Ovary
P: Peritoneum

an association with aggressive growth, liver metastasis, and post-surgical recurrence of liver metastasis. The specific genes involved were not identified. These studies indicate a worse prognosis for patients with chromosome 1 aberrations. Overall, 56% of 273 metatsatic PENs analysed in several different studies were found to show various chromosomal aberrations as opposed to 39% of 264 non-metastatic PENs (Table 17.7). These data point toward a potential association between chromosomal aberrations and progression of PENs.

Chromosome 3 is the location of the *vHL gene* (3p25.3) [120], which has been associated with vHL syndrome-associated PENs. Recent studies have shown LOH at loci proximal to vHL locus [159] and LOH at 3p14.2-3p21 more often in malignant insulinomas than in benign insulinomas [160]. Barghorn et al. [161] found an increased frequency of LOH at 3p25.3-p23 in malignant as compared to benign PENs (70.2% versus 28.0%; p=0.001) and in metastasizing as compared to non-metastasizing PENs (73.7% versus 41.5%; p=0.008). Additionally, a strong correlation was found between the loss of alleles on chromosome 3p and clinically metastatic disease (LOH 73.7% in metastasizing versus 41.5% in non-metastasizing tumors; p=0.008). These findings suggest a tumor suppressor gene at 3p25.3–p23 that may be associated with sporadic PEN development and that losses of larger centromeric regions are associated with metastatic progression.

In another study, *LOH at 3q* was found in half of sporadic PENs with hepatic metastases, while PENs without hepatic metastasis showed no LOH at this location [162]. Microsatellite markers demonstrate the smallest common deleted region at 3q27-qter, the region of p51 (a member of the p53 tumor suppressor family) [163]. These findings are suggestive of a late event in the tumorigenesis of PENs, consistent with advanced stage of tumor development [16].

Chromosome 6, studied in sporadic PENs, found a loss at 6q in 39% of tumors overall and in 100% of insulinomas, suggesting a chromosomal aberration specific to this type of PEN [146]. Further analysis found the smallest regions of allelic deletions at 6q22 (50%) and 6q23–24 (41.2–56.3%). Also, fluorescent in-situ hybridization analysis showed more aberrations in metastatic tumors than in benign PENs [164]. Thus, chromosome 6 alterations may play a specific role in the genesis of β-cell tumors (insulinomas) and may also have prognostic significance in these neoplasms.

LOH on chromosomal arm 9p, the home of p16, is a frequent finding in PENs. However, Moore et al. [165] found p16 mutation in only one insulinoma out of 41 PENs none of which showed methylation. Current studies seem to indicate p16 inactivation by promoter methylation may be restricted to functional gastrinomas. Although homozygous p16 gene deletions have also been observed in PENs [166], they seem to be rare events. Using comparative genomic hybridization, Speel et al. [167] found 9q gain to be the most common gain in insulinomas (50%).

Chromosome 10q23 is home to the tumor suppressor gene, PTEN. Perren et al. [168] performed a mutation analysis of the entire coding region of PTEN in 33 PENs but revealed only 1 tumor with a somatic mutation in exon 6. Although an intragenic PTEN mutation is rare in PENs, 10q23 region LOH was detected in 8 of 15 malignant (53%) and in 0 of 7 benign PENs. All samples with LOH were malignant PENs. This suggests that allelic loss of this region could be associated with malignant behavior. PTEN immunohistochemical expression in nonneoplastic islets is localized to the nucleus. PTEN expression was lost in the single malignant PEN with two structural hits; however, all of the PENs with LOH remained PTEN-immunopositive but were localized predominately in the cytoplasm and cell membrane in 23 of 24 (96%) PENs. No increase in malignant behavior is associated with this shifting of PTEN from the nucleus but is associated with the neoplastic state in general. Perren et al. [168] hypothesized that inappropriate compartmentalization of PTEN could be an initiating event in PENs with resultant neoplasia, whereas physical loss of 10q leads to progressive malignancy.

Chromosome 11q13, discussed previously, is associated with *MEN1* and the development of most MEN1-associated PENs as well as some sporadic PENs.

Chromosome 11p13–15 was studied in a comparative genomic hybridization investigation of 25 PENs from 23 patients. 11p13–15 loss was found in 24% of cases, likely representing uncharacterized tumor suppressor genes in this region [147].

Chromosome 11q23 harbors the tumor suppressor gene *succinate dehydrogenase subunit D (SDHD)* [169], a hydrophobic membrane anchor for the catalytically active subunits of cytochrome II. SDHD

also participates in electron transport and interacts with quinones [170]. SDHD is responsible for familial paraganglioma type 1. A number of studies have shown significant allelic loss of 11q extending to 11q23, or distal to 11q13, and have thus postulated that a previously unrecognized tumor suppressor in this region plays a role in PEN development [107, 146, 171]. Perren et al. [172] studied neuroendocrine tumors including PENs and found no somatic SDHD mutations. However, LOH rates ranged from 20 to 50%. These findings do not exclude SDHD from a role in the tumorigenesis of endocrine tumors since evidence exists that the gene is potentially imprinted in these tissues [173, 174].

Chromosome 12p12 is the location of the K-ras gene, which is commonly mutated in pancreatic ductal adenocarcinomas but is found only rarely in PENs [165], supporting the idea that exocrine and endocrine tumorigenesis involve different genetic pathways.

Chromosome 15q has been implicated to be the location of a tumor suppressor gene important in PENs, based on the findings of comparative genomic hybridization studies. SMAD3 localizes to 15q, and LOH at DNA markers surrounding SMAD3 was found in 20% of enteropancreatic tumors. However, further study revealed no acquired clonal mutations, insertions, or microdeletions in SMAD 3 in any tumor, making it an unlikely tumor suppression gene in PENs [175].

Chromosome 17p13 is home to TP53, which plays a significant role in the tumorigenesis of pancreatic ductal carcinomas, but not PENs. A study by Moore et al. [176] supported previous suggestions that the presence of a tumor suppressor gene other than TP53 on chromosomal arm 17p is involved in tumorigenesis of nonfunctional PENs.

Chromosome 18q21 mutations may play a role in the tumorigenesis of nonfunctional PENs, whereas select functional tumors lack this change [177]. 18q21 is the location of the *DPC4/Smad4 gene*, a cell cycle regulator [178]. However, in another series of PENs, these chromosomal aberrations were not detected in any of the 19 nonfunctional PENs analyzed [179].

Chromosome 22 was studied in gastrinomas, insulinomas, VIPomas, and nonfunctional PENs, and LOH was found on chromosome 22q in 22 of 23 tumors [180]. Another study of insulinomas found LOH in 57% of tumors at 22q12.1–q12.2 [181]. This site is the location of the hSNF5/INI1 gene, implicated in medulloblastoma and other pediatric central nervous system tumors [182]. Further studies could not find an alteration in this gene suggesting it is not the cause of tumor development [16, 181].

X chromosome losses were seen in patients with functional and nonfunctional PENs and were associated with shorter patient survival [11] and clinically aggressive behavior [152, 183, 184].

Y chromosome losses were found frequently in PENs from males (36%) and were associated with metastasis, local invasion, and high proliferation rates [184].

17.11.3 Cell Cycle Regulators

Regulation of the cell cycle, simply put, keeps cell death (apoptosis) in balance with cell growth (proliferation). Loss of cell cycle regulation is one of the hallmarks of neoplasia. Understanding the regulatory mechanisms of the cell cycle are complex, as multiple, often repetitive pathways may be involved. A number of studies have shown that common cell cycle regulators are involved in the tumorigenesis of PENs.

P27KIP1 is a cell kinase inhibitor that opposes cell cycle progression and is located on chromosome 12p12–p13.1 [185]. A study by Guo et al. [186] found overexpression of P27KIP in sporadic PENs. An elevation of P27KIP1 expression was found to be inversely related to Ki-67 in a study of 109 gastroenteropancreatic neuroendocrine tumors, suggesting that P27KIP1 may decrease proliferative rates in tumors [187].

Loss of *p16INK4/p14ARF*, a tumor suppressor gene located on 9p21 [188], leads to tumorigenesis as a result of deregulation of p53 and cyclin-dependent kinase/retinoblastoma pathways [189]. Reports found inactivating p16INK4 gene alterations (such as homozygous deletion and methylation at the 5'CpG islands of promoter regions) in 92% of gastrinomas and nonfunctional PENs. Loss of expression of genes in the 9p21 region was found in 57% of nonfunctional PENs, 30% of insulinomas, and 22% of gastrinomas. This study also found CpG promoter methylation of the p16 gene [190].

Cyclin D1, on chromosome 11q13 [191, 192], plays an important role in cell cycle regulation. Nuclear expression of Cyclin D1 was found to be increased in almost half of the PENS [193]. Sporadic PENs

were specifically studied and Cyclin D1 overexpression was found in 65% (20 out of 31) of the PENs studied compared to normal pancreatic tissue [194]. Pathways associated with Cyclin D1, specifically the P38/mitogen-activated protein kinase and Akt/PKB pathways, were activated in PENs, whereas down-regulation of the extracellular signal-regulated kinase pathway was also found with overexpression of Cyclin D1 [195–197].

K-RAS2, on chromosome 12p12.1 [198], is an important oncogene that transduces cell growth signals, mutations of which lead to growth factor-independent stimulation of cell proliferation [199]. K-RAS2 mutations were not detected in PENs in a study by Yashiro et al. [200]. Another study found strong K-RAS2 immunoreactivity and mutations in 4 of 6 insulinomas studied [201].

As described above, the *PI3K-Akt/PKB pathway* participates in the mediation of β-cell mass up-regulation [22, 202, 203]. This pathway activates downstream messengers and transcription factors such as PDX-1, Ngn-3, Isl-1, NeuroD/Beta2, and Nkx2.2, known to act during pancreatic embryogenesis. Glucose and insulin-like growth factor induce activation of the PI3K-Akt/PKB pathway and promote in vitro proliferation of insulinoma cells [202]. A persistant stimulus that promotes proliferation is seen in other tumors, including the persistence of achlorhydria inducing gastrinomas. Mouse studies found that up-regulation of the PI3K-Akt/PKB pathway is not sufficient for neoplastic transformation [22].

Somatostatin (SST) and G-protein-coupled transmembrane receptors (SSTRs) seem to have a role as regulators of islet morphology and cell proliferation in the endocrine pancreas [1]. Loss of SST/SSTR signaling may be a necessary but insufficient step in the pathogenesis of islet cell tumors. In support of this theory, MEN-1 studies showed decreased expression of SST and islet amyloid polypeptide in multiple endocrine neoplasia type 1 [204], providing a link between loss of menin, suppression of SST and islet amyloid polypeptide expression, and oncogenesis.

The dominant effect of the protooncogene *c-Myc* is apoptosis of the islet cells. However, increased expression of c-Myc has been demonstrated in glucose-induced hyperplasia of islets. Thus, the effects of this gene depend on the environmental effects and the influences of other genes and proteins. Pelengaris and Khan [205] proposed a model of accumulating mutations leading to progression from hyperplasia to neoplasia. c-Myc is thought to be an early event in hyperplastic islets. c-Myc expression was found to be increased in hyperplastic islets and benign and malignant insulinomas.

RIP-Tag2 oncogene expression in transgenic mice leads to islet cell hyperplasia and neoplasia. Tag oncoprotein inactivates tumor suppressor proteins p53 and pRb. Decreased apoptosis is also seen due to overexpression of antiapoptotic protein Bcl-XL and Bcl-2. These antiapoptotic proteins counteract the effects of proapoptotic c-Myc [205] (Fig. 17.9) [206].

HER-2 neu, found on chromosome 11q21 [207], is a well-known oncogene that is overexpressed in some cases of breast carcinoma and is associated with increased malignant behavior, proliferation, and metastasis [208]. HER-2 neu has been studied in gastrinomas, where its overexpression was found in a minority of these tumors, and was associated with liver metastasis [209].

The CpG island methylation of the estrogen receptor gene, located on chromosome 6q24 [210], has also

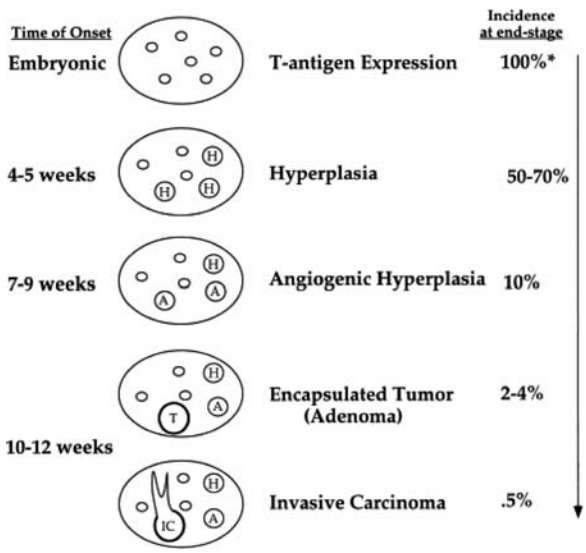


Fig. 17.9 [206] A schematic representation of RIPTag tumorigenesis. T-antigen expression commences during embryogenesis but is without apparent effect until 4–5 weeks when sporadic islets become hyperplastic (H); over time 50–70% become hyperplastic. At 7–9 weeks, angiogenic islets (A) appear. From these angiogenic islets emerge encapsulated tumors (10–12 weeks; T) (adenomas) of which a subset develop into invasive carcinoma (IC). *Incidence at all stages

been described in breast carcinoma and has significant therapeutic implications, indicating tamoxifen resistance [211]. Estrogen receptor gene methylation was found in 64% of PENs in one study [212].

Other cell cycle regulators have been studied with controversial results, including the retinoblastoma tumor suppressor gene initially found to be deleted in insulinomas [213, 214] but without further confirmation [135, 215, 216] and, as mentioned previously DPC4/Smad4 on chromosome 18q21 [178].

Cell cycle regulators studied and found not to be important contributors to PEN development to date include P53 located on chromosome 17p13.1 [176, 217–219], β-catenin located on chromosome 3p21 [220, 221], phospholipase CB3 located on chromosome 11q13 [222], and retinoic acid receptor β located on chromosome 3p24 [212, 223].

17.11.4 Gene Expression Profiling

Gene expression profiling using microarray analysis has identified a number of genes typical of PENs [204, 224–227]. Normal islets were compared with PENs, showing overexpression of 66 genes, especially IGFBP3 (a growth factor), fibronectin (a cell migration/adhesion molecule), and oncogenes MLLT10/AF10. 119 genes were underexpressed, including p21CIP1 (a cell cycle regulator), JunD (a transcription factor), and NME3 (a metastasis suppressor gene). A second study of gene expression in PENs compared normal islet cells and 3 neuroendocrine tumor cell lines; 667 genes were up-regulated, and 323 were down-regulated [224, 225]. Using well-characterized subsets of PENs and adjacent histologically normal pancreatic islets, we discovered a number of genes that were differentially expressed between normal pancreatic islets and PENs. These include RUNX1T1, paladin, and p21 [228, 229]. Subsequently, we have also validated these candidate genes as predictors of liver metastases on independent test sets of PENs with and without liver metastases [230]. Whether these genes are also important in the tumorigenesis is currently under investigation. Although gene expression profiling studies are identifying new candidate genes that may prove important in the pathogenesis of PENs, comparison among these studies is becoming difficult due to variations in study designs, patient populations, and tumor samples studied.

17.12 Additional Evidence in Support of Pancreatic Endocrine Tumorigenesis

Cathepsins are likely to mediate a proangiogenic change of hyperplastic islets, which is an important step in the progression from hyperplasia to neoplasia (Fig. 17.10) [231–233]. *CD44*, a chief component of T-cell activation signaling, plays a role in tumor progression through growth and migration [234, 235]. Imam et al. [236] found "strong" staining of v6 and v9 isoforms associated with benign PENs, decreased proliferation, and longer survival. *Neuroendocrine secretory peptide 55* (NESP-55), a chromogranin family member, is located on chromosome 20q13 [237]. Srivastava et al. [238] distinguished gastrointestinal and pulmonary carcinoids from PENs and pheochromocytomas by examining the expression of NESP-55. PENs and pheochromocytomas stained positive for the protein, whereas gastrointestinal and pulmonary carcinoids did not. This is just one of several recently discovered differences between carcinoids and PENs. NESP-55 may be useful in establishing the origin of metastatic endocrine tumors.

Human MutL homologue 1 (hMLH1), found on chromosome 3p21.3 [239], is a mismatch repair gene. One study found hMLH1 to be hypermethylated in 23% of PENs with evidence of microsatellite instability [240]. Promoter hypermethylation (gene silencing) was associated with an improved 5-year survival (100% versus 56%).

Telomerase, on chromosome 5p15.33 [241], is an enzyme that maintains the chromosomal telomere. Telomere degradation is a normal part of the cell cycle, but aberrations of telomerase can lead to tumorigenesis [16]. Telomerase activity may predict an unfavorable outcome in PENs [11, 242]. *Thrombomodulin*, an endothelial anticoagulant, when overexpressed, reduces cellular proliferation and promotes cellular adhesion in vitro, while expression of thrombomodulin in vivo is inversely correlated with metastatic spread [243, 244].

E-cadherin functions to promote cell-cell adhesion. Loss of E-cadherin is associated with invasion and metastasis in many malignancies. Chetty et al. [18] found aberrant E-cadherin expression in more than 50% of PENs, which strongly correlated with lymph node and liver metastasis. In addition, nuclear

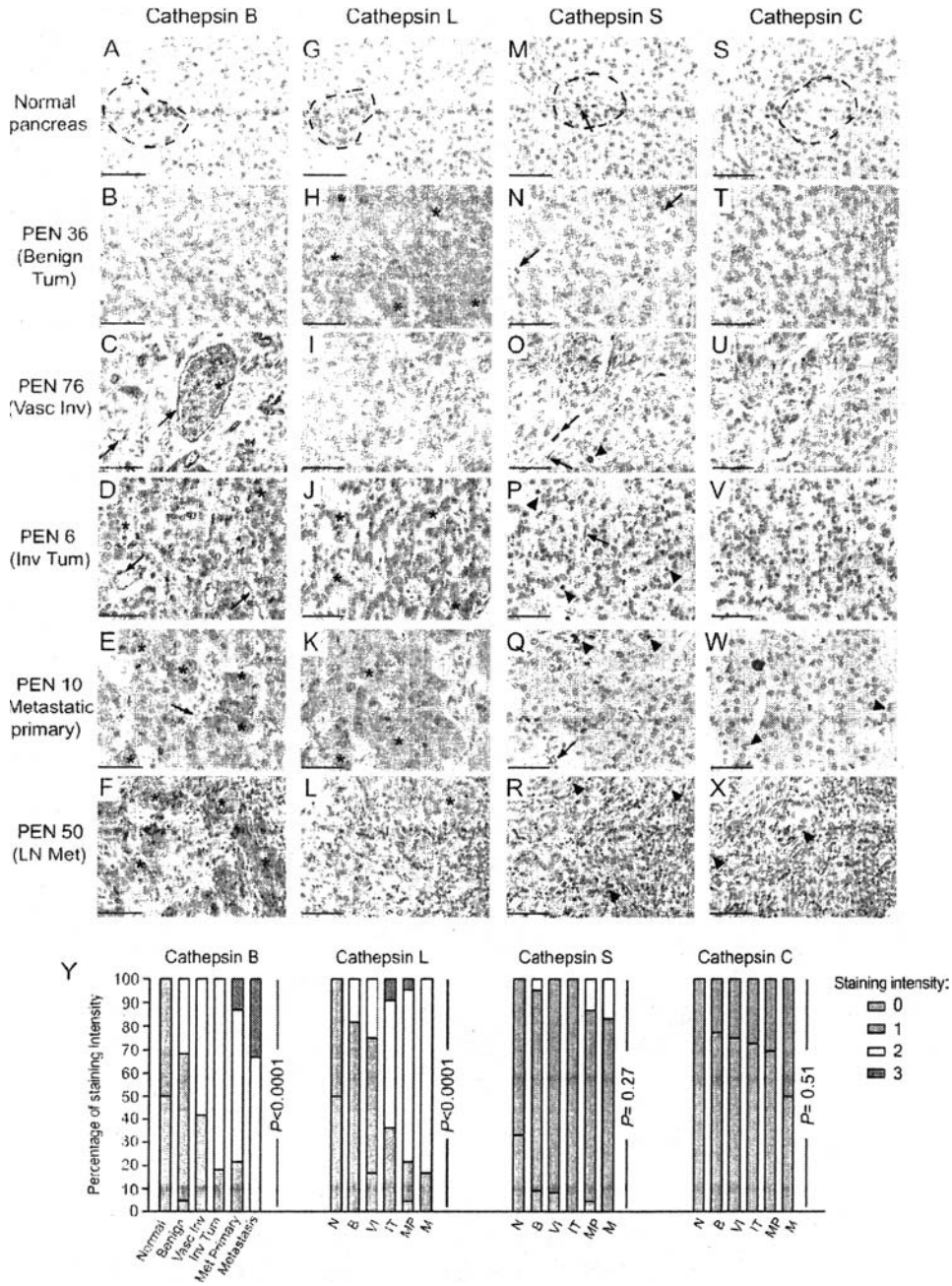


Fig. 17.10 Increased levels of cathepsins B and L are positively associated with tumor progression in human PEN lesions and associated metastases. A TMA was constructed from a panel of human PEN and normal pancreas tissues. (**A–X**) Tissue arrays were stained with antibodies against cathepsins B, L, S, and C as indicated. Cathepsin-positive cells are stained in brown, and hematoxylin (blue) was used as a counterstain. Representative images of normal human pancreas ($n = 6$) stained for each antibody are shown in the first row, with normal islets indicated with a dotted black line, surrounded by normal exocrine cells. Representative images for each of the tumor stages – Benign Tumor ($n = 22$), Vascular Invasive Tumor ($n = 12$), Invasive Tumor ($n = 11$), Metastatic Primary ($n = 23$), and Metastasis ($n = 6$) – are shown in the rows *below*. The PEN number corresponds to the position on the tissue array. Tumor cell staining is indicated by asterisks, endothelial cell staining by arrows, and immune cell staining by arrowheads. (**Y**) The cathepsin staining for each tissue specimen was scored as negative (0) or positive [three levels: weak (1); moderate (2); strong (3)] and graphed as the percentage of staining intensity for each stage. For each cathepsin, an overall test of differences among any of the groups (normal and tumor) was performed. An exact version of Mantel Haenszel's test for trend was performed to look for differences in staining in each tumor group compared with the normal controls and to calculate *P* values, which are shown next to each data set. Bars, 50 μm

E-cadherin was seen in 18/57 cases when stained with antibodies detecting the cytoplasmic fragment of E-cadherin. This is a previously undescribed staining pattern in PENs.

Cell signaling pathways influence tumor growth and hormonal activity. Neuroendocrine cells can express the *insulin-like growth factor (IGF) and its receptor (IGFR)* [245]. Cell line studies indicate that IGF-1 can act in autocrine and paracrine fashion to inhibit apoptosis and stimulate secretion of chromogranins, possibly by activating the P13K-AKT pathway. VEGF is also expressed by neuroendocrine tumors, and elevated levels of VEGF have been associated with tumor progression [246, 247].

17.13 Summary

While some consider that the pancreas may be the least understood organ after the brain, there is a rapid increase in our knowledge-base, as evidence is accumulating to construct a working hypothesis of the pathogenesis of PENs. We have learned, mainly through the study of β-cell regulation, that neogenesis and transdifferentiation, replication, hypertrophy, and apoptosis work together to control endocrine cell mass. However, the pluripotent stem cells thought to play a role in neogenesis and transdifferentiation have yet to be discovered. Studies have found many of the same mechanisms that operate during pancreatic development in the embryo regulate β-cell mass in adults. One could imagine how deregulation of these processes could lead to oncogenesis. All of these processes are steered by cell regulators such as glucose, c-Myc, P13K-AKT/PKB, PDX-1, Ngn-3, and others. Many of the members of these complex molecular pathways have been implicated in the pathogenesis of PENs. Genetic syndromes, which include PENs as one of their components, allow for the identification of genes associated with the genesis of PENs, including the MEN1 gene, vHL gene, NF-1 gene, TSC1 gene, and TSC2 gene. Advanced molecular testing is currently making it more feasible to pursue newer lines of genetic studies to unravel an increasing number of chromosomal aberrations associated with genesis and progression of PENs. Comparative genomic hybridization studies revealed the most frequent chromosomal gains were on chromosomes 7 and 20. The most frequent losses were on chromosomes 2, 6q, 21q, and Y. The chromosome aberrations most frequently associated with metastasis included gains of chromosomes 7, 14q, 4, and Xq, as well as losses of chromosomes 6p, 3p, 6q, and 21q. LOH analyses have identified multiple tumor suppressor gene loci that contribute to the pathogenesis of PENs. Gene expression profiling using microarray analysis has identified a number of genes differentially expressed in PENs when compared with normal islets and include IGFBP3, fibronectin, oncogene MLLT10/AF10, p21C1P1, JunD, NME3, RUNX1T1, paladin, and p21. One of the challenges is that comparison among these studies is becoming increasingly difficult due to variations in study designs, patient populations, and tumor samples (fresh-frozen versus archival) and variation in the technical platform used. A number of interesting lines of investigation are lending credence to various hypotheses regarding the pathogenesis of PENs. Some the recent studies have focused on cathepsins, CD44, NESP-55, hMLH1, telomerase, thrombomodulin, and E-cadherin expression/activity.

All in all, many studies have contributed significantly to our current knowledge of the tumorigenesis and progression of PENs. As understanding of the mechanisms and mediators of islet cell neogenesis increases, understanding the deregulation of various biologic pathways contributing to the pathogenesis of PENs will also improve. Recent findings suggest that PENs have as diverse a spectrum of genetic aberrations as are their clinical presentations. Nonfunctional PENs exhibit more molecular aberrations than functional PENs. Also, malignant behavior seems to be associated with increasing genetic aberrations, suggesting specific genes may be associated with metastases in PENs [16]. Multiple molecular alterations, involving migratory, cell cycle, and angiogenic functions, have been found to promote PEN development/growth, invasion, and metastases [226, 243, 248]. As a result of these findings, phase III trials of novel therapies targeting mTOR, VEGF and other target are in progress. Focused investigation of various mechanisms of tumorigenic pathways of PENsorigenesis will contribute to novel diagnostic, therapeutic and preventive stratagies, as well as facilitate the development of prognostic and predictive markers, while continuing to advance our understanding of the pathogenesis of PENs.

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Chapter 18

Mechanisms of Carcinogenesis in Colorectal Cancer

Shaokun Shu and Jin Q. Cheng

Contents

18.1 Introduction

Colorectal cancer (CRC) is the third most common malignancy and the fourth cause of cancer mortality, with over 1 million new cases diagnosed worldwide each year. The lifetime incidence for patients at average risk is 5%, with 90% of cases occurring after age 50. Approximately 1 in 3 people who develop CRC die of this disease [1, 2]. The prognosis of CRC is closely associated with disease stage at the time of diagnosis. While early stage CRC is frequently curable with surgery, unresectable metastatic CRC is a fatal disease. Compelling evidence indicates that early detection and prevention by removal of premalignant polyps can reduce colorectal cancer mortality.

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The mechanism of colorectal carcinogenesis is complex, influenced by genetic and environmental factors. These different risk factors reflect the mode of presentation of CRC that follows one of the three: inherited, familial and sporadic. The patients with inherited predisposition to CRC account for less than 10% of cases, and these patients are subdivided according to whether or not colonic polyps are a major disease manifestation. The diseases with polyposis include familial adenomatous polyposis (FAP) and the hamartomatous polyposis syndromes (e.g., Peutz-Jeghers, juvenile polyposis), those without polyposis include hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome I), and the cancer family syndrome (Lynch syndrome II). These conditions are associated with a high risk of developing CRC, and the genetic mutations underlying many of them have been identified. Familial CRC is the least understood subgroup. Up to 25% of affected patients have a family history of CRC, but the pattern is not consistent with one of the inherited syndromes. Individuals from these families are at increased risk of developing CRC, although the risk is not as high as with the inherited syndromes. Genome-wide association studies might offer the potential to uncover the genetic alterations for familial CRC [3, 4]. Sporadic disease, in which there is no family history, accounts for approximately 70% of all CRC. The patients are usually older than 50 years of age. The dietary and environmental factors, as well as genetic changes, have been etiologically implicated. In general, two essential requirements are needed for an intestinal cell to develop into a cancer: a selective advantage to allow for the initial clonal expansion, and genetic instability to permit for multiple hits in other genes that are responsible for tumor progression and malignant transformation.

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18.2 Chromosomal Instability and Microsatellite Instability Pathways

In 1990, Fearon and Vogelstein described the molecular basis for CRC as a multistep process in which each accumulated genetic event conferred a selective growth advantage to the colonic epithelial cell [5]. A progression from normal mucosa to adenoma to carcinoma was supported by the demonstration of accumulating mutations in genes of K-ras, adenomatous polyposis coli (APC), tumor protein P53 (TP53), and deleted in colorectal carcinoma (DCC), all of which are thought to be of significance, but are not able successfully to account for all CRCs. There is heterogeneity in the pathogenetic pathway leading to CRCs, and there are two major tumorigenic pathways. The first is driven by chromosomal instability (CIN), the progress of which involves both oncogenes and tumor-suppressor genes residing on chromosomes 5q, 17p, and 18q [6, 7]. Chromosome 5q genes are responsible for APC, 17p for TP53, and 18q for DCC or SMAD4, respectively. K-ras is the most common oncogene following this pattern. As far as tumor-suppressor genes are concerned, genes of APC, TP53, DCC/SMAD4 play important roles in this sequential adenoma to carcinoma pattern. The second pathway is a pathway that involves microsatellite instability that has well be depicted as a consequence of the alteration in mismatch repair (MMR) genes [8, 9].

Interestingly, the first pathway is prevalent in the distal colon. It was reported that the frequency of allelic loss on the three chromosomes, 5q, 17p, and 18q, was more than double in distal tumors as compared to proximal CRC [6], and that close to 100% of FAP individuals will develop CRC in the left colon [10]. However, as for the proximal colon, the second pathway is predominant, and this is reflected in the high incidence of MSI phenotype in the proximal colon [8, 9], which is up to as much as ten times higher than that in distal tumors in sporadic CRCs. FAP and HNPCC, the two major familial forms of CRC, exhibit a distal location preference and a proximal location preference, respectively. The former has an involvement in the CIN pathway, while the latter in the MSI pathway [8, 9, 11]. It has been reported that 60%~70% of HNPCC carcinomas are located proximal to the splenic flexure, compared with 30% among the sporadic cases.

18.3 APC and β-Catenin

Homozygous Apc knockout mouse is embryonic lethal and heterozygous ($Apc^{+/Min}$) mice developed multiple polyps throughout the intestinal tract, mostly in the small intestine [12, 13]. The earliest polyps arose multifocally during the third week after birth, and new polyps continued to appear thereafter. Surprisingly, every nascent polyp consisted of a microadenoma covered with a layer of normal villous epithelium. Genotyping showed that all microadenomas had lost the wildtype Apc allele, whereas the mutant allele remained unchanged. These results indicated that loss of heterozygosity, followed by formation of intravillous microadenomas, was responsible for polyposis in the intestinal mucosa. Further, conditional targeted deletion of exon 3 in mice, which encodes serines and threonines phosphorylated by GSK3β, caused adenomatous intestinal polyps resembling those in APC knockout mice [14]. Some nascent microadenomas were also found in the colon. Thus, the APC/β-catenin pathway plays a major role in early colorectal carcinogenesis.

Mutations of the APC are common to most sporadic colorectal cancers and are also responsible for FAP. APC encodes a large (312 kDa) protein that forms a multiprotein complex containing β-catenin, axin/axin2, casein kinase I, and glycogen synthase kinase 3β [15–17], where β-catenin is phosphorylated by GSK3β and CKI. Phosphorylation of β-catenin leads to its ubiquitination and subsequent degradation. Inactivation of APC results in the accumulation of β-catenin protein and resembles constitutively active Wnt signaling [10–20]. The accumulated β-catenin protein translocates into the nucleus, where it forms a complex with TCF/LEF-family nuclear proteins and drives the transcription of multiple genes (e.g., c-Myc, cyclin D1) implicated in tumor growth and invasion. In addition, 50% of sporadic tumors with intact APC are reported to show mutations of β-catenin itself. Recent data suggest that β-catenin mutations largely occur in the context of the hereditary nonpolyposis colon cancer syndrome, which is caused by germline mutations in DNA mismatch repair genes MSH2 and MLH1 [21]. Mutation of the β-catenin gene is often detected in the casein kinase I and glycogen synthase kinase 3β phosphorylation sites of the β-catenin protein, which results in the accumulation of β-catenin and activation of LEF/TCF.

18.4 K-ras and p53

Approximate 15–68% of sporadic colorectal adenomas and in 40–65% of cancers carry K-ras mutations [22–26]. The majority of K-ras mutations occur as an activating point mutation in codons 12, 13, and 64 [27, 28]. Several signal transduction pathways could be activated by actively mutated K-ras, including phosphatidylinositol 3-kinase (PI3K)/Akt, MAPK and Ral cascades, leading to cell survival, growth and angiogenesis [29]. The p53 mutations have been identified in 40–50% of sporadic colorectal cancers [30]. The frequency of p53 mutations is higher in distal colon and rectal cancers than in proximal colon cancers [31]. Further, p53 mutations are associated with poor prognosis [32]. Tumor suppressor function of p53 has been well documented. However, p53-knockout mice predominantly develop lymphoma rather than epithelial tumors. The underlying mechanism needs to be further investigated.

18.5 DCC

Loss of heterozygosity on chromosome 18q21.3 have been detected in approximately 70% of primary colorectal cancers, particularly in advanced colorectal cancers with hepatic metastasis, suggesting that a gene on 18q plays a significant role in CRC progression. The DCC (deleted in colorectal cancer) gene was long ago proposed as a candidate tumor suppressor gene on 18q [33]. However, point mutations of the DCC gene are only identified in approximately 6% of sporadic colorectal cancers [34]. Mice heterozygous for DCC have been reported to lack the tumor predisposition phenotype [35], suggesting that other candidate gene(s) should be examined in this region.

18.6 DNA Mismatch Repair (MMR) Genes

Accumulated studies have shown that MMR enzymes, hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6, are responsible for microsatellite instability (MSI) in CRC [36–39]. Tumors with instability at two or more of these markers were defined as being MSI-H (high-frequency MSI), whereas those with instability at one or showing no instability were defined as MSI-L (low-frequency MSI) and MSS (microsatellite stable) tumors, respectively. MSI-H cancers have distinct clinicopathological features from MSI-L and MSS tumors. They can occur in the context of the HNPCC syndrome [35, 36, 40, 41] and as many as 10–15% of sporadic CRC [42]. These cancers are also characterized by distinct histopathological features, including mucinous or signet-ring cell differentiation, medullary features, and excess lymphocyte infiltrations. However, neither MSI-L nor MSS tumors demonstrate such characteristic features. Because the simple inactivation of an MMR gene is not enough for a transforming event, additional genetic changes are believed to be necessary for cells to become malignant. To date, most of these have been found in genes containing coding repeat sequences that are particularly prone to alterations in MSI-H cancers. Accumulation of such alterations appears to be the main molecular mechanism by which MSI-H cells accumulate functional changes with putative oncogenic effects. These mutations occur in many genes at variable frequencies. They can affect genes with a putative role in human carcinogenesis involved in different or similar pathways, and are thus thought to be inactivating or activating events selected for in these cancers in a recessive or dominant manner. In 1995, frameshift mutation in repeat sequence of TGFßRII was first reported in human colorectal MSI-H tumors [43]. More recently, other genes containing coding repeats were shown to be altered at various frequencies in MSI-H cancers. These include BAX, hMSH3, hMSH6, IGFIIR ACTRII, AIM2, APAF-1, AXIN-2, BCL-10, BLM, Caspase-5, CDX-2, CHK-1, FAS, GRB-14, cell cycle protein hG4-1, KIAA0977, MBD-4, hMLH3, NADH ubiquinone oxidoreductase, OGT, PTEN, RAD-50, RHAMM, RIZ, SEC63, SLC23AT, TCF-4, and WISP-3 [44–62]. In addition, approximately 30–40% of sporadic MSI-H cancers have APC mutations. Similarly, approximately 36% of sporadic MSI-H cancers have p53 mutations. Thus, a subset of CRC are associated with both MSI and mutations of the APC, p53 and other genes. Recent studies have shown that epigenetics is an important mechanism of colorectal carcinogenesis. HNPCC is mostly due to mutations of MMR genes which show MSI phenotype. Epigenetic, rather than genetic silence of the transcription of MMRs and a number of tumor

suppressor genes, has been detected in sporadic colorectal cancers with MSI [63]. Methylation is believed to be a crucial epigenetic regulation in colorectal carcinoma.

18.7 TGFβ Pathway

The TGFβ family of cytokines has 33 members in humans [64], including TGF-β isoforms, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs). TGFβ family members exert their cellular effects by forming heterotetrameric complexes of type I and type II serine/threonine kinase receptors. In the complex, the type II receptor phosphorylates and activates the type I receptor, which thereafter phosphorylates downstream effectors of the Smad family [65, 66]. The Smad family consists of eight members which form three subfamilies; receptor-activated (R-)Smads (Smad2 and Smad3 are phosphorylated by TGF-β and activin receptors, and Smad1, Smad5, and Smad8 by BMP receptors), a single common-mediator (Co-)Smad (Smad4), and two inhibitory (I-)Smads (Smad6 and Smad7). After R-Smads have been phosphorylated in their C-terminals by type I receptors, they form oligomeric complexes with Smad4, which are translocated to the nucleus where they in collaboration with other nuclear factors regulate the expression of specific genes [65–67]. I-Smads are induced by Smad signaling and act in negative feedback control mechanisms [68].

TGF-β inhibits growth of normal intestinal epithelium and colonic adenoma cells in culture. However, conversion of adenoma to an adenocarcinoma and a metastatic lesion is associated with loss of growth-inhibitory responses to TGF-β. Rather, TGFβ promotes cell proliferation, epithelial-mesochymal transition (EMT), invasion and metastasis. One mechanism by which tumor cells become resistant to the growth inhibitory actions of TGF-β is through downregulation or mutation of the TβRII. TβRII has been shown to be inactivated in a subgroup of colorectal carcinomas associated with the MSI [69]. Mutations of TβRII have also been identified in 15% of MSS colorectal cancers [70]. A recent report shows that conditional loss of TβRII in breast cancer cells resulted in chemokine-mediated recruitment of myeloid cells into the tumor stroma and promotion of invasion and metastasis [71]. Furthermore, Smad2 mutations have been identified in a small subset of colorectal cancers [72, 73]. The most commonly altered Smad mediator in CRC is Smad4. Mutations of Smad4 have been detected in 20–30% of CRC [74, 75]. Downregulation of Smad4 is correlated with loss of E-cadherin expression [76], liver metastasis, and poor prognosis in CRC [77, 78], suggesting that loss of Smad4 expression could be a causal factor for tumorigenesis in CRC.

In addition, it have been well documented that TGFβ induces CRC metastasis by promoting EMT and cell motility and invasion. In response to TGFβ, the type II receptor kinases phosphorylates the type I receptors, which then leads to activation of the cellular responses to TGFβ. Inhibition of TGFβ type II receptor function reverses EMT in colon cancer cells and inhibits EMT in skin and mammary cancer models in vivo [79–81]. The type I receptor also plays a critical role in TGFβ-induced EMT. Expression of an activated version of the TβRI receptor ALK-5 (e.g., the major TGFβ type I receptor) or ActRIB/ALK-4 (e.g., the major type I receptor for activin and nodal) recapitulates TGFβ induced EMT in NMuMG cells [82, 83], whereas dominant negative type I receptor block TGFβ-induced EMT [83]. Further, increased expression of Smad2 or Smad3 with Smad4 induces EMT, or enhances the induction of EMT by the activated form of TβRI, in NMuMG cells, whereas expression of dominant negative versions of Smad2 or Smad3 blocks TGFβ-induced EMT. Similarly, Smad4 is indispensable for EMT. Knockdown of Smad4 expression or expression of a dominant negative mutant of Smad4 abrogates TGFb-induced EMT phenotype. Furthermore, genetic ablation of Smad4 leads to preservation of epithelial markers and a lower degree of EMT in adenocarcinoma [84]. In contrast, the inhibitory Smads (Smad6 and 7) function as negative regulators and thus repress TGFβ-induced EMT. Underlying mechanisms include induction of expression of three families of transcription factors, the Snail, ZEB and bHLH families, by TGFβ, either through a Smad-dependent mechanism (in the case of Snail proteins) or indirectly through activation of other transcription factors or relief of repression. Upon activation these transcription factors in turn repress epithelial marker gene expression and concomitantly activate mesenchymal gene expression.

18.8 MicroRNAs

MicroRNA (miRNAs) are transcripts of 19–25 nucleotides that are conserved among invertebrates, vertebrates, and plants, suggesting that these molecules participate in essential processes [85]. MiRNAs function as negative regulators of gene expression and each miRNA regulates hundreds, even thousands of genes. Accumulated studies showed that miRNAs have been deregulated in various types of human malignancy including CRC. The expression profiles of miRNAs can be used for the classification, diagnosis and prognosis of human malignancies. Further, miRNAs could function as oncogenes or tumor suppressors to regulate cell survival, growth, migration, invasion, angiogenesis and metastasis.

The first miRNA profile of CRC showed consistently reduced accumulation of the specific mature miR-143 and miR-145 in the adenomatous and carcinoma stages of colorectal neoplasia [86]. Since consistent levels of the ~70-bp pre-miR-143 present in each of the cell lines examined, the different levels of mature miR-143 in these cells were controlled by a post-transcriptional mechanism, suggesting that abnormal processing might affect miRNAs expression in colon cancer cells. A further miRNA profiling study evaluated the expression of miRNAs in CRC samples characterized by microsatellite stability (MSS) or by high levels microsatellite instability (MSI-H). Their analysis of miRNA expression profiles of MSI-H ($n = 16$) and MSS CRCs ($n = 23$) identified 14 differentially expressed miRNAs [87]. The most prominent class of differentially expressed miRNAs is various members of the oncogenic miR-17-92 family, suggesting that these miRNAs have a role in the bio-pathologic characteristics that distinguish MSS from MSI-H CRCs. Moreover, miR-17-5p, miR-20, miR-25, miR-92-1, miR-92-2, miR-93-1, and miR-106a were significantly up-regulated in MSS CRCs relative to MSI-H CRCs [87]. Because members of the miR-17-92 family can act as oncogenes to promote cell growth and inhibit apoptosis [88], up-regulation of these miRNAs may be involved in the more aggressive clinical behavior of MSS tumor than MSI-H neoplasm. Lu et al. [89] and Volinia et al. [90] were able to classify the tissues of origin for metastases from poorly differentiated tumors as well as categorize human CRC and normal colon tissues with low rates of misclassification using upregulated expression of 21 miRNAs and the downregulated expression of one miRNA (miR-9-3).

Accumulated studies have demonstrated that miRNAs play a critical role in cancer initiation and progression by negative regulation of their target genes. MiR-10b has been shown to initiate breast cancer invasion and metastasis [91], whereas miR-335 suppresses breast cancer metastasis and migration by targeting the transcription factor SOX4 and tenascin C, an extracellular matrix component with anti-adhesive properties [92]. MiR-15a and miR-16 exert their tumor suppressor function by targeting multiple oncogenes, including BCL2, MCL1, CCND1, and WNT3A [93]. MiR-214 induces cell growth and survival by inhibition of PTEN, Patched and Sufu expression [94–96]. A recent report showed that miR-135a and miR-135b function as oncoemiRs by direct targeting the 3′ untranslated region of the adenomatous polyposis coli gene (APC), suppress its expression, and induce downstream activity in the Wnt signaling pathway [97]. Underlying mechanisms of dysregulation of miRNAs in human cancer include chromosomal alterations, epigenetic silencing, aberrant processing and transcriptional regulation. It was shown that upregulation and downregulation of a number of miRNAs correlate with chromosomal gain and lose or epigenetic changes. Imbalance of pri-, pre- and mature miRNAs is due to altered miRNA processing. A growing list of publications showed that alterations of transcriptional factors are responsible for a number of miRNA dysregulation in human cancer. For example, miR-34 is controlled by p53 [98–100] and NF-kappa B induces miR-155 [101]. Further, c-Myc has been shown to transcriptionally upregulate miR-17-92 family [102]. However, the extent of miRNA regulation by various transcription factors in colon cancer cells, as well as miRNA as diagnostic/prognostic marker and therapeutic targets, remain to be further investigated.

18.9 Conclusion Remarks

During the last 2 decades, several important breakthroughs have been achieved in understanding the molecular basis of colorectal cancer. Mutation of the APC gene makes a significant contribution to tumor initiation and progression in CRC. Likewise, the DNA

MMR genes have gatekeeper and caretaker function in the development of CRC. While stepwise of CRC progression model is valuable, each step could require for multiple genetic alterations, some of which might be overlapped. Further, CRC from different anatomic sites, i.e., proximal colon, distal colon, and rectum, have unique genetic changes and should not be assumed to be constant in their biological behavior or relative risk factors. Animal models are needed to document the importance of miRNAs in CRC carcinogenesis. Ultimately, major challenge is how these genetic changes translate to therapeutic approach to improve the survival of CRC patients.

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Chapter 19

Carcinogenetic Pathway of Superficial Low-Grade Urothelial Carcinoma

Shang-Tian Chuang, Robert A. Tracy, and Ximing J. Yang

Contents

19.1 Introduction

Bladder cancer is a major cause of health expenses. It is estimated to be the seventh most prevalent malignancy worldwide and accounts for approximately 3.2% of the international cancer burden [1]. Bladder cancer is more common in men than in women [2] and more prevalent in industrialized than in developing countries [3].

The bladder is lined by urothelium, so it is no surprise that urothelial carcinoma represents greater than 90% of the tumors within the bladder [4]. Other carcinomas involving the bladder include adenocarcinoma and squamous cell carcinoma. Most cases of urothelial carcinoma are sporadic. Risk factors, such as, cigarette smoking, exposure to arylamines (particularly 2-naphthylamine), Schistosoma haematobium infection, and radiation therapy, have been strongly associated with urothelial carcinoma [5].

Urothelial carcinoma of the bladder is classified into superficial (stage Ta, Tis, and T1) and muscle invasive (T2, T3, T4) tumors. Superficial tumors include non-invasive papillary carcinoma, carcinoma in situ, and tumors that invade the subepithelial connective tissue (lamina propria). They account for 75–85% of urothelial carcinoma of the bladder [6]. More than 70% of patients with superficial tumors will have one or more recurrences after initial treatment [7]. Progression to muscle invasive disease will develop in 10–20% of these patients with superficial tumors [7].

Muscle invasive tumors, which include tumors that invade the muscularis propria, perivesical tissue, and adjacent organs, comprise the remaining 15–25% of urothelial carcinoma of the bladder [6]. Unfortunately, regardless of radical cystectomy and/or systemic therapy, approximately 50% of patients with muscle invasive urothelial carcinoma die from metastases within 2 years of diagnosis [8, 9].

In addition, urothelial carcinoma of the bladder is classified into papillary with low and high grade and non-papillary (flat) tumors. Histologically, urothelial papillary tumors are those that generally consist of fibrovascular cores lined by neoplastic urothelial cells. Low grade papillary carcinoma can have fused papillae lined by predominantly ordered neoplastic urothelial cells that exhibit enlarged nuclei which vary in size and shape. High grade papillary carcinoma demonstrates fused papillae that are branched and lined by neoplastic urothelial cells that show marked variation in size and shape of the nuclei. Non-invasive low grade papillary tumors account for approximately 80% of urothelial carcinoma [10]. These lesions often recur multiple times but are limited in their potential to

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become muscle invasive. The 5 year survival rate is about 90% if these lesions are treated early by surgical resection and intravesical immunotherapy [11].

On the other hand, non-papillary (flat lesions), such as, urothelial carcinoma in situ (CIS), are lesions in which the urothelium contains cells that are cytologically malignant as defined by a neoplastic urothelial cell with a nuclear size of five times or greater than that of a lymphocyte's nucleus. De novo (primary) CIS accounts for about 1–3% of urothelial neoplasms [10] and can present as invasive tumors.

Interestingly, urothelial carcinoma of the bladder is distinct from other epithelial carcinomas in that it is thought to have two divergent pathways of carcinogenesis. Studies have shown that superficial/low grade papillary tumors develop along one molecular pathway while muscle invasive tumors and CIS develop along a different molecular pathway. Deletions of chromosome 9 are more commonly associated with superficial/papillary tumors while loss of heterozygosity on chromosome 17 is more frequently seen in carcinoma in situ and invasive tumors [12–16]. In addition, low grade papillary tumors are shown to have activating mutations involving tyrosine kinase receptors, such as, fibroblast growth factor receptor 3 (FGFR3) [17, 18], and its pathways, such as, Ras (19) and phosphoinositide 3-kinase (PI3K) pathways [19]. In contrast, most CIS and high grade invasive tumors have defects in the p53 and retinoblastoma (RB) protein genes and their pathways [20].

Chromosomal 9 alterations and activating mutations of tyrosine kinase receptors and its pathways involving superficial/papillary tumors will be discussed in this chapter.

19.2 Chromosomal Aberrations

Chromosomal aberrations, which include deletions, amplifications, and aneusomies, are common in urothelial carcinoma and appear to involve almost all the chromosomes [21]. Chromosome 9 monosomy can be seen in non-invasive papillary tumors [22]. However, there are also more localized deletions of various chromosomal regions. Deletions of chromosome 9, although identified in urothelial carcinomas of all grades and stages, is often the only genetic alteration found in low grade tumors [22]. Deletions of both arms of chromosome 9 (9p-/9q-) have been shown to occur during early urothelial carcinogenesis and are frequently present in superficial low grade papillary tumors [13, 23]. According to Simoneau et al., 48% of superficial tumors had at least one deletion in chromosome 9 [24].

The 9p deletion (9p21) affects the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene. This CDKN2A gene encodes for the tumor suppressor proteins p16 and alternative reading frame (ARF). p16, also known as, inhibitor of cyclin-dependent kinase 4A (INK4A) is a member of the INK4 family. It arrests the G1/S cell cycle transition by preventing the phosphorylation of the retinoblastoma protein (pRB). Loss of p16 expression would, therefore, result in lack of regulatory control of the cell cycle [25].

In addition, loss of heterozygosity of 9q is more common in non-invasive low-grade papillary tumors than in CIS and muscle invasive tumors [13]. Deletions on 9q (9q22.3, 9q31–32, 9q33, and 9q34) are found to be twice as common as deletions on 9p, which so happen to be mostly associated with 9q deletions [26]. This suggests the possibility that gene alterations on 9q may be an early event in superficial papillary tumors [26]. Of interest, even deletions of chromosome 9 are described in normal-appearing urothelium adjacent to areas demonstrating early precursor changes [27]. Furthermore, chromosome 9 deletions are seen in cells taken from voided urine of patients who currently have no detectable tumor and negative urine cytologies [27]. These chromosomal aberrations found in normal-appearing urothelium adjacent to precursor lesions could explain the frequent recurrence of papillary urothelial carcinoma.

However, as previously mentioned, although chromosome 9 deletions may be the only genetic alteration identified in superficial papillary tumors, chromosome 9 deletions have been demonstrated in both urothelial dysplasia and CIS. This would imply that chromosome 9 deletions do not distinguish between the two tumorigenesis pathways [28].

19.3 Activation of Tyrosine Kinase Receptor and Pathway

In addition to chromosomal aberrations, mutations in tyrosine kinase receptors and pathways, such as, FGFR3, PI3KCA, and Ras have been identified in low grade papillary urothelial tumors.

19.3.1 Fibroblastic Growth Factor Receptor 3 (FGFR3)

Typically, when a ligand binds to a cell surface receptor, an extracellular signal is tranduced into the cell creating changes in gene expression. Tyrosine kinase is a family of cell surface receptors and consists of an extracellular ligand-binding domain, a transmembrane region, and a cytoplasmic tail that has intrinsic tyrosine kinase activity. Fibroblast growth factor receptor 3 (FGFR3) is a member of the tyrosine kinase family. It is involved in cell growth and differentiation, angiogenesis, and embryogenesis [29]. Specific point mutations in FGFR3 have been associated with human skeletal dysplasias with severe impairment in cranial, digital and skeletal development [30]. Somatic FGFR3 mutations have also been identified in urothelial carcinoma. Seventy percent of low grade papillary non-invasive tumors exhibit FGFR3 mutations [18, 31, 32]. In contrast, only 10–20% of invasive tumors harbor FGFR3 in genes, suggesting that low grade papillary non-invasive tumors have an alternative pathogenesis than invasive tumors [18, 31, 32].

Most of the mutations identified in FGFR3 have been missense mutations that cause amino acid substitutions that involve the extracellular domain, transmembrane region, and cytoplasmic tail [31, 33, 34]. The extracellular ligand-binding domain of FGFR3 consists of three extracellular immunoglobulin-like domains which are connected by loops. The most common mutation results in the conversion of a non-cysteine residue into a cysteine in these loops, with the loop between the extracellular immunoglobulin I and immunoglobulin II being the most common [11]. These mutations can result in autophosphorylation of the intracellular kinase region and decreased translocation to the lysosomal degradative pathway which would could result in increased and prolonged activation of the receptor [35, 36].

19.3.2 Phosphatidylinositol 3 Kinase p110 α (PI3KCA)

Activated FGFR3 can trigger the downstream phosphoinositide 3-kinase (PI3K) pathway. PI3K generates 3′-phosphoinositides which bind to the pleckstrin homology domain of 3′-phosphoinositides-dependent kinase 1 and Akt with subsequent activation of this pathway [37]. Depending on the substrate specificity, activation mechanisms, and expression patterns, the PI3K family is separated into three classes. Class I is further divided into class 1A subgroup which are coupled to signal transduction by receptor tyrosine kinase upon growth factor binding and class 1B subgroup which signal from G-coupled receptors [38]. Class I PI3K consists of a catalytic (p110) and a regulatory subunit. There are four (α, β, γ, and δ) different catalytic subunits of which the catalytic p110α subunit is encoded by the PI3KCA locus [38]. Activating somatic mutations in the PI3KCA have been identified in cancers of the breast, colon, ovary, and stomach [39]. Recently, PI3KCA hotspot mutations in codons 542, 545, and 1047, have been found in approximately 20% of superficial bladder tumors in contrast to a very low prevalence in muscle invasive tumors [19]. In addition, a subset of the superficial tumors with PI3KCA has FGFR3 mutations [19]. Therefore, it is quite possible that FGFR3 and PI3KCA may represent a similar pathway of tumor progression. It has been postulated by Lopez-Knowles et al. that activation of PI3K pathway in bladder cancer may enhance malignant behavior in FGFR3-mutant tumors [19].

19.3.3 Ras

In the tyrosine kinase pathway, Ras proteins are also downstream from FGFR3. Ras genes encode membrane-bound guanine nucleotide-binding proteins that are responsible for the transduction of signals that regulate cell growth and differentiation. Ras proteins are activated when bound by GTP and with subsequent hydrolysis of the bound GTP to GDP and phosphate is inactivated. GTP binding can be catalyzed by guanine nucleotide exchange factors. In addition, the rate of conversion from GTP to GDP can be accelerated by guanine nucleotide activating proteins (GAPs). Proto-oncogenes in the Ras family include HRAS, KRAS, RRAS, and NRAS [40].

HRAS was the first human oncogene identified in the bladder cancer cell line T24 [41]. HRAS mutations, which have been found on codons 12, 13 and 61 [42], occur in about 30–40% of low grade non-invasive papillary tumors [43–45]. One specific mutation

frequently found in bladder tumors substitutes the amino acid glycine with amino acid valine at position 12 (G12V) [7]. With this substitution, the HRAS gene is constantly activated which may result in uncontrolled cell division and subsequent tumor formation.

Mutations have not only been found in HRAS but also two other Ras genes, NRAS and KRAS2 [7]. Mutations found in NRAS were G12R, Q61L, and Q61R while mutations found in KRAS2 were G12A and G12V [7]. It is unclear whether both Ras and FGFR3 mutations can co-exist in the same tumor. However, Jebar et al. recently discovered that in no cases were Ras and FGFR3 mutation found together, suggesting mutual exclusion [7].

19.3.4 Other Tyrosine Kinase Receptors

In addition to FGFR3, other tyrosine kinase receptors, such as, the ErbB family can be over-expressed in urothelial carcinoma. The ErbB family includes epidermal growth factor receptor (EGFR or ErbB-1), ERBB2 (HER2/c-neu or c-ErbB-2), ERBB3 (HER3 or c-ErbB-3), and ERBB4 (HER4 or c-ErbB-4). In general, binding of specific ligands leads to dimerization followed by activation of the receptor. Activated receptors are responsible for DNA synthesis and proliferation [46].

Similar to other tyrosine kinase receptors, EGFR is composed of an extracellular ligand-binding domain, a transmembrane region, and an intracellular domain with intrinsic tyrosine kinase activity [47]. Mutations in EFGR may result in persistent activation of the cascades which may lead to uncontrolled cell division [48]. ERBB2 has no external ligand; however, it is believed to be the preferred dimerization partner for other receptors [49]. ERBB3 does not have tyrosine kinase activity, and is therefore, restricted in activation of downstream pathways alone [50]. ERBB4 is more direct in activating the transcription of target genes by moving a portion of its intracellular domain to the nucleus [51].

Interestingly, over-expression of ERBB3 and ERBB4 has been found to be associated with superficial low grade tumors [52]. In contrast, the over-expression of EGFR and ERBB2 are associated with muscle invasive tumors [53–55]. These findings would once again support the two distinct pathways of urothelial carcinoma.

19.4 Conclusion

Even though superficial/low grade papillary tumors are generally are not life-threatening, the disease still places a heavy burden on patients and healthcare providers. Following surgical resection of these tumors, patients typically require long-term follow up with invasive procedures. Although their mutual exclusivity is still debatable, tumorigenesis of urothelial carcinoma of the bladder is believed to develop through divergent pathways with division between superficial/low grade papillary tumors and muscle invasive tumors and CIS. With this knowledge, possibly potential markers for non-invasive disease monitoring and for targeted therapy for patients with superficial/low grade papillary tumors may be discovered.

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Chapter 20

Carcinogenetic Pathway of Urothelial Carcinoma

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Contents

20.1 Introduction

Urothelial carcinoma (UC) is the second most common cancer of the genitourinary tract, representing the fourth most common malignancy in males and the ninth most common in females in the United States [1, 2]. An average of 260,000 new cases are diagnosed worldwide yearly, with approximately 68,810 new cases in 2008 in the USA with corresponding 14,100 deaths [3]. UC has a high recurrence rate and generally does not present as metastatic disease. The current treatment for UC is based on pathological staging. For the past two decades, the molecular pathways of progression and evolution of bladder cancer have been the center of investigation [4].

Two discrete biologically significant pathways involving bladder carcinogenesis are recognized, one leading to superficial papillary carcinomas and the other leading to more aggressive either flat (carcinoma in situ) or invasive carcinoma.

Approximately 70% of urothelial neoplasms are superficial papillary carcinomas with a tendency to frequently recur locally, however, rarely invade or metastasize. In contrast, 30% are the more aggressive non-papillary flat, carcinomas that have a higher propensity to invade and metastasize [2, 4–6]. For patients with invasive or metastatic disease, despite aggressive therapeutic intervention, the overall cure is 20–50% [7]. Papillary and the more aggressive non-papillary flat urothelial carcinomas (UC) have unique molecular profiles and appear to develop and advance through two distinct molecular pathways. However, it is not known if these two pathways are mutually exclusive [6, 8, 9].

The majority of non-papillary flat urothelial carcinomas (UC) show alterations in the p53 (TP53)-Mdm2-p14 and the retinoblastoma-p16 (Rb) genes and pathways [6, 8, 10–14]. Both p53 and Rb maintain cellular homeostasis and control normal cell cycle, cellular growth and proliferation [5, 6, 15]. Cell cycle alterations are the most common cause of molecular modifications in UC [8]. The retinoblastoma (Rb) pathway regulates cell cycle by receiving extracellular growth signals via the Ras-mitogen activated protein kinase (Ras-MAPK) signal transduction pathway. The Ras-MAPK pathway transfers extracellular growth signals to the nucleus, where cyclin/CDK (cyclin-dependent kinase) complexes induce phosphorylation of Rb. In response to cellular stress, Rb is the main regulator of cell cycle progression, while p53's main function is to trigger apoptosis or growth arrest in the G1 phase. Rb is regulated by factors such as cyclin D1 and p16 (a CDK inhibitor) [5, 15]. Mutations of p53,

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or the components of its pathway, ie $p21^{WAF1/CIP1}$ gene, interrupt cellular growth and apoptosis, leading to neoplastic transformation [5, 16]. p53 is regulated by the p14/ARF (a CDK inhibitor) and the oncoprotein Mdm2 [16]. P14 is known to inhibit the function of Mdm2. Mdm2 suppresses the activity of p53 and triggers its degradation [8]. Clinically aggressive UC also exhibits discrete molecular gene alterations involving cell-stroma interactions [5, 9]. Tumor angiogenesis plays a role in UC progression by providing oxygen, nutrients and growth factors to the neoplastic cells [5, 6].

Alterations in the p53 pathway are reported to be a significant independent predictor of survival [16]. p53 mutations have been linked as the major contributory factor for the proclivity of in situ carcinoma to invade the lamina propria and urinary bladder muscle wall [6, 17]. Patients with normal p53 pathways are reported to show significantly low death rate and therefore considered low-risk. However, patients with abnormalities of the p53 pathway have significantly aggressive clinical course, high death rate, decreased overall survival and considered high-risk [15, 16]. In UC, Rb and p53 are commonly both altered [18]. Molecular alterations of p53 and Rb genes and their pathways in conjunction with overexpression of Mdm2 and loss of p21 have been found to be significantly associated with poor prognosis and advanced stage in UC [6, 8, 16]. Distinct genetic events portray the interaction between the molecules involved in these pathways, lending to their use as prognostic indicators. Alterations of p53 and Rb may help identify patients with high risk superficial cancers more likely to progress to invasive carcinoma, identifying patients who may be managed without radical cystectomy [16]. Aberrant levels of p53 and Rb may also identify individual patients who may fail conventional treatment and who may benefit from therapies targeting specific markers of carcinogenesis. Novel pharmacologic agents targeting altered pathway-specific molecules are currently in development. There is an increased risk of bladder cancer recurrence and disease progression with increasing number of unregulated altered markers. Patients with unaltered wild type p53 and Rb bladder cancers show significant decreased risk of recurrence and mortality when compared to those who have mutational alteration in both p53 and Rb. Future therapeutic modalities for urothelial carcinoma will take advantage of multimarker panels using a combination of altered markers exerting synergistic action [6, 12–15, 18–22].

20.2 p53 Cell Cycle Regulation Pathway

The p53 oncoprotein is a crucial molecule involved in cell cycle control in urothelial carcinoma (UC). The p53 protein is encoded by the tumor suppressor gene TP53 located on the short arm of chromosome 17 p13.1. Inactivation of the p53 tumor suppressor gene is important for cancer development, progression and therapeutic response. Loss of heterozygosity of one allele, followed by mutation of the remaining allele, is an important mechanism for gene inactivation [6, 8, 10, 23, 24]. p53 protein is a transcription factor activating genes involved in apoptosis (BAX gene), cell cycle arrest (p21/WAF1 genes) [6, 15] and identifying DNA damage [5, 16, 22]. p53 also plays an important role in angiogenesis [6, 8]. p53 pathway genes preside over the programs of cell growth and death, playing a critical role in G_1-S cell cycle transition in response to cellular stress [6, 16, 25, 26]. Mutations of p53 result in loss of its control over apoptosis, cell cycle progression and transcription of genes involved in DNA repair [6, 22]. Tumors exhibiting uninhibited proliferation and lack of apoptosis show a selective growth advantage and typically are resistance to treatment [6, 16, 27, 28].

Mutation in the TP53 gene is accepted as a critical event in numerous cancers, including UC [6, 15]. These mutations are in general missense point mutations. Mutated p53 is more stable with a longer half-life that is resistant to standard regulatory degradation by the ubiquitin pathway and thus accumulates in the nucleus. Both nuclear accumulation and gene mutations are a factor in tumor progression. Determining the status of both the gene and the protein can give added synergistic data concerning prognosis. The site of mutation may also be imperative in understanding tumor behavior [6]. Accumulation of p53 within the nucleus and TP53 gene mutations are associated with aggressive clinical behavior, a greater risk of recurrence and progression and decreased overall survival in UC [9, 16]. This is particularly observed in patients with invasive, organ-confined, node-negative (T1-2bN0) tumors [6, 8]. Chatterjee, et al. [15],

reported significantly better overall survival and lower rates of recurrence for normal wide type p53 UC than mutated p53 UC [15]. The 5-year recurrence rates for wide type p53 UC versus mutated p53 UC are reported as 30% versus 70% ($P < 0.001$), whereas the 5-year survival rates as 61% versus 26% ($P < 0.001$) [15].

Cellular transition through the G1 to S phase is regulated by cyclin-dependent kinases (CDKs). P53 protein inhibits cell cycle progression at G1-S cell phase thereby arresting cell growth. P53 implements its control and influences tumor progression through the transcriptional activation of the $p21^{WAF1/CIP1}$ [6, 8, 15, 16, 23] gene, an integral part of the p53 pathway. The $p21^{WAF1/CIP1}$ gene encodes the p21 protein, a universal cyclin-dependent kinase inhibitor *(*CDKI) that can arrest cells in the G1 phase by inhibiting DNA replication [6, 15, 22, 29, 30] (Fig. 20.1). $p21^{WAF1/CIP1}$ is upregulated by p53 in response to DNA damage and cellular stress. p21 arrests the cell cycle by inhibiting cyclin/CDK complexes, and in this manner prevents Rb phosphorylation [6, 15, 16, 23, 31]. Unphosphorylated Rb can then bind and sequester the transcription factor E2F thus preventing it from transcribing genes necessary for DNA synthesis [6, 15, 32]. Hence, there is an essential interaction between the components of the p53, p21 and Rb pathways [6, 15]. Rb also arrests cells in the G1/S phase in response to DNA damage, suggesting a possible link between Rb and p21 [6, 16, 22]. Loss of expression of p21 is considered to be abnormal [6, 16, 33, 34]. Loss of p21 expression has been shown to be a predictor of UC progression, tumor recurrence and decreased overall survival [6, 8, 15, 16, 33, 34]. The p21/WAF1 gene, itself, is not a target for mutations [35]. A p21-negative phenotype likely represents the existence of a nonfunctional, mutated p53 imparting an aggressive clinical course [25].

The Mdm2 proto-oncogene, encoded on chromosome 12q14.3–q15, is also an integral part of the p53 pathway. Mdm2 is involved in an autoregulatory feedback loop with p53 and is known to stabilize p53 [6, 8, 36]. p53 upregulates expression of Mdm2. Activated mdm2 then binds to p53 and serves as a negative regulator, inactivating its function by inhibiting its transcriptional activity [6, 16, 36–38]. Mdm2 is amplified in UC and the frequency of nuclear amplification increases with tumor grade and stage. Mdm2-positive tumors are associated with early tumor stage and poor survival [6, 16, 33, 34]. Deregulation of the p53 pathway, associated with overexpression of mdm2 (mdm2-positive) and loss of p21 (p21-negative) phenotypes, influences prognosis and outcome in UC [16]. Lymph node negative patients with p21-negative, p53-mutated, mdm2-positive tumors show a greater recurrence and lower survival rate than those with p21-positive, p53-negative, mdm2-negative tumors, independent of tumor grade or pathologic stage [6, 8, 16]. Studies have shown that p21 mutations and mdm2 amplification, by themselves, may not be clinically significant. However, in conjunction with mutated p53, it has been shown that p21 and mdm2 can exert a cooperative effect impacting tumor progression and survival [16]. It is postulated that alterations of p53 and mdm2 influence clinical outcome during initial stages of UC, possibly accredited to their inhibition of apoptosis and cell growth. On the other hand, additive effects of other molecular defects, such as lack of p21, are postulated to influence later stages of UC progression [16].

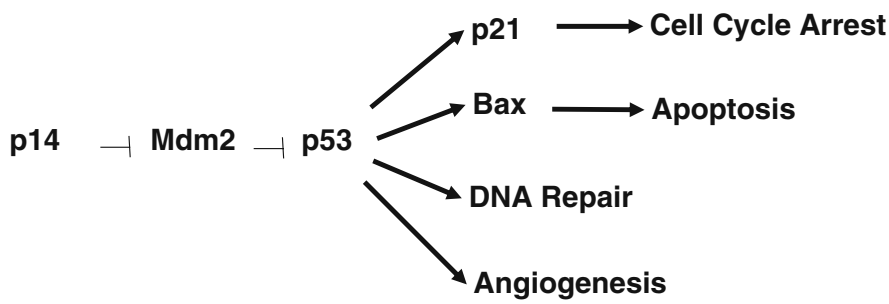

Fig. 20.1 The p53 pathway. Mutations of p53 or components of its pathway, i.e., p21 or Bax, interrupt cellular growth and apoptosis, leading to neoplastic transformation. The p53 gene is regulated through p14 which inhibits Mdm2 function. Mdm2 is known to suppress p53 activity and trigger its degradation. P53 also plays a role in angiogenesis and the transcription of genes involved in DNA repair

Patients with organ-confined invasive bladder cancer who show p53 mutations may benefit from adjuvant chemotherapy containing DNA-damaging agents such as cisplatin since DNA damage to p53-altered urothelial cells results in apoptosis [6, 39]. There has been an ongoing effort in the investigation of molecular and viral vectors that can store functional TP53. Adenoviral vectors containing a functional wild-type TP53 gene have inhibited tumor growth in bladder cancer cell lines. Initial clinical trials, using a combination of cisplatin with TP53 containing adenovirus, have been shown to have a synergistic effect leading to increased apoptosis, thus implying that the combination of adenoviral vector-mediated TP53 delivery with DNA-damaging agents should be further studied in the treatment UC [6].

20.3 Ras-MAPK Signal Transduction Pathway

The key target in the signal transduction pathway in UC is epidermal growth factor receptor (EGFR) protein, a receptor tyrosine kinase. In invasive UC, there is continuous activation of the Ras-MAPK (mitogen activated protein kinase) pathway, typically through the activation of receptor tyrosine kinases such as EGFR. EGFR expression is associated with a more aggressive clinical course. Under consideration for the targeted therapy for UC are inhibitory monoclonal antibodies raised against the extracellular domains of EGFR. Two members of the EGFR family, Erb-B-1 and Her-2/neu (Erb-B-2), have been the focus of several targeted therapies in UC [6, 9].

MAPK regulates cell proliferation and survival. Binding of epidermal growth factor (EGF) causes activation of the already overexpressed EGFR. The activated EFGR receptor then recruits proteins that activate Ras. This activated Ras protein can then transmit a mitogenic signal via the Ras-MAPK pathway through the MAPK/ERK (extracellular signal-regulated kinase) system.

RASSF1A (Ras association domain family 1), a tumor-suppressor gene, encodes a protein that inhibits the function of activated Ras protein. RASSF1A is commonly highly methylated in bladder cancer, and its increased methylation is associated with increasing tumor stage. The death-associated protein kinase (DAPK), an apoptosis promoter, prevents the transfer of extracellular-signal regulated kinase (ERK) protein from the cytoplasm into nucleus, thus inhibiting signal transduction. It is controversial as to whether ERK methylation level is a prognostic indicator by itself, since several studies have shown varying levels of methylation for the ERK gene in UC [6]. Transfer of ERK into the nucleus from the cytoplasm activates MSK1 (mitogen-activated and stress-activated protein kinase1), a histone H3 kinase, that can relax chromatin, causing it to be more transcriptionally available. This alteration in the chromatin stimulates MYC, a gene that encodes the c-Myc protein, a transcription factor that controls the cell cycle. Correlation has not yet been found between the MYC methylation pattern and clinical stage of UC, and there is conflicting data on the significance of c-Myc protein expression relating to prognosis. C-Myc gene promotes expression of cyclins that complex with cyclin-dependent kinases (CDKs), which in conjunction regulate the RB pathway [6].

20.4 Retinoblastoma Pathway

Nuclear phosphoprotein (Rb) is encoded by the retinoblastoma (Rb) gene located on chromosome 13q14 [6, 8, 9, 23, 24, 40]. Rb is involved in senescence, cell-cycle regulation and apoptosis and is regarded as a key tumor suppressor in UC [6, 8]. Rb is essential in cell cycle regulation typically at the G1/S transition. The active, dephosphorylated form of Rb binds transcription factor E2F, preventing it from transcribing genes required for DNA synthesis during the S phase of the cell cycle [5, 6, 8, 15, 23, 24, 32]. Rb releases E2F after phosphorylation by cyclin-dependent kinases (CDKs). Unbound released E2F can then transcribe genes required for DNA synthesis and activate genes needed for G1 to S cell cycle phase transition [15, 23, 41] (Fig. 20.2).

Both high Rb protein expression and loss of Rb function, even in the presence of detectable nuclear RB protein, have been implicated in high-grade and invasive UC. Gene deletions and dysfunctional mutations of Rb are mainly associated with more aggressive tumor behavior [6, 13, 14, 42, 43]. Deletion of chromosome 13q is the most common cause of RB gene inactivation. Chatterjee, et al. [15], reported significantly increased recurrence ($P < 0.001$) and decreased

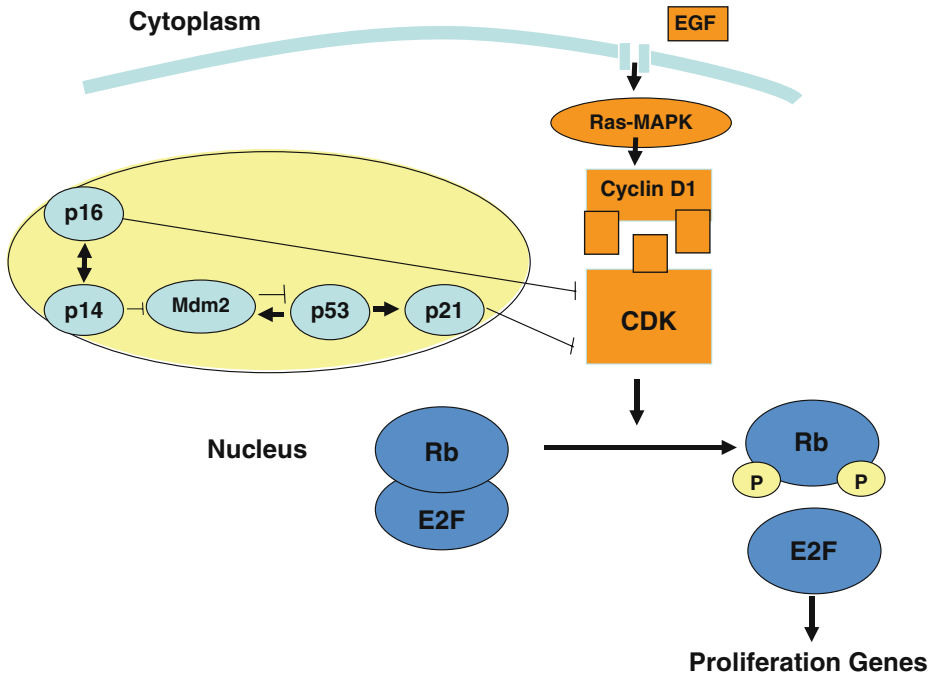

Fig. 20.2 Interactions of the p53 (*yellow*) and retinoblastoma (*blue*) pathways in urothelial carcinogenesis. Extracellular growth signals, i.e., EGF, stimulate the Ras-MAPK signal transduction pathway. The Ras-MAPK pathway then transfers signals to the nucleus, where cyclin/CDK complexes induce phosphorylation of Rb. Phosphorylated Rb releases E2F which then causes transcription of genes that promote proliferation. This process is also regulated by the p53 pathway. EGF, epidermal growth factor; Ras-MAPK, Ras-mitogen activated protein kinase; CDK, cyclin-dependent kinase; Rb, retinoblastoma

overall survival ($P = 0.001$) for UC patients with altered Rb, with estimated 5-year recurrence rates for wide type Rb versus altered Rb as 29% versus 57%, and estimated 5-year survival rates as 67% versus 33%, respectively [15].

Poor prognosis in UC is associated with genetically altered Rb, hyperphosphorylated Rb or increased Rb protein expression. Hyperphosphorylation (inactivation) of Rb and increased Rb protein expression results from decreased $p16^{ink4a}$ expression (encoded on CDKN2a gene located on chromosome 9q21) and/or overexpression of cyclin D1 (Fig. 20.3). Cyclin D1 is crucial positive regulator of the G1-S cell cycle transition and is activated by CDKs [8]. Phosphorylation of Rb promotes release of bound E2F resulting in cell cycle progression. Increased p16 expression, a cyclin dependent kinase inhibitor (CDKI), has been shown to result in hypophosphorylation of Rb, decreased cell proliferation, and decreased levels of Rb expression therefore acting as a negative cell cycle regulator. Hypophosphorated Rb remains complexed to E2F resulting in G1 cell cycle arrest resulting in lack of regulatory control of cell cycle progression [6].

Since hyperphosphorylation of Rb has been demonstrated to be a means of tumor suppressor pathway inactivation in UC, it may be conceivable that hypophosphorylation of the wild-type Rb protein using cyclin-dependent kinase inhibitors (CDKIs), may improve prognosis. Recently, INK4 and KIP, the genes encoding CDKI's, have been identified. Initial experiments using the protein kinase inhibitor staurosporine, which stimulates G_1 and G2 phase arrest in normal urothelium, have shown growth arrest after transfection with retrovirus containing functioning Rb [8]. Currently, CDKIs such as flavopiridol (L86-8275), a semisynthetic flavonoid that is a derivative of an indigenous Indian plant, and UCN-01 (7-hydroxystaurosporine) have been successful in phase I and II clinical trials for the treatment of UC. Flavopiridol directly inhibits cyclin dependent kinases, and additionally decreases cyclin D1 levels which may be elevated in UC [6, 8].

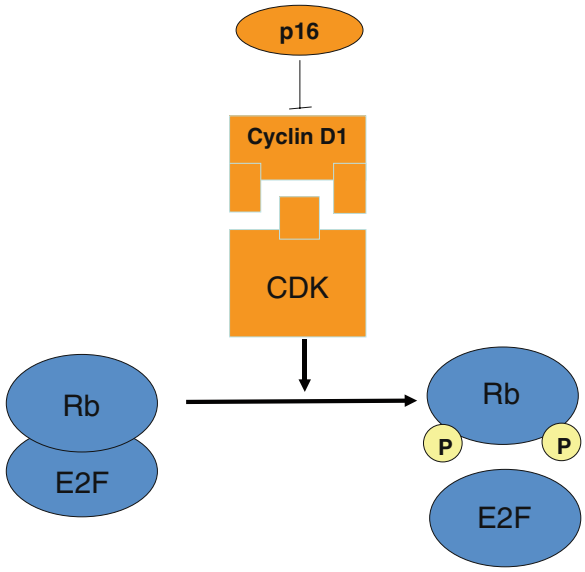

Fig. 20.3 The Rb pathway. Active dephosphorylated Rb binds transcription factor E2F. Rb releases E2F after phosphorylation by CDKs. Unbound released E2F can then transcribe genes required for DNA synthesis and activate genes necessary for G1 to S cell cycle phase transition. Rb, retinoblastoma; E2F, transcriptional factor; CDK, cyclin dependent kinases

20.5 Combined Effects of p53, p21 and Rb

Various studies have proposed that a combination of complementary markers such as p53, Rb and cyclin dependent kinase inhibitors such as p21 and p16 may give a more accurate prediction of outcome than a single marker [6, 12–15, 19–22]. In the absence of molecular alterations of all markers, patients show extremely low recurrence rates and increased survival. A significant number of patients with alterations in one of the markers show increased recurrence and decreased survival, [12–15, 19–22]. However, alterations in two or three markers cause a significant reduction in survival and increase in recurrence. The majority of patients with all three markers altered show recurrence and die within 5 years. Mitra et al. reported on the 5 year survival and recurrence rates of patients with no altered markers as 70 and 23% respectively, in contrast to patients with all three markers altered as 8 and 93% respectively [6, 8]. Thus, analysis of all markers p53, p21 and pRb provides additive prognostic data than of a single marker [15].

Chatterjee et al. reported on the 5-year recurrence rates in patients with UC who exhibit three altered markers, two altered markers, one altered marker and no altered markers as follows: 23, 32, 57, and 93%, respectively ($P < 0.001$). The 5-year survival rates were reported as 70, 58, 33, and 8%, respectively ($P < 0.001$). When stratified by stage, there remained a significant association of combined altered marker expression with recurrence rates and overall survival [6, 9, 15]. Alterations in two or more markers exert the greatest impact on recurrence and disease free survival than one marker which has a lessor effect. In patients whose tumors are altered in all three markers, more than 90% show tumor recurrence, and only 8% of survived at 5 years [15]. Therefore, multiple individual molecular alterations act synergistically in multiple interacting molecular pathways in bladder carcinogenesis and progression [6, 9, 15, 19, 21, 22].

20.6 Angiogenesis

Tumor angiogenesis has been shown to an independent prognostic factor in UC, showing significant association with disease-free and overall survival. VEGF (vascular endothelial growth factor), a pro-angiogenic factor, is a main molecule in the tumor angiogenesis pathway. VEGF is associated with early recurrence and progression to invasive tumor. High serum VEGF levels are associated with high stage, high grade, vascular invasion, and poor disease-free survival. VEGF stimulates the formation of urokinase-type plasminogen activator, which degrades extracellular matrix, aiding endothelial cell migration and invasion. Therapeutic agents have been designed against pro-angiogenic factors that induce endothelial cell apoptosis and inhibit tumor growth [6].

The p53 protein also plays an important role in tumor angiogenesis. The p53 has been reported to inhibit angiogenesis by upregulating thrombospondin-1 (TSP-1), a potent angiogenesis inhibitor. The p53 molecular alterations are associated with low TSP-1 expression and increased tumor angiogenesis. Decreased TSP-1 levels are associated with increased recurrence and reduced overall survival rates in UC. Thus, tumor angiogenesis is a complex interaction of stimulatory and inhibitory molecules [6].

20.7 DNA Methylation

DNA methylation in the promoter regions of tumor suppressor genes is an important mechanism of transcriptional suppression in UC. DNA methylation inhibits tumor suppressor gene expression by suppressing transcription through modifications in chromatin structure [4, 6]. It involves adding a methyl group to the cytosine ring of CpG dinucleotide. Specific proteins attached to methylated DNA trigger compounds containing histone deacetylases. Histone deacytylation results in chromatin compression and thus transcriptional inhibition. Many tumor suppressor genes contain CpG dinucleotides and demonstrate evidence of DNA methylation. Aberrant promoter hypermethylation has been shown to be an early event in UC carcinogenesis and significantly associated with advanced stage, tumor progression, tumor recurrence, and increased mortality compared to tumors without methylation. Targeting DNA hypermethylation in UC by novel demethylating agents has been suggested to be a critical therapeutic approach. Demethylating agents can be used to reverse the hypermethylation of tumor suppressor gene promoters in UC, thus making these genes functionally active [4, 6].

20.8 Conclusion

In UC, carcinogenesis and tumor progression result from a variety of genetic mutations such as oncogene activation, gene deletions and inactivation of tumor suppressor genes affecting the signal transduction and cell cycle pathways [15, 17, 44–46]. Detecting these genetic mutations may possibly help predict clinical outcome, recurrence rate, survival and therapeutic response in treating individual patients [15]. The mechanisms involved in the control of Ras-MAPK signal transduction (cyclin-dependent kinase and their inhibitors), p53 cell cycle regulation, Rb (retinoblastoma) and angiogenesis pathways are currently implicated in the carcinogenesis of aggressive flat and invasive bladder carcinomas. Mutations in tumor suppressor genes such as p53 and Rb are associated with UC tumor progression [11–14, 19–21, 47, 48]. Within the past several years, components of these pathways have been shown to be important prognostic and therapeutic response indicators and probable therapeutic targets. No single marker has been shown to be exclusively responsible for disease outcome since bladder cancer develops along multiple molecular pathways. Abnormal nuclear accumulations of p53-mdm2, loss of p21 expression, and alterations of Rb (either absent Rb or overexpressed Rb) are reported to have synergistic effects promoting UC progression than a single marker alone [15, 19, 21].

Interactions between the molecular pathways involved in UC will allow clinicians to identify key molecules that can be targeted therapeutically based on molecular alterations that are biologically and prognostically important. The ultimate goal is to elucidate targeted specific therapies to which the tumor is most and least likely to respond to, thereby individualizing treatment. Currently, investigators are studying numerous combinations of genes controlling cell cycle, apoptosis, angiogenesis, transcription, signal transduction and cell growth in order to develop new molecular targets which may enhance pathological staging, correlation with prognosis, therapeutic response, and overall clinical outcome in UC. Novel therapeutic agents, including viral vectors carrying wild-type genes, small molecule inhibitors and monoclonal antibodies are under development targeted to specific pathways and molecules. Clinical trials are being conducted on many of these agents. Therapeutic use of combined markers targeting multiple pathways may lead to synergistic tumor-suppressing effects, thus improving response to therapy.

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Chapter 21

Mechanisms of Carcinogenesis in Prostate Cancer

Jose J. Correa and Julio Pow-Sang

Contents

21.1 Introduction

Prostate cancer (Pc) is the most common non-cutaneous, malignant neoplasm in men in Western countries. The estimated new cases in 2007 are 218.890 and there will be 27.050 deaths by this disease, in the same year, in the United States [1]. It is the fourth most common male malignant neoplasm world wide, with an incidence variation between countries and ethnic populations. The lowest incidence is reported in Asia (1.9 cases per 100.000/year in Tianjin, China) and the highest in North America and Scandinavia (272 cases per 100.000/year in African Americans) [2].

Cancer represents the dysregulation of cell growth, which is normally tightly controlled. In normal, non-cancerous cells, the cell cycle integrates the numerous growth-regulating signals acting on the cell and determines when the cell should undergo division. In cancerous cells, the process of cell division is disrupted and unregulated, resulting in cell proliferation and tumor growth.

The development of cancer is a complex process that involves genetic and biochemical steps. The first event in carcinogenesis is termed "initiation", in which a genetic change occurs and the cell gains a malignant potential. The second event, "promotion," is an additional genetic change that is irreversible and gives abnormal cell growth and "progression" of the tumor. The causes of these events appear to be multifactoral: genetic predisposition, environmental factors. Genes and environment interact to cause the cancer.

21.1.1 Prostate Cancer Subtypes

We can divide Pc in subtypes [3].

21.1.1.1 Hereditary (Genetic Material Damage): Familial, Racial

In the hereditary type usually the age of onset is younger than 55 years of age and there are one or more

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first-degree relatives with the diagnosis. Forty percent of patients younger than 55 years have the hereditary type compared with nine percent of patients at 85 years old [4].

21.1.1.2 Sporadic (Genetic Material Damage): Diet, Age, Occupation

Sporadic types constitute approximately 85% of cases with, 15% being hereditary.

Despite the recognition of various events during prostate cancer, such as deregulation of receptors, oncogenes and tumor suppressor genes, the molecular events involved in neoplastic initiation and progression are poorly understood. Also there are other unsolved questions about prostate cancer:

- Why is it so common?
- Why the selectivity to this organ, but less common to organs in the vicinity (e.g., Seminal vesicles or bulbourethral gland)?
- Why the geographic variation (less common in Asian countries)?
- Why the zonal predilection in the prostate (more common in peripheral zone)?
- Why the racial differences (more common in African Americans)?

Given the heterogeneity of prostate cancers a unifying etiology for the disease may not exist, but different mechanisms interact to produce the disease.

In this chapter we are going to discuss some of the genetic and molecular theories involved in prostate carcinogenesis.

21.2 Genetics

Pc is more associated with a strong hereditary component than any other type of cancer in humans (different studies in twins confirm this issue) [5, 6]. According to the study by Steinberg et al., men with a family history of prostate cancer are at an increased risk of having the disease. This risk increases with the number of first degree relatives with Pc: with one relative, the risk increases by 2; with two, by 5; and with three, by 11 [7]. Different germline prostate cancer susceptibility genes and somatic genome alterations have been identified. Several loci are likely to have dominant susceptibility genes, suggesting that it is a genetically heterogeneous disease. The chromosomal region 1q25–25 is called the hereditary prostate cancer gene (HPC1 gene) and is one of the most investigated [8]. The HPC1 locus was the first prostate-cancer locus to be reported and has been found to predict risk of prostate cancer in families with a high frequency of the disease [9].

21.2.1 RNASEL

The ribonuclease L gene (RNASEL) encodes a widely expressed latent endoribonuclease that is involved in interferon inducible RNA degradation. It has been linked to HPC1. HPC1 encodes the RNASEL enzyme [10]. The RNASEL/HPC1 gene has proapoptoic activity and it has a role in the mediation of the antiviral and proapoptoic effect induced by interferon [11]. When activated by interferon, cells containing a functional RNASEL/HPC1 gene produce an enzyme that degrades single stranded RNA, leading to apoptosis. This pathway is thought to be one method that cells utilize to combat viral infections [12]. In research on mice with homozygous deletion in the RNASEL gene, they display diminished anti-viral activity in response to interferon alpha [13]. This may lead to infectious agent mediated damage and persistent infection, chronic inflammation and histologic change in the prostatic epithelium that we will discuss later in this chapter and that potentially ends in Pc.

21.2.2 MSR1

The macrophage-scavenger receptor 1 (MSR1) gene is located on 8p22, an area associated with frequent allelic loss in prostate cancer. Mutations in the gene have been reported in some families with hereditary prostate cancer [14]. Studies, in mice deficient in MSR, show that they are highly susceptible to infection by Listeria monocytogenes, Staphylococcus aureus, Escherichia coli and HSV1 [15]. Other studies have not found any association between MSR1 mutations and prostate cancer risk [16]. MSR1 is a similar mechanism to RNASEL, with the alteration causing inadequate ability to fight infections leading to persistent infection and inflammation.

21.3 Inflammation and Prostate Cancer

Approximately twenty percent of all human cancers, in adults, result from chronic inflammatory states or recurrent inflammation [17–19]. The potential causes of inflammation are diverse, including infectious diseases (e.g., Prostatitis) and environmental exposure.

Different infectious diseases can compromise the prostate, including bacteria (sexually and non-sexually transmitted diseases), viruses such as Human papillomavirus (HPV), cytomegalovirus (CMV), human herpes virus (HHV), and human herpes simplex virus type 2 (HSV2). Not all of them produce inflammation of the prostate, and it is still unknown if there is a role of the different pathogens that can infect the prostate in the development of Pc [20, 21].

Apparently, it is not the infection or environmental exposure, per se, that derives in Pc, but the response to these events that induces the inflammatory cells to generate oxidative damage to the DNA in the epithelial cells or to initiate a free-radical chain reaction that finally will create the environment to produce Pc. The "Injury and regeneration" hypothesis by De Marzo, Nelson et al., suggests that repeated injury to the prostate epithelium by oxidative or nitrosative damage from inflammatory cells, in response to pathogens or autoimmune disease from direct injury from circulating carcinogens and toxins derived from the diet or from urine reflux to the prostate, causes morphological change called proliferative inflammatory atrophy (PIA). The association between PIA and chronic inflammation suggests that the lesions, caused by regenerative proliferation of the epithelial cells in response to injury caused by inflammatory oxidants and the hyperproliferative state, may lead to cancer. They have demonstrated transition between areas of PIA with Prostatic intraepithelial neoplasia (PIN) [22–24]. Proliferative inflammatory atrophy (PIA) areas have epithelial cells that fail to differentiate into columnar secretory cells, and are usually located in the peripheral area of the prostate and often near PIN or cancer areas [25].

21.3.1 Proliferative Inflammatory Atrophy (PIA)

Prostate atrophy can occur in two forms: diffuse and focal. The diffuse form results from decreased levels of androgens and involves the entire prostate. The focal form is not related to decreased androgens and occurs in patches of atrophic epithelium surrounded by normal epithelium. They are also located mainly in the prostatic peripheral zone [26].

An interesting article by Putzi and De Marzo et al., suggested that there are morphologic transitions within the same acinar/duct unit between high-grade prostatic intraepithelial neoplasia (HGPIN) and PIA, which occur frequently. This finding supports a model in which the proliferative epithelium in PIA may progress to HGPIN [27].

In a recent European study by Tomas et al., analyzing different types of atrophy in normal of benign hyperplastic prostatic tissue and in Pc tissue, they found an association between PIA and Pc. PIA was significantly more frequent in prostates with carcinoma (1.63 vs 1.27 atrophic lesions per slide) ($p < 0.001$), whereas Proliferative Atrophy (PA) displayed an increased frequency in BPH (2.28 vs 0.76 atrophic lesions per slide) ($p < 0.001$) [28].

Also, molecular pathways involved in prostate cancer have been shown to be altered in PIA lesions:

21.3.2 GSTP1 (π Class Gluthatione S-Transferase Gene)

GSTP1 is a gene that encodes an enzyme that acts as a reactive oxygen species scavenger, and as a carcinogen detoxifier [29]. GSTP1 is expressed in basal cells; but under environmental stress may be expressed by benign luminal or columnar cells, a finding of the proliferative inflammatory atrophy (PIA) [23]. When there is hypermetilation of the cytosine residues in the CpG islands, the protective effect of the GSTP1 is lost and that change prevents the transcription of GSTP1. The CpG island hypermetilation of sequences in the promoter region of the GSTP1 gene is a common finding in prostate cancer cells [29, 30]. Cells with defective GSTP1 genes become vulnerable to oxidants that cause genomic damage and change in the epithelium, with transforming potential into prostatic intraepithelial neoplasia (PIN) and cancer.

Interestingly, in invitro studies, the heterocyclic amines produced by charred protein products such as meat, specifically 2-amino-1-methyl-6-phenylimidazol (4, 5-b) pyridine (PhIP), have shown that it produces changes in certain cancer cell lines.

In the prostate cancer cell-line LNCaP, modified to express GSTP1 when exposed to PhIP, expresses less GSTP1 than the unexposed cells [31]. Others have found that PhIP induce inflammation and atrophy before inducing PIN and cancer [32].

21.3.3 NKX3.1

NKX3.1 is located at 8p21, encodes a prostate-specific homebox gene that is necessary for normal prostate development, and represses PSA gene transcription [33]. In mice research, NKX3.1 homozygous or heterozygous deletion produces PIN like lesions [34]. In humans, the loss has also been detected in PIN lesions and in cancer lesions [35]. However, it is not clear that NKX3.1 is a somatic target for inactivation during prostate carcinogenesis. The reason is that somatic mutations have been detected in one of the alleles in prostate cancer cells, but not in both alleles [36]. However, loss of NKX3.1 expression in Pc has been reported when there is cancer progression (e.g., High grade Pc, androgen independent metastases) [37].

As mentioned above, some molecular pathways involved in prostate cancer are also altered in PIA lesions. The gene for phosphatase and tensin homologue (PTEN) is a tumor suppressor gene, suppressing cell proliferation and increasing apoptosis. It is present in normal prostatic cells and PIN cells, but is reduced in high grade Pc. As in NKX3.1, somatic allelic losses are common in prostate cancer cells, but the remaining allele are not frequently affected. More mutations are found in metastatic lesions.

21.3.4 Additional Genes

Other genes that have been identified as being linked to prostate cancer are:

21.3.4.1 OOG1

This gene is located in 3p26.2 and works in DNA repair, caused by oxidative damage and polymorphisms at this locus, and has been associated with increased risk of prostate cancer [38].

21.3.4.2 CHECK2

This gene, located in 22q12.1, is also linked to DNA repair. It prevents DNA replication when defective [39].

21.3.4.3 BRCA2

BRCA2, located in 13q12.3, is also linked to DNA repair. According to the inflammation model of prostate carcinogenesis, the oxidative stress caused by inflammation is the first step in causing mutations and DNA damage; and, if the defense mechanisms against the oxidative stress are defective by different causes (inherited or acquired alterations), prostate cancer may develop.

21.4 Cyclooxygenase 2(COX-2) Pathway and Pc Relation

Another inflammatory pathway that has been implicated in Pc is the production of prostaglandins; in this pathway the Cyclooxygenase (COX) enzyme converts arachidonic acid to prostaglandin endoperoxide synthase (PGG2), an intermediate prostaglandin [40]. Two isoforms of COX have been identified. COX1 is a constitutive enzyme involved in "protective mechanisms:" renal blood flow, platelet aggregation, water re-absorption, mucosa protection, and acid secretion [41].

The COX2, on the other hand, is an inducible and pro-inflammatory (mediates acute and chronic inflammation) enzyme that can be induced by cytokines, growth factors, mitogens and tumor promoters. The relation of COX2 to different types of cancer is based on studies that demonstrate that overexpression of COX2 leads to cancer in different tissues [42, 43]. In prostate tissue, specifically different studies have demonstrated how high levels of COX2 are found in prostate cancer cells [44, 45].

Other studies have even found low or no expression of COX2 in benign prostatic tissue; and inconsistently high expression, not in PIN and malignant prostatic tissues, but in prostatic inflammation and PIA areas, is up regulated [46, 47]. This can be related to the malignant

potential of the inflammation theory and PIA areas exposed by De Marzo et al. as discussed previously.

Epidemiological studies have found a diminished risk of prostate cancer, associated with the use of non-steroidal anti-inflammatory drugs (NSAIDs), which suggest that blocking the inflammation pathway controlled by COX2 may prevent the Pc development [48–51]. In one in vitro study using two prostate cancer cell lines PC3 and LNCaP, Celecoxib (a selective COX-2 inhibitor) inhibited the cell growth by blocking the cell cycle in G1 stage and reducing DNA synthesis. With the highest dose, there was also a 52% decrease in tumor volume and a 50% decrease in cell proliferation and microvessel density [52].

But, because of cardiovascular risk associated with COX-2 inhibitors [53–56], the clinical studies using this agent to prevent Pc or other type of tumors were terminated early [57].

21.5 Hormonal Related Theories of Prostate Carcinogenesis

The precise role of androgens in the etiology of human prostate cancer is unclear, but different epidemiological data have related serum androgen levels and an increased prostate cancer incidence in some populations. African Americans have higher levels of total serum testosterone than the Japanese population [58]. Also, men with 5-alpha reductase (5-AR) deficiency do not develop prostate cancer [59]; and prostate cancer is rare in men castrated before puberty or early in adulthood. According to epidemiological studies, direct or indirect influences of androgens can have a role in the transformation of normal prostatic tissue in cancer. Despite some findings, the precise role of androgens in Pc development is unclear. Some genes having been involved:

21.5.1 Androgen Receptor Gene (AR)

AR is part of the superfamily of nuclear hormone receptors, which are ligand inducible regulators of gene expression. AR is located on the long arm of the X chromosome.

The AR functions as a ligand activated transcription factor by inducing the expression of numerous mitotic gene products which are important signaling elements for the normal and abnormal prostate development. Also, AR integrates cellular signals by interacting with central signal transduction pathways [60, 61].

Different polymorphisms of the AR gene have been identified. One of the most studied ones is the exon 1 of the AR gene, where a highly conserved CAG (glutamine) repeat exists. The length of this CAG repeat is variable depending on the population studied, with the longest one in Asian Americans (22.4) and the shortest in African Americans (20.1) [62, 63]. AR, containing shorter repeats, have higher transactivating potentials, which can explain the differences in prostate cancer incidence between races: according to epidemiological studies, African Americans have a relatively high risk of prostate cancer and Asians have a relatively low risk. Some studies have found that short CAG repeats are associated with an increased risk of prostate cancer [64–66]. Other studies have failed to demonstrate this association [67–69]. The variability of results between studies can be explained by another unidentified genetic change, as well as potential confounding variables that can be present such as the diets or androgen levels between populations.

21.5.2 Cytochrome P-450c17 (CYP17) Gene

This gene is located on chromosome 10 and is involved in the synthesis of androgens. It encodes the cytochrome p450c17-alpha that mediates 17-alpha hydroxylase and 17–20-lyase activities in the testosterone biosynthesis in gonads and adrenals, being the last step in the production of testosterone [70].

A T-to-C polymorphism in the 5′ promoter region of the CYP17 gene that encodes the cytochrome P450c17-alpha has been implicated as a risk factor for prostate cancer. Different studies have found a correlation between the T-to-C polymorphism and increased prostate cancer risk [71, 72]. Others found no association [73, 74]. The meta-analysis by C. Ntais, A. Polycarpou, et al. suggests that the CYP17 polymorphism is unlikely to considerably increase the risk of sporadic prostate cancer on a wide population basis,

and specifically in European descent patients; but they also consider that it is possible that the polymorphism may be important in subjects of African descent [73].

21.5.3 5-Alpha-Reductase Type II (SRD5A2) Gene

There are two known 5-alpha reductase isoenzymes: 5-alpha reductase-1 and 2. Type 1 is present in low levels in different tissues, and type 2 is found in androgen sensitive cells of the skin and prostate.

The SRD5A2 gene codes for the SRD5A2 protein. This protein converts testosterone to the more active form, dihydrotestosterone. A polymorphism that is associated with prostate cancer is the A49T variant (alanine by threonine at codon 49). This variant increases concentrations of SRD5A2 by five times and, according to some studies, increases the risk of prostate cancer especially in African Americans and Hispanics, also giving a poor prognosis [75, 76].

In theory, decreasing the androgenic stimulation of prostate cells may lower the probability for entering in a carcinogenic process, but it has been difficult to test this hypothesis.

In the Prostate Cancer Prevention Trial, finasteride a 5-alpha reductase inhibitor was compared to a placebo in 18,000 healthy men treated for 7 years, either with a placebo or finasteride. Prostate biopsy was performed at the end of the trial, for prostate specific antigen changes (PSA changes), or for suspicious rectal examination. At the end of the study, 24.4% of men who received placebo were diagnosed with Pc as compared with 18.4% who received Finasteride: a 25% reduction in diagnosis. The risk of being diagnosed with higher grade Pc was increased in the finasteride group, 37% with a Gleason score of 7 or higher as compared to 22.2% in the placebo group. When analyzing the group of patients who had a full seven years of treatment with finasteride, there was essentially no difference in the number of high grade tumors: 89 with placebo and 92 with finasteride. Patients with the shortest exposure to finasteride (between 1 and 7 years), have the greatest risk of high grade disease than the placebo treated group [76]. The hypothesis that finasteride really reduces the numbers of low grade prostate cancer and/or unmasks the high grade ones is under discussion.

There is another trial under development that compares dutasteride, another 5-alpha-reductase inhibitor, with a placebo. This is a 4-year, phase III, placebo controlled study to determine whether 0.5 mg dutasteride daily decreases the risk of biopsy detectable prostate cancer. All men underwent biopsy before study entry, allowing review of the relationship between histological prostate inflammation and prostatitis symptoms. Dutasteride blocks both isoforms of 5-alpha-reductase [77].

21.5.4 Estrogens and Prostate Cancer

Estrogens also have been implicated in prostate carcinogenesis. The two main estrogen receptors (ER): alpha (ER-α) and Beta (ER-β) are expressed in the adult human prostate. ER-β is mostly localized to the basal epithelial compartment and, to a much lesser extent, in stromal cells and ER-α predominantly in the stromal compartment. Also, aromatase, the enzyme required for the metabolism of androgens to estrogen, is expressed in the stroma of the normal prostate [78, 79]. As men get older, the androgen to estrogen ratio decreases: testosterone decreases, while estrogens (estradiol 17-β) maintain a sustained level suggesting that estrogens may have a role in prostate carcinogenesis.

In animal models, using Noble rats and giving testosterone and estradiol results in high incidence of adenocarcinomas, the authors in these studies suggest that androgen supported estrogen enhanced stimulation of cell proliferation may be required for dysplasic lesions to develop [80]. Also, when androgens and estrogens were administered independently they could not produce malignancy [80–82].

The estrogen effect in the prostate is dual: ER-α mediates the "adverse" effects or promotes abnormal proliferation, while ER-β may be protective against abnormal proliferation of epithelial cells. In studies using knockout mice for ER-β receptor, they develop prostate hyperplasia; while ER-α knockout mice do not [83]. Also, some studies have shown that ER-β expression is reduced or lost in cancer compared to benign tissues, while ER-α persists in malignant tissues [84, 85]. According to these studies, the stimulation of the ER-α promotes aberrant proliferation, inflammation and cancer, while ER-β

stimulation prevents hypertrophy and hyperplasia [86]. With regard to ER-β stimulation, studies show that consumption of genistein and other phytoestrogens apparently act as ER-β agonists [87, 88]. Also, because of the activity of ER-α, there is a rationale for the use of ER-α-specific antagonists in the chemoprevention of Pc [89].

As mentioned above, estrogen can also cause inflammation of the prostate gland which links this hypothesis to the one on inflammation, explained previously. In the studies by Bianco et al., using hypogonadal (hpg) mice exposed to estradiol for 6 weeks, additional to the proliferative response, they identified neutrophils in the stroma that migrate through the epithelium to the lumen where accumulated cellular debris, inflammatory cells and anuclear keratinized deposits where found. The hpg mice, deficient in pituitary gonadotropins and sex steroids, avoid the confounding effect of the androgen withdrawal and, thus, the inflammatory pathology must be a response of the tissue to estrogen [90].

The imprinting theory of prostate cancer hypothesises that a predisposition to develop prostate cancer occurs through estrogen-mediated embryonic events that in later life, "trigger" aberrant growth. The exposure of male rats or mice to high levels of estrogens during the neonatal period leads to permanent alterations in growth and function of the prostate gland and a reduced responsiveness to androgens during adulthood. This process, referred to as neonatal imprinting, is associated with an increased incidence of prostatic lesions with aging, which include extensive immune cell infiltrate and epithelial cell hyperplasia and severe dysplasia similar to high grade prostatic intraepithelial neoplasia [91]. In another study by Bianco et al., androgen receptor knock out mice were treated with diethyl stilboestrol (DES) in neonatal life, and they developed prostatic epithelial dysplasia and inflammatory cell infiltrates in the ventral and dorsolateral prostate lobes, upon aging. This may also link the imprinting theory with the inflammation theory [92]. Maternal exposure to pharmacological levels of DES has been shown to induce prostatic abnormalities in human offspring [93]. With regard to imprinting, different theories try to explain the phenomenon: one through enhanced AR expression; and others through findings of estrogenized prostate that has a thick layer of fibroblasts beneath the basement membrane and a continuous layer of basal epithelial cells between the basement membrane and the luminal cells, creating a physical barrier that impedes differentiation and inhibiting paracrine signalling between stroma and epithelium [93, 95].

21.6 Conclusion

Prostate carcinogenesis is complex. Epidemiologic and research studies have being trying to find the etiology of prostate cancer that appears multifactorial. Different factors have being associated to the carcinogenesis process: ethnicity, geographic area, diet, lifestyle; genetic and heritable factors. The environmental factors contribute to induce changes in the prostatic environment that may eventually lead to prostate cancer development. Hormonal and inflammation hypothesis, with studies demonstrating how they can induce Pc, are interesting models of the disease. Even hormonal and inflammation correlate when estrogenic inflammation of the prostate is demonstrated in some studies.

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Chapter 22

HPV in Cervical Carcinoma

Elizabeth Sagatys and Ardeshir Hakam

Contents

22.1 Introduction

When Papanicolaou described the diagnostic value of vaginal smears in the evaluation of carcinoma of the uterus in 1941, he estimated that the death rate for uterine cancers (cervical cancer included) to be approximately 26,000 per year [1]. That number has been reduced dramatically due to Papanicolaou's introduction of the cervical/vaginal smear. Today, approximately 11,000 women in the United States and 500,000 women worldwide are diagnosed with cervical cancer annually. Each year cervical cancer is responsible for more than 250,000 deaths worldwide and is the second most common cancer in women worldwide [2, 3]. In some locations, particularly Central America and Southern Africa, it is the most prevalent cancer in women [4].

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Human papillomavirus (HPV) has been strongly associated with the development of skin and mucosal carcinomas, particularly cervical carcinoma. In the course of this chapter, we will explore the HPV virus, its transmission, detection and role in cervical carcinogenesis.

22.2 Human Papillomavirus (HPV)

Human papillomavirus (HPV) is a member of the nonenveloped DNA papovavirus family. HPV is mainly transmitted by direct skin or genital contact. HPV DNA is double stranded and circular with approximately eight thousand bases. Within the genome are 9 important genes – 7 "early" genes (E genes) and 2 "late" genes (L genes) [5]. The E genes are transcribed early in the replication process and are involved in controlling DNA replication and transcription [5]. In contrast, the L genes are transcribed later in the replication process and encode proteins that form the intact viral capsid structures [5].

Of the E genes, three (E2, E6 and E7) have been shown to be involved in HPV integration. Studies have shown that E6 and E7 are conserved when incorporated into the host genome [6, 7]. E6 and E7 are involved in alterations of the cell cycle, while E2 normally acts as a suppressor gene for E6 and E7. However, when HPV is incorporated into the host genome, the E2 locus is broken [5]. The E6 product then binds and suppresses the activity of p53. This allows the cell to enter the S phase (DNA replication) [5]. There is also loss of normal G1 arrest for DNA repair. On the other hand, the E7 product binds to the retinoblastoma (Rb) suppressor gene product. This binding frees transcription promoter E2F

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to bind to DNA, which moves the cell through S phase [5]. Both E6 and E7 work simultaneously to disregulate the cell cycle; thereby promoting cellular transformation and subsequent uncontrolled growth (malignancy) [8, 9].

Currently there are about one hundred genetically distinct subtypes of HPV identified [10]. These subtypes have been divided into "high risk" and "low risk" subtypes according to their propensity to lead to high-grade lesions and invasive carcinoma. High-risk HPV subtype infections typically resolve spontaneously (>90%). However, they can lead to high-grade dysplasia or malignancy, but it usually takes 20–30 years for infection with HPV to result in invasive carcinoma [11–13]. Common high risk strains include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 [10]. Of these, HPV 16 and 18 are the most prevalent [14]. Low risk HPV subtype infections are usually asymptomatic and most commonly cause benign lesions such as warts and condyloma acuminatum. Common low risk strains include HPV 6, 11, 42, 43, and 44 [11, 15].

HPV is transmitted by direct skin contact. Microtrauma allows the virus to initially reside in the basal epithelial cells [16]. As the epithelial cells differentiate and migrate toward the surface, HPV genes, particularly E6 and E7, are expressed and the cells begin to manifest the viral changes seen under microscopy. These changes include perinuclear vacuolization (koilocytosis) of the upper spinous layers. Mature virions are produced in the cells within the granular layer. As the cell move into the stratum corneum, the virus is shed [17].

22.3 Detection of HPV and Cervical Lesions

22.3.1 Papanicolaou (PAP) Smear

Since its introduction in the early 1940s, the Papanicolaou (PAP) smear has drastically reduced the death rate due to cervical cancer through early detection of dysplastic lesions. Normal superficial squamous cells have a nucleus the size of a lymphocyte with a large amount of cytoplasm. A progressive increase in the nuclear to cytoplasmic (N:C) ratio correlates with a progression from low to high-grade dysplasia. Smears that are designated as atypical squamous cells of undetermined significance (ASCUS) should be promptly followed with HPV testing in patients over 21 years of age [18]. The main goal of this testing is to identify high risk strains. There is no need to do HPV testing on high-grade lesions because they are nearly always associated with a high-risk HPV strain.

The American Society for Colposcopy and Cervical Pathology (ASCCP) recently released updated consensus guidelines for the pathologic classification of cervical lesions and for HPV testing. Cytologic samples should still be classified using the 2001 Bethesda System. For histologic specimens, a two-tiered system is recommended for precursor lesions: CIN1 and CIN2, 3. CIN 2 and 3 should be classified together except in adolescents, where every attempt at discriminating CIN2 from CIN3 should be made. CIN2 lesions in adolescents should be followed conservatively initially [19].

22.3.2 Immunohistochemistry

HPV can be detected using immunohistochemical methods. These stains are performed on formalin-fixed paraffin embedded tissues. The monoclonal or polyclonal antibodies are targeted at the L1 capsid protein for most known papilloma viruses.

Other immunohistochemical stains used in evaluating cervical lesions for HPV and dysplasia are Ki-67

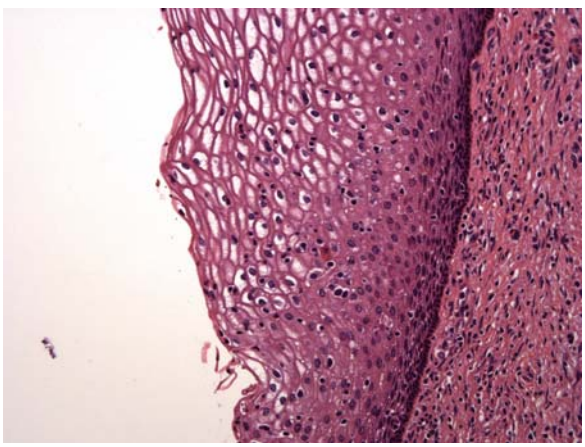

Fig. 22.1 Koilocytic atypia (HPV changes) involving cervical squamous mucosa, H&E stain

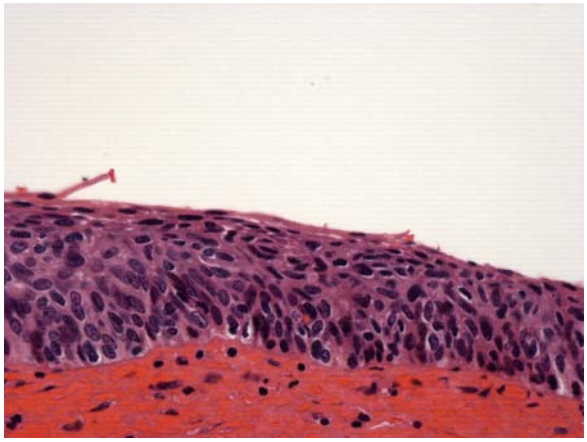

Fig. 22.2 Ki-67, the Koilocytes demonstrate nuclear staining

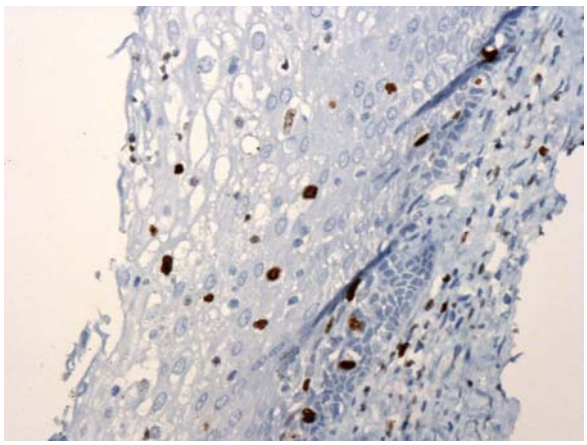

Fig. 22.3 Severe dysplasia of the cervix, H&E stain

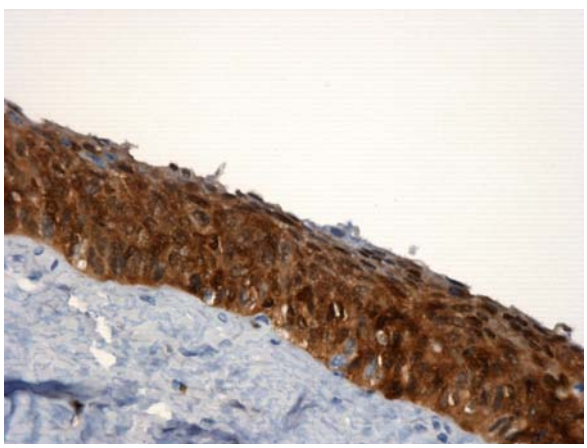

Fig. 22.4 P-16 staining of the dysplastic epithelium

(MIB1) (Figs. 22.1 and 22.2) and p16 (Figs. 22.3 and 22.4). The p16 immunohistochemical stain is directed toward p16, a cyclin-dependant kinase inhibitor that has been shown to positively correlated with HPV integration [20]. p16 is thought to be involved in the early events of HPV associated cervical carcinogenesis and could be useful in the early diagnosis of cervical cancer [21]. As such, p16 is used as a surrogate marker for HPV genome integration.

22.3.3 In Situ Hybridization (ISH) Assays

In situ hybridization (ISH) assays can be utilized to detect HPV DNA in tissue [22]. This technique localizes the HPV inside individual cells. The intensity of the signal is directly proportional to the HPV copy number present. Some authors contend that low copy numbers of HPV16 due to high level of viral integration may lead to false negatives [22]. ISH assays also preserves cell and tissue morphology, making correlation with associated histomorphology easier [23]. This process utilizes a flouorescin-labeled probe specific for the target HPV DNA and primary antibody. A secondary antibody binds with associated colorimetric indicator, allowing for identification under the microscope [24]. ISH assays allow for the detection of HPV DNA in tissue and liquid based PAP samples. It also has a high sensitivity (10–50 copies of target DNA per nucleus). The type of signal seen (confluent vs. punctate) demonstrates either episomal or integrated HPV DNA, respectively [25, 26].

Another HPV test currently used is the Hybrid Capture II test. This test utilizes an RNA probe cocktail to detect HPV. The cocktail detects HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 [27]. The DNA is released from the cells and denatured. The RNA probe cocktails are added and are allowed to hybridize with the target DNA. These hybrids are then bound to antibodies coating detection wells/tubes and detection antibodies are added. If there is successful binding, the detection antibodies release a chemiluminescent signal and the sample is positive for high risk HPV [28, 29].

Another detection method for HPV is the Linear Array HPV Genotyping Test from Roche Molecular Systems. This test uses a four step process: preparation of the specimen, PCR amplification, hybridization

with specific probes and colorimetric detection using hybrid strips to identify 37 highly prevalent strains of HPV [30]. These strains include low, intermediate and high risk strains: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108 [27].

HPV can also be detected using polymerase chain reaction (PCR). There are currently two types of probes utilized for HPV detection – general and type specific. The general probes target the more conserved regions of the L1 capsid gene [31–33]. The type specific probes target the more specific variations in E6 and E7 genes [34]. PCR is sensitive to about 10–200 copies of HPV DNA. However, currently it is predominantly used in the research setting. There are problems using PCR for general probes –as deletions in the L1 capsid gene can lead to false negatives [35].

22.4 Dysplasia and the Progression to Carcinoma

Cervical squamous intraepithelial lesions are divided into low grade and high-grade lesions. Low-grade lesions are predominantly transient and true neoplastic transformation only occurs with persistent infections in these cases. High-grade lesions result from integration of portions of the HPV genome into the host genome. The actions of E6 and E7 on p53 and Rb respectively lead to a loss of normal presynthesis repair mechanisms and uncontrolled DNA synthesis and cell proliferation with the host genes [4]. As the uncontrolled DNA synthesis continues, DNA mutations accumulate leading to dysplasia. It has been shown that the entire HPV genome is not present in high-grade lesions. Only a portion of the high risk HPV genome is incorporated into the host cell genome [5]. High-grade lesions show no evidence of koilocytic viral cytopathic effect or intact virions.

E6 and E7 oncoproteins alter the expression and function of p16INK4A, p21waf1 and p27KIP1 [36]. These are cyclin dependent kinase inhibitors (CDKI) that alter the expression of G1 cyclin dependent kinases [21]. Aberrations in these CKDI are associated with lower grade lesions (CIN1 and CIN2) [21]. When functioning normally, the CDKI prevent the progression from G1 to S phase [21]. P16INK4A has been shown to be increased, while p27KIP1 is decreased, following incorporation of HPV in the normal benign epithelium to progression to neoplastic epithelium [21]. Other studies have reported that p27KIP1 is actually increased in invasive squamous carcinoma of the cervix. This is believed to be associated with cdk2 and cyclin E binding and sequestration [21, 37].

Not only are CDKI linked with the development of cervical cancer, but cyclin D1 is as well. Bahnassy et al. found that cyclin D1 levels were upregulated in CIN3 and invasive squamous carcinoma of the cervix. Overexpression of cyclin D1 was seen in approximately 46.5% of invasive squamous carcinoma and 18.4% of CIN3 [21].

In addition, E6 and E7 oncoproteins have been shown to cause polyploidy quickly after their introduction into squamous epithelial cells. It is believed that this is due to deregulation of polo-like kinase 1 (Plk1) by the loss of p53 and the phosphorylation of Rb [38].

Recent studies have shown that the E7 gene has three conserved regions, CR1, CR2 and CR3. These regions are necessary for the virus' oncogenic activities [16]. E7 binds to Rb in both high and low risk strains. However, the binding of E7 in high risk strains is approximately 10-fold that of low risk strains [39]. After CR2 binds to the Rb gene, there is an exposure of two areas in CR3, which help potentiate the displacement of E2F from Rb [40]. The release of E2F leads to subsequent progression of the cell cycle, stimulation of proliferation of basal cells, and inhibition of cellular differentiation and death [41, 42].

Rb and p53 are not the only genes affected by HPV. Matrix metalloproteinase-2 (MMP-2) expression has been found to be upregulated in high grade CIN and squamous cell carcinoma (SCC) [43]. MMP-2 is type IV collagenase that cleaves collagen type IV and other extracellular membrane glycoproteins. Collagen type IV is a major component of the basement membrane. The level of activation of MMP-2 was also found to be increased in cervical carcinogenesis; thereby facilitating cervical invasion by in situ lesions [43].

Matrix metalloproteinase-9 (MMP-9) has also been linked to invasion by in situ cervical lesions [44]. Like MMP-2, MMP-9 acts to cleave type IV collagen and other basement membrane components including laminin-5. MMP-9 has been shown recently to be increased in patients with high grade CIN or SCC when compared to patients without cervical lesions [44].

This has clinical applications as MMP-9 and MMP-2 plasma levels can be measured and utilized as a testing method to separate those with significant lesions from those without [44].

22.5 Prevention and Therapy

Regular screening has markedly decreased the incidence of cervical carcinoma in the United States and worldwide. Recent attention has turned to prevention of cervical lesions through a vaccine. The development of the vaccine was met with many issues, both scientific and social [45–47]. Since cervical carcinoma has been linked to several of the known types of HPV a multivalent vaccine, targeting several different strains, would be necessary for better disease suppression. HPV 16, 18, 31 and 45 are the four most common strains associated with cervical carcinoma, linked to approximately 80% of cervical carcinoma in 22 countries [4]. Currently, vaccines the FDA approved cervical vaccine available in the United States includes HPV 6, 11, 16 and 18 [47]. With a reduction in cervical carcinomas associated with HPV 16 and 18 possible in this age of vaccination, continued cytologic cervical screening must still be performed. The suppression of the most common types of carcinogenic HPV may lead to an increased prevalence of other, currently less common, HPV strains. As such, future vaccines may require additions of these HPV types as the prevalence increases.

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