Histopathology, Immunohistochemistry, and Molecular Biology

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2.1 Introduction

The pathology of thyroid carcinoma is characterized by a defined histopathological classification system that has only undergone minor changes in the past few years, which is the basis of clinical diagnostic and treatment modalities as well as by a rapid progress of application of new methods and increasing knowledge of basic mechanisms in molecular pathology. However, at least in the field of C-cell carcinoma, thyroid pathology represents an impressive example of the successful application of molecular pathology in clinical practice. The aim of this review is to summarize the principles of clinical histopathology in thyroid carcinoma followed by a brief analysis of recent work in molecular pathology concentrating on original recent articles on surgical material and revealing some correlations to the questions of diagnostic clinical histopathology.

2.2 Principles of Histopathological Diagnosis and Classification

2.2.1 The Rules and Their Problems

According to the World Health Organization classification (Fig. 2.1) malignant tumors of the thyroid are subdivided into thyroid-specific, which are unique to the thyroid (for example follicular, papillary, and medullary carcinoma), and tumors commonly found also in other organs, but still have some particular characteristics when they occur in the thyroid gland (for example lymphoma, some types of sarcoma). Thyroid-specific tumors are thought to be derived from follicle cells (follicular and papillary carcinoma) and from parafollicular, calcitonin-producing C cells (medullary carcinoma). Interestingly, and in contrast to other organ systems, each of these tumors has its own rules of histopathological diagnosis, referring not only to the subclassification, but also to the histopathological establishment of malignancy. Interestingly, a change has been noted in the distribution of the subtypes of differentiated carcinoma, with a relative increase in papillary carcinoma in several countries, thought to be a consequence of altered iodine uptake [27, 59].

WHO classification of thyroid carcinoma[a]			
1	Epithelial tumors		
1.1.	Benign		
1.1.1.	Follicular adenoma		8330/0
1.1.2.	Others		
1.2.	Malignant		
1.2.1.	Follicular carcinoma	Minimally invasive (encapsulated) Widely invasive Oxyphilic cell type Clear cell variant	8330/3
1.2.2.	Papillary carcinoma	Papillary microcarcinoma Encapsulated variant Follicular variant Diffuse sclerosing variant Oxyphilic cell type	
1.2.3.	Medullary (C-cell) carcinoma	Mixed medullary-follicular carcinoma	
1.2.4.	Undifferentiated (anaplastic) carcinoma		8020/3
1.2.5.	Others		
2	Nonepithelial tumors		
3	Malignant lymphomas		
4	Miscellaneous tumors		
5	Secondary tumors		
6	Unclassified tumors		
7	Tumor-like lesions		

[a] Hedinger Chr. (1998): Histological typing of thyroid tumours. Springer, Heidelberg New York

Fig. 2.1. World Health Organization classification of thyroid carcinoma [54]

2.2.2 Papillary Carcinoma

Papillary carcinoma is the most frequent type of follicle cell-derived carcinoma. The histopathological diagnosis was originally based on microscopic detection of papillae. These are delicate stalks of epithelial cells situated on basal membranes covering stromal fibers and thin capillaries. Often the tumors contain round laminated calcifications (psammoma bodies). The papillary structures often have a follicular pattern. These tumors have been called "mixed carcinomas". Since these tumors show a clinicopathological behavior identical to that of pure papillary carcinoma, the histostructural component of the papilla has been replaced as the primary criterion of this tumor type, and nuclear characteristics have been defined, which are now the main tool used for diagnosis. These nuclei (ground-glass nuclei) are enlarged, round-to-oval structures, with a pale karyoplasm condensing continuously to the nuclear membrane. This is an optical phenomenon caused by cytoplasmatic pseudoinclusions. The nuclei are densely arranged and often overlap each other (shingle roof pattern). The occurrence of ground-glass nuclei is the main criterion for diagnosing papillary carcinoma. For technical reasons this phenomenon can-

not be detected in frozen material, i.e., frozen sections or paraffin sections after frozen section procedures. The nuclear criterion of ground-glass nuclei overrules the histoarchitectural structure (follicular/papillary) in the differential diagnosis of follicle cell-derived tumors and so-called mixed tumors. Additionally, the papillary carcinomas have specific features, which may further substantiate the diagnosis. They are often accompanied by a lymphocytic-type thyroiditis, a phenomenon that may give rise to analyses both to pathogenetic mechanisms and to prognostic implications (see the respective chapters). The surrounding stroma may show a dense fibrosis (with or without coexisting lymphocytic thyroiditis). This phenomenon can be seen regularly in the group of small (>10 mm, mostly 1- to 3-mm) papillary carcinomas (occult sclerosing papillary carcinoma [71] or papillary microcarcinoma [53]), which are frequent incidental findings in surgical specimens removed for reasons unrelated to malignancy (e.g., multinodular goiter) but also in large, clinically overt carcinomas with a diffuse type of sclerosis. This fibrotic reaction of the stroma also gives rise to investigations into both pathogenetic mechanism and prognostic factors. Papillary microcarcinoma may occur in a familial form and these tumors show more aggressive clinical behavior than sporadic cases [83].

Papillary carcinoma may be encapsulated, i.e., surrounded by a collagenous capsule, often with large venous vessels inside and outside the capsule. The carcinoma may infiltrate the capsule or may diffusely infiltrate the surrounding parenchyma without any capsule formation. Additionally, it may infiltrate the surrounding veins, but this is not a necessary basis for the diagnosis of malignancy in this tumor type. This is in sharp contrast to follicular carcinoma, where vascular infiltration is one of the main criteria of malignancy.

Comparable with follicular carcinoma, papillary carcinoma of the thyroid can show variations of the cytoplasm of the tumor cells. These are the oncocytic cell type (Hürthle or eosinophilic cell) based upon an enormous increase in the number of mitochondria (or in rare cases rough endoplasmatic reticulum) in the cytoplasm, or rare, clear-cell types with an increase in lipid (or other) vacuoles. The oncocytic-cell type of papillary carcinoma causes diagnostic problems because it obscures the pattern of ground-glass nuclei. Nuclei of oncocytes are hyperchromatic, often with condensed chromatin structures. Therefore, diagnosis cannot depend only on nuclear criteria in oncocytic-cell variation, but must depend on papillary structure and/or infiltrating growth. However, papillary structures are difficult to detect in highly cellular oncocytic tumors because of very similar technical artifacts in microfollicular adenomas. Several specific subtypes of papillary carcinoma have been investigated and described in recent years. They are be discussed in a separate chapter.

2.2.3 Follicular Carcinoma

Second in frequency of occurrence is follicular carcinoma. Nuclear characteristics do not play a role in the diagnosis of follicular carcinoma apart from the

exclusion of ground-glass nuclei. The diagnosis of follicular carcinoma is based on the histopathological demonstration of infiltrative growth. There are two criteria: (1) true infiltration of the venous vessels outside the tumor capsule, and (2) fungus-like infiltration through the tumor capsule into the surrounding parenchyma. There is intensive debate among pathologists as to how indisputable vessel infiltrations can be demonstrated. The staining of vascular components (elastic fibers, endothelial cells) may be helpful in difficult cases. It is not easy to discern follicular proliferations adjacent to enlarged (originally perifollicular) capillaries and seemingly infiltrating the capillary lumen. As a rule, the infiltrated vessel must be a vein and must be situated outside the tumor capsule. Also the interstitial infiltrative growth into the surrounding parenchyma may be difficult to evaluate. The vessels have to be separated from artificial clefts at the tumor capsule made during surgical or pathological preparation. Therefore, intracapsular (tumor capsule) enucleated tumor specimens cannot be analyzed histopathologically for infiltrative growth characteristics. There is ongoing debate in the literature as to whether infiltrative growth alone without vascular infiltration is sufficient for the diagnosis of malignancy. The criteria for histopathological vascular infiltration analysis were described precisely by Schmid et al. [122].

Cytoplasmatic variations also raise specific diagnostic problems in follicular carcinoma. The most frequent – as in papillary carcinoma – is the oncocytic variant (Hürthle-cell type, eosinophilic-cell type). Eosinophilic cells usually show low cytoplasmatic coherence and thus are artificially disseminated into the surrounding parenchyma. This phenomenon may create problems, particularly in intraoperative frozen sections. Additionally, there are two main questions discussed in the literature concerning the oncocytic-type tumors: firstly, are large oncocytic (follicular structured) tumors malignant even without vascular/parenchymal infiltration? Secondly, is the prognosis of eosinophilic carcinoma equal to, worse than or better when compared with their follicular counterparts with regular cytoplasm? These questions will be discussed in the chapter concerning prognosis. A second cytoplasmic subtype is the clear-cell variant [59, 123].

The rules of histopathological diagnosis in the field of follicular carcinoma described above clearly point toward a problem in clinical pathology of the thyroid: the evaluation of capsular infiltration (better extracapsular extension) and venous infiltration of the tumor presupposes the investigation of the whole tumor capsule, when infiltrating growth is not detectable by gross examination. Intraoperative frozen sections therefore cannot rely on classic cytological features such as nuclear atypia to rule out an infiltrative growth pattern. This may be difficult in cases of encapsulated follicular tumors, because the whole capsule is not available in the intraoperative situation in large tumor specimens. Therefore, the vascular infiltration may be missed during frozen sectioning. This problem has raised the problem of whether frozen sections on the whole should be performed for follicular tumors. This will be discussed later.

For follicular carcinoma – as for papillary carcinoma – several subtypes have been described which differ from the main type regarding prognosis. These

subtypes are situated mainly at the border with anaplastic carcinoma as poorly differentiated carcinoma and will be described in detail.

2.2.4 Anaplastic (Undifferentiated) Carcinoma

Anaplastic carcinoma is mostly detected by the pathologist by fine-needle aspiration biopsy (FNAB) or tumor reduction specimen. Complete resection specimens are rare. The diagnosis of malignancy is evident by cytological polymorphism and histological dedifferentiation. Most tumors show large areas of necrosis; in cases of hemorrhagic necrosis, hemangioendothelioma has to be excluded. Some cases of anaplastic carcinoma show remnants of differentiated (mostly follicular) carcinomas, indicating a dedifferentiation pathway from differentiated to anaplastic carcinoma. Histopathologically the tumors are solid sheets of highly anaplastic cells or spindle cells with morphologically sarcoma-like areas and frequent appearance of giant cells. There is now general agreement that these tumors represent carcinomas and true sarcomas are rare in the thyroid.

Small-cell anaplastic carcinomas were diagnosed frequently many years ago but now there is general agreement that most cases of "small-cell carcinoma" are in fact non-Hodgkin lymphomas. Curative treatment of anaplastic carcinoma is extremely rare [80], but there are reports including patients with 5-year survival after R0 resection [104]. Even in this highly aggressive tumor, statistically independent prognostic factors have been elucidated [147].

2.2.5 Medullary (C-Cell) Carcinoma

The histopathological hallmarks of medullary thyroid carcinoma are more variable than originally supposed. Characteristically the tumor is composed of solid nests and infiltrating formations of polygonal or spindle-shaped cells. Amyloid deposits within the stroma are found in about the half of the tumors. Several subtypes have been described, demonstrating a large variety of this tumor type. These include papillary, giant-cell, squamous differentiation or classic carcinoid patterns. Even mucus production and melanin pigmentation have been observed [2]. According to general agreement, no preexisting adenoma exists; all tumors exceeding 50 cells are considered malignant and separate from C-cell hyperplasia. Immunohistochemistry is strongly indicated for all cases of solid tumors without typical features of papillary or follicular carcinoma to prevent underdiagnosis of medullary carcinoma. Thyroid paraganglioma [75], hyalinizing trabecular adenoma, and metastatic neuroendocrine tumor are typical differential diagnoses.

T[a]	Primary tumor
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor 2 cm or less maximum dimension, limited to the thyroid
T2	Tumor more than 2 cm but not more than 4 cm maximum dimension, limited to the thyroid
T3	Tumor more than 4 cm maximum dimension limited to the thyroid or any tumor with minimal extrathyroid extension (e.g., extension to sternothyroid muscle or perithyroid soft tissues
T4a[b]	Tumor of any size extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, esophagus, or recurrent laryngeal nerve
T4b[b]	Tumor invades prevertebral fascia or encases carotid artery or mediastinal vessels
N[a]	Regional lymph nodes
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
N1a	Metastasis to level VI (pretacheal, paratracheal, and prelaryngeal/Delphian lymph nodes)
N1b	Meastasis to unilateral, bilateral, or contralateral cervical or superior mediastinal lymph nodes
M[a]	Distant metastasis
MX	Distant metasasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Fig. 2.2 TNM classification of thyroid carcinoma

2.3 Histopathology and Prognosis

The histopathological classification into the main types of thyroid carcinoma (papillary, follicular, medullary and anaplastic) has been shown to be the most powerful prognostic factor concerning overall survival, disease-free survival, recurrence and metastasis rate [43]. To improve the accuracy of prognosis and to allow specific treatment modalities histopathology has been combined with other prognostic factors to establish a more individual scoring system. Additionally, within the histopathological classification system, several subtypes have been described which are proposed to have prognostic implications. However, these studies are often based on a small number of cases and most studies are performed retrospectively.

2.3.1 Histopathology and Prognostic Scores

Several clinical staging and prognostic scoring systems have been proposed and all of them include the histopathological type as a major component. The tumor

Stage grouping[c]				
Papillary or follicular carcinoma				
Under 45 years	Stage I	Any T	Any N	M0
	Stage II	Any T	Any N	M1
45 years and older	Stage I	T1	N0	M0
	Stage II	T2	N0	M0
	Stage III	T3	N0	M0
		T1	N1a	M0
		T2	N1a	M0
		T3	N1a	M0
	Stage IVA	T4a	N0	M0
		T4a	N1a	M0
		T1	N1b	M0
		T2	N1b	M0
		T3	N1b	M0
		T4a	N1b	M0
	Stage IVB	T4b	Any N	M0
	Stage IVC	Any T	Any N	M1
Medullary carcinoma	Stage I	T1	N0	M0
	Stage II	T2	N0	M0
	Stage III	T3	N0	M0
		T1	N1a	M0
		T2	N1a	M0
		T3	N1a	M0
	Stage IVA	T4a	N0	M0
		T4a	N1a	M0
		T1	N1b	M0
		T2	N1b	M0
		T3	N1b	M0
		T4a	N1b	M0
	Stage IVB	T4b	Any N	M0
	Stage IVC	Any T	Any N	M1
Anaplastic carcinoma[d]	Stage IVA	T4a	Any N	M0
	Stage IVB	T4b	Any N	M0
	Stage IVC	Any T	Any N	M1

[a] All categories may be subdivided: (a) solitary tumor; (b) multifocal tumor (the largest determines the classification)
[b] All anaplastic carcinomas are considered T4 tumors: T4a intrathyroidal anaplastic carcinoma – surgically resectable; T4b extrathyroidal anaplastic carcinoma – surgically unresectable
[c] Separate stage groupings are recommended for papillary or follicular, medullary, and anaplastic (undifferentiated carcinoma)
[d] All anaplastic carcinomas are considered Stage IV

Fig. 2.2 Continued

node metastasis (TNM) system [129] (Fig. 2.2) of thyroid carcinoma follows a strategy differing from that of other tumors: there is a general classification schedule (T1–4, N, M) applying to all four histological main types (follicular, papillary, medullary, anaplastic). The specific influence of cell types and other

prognostic factors (age >45 years) are taken into consideration at the specific tumor stage grouping. Thus, independent from the specific T (or N, M) category each anaplastic carcinoma is considered as stage IV. Most other staging systems refer only to differentiated follicular/papillary (EORTC [14]; AMES [15]; Ohio State University [85]) or to papillary carcinoma (AGES [51]; MACIS [52]; University of Chicago [28]) and include size (except EORTC), age (except University of Chicago and Ohio State University), sex (EORTC and AMES), and lymph-node metastasis (University of Chicago and Ohio State University, TNM depending on age). Extrathyroid extension and distant metastasis are considered in all scoring systems, completeness of resection only by MACIS and TNM by the use of R category. The TNM classification has been shown to be useful in distinguishing patients with different prognostic outcomes, but in large retrospective series its value for therapy decisions is diminished by the relatively small proportion of patients in stages other than stage I [82]. TNM principally recommends the application of grading, but there is agreement that a general differentiation grading such as that for squamous carcinoma in the head and neck region is not applicable to thyroid carcinoma. Some subtypes of follicle-cell tumors (Chap. 3) as defined by their infiltration or differentiation type may be candidates for grading steps between well-differentiated and anaplastic carcinoma, but presently they do not fit into the TNM definition. Brierley et al. [12] compared the discriminating ability of ten different staging systems on 382 patients and recommended the use of the TNM classification.

Major changes in the new TNM classification system include: (1) new metrical dimensions in the T category, with an increase from 1 to 2 cm in the smallest tumor category and new definitions for local tumor growth beyond the thyroid capsule; (2) new definitions for lymph node metastasis; (3) anaplastic carcinomas are subdivided into resectable and unresectable ones; (4) regrouping of papillary and follicular carcinomas of patients aged 45 years or older. The new classification has been critized and therefore it seems important to state the exact tumor diameter in the pathological report, as well as giving a correlation with the old classification to avoid misunderstanding.

Additional prognostic factors have been detected also in medullary carcinoma patients. Age and stage (especially extrathyroidal extension) have been shown to be important variables in all recent series [40, 44]. Several histological findings have been shown to be prognostically relevant, such as lack of amyloid and heterogeneous calcitonin staining [124] and necrosis, focal squamous pattern and presence of oxyphilic cells [38]. In the specific group of sporadic medullary microcarcinoma (<1 cm), the preoperative calcitonin level and clinical symptoms are predictors of an unfavorable outcome [47].

2.3.2 Histological Subtypes Influencing Prognosis

In both the papillary and the follicular carcinoma groups, several subtypes or variants have been described. The most frequent and first-described variant is the Hürthle-cell carcinoma. The diagnostic problems of the oncocytic variant

of papillary carcinoma are described above. Berho and Suster [11] described 15 cases of unquestionable oncocytic papillary carcinomas and concluded that these tumors do not appear to behave more aggressively than usual papillary carcinomas. In follicular oncocytic carcinomas, Khafif et al [68] demonstrated that the prognosis of Hürthle-cell carcinoma did not differ from that of pure follicular carcinoma.

In a large series of patients, McDonald et al. [87] showed that the behavior of Hürthle-cell carcinoma follows the rules of prognostic scores such as AMES risk stratification used by the authors. Papotti et al. [102] have investigated a series of 60 cases of Hürthle-cell carcinomas and defined a subgroup with predominant solid or trabecular pattern resembling variants of poorly differentiated follicular carcinoma. This group showed a significantly worse clinical outcome than cases with a predominantly follicular structure. The results of this study also show that the prognostic factors of the general classification (specifically the histostructural differentiation) are valid within the group of oncocytic carcinomas.

Both follicular and papillary carcinoma can be subdivided concerning the degree of invasive growth into an encapsulated (minimally invasive) or widely invasive type. However, in both cases the exact differentiation between the two types is under discussion and not fully standardized. In most studies that use this criterion, the prognosis of encapsulated follicular carcinoma is excellent [76, 122]. Goldstein et al. [45] used a semiquantitative approach to quantify the number of vascular infiltrations and/or complete capsular penetrations in metastatic encapsulated follicular and Hürthle-cell thyroid carcinoma. They find no differences either between follicular and Hürthle-cell carcinoma or between metastatic and nonmetastatic carcinomas.

There are correlations between widely invasive follicular carcinomas and insular carcinoma as described by Carcangiu et al. [19]. These tumors are characterized by infiltrating, but well-defined nests of small, uniform cells with frequent areas of necrosis and resemble the "*wuchernde Struma Langhans*" [77]. Pilotti et al. [106] compared 27 cases of insular carcinoma with 29 widely invasive follicular carcinomas and found statistically more frequent extrathyroidal extension and lymph-node metastasis in the group of insular carcinomas. However, the survival data were identical between the two groups investigated. Interestingly, both tumor groups share a frequent point mutation at codon 61 of the *ras* gene. Sasaki et al. [119] identified insular components both in follicular and papillary carcinomas. Besides age, tumor size, vascular invasion, necrosis and capsule formation, the insular component was an independent prognostic marker both in follicular and papillary carcinoma. This correlates well with earlier series indicating a group of poorly differentiated carcinomas (with both elements of follicular and papillary carcinomas), indicating prognosis midway between differentiated and anaplastic carcinoma [86, 118, 142]. Nishida et al. [98] subdivided poorly differentiated carcinoma into diffuse and focal types and found significant differences in the outcome of patients concerning frequency of tumor relapse and overall survival.

Several subtypes have also recently been described in the papillary group of carcinomas. Tall-cell variant was originally described by Hawk and Hazard

[50]. The tumor cells covering the papillary stalks are by definition twice as tall as they are wide. There is some overlap [125] with the columnar-cell variant described by Evans [33]. The nuclei show striking stratification and lack typical cytomorphological features such as the ground-glass appearance. Ostrowski and Merino [100] showed by immunohistochemical analyses that the tall-cell variant is phenotypically different from classical papillary carcinoma. The immunohistochemical overexpression of *p53* has been shown to be significantly more frequent in the tall-cell variant than in age- and sex-matched common papillary carcinomas [117]. Yunta et al. [155] and Evans [34] stressed the importance of a tumor capsule in columnar-cell carcinoma. This raises the question of whether the worse prognosis in tall-cell and columnar variants reflects interrelationships with other relevant prognostic findings or represents an independent prognostic factor. Also, extensive lymphocytic infiltration seems to influence the prognosis [101] as it is under discussion in the common types of papillary carcinoma. In contrast to these variants, which are suggested to worsen the patient's prognosis, the macrofollicular variant implies a good prognosis even when accompanied by a small, insular component [3].

The influence of concomitant thyroiditis on the pathogenesis or prognosis of thyroid tumors has been discussed. Whereas severe lymphocytic thyroiditis is now accepted as a disease pathogenetically related to non-Hodgkin's lymphoma of the thyroid, the pathogenetic influence on papillary carcinoma has not been proved. However, an influence has been shown of lymphocytic infiltration and fibrous reaction on the prognosis of papillary carcinoma. Coexisting lymphocytic thyroiditis has been shown to be associated with lower pT stages in 153 thyroid carcinomas [121]. By means of a multivariate approach Kashima et al. [66] showed that apart from age (45 years or more), vascular invasion, and lymph-node metastasis, the absence of chronic thyroiditis represents an independent prognostic indicator both for relapse-free and overall survival.

This has been confirmed by Loh et al. [81] in a retrospective study on a large series of patients. However, a diffuse lymphocytic infiltration combined with extensive fibrosis (diffuse sclerosing variant according to Vickery et al., [146] has been shown to have worse prognostic signs (lymph-node metastasis, pulmonary metastasis). Albareda et al. [1] also found a greater degree of lymph-node metastases in this group of patients, but the authors found no difference for overall survival. In recent years variants with exuberant fibrosis (nodular fasciitis-like) have been described and correlated with increased transforming growth factor (TGF)-beta production [140]. An interesting theory was contributed by Mitsiades et al. [90], who showed that the expression of the apoptosis-inducing FAS ligand was correlated with a more aggressive phenotype of papillary thyroid carcinoma, suggesting that these tumors induce apoptosis of infiltrating lymphocytes and escape immune surveillance.

2.4 Histo-/Cytopathology in Preoperative and Intraoperative Diagnosis (Problem of Frozen Section)

As can be clearly seen from the microscopy principles discussed above, a preoperative diagnosis of thyroid carcinoma by FNAB is affected by two thyroid-specific phenomena: Firstly, many of the tumors are clinically small, indolent tumors (occult carcinoma) or carcinomas arising in multinodular goiter. Therefore, FNAB in such cases cannot be guaranteed to show the relevant cellular material. This is mainly a problem of the clinical detection systems. However, Sugino et al. [133], in their series of 112 patients with papillary microcarcinoma (10 mm or less), were able to confirm the diagnosis in 100 patients (89.3%). The second problem is based exclusively on microscopic factors: Whereas papillary, medullary and anaplastic carcinomas show well-defined, clearly detectable cytological findings in most cases, follicular carcinomas are by definition characterized by their infiltrative growth pattern not detectable in FNAB specimens and causing problems even in intraoperative frozen sections, when only a limited number of sections of the tumor capsule are available for analysis.

There has been much controversy in the literature about the diagnostic impact of intraoperative histopathologic diagnosis by frozen sectioning [73, 113]. Intraoperative cytology may be an additional help especially in cases of encapsulated papillary carcinoma [8, 145]. The size of the respective lesion has been shown to be predictive of malignancy in Hürthle-cell neoplasms [23], but not in the general differential diagnosis between follicular adenoma and follicular carcinoma [42]. Even concerning cost-effectiveness the results are contradictory. Whereas McHenry et al. [88] found frozen-section examination to change the intraoperative management in only 3% of patients and therefore not to be cost-effective, Paphavasit et al. [103] found in 1,023 patients with follicular and Hürthle-cell neoplasms that intraoperative frozen section evaluation was highly accurate and cut costs considerably by reducing the number of two-stage operations. We agree with Rosai et al. [112] that frozen sections are helpful in widely invasive follicular carcinoma, papillary carcinoma, anaplastic carcinoma and medullary carcinoma. In cases of conspicuous follicular structured lesions, the diagnosis of a follicular lesion has to be made and the diagnosis has to be deferred to permanent sections of paraffin-embedded tissues. This has to be performed within 3 days for surgical reasons. Perhaps new fixation techniques may significantly shorten this time period until final diagnosis [109].

2.5 Auxiliary Techniques (Cytometry, Immunohistochemistry, Molecular Pathology)

Beyond classic histopathology and cytopathology additional techniques have been developed in tumor pathology and have been used for thyroid carcinoma specimens to improve both the accuracy of preoperative cytological diagnosis

and the precision of prediction of the biological behavior of the respective tumors. These techniques include morphometric and cytometric as well as immunohisto-/cytochemical and molecular pathology approaches.

2.6 Preoperative Diagnosis (Fine-Needle Aspiration Biopsy)

Descriptive cytomorphometric approaches [29, 94] and DNA cytophotometry have been investigated for several years for their ability to assist in the differential diagnosis of follicular adenoma and follicular carcinoma in FNAB specimens. More recently Horii et al. [60] have combined DNA cytometry (ploidy pattern) with Ki67 staining and found an accuracy of over 80%, but their work was performed on surgical material. Also AgNOR staining has been shown to be of some discriminatory value [115]. Immunostaining of both FNAB specimens and the corresponding surgical material with antibodies against galectin-3 (a carbohydrate-binding protein involved in cell-cell and cell-matrix interactions) has been shown by Orlandei et al. [99] to be highly discriminative with positive staining of all follicular carcinoma specimens but galectin-3 expression in only 3 of 29 follicular adenomas. An interesting approach has been used by Winzer et al. [152] with the successful application of reverse transcription polymerase chain reaction (RT-PCR) on FNAB specimens detecting mRNA in cell numbers as small as ten for some genes and opening the possibility for molecular genetic analyses for preoperative diagnosis on such specimens. Also by RT-PCR from FNAB, Zeiger et al. [156] were able to demonstrate the expression of telomerase transcriptase in 2 out of 3 follicular carcinomas but not in 3 follicular adenomas and 5 hyperplastic nodules. For all specimens they achieved predictive values of more than 90%. In contrast, Haugen et al. [49], by using a telomeric repeat amplification protocol (TRAP) on surgical material, found no telomerase activity in three follicular carcinomas, but a positive reaction in 10 of 14 papillary carcinomas.

Although papillary carcinoma has – in contrast to well-differentiated follicular carcinoma – clearly defined cytomorphologic patterns detectable in FNAB specimens, there are also some problems in making a differential diagnosis between papillary hyperplasia in nodular goiter; therefore, new techniques have been used to solve this problem. Immunocytochemistry with antibodies against CD 57 [69] and CD 44 [25] have been shown to be of diagnostic value. Takano et al. [135] have used a real-time quantitative RT-PCR technique to measure the copy number of oncofetal fibronectin mRNA in FNAB specimens and found significant differences between papillary carcinomas and adenomatous goiter. The expression of *MAGE-1* and *GAGE-1/-2* genes in FNAB has been shown by Ruschenburg et al. [116] to give additional information to delineate papillary carcinoma from papillary hyperplasia.

These molecular techniques may be the basis of a clinically useful method to solve this diagnostic problem that hampers the application of FNAB in presurgical decision making.

2.7 Prognosis

Several attempts have been made to improve the accuracy of prognosis beyond the scope of classic histopathology and its combinations with clinicopathological scoring systems. These attempts refer to cell-biological mechanisms such as cellular proliferation or differentiation and tumor-stromal interaction (or combinations thereof). Nuclear morphometry and cytometry (both image cytometry and flow cytometry) have been used for many years. Recently, Sturgis et al. [131] readressed this method and showed that DNA image cytometry on fine-needle aspirates from 26 primary and metastatic papillary thyroid carcinomas by the detection of aneuploidy predicts distant metastasis and death from the tumor. Tseleni et al. [143] showed a correlation of descriptive nuclear morphometric patterns (such as area, perimeter, axis length and roundness) with clinical prognostic factors (age, tumor size, and thyroid capsule infiltration). More precisely defined proliferation markers have been studied by several authors. The antibody against proliferating cell nuclear antigen (PCNA) has given contradictory results. Ando et al. [4] found a correlation of PCNA staining index with age and sex, but Moreira Leite et al. [92] found PCNA to be independent from the prognostic MACIS score.

The best standardized proliferation marker *Ki67* (or *Mib1*) has also been used alone or in combination with other cell biological markers. Tallini et al. [137] compared the immunohistochemical expression of *Ki67/Mib1* and cyclin-dependent kinase inhibitor *p27/KIP1* with morphologically based prognostic groups (well-differentiated papillary or follicular carcinoma, papillary or follicular carcinoma with unfavorable pathologic features: poorly differentiated or tall-cell variant, and undifferentiated carcinomas). Whereas both tested parameters showed a clear correlation with the histological groups and with some clinical prognostic factors, no significant association could be found within any of the histological groups. Resnick et al. [110] came to similar conclusions, but in contrast to Tallini et al. [137] they found different levels of p27 staining between papillary and follicular carcinoma. In the papillary microcarcinoma group, Sugitani et al. [134] demonstrated that besides bulky lymph-node metastases, *Ki67* and TGF beta3 labeling indices may be indicators of a worse outcome for the patients. Also, p53 overexpression and growth factor receptors (such as epidermal growth factor receptor [EGFR]) have been shown to be correlated with classic prognostic factors [22]. CD97 originally found on the cell surface of leukocytes has been shown to be a marker of dedifferentiation in thyroid carcinoma [5]. Two pathogenetically interesting groups of papillary carcinoma have been analyzed as to whether they represent prognostic specific entities: carcinomas with sporadic ret oncogene rearrangement and carcinomas arising in patients with familial adenomatous polyposis. Soares et al. [128] demonstrated ret rearrangement by Southern blot analysis in 24.2% of sporadic papillary carcinomas and found no correlation with several pathological and clinical parameters, but with significantly younger age and lower proliferation rate.

Interesting results have been obtained by investigations on mechanisms of cellular interactions. Walgenbach et al. [149] showed that the immunohistochemical downregulation of E-cadherin was associated with advanced

T categories and higher rates of lymph-node involvement and distant metastasis and represents a significant prognostic factor for worse survival. CD44-v6 was shown by Kurozumi et al. [74] to be correlated with lymph-node metastasis. Angiogenesis, as a promising field of research, has also been investigated in thyroid carcinoma by several groups. Ishiwata et al. [61] demonstrated that the counting of factor VIII-related antigen-stained microvessels represents an independent prognostic factor in papillary thyroid carcinomas. Accordingly, Dhar et al. [30] found that microvessel density was significantly correlated with recurrence-free survival. In contrast, Fontanini et al. [37] found an association between newly formed vessels and survival in medullary carcinoma patients but not in the groups with well-differentiated or undifferentiated carcinomas. These discrepancies may also be influenced by methodology aspects: Wong et al. [153] showed, by differentiation between systematic measurements across one dimension of the tumor (systematic field analysis) and assessment from the three most vascularized fields of the tumor (hot spot analysis) that only hot-spot analysis was correlated with prognosis in cases of follicular carcinoma. In contrast, vascularity was not correlated with outcome in cases of papillary carcinoma, regardless of the method of assessment. However, Miki et al. [89], using an immunohistochemical approach, showed a higher expression in clinically evident tumors than in occult carcinomas and higher expression in tumors with extrathyroidal extension and concluded that the *ret/PTC* oncogene may be involved in the local invasion of papillary carcinomas. The second molecular pathological pathway also under investigation for specific prognostic characteristics is the rare but well-documented occurrence of papillary carcinoma at the familial adenomatous polyposis (FAP) syndrome. These tumors frequently show cribriform structures and multicentricity and bilateral disease and occur at young age, but the long-term prognosis is good according to Perrier et al. [105].

2.8 Pathogenesis

Molecular biology and pathology have supported us with an enormous arsenal of molecular tools and mechanisms to study thyroid cancer and to evolve solutions to many of the problems in clinical pathology. These new data enable us to analyze the anatomical and molecular histogenesis of the tumors, the cytogenetic development from benign tumors to highly aggressive neoplasms and complex regulation systems of cellular growth and differentiation, including thyroidcarcinoma-specific interactions with stromal elements. Some of these data are represented where close associations with clinical pathology are obvious.

2.8.1 Anatomical Histogenesis

Tumors with both follicular and C-cell differentiation have been recognized for several years [48]. Trapping of thyroglobulin-positive preexisting follicles within the tumor areas has always been a problem. Recently, neuroendocrine differentiation in follicle-cell thyroid carcinoma has been observed by several authors, comparable with similar observations in other nonneuroendocrine organ tumors [64]. Tseleni-Balafouta et al. [143] found a statistically significant correlation between very frequent (46.6%) focal neuroendocrine differentiation of papillary carcinomas and some prognostically relevant factors such as old age, tumor size, infiltration of the tumor capsule, or lymph-node involvement.

2.8.2 Molecular Pathogenesis

2.8.2.1 Genetics

Medullary thyroid carcinoma in its inherited form (about 20% of all thyroid C-cell carcinomas) is now one of the best accepted and standardized examples of the application of molecular tumor pathology. It occurs in three distinct clinical syndromes [MEN 2a, MEN 2b and familiar medullary carcinoma (FMTC)] and is based upon germline mutations of the *RET* proto-oncogene. The entity of FMTC was criticized by Moers et al. [91], who found that the specific type of the respective germline mutation rather than the actual predominating phenotype should be the basis of classification. By screening a large family with FMTC over a long period of time, the authors found a similar phenotypic course of the disease with MEN 2a families with the same mutation of the *RET* oncogene (Cys 618), but different results from that in families with a Cys634 mutation. Prophylactic thyroidectomy is justified in gene carriers. Hinze et al. [56] investigated the thyroids of patients at risk of hereditary medullary carcinoma after prophylactic thyroidectomy. The youngest patient with carcinoma was 6 years, the youngest with lymph-node metastasis, 17 years. Kebebew et al. [67] presented three cases of children who underwent preventive total thyroidectomy who had no evidence of medullary carcinoma or C-cell hyperplasia. According to their review of the literature, 3.4% of patients have normal glands, indicating that the intervention occurred before the appearance of hyperplasia.

Interestingly, a proportion of sporadic medullary carcinomas are associated with somatic mutations of the *ret* proto-oncogene indistinguishable from the MEN 2b syndrome (codon-918, and very rarely codon 883). Eng et al. [32] detected codon 918 mutations in 80% of sporadic medullary carcinomas in at least one subpopulation of the tumor.

Both papillary and follicular carcinoma may also occur in a familial form. Papillary carcinoma is a rare manifestation of familiar adenomatous polyposis and occurs in about 1–2% of patients. These tumors have been shown to pres-

ent "unusual" histology in the majority of cases [105]. Comparable histological findings were described in non-FAP cases by Cameselle-Teijeiro and Chan [17], suggesting that this cribriform-molecular variant may represent the sporadic counterpart of FAP-associated carcinoma. Cetta et al. [21], in a large series of patients with FAP-associated thyroid carcinomas, found germline mutations of the *APC* gene frequently in exon 15 in the genomic area associated with congenital hypertrophy of the retinal pigment epithelium (CMPE). Interesting types of familial carcinoma have been described by Canzian et al. [18], with the mapping of a gene site on chromosome *19p* and with cellular oxyphilia of the tumors. Lupoli et al. [83] described a familial papillary microcarcinoma with unfavorable behavior. The occurrence of follicular carcinoma in patients with Cowden's disease has long been well known. Recently, *PTEN* gene germline mutations have been detected and *PTEN* inactivation in transgenic mice developed spontaneous thyroid tumors besides tumors at other sites [31].

2.8.2.2 Malignant Transformation

In papillary carcinoma the *ret* proto-oncogene activation has been intensively studied. The *PTC/ret* oncogene arises through an intrachromosomal inversion or translocation of the tyrosine-kinase domain of the *ret* proto-oncogene with different activating genes. Three transforming fusion proteins are known (rcVPTC 1–3). The *ret/PTC 1* rearrangement has been shown to occur in children suffering from Chernobyl-associated papillary thyroid carcinomas in 29% [107]. Nikiforov et al. [96] compared the ret oncogene rearrangements and histomorphology in post-Chernobyl papillary carcinomas in children with children without history of radiation exposure. Both the histopathology and molecular findings showed interesting differences. Whereas in the sporadic group a typical papillary pattern was prevalent, among radiation-induced tumors solid variants of papillary carcinoma were found in 37% and typical papillary carcinoma only in 18%. Among radiation-induced tumors the distribution pattern of the *ret* oncogene subtypes (pTC1-3) was 16.2 and 58%, whereas in the sporadic group 47% showed PTC1 and only 18% pTC3. The NTRKI tyrosine kinase/tropomyosin (TPM) rearrangement has been found in only 5 of 81 tumors without *ret* rearrangement from children after the Chernobyl reactor accident [9]. Waldmann and Rabes [148] demonstrated that, in contrast to thyroid neoplasia in adults, *G(s) alpha* gene mutations do not play a role in the development of childhood thyroid tumors. Nikiforov et al. [97] have investigated the breakpoints of the two genes involved in the fusion of the *ret/PTC3* oncogene in radiation-induced post-Chernobyl papillary thyroid carcinomas (*ELEI* and *RET*) and found them distributed in a relatively random fashion, except for clustering in the ALU region of ELEI. The alignment of *ELEI* and *RET* introns in the opposite orientation showed that the position of the break in one gene corresponded to the break in the other gene. Their suggestion is that a single radiation track could produce concerted breaks in both genes leading to inversion and fusion due to recipro-

cal exchange via end-joining of the gene fragments. Animal models have been used to study the pathogenetic mechanisms of ret oncogene activation leading to papillary carcinoma. Cho et al. [26] demonstrated increased follicle-cell proliferation rate, distorted follicle formation, and reduced radioiodide-concentrating activity after targeted expression of *RET/PTC 1* in the thyroid gland in transgenic mice. Interestingly, Fischer et al. [36] were able to demonstrate by the use of a *RET/PTC* retroviral construct infection of human thyroid epithelial cells, that the *RET/PTC*-infected cells showed an altered nuclear morphology with an irregular nuclear contour and a euchromatic appearance similar to papillary carcinoma in vivo. The growth pattern was also changed in vitro following infection with *RET/PTC*. In a large series from 27 regions of the Ukraine, Tronko et al. [141] in agreement with molecular pathology data, have shown a high frequency of papillary carcinomas with solid growth pattern, lymph-node metastasis, and extrathyroidal spread. In contrast, in sporadic papillary carcinoma in adult patients, *ret/PTC* activation did not correlate with clinical markers of increased morbidity (large tumor size, extrathyroidal extension, and metastases) [136].

Besides these thyroid-specific mechanisms the role of many oncogenes and growth-regulating proteins also active in other tumors has been investigated. *Ras* point mutations have been shown to occur very early in tumorigenesis (reviewed by Wynford-Thomas [154]). Even follicular adenomas have revealed one of the three known point mutations in up to 33%. In contrast, by the use of a highly sensitive single-stranded conformation polymorphism (SSCP) approach combined with DNA sequencing, Ezzat et al. [35] found one H *ras* mutation (codon 13) and two discrete alterations on codon 17, and 22 N61 mutations in two papillary carcinomas and one follicular adenoma. K *ras* mutations were not present in any of the tumors examined (n=45). Bartolone et al. [7] have investigated the frequency of activating mutations of the three ras mutations in thyroid tumors from patients from a iodine-deficient and from a relatively iodine-sufficient area and found no mutations at the three known mutation spots. Sugg et al. [132] have compared the appearance of H, N, K *ras* mutations with *ret/PTC* rearrangement and *erbB- 2/neu* mutations. They also found a relatively low frequency of *ras* mutation in papillary carcinoma. *ErbB-2/neu* gene amplification and activating mutations have not been detected, but elevated mRNA levels have. The lack of correlation among the three oncogenes was interpreted as suggesting that they are not cumulative factors in the pathogenesis of papillary carcinoma. A comparative analysis of c-erbB-2, bcl-2, p53 and p21 was performed by Soda et al. [130] by immunohistochemical staining. Be1-2 was expressed only in well-differentiated tumors, with only some poorly differentiated tumors staining positive. p21 was detected in about the half of the tumors and p53 in 10% with strong reaction in poorly differentiated tumors. Bel-2 and Bax as apoptosis-repressing and -promoting proteins were also investigated by Manetto et al. [84]. In their immunohistochemical and Western blot analysis, the authors have shown Bcl-2 expression in benign lesions and well-differentiated carcinomas, expression of both proteins in cases of tall-cell variant papillary carcinoma and poorly differentiated carcinoma, and sole Bax expression in anaplastic carcinoma.

2.8.2.3 Mechanisms of Invasion and Metastasis

The *met* oncogene encodes for a protein with tyrosine kinase activity, which serves as a receptor for hepatocyte growth factor/scatter factor, which stimulates cell motility and invasion in particular. This complex has been investigated especially in papillary carcinoma. Ruco et al. [114] found Met protein expression immunohistochemically in 77% of papillary carcinomas. By functional in vitro investigations on primary cultures of papillary carcinomas, the same group has demonstrated the involvement of the HGF/*Met* system in the invasiveness of tumor cells. Another mechanism of invasion investigated is cathepsin B activity. Shuja et al. [126] found a ninefold increase of cathepsin B in papillary carcinoma. Altered patterns of immunohistochemical staining and additional protein bands on Western blots led to the suggestion that Cathepsin B may play a role in invasion and metastasis. Inactivation of E-cadherin, a suppressor of invasion and metastasis has been shown by Graff et al. [46] to be caused not by mutations but by hypermethylation of the 5 CpG island frequently in papillary carcinoma. Beta-catenin mutations were frequently detected in anaplastic carcinomas by Garcia-Rostan et al. [41]. The role of integrins in particular in bone metastasis has been investigated. Smit et al. [127] demonstrated an effect of synthetic RGD peptides on the attachment of cell lines of primary and metastatic follicular carcinomas in vitro. The attachment could be inhibited by anti-integrin antibodies. Bellahcene et al. [10] demonstrated the expression of bone sialoprotein in the majority thyroid carcinomas with significantly higher expression in poorly differentiated carcinomas. Bone sialoprotein is found physiologically in the mineral compartment of the developing bone. Interestingly, this protein is expressed ectopically in tumors known to metastasize to the skeleton. The proto-oncogene ets-1, a transcription factor controlling a number of genes involved in remodeling of the extracellular matrix, was detected in the majority of thyroid carcinomas, but also in 40% of follicular adenomas by Nakayama et al. [95].

2.8.2.4 Cell Cycle Regulation

Many cell cycle regulators have been investigated in thyroid carcinoma. By semiquantitative immunohistochemical staining of follicular adenomas and follicular variants of papillary carcinomas Wang et al. [150] demonstrated similar staining results of cyclin DI and E, but a significant increase in staining intensity of p27 in adenomas when compared with papillary carcinoma (follicular variant). Muro-Cacho et al. [93] found an increase in cyclin D1 and down regulation of p27kip by immunohistochemical staining of papillary carcinomas. This was explained by functional abnormalities in type 11 receptors of transforming growth factor beta. In contrast, Baldassarre et al. [6] found an abnormal cytoplasmic localization of p27, which was explained by overexpression of cyclin D3. These mechanisms were analyzed by in vitro transfection of

a mutant p27 devoted to its nuclear localization signal and thereby intermitting the interaction with nuclear cyclin-dependentkinase 2. The Axl protein as a new family of receptor tyrosine kinase has been shown to play a crucial role in regulating thyroid-cell growth and differentiation. The respective ligand Gas6, a protein S-related molecule, is a mitogenic factor for thyroid follicle cells. Ito et al. [63] have demonstrated increased Axl expression by immunohistochemistry and mRNA in situ hybridization in papillary and anaplastic carcinomas.

The frequency of p53 mutations is generally low in differentiated thyroid carcinoma. Ho et al. [57] combined immunohistochemical staining of *p53* with genotypic analyses and found nuclear overexpression only in poorly differentiated (10.5%) and undifferentiated carcinomas (25%). Mutations occurred in 4.35% of well-differentiated carcinomas and in 17.2% of poorly differentiated carcinomas. The mutation rate in undifferentiated carcinoma is high [62].

2.8.2.5 Cytogenetics and Clonality

Chromosomal and cytogenetic studies are of interest both for diagnostic and basic reasons apart from analyses of the known genes. Clonality was studied by Kim et al. [70] using a PCR assay in the X-linked human androgen receptor (*HUMARA*) gene by random X chromosome inactivation in women. All papillary carcinomas and follicular adenomas investigated were monoclonal, but also 3 of 13 follicular nodules from nodular goiters were monoclonal. This technique was successfully applied by Kakudo et al. [64] to the differentiation between aberrant thyroid tissue (tongue and bilateral neck lymph nodes) from true metastases of thyroid carcinoma. On a chromosomal level, Califano et al. [16] investigated 30 papillary carcinomas for loss of heterozygosity (LOH) and found LOH in 15 cases with frequent loci at 4q, 5p, 7p and 11p suggesting putative tumor-suppressor genes at these chromosomal arms. Polysomies of chromosomes 7 and 12 were detected by Roque et al. [111] by conventional and fluorescence in situ hybridization (FISH) cytogenetic studies. With the FISH technique they found gains with increasing frequency from goiters to adenomas and follicular carcinomas (18.2%, 52.4% and 66%). By comparative genomic hybridization (CGH) analyses, Hemmer et al. [55] found mostly gains in adenomas (chromosomes 7, 5, 12, 14, X, 18, 17) but losses in follicular carcinomas (chromosome 22, 1). Loss of chromosome 22 has been shown to be common in widely invasive follicular carcinoma. In Hürthle-cell neoplasms Tallini et al. [136], by the use of CGH, found two separate groups of tumors, one with gains of chromosomes 5 and 7, the other by loss of chromosome 2. Pathological and clinical features were similar in the two groups and the chromosomal unbalance was found to be independent from the ras-mutation (only one case in this series with a balanced karyotype). Recently Wilkens et al. [151] have used FISH and CGH and found aberrations of *5p*, *8p* and *8q* to play a role in the development of anaplastic thyroid carcinoma, whereas Komoike et al. [72] also found frequent loss of 16p by CGH techniques on tumor-cell lines. Microsatellite instability was detected by Lazzereschi et al. [78] in 21.5% of thyroid tumors and tumor He le-

sions investigated, including 9.8% of cases with instability at three or more loci. Instability was significantly more frequent in follicular adenoma and carcinoma than in papillary carcinoma. In the group of familial nonmedullary thyroid cancer (FNMTC) Canzian et al. [18] mapped a chromosomal gene locus to chromosome 19p by linkage analyses.

2.8.2.6 Receptor Activation

Mutations of the TSH receptor have been shown to be a major cause of toxic adenoma of the thyroid. Tonacchera et al. [139] demonstrated activating mutations in 12 of 15 hyperfunctioning thyroid adenomas. In one adenoma, which was negative for *TSH-R* mutations, a mutation of the *Gs alpha* gene was identified. In contrast, in nonfunctioning adenomas (and including two cases with malignant transformation) no mutations of the *TSH-R* or the *Gs alpha* gene could be identified. In a larger series of carcinomas, the same group has corroborated these data and suggested that clonal somatic mutations of the *THS-R* gene do not play a role in the pathogenesis of differentiated thyroid carcinoma [20]. The insulin receptor has been demonstrated by immunohistochemistry and functional assays [39] to be significantly increased in follicular and papillary thyroid carcinoma, but also in nonfunctioning benign adenoma.

2.8.2.7 Telomerase

Much interest has been concentrated on telomerase in thyroid neoplasms. Some of the diagnostic aspects have been discussed above. Brousset et al. [13] detected telomerase activity in 20% of papillary carcinomas and 4 of 6 follicular and 2 of 3 undifferentiated carcinomas. One case out of 12 adenomas was positive. Similar results were reported by Cheng et al. [24]. They found 52% of papillary carcinomas and 91% of follicular carcinomas to be positive by the use of telomeric repeat amplification protocol and 4 out of 14 adenomas. The cancers negative for telomerase activity were mostly in the early stages.

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