Clinical Significance of Molecular Expression Profiles of Hürthle Cell Tumors of the Thyroid Gland Analyzed via Tissue Microarrays

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Hürthle cell tumors are rare thyroid neoplasms for which disease biology is poorly understood and diagnosis of carcinoma can be challenging. The aim of the study was to characterize molecular expression profiles of Hürthle cell tumors and to determine the clinical significance of identified phenotypes. Paraffin-embedded tissue cores of normal thyroid ($n = 18$), and histopathologically well-defined Hürthle cell adenomas ($n = 27$), Hürthle cell tumors of unknown malignant behavior ($n = 7$), and minimally ($n = 14$) and widely ($n = 21$) invasive Hürthle cell carcinomas were arrayed in triplicate on tissue microarrays. Expression profiles of p53, mdm-2, p21, Bcl-2, cyclin D1, and Ki-67 were detected by immunohistochemistry and correlated with clinicopathological data and patient outcome using standard statistical methodology. Median follow-up time was 8 years. High Ki-67 proliferative index was evident only in the clinically aggressive widely invasive Hürthle cell carcinomas and was associated with significantly reduced relapse-free ($P = 0.001$) and disease-specific ($P < 0.001$) survival. The molecular phenotype of Hürthle cell tumors, independent of histopathological subtype diagnosis, was characterized by p53(-), mdm-2(+), p21(±), cyclin D1(-), and Bcl-2(±). Normal thyroid tissue demonstrated a p53(-), mdm-2(-), p21(-), cyclin D1(-), and Bcl-2(+) phenotype. The Bcl-2(+) phenotype was associated with improved relapse-free survival ($P = 0.04$) and disease-specific survival ($P = 0.01$) in widely invasive carcinomas and the Ki-67(+)/Bcl-2(-) phenotype was associated with the diagnosis of widely invasive Hürthle cell carcinoma ($P < 0.001$). This study demonstrates that tissue microarray-based profiling allows identification of molecular markers that are associated with patient prognosis. High Ki-67 proliferative index was associated with adverse outcome in Hürthle cell neoplasms. Together with down-

regulation of Bcl-2, high Ki-67 proliferative index may be useful for diagnosing widely invasive Hürthle cell carcinomas. Molecular alterations in the p53 pathway play a role in Hürthle cell tumorigenesis, but other unidentified molecular changes seem to be required to induce the malignant phenotype. (*Am J Pathol* 2002, 160:175–183)

Oncocytic or Hürthle cell (HC) neoplasms are rare entities comprising ~5% of epithelial thyroid tumors. The natural history of HC tumors spans a continuum that includes benign oncocytic adenomas, tumors of unknown malignant behavior (UMB), minimally invasive nonthreatening malignancy, and aggressive carcinomas demonstrating widespread invasion. A pivotal issue in the treatment approach to these tumors is the correlation of diagnostic histopathological criteria and tumor biology. Although HC carcinomas were previously considered as follicular carcinomas and were classified as such by the World Health Organization, they are now recognized as a distinct clinicopathological entity.^{1,2} A recent study demonstrated that the clinical behavior of HC tumors may be predicted on the basis of well-defined histopathological criteria. In this study, HC tumors were defined as thyroid neoplasms composed of follicular cells exhibiting oncocytic features in >75% of the tumor.¹ The diagnosis of HC carcinoma can be challenging and diligent scrutiny of multiple histopathological sections is required to define the nature and extent of capsular and vascular invasion, the hallmarks of malignancy for this disease. This has prompted investigators to examine the biology of HC neoplasms on a molecular level.

Mutations in the p53 tumor suppressor gene are among the most frequently detected mutations in human cancer.³ A number of studies have reported an increased prevalence of p53 mutations in poorly differentiated and undifferentiated thyroid carcinomas.^{4–7} However, few studies have addressed the role of p53 gene expression in oncocytic neoplasms with divergent findings.^{8,9} Studies analyzing p53 alterations in thyroid carcinomas have

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not considered other molecular components that are part of the p53 pathway. Murine double-minute-2 (mdm-2) overexpression is a common mechanism of p53 inactivation in human cancers, as it inhibits p53-mediated transactivation and shuttles the p53 protein into degradative pathways.^{10–12} One indirect indicator of p53 activity is the nuclear protein p21 (WAF-1). Wild-type p53 along with other cellular growth factors activate p21 gene expression and the corresponding p21 protein triggers cell-cycle arrest in the G₁ phase.¹³ In addition to cell-cycle control, p53 mediates programmed cell death through the Bcl-2/BAX apoptotic pathway.¹⁴ The patterns of p53 expression and those of important related molecules, mdm-2, p21, and Bcl-2 have not been collectively studied in HC neoplasms. Cyclin D1 is a regulator of cell-cycle progression and may have a role in thyroid carcinogenesis.^{15,16} As a marker of cellular proliferation, Ki-67 stands at the end of various pathways controlling cell division¹⁷ and holds potential for prognostic stratification of patients with various cancers. A recent study found Ki-67 and cyclin D1 to be useful in distinguishing HC adenoma from carcinoma.¹⁸

To efficiently investigate the various molecules potentially relevant for HC tumor biology and to determine their potential clinical significance, large-scale analysis of multiple molecules in the same tumor tissues is required. The newly evolved and recently validated tissue microarray technique allows such molecular profiling of cancer specimens by immunohistochemistry.^{19,20} In the present study we use tissue microarrays, following recently established criteria,²⁰ and immunohistochemistry analysis to characterize the significance of alterations in the p53 pathway and other cell cycle-related molecules in a histopathologically well-characterized cohort of patients with HC neoplasms. This molecular data were correlated with clinicopathological parameters and patient outcome to determine their potential prognostic value.

Materials and Methods

Patients and Histopathology

The study consisted of patients with HC adenomas ($n = 27$), HC tumors of UMB ($n = 7$), and minimally ($n = 14$) and widely invasive ($n = 21$) HC carcinomas. Samples of normal thyroid tissue from eighteen patients served as controls. A total of 87 cases were therefore studied. Median age of the study cohort was 53 years (range, 9 to 87 years). There were 63 females (72%) and 24 males (28%). Median follow-up time for the study cohort was 8 years (range, 2 to 22 years).

To confirm the diagnosis of HC tumors, all available histological slides were reviewed (mean of 16 histological slides per patient).¹ HCC tumors were defined as such if they were composed of >75% follicular cells with oncocyctic characteristics.^{1,2} Nodular nonencapsulated aggregates of oncocytes in the setting of nonneoplastic or inflammatory thyroid pathology were not considered HC tumors. The identification of a cell as an oncocyte was based on the presence of acidophilic, granular cyto-

plasm, and nuclei lacking the nuclear features of papillary thyroid carcinomas.

HC adenomas were defined as encapsulated lesions growing in a follicular pattern. HC tumors of UMB were diagnosed based on a solid/trabecular growth pattern with no or incomplete capsular invasion and without vascular invasion. HC carcinoma was diagnosed if the tumor displayed complete capsular invasion and/or vascular invasion. Incomplete capsular penetration and any degree of vascular invasion were considered carcinoma. The presence of incomplete capsular invasion alone was insufficient for the diagnosis of HC carcinoma. Tumors with a single focus of vascular invasion and/or a single focus of complete capsular invasion were classified as minimally invasive HC carcinomas. HC tumors with more than one focus of intra- or extra-capsular vascular invasion, and/or more than one focus of complete capsular invasion were classified as widely invasive HC carcinomas. The majority (88%) of widely invasive HC carcinomas demonstrated both extracapsular angioinvasion and complete capsular penetration. All widely invasive HC carcinomas had more than two foci of invasion. Histopathological review was conducted without the knowledge of clinical characteristics or outcome.¹

No patient with UMB or minimally invasive HCC developed recurrence or died of disease. Seventy-three percent of patients with widely invasive HCC recurred and 55% died of disease. Primary tumor size in widely invasive HCC did not significantly correlate with patient outcome.¹

Tissues, Array Construction, and Immunohistochemistry

One reference pathologist (RAG) conducted a critical review of all available histological slides. In addition to normal thyroid tissue, only encapsulated lesions demonstrating >75% follicular cells with oncocyctic characteristics were included in the study group.² HC tumors were defined by previously established criteria as described above.¹

Normal thyroid tissue, and tissue from all types of HC tumors were embedded in paraffin. Five- μ m sections stained with hematoxylin and eosin were obtained to confirm the diagnosis and to identify different viable, representative areas of the specimen. From these defined areas core biopsies were taken with a precision instrument (Beecher Instruments, Silver Spring, MD) as previously described.¹⁹ Tissue cores with a diameter of 0.6 mm from each specimen were punched and arrayed in triplicate on a recipient paraffin block.²⁰ Immunohistochemistry analysis performed on triplicate cores taken from one representative tumor block was demonstrated to be concordant with full section analysis and can be reliably used to overcome the problem of tumor heterogeneity in tissue microarray-based analyses if based on recently established criteria.²⁰ Five- μ m sections of these tissue array blocks were cut and placed on charged poly-L-lysine-coated slides (Figure 1A). These sections were used for immunohistochemical analysis.²¹ Tissues

Figure 1. A: Multitissue paraffin block consisting of 0.6-mm cores of HC tumors and normal tissues (**left**). A 5-μm section of this tissue microarray stained with H&E is shown at the **center**. Immunophenotypes with strong (**top**) and moderate (**bottom**) numbers of Hürthle cells staining positive for the respective antigen (**right**). **B:** Representative immunophenotypes of the investigated antigens in HC tumors of the thyroid (quarter cores of the tissue array depicted). Original magnifications, ×400.

known to express the antigens under study were used as positive controls. Arrayed normal tissues served as baseline controls.

Sections from tissue arrays were deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin-immunoperoxidase method. Briefly, sections were submitted to antigen retrieval by microwave oven treatment for 15 minutes in 0.01 mol/L of citrate buffer at pH 6.0. This procedure was performed for all antibodies under study. For Ki-67 antibody, an additional step of incubation in preheated 0.05% trypsin and 0.05% CaCl₂ in Tris-HCl (pH 7.6) for 5 minutes at 37°C before microwave treatment was performed. Slides were subsequently incubated in 10% normal horse serum for 30 minutes. The slides were then incubated overnight at 4°C in appropriately diluted primary antibody. Mouse anti-human monoclonal antibodies to p53 (Ab-2, clone 1801, 1:500; Calbiochem, Cambridge, MA), mdm-2 (clone 2A10, 1:500; kindly provided by Dr. A. Levine, Rockefeller University, New York, NY), p21 (Ab-1, clone EA10, 1:100; Calbiochem), cyclin D1 (Ab-3, clone DCS-6, 1:100; Calbiochem), Ki-67 (Mib-1, 1:50; Immunotech, Marseille, France) and Bcl-2 (clone 124, 1:72; DAKO, Glostrup, Denmark) were used. The anti-p53 antibody detects wild-type and mutated p53. Samples were then incubated with biotinylated anti-mouse immunoglobulins at 1:500 dilution (Vector Laboratories, Inc., Burlingame, CA) followed by avidin-biotin-peroxidase complexes (1:25; Vector Laboratories, Inc.) for 30 minutes. Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain.

Tissue loss is a significant factor for tissue array-based analysis with previously reported rates of tissue damage ranging from 15 to 33%.^{20,22–24} In our analysis rates of lost cases attributable to tissue damage ranged between 3% and 18% for the different markers.

Immunoreactivity was classified as continuous data (undetectable levels or 0% to homogeneous staining or 100%) for all markers. Several investigators (RAG, AH, AS) reviewed and scored slides independently by estimating the percentage of tumor cells showing characteristic staining in a semiquantitative manner. For every marker, the entire tumor tissue of the three core sections was evaluated. A consensus was obtained between investigators by reading slides under a multiheaded microscope. Cut-off values used in this study were based on previously established cut-off values for well-characterized antibodies used in our laboratory.^{11,20,25–27} These cut-off values were modified according to specific clinicopathological correlations in HC tumors. The cut-off values for tumor cell staining were defined as follows: 1) high Ki-67 proliferative index if >5% tumor nuclei stained; 2) p53 nuclear overexpression if >5% tumor nuclei stained; 3) mdm-2 overexpression if >50% tumor nuclei stained; 4) cyclin D1 overexpression if >5% of tumor nuclei stained; 5) Bcl-2 overexpression if >50% of tumor cells demonstrated cytoplasmic staining; 6) p21 overexpression if >10% of tumor nuclei stained. Tumors were then grouped into two categories defined as follows: normal expression (neoplasms below defined cut-off value of immunoreactivity in normal, benign, and tumor

cells) and abnormal expression (normal and neoplastic tissues above defined cut-off values of immunoreactivity). Representative immunophenotypes for each investigated marker in tumor tissue are demonstrated in Figure 1A.

Statistical Analysis

Summary statistics were obtained using established methods.²⁸ Associations between categorical variables were evaluated using the Fisher's exact test.²⁹ Hypothesis testing was performed using the chi-square test with Yate's correction²⁸ when variable size or frequency was large enough to justify its use. Outcome was classified according to sites of first disease recurrence. Time to recurrence and tumor-related mortality were calculated from the date of primary surgery. Deaths resulting from disease were treated as an endpoint for disease-specific survival. Those patients who died of other causes free of disease were considered to have been alive without evidence of disease and were censored. Relapse-free survival was calculated from the time of surgery to any local, regional nodal, or distant disease recurrence. The rate of recurrence or death was estimated using the Kaplan-Meier product limit method.³⁰ Univariate survival comparisons were performed using the log-rank test.³¹ Patients with adenomas were excluded from survival analysis because none of them developed recurrence or died of disease.

In widely invasive tumors, capsular invasion alone was a rare event and vascular invasion alone did not occur: all widely invasive tumors analyzed had capsular invasion and only two of them had no vascular invasion. For that reason, a statistical evaluation of differences between tumors with capsular invasion alone *versus* tumors with vascular invasion alone was not feasible.

In all statistical analyses, a two-tailed *P* value ≤ 0.05 was considered statistically significant. All analyses were performed using JMP statistical software (SAS Institute, Inc., Cary, NC).

Results

Cell Proliferation

No patient in the normal, adenoma, UMB, or minimally invasive carcinoma group demonstrated a Ki-67-positive phenotype (high Ki-67 proliferative index), defined as >5% of tumor cells demonstrating nuclear immunoreactivity. High Ki-67 proliferative index was present only in patients with widely invasive HC carcinoma (7 of 14, 50%; Table 1). This phenotype was associated with larger tumor size, capsular and vascular invasion, and extrathyroidal disease extension. Of the seven widely invasive carcinomas with this phenotype, two had two to four foci and five had more than four foci of major capsular invasion. Of these seven Ki-67-positive carcinomas two had two to four foci and four had more than four foci of vascular invasion.

The Ki-67-positive phenotype was associated with significantly lower relapse-free survival than the Ki-67-neg-

Table 1. Clinicopathological Features and Patient Outcome According to Ki67 Expression in Hürthle Cell Neoplasms

Characteristic	Ki67 expression		P value
	Negative (n = 70)	Positive (n = 7)	
Diagnosis			<0.001
Normal	18 (100%)	0 (0%)	
Adenoma	26 (100%)	0 (0%)	
UMB	7 (100%)	0 (0%)	
Mi HCC	12 (100%)	0 (0%)	
Wi HCC	7 (50%)	7 (50%)	
Vascular invasion			<0.001
No	39 (98%)	1 (2%)	
Yes	13 (68%)	6 (32%)	
Capsular invasion			<0.001
No	37 (100%)	0 (0%)	
Yes	15 (68%)	7 (32%)	
Extrathyroidal extension			0.001
No	49 (94%)	3 (5%)	
Yes	3 (43%)	4 (57%)	
Status			<0.001
NED	66 (97%)	2 (3%)	
AWD	2 (50%)	2 (50%)	
DOD	2 (40%)	3 (60%)	

Mi, minimally invasive; Wi, widely invasive; HCC, Hürthle cell carcinoma.

Total number of patients < 87 reflects tissue loss during specimen microarray processing. Normal tissue not included in estimates of size, invasion, and extrathyroidal extension.

ative phenotype by univariate analysis; the 8-year relapse-free survival of patients with high Ki-67 proliferative index and no Ki-67 expression was 44% and 75% ($P = 0.001$), respectively (Figure 2A). High Ki-67 proliferative index was also associated with significant tumor-related mortality (8-year disease-specific survival 37% versus 85%, $P < 0.001$; Figure 2B).

Bcl-2 Expression

Overexpression of the Bcl-2 protein defined by >50% tumor cell cytoplasmic staining was evident in all normal and the majority of adenomas and UMB (78% and 71%). One fourth of HC carcinomas demonstrated down-regulation of Bcl-2 expression (Table 2). Along the continuum of normal thyroid tissue [94%, Bcl-2(+)], HC tumors with benign clinical behavior (oncocytic adenomas [78%, Bcl-2(+)], tumors of unknown malignant behavior [71%, Bcl-2(+)], minimally [43%, Bcl-2(+)] invasive carcinomas), and widely [57%, Bcl-2(+)] invasive HC carcinomas a progressive decline in Bcl-2 expression was identified. Among the widely invasive carcinomas, Bcl-2 expression >50% was associated with favorable relapse-free survival and disease-specific survival. Actuarial 8-year relapse-free survival among patients with Bcl-2-positive and -negative widely invasive carcinomas was 51% and 18% ($P = 0.04$), respectively. Corresponding 8-year disease-specific survival for patients with Bcl-2-positive and -negative tumors was 86% and 18%, respectively ($P = 0.01$).

Combined analysis of Ki-67 and Bcl-2 identified the Ki-67(+), Bcl-2(-) phenotype as being significantly as-

Figure 2. Eight-year recurrence-free and disease-specific survival for patients with HC neoplasms (UMB, minimally and widely invasive carcinomas) stratified by Ki-67 status. The Ki-67-positive phenotype was significantly associated with a higher risk to develop recurrent disease (A) or to die of disease (B) than the Ki-67-negative phenotype.

sociated with the diagnosis of widely invasive carcinoma when compared to normal tissue or all other diagnoses combined ($P = 0.002$).

Expression of Cell-Cycle Regulatory Proteins

No overexpression of p53 was identified in normal thyroid tissue or HC UMB (Table 2). p53 protein half-life is short and expression levels are low in normal cells and therefore immunohistochemistry cannot detect these wild-type p53 levels. In cancer cells, most p53 mutations lead to products that accumulate in the nuclei and can be demonstrated by immunohistochemistry. Positive immunostaining represents rarely accumulation of wild-type p53, or, more commonly, the stable protein product of a mutated p53 gene that has lost its cell-cycle regulatory function. Nuclear p53 expression was present in a small number of patients with HC adenomas (1 of 27), minimally (1 of 14), and widely (4 of 41) invasive carcinomas suggesting inactivation of the p53 protein. In contrast, the majority (57 to 71%) of HC neoplasms demonstrated nuclear staining for mdm-2 (Table 2). All HC tumors with p53-positive phenotype demonstrated mdm-2 overexpression. p21 protein expression was present in all types of HC neoplasms (range of positive tumors, 43 to 63%).

Table 2. Molecular Expression Profiles of Cell-Cycle Regulators in Hürthle Cell Neoplasms ($n = 69$) and Normal Thyroid Tissues ($n = 18$)

Marker	Normal ($n = 18$)	Adenoma ($n = 27$)	UMB ($n = 7$)	Mi ($n = 14$)	Wi ($n = 21$)
p53					
Negative	18 (100%)	25 (92.6%)	7 (100%)	12 (85.7%)	15 (71.4%)
Positive	0 (0%)	2 (7.4%)	0 (0%)	1 (7.1%)	4 (19%)
mdm-2					
Negative	16 (88.9%)	5 (18.5%)	0 (0%)	2 (14.3%)	5 (23.8%)
Positive	1 (5.6%)	19 (70.4%)	5 (71.4%)	8 (57.1%)	12 (57.1%)
p21					
Negative	17 (94.4%)	7 (25.9%)	3 (42.9%)	4 (28.6%)	8 (38.1%)
Positive	0 (0%)	17 (63%)	4 (57.1%)	6 (42.9%)	9 (42.9%)
Bcl-2					
Negative	0 (0%)	3 (11.1%)	2 (28.6%)	4 (28.6%)	5 (23.8%)
Positive	18 (100%)	21 (77.8%)	5 (71.4%)	6 (42.9%)	12 (57.1%)
Cyclin D1					
Negative	18 (100%)	25 (92.6%)	7 (100%)	11 (78.6%)	17 (81.0%)
Positive	0 (0%)	1 (3.7%)	0 (0%)	0 (0%)	1 (4.8%)

Mi, minimally invasive carcinoma; Wi, widely invasive carcinoma; UMB, Hürthle cell tumor of unknown malignant behavior.
 Number of patients in subgroups less than total number in respective group reflects tissue loss during tissue microarray processing.

The p21-positive phenotype was not identified in any of the normal thyroid cases (Table 2). All tumors demonstrating p21 overexpression were also overexpressing mdm-2. Although 48% of mdm-2-positive tumors were p21-negative, no p21-negative tumor showed mdm-2 overexpression. Cyclin D1 expression was evident in 2 of 87 cases, one each with adenoma and widely invasive carcinoma. mdm-2 overexpression was significantly more common in neoplastic tissues ($P < 0.001$) compared to normal thyroid tissues. No other cell cycle regulator showed any meaningful association with tissue type.

Multimarker Phenotypes

To further describe the differential expression of cell growth promoters and inhibitors in the same tissues we characterized these neoplasms according to their multimarker phenotypes including all investigated molecules. The phenotype of normal thyroid tissue was Ki-67(-), p53(-), mdm-2(-), p21(-), cyclin D1(-), Bcl-2(+). Multimarker phenotypes in tumor tissues were heterogeneous and could not distinguish between tumor types. Widely invasive carcinomas differed from all other tumors primarily through the Ki-67(+) phenotype. Independent from Ki-67, the most frequently observed molecular phenotypes in all HC tumors were p53(-), mdm-2(+), p21(±), cyclin D1(-), Bcl-2(±). This information is summarized in Table 2.

Discussion

HC tumors are rare epithelial thyroid neoplasms with variable biological behavior. Despite well-defined histopathological criteria¹ the biology of this disease is poorly understood. The treatment approach to these tumors, especially for the clinically aggressive variant of widely invasive HC carcinoma, may be improved through the correlation of criteria reflecting histomorphology and tumor biology. In addition, investigation of molecular

changes in tumors representing the entire disease spectrum may enhance our understanding of mechanisms involved in thyroid tumorigenesis. This study was designed to investigate the protein expression profiles of p53, mdm-2, Bcl-2, p21, cyclin D1, and Ki-67 in a large, well-defined cohort of HC tumors ($n = 69$). For these analyses we arrayed triplicate 0.6-mm core biopsies from each tumor on two tissue microarrays and determined their expression with well-characterized monoclonal antibodies following recently established criteria.²⁰ Eighteen normal thyroid tissues also included on the same tissue arrays were used to define the expression of these markers in normal thyroid tissue. These molecular profiles were correlated with clinicopathological parameters and patient outcome to determine their prognostic value.

Despite the fact that p53 mutations are among the most frequently detected mutations in human cancers³ they are heterogeneously detected in different types of thyroid carcinoma⁴⁻⁷ and are particularly rare in HC tumors.^{8,9,32,33} Our study found only seven cases displaying p53 overexpression in a cohort of 69 HC tumors (Table 2). There were more tumors that overexpressed p53 in the life-threatening widely invasive carcinoma group than in the other groups characterized by benign clinical behavior. This further elaborates earlier findings of higher p53 immunoreactivity in oncocytic carcinoma compared to adenoma from a set of carcinomas that were not stratified according to degree of tumor invasiveness (minimally versus widely invasive carcinoma).⁹ However, in our cohort, overexpression of mdm-2, a p53-binding protein that inactivates p53 function,¹⁰⁻¹² was a frequent event (57 to 71%, Table 2) across all groups of HC tumors, but was only seen in one case of normal thyroid tissue. This finding puts the relevance of different members of the p53 pathway for thyroid tumorigenesis into perspective. The frequent overexpression of mdm-2 in a cohort that rarely shows p53 mutations suggests that mdm-2 inhibits wild-type p53, thus contributing to the proliferative potential of these cells. The fact that this is common in benign and malignant disease suggests that

there are other relevant molecular factors that contribute to the malignant phenotype.

p21 (WAF-1) expression can be an indirect indicator of p53 activity because it is activated by wild-type p53 and other cellular growth factors.¹³ Expression of p21 was negative in normal thyroid tissue but positive in 43 to 63% of HC tumors of benign and malignant behavior (Table 2). No correlation with p53 expression was observed. Interestingly, all p21-positive tumors, but no p21-negative tumors overexpressed mdm-2. This suggests that expression of p21 has a role in thyroid tumorigenesis. However, the fact that p21 expression can also be induced independent of p53 via growth factor-related mechanisms that were not investigated in this analysis complicates the mechanistic interpretation of this finding. The synchronous up-regulation of mdm-2 and p21 expression may be attributable to DNA damage-induced phosphorylation of p53, which has been shown to induce mdm-2 and p21 *in vivo*.³⁴ Neither p53, mdm-2, nor p21 expression were significantly associated with clinical outcome in this study.

In addition to cell-cycle control, p53 mediates programmed cell death through the Bcl-2/BAX apoptotic pathway.^{14,35} Bcl-2 is the anti-apoptotic component in this balanced system regulating programmed cell death and its overexpression has been shown to promote tumorigenesis. Bcl-2 was strongly expressed in all normal thyroid tissues analyzed (up to 100% of cells positive for Bcl-2) contrary to observations made in other normal tissue types.²⁵ In conjunction with this observation, a cut-off value for Bcl-2 down-regulation was defined as <50% tumor cells being positive for Bcl-2. Reduction of Bcl-2 levels, as compared to normal thyroid, ranged between 11% and 29% in benign and malignant HC tumors but was more common in malignant tumors. Importantly, Bcl-2 down-regulation was associated with poorer relapse-free and disease-specific survival for patients with widely invasive carcinoma. This is consistent with reports describing the prognostic significance of the Bcl-2-negative phenotype in patients with colorectal cancer.^{36,37} Hypothetically, this paradoxical finding could be explained by homogeneously strong expression patterns of pro- and anti-apoptotic components of the apoptotic pathway in normal thyroid tissue. In tumors, the expression levels of these counterbalancing molecules may be shifted in favor of inhibiting apoptosis. This hypothesis is supported by recent *in vivo* studies in mice using gain and loss of function models of BAX and Bcl-2,³⁵ but needs to be confirmed in the context of thyroid tissue in humans. We chose not to investigate the BAX protein in this analysis because of lack of proper reagents for immunohistochemical analysis.

The Ki-67 antigen is a nuclear protein associated with cellular proliferation.¹⁷ Its immunohistochemical detection correlates with the growth fraction of tumors.³⁸ High Ki-67 proliferative index has been reported to correlate with prognosis in patients with various cancers.^{25,26,39,40} We chose to investigate Ki-67 expression as a marker at the endpoint of multiple pathways controlling cellular proliferation. Our data show that no patient in the normal, adenoma, UMB, or minimally invasive carcinoma group

expressed a high Ki-67 proliferative index defined as >5% of tumor cells having nuclear immunoreactivity. High Ki-67 proliferative index was present only in patients with widely invasive HC carcinoma (Table 1) and was associated with the morphological criteria of aggressive behavior. In addition, the Ki-67-positive phenotype was associated with reduced relapse-free survival (Figure 2A) and higher tumor-related mortality (Figure 2B) than the Ki-67-negative phenotype. The expression of Ki-67 is present in a lower percentage of tumor cells than reported for other malignancies^{26,39,40} and the positive cells are scattered throughout the tumor specimen. Our data demonstrate that high Ki-67 proliferative index reflects a malignant phenotype. Ki-67 status adds relevant information to the histopathological criteria of invasiveness and holds potential to identify patients at risk of disease recurrence and tumor-related death.

An additive effect of Ki-67 and Bcl-2 for prediction of patient prognosis was observed for adverse outcome. The Ki-67(+)/Bcl-2(-) phenotype was associated with the diagnosis of widely invasive HC carcinoma.

Cyclin D1 is a regulator of the G₁ checkpoint of the cell-cycle and may have a role in thyroid carcinogenesis.^{15,16} Erickson and colleagues¹⁸ found cyclin D1 to be useful in distinguishing HC adenoma from carcinoma. In our analysis, cyclin D1 was negative in all normal thyroid tissues and, with the exception of one adenoma and one carcinoma, also negative in tumor tissues. This suggests that cyclin D1 overexpression, although a significant marker of tumor aggressiveness in other malignancies²⁷ has a marginal role in HC tumors. Differences between the two studies may be attributable to the use of different reagents, especially the use of a polyclonal antibody in the first study compared to our monoclonal antibody.

In an effort to further define multimolecular phenotypes that identify HC neoplasms, we characterized the expression of all investigated molecules across tissue types. A distinct multimarker phenotype was identified that was representative for normal thyroid tissue: Ki-67(-), p53(-), mdm-2(-), p21(-), cyclin D1(-), Bcl-2(+). Multimarker phenotypes in tumor tissues were heterogeneous and could not distinguish between tumor types. Widely invasive carcinomas differed from all other tumors primarily through the Ki-67(+) phenotype. Independent from Ki-67, the most frequently seen molecular phenotypes in all HC tumors were p53(-), mdm-2(+), p21(±), cyclin D1(-), Bcl-2(±) (Table 2). This demonstrates the complexity of the neoplastic process that includes multiple molecular changes that can vary greatly between tumors. Ki-67 represents the endpoint of multiple growth regulatory pathways and is the only discriminating marker that correlates with tumor biology.

In summary, our data demonstrate the feasibility of tissue array-based profiling of protein expression patterns in tumor tissues, allowing identification of molecular phenotypes that are associated with patient prognosis. High Ki-67 proliferative index correlates with recurrence and tumor-related mortality among HC tumors. Down-regulation of Bcl-2 also contributes to the aggressiveness of these tumors and may be useful, together with high

Ki-67 proliferative index, for diagnosing aggressive widely invasive HC carcinomas. Molecular alterations in the p53 pathway play a role for HC tumorigenesis, but, alone, seem to be insufficient to trigger the development of the malignant phenotype. Further studies addressing different molecular pathways will likely identify factors that contribute further to HC carcinogenesis.

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References

- Stojadinovic A, Ghossein R, Hoos A, Urist MJ, Spiro RH, Shah JP, Brennan MF, Shaha AR, Singh B: Hürthle cell carcinoma: a critical histopathological appraisal. *J Clin Oncol* 2001, 19:2616–2625
- Rosai J, Caracangui M, DeLellis R: Tumors of the thyroid gland. Atlas of Tumor Pathology, 3rd series, fascicle. Edited by J Rosai, L Sobin. Washington, DC, Armed Forces Institute of Pathology, 1992
- Levine AJ, Momand J, Finlay CA: The p53 tumor suppressor gene. *Nature* 1991, 351:453–456
- Ito T, Seyama T, Mitzuno T, Tsuyama N, Hayashi T, Hayashi Y, Dohi K, Nakamura N, Akiyama M: Unique association of p53 mutation with undifferentiated but not differentiated carcinomas of the thyroid gland. *Cancer Res* 1992, 52:1369–1371
- Dobashi Y, Sakamoto A, Sugimura H, Mernyei M, Mori M, Oyama T, Machinami R: Overexpression of p53 as a possible prognostic factor in human thyroid carcinoma. *Am J Surg Pathol* 1993, 17:375–381
- Fagin JA, Matsuo K, Karmakar A, Chen DL, Tang SH, Koeffler HP: High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas. *J Clin Invest* 1993, 91:179–184
- Donghi R, Longoni A, Pilotti S, Michieli P, Della Porta G, Pierotti MA: Gene p53 mutations are restricted to poorly differentiated and undifferentiated carcinomas of the thyroid gland. *J Clin Invest* 1993, 91:1753–1760
- Papotti M, Torchio B, Grassi L, Favero A, Bussolati G: Poorly differentiated oxyphilic (Hürthle cell) carcinomas of the thyroid. *Am J Surg Pathol* 1996, 20:686–694
- Muller-Hocker J: Immunoreactivity of p53, Ki-67, and Bcl-2 in oncocytic adenomas and carcinomas of the thyroid gland. *Hum Pathol* 1999, 30:926–933
- Momand J, Zambetti GP, Olson DC, George D, Levine AJ: The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992, 69:1237–1245
- Cordon-Cardo C, Latres E, Drobnjak M, Oliva MR, Pollack D, Woodruff JM, Marechal V, Chen J, Brennan MF, Levine AJ: Molecular abnormalities of mdm-2 and p53 genes in adult soft tissue sarcoma. *Cancer Res* 1994, 54:794–799
- Cordon-Cardo C: Mutation of cell cycle regulators. Biological and clinical implications for human neoplasia. *Am J Pathol* 1997, 147:545–560
- El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993, 75:817–825
- Chao DT, Korsmeyer SJ: BCL-2 family: regulators of cell death. *Annu Rev Immunol* 1998, 16:395–419
- Zou M, Shi Y, Farid NR, Al-Sedairy ST: Inverse association between cyclin D1 overexpression and retinoblastoma gene mutation in thyroid carcinomas. *Endocrine* 1998, 8:61–64
- Lazzereschi D, Sambuco L, Carnovale Scalzo C, Ranieri A, Mincione G, Nardi F, Colletta G: Cyclin D1 and cyclin E expression in malignant thyroid cells and in human thyroid carcinomas. *Int J Cancer* 1998, 76:806–811
- Gerdas J, Li L, Schlueter C, Duchrow M, Wohlenberg C, Gerlach C, Stahmer I, Kloth S, Brandt E, Flad HD: Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. *Am J Pathol* 1991, 138:867–873
- Erickson LA, Jin L, Goellner JR, Lohse C, Pankratz VS, Zukerberg LR, Thompson GB, van Heerden JA, Grant CS, Lloyd RV: Pathologic features, proliferative activity, and cyclin D1 expression in Hürthle cell neoplasms of the thyroid. *Mod Pathol* 2000, 13:186–192
- Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP: Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998, 4:844–847
- Hoos A, Urist MJ, Stojadinovic A, Mastorides S, Dudas M, Kuo D, Brennan MF, Lewis JJ, Cordon-Cardo C: Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. *Am J Pathol* 2001, 158:1245–1251
- Cordon-Cardo C, Richon VM: Expression of the retinoblastoma protein is regulated in normal human tissues. *Am J Pathol* 1994, 144:500–510
- Mucci NR, Akdas G, Manely S, Rubin MA: Neuroendocrine expression in metastatic prostate cancer: evaluation of high throughput tissue microarrays to detect heterogeneous protein expression. *Hum Pathol* 2000, 31:406–414
- Schraml P, Kononen J, Bubendorf L, Moch H, Bissig H, Nocito A, Mihatsch MJ, Kallioniemi OP, Sauter G: Tissue microarrays for gene amplification surveys in many different tumors types. *Clin Cancer Res* 1999, 5:1966–1975
- Richter J, Wagner U, Kononen J, Fijan A, Bruderer J, Schmid U, Ackermann D, Maurer R, Alund G, Knönel H, Rist M, Wilber K, Anabitaré M, Hering F, Hardmeier T, Schonenberger A, Flury R, Jager P, Fehr JL, Schraml P, Moch H, Mihatsch MJ, Gasser T, Kallioniemi OP, Sauter G: High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. *Am J Pathol* 2000, 157:787–794
- Hoos A, Lewis JJ, Antonescu CR, Dudas ME, Leon L, Woodruff JM, Brennan MF, Cordon-Cardo C: Characterization of molecular abnormalities in human fibroblastic neoplasms—a model for genotype-phenotype association in soft tissue tumors. *Cancer Res* 2001, 61:3171–3175
- Hoos A, Stojadinovic A, Mastorides S, Urist MJ, Polsky D, Di Como CJ, Brennan MF, Cordon-Cardo C: High Ki-67 proliferative index predicts disease-specific survival in patients with high-risk soft tissue sarcomas. *Cancer* 2001, 92:869–876
- Kim SH, Lewis JJ, Brennan MF, Woodruff JM, Dudas M, Cordon-Cardo C: Overexpression of cyclin D1 is associated with poor prognosis in extremity soft tissue sarcomas. *Clin Cancer Res* 1998, 4:2377–2382
- Altman DG: Practical Statistics for Medical Research. London, Chapman & Hall, 1996, pp 404–408
- Mehta C, Patel N: A network algorithm for performing Fisher's exact test in rXc contingency tables. *J Am Stat Assoc* 1983, 78:427–434
- Kaplan E, Meier P: Nonparametric estimation from incomplete observations. *J Am Statist Assoc* 1958, 53:457–481
- Peto R, Peto J: Asymptotically efficient rank invariant procedures. *J R Stat Soc A* 1972, 135:185–207
- Jossart GH, Epstein HD, Shaver JK, Weier HU, Greulich KM, Tezelman S, Grossman RF, Siperstein AE, Duh QY, Clark OH: Immunocytochemical detection of p53 in human thyroid carcinomas is associated with mutation and immortalization of cell lines. *J Clin Endocrinol Metab* 1996, 10:3498–3504
- Grebe SK, McIver B, Hay ID, Wu PS, Maciel LM, Drabkin HA, Goellner JR, Grant CS, Jenkins RB, Eberhardt NL: Frequent loss of heterozygosity on chromosomes 3p and 17p without VHL or p53 mutations suggests involvement of unidentified tumor suppressor genes in follicular thyroid carcinoma. *J Clin Endocrinol Metab* 1997, 11:3684–3691
- Jabbur JR, Huang P, Zhang W: DNA damage-induced phosphorylation of p53 at serine 20 correlates with p21 and Mdm-2 induction in vivo. *Oncogene* 2000, 19:6203–6208
- Knudson CM, Korsmeyer SJ: BCL-2 and BAX function independently to regulate cell death. *Nat Genet* 1997, 16:358–363
- Schwandner O, Schiedeck THK, Bruch HP, Duchrow M, Windhoevel U, Broll R: p53 and Bcl-2 as significant predictors of recurrence and survival in rectal cancer. *Eur J Cancer* 2000, 36:348–356
- Buglioni S, D'Agnano I, Cosimelli M, Vasselli S, D'Angelo C, Tedesco M, Zupi G, Mottolese M: Evaluation of multiple bio-pathological fac-

- tors in colorectal adenocarcinomas: independent prognostic role of p53 and Bcl-2. *Int J Cancer* 1999, 84:545–552
38. Kroese MC, Rutgers DH, Wils IS, Van Unnik JA, Roholl PJ: The relevance of the DNA index and proliferation rate in the grading of benign and malignant soft tissue tumors. *Cancer* 1990, 65:1782–1788
39. Borre M, Bentzen SM, Nerstrom B, Overgaard J: Tumor cell proliferation and survival in patients with prostate cancer followed expectantly. *J Urol* 1998, 159:1609–1614
40. Molino A, Micciolo R, Turazza M, Bonetti F, Piubello Q, Bonetti A, Nortilli R, Pelosi G, Cetto GL: Ki-67 immunostaining in 322 primary breast cancers: associations with clinical and pathological variables and prognosis. *Int J Cancer* 1997, 74:433–437