β -Catenin Dysregulation in Thyroid Neoplasms

Down-Regulation, Aberrant Nuclear Expression, and CTNNB1 Exon 3 Mutations Are Markers for Aggressive Tumor Phenotypes and Poor Prognosis

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β -catenin has a role in cell adhesion and Wnt signaling. It is mutated or otherwise dysregulated in a variety of human cancers. In this study we assess β -catenin alteration in 145 thyroid tumors samples from 127 patients. β -catenin was localized using immunofluorescence and mutational analysis was performed by single-strand conformational polymorphism. Membrane β -catenin expression was decreased in eight of 12 (66%) adenomas and in all 115 carcinomas ($P < 0.0001$). Among carcinomas, reduced membrane β -catenin was associated with progressive loss of tumor differentiation ($P < 0.0001$). *CTNNB1* exon 3 mutations and nuclear β -catenin localization were restricted to poorly differentiated [7 of 28 (25%) and 6 of 28 cases (21.4%), respectively] or undifferentiated carcinomas [19 of 29 (65.5%) and 14 of 29 (48.3%) cases, respectively]. Poorly differentiated tumors always featured mutations involving Ser and Thr residues and were characterized by Thr to Ile amino acid substitutions ($P = 0.0283$). The association between *CTNNB1* exon 3 mutations and aberrant nuclear immunoreactivity ($P = 0.0020$) is consistent with Wnt activation because of stabilizing β -catenin mutations. Low membrane β -catenin expression as well as its nuclear localization or *CTNNB1* exon 3 mutations are significantly associated with poor prognosis, independent of conventional prognostic indicators for thyroid cancer but not of tumor differentiation. Analysis of β -catenin dysregulation may be useful to objectively subtype thyroid neoplasms and more accurately predict outcomes. (*Am J Pathol* 2001, 158:987–996)

β -catenin is a multifunctional protein with an important role in cell adhesion and signal transduction. It is a member of the armadillo (arm) family of proteins and a downstream effector of the Wnt signaling pathway.^{1–3} In nor-

mal resting cells in the absence of Wnt activation, β -catenin is localized to the adherens junctions on the cell membrane and free cytoplasmic β -catenin levels are very low because β -catenin is rapidly destroyed by ubiquitin-proteasome degradation^{1–3}. Wnt binding to cell surface receptors of the Frizzled protein family antagonizes degradation of β -catenin that is diverted to the nucleus where it stimulates expression of specific target genes.^{1–3} β -catenin degradation requires phosphorylation of critical Ser and Thr residues and a multiprotein destruction complex that includes the adenomatous polyposis coli protein (APC) and Axin. Both are necessary to facilitate the addition of phosphate groups by the glycogen synthase kinase-3 β (GSK3 β) or other kinases leading to β -catenin proteolysis.^{1–3} Defects in APC that result in its inability to functionally interact with the β -catenin destruction complex result in increased free β -catenin and Wnt activation.^{4–6} Also, mutation or deletion of the putative GSK3 β phosphorylation sites result in stable β -catenin forms that are insensitive to degradation and therefore accumulate, leading to aberrant Wnt signaling.^{4–6} These mechanisms of β -catenin dysregulation have been shown to play an important role in human tumorigenesis. In fact, inactivating germ-line APC mutations lead to familial adenomatous polyposis whereas somatic APC mutations are present in >80% of sporadic colorectal adenomas and carcinomas.² Also, activating β -catenin mutations that stabilize the protein have been discovered in a variety of human neoplasms ranging from colonic adenocarcinoma, where they are present in approximately half of the tumors with wild-type APC,⁷ to malignant fibrous histiocytoma.² Although a reduction of β -catenin bound to the cell surface has been recently demonstrated in thyroid carcinoma,^{8,9} the biological and clinical relevance of

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Table 1. Pathological and Clinical Features of the Thyroid Tumors Analyzed for β -Catenin Dysregulation

Tumor differentiation	Sex	Age (median)	Histologic diagnosis	Size (median)
Benign tumors (n = 12)	F: 11 (91.6) M: 1 (8.3)	47	Follicular adenoma	3.0 cm
Well-differentiated carcinoma (n = 58)	F: 33 (56.9) M: 25 (43.1)	43	Papillary carcinoma 46 (79.3) Follicular carcinoma 12 (20.7)	3.0 cm
Poorly differentiated carcinoma (n = 28)	F: 15 (53.6) M: 13 (46.4)	56	Poorly differentiated carcinoma NOS 16 (57.1) Insular carcinoma 12 (42.9)	6.7 cm
Undifferentiated carcinoma (n = 29)	F: 19 (65.5) M: 10 (34.4)	69	Anaplastic carcinoma	8.0 cm

(Table continues)

F; female; M; male; NOS; not otherwise specified; NED; no evidence of disease; AWD; alive with disease; DOC; dead of other causes; DOD; dead of disease; UNK; follow-up unknown; AJCC, American Joint Committee on Cancer.

The numbers in parentheses indicate percentage values

*Patients were followed for 1 to 17 years (5.5 years median follow-up time) or until death.

β -catenin dysregulation in thyroid neoplasia is primarily unknown. We have previously shown that activating β -catenin mutations are very common among undifferentiated (anaplastic) thyroid carcinoma, a rare but highly lethal form of thyroid cancer.¹⁰ In this report we analyze β -catenin expression in a series of 145 thyroid tumor samples covering the entire spectrum of tumor differentiation and we study its relationship with β -catenin gene (*CTNNB1*) mutation, clinicopathological features, and patient survival.

Materials and Methods

Tumor Cases

We analyzed 145 tumor samples: 133 from a cohort of 115 patients who underwent surgery for thyroid carcinoma and, for comparison with the malignant cases, 12 samples of thyroid follicular adenoma. Formalin-fixed paraffin-embedded specimens were obtained from the files of the Pathology Departments at Yale New Haven Hospital, Yale University, and the Covadonga Hospital, University of Oviedo, Spain. Thyroid carcinomas were chosen to cover the entire spectrum of differentiation for tumors of follicular cell derivation and based on the availability of detailed clinical and follow-up information. The pathological and clinical features of the tumors are summarized in Table 1. All histological diagnoses were reviewed according to established histological criteria¹¹ and, similar to what was previously described,¹² the carcinomas were divided into three groups based on their degree of differentiation (Table 1). Group 1 consisted of well-differentiated thyroid tumors of either papillary (27 classical papillary carcinomas, nine follicular variants, 10 microcarcinomas) or follicular type. Group 2 consisted of poorly differentiated tumors exhibiting the features of insular carcinoma¹³ or consisting of neoplasms with a trabecular or solid (comedo-type) growth pattern with nuclear hyperchromasia, high mitotic activity, and necrosis (poorly differentiated thyroid carcinoma not otherwise

specified).¹¹ Group 3 included a series of undifferentiated (anaplastic) carcinomas that had been previously investigated for β -catenin dysregulation.¹⁰ Patients were staged following the recommendations of the American Joint Committee on Cancer¹⁴ and managed according to standard clinical protocols. Follow-up information was previously available for 45 patients.¹² The follow-up status in the remaining patients was determined by clinical examination, analysis of serum TG values, and radiographic and/or ¹³¹I-uptake studies. Patients were followed for 1 to 17 years (5.5 years median follow-up time) or until death. Processing of samples and clinical information proceeded in agreement with review board approved protocols. Histological classification of thyroid carcinoma in three groups according to their degree of differentiation successfully stratified the mortality risk for thyroid carcinoma in the cohort of patients selected for the study ($P = 0.0001$), thus justifying the validity of this approach.

Immunohistochemistry

Representative paraffin-embedded blocks were selected from each tumor sample and sections cut for fluorescence immunohistochemistry. This was performed as previously described.¹⁰ Briefly, after antigen retrieval sections were incubated overnight with anti- β -catenin monoclonal antibodies (Transduction Labs., Lexington, KY) diluted to 2 to 7 μ g/ml. Sections were then washed and incubated for 1 hour with Cy3-conjugated goat anti-mouse immunoglobulin antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) at a 1:500 dilution. Fluorescent Cy3-conjugated secondary antibodies were used instead of conventional chromogens to increase sensitivity and to better define the subcellular localization of β -catenin. Immunoreactivity was expressed as the percentage of positively stained target cells in each of four intensity categories (0, no staining; 1+, weak but detectable above control; 2+, distinct; 3+, intense). For each

Table 1. Continued

Extrathyroid extension	Vascular invasion	Lymph node metastasis	Distant metastasis	Stage AJCC	Follow-up*
No	No	No	No	Benign tumors	NED 12 (100)
24 (41.4)	24 (41.4)	30 (51.7)	7 (12.0)	I 33 (56.9) II 8 (13.8) III 14 (24.2) IV 3 (5.1)	NED 42 (72.4) AWD 10 (17.3) DOC 1 (1.7) DOD 5 (8.6)
16 (57.1)	27 (96.4)	11 (39.3)	17 (60.7)	I 4 (14.3) II 6 (21.4) III 10 (35.7) IV 8 (28.6)	NED 10 (35.7) AWD 5 (17.9) DOD 12 (42.9) UNK 1 (3.5)
29 (100)	19 (65.5)	11 (37.9)	8 (27.5)	IV 29 (100)	DOD 29 (100)

tissue section, a numerical value (the *H* or histology score) was derived by summing the percentages of cells staining at each intensity multiplied by the weighted intensity of staining¹⁵ after random high-power field observations ($\times 400$) corresponding to at least 4,000 tumor cells. The intense membranous staining of normal thyroid follicles adjacent to the tumor was used as internal standard for the scoring of β -catenin expression. Negative controls were performed by omitting the primary antibody. Membranous, cytoplasmic, and nuclear immunoreactivity were evaluated by separate *H* scores but, given the relative paucity of tumor cells with nuclear or cytoplasmic immunostaining and the resulting low *H* scores, nuclear and cytoplasmic immunoreactivity patterns were simply recorded as positive or negative with no cut-off values for statistical analysis. *H* score values were independently estimated by two of the authors (GGR and GT) without knowledge of the clinicopathological parameters and a consensus was reached on all values used for computation. In 15 cases (11.3% of the total) the discrepancy among the authors was $>10\%$ of the individual values but in none of them did the definitive scores cross the cutoffs chosen for statistical analysis.

DNA Isolation and Mutational Analysis

DNA was isolated from 91 formalin-fixed tumor specimens corresponding to the paraffin blocks analyzed immunohistochemically. These were obtained from 66 patients with thyroid carcinoma and seven patients with follicular adenoma. Nine of the patients with carcinoma had well-differentiated tumors, (four follicular and five papillary carcinomas), 28 had poorly differentiated, and 29 had undifferentiated carcinomas. To perform mutational analysis in areas with different histological appearance, tumor differentiation and/or tumor recurrences multiple DNA samples were studied in eight cases of poorly differentiated carcinoma and in six cases of undifferentiated carcinoma. When necessary tumor material was manually microdissected to increase the proportion of neoplastic cells that always represented at least 80% of the total. DNA extraction was performed accordingly to

standard procedures. Tumor DNA was evaluated for mutations in the *GSK3 β* phosphorylation consensus motif of the β -catenin gene (*CTNNB1* exon 3) by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) using primers specific for the third coding exon of the β -catenin gene (forward: 5'-primer GCTGATTGATGGAGTTGGAA; reverse: 3'-primer GCTACTTGTCTTGAGTGA). PCR-SSCP was conducted as previously described.¹⁰ Briefly, PCR was performed in a 30- μ l reaction mixture containing 20 to 100 ng of genomic DNA, 20 pmol of each primer, 250 μ mol/L of each dNTP, 2 mmol/L MgCl₂, 10 $\times$ Perkin Elmer buffer II, and 2.5 U of AmpliTaq Gold (Perkin Elmer Applied Biosystems Division, Foster City, CA). The mixture was heated for 10 minutes at 94°C for initial DNA denaturation, followed by 35 cycles of denaturation (94°C for 1 minute), annealing (55°C for 2 minutes), and extension (72°C for 3 minutes), on the GeneAmp PCR system 9600 (Perkin Elmer Applied Biosystems Division). For SSCP, aliquots of the PCR products were denatured, loaded onto a 40% mutation detection enhancement gel, and run in a SE 600 vertical gel apparatus (Hoefer Scientific, San Francisco, CA) at 400 V. All samples exhibiting mobility shifts by SSCP were excised from the gel, eluted, and re-amplified with the same set of primers and PCR conditions described above. The products were separated, purified, and sequenced using an Applied Biosystems 377 DNA sequencer (Perkin Elmer Applied Biosystems Division). Nucleotide sequencing from both the sense and antisense orientation was performed for confirmation. All mutated cases were verified by repeated PCR-SSCP.

Statistical Analysis

CTNNB1 exon 3 mutations, β -catenin nuclear, or cytoplasmic immunoreactivity were coded as "yes" or "no" for categorical data analysis (chi-square and Fisher's exact tests), whereas the mean β -catenin *H* score values were compared using one-way analysis of variance with a Bonferroni correction made for multiple comparisons across groups. All of the patients investigated were studied according to: 1) histological type and degree of dif-

Table 2. Correlation of β -Catenin Expression and β -Catenin Exon 3 *CTNNB1* Mutations with Histological Diagnosis and Unfavorable Prognostic Parameters for Thyroid Carcinoma

Diagnosis and clinical pathological parameters	Number of cases	β -Catenin membrane immunoreactivity (mean <i>H</i> Score $\pm$ SD)		β -Catenin exon 3 mutation and/or nuclear immunoreactivity (%)	
Well-differentiated carcinoma	58	199 $\pm$ 90	$P < 0.0001$	0 (0%)	$P < 0.0001$
Papillary carcinoma	46	208 $\pm$ 87	NS	0 (0%)	
Follicular carcinoma	12	166 $\pm$ 99		0 (0%)	
Poorly differentiated carcinoma	28	120 $\pm$ 68	$P < 0.0001$	9 (32.1%)	$P < 0.0001$
PD carcinoma NOS	16	114 $\pm$ 70		5 (31.2%)	
PD carcinoma Insular	12	130 $\pm$ 68	NS	4 (33.3%)	NS
Undifferentiated carcinoma	29	3 $\pm$ 7	$P < 0.0001$	23 (79.3%)	$P < 0.0001$
Male sex	48	146 $\pm$ 109	NS	14 (29.2%)	NS
Age ≥ 45	78	96 $\pm$ 97	$P < 0.0001$	29 (37.2%)	$P = 0.0008$
Size ≥ 5 cm	68	91 $\pm$ 101	$P = 0.0019$	31 (45.6%)	$P < 0.0001$
Extrathyroidal extension	69	89 $\pm$ 100	$P < 0.0001$	28 (42.4%)	$P < 0.0001$
Vascular invasion	70	113 $\pm$ 103	$P = 0.0311$	24 (34.3%)	NS
Lymph node metastases	52	151 $\pm$ 115	NS	15 (28.8%)	NS
Distant metastases	32	95 $\pm$ 77	$P = 0.0366$	12 (37.5%)	NS
High stage	64	84 $\pm$ 97	$P < 0.0001$	29 (45.3%)	$P < 0.0001$
Alive with disease or dead of disease	61	79 $\pm$ 96	$P < 0.0001$	28 (46.6%)	$P < 0.0001$
Dead of disease	46	51 $\pm$ 80	$P < 0.0001$	25 (54.3%)	$P < 0.0001$

ferentiation of the tumor, 2) age, 3) sex, 4) tumor size, 5) vascular invasion, 6) extrathyroidal extension (confirmed histologically on all cases), 7) lymph node metastases, 8) distant metastases, and 9) pathological stage (AJCC¹⁴). β -catenin membrane immunoreactivity as well as *CTNNB1* exon 3 mutations or β -catenin nuclear expression were compared with the presence of H-, K-, or N-Ras mutations and proliferative activity (Ki67/MIB1 labeling index) in subsets of the cases analyzed. Logistic regression statistics was performed using *CTNNB1* exon 3 mutation, β -catenin nuclear localization, and low or high membrane β -catenin immunoreactivity as dependent variables. Survival was evaluated on all 115 patients with carcinoma using Kaplan-Meier plots and log-rank tests. Prognostic models containing the accepted clinicopathological indicators of poor outcome listed in Table 2 as independent covariates were devised using Cox's proportional hazards analysis. The 25th percentile of membranous β -catenin *H* scores that provided optimal stratification of data were chosen as cutoff for comparison with the Ki67/MIB1 labeling index, logistic regression, and survival analyses. Computing was performed using Statview (SAS Institute Inc., Cary, NC) and GraphPad Prism (GraphPad, San Diego, CA) software.

Results

β -Catenin Expression Pattern

Normal thyroid follicular cells from perilesional tissue showed strong membrane β -catenin immunoreactivity with no nuclear or cytoplasmic localization. This strong membranous staining of normal tissue provided an internal control for staining distribution and intensity. Three patterns of immunoreactivity were observed in the thyroid tumors: 1) membranous, 2) nuclear, and 3) cytoplasmic (Figure 1). Membrane β -catenin expression was decreased in eight of 12 (66%) follicular adenomas (331 $\pm$ 68, mean *H* score $\pm$ SD) and in all of the thyroid carci-

nomas analyzed (131 $\pm$ 109, mean *H* score $\pm$ SD) (Figure 2). Analysis of variance comparison of the means of the raw β -catenin *H* scores demonstrated a significant difference between follicular adenomas and all carcinomas ($P < 0.0001$) or the well-differentiated follicular carcinoma group ($P = 0.0001$). Membrane β -catenin reduction correlated with progressive loss of tumor differentiation ($P < 0.0001$, analysis of variance) (Table 2). Nuclear β -catenin expression was observed only in poorly differentiated (6 of 28 cases, 21.4%) or undifferentiated carcinomas (14 of 29 cases, 48.3%) indicating that also aberrant nuclear localization of β -catenin is a marker for loss of tumor differentiation ($P < 0.0001$, chi-square test for trend). Nuclear expression was associated with marked reduction or complete loss of membrane immunoreactivity among thyroid carcinomas ($P < 0.0001$, analysis of variance). In eight cases there was focal but distinct cytoplasmic immunoreactivity. Except for one papillary carcinoma, these cases were poorly differentiated or undifferentiated tumors. There was no significant difference in β -catenin expression among different histological types of thyroid carcinoma such as well-differentiated papillary *versus* follicular carcinoma (Table 2). Although oncocytic tumors usually featured relatively low membrane *H* scores, the correlation with β -catenin expression patterns was not statistically significant.

CTNNB1 Exon 3 Mutations

SSCP analysis displayed characteristic mobility shifts (Figure 3) and nucleotide sequencing of individual aberrant bands revealed somatic alterations only in poorly differentiated (7 of 28 or 25.0%) or undifferentiated (19 of 29 or 65.5%) carcinomas. The presence of *CTNNB1* exon 3 mutations correlated with a diagnosis of carcinoma ($P = 0.0456$, Fisher's exact test) and among the carcinomas with progressive loss of tumor differentiation ($P < 0.0001$, chi-square test for trend). Figure 4 shows a schematic summary of the location of all mutations detected

and the corresponding β -catenin immunoreactivity patterns in thyroid cancer. The number as well as the type of mutation or the specific amino acid substitutions did not correlate with clinicopathological parameters other than the degree of tumor differentiation.

Potential phosphorylation target residues (Ser/Thr) important for β -catenin degradation were mutated in all poorly differentiated carcinomas bearing *CTNNB1* exon 3 mutations. There often were several *CTNNB1* exon 3 mutations in a given tumor (the average number of β -catenin mutations per case was 1.8). The most common type of mutations were C:G>T:A transitions (11 of 13, 84.6% of mobility shifts on SSCP gels) that always involved the second nucleotide position of the altered codon. There was a significant correlation between Thr to Ile amino acid substitutions, all because of C:G>T:A transitions at codons 40 or 41, and poorly differentiated carcinomas ($P = 0.0283$, Fisher's exact test). Undifferentiated carcinoma had a higher prevalence of *CTNNB1* exon 3 mutations compared to poorly differentiated thyroid cancers ($P = 0.0033$, Fisher's exact test). Multiple distinct *CTNNB1* exon 3 mutations (not uncommonly three or four different types of them) were observed and 2.4 was the average number of β -catenin mutations per case. Mutations at putative Ser and Thr phosphorylation sites critical for β -catenin degradation represented less than half of all β -catenin exon 3 mutations (20 of 46, 43.5%).

In the whole cohort of cases analyzed *CTNNB1* exon 3 mutations were associated with aberrant β -catenin nuclear immunoreactivity ($P = 0.0020$, Fisher's exact test) and with low membrane expression ($P < 0.0001$, analysis of variance). Although cytoplasmic β -catenin immunoreactivity was commonly detectable in tumors with *CTNNB1* exon 3 mutations, the correlation was not statistically significant.

Mutational analysis was performed on multiple samples microdissected from the same tumor or its recurrences on all cases in which a better differentiated component could be demonstrated histologically. β -catenin mutations were identified in the anaplastic but not in the well-differentiated component associated with two undifferentiated carcinomas. Similarly, SSCP analysis of seven separate samples from three different tumors in which a well-differentiated component was associated with areas featuring poorly differentiated histological appearance revealed β -catenin exon 3 mutations only in the less differentiated portions of the tumor. In all three cases, β -catenin mutation was associated with markedly decreased membrane immunoreactivity and aberrant nuclear localization that were detected only in the poorly differentiated areas, whereas the better differentiated components did not exhibit nuclear staining and expressed significantly higher levels of membrane β -catenin.

β -Catenin Dysregulation, Clinicopathological Parameters, and Survival

Given the association ($P = 0.0020$, Fisher's exact test) between aberrant nuclear immunoreactivity and *CTNNB1*

exon 3 mutations and because both reflect Wnt activation, these two variables were combined for comparison with the clinicopathological parameters and survival. Aberrant nuclear localization/*CTNNB1* exon 3 mutations and decreased membrane β -catenin immunoreactivity correlated with unfavorable prognostic factors (Table 2). Well-differentiated papillary or follicular carcinomas did not show aberrant nuclear localization/*CTNNB1* exon 3 mutations but low membrane β -catenin expression was in general associated with unfavorable prognostic factors in this tumor group. The correlation was independent and significant for old age ($P = 0.0402$, analysis of variance), high tumor stage ($P = 0.0380$, analysis of variance), and distant metastases ($P = 0.0304$, analysis of variance). Within the poorly differentiated carcinoma group, unfavorable prognostic factors were commonly associated with tumors showing low membrane β -catenin *H* scores or aberrant nuclear localization/*CTNNB1* exon 3 mutations but only the correlation between aberrant β -catenin nuclear localization and lymph node metastases reached statistical significance ($P = 0.0221$, Fisher's exact test). The lack of statistical correlation between unfavorable prognostic indicators and β -catenin expression patterns or *CTNNB1* mutations within the undifferentiated carcinoma group was consistent with the uniformly poor prognosis of anaplastic carcinomas in this series.

Survival analysis performed using the Kaplan-Meier survival estimates method, revealed a significant correlation between low membrane β -catenin *H* score (log-rank, $P < 0.0001$) or aberrant β -catenin nuclear localization/*CTNNB1* exon 3 mutations (log-rank, $P = 0.0069$) and patient survival (Figure 5). Univariate and multivariate analysis were performed to assess the relative risk of disease-related death and to establish whether β -catenin dysregulation was an independent predictor of tumor-related death. Both low membrane β -catenin *H* score and the finding of aberrant β -catenin nuclear localization/*CTNNB1* exon 3 mutations predicted survival independently of the clinicopathological indicators of poor outcome for thyroid cancer listed in Table 2, with the exception of tumor differentiation.

Correlation of β -Catenin Dysregulation with Proliferative Activity

Cellular proliferation was previously analyzed in a subset of the thyroid carcinomas studied in this report to include 31 well-differentiated, 18 poorly differentiated, and 20 undifferentiated tumors.¹² Proliferative activity was evaluated immunohistochemically after computation of the Ki67/MIB1 labeling index (ie, the percentage of tumor cell nuclei positive for the proliferation marker Ki67/MIB1)¹² on sections cut from the same paraffin blocks used for the analysis of β -catenin. Carcinomas with low membrane immunostaining for β -catenin had higher Ki67/MIB1 labeling index (16.43 ± 22 , mean $\pm$ SD) compared with those featuring high membrane immunoreactivity (5.48 ± 5 , mean $\pm$ SD) ($P = 0.0025$, analysis of variance). Tumors with *CTNNB1* exon 3 mutations or β -catenin nuclear localization also featured higher Ki67/MIB1

Figure 2. Distribution of β -catenin membrane immunoreactivity (H score values) among thyroid tumors. **Filled circles** correspond to cases with no aberrant nuclear expression. **Open circles** indicate cases with aberrant nuclear localization of β -catenin. **Horizontal bars** indicate the median H score values.

labeling index (12.65 ± 12 compared with 7.245 ± 14 , mean $\pm$ SD) although the difference did not reach statistical significance ($P = 0.1515$, analysis of variance).

Correlation of β -Catenin Dysregulation with Oncogenic Ras

Thirty of the well-differentiated as well as all of the poorly-differentiated and all of the undifferentiated tumors have been analyzed for activating H-, K-, and N-Ras mutations at codons 12, 13, and 61.¹⁶ Ras genotyping was performed by PCR/SSCP using DNA extracted from the same paraffin blocks also analyzed for β -catenin. Tumors with mutated H-, K-, or N-Ras had lower membrane β -catenin H score values (70.80 ± 81 , mean H score $\pm$ SD) compared with those without Ras mutations (133.80 ± 115 , mean H score $\pm$ SD) ($P = 0.0068$, analysis of variance). H-, K-, or N-Ras mutations often coexisted with *CTNNB1* exon 3 mutations or β -catenin nuclear localization but the association did not reach statistical significance ($P = 0.1113$, Fisher's exact test). Interestingly, H-, K-, or N-Ras mutations were detected in four of the six cases with aberrant β -catenin nuclear localization without *CTNNB1* exon 3 mutation (samples 8, 20, 22, and 98).

No statistical effects were obtained by adding H-, K-, and N-Ras mutations to the analysis of the relationship between β -catenin dysregulation, clinicopathological parameters, and survival beyond what already shown above for membrane β -catenin H score values, aberrant nuclear β -catenin expression, and *CTNNB1* exon 3 mutations. However, after the exclusion of the undifferentiated carcinomas, all of which were invariably fatal, statistical analysis demonstrated decreased survival for patients with tumors featuring activated Ras or aberrant nuclear β -catenin expression/*CTNNB1* exon 3 mutations (log-rank test, $P = 0.0001$) (Figure 6). In the same group of patients, univariate analysis revealed a high likelihood of tumor-related death if activating Ras mutations or β -catenin nuclear localization/*CTNNB1* exon 3 mutations were detected ($P = 0.0010$; relative risk, 6.53; 95% confidence interval, 2.12 to 20). Activating Ras mutations or aberrant β -catenin nuclear localization/*CTNNB1* exon 3 mutations represented an independent and better predictor of tumor-related death than loss of tumor differentiation, large tumor size, extrathyroidal extension, vascular invasion, lymph node metastases, and advanced age.

Discussion

This study defines the role played by alterations of β -catenin in thyroid tumorigenesis by systematic analysis of a large series of thyroid tumors of follicular cell derivation and identifies a novel pathway for thyroid tumor progression. The results demonstrate two main patterns of β -catenin expression, membranous and nuclear, reflecting β -catenin multifunctional role in cell surface adhesion and in signal transduction. They clearly show that thyroid tumors are associated with decreased membrane expression of β -catenin. We have seen a dramatic down-regulation of membrane bound β -catenin in anaplastic thyroid cancer that represents the least differentiated form of carcinoma and the end point of tumor progression.¹⁰ This study expands this preliminary observation and demonstrates that reduced membranous immunoreactivity closely parallels progressive loss of tumor differentiation. The reduction is significantly higher in carcinomas compared with follicular adenomas. Although the number of cases analyzed is small, this observation may prove useful in the distinction between follicular adenoma and well-differentiated follicular carcinoma, a differential diagnosis that is extremely difficult on fine-needle aspiration specimens and can sometimes be problematic also on histology sections. Reduced membrane β -catenin has been documented in numerous human cancers¹⁷ where it was sometimes observed in high-grade or poorly differentiated tumors. In a few studies (see Ramesh et

Figure 1. Immunofluorescence staining for β -catenin in follicular adenoma (**a**) showing intense membrane immunoreactivity. Membrane β -catenin immunoreactivity is reduced in well-differentiated papillary carcinoma (**b**) and well-differentiated follicular carcinoma (**c**); there is no evidence of aberrant nuclear localization in the follicular adenoma or well-differentiated thyroid carcinomas (**a-c**). H&E section of a poorly differentiated thyroid carcinoma (NOS) (**d**) and corresponding immunofluorescence stain showing cytoplasmic accumulation of β -catenin (**e**). H&E section of a poorly differentiated thyroid carcinoma (insular type) (**f**) and corresponding immunofluorescence stain demonstrating marked reduction of membrane β -catenin immunoreactivity without aberrant nuclear expression (**g**). H&E section of a poorly differentiated thyroid carcinoma (NOS) (**h**) and corresponding immunofluorescence stain showing aberrant nuclear immunoreactivity of β -catenin and markedly reduced membrane expression (**i**); the same section is stained with the nuclear stain DAPI (4',6'-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA) to confirm β -catenin nuclear localization (**j**).

Figure 3. a: SSCP analysis of *CTNNB1* exon 3 in five poorly differentiated thyroid carcinomas (cases 82, 83, 84, 85, and 86; WT, wild-type). **b:** Sample sequence trace for case 85 showing C: G>T: A transitions at codons 40 and 41, both corresponding to the replacement of Thr with Ile amino acid residues. The remaining mutated tumors were case 7 (at codons 33, TCT→TTT and 40 ACT→ATT), case 11 (at codon 41, ACC→ATC), case 65 (at codons 40, ACT→ATT and 43, GCT→GTT), case 68 (at codon 40, ACT→ATT), case 82 (at codons 47, AGT→AAT and 69, GGA→AGA), case 86 (at codons 40, ACT→ATT, 44, CCT→CTT and 60, TCC→TTC).

al¹⁸) the correlation has been shown to be statistically significant. This reduction is consistent with progressive deregulation of intercellular adhesion in cancer that promotes tumor detachment from the primary site and facilitates tumor spread.¹⁷ In this series, reduced membrane β -catenin immunoreactivity parallels high tumor stage and an increased propensity of the cancer to spread locally outside the thyroid, to invade blood vessels, or give rise to distant metastases. These findings validate experimental models showing an inverse correlation between β -catenin levels and hematogenous spread of murine carcinoma cells¹⁹ and are similar to reports in other tumor types where reduced β -catenin expression was also associated with the capacity to invade²⁰ and metastasize.²¹

The observation that β -catenin is the nodal point of the Wnt pathway has enlarged the perspective in the analysis of E-cadherin/catenin adhesion complexes in tumor progression. We show that aberrant Wnt signaling is restricted to aggressive phenotypes of thyroid neoplasms and analysis of multiple samples from several tumors with areas featuring a distinct well-differentiated component clearly demonstrates Wnt activation only in the foci with poorly or undifferentiated morphology. Although Wnt activation is not a necessary event, it may be sufficient for neoplastic progression. In fact, in at least two of the undifferentiated carcinomas in this series (samples 21 and 119, data not shown) we identified mutations in the *CTNNB1* coding region (exon 3) of the β -catenin gene but did not identify alterations of other genes relevant for thyroid tumorigenesis (Ras, RET, p53).

The overall features of β -catenin mutations identified in this series are comparable with those reported for other cancers, although the prevalence of *CTNNB1* exon 3 mutations in poorly and particularly in undifferentiated thyroid carcinomas is generally higher.² Comparison of the pattern of *CTNNB1* exon 3 mutations shows interesting differences between poorly and undifferentiated tumors some of which may find useful diagnostic applications. Undifferentiated carcinomas have a significantly

higher prevalence of β -catenin mutations, with mutations to nonphosphorylatable amino acids exceeding those found in serine or threonine. Poorly differentiated carcinomas, on the other hand, exhibit a lower prevalence of β -catenin mutations. These involve putative Ser and Thr phosphorylation residues and often result in Thr to Ile amino acid substitutions.

Activation of Wnt signaling may occur because of different molecular events in different tumor types.² This study demonstrates that aberrant nuclear localization of β -catenin is significantly associated with *CTNNB1* exon 3 mutations. Although the correlation is not perfect, particularly among undifferentiated carcinomas, the finding indicates that Wnt signaling in thyroid neoplasms is primarily due to of β -catenin mutations and is compatible with the finding that APC is active in sporadic thyroid tumors.²² Our study also shows that activation of Wnt is statistically associated with loss of membrane β -catenin expression. This is consistent with interrelated but functionally independent roles for β -catenin in adherens junctions and in the Wnt pathway²³ in thyroid cancer. In fact,

Figure 4. Schematic illustration of the location of all of the mutations detected in the *CTNNB1* coding region (exon 3) of the β -catenin gene in thyroid tumors. Ser/Thr residues are underlined, cases with β -catenin cytoplasmic immunoreactivity are in **italics**, and cases with aberrant β -catenin nuclear localization are in **bold** characters.

Figure 5. Overall survival of 115 patients with thyroid carcinoma dichotomized according to membrane β -catenin immunoreactivity (**a**). Eighty-five of the 115 cases had membrane β -catenin H scores >20 . Survival in the same cohort according to aberrant Wnt activation (β -catenin nuclear immunoreactivity and/or CTNNB1 exon 3 mutation detected by SSCP) (**b**). β -catenin nuclear immunoreactivity and/or CTNNB1 exon 3 mutation by SSCP were detected in 32 of the 115 cases.

laboratory models indicate that mutant β -catenin forms active in Wnt signaling show little accumulation in the cell junctions or in the cytoplasm²³ and that a reduction of E-cadherin enhances β -catenin's role in Wnt activation.²⁴ Interestingly, decreased E-cadherin expression in thyroid tumors is also associated with loss of tumor differentiation²⁵ and its reduction may therefore facilitate the aberrant Wnt signaling in poorly and undifferentiated thyroid cancers. Although the consequences of E-cadherin inactivation are conceivably distinct from those of β -catenin mutation,²⁶ a similar relationship between reduced β -catenin expression and nuclear localization has been demonstrated for colorectal carcinomas.²⁷

Consistent with their putative role in tumor growth, cellular proliferation is associated with both aberrant Wnt signaling and decreased membrane β -catenin in thyroid carcinomas. However, and despite similar results in other tumor types,²⁸ the relationships between β -catenin mutations, tumor size, cellular proliferation, and β -catenin expression patterns appear far from linear and requires further investigation.^{29,30} Several oncogenes are known to influence the development and progression of thyroid

Figure 6. Overall survival of 58 patients with well-differentiated and poorly differentiated thyroid carcinoma dichotomized according to the presence of H-, K-, N-Ras mutation and/or aberrant Wnt activation (β -catenin nuclear immunoreactivity and/or CTNNB1 exon 3 mutation detected by SSCP). Thirty-five cases were negative for both Ras and Wnt activation, the remaining 23 cases showed either oncogenic Ras or aberrant Wnt signaling.

tumors and the important role played by activating Ras mutations in thyroid tumorigenesis has long been recognized.³¹ Tumors with activated Ras feature significantly lower membrane β -catenin expression. This validates the *in vitro* observation that Ras influences localization and activity of β -catenin by dismantling cadherin-catenin complexes, stabilizing β -catenin and promoting its nuclear accumulation.³² The finding that Ras oncogenes facilitate aberrant Wnt signaling in thyroid cancer seems further supported by the detection of mutated Ras in the majority of cases with aberrant nuclear β -catenin in the absence of CTNNB1 exon 3 mutation. The possible co-operation of Ras with Wnt signaling may have important clinical implications as indicated by their seemingly complementary role in determining the behavior of thyroid carcinoma subset.

Pathologists have often pointed out how many types of cancer, including poorly differentiated thyroid neoplasms,³³ bear morphological similarities to the corresponding developing tissues. Alterations of the Wnt signaling pathway seem to provide a paradigm for the connection between embryogenesis and cancer because activating β -catenin mutations have also been shown in medulloblastoma,³⁴ hepatoblastoma,³⁵ and Wilms tumor³⁶ that are closely reminiscent of differentiating embryonal tissues. The close parallel between dysregulation of β -catenin and neoplastic progression is consistent with the recent concept of a continuum in the spectrum of thyroid tumor differentiation. In contrast with neoplasms of most other epithelial organs, thyroid carcinomas of follicular cell origin have traditionally been viewed in terms of extremes, ie, well-differentiated carcinomas with papillary or follicular morphology and undifferentiated carcinomas and this approach is still reflected in the current staging system for thyroid cancer.¹⁴ The term poorly differentiated carcinoma was introduced only in the 1980s.¹³ Although the validity of the notion of a poorly differentiated neoplasm occupying an intermediate position in the pathological spectrum of thyroid tumors has subsequently been supported by additional studies,¹¹ the

morphological criteria used in the different case series are not always comparable. The correlations shown in this study between loss of tumor differentiation and β -catenin dysregulation suggest this marker may prove an objective and useful adjunct to the diagnosis of these thyroid tumors.

In summary, this study identifies β -catenin dysregulation as a pathway in the progression of thyroid tumors. Altered β -catenin is a marker for aggressive tumor phenotypes among neoplasms of thyroid follicular cell derivation because both reduction of β -catenin membrane immunoreactivity as well as its aberrant nuclear localization closely parallel loss of tumor differentiation and poor prognosis. The analysis of β -catenin expression or mutation status may ultimately be very useful to objectively subtype thyroid neoplasms and more accurately predict outcomes.

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