Role of p53, CD44V6 and CD57 in Differentiating Between Benign and Malignant Follicular Neoplasms of the Thyroid

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Abstract. The distinction between follicular adenoma (FAD) and follicular carcinoma (FCA) of the thyroid can be particularly challenging in routine practice of diagnostic surgical pathology. It often requires examination of several histologic sections in order to identify the presence of unequivocal capsular and/or vascular invasion. To investigate the role of immunohistochemical markers in the differential diagnosis of follicular lesions of the thyroid, we studied the pattern of expression of p53, Bcl-2 and of the adhesion molecules CD44V6 and CD57, in 20 FADs and 21 FCAs of the thyroid. Ninety percent of FCAs exhibited strong nuclear p53 expression. p53 stain was seen in only 15% of FADs (p<0.0001), and it was weak. Bcl-2 cytoplasmic immunoreactivity was observed in 57% of FCAs and 60% of FADs (p=1.00). Similarly, membranous CD44V6 staining was seen in 81% of FCAs, but in only 20% of FADs (p=0.0001). CD57 was present in the cytoplasm of 71% of FCAs and 15% of FADs (p=0.0004). None of the markers studied correlated with tumor size. The results of this study indicate that immunohistochemical detection of p53, CD44V6 and CD57 may have practical utility in the differential diagnosis of FCAs from FADs in routine surgical pathology.

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esophageal and gastric tumors, CD44 variants expression has been associated with aggressive tumors and poor outcome (22-24). In thyroid cancers, aberrant alternatively spliced CD44 proteins, including CD44V6, are expressed in papillary carcinomas but not in normal thyroid tissue (25,26). Despite a single report of CD44 expression in 9 out of 16 follicular adenomas (27), to our knowledge the expression of CD44V6 in follicular neoplasms of the thyroid has not been studied. Since the CD44V6 isoform is thought to play a role in tumor progression (28), and to modify the metastatic potential of tumor cells (29-30), one would anticipate its expression in FCAs but not in follicular adenomas.

Bcl-2 protein is a modulator of programmed cell death and is involved in both lymphoid and epithelial malignancies. To date the reporting of Bcl-2 protein expression in thyroid cancer has been sporadic and some have shown a down-regulation of Bcl-2 in papillary carcinomas (31,32). Anti-Leu-7 (CD57) antibody is a marker of natural killer lymphocytes (anti HNK-1) (33,34). It recognizes a carbohydrate epitope of N-CAM, a neural cell adhesion molecule (35). This antibody has been shown to react strongly with thyroid carcinomas (36). According to different studies, CD57 immunoreactivity is detected in 82-100% of follicular carcinomas but in only 33-43% of follicular adenomas (37-39).

Any of the above molecular markers exhibiting preferential expression for either follicular adenoma or carcinoma could be used as a discriminator between these two lesions. To test this hypothesis, we compared and statistically analyzed the expression of p53, CD44V6, Bcl-2 and CD57 in 20 FADs and 21 FCAs.

### Materials and Methods

**Case selection.** Twenty cases of FADs and 21 cases of FCAs were selected from the files of the Departments of Pathology at the Moffitt Cancer Center and Research Institute at the University of South Florida, and at Cooper University Hospital, USA. The FCAs included 15 microinvasive encapsulated follicular carcinoma of the thyroid and 6 cases of metastatic follicular carcinomas of the thyroid. The hematoxylin and eosin stains of all the cases were included 15 microinvasive encapsulated follicular carcinoma of the thyroid and 6 cases of metastatic follicular carcinomas. The latter tumors ranged in size between 1.2 and 8 cm (mean 4.1 cm., median 6.1 cm.). All these tumors were metastatic and 15 were microinvasive encapsulated follicular neoplasms of the thyroid has not been studied. Since the CD44V6 isoform is thought to play a role in tumor progression (28), and to modify the metastatic potential of tumor cells (29-30), one would anticipate its expression in FCAs but not in follicular adenomas.

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**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue sections, 4 μm thick, were applied to positively-charged slides. After deparaffinization and rehydration they were stained using the following primary antibodies: murine monoclonal antibody p53 (DO-7) (Cat. No. M7001, Dako, Carpinteria, CA, USA, dilution 1:100), recognizing an epitope lying in between amino acid 19 and 26 in the N-terminus of the human p53; mouse monoclonal antibody against CD44V6 (clone VFF-7) (Biosource International, Camarillo, CA, USA, dilution 1:80); and anti-HNK-1 monoclonal antibody (CD57) (Cat. No. 550010, CAMFolio-Becton Dickinson Lab., Bedford, MA, USA, dilution 1:25).

Non-enzymatic antigen retrieval was performed as previously described (40). The immunohistochemical stains were performed using the avidin-biotin peroxidase complex technique (ABC Kit, Vector Laboratories, Burlingame, CA, USA), at room temperature. Endogenous peroxidase and non-specific background staining were avoided by incubating the slides with 3% aqueous hydrogen peroxide for 10 min. After washing with phosphate-buffered saline (PBS) for 5 min, the slides were blocked with normal serum for 20 min and incubated for 1 h with the primary antibody at the dilution given above. After PBS washing, the secondary biotinylated antibody was applied for 20 min. The avidin-biotin peroxidase reaction was developed in the presence of a chromogen (3,3′ dianinobenzidine supplemented with hydrogen peroxide). Sections of colon carcinoma (p53), skin (CD44V6), lymph node (Bcl-2) and malignant melanoma (CD57) were used as positive controls.

**Analysis of immunohistochemical data.** The stained slides were examined microscopically by three independent observers using the following methods: primary antibodies: murine monoclonal antibody p53 (DO-7) (Cat. No. M7001, Dako, Carpinteria, CA, USA, dilution 1:100), recognizing an epitope lying in between amino acid 19 and 26 in the N-terminus of the human p53; mouse monoclonal antibody against CD44V6 (clone VFF-7) (Biosource International, Camarillo, CA, USA, dilution 1:80); and anti-HNK-1 monoclonal antibody (CD57) (Cat. No. 550010, CAMFolio-Becton Dickinson Lab., Bedford, MA, USA, dilution 1:25).

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**Results**

**Clinicopathologic features.** The age of the patients with FAD ranged between 28 and 73 years (mean 47 years, median 50 years). Sixteen were female and only 4 were male. The FADs ranged in size between 1 and 6 cm (mean 3.2 cm., median 3.5 cm.). All these tumors were encapsulated, exhibited a follicular architecture and lacked either vascular or capsular invasion. Conversely, the age of the patients with FCA ranged between 24 and 77 years (mean 58 years, median 50.5 years). Fifteen of these patients were female and 6 were male. Six of the FCAs were metastatic and 15 were microinvasive encapsulated follicular carcinomas. The latter tumors ranged in size between 1.2 and 8 cm (mean 4.1 cm., median 6.1 cm.) and all of them had a capsule. Five of them had vascular invasion only, 2 had capsular invasion only and 8 had both.

**Table I. Summary of immunohistochemical results.**

<table>
<thead>
<tr>
<th>Stain</th>
<th>Follicular adenoma</th>
<th>Follicular carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>3/20 (15%)</td>
<td>19/21 (90%)</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>12/20 (60%)</td>
<td>12/21 (57%)</td>
</tr>
<tr>
<td>CD44V6</td>
<td>4/20 (20%)</td>
<td>17/21 (81%)</td>
</tr>
<tr>
<td>CD57</td>
<td>3/20 (15%)</td>
<td>15/21 (71%)</td>
</tr>
</tbody>
</table>
Figure 1. p53 immunostain intensely decorating the nuclei of the tumor cells in a case of follicular carcinoma. The nuclei of the cells lining the adjacent normal follicles are negative (right side of the figure).

Figure 2. A follicular carcinoma staining strongly positive with CD44V6 antibody. Note the characteristic "membranous" stain.
Immunohistochemical analysis (Table I). Immunohistochemically, p53 nuclear positivity was detected in 19 cases of FCAs (90%) and weakly in only 3 FADs (15%) ($p<0.0001$). In the FCAs the intensity of the stain was strong in 9 cases (43%). These cases included all of the metastatic FCAs. Characteristically, the normal thyroid parenchyma was p53-negative (Figure 1). Moderate to weak Bcl-2 immunoreactivity was found in the cytoplasm of 12 FCAs (57%) and 12 FADs (60%) ($p=1.00$). Ten FCAs and 2 FADs expressed both markers. The adjacent normal thyroid follicles, when present, stained strongly for Bcl-2. CD44V6 staining was identified in 17 (81%) FCAs, but in only 4 (20%) FADs ($p=0.0001$). In all of the CD44V6-positive cases the staining was characteristically membranous (Figure 2). Four of the 6 metastatic FCAs were CD44V6-negative, the remaining 2 exhibited only weak CD44V6 staining. CD57 was present in the cytoplasm of 15 FCAs (71%) and of only 3 FADs (15%). This stain was cytoplasmic and consistently of strong intensity in all the positive cases (Figure 3).

No significant correlation was detected between the expression of any of the molecular markers studied and tumor size.

Statistical analysis (Table II). The data obtained were subjected to univariate and multivariate logistic regressions in order to study the relationship of the immunohistochemical variables to tumor type. Examination of the classification table summarizing the characteristics of various cut-offs that might be used to predict tumor type revealed that a rule based on predicting carcinoma when the p53 value exceeded one, or the sum of p53, CD44V6 and CD57 values exceeded two would have an estimated sensitivity of 83% and an estimated specificity of 83%.

Discussion

The differential diagnosis between benign and malignant follicular lesions of the thyroid is difficult and it is often based on the presence of definitive capsular infiltration and/or vascular invasion. Application of these histomorphologic criteria requires extensive sampling of the specimen, frequently with the need for additional deeper tissue sections. Such practice can be expensive and time-consuming, but often necessary. In this paper we demonstrated that the immunohistochemical detection of p53, CD44V6 and CD57 can accurately differentiate between FADs and FCAs with a sensitivity of 83% and a specificity of 83% as well.

The p53 gene is a tumor suppressor gene located on the short arm of chromosome 17. In the wild-type form, p53 protein induces a variety of effects through the transcriptional activation of genes involved in the modulation of cell cycle progression (23-25). This protein is also involved in cell growth control, DNA repair and can induce irreversible programmed cell death. It is thought that allelic losses and mutations of this gene can either modify the DNA binding
domain of the protein (exon 5-9), or unfold its conformational structure, interfering with the p53 tumor suppressor activity (1,2). In fact, the mutated p53 protein is unable to control important check points of the cell cycle, to induce apoptosis and to modify the sensitivity of the cells to chemotherapeutic agents (26-29). p53 represents a late genetic event in human thyroid carcinogenesis. It has only rarely been identified in thyroid adenomas (41), but has repeatedly been detected in poorly-differentiated and undifferentiated thyroid carcinomas (13-16),(42-45). In our study, p53 oncoprotein was strongly expressed in 90% of the FCAs but it was only found to be weakly expressed in 15% of FADs. This differential expression was statistically significant (p≤0.0001).

CD44V6 is a transmembrane glycoprotein involved in cell to cell and cell to matrix interactions. CD44V6 is an isoform of CD44, originating by alternative splicing of its gene, occurring between exon 5 and 16 (20,21,46). The variation at the gene level translates to variations on the most proximal portion of the CD44 receptor. Expression of CD44 isoforms appear to play a role in tumor growth (28), and to modify the metastatic potential of tumor cells (29,30). This molecule is a cell surface receptor for hyaluronan, a very large molecule containing numerous negative charges and, therefore, having high affinity for water (47). CD44 is used as “space holding” compound and it is potentially capable of entrapping tumor cells within the hyaluronic acid at the primary site. This view is also supported by the recent finding that, in cells triggered to undergo apoptosis, shedding of surface CD44 is the main mechanism by which cells detach from adjacent structures (48). We noted that 4 of the 6 metastatic FCAs were CD44V6-negative and the other two were weakly positive. This finding suggests that, in thyroid cancer as in other cancers, the expression of CD44V6 is important early during the development of cancer, but may be lost during the acquisition of the migratory function by the tumor cells. It is possible that the loss of CD44V6 induces a defective binding of the tumor cells to the extracellular matrix, increasing their mobility and metastatic potential. Studies of CD44 and CD44 isoforms expression in thyroid neoplasms have been scarce. Figge et al., using immunohistochemical and RT-PCR techniques, have reported higher levels of CD44 and CD44V6 in papillary carcinomas as compared to normal thyroid tissue (26,27). These authors also described the expression of CD44V6 in 1 case of thyroid follicular carcinoma. However, the number of cases evaluated was not specified. In our study CD44V6 revealed to be a helpful differential marker in the distinction between FADs and FCAs. Its expression was detected in 81% of FCAs and in only 20% of FADs (p≤0.0001).

In this study we also reported the use of anti-Leu 7 (HNK-1/N-CAM, CD57) as a marker of discrimination between follicular lesions of the thyroid. This is a membranous 110 kD glycoprotein first identified on subsets of natural killer lymphocytes and T lymphocytes lacking NK activity (33,34). Further characterization of this glycoprotein revealed that its activity is also present on a variety of solid tumors including neural and neuroectodermal tumors (49,50), malignant melanoma (51), Ewing sarcoma (52), PNET (53) and in small lung carcinoma (54). Anti-Leu (7) antibody also detects a carbohydrate epitope present on a subpopulation of cell-adhesion molecules including N-CAM and the myelin associated glycoprotein (MAG) (35). Ghali et al. first reported anti-Leu 7 immunoreactivity in follicular and papillary carcinomas of the thyroid (36). In their study the authors also described Leu 7 positivity in 6 out of 14 follicular adenomas. Others have confirmed anti-Leu 7 (CD57) reactivity of follicular and papillary thyroid carcinomas (55-57). Fucich et al. noted that the anti-Leu 7 antibody did not stain the benign thyroid tissues adjacent to the carcinomas, and detected CD57 positivity in 93% of 14 follicular carcinomas, but also in 50% of the 16 follicular adenomas (57). In our study, 71% of FCAs but only 3 out of 17 FADs (15%) showed cytoplasmic immunoreactivity for CD57. This difference was statistically significant (p=0.0004).

Bcl-2 oncoprotein is found on the inner mitochondrial membrane and perinuclear membrane (58), and it functions by blocking apoptosis and cooperating with c-myc in cell transformation (59). High levels of Bcl-2 protein expression have been detected in normal as well as non neoplastic thyroid tissues, including cases of Hashimoto thyroiditis and of Grave’s disease (31,32). Conversely, Bcl-2 expression is usually down-regulated in thyroid carcinomas, but mostly in those of the papillary or undifferentiated type (31,32). Fucich et al. noted that Bcl-2 expression is usually down-regulated in thyroid carcinomas, but mostly in those of the papillary or undifferentiated type (31,32,59-62). Furthermore, Okayasu et al. in their study comparing Bcl-2 stain to Ki67 labeling index, observed down-regulation of Bcl-2 protein expression in papillary carcinomas (N=42), but not in follicular adenomas (N=20), or in normal thyroid (N=14). The decreased Bcl-2 expression in papillary carcinomas correlated with enhanced apoptotic death rate (60). In this study we found no significant difference in Bcl-2 expression between FCAs and FADs (p=1).

Univariate and multivariate logistic regression analysis of our data revealed that p53, CD44V6 and CD57 can be used singly or in combination as a valuable armamentarium in the histologic differential diagnosis of difficult follicular thyroid neoplasms.

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Table II. Results of logistic regressions.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
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<tbody>
<tr>
<td></td>
<td>OR* 95% CI</td>
<td>p-value</td>
</tr>
<tr>
<td>p53</td>
<td>5.1 1.4, 18.9</td>
<td>0.02</td>
</tr>
<tr>
<td>CD44</td>
<td>2.3 1.1, 4.9</td>
<td>0.03</td>
</tr>
<tr>
<td>CD57</td>
<td>2.8 1.4, 5.5</td>
<td>&lt;0.01</td>
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*Estimated odds ratio associated with increase of one unit. The square of this value is the estimated odds ratio associated with an increase of two units.

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In conclusion, we studied the expression of p53, Bcl-2, CD44V6 and CD57 in follicular neoplasms of the thyroid. Our data indicated that the detection of p53, CD44V6 and CD57, but not of Bcl-2, may be of potential diagnostic utility in the histologic differential diagnosis of FCAs from FADs.

References

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