Underexpression of p27/Kip in Thyroid Papillary Microcarcinomas With Gross Metastatic Disease

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Objective: Papillary microcarcinomas (PMCs) of the thyroid (measuring less than 1 cm in maximum dimension) are extremely common incidental histologic findings, and most of these tumors are not considered clinically significant. However, rare PMCs behave aggressively and metastasize early, giving rise to clinically significant metastatic disease. We hypothesized that p27 and MIB-1/Ki-67 immunoreactivity would allow us to identify this small subgroup of PMCs that have the potential to behave aggressively.

Methods: We reviewed the histopathology reports of 2000 patients who underwent thyroid surgery at our institution between 1995 and 1999 and identified 22 patients who presented with gross regional metastases from a primary PMC. The primary and metastatic tumors were stained for ret, p53, p27, and MIB-1 using the avidin-biotin-peroxidase complex technique. A control group of 33 nonmetastasizing PMCs was also analyzed.

Results: Immunoreactivity for ret, p53, and MIB-1 showed no difference between metastasizing and nonmetastasizing PMCs. In most tumors, ret was present, while p53 immunoreactivity was absent in all tumors. MIB-1 staining was present in a small number of cells in both groups of tumors. Immunoreactivity for p27 was quantitated by the intensity of expression as well as the distribution of positive cells within each tumor. All tumors showed lower p27 expression than normal thyroid tissue. However, metastasizing PMCs demonstrated a significantly lower expression of p27 than nonmetastasizing PMCs (P<.001).

Conclusion: Our results suggest that p27 immunohistochemical analysis may be a valuable diagnostic tool in predicting aggressive potential in PMCs.

METHODS AND MATERIALS

We reviewed our database on thyroid cancer and the histopathology reports of 2000 patients who had undergone thyroid surgery at our institution between 1995 and 1999 and identified 30 patients who had pathologically proven lymph node metastases from a primary PMC in the thyroid gland (T1 N1 disease). Among these, 22 patients had gross (≥2 cm) nodal metastases from an occult PMC of the thyroid gland. These patients initially presented with clinically evident lymph nodes metastases from an unknown primary tumor. Only after fine needle aspiration cytology, and in some cases lymph node excision biopsy, were these metastases shown to be of thyroid origin. Subsequent thyroidectomy then revealed the primary lesion to be a PMC.

The other 8 patients had undergone thyroidectomy for various nonmalignant indications and had been incidentally found to have a PMC as well as microscopic metastatic deposits in a parathyroid lymph node. Because the significance of these lesions with micrometastatic foci remains unknown, we elected to exclude these from further analysis.

We immunohistochemically analyzed archival paraffin-embedded tissue from the 22 tumors with gross metastatic disease for ret, p53, MIB-1, and p27 staining. The control group, 33 randomly selected nonmetastasizing PMCs, underwent similar analysis. These PMCs were selected from thyroid glands that had been excised for nodular hyperplasia. Papillary microcarcinomas from glands containing malignancy were not included. To extend our analysis on p27, we studied 2 groups of larger papillary carcinomas as well, 14 with gross nodal metastases and 20 without nodal involvement. We hypothesized that p27 expression might predict the metastatic potential of papillary carcinomas in general, and we included these larger tumors as an additional control. In selecting these papillary carcinomas, we selected typical papillary cancers between 2 and 4 cm in size without evidence of extrathyroidal extension. We also excluded tumors with poor histologic features such as tall-cell or columnar-cell differentiation and tumors with poorly differentiated or anaplastic foci.

IMMUNOHISTOCHEMICAL ANALYSIS

The metastasizing and nonmetastasizing PMCs were analyzed by immunohistochemical analysis using antibodies for ret, p53, MIB-1, and p27. The 2 groups of larger papillary carcinomas were analyzed for MIB-1 and p27.

Formalin-fixed, paraffin-embedded sections 3 μm thick were dewaxed in toluene and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide. Antigen retrieval was performed in 10mM citrate buffer (pH, 6.0) inside a microwave pressure cooker for p53, MIB-1, and p27, and by formic acid pretreatment for ret. Endogenous biotin detection was blocked with the Avidin-Biotin Blocking Kit (Vector Lab Inc, Burlingame, Calif). Primary antibody incubations were carried out at room temperature as follows: ret, rabbit polyclonal (C-19) (Santa Cruz Biotechnology Inc, Santa Cruz, Calif), 1:1000 dilution, overnight incubation; p53, mouse monoclonal (D0-7) (Novocastra Ltd, Newcastle upon Tyne, ©2002 American Medical Association. All rights reserved.

RESULTS

ret AND p53

There was no difference in the immunoreactivity for ret and p53 between the metastasizing and nonmetastasizing PMCs. In most of the tumors ret was present in both groups, while p53 immunoreactivity was absent in all tumors in both groups.

MIB-1 (Ki-67)

MIB-1 immunoreactivity was present in a small number of cells in both metastasizing and nonmetastasizing PMCs. In the nonmetastasizing microcarcinomas, the MIB-1 labeling index ranged from 0 to 30, with a mean of 6.89 and a median of 4.5. In the metastasizing microcarcinomas, the MIB-1 labeling index ranged from 2 to 37, with a mean of 10.17 and a median of 6.5. As is apparent, there was considerable overlap in the labeling indices between the 2 groups, and while the mean and median labeling indices were slightly higher in the group with metastases, these differences were not statistically significant.

p27/Kip1

Nuclear p27 immunoreactivity was very strong in normal thyroid follicular cells, fibrovascular stromal cells, and lymphocytes. Almost all the tumors, including both larger carcinomas and microcarcinomas, showed lower than normal p27 expression, though the degree of underexpression varied. Of 89 tumors, only 4 showed normal (grade 4) p27 expression. Two of these were nonmetastasizing PMCs, while the other 2 were nonmetastasizing papillary carcinomas.

For the PMCs as well as the larger papillary cancers, the tumors with metastases showed significantly less p27 expression than the tumors without metastases (P<.001 and P<.005, respectively). Of the 22 metastasizing PMCs, 15 tumors (68.2%) showed very faint (grade 0 and 1) p27 expression (Figure 1) compared with only 6 tumors with moderate (grade 2) staining (Figure 2) and 1 tumor with strong (grade 3) staining. Conversely, of the 33 nonmetastasizing PMCs, only 6 tumors (18.2%) showed faint (grade 1) p27 expression compared with 15 tumors with moderate (grade 2) staining, 10 tumors with strong (grade 3) staining (Figure 3) and 2 tumors with very strong (grade 4) staining.

For the larger papillary carcinomas, the results were similar. Of the 14 papillary carcinomas with metastases, 7 (50%) showed faint (grade 1) p27 expression while 7 (50%) showed moderate (grade 2) p27 expression. However, of the 20 papillary carcinomas without metastases,
Papillary thyroid microcarcinomas with gross metastatic disease are extremely uncommon. However, a small subgroup of PMCs have the potential to behave aggressively and metastasize early, resulting in increased morbidity and mortality.\(^3\) 

Routine histopathologic analysis is unable to distinguish between the typical PMC, which tends to remain quiescent, and the rare PMC with the potential for aggressive behavior. We undertook this study to try to identify immunohistochemical markers that might allow us to recognize the potentially aggressive lesions. We hypothesized that one or several tumor genes might influence the behavior and metastatic potential of PMCs.

A small number of PMCs are considered incidental findings yet are found to be associated with micrometastatic disease in lymph nodes on careful histologic examination. We identified 8 such cases in our series. The true incidence of this phenomenon is not known, and...
genes that are expressed in follicular epithelium. Rear-
of the other tumors of thyroid follicular cell origin. These re-
lar carcinomas of the thyroid gland7 and is not seen in
rangement of several forms, has been shown to occur only in papil-
static spread of larger papillary thyroid carcinomas re-
terestingly, the incidence of ret/PTC rearrangement seems
to be higher in PMCs than in larger papillary carcinomas. Members of our group7 have reported that the incidence of ret/PTC rearrangement in PMCs was almost 80% vs 40%
for larger papillary carcinomas.

The significance of ret rearrangements in papillary thy-
roid carcinoma is still unclear. Several reports have sug-
ggested that ret rearrangement may predict aggressive beh-
vior, including local invasion and the potential for metastases.8 However, expression of ret/PTC gene prod-
ucts seems to be reduced in papillary carcinomas with ag-
gressive histologic features such as columnar and tall-cell

carcinomas stained for ret under immunohistochemical analy-
and there was no difference between the 2 groups. These
results are similar to those previously reported.7 This in-
dicates that while ret rearrangement plays a role in the de-
velopment of papillary carcinoma, it does not influence the meta-
static potential of early papillary thyroid carcinomas.
p53 Mutation resulting in protein overexpression is a
common feature of poorly differentiated and anaplastic thy-
roid carcinomas but is uncommon in well-differentiated thyroid carcinomas. However, p53 overexpression is seen
with increasing frequency in papillary carcinomas with poor
histologic features such as tall-cell or columnar-cell mor-
phologic characteristics11 and in tumors with evidence of
dedifferentiation.6 Overexpression of p53 has also been
linked to the potential for papillary carcinomas to meta-
tase to regional lymph nodes.3,12

We studied p53 immunoreactivity in both metastasiz-
ing and nonmetastasizing PMCs. None of the tumors showed
nuclear accumulation of p53 protein. Therefore, while p53
inactivation may play a role in the dedifferentiation of well-
differentiated thyroid carcinoma, it does not seem to play
a role in the potential for PMCs to metastasize.

MIB-1/Ki-67 expression is a marker of proliferative activity and has been shown to be significantly higher in poorly differentiated and anaplastic carcinomas than in well-differentiated carcinomas.13-15 The relationship be-
tween MIB-1 labeling index and the risk of metastases is, however, less clear. In a study of follicular thyroid car-
cinoma, Erickson et al13 found significantly higher MIB-1 expression in patients with distant metastases. Simi-
larly, Sugitani et al13 found a significantly higher MIB-1 labeling index in patients with PMCs who died of dis-
tant metastases. However, Tallini et al14 found no asso-
ciation between MIB-1 expression and metastases in their
patients with well-differentiated thyroid carcinomas. In our study, the mean and median MIB-1 labeling
indices were higher in the metastasizing PMCs than in the
nonmetastasizing microcarcinomas. However, the dif-
fferences were not statistically significant.

The p27/Kip-1 gene is a tumor suppressor gene that
encodes a 27-kd protein, a cyclin-dependent kinase inhibi-

Figure 3. Incidentally discovered papillary microcarcinoma with no evidence of metastasis showing strong (grade 3) p27 expression at ×20 magnification (A) and ×40 magnification (B).

![Image](image_url)

Table 1. Grading of p27/Kip-1 Immunoreactivity
According to Intensity of Nuclear Staining*

<table>
<thead>
<tr>
<th>Type of Carcinoma</th>
<th>p27/Kip-1 Immunoreactivity Grade</th>
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<tbody>
<tr>
<td>PMC</td>
<td></td>
</tr>
<tr>
<td>Met (n = 22)</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>No met (n = 33)</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>PTC</td>
<td></td>
</tr>
<tr>
<td>Met (n = 14)</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>No met (n = 20)</td>
<td>0 0 0 0 0</td>
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</tbody>
</table>

*PMC indicates papillary microcarcinoma; PTC, papillary thyroid carcinoma; and Met, metastases.

Table 2. Statistical Results of p27/Kip-1 Staining*

<table>
<thead>
<tr>
<th>Type of Carcinoma</th>
<th>p27/Kip-1 Immunoreactivity Grade</th>
<th>( \chi^2 )</th>
<th>df</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met (n = 22)</td>
<td>0 1 2 3 4</td>
<td>0.001</td>
<td></td>
<td>.001</td>
</tr>
<tr>
<td>No met (n = 33)</td>
<td>0 0 0 0 0</td>
<td></td>
<td></td>
<td>.001</td>
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<tr>
<td>PTC</td>
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*PMC indicates papillary microcarcinoma; PTC, papillary thyroid microcarcinoma; Met, metastases; and ellipses, not applicable.

the clinical significance of these lesions remains un-
clear.3 Indeed, the clinical significance of micrometa-
static spread of larger papillary thyroid carcinomas re-
ains controversial. We therefore elected to omit these
cases from our analysis. However, it will be interesting
to apply the results of our study to larger groups of thy-
roid carcinomas with micrometastases.

The ret/PTC gene rearrangement, which may take one of several forms, has been shown to occur only in papil-
ary carcinomas of the thyroid gland8 and is not seen in
other tumors of thyroid follicular cell origin. These re-
arangements result in fusion of the intracellular domain
of the ret protooncogene with N-terminal portions of other
genes that are expressed in follicular epithelium. Rear-
rangements of ret in papillary thyroid carcinoma have been
shown to occur early in the carcinogenetic process.4 In-
terestingly, the incidence of ret/PTC rearrangement seems
to be higher in PMCs than in larger papillary carcinomas.

We hypothesized that immunoreactivity for ret might
help distinguish between metastasizing and nonmetastasiz-
ing PMCs. However, we found that most of the microcar-
cinomas stained for ret under immunohistochemical analy-
sis, and there was no difference between the 2 groups. These
results are similar to those previously reported.7 This in-
dicates that while ret rearrangement plays a role in the de-
velopment of papillary carcinoma, it does not influence the meta-
static potential of early papillary thyroid carcinomas.
This protein acts as a negative regulator of the cell cycle, controlling G1 to S phase transition. Reduced p27 function has been implicated in the pathogenesis of several malignancies. While rearrangements and mutations of the p27 gene are uncommon, underexpression of nuclear p27 protein has been shown to occur in cancers of several organs. Thyroid tumors have been shown to express significantly less p27 than hyperplastic nodules, and malignant tumors express significantly less p27 than do benign tumors. Poorly differentiated thyroid carcinomas also express less p27 than well-differentiated thyroid carcinomas. Expression of p27 has also been reported to show an inverse correlation with MB-1 expression.

In this study, we hypothesized that PMCs that demonstrated aggressive potential and early metastases might have lower p27 expression than typical PMCs. We quantified the expression of p27 by assessing the intensity of nuclear staining in thyroid tumor cells. Previous studies have quantified p27 expression based on a labeling index of positive cells per 1000 cells counted. This strategy was effective when comparing groups with different thyroid diseases (e.g., hyperplasia vs neoplasia or benign tumors vs malignant tumors). However, in our study we were comparing groups with similar pathologic characteristics, and we found that the percentage of cells expressing p27 was very similar between the 2 groups of microcarcinomas and the 2 groups of papillary carcinomas. It was the intensity of p27 expression that varied between tumors. We therefore graded the tumors according to the intensity of p27 expression.

Our results show that p27 is significantly underexpressed in metastasizing PMCs compared with nonmetastasizing PMCs. Thus, p27 immunohistochemical analysis seems useful to distinguish the rare PMCs that have the potential to behave aggressively from the typical PMCs that tend to remain quiescent. Our results also show that p27 underexpression predicts an increased risk of lymph node metastases in larger papillary thyroid carcinomas. This further illustrates the value of p27 immunohistochemical analysis in predicting behavior in papillary thyroid cancer. Our data indicate that PMCs that underexpress p27 should no longer be regarded as incidental findings of little significance, but as true papillary carcinomas with the potential for aggressive behavior.

In conclusion, p27 seems to be significantly underexpressed in PMCs as well as in larger papillary carcinomas of the thyroid that metastasize to regional lymph nodes. Immunohistochemical analysis for p27 may be a valuable diagnostic tool in distinguishing PMCs of the thyroid with aggressive potential from the more typical indolent lesions and may also prove useful for predicting the metastatic potential of larger papillary cancers of the thyroid.

**REFERENCES**