Expression of Galectin-3, nm-23, and Cyclooxygenase-2 Could Potentially Discriminate Between Benign and Malignant Pheochromocytoma

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Key Words: Cyclooxygenase-2; Galectin-3; nm-23; Pheochromocytoma

Abstract

Currently, the only reliable indicator of malignancy in pheochromocytoma is the presence of distant metastasis or extensive local invasion; predicting behavior of pheochromocytoma remains challenging. We aimed to correlate the behavior of pheochromocytoma with its expression of nm-23, cyclooxygenase (COX)-2, and galectin-3 (genes used to predict the course of some neoplastic diseases), evaluated immunohistochemically in 55 paraffin blocks of formalin-fixed pheochromocytoma specimens with confirmed behavior.

In 3 (7%) of 44 benign and 7 (64%) of 11 malignant pheochromocytomas, there was negative nm-23 expression (P = .000). COX-2 immunoreactivity was positive in 10 (23%) of benign and 9 (82%) of malignant tumors (P = .000). Galectin-3 was expressed in 5 (11%) of benign and 9 (82%) of malignant pheochromocytomas (P = .000).

Negative nm-23, along with positive COX-2 or galectin-3, predicted malignancy with 100% specificity. Dual negativity for galectin-3 and COX-2, along with nm-23 positivity, indicated benign behavior with 100% sensitivity.

In early pheochromocytoma, evaluation of nm-23, galectin-3, and COX-2 expression could predict the outcome. Larger studies seem necessary to confirm the potential practical value of our findings.

Pheochromocytoma, with an incidence of about 2 per million person-years,1 occurs in all age groups; however, it is most common in the fourth and fifth decades of life.1,2

Signs and symptoms of the tumor are mostly related to excessive release of catecholamines, including epinephrine and, particularly, norepinephrine; signs and symptoms include sustained or paroxysmal hypertension, postural hypotension, hypermetabolic state, tachycardia, bradycardia, palpitations, headaches, anxiety, and chest pain.3-5 The diagnosis is usually confirmed by measuring the level of catecholamines or their metabolites in urine or plasma.3

Approximately 10% of pheochromocytomas are malignant.1,4 Features such as greater tumor size, confluent tumor necrosis, small individual tumor cells, increased proliferative activity, extensive local invasion, and an extra-adrenal location have been proposed to be associated with malignancy.3,4,6 However, it can be stated that currently, based on conventional histomorphologic, genetic, molecular, and immunohistochemical methods, no reliable criteria exist to distinguish between malignant and benign lesions.7 The only reliable absolute indicator of malignancy in pheochromocytoma is the presence of metastasis.2,4

Metastasis suppressor genes have a role in preventing the extension of tumors. nm-23 is a metastasis suppressor gene for which the expression level depends on the state of cell growth.8 Decreased expression of nm-23 has been associated with increased invasiveness in different cancers, including those of the breast, hepatocytes, ovary, bladder, and renal cells.9-21

Another gene involved in cancer progression through promotion of tumor cell proliferation, tumor angiogenesis, and resistance to apoptosis is cyclooxygenase (COX)-2.22 The
role of COX-2 has been shown in early stages of carcinogenesis beginning in premalignant adenomatous lesions and in the course of their progression to colon adenocarcinoma, as well as in esophageal, breast, papillary thyroid, medullary thyroid, prostatic, and pulmonary carcinomas, and most relevant, in pheochromocytoma.22-33

Galectins are a family of β-galactosidase-binding lectins that are involved in transformation to and progression of malignancy. Although not highly specific, their expression pattern in follicular thyroid neoplasms has been proposed as an ancillary tool for distinction between benign and malignant lesions.34 Expression of galectin-3 in non–small cell lung carcinoma is associated with shorter survival.35,36 Similar associations of galectin-3 expression and outcome have also been observed in uterine, breast, and colorectal carcinomas.37-39 Moreover, it has been suggested that the expression of galectin-3 is distinct in various types of pheochromocytomas, although the proposal needs to be verified by further study.40

In this study, we evaluated the immunohistochemical expression of nm-23, COX-2, and galectin-3 in 55 cases of benign and malignant pheochromocytoma and attempted to correlate the pattern of expression of these markers with tumor behavior. Because most of the published studies are about the relationship between immunohistochemical or molecular expression of markers in pheochromocytoma and tumor behavior, we also studied adrenal and extra-adrenal pheochromocytomas as a single group.7

Our aim was to propose immunohistochemical evaluation of certain markers as a tool to discriminate between potentially benign and malignant pheochromocytomas at the time of diagnosis, independent of tumor location, histomorphologic features, genetic findings, and familial predisposition. Application of such prognostic criteria to categorize patients into high- and low-risk groups could be used to guide their treatment and follow-up. This means choosing more aggressive treatment and strict follow-up for the high-risk group and offering more reassurance and avoiding undue expenses for the low-risk group.

Materials and Methods

Paraffin blocks of formalin-fixed samples representing 55 cases of pheochromocytoma diagnosed during 20 years (1986-2006) in the Department of Pathology, Shariati Hospital, Tehran, Iran, were examined to determine the simultaneous expression of galectin-3, COX-2, and nm-23. According to the course and outcome, observed during 4 to 20 years of follow-up, 44 (35 adrenal and 9 extra-adrenal) cases were considered benign and 11 (7 adrenal and 2 extra-adrenal) cases were regarded as malignant. Distant metastases or extensive invasion to the surrounding organs and, in 1 case, lymph node metastasis were considered evidence of malignancy.

In all cases, H&E-stained slides were reviewed, and the tumor histologic pattern was determined Table 1. All cases were graded according to the PASS (Pheochromocytoma of the Adrenal gland Scaled Score) system proposed by Thompson.41

Complete clinical data were available for all cases, including information about the patients’ follow-up. The study was conducted according to the protocol of the Ethics Committee of the Endocrinology & Metabolism Research Center, Shariati Hospital.

The paraffin blocks containing minimal necrosis and hemorrhage and a representative amount of the tumoral tissue were selected for immunohistochemical study. Three-micrometer sections from representative blocks of every tumor were deparaffinized with xylene and alcohol and then rehydrated. The slides were immersed in 3% hydrogen peroxide for 15 minutes. Antigen retrieval was done in an appropriate pH for each marker Table 2. Subsequently, sections were incubated with the first antibody for 1 hour, washed, and then incubated with the second antibody (EnVision+ system HRP-labeled polymer, DAKO, Carpinteria, CA) for 30 minutes. Finally, the antigens were detected by using the chromogen (Table 2).

A semiquantitative scoring method (score 1 to 4) based on the extent of staining of tumor cells was used for evaluation of immunopositivity for the 3 markers Table 3. The expected staining pattern was nuclear and/or cytoplasmic for nm-23 and cytoplasmic for COX-2 and galectin-3. To ensure the validity of the technical method, positive control samples, which consisted of adrenal cortex for nm-3 and COX-2 and prostate for galectin-3, as recommended by the manufacturer, were used.

The slides were independently reviewed by 2 pathologists having no information about the clinical data. In cases of discordant diagnosis, the 2 pathologists would discuss the case to reach consensus; otherwise, the case was reviewed by a third pathologist.

Table 1
Histologic Patterns of the Tumors and Their Behavior

<table>
<thead>
<tr>
<th>Histologic Tumor Pattern</th>
<th>Benign</th>
<th>Malignant</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nesting</td>
<td>22 (40)</td>
<td>1 (2)</td>
<td>23 (4)</td>
</tr>
<tr>
<td>Trabecular</td>
<td>13 (24)</td>
<td>1 (2)</td>
<td>14 (25)</td>
</tr>
<tr>
<td>Solid</td>
<td>7 (13)</td>
<td>1 (2)</td>
<td>8 (15)</td>
</tr>
<tr>
<td>Spindle cell</td>
<td>1 (2)</td>
<td>4 (7)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Small cell</td>
<td>1 (2)</td>
<td>4 (7)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Total</td>
<td>44 (80)</td>
<td>11 (20)</td>
<td>55 (100)</td>
</tr>
</tbody>
</table>
After data collection, the analysis of qualitative and quantitative data was done using the Statistical Package for Social Sciences, version 15.0 (SPSS, Chicago, IL). A P value of less than .05 was considered significant.

Results

Of 55 cases of pheochromocytoma in this study, 44 and 11 cases were categorized as benign and malignant, respectively. Of the 44 benign, 9 (20%) and 2 (18%) of 11 malignant cases had extra-adrenal locations. There was no significant relationship between tumor location and tumor behavior (P = .866).

The female/male ratio was 25:19 in the benign category and 5:6 in the malignant category. No relationship was observed between sex of the patients and tumor behavior.

The age groups ranged from 10 to 19 years old to 70 to 79 years old. Benign and malignant tumors were most commonly encountered in the 40- to 49-year-old group; however, the age distribution in the benign category was wider compared with the malignant category.

The mean ± SD diameters of benign and malignant tumors were 6.92 ± 2.67 cm and 9.18 ± 5.02 cm, respectively (P = .044). Tumor necrosis was present in 14 (32%) of benign and 7 (64%) of malignant cases with no statistically significant difference (P = .054).

The histologic patterns of benign and malignant tumors are shown in Table 1. Tumor patterns such as small cell and spindle cell were associated with malignant behavior, independent of immunohistochemical results (P = .04). The mean ± SD PASS scores in benign and malignant cases as 2 groups were 1.70 ± 1.76 and 3.64 ± 1.86, respectively (P = .002). The most remarkable finding was that the PASS score was 5 or less in all benign tumors.

The results of immunoreactivity of the pheochromocytomas for the 3 markers and the proportion of tumors with a certain histologic pattern in each immunohistochemical score category are shown Table 4 and Table 5. Considering a cutoff of 10% (percentage of tumor cells with positive staining) to discriminate between negative and positive expression of the markers, the specificity, sensitivity, negative predictive value (NPV), and positive predictive value (PPV) were calculated for each marker Table 6.

In 2 benign tumors, local invasion to the capsule but no extracapsular extension had been observed. In 1 such tumor with no tumor necrosis and 16 years of follow-up, an immunoreactivity score of 1 for all 3 markers was observed; in the other, which contained necrosis and had been followed up for 10 years, the immunoreactivity scores for nm-23, COX-2, and galectin were 2, 1, and 4, respectively. In both mentioned...
tumors, the largest tumor diameter was 8 cm. Neither of them showed evidence of recurrence or metastasis during the follow-up period.

An attempt was also made to find a relationship between immunoreactivity for the 3 markers and the tumor histologic pattern categorized in order of organization as nesting, trabecular, solid, spindle cell, and small cell. We observed that increased immunoreactivity for nm-23 was associated with nesting and trabecular histologic patterns (P for trend = .053: borderline), while in the case of COX-2 and galectin-3, increased immunoreactivity corresponded to spindle cell and small cell histologic patterns (P for trend = .02 and P for trend = .000, respectively [significant]). Thus, the presence of small cell and spindle cell histologic patterns is associated with increased expression of COX-2 and galectin-3 and decreased nm-23 expression, which corresponds to malignant tumor behavior.

**Discussion**

Pheochromocytoma, a tumor of the sympathoadrenal system, has malignant behavior in about 10% of the cases.\(^1\),\(^4\),\(^7\) Currently, no absolute histomorphologic, genetic, molecular, or immunohistochemical finding is available to differentiate between malignant and benign pheochromocytomas,\(^7\) except for the presence of distant metastases.\(^2\),\(^4\) However, extensive local invasion and confluent tumor necrosis are “suspicious” features.\(^2\) Few studies have investigated the correlation between different histomorphologic patterns and tumor behavior.\(^6\)

We observed that the less common small cell and spindle cell histologic patterns are more prevalent in tumors with malignant behavior (P = .04); however, we could not establish any relationship between malignancy and an extra-adrenal tumor location.

**Table 5**

<table>
<thead>
<tr>
<th>Marker/Immunohistochemical Score</th>
<th>Nesting</th>
<th>Trabecular</th>
<th>Solid</th>
<th>Spindle Cell</th>
<th>Small Cell</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galectin-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 41)</td>
<td>20 (49)</td>
<td>12 (29)</td>
<td>8 (20)</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>.000</td>
</tr>
<tr>
<td>2 (n = 4)</td>
<td>2 (50)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (50)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>3 (n = 4)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>2 (50)</td>
<td></td>
</tr>
<tr>
<td>4 (n = 6)</td>
<td>1 (17)</td>
<td>1 (17)</td>
<td>0 (0)</td>
<td>2 (33)</td>
<td>2 (33)</td>
<td></td>
</tr>
<tr>
<td>Cyclooxygenase-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 36)</td>
<td>17 (47)</td>
<td>11 (31)</td>
<td>5 (14)</td>
<td>1 (3)</td>
<td>2 (6)</td>
<td></td>
</tr>
<tr>
<td>2 (n = 4)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>2 (50)</td>
<td>1 (25)</td>
<td>0 (0)</td>
<td>.022</td>
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<td>3 (n = 3)</td>
<td>1 (33)</td>
<td>1 (33)</td>
<td>1 (33)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>4 (n = 12)</td>
<td>5 (42)</td>
<td>1 (8)</td>
<td>0 (0)</td>
<td>3 (25)</td>
<td>3 (25)</td>
<td></td>
</tr>
<tr>
<td>nm-23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 10)</td>
<td>3 (30)</td>
<td>1 (10)</td>
<td>1 (10)</td>
<td>1 (10)</td>
<td>4 (40)</td>
<td></td>
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<tr>
<td>2 (n = 7)</td>
<td>5 (71)</td>
<td>1 (14)</td>
<td>0 (0)</td>
<td>1 (14)</td>
<td>0 (0)</td>
<td>.053</td>
</tr>
<tr>
<td>3 (n = 8)</td>
<td>3 (38)</td>
<td>3 (38)</td>
<td>2 (25)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>4 (n = 30)</td>
<td>12 (40)</td>
<td>9 (30)</td>
<td>5 (17)</td>
<td>3 (10)</td>
<td>1 (3)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
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<tr>
<td>nm-23</td>
<td>63</td>
<td>93</td>
<td>70</td>
<td>91</td>
</tr>
<tr>
<td>Cyclooxygenase-2</td>
<td>81</td>
<td>77</td>
<td>47</td>
<td>94</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>81</td>
<td>88</td>
<td>64</td>
<td>95</td>
</tr>
</tbody>
</table>

NPV, negative predictive value; PPV, positive predictive value.

Different genes involved in different stages of the cell cycle, induction of cell differentiation, adhesion, or apoptosis have been evaluated regarding their potential for prediction of the course of neoplastic lesions and have sometimes proved useful. Expression of nm-23, a metastasis suppressor gene,\(^7\) is variable depending on the state of cell growth; highest and lowest nm-23 expression occur in the S phase and in the G\(_0\)/G\(_1\) phase, respectively.\(^8\) Decreased expression of this gene in different cancers, including breast carcinoma,\(^9\),\(^11\) hepatocellular carcinoma,\(^12\),\(^13\) non–small cell carcinoma of lung,\(^14\),\(^16\) ovarian carcinoma,\(^17\),\(^18\) bladder carcinoma,\(^19\),\(^20\) and renal cell carcinoma,\(^21\) has been associated with an increase in tumor invasiveness and prevalence of metastasis. A study by Ohta et al\(^7\) on 15 benign and 10 malignant pheochromocytomas showed that down-regulation of the nm-23 gene evaluated by real-time polymerase chain reaction was significantly higher in malignant tumors. Our study also demonstrated a greater extent of immunoreactivity for nm-23 in benign pheochromocytomas compared with the malignant group, the difference being statistically significant (P = .000).

The role of COX-2 in early stages of carcinogenesis has particularly been shown in premalignant adenomatous lesions of colon.\(^22\) The product of COX-2 is a membrane glycoprotein that undergoes transcriptional and posttranscriptional regulation
by proinflammatory agents, cytokines, growth factors, onco-
genesis, and tumor promoters. In the present study, malignantpheochromocytomas showed statistically significantly more
extensive immunoreactivity for COX-2 than benign pheochro-
mocytomas ($P = .000$). Salmenkivi and colleagues evaluated
the expression of COX-2 in pheochromocytoma in a similar
study in 2001. Assuming 20% immunoreactivity as the cutoff,
all malignant cases had positive immunoreactivity (strong or
moderate immunoreactivity, >20% of tumor cells), and 75% of
the benign cases had negative or weak immunoreactivity. This
study eventually concluded that negative or weak reactivity for
COX-2 could be in favor of benign pheochromocytoma.33 The
present study, similar to that of Salmenkivi and colleagues,33
showed differential expression of COX-2 in benign and malig-
nant pheochromocytomas.

Galectin-3 is an inhibitor of apoptosis and involved in
cell growth, adhesion, angiogenesis, and metastasis.40 This
marker could help in the differentiation of benign from malig-
nant follicular thyroid neoplasm.34 In our study, the difference
between the extent of immunostaining in benign and malig-
nant categories was statistically significant ($P = .000$). Gimm
and colleagues evaluated 4 malignant and 24 benign pheo-
chromocytomas for galectin-3 expression. In their study, the
cases considered malignant owing to the presence of meta-
tasis and extensive invasion proved to have strong reactivity
(diffuse or partial) for the marker, but the cases that were
considered malignant owing to lymph node metastasis did not
show strong immunoreactivity. In benign cases, the reactivity
was mostly weak or negative. One sporadic case had moder-
ate cytoplasmic reactivity, but subsequent follow-up of the
case was not available. The fact that lymph node metastasis
in pheochromocytoma is a controversial indicator of malignancy
might be an explanation for the paradoxical strong reactivity
observed in the malignant cases. In our study, however, both
of the malignant cases (of 11) that had negative immunoreac-
tivity for galectin-3 had been considered malignant owing to
distant metastasis. Moreover, in 1 case regarded as malignant
by reason of lymph node metastasis, the staining score was 2,
again being different from the mentioned study.

To increase the diagnostic sensitivity and specificity and
with the aim of arriving at a practical conclusion, the simulta-
aneous reactivity for all 3 markers was evaluated Table 7.

Considering the obtained results, to avoid missing
malignant cases, it is best practice to evaluate the expres-
sion of all 3 markers in the first step. Possible conditions
encountered and the proper approaches to them are as follows
Figure 1.

<table>
<thead>
<tr>
<th>Combined Markers</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative nm-23 and positive COX-2</td>
<td>45.5</td>
<td>100</td>
<td>100</td>
<td>88.0</td>
</tr>
<tr>
<td>Negative nm-23 and positive Gal-3</td>
<td>45.5</td>
<td>100</td>
<td>100</td>
<td>88.0</td>
</tr>
<tr>
<td>Positive COX-2 and positive Gal-3</td>
<td>72.7</td>
<td>97.7</td>
<td>88.9</td>
<td>93.5</td>
</tr>
<tr>
<td>Negative nm-23 and positive COX-2 and positive Gal-3</td>
<td>36.0</td>
<td>100</td>
<td>100</td>
<td>86.3</td>
</tr>
<tr>
<td>Negative nm-23 or positive COX-2 or positive Gal-3</td>
<td>100</td>
<td>61.0</td>
<td>39.2</td>
<td>100</td>
</tr>
<tr>
<td>Negative nm-23 and positive COX-2 or Gal-3</td>
<td>54.5</td>
<td>100</td>
<td>100</td>
<td>88.7</td>
</tr>
<tr>
<td>Positive nm-23 and COX-2 and Gal-3</td>
<td>39.3</td>
<td>97.7</td>
<td>80</td>
<td>86.0</td>
</tr>
</tbody>
</table>

COX-2, cyclooxygenase-2; Gal-3, galectin-3; NPV, negative predictive value; PPV, positive predictive value.
Condition 1 includes the tumors with a positive reaction for COX-2 or galectin-3 or a negative reaction for nm-23; this warrants further evaluation of the patient. Otherwise (namely, condition 2), the tumor is considered benign and there would be no necessity for more investigations. Accordingly, in our study, in all malignant cases and 17 (39%) of 44 benign cases, further investigation would have been indicated (condition 1). In condition 1, by evaluation of the pattern of reactivity of the markers, behavior of the tumor could be predicted to some extent.

In condition 1A, the tumor is positive for COX-2 or galectin-3 and simultaneously shows negative staining for nm-23; the tumor must be considered malignant (specificity, 100%; and PPV, 100%). This necessitates constant and frequent follow-up investigations for early detection of metastasis or recurrence and application of timely and appropriate therapeutic measures and probably prophylactic treatments. In the present study, 6 (55%) of 11 malignant tumors had this condition.

In condition 1B, the tumor shows positive staining for nm-23 and simultaneous positivity for COX-2 and galectin-3; the course of the tumor would be malignant (specificity, 98%; and PPV, 80%).

In our study, 4 of 11 malignant and 1 of 44 benign tumors had this condition (not overlapping with the tumors having condition 1A). However, it would be reasonable to adopt a similar strategy in this group as well. Of all malignant tumors, 91% were categorized as having condition 1 (1A and 1B).

In condition 3, the tumor has none of the mentioned conditions (as 1 [9%] of 11 malignant and 16 [36%] of 44 benign tumors in the present study); although there is higher probability of being benign than malignant (NPV, 94%), it would be prudent to perform periodic follow-up investigations to avoid missing lesions with malignant behavior. A less intensive and less frequent follow-up program would be more appropriate in the latter case. In tumors having the condition 3, taking the tumor size and presence or absence of necrosis into consideration could help more in predicting the probable behavior of the tumor. In our study, only 1 of the tumors having condition 3 was malignant, and it had a maximum diameter of 8 cm and contained necrotic foci.

It appears that early in the course of disease, evaluation of expression of the 3 markers, nm-23, galectin-3, and COX-2, and the presence of tumor necrosis and tumor size could predict the outcome of pheochromocytoma with reasonable specificity and sensitivity. However, performing more studies would seem necessary to confirm the potential practical value of the findings of this study.

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References