Immunohistochemical expression of Ki67, c-erbB-2, and c-kit antigens in benign and malignant pheochromocytoma

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Abstract

In this study, we evaluated the immunohistochemical expression and the possible advantages of Ki67 antigen, c-erbB-2, and c-kit proto-oncogenes in the differentiation between benign and malignant pheochromocytomas.

Paraffin-embedded tissue blocks from 44 benign (35 adrenal and 9 extra-adrenal) and 11 malignant (9 adrenal and 2 extra-adrenal) cases of pheochromocytoma were selected for immunohistochemical staining using antibodies against Ki67, c-erbB-2, and c-kit antigens. We investigated the relationship between the expression of these antigens and age, gender, tumor size, histologic patterns and necrosis, as well as tumor behavior.

The risk of malignancy was higher when tumor size was increased. We found out that more uncommon tissue patterns, such as small cell and spindle cell patterns, are mostly indicators of malignancy. There was a statistically significant relationship between Ki67- and c-erbB-2-positive staining of the tumor cells and the malignant behavior of the pheochromocytomas (p-value=0.000), while cytoplasmic c-kit staining did not show any correlation with tumor malignancy (p-value=0.087).

We concluded that tumor size and the histomorphologic patterns (spindle cell and small round cell) are significantly associated with tumor behavior. In addition, Ki67 positivity and c-erbB-2 expression can be used as immunohistochemical markers for predicting the malignant behavior of pheochromocytoma.

Introduction

Pheochromocytoma is a rare neuroendocrine neoplasm with an annual prevalence rate of about 1–2 per 100,000 adults [1]. About 10% of these tumors can be referred to as malignant pheochromocytoma, which is a life-threatening tumor [2]. It is generally accepted that predicting a malignant potential in pheochromocytoma (like other neuroendocrine tumors) only on the basis of histologic criteria is often difficult, and other criteria, such as the presence of metastasis, should be considered [2,3]. Because of the lack of accurate histologic criteria, a lifelong follow-up of these patients is necessary. In recent years, several studies have investigated biochemical and ultrastructural factors which could influence the behavior of pheochromocytoma. The evaluation of the proliferative index [4–6], the immunohistochemical analysis of c-erbB-2 [2,5,7], tumor angiogenesis [6], p53 [5,8], and many other markers are some examples of recent research, the results of which are conflicting and controversial.

In this study, we evaluated the immunohistochemical expression and the possible advantage of Ki67 antigen, c-erbB-2, and c-kit proto-oncogenes in the differentiation between benign and malignant pheochromocytoma.

The immunohistochemical evaluation of the proliferative activity on the basis of nuclear expression of Ki67 is commonly used as a prognostic value in various tumors, including breast carcinoma and lymphoma, in which high proliferative activity indicates poor prognosis. Some studies have investigated the role of Ki67 in pheochromocytoma, the results of which are vague and conflicting [4–6,9–12].

C-erbB-2 proto-oncogene encodes a membrane receptor with similarities to the epidermal growth factor receptor. Malignancies of many organs, such as breast, stomach, ovary, and prostate, are associated with poor prognoses [7,13,14]. On the other hand, Herceptin, a monoclonal antibody against c-erbB-2 antigen, can be a therapeutic choice in these cancers [15]. Only few studies have evaluated the membranous expression of this oncogene in benign and malignant pheochromocytoma [2,5,7].
c-kit proto-oncogene encodes a transmembrane tyrosine kinase receptor, which plays a target role in cancer molecular therapy. The mutation of c-kit causes it to function as an oncogene in mastocytosis, melanoma, germ cell tumors, and gastrointestinal stromal tumors (GIST). The expression of this antigen in normal and non-neoplastic adrenal medullary cells is uncertain; the nuclear protein related to c-kit was found in one study [16] while no c-kit immunoreactivity was detected in normal adrenal medullary cells by another group [17]. In addition, studies investigating the immunohistochemical expression of this oncogene in benign and malignant pheochromocytoma are limited [17,18].

The aim of the present study was to evaluate the immunohistochemical expression of the three mentioned markers in benign and malignant pheochromocytomas which had a 2- to 20-year follow-up period. If any relationship exists, tumor behavior could be predicted at the time of diagnosis, and fatal metastasis of the malignant pheochromocytoma can be prevented by making more precise follow-ups and by applying more invasive treatments for high-risk patients.

Materials and methods

Paraffin-embedded, formalin-fixed tissue blocks from 55 cases of pheochromocytoma, 44 benign (35 adrenal and 9 extra-adrenal) and 11 malignant (9 adrenal and 2 extra-adrenal) were obtained from the surgical pathology archive at Shariati Hospital, which is affiliated to Tehran University of Medical Science. The criterion for malignancy was histologic evidence of a lymph node or distant organ metastasis in the 2- to 20-year follow-up period (1985–2004). The presence of multiple tumors or the recurrence of pheochromocytoma in adrenal or extra-adrenal location was not considered a malignancy.

In all cases, Hematoxylin/Eosin-stained slides were reviewed. The appropriate paraffin-embedded blocks were selected for immunohistochemical (IHC) staining, taking into account the minimal level of hemorrhage and necrosis, as well as proper formalin fixation. IHC staining (Avidin–Biotin peroxidase method) was performed using antibodies against Ki67, c-erbB-2, and c-kit antigens (Dako, Denmark). The 3 μm sections, obtained from formalin-fixed, paraffin-embedded specimens, were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Endogenous peroxidase activity was blocked by hydrogen peroxide 3% for 15 min. The slides were immersed in an antigen retrieval solution to improve the quality of staining, and were then incubated overnight with a 1:25–1:50 dilution of the primary monoclonal antibody. Then, conjugation with the antibody was conducted using 30-min incubation with biotin and another 30-min incubation with avidin. Diaminobenzidine was used as a chromogen for color development. Burkitt lymphoma, breast cancer, and GIST were considered as positive controls for Ki67, c-erbB-2, and c-kit, respectively.

A standard light microscope was used. The IHC slides were interpreted in a blind fashion, without being aware of the benign or malignant nature of the tumor to minimize the bias error in studying the slides.

Ki67 expression in tumor cell nuclei, c-erbB-2 staining as the continuous circumferential membranous [5], and/or diffuse granular cytoplasmic [2] and cytoplasmic staining of c-kit were considered as positive immunoreactivity. The percentage of each antigen expression in the area of highest staining was considered as an average in 10 high-power fields (×400), which included at least 1000 tumor cells [5,6]. The cutoff point for the significant positive staining of Ki67 [6,10], c-erbB-2 [2], and c-kit [18] was considered 5%, 50%, and 5%, respectively.

Finally, the following parameters were noted: patient age at the time of diagnosis, gender, side (left or right), and the exact site of tumor (adrenal or extra-adrenal), tumor size, different histologic patterns (small cell, spindle cell, nestling, solid, and trabecular), and necrosis. The relationship between the expression of the studied antigens and the above-mentioned parameters, as well as tumor behavior, was analyzed using Pearson’s chi-square test (SPSS 15 software). The level of significance was set at 0.05.

Results

Age and gender

Table 1 shows the data related to age and sex of the patients. Patient age ranged from 8 to 70 years. There was no significant relationship between age or sex of the patients and tumor behavior (p-value > 0.05).

Location

The data on tumor location is presented in Table 1. Seven tumors, all of which were benign, showed bilateral adrenal involvement at the time of diagnosis. According to the patients’ files and family histories, we found out that none of these bilateral benign tumors was familial. Unfortunately, genetic analysis was not performed. There was no significant relationship between tumor location and its behavior (p-value = 0.866).

Histologic pattern

Table 2 presents the different histologic patterns in relation to the benign or malignant nature of the tumor. We found out that...
more uncommon tissue patterns, such as small cell and spindle cell patterns, are mostly indicators of malignancy \((p\text{-value}=0.04)\). It is worth mentioning that the tumors were categorized as small or with spindle cell histology if they consisted of at least 90 percent of small or spindle-like morphology. Only a careful examination revealed a more typical nesting pattern elsewhere in the tumor.

**Tumor necrosis**

The presence of necrosis in benign and malignant tumors was 31.8% and 63.6%, respectively. The difference was not statistically significant \((p\text{-value}=0.054)\).

**Immunohistochemistry**

Table 3 shows the frequency of positive IHC staining for Ki67, c-erbB-2, and c-kit antigens in benign and malignant tumors. All of the benign tumors showed less than 1% of proliferative activity, and tumors with a malignant behavior demonstrated more than 5% of Ki67-positive nuclei. There was a statistically significant relationship between Ki67- and c-erbB-2-positive staining of the tumor cells and the malignant behavior of the pheochromocytomas \((p\text{-value}=0.000)\), while cytoplasmic c-kit staining did not show any correlation with tumor malignancy \((p\text{-value}=0.087)\).

The increase in tumor size was correlated with the high possibility of Ki67-positive staining \((p\text{-value}=0.044)\). However, there was no relationship between tumor size and c-erbB-2 \((p\text{-value}=0.339)\), and c-kit \((p\text{-value}=0.679)\) markers.

Table 4 displays the frequency of positive staining of the studied markers, according to different histologic patterns. As it is obvious, Ki67-positive staining showed the best correlation with the spindle cell and small cell patterns \((p\text{-value}=0.000)\) while c-erbB-2- and c-kit-positive staining was mostly noted in the spindle cell histologic pattern \((p\text{-value}=0.01\) and 0.04, respectively).

Figs. 1 and 2 show different histologic patterns, as well as immunohistochemical staining of pheochromocytoma.

**Discussion**

For many years, the prediction of the behavior of adrenal or extra-adrenal pheochromocytomas has been a critical and controversial subject for both clinicians and pathologists. In this study, we evaluated the demographic (age and sex), gross (tumor side and size), and microscopic (tumor necrosis, histopathologic pattern) features, as well as immunohistochemical staining (Ki67, c-erbB-2, and c-kit), in order to predict the final destination of the tumor.

As revealed in our study, there are several reports that have also shown a positive correlation between the clinically malignant pheochromocytoma and tumor size \([4,19]\). However, Brown et al. \([9]\), who focused on and confirmed the relationship between Ki67 expression and the malignant behavior of pheochromocytoma, did not find any association between tumor size and metastatic potential.

Some studies focusing on the morphologic aspects of a large number of pheochromocytomas (a total of 110 cases) showed that tumor necrosis was significantly associated \((p=0.03)\) with its malignant behavior \([4,22]\). In our study, the lack of a prominent relationship \((p=0.054)\) may be due to the lower number of studied patients in proportion to the previous reports.

Studies investigating the correlation between different histomorphologic patterns and tumor behavior are limited. In one study, polygonal and round cells were the major cell types in benign and malignant pheochromocytomas, respectively \([8]\). According to our results, more uncommon microscopic patterns, such as small round and spindle cell types, were more prevalent in tumors with a malignant behavior. These features are the alarming signals for the pathologists, and clinicians should be more cautious regarding the possibility of malignancy. Strong et al. \([22]\) also stated that tumor cell spindling is significantly correlated with malignant behavior.

In recent years, the estimation of proliferative activities by Ki67 immunostaining has become noteworthy, especially in endocrine tumors. As the reproducibility of simple mitotic count is low and unacceptable, the evaluation of proliferative activities by DNA analysis or Ki67 immunostaining is more popular. In the case of pheochromocytoma, our recent findings agree with several previously published studies \([4,6,9–12,22,23]\), and this indicates a definite and significant association between Ki67 proliferative index and malignancy. In addition, we found out that tumors with a larger size and small round or spindle cell histologic patterns have a higher probability of Ki67-positive immunostaining. According to our study, both sensitivity and specificity of Ki67 for determining benignancy or malignancy are 100%.

The overexpression of c-erbB-2 gene has been shown to play an important role in the pathogenesis of some tumors, and is essentially correlated with poor prognoses. For example in breast cancer, overexpression of c-erbB-2 is one of the most valuable prognostic factors. Similarly, this gene has been found to be central in some endocrine tumors \([20,21]\). Evers et al. \([21]\) reported amplification of c-erbB-2 proto-oncogene in endocrine tumors of the gastrointestinal tract and postulated that the number of gene copies is an important prognostic factor.

In case of pheochromocytoma, the studies dealing with the significance of c-erbB-2 gene are limited and controversial. Castillo-Guerra et al. \([2]\) showed a statistical correlation between the expression of this oncogene and tumor behavior. Similar to our study, they noted high c-erbB-2 immunostaining positivity in pheochromocytomas with malignant behavior. On the other hand, Gupta et al. reported that c-erbB-2 overexpression was not seen in

### Table 3

<table>
<thead>
<tr>
<th>Tumor behavior</th>
<th>Tumor marker</th>
<th>Ki67 (No. (%))</th>
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<tbody>
<tr>
<td></td>
<td>c-erbB-2 (No. (%))</td>
<td>c-kit (No. (%))</td>
</tr>
<tr>
<td>Benign</td>
<td>39 (70.90)</td>
<td>5 (9.09)</td>
</tr>
<tr>
<td>Malignant</td>
<td>3 (5.45)</td>
<td>8 (14.54)</td>
</tr>
<tr>
<td>Total</td>
<td>42 (76.36)</td>
<td>13 (23.63)</td>
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* The percentage is calculated for the total of 55 cases.

### Table 4

<table>
<thead>
<tr>
<th>Tumor markers</th>
<th>Histologic patterns</th>
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<tbody>
<tr>
<td></td>
<td>Trabecular (%)</td>
</tr>
<tr>
<td>Ki67</td>
<td>7.1 12.5 4.3 80 80</td>
</tr>
<tr>
<td>c-erbB-2</td>
<td>7.1 50 8.7 80 40</td>
</tr>
<tr>
<td>c-kit</td>
<td>0 12.5 4.3 60 20</td>
</tr>
</tbody>
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Fig. 1. Different histologic patterns of pheochromocytoma, including: nesting (A), trabecular (B), solid (C), small cell (D), and spindle cell (E) patterns. (H&E, × 400).

Fig. 2. Positive immunohistochemical staining of pheochromocytoma for Ki67 (A), c-erbB-2 (B), and c-kit (C) markers.
any of the benign or malignant adrenal or extra-adrenal pheochromocytomas. In their study, c-erbB-2 gene did not seem to be of any use in predicting tumor behavior [5].

In the present study, c-erbB-2 gene was not only correlated with tumor behavior, but also was associated with the histologic pattern, i.e., pheochromocytomas with spindle cell morphology showed stronger c-erbB-2 positivity. Therefore, tumors with both of those features need more consideration regarding the possibility of their malignant behavior.

Positive and negative predictive values, as well as the efficacy of c-erbB-2 to differentiate benign and malignant adrenal and extra-adrenal pheochromocytomas, were estimated at 61.5%, 92.8%, and 85.4%, respectively.

Only few studies have evaluated c-kit expression in adrenal medullary tumors. In the study of Zhang et al., 20 adrenal and 20 extra-adrenal pheochromocytomas were immunostained for a c-kit proto-oncogene in which the positive staining was 14% and 8%, respectively [17]. Similar to our study, they did not find any statistically significant correlation between c-kit expression and tumor behavior. Gross et al. evaluated the role of Glivec, a selective inhibitor of c-kit tyrosine kinase, for treating patients with malignant endocrine tumors which expressed c-kit proto-oncogene, including 2 cases of malignant pheochromocytoma. They found out that this medication was not effective for the treatment of this group of patients, and also caused severe toxicity [24].

In summary, we evaluated adrenal and extra-adrenal benign and malignant pheochromocytomas. We concluded that tumor size and the histomorphologic patterns (spindle cell and small round cell) are significantly associated with tumor behavior. In addition, Ki67 positivity and c-erbB-2 expression can be used as immunohistochemical markers for the prediction of the malignant behavior of pheochromocytoma.

Most of the patients diagnosed as having pheochromocytoma usually leave without any special follow-up although it is known that 10% of this type of tumor is malignant. Therefore, in such cases with high probability of malignancy according to the above criteria, it is recommended to follow the patient closely and accurately or even to administer preventive therapies.

References