An approach to malignant mammary phyllodes tumors detection

Pristup otkrivanju malignih filodnih tumora dojke

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Abstract

Background/Aim. Mammary phyllodes tumors (MPT) are uncommon fibroepithelial (biphasic) neoplasms whose clinical behavior is difficult to predict on the basis of histological criteria only. They are divided into benign, borderline malignant and malignant groups. Sometimes it appears difficult to distinguish these tumors from other types of soft tissue sarcomas. Because of the relatively scant data on the role of biological markers in MPT histogenesis, we have decided to undertake the following study, trying to shed more light on the issue by investigating the following elements that make up MPT: their histological patterns, biological behavior, enzymohistochemical, histochemical and immunohistochemical characteristics (ICH) together with the mast cell analysis. Methods. We examined the biopsy material of 35 MPT in our laboratory. Enzymohistochemistry was performed on frozen sections (method of Crowford, Nachlas and Seligman). The used methods were classical hematoxylin-eosin (H&E); histochemical Masson-trichrome, Alcian-blue, Periodic acid Schiff and immunohistochemical LSAB2 method (DacoCytomation). Ki-67, c-Kit, vimentin, estrogen receptor (ER), progesterone receptor (PR) and Her-2 oncoprotein immunohistochemistry was performed on all tumors. Results. The patients were ranged per age from 30–62 years (mean 43.3 years, median 39 years). A total of 35 cases of MPT were included: 20 benign, 6 borderline malignant (17%) and 9 malignant (26%). Twenty-two patients (62.8 %) underwent segmental mastectomy, while 13 (37.2%) had total mastectomies. Twenty-eight patients had negative surgical margins at original resection. The mean size of malignant MPT (7.8 cm) was larger than that of benign MPT (4.5 cm). Significant features of the malignant MPT were: stromal cellularity, stromal cellular atypism, high mitotic activity, atypical mitoses, stromal overgrowth, infiltrative tumor contour and heterologous stromal elements. Benign MPT showed strong enzymohistochemical Leucine Amino Peptidase (LAP) activity in both epithelial and stromal components while it was weak or absent in the epithelial parts of the malignant tumors. Acid mucopolysacharides were present in the stromal component of all types of these tumors. Benign MPT had a lower Ki-67 than did borderline malignant MPT (4 versus 28). Malignant MPT had a greater than 8-fold higher Ki-67 activity than did benign tumors (35 versus 4). Intracytoplasmic c-kit expression was associated with a pathological diagnosis of malignant MPT, correlating with increasing grade (p < 0.05). In hypercellular stroma of borderline malignant and especially malignant forms of MPT, high activity of ER in mast cells was confirmed. Oncoprotein Her-2 activity, mostly in epithelial components, correlated with the degree of malignant progression of MPT (p < 0.05). Conclusion. Besides the well-known malignant features additional parameters have been found to be high Ki-67 and c-kit stromal expressions, and weak LAP activity in the epithelial part of malignant MPT, as well as mast cells with a high expression of ER.

Key words: phyllodes tumor; Ki-67antigen receptors, estrogen; receptors, progesterone; genes, erbB-2; mast cells; leucyl aminopeptidase; breast neoplasms; diagnosis

Apstrakt

Uvod/Cilj. Filodni tumori dojke su retki bifazični tumori, građeni od unmaženog epitela i vretenastočelijske strome. Teška diferencijalna dijagnoza maligne varijante od drugih sarkoma, kao i retki podaci u literaturi o dijagnoznom značaju onkogenih markera, opravdavaju proučavanje histoloških osobina, biološkog potencijala, enzimohistochemijskih, histochemijskih i imunohistochemijskih karakteristika filodnih tumora dojke. Metode. Histološkom, histochemijskom i imunohistochemijskom metodom na parafinskim presecima hirurških biopsija, kao i enzimohistochemijskom metodom na kriostatskim presecima, analizirano je 35 filodnih tumora dojke. Za imunohistochemijsko proučavanje korišćena su antitela na Ki-67, c-kit, vimentin, estrogenске receptore (ER), progesteronske receptore (PR) i Her-2 onkoproteine. Rezultati. Prosječna starost bolesnika iznosila je 43,3 godine. Od 35 filodnih tumora, bilo je 20 benignih, 6 graničnomalignih a 9 malignih. Kod 22 bolesnika uradena je segmentalna, a kod 13 totalna mastektomija. Kod 28 slučaje-
Introduction

Mammary phyllodes tumor (MPT) is a specific variant of breast tumor with benign, semi malignant and malignant features. It contains two predominant stromal or parenchymal parts of the tumor, thus phyllodes tumor is classified as biphasic tumor. The tumor was first reported with much more details in 1883 by Johannes Müller, but the first report regarding to its metastases was reported in 1993 by Lee and Pack. Modern molecular pathology, especially genetic studies, have been pointed out biphasic behavior of some breast tumor (fibroadenomas, phyllodes tumors). These investigations cleared up the first steps of tumorous stromal/epithelial proliferations. Both histochemical and immunohistochemical examinations of numerous authors confirmed the suggestion that in the pathogenesis of these tumors, very important role have an inactivity of differentiation factors, like the factors in embryogenesis (Wnt signaling), as well as the tumor suppressor genes. The result of these events is unusual expression of stromal enzymohistochemical parameters, for example an intensive leucine aminopeptidase (LAP) activity. The result of “wondering” in the differentiation during the genesis of phyllodes tumor is a true menagerie of ductal form, producing cysts. The “leaf-like” processes of mixoid stromal-epithelial tissue point out to wrong differentiation. The above pointed out facts are the reason for studding histological patterns, biological behavior and enzymohistochemical, histochemical and immunohistochemical characteristics of MPT including stromal mast cells analysis.

Methods

We examined the biopsy material of 35 MPT in our laboratory. Histological, histochemical and immunohistochemical examination was applied on formalin-fixed, paraffin-embedded tissue sections from MPT. Enzymohistochemistry was performed on frozen sections. The cases spanned a period from 1997 through 2007. The standard criteria used for distinguishing between benign, borderline malignant, and malignant MPT were applied. In addition to histological features, including the presence of stromal overgrowth, the presence of metaplasia, and mitotic activity, proximity to the resection margin was recorded as positive when there was tumor at the inked margin; as negative when the tumor was more than 1 cm from the margin; and as close when the tumor was within 1 cm of the margin. The frozen cryostat sections – 20 μC were used for the detection of LAP activity. Method of Crawford, Nachlas and Seligman, pH=6.5, by using L-Leucine-alpha-Naphtylamide as substrate, was applied for the examination of LAP activity. Positive reaction had intense red color inside of tumor cell cytoplasm. The following methods were used: classical hematoxylin-eosin (H&E); histochemical Masson-trichrome, Alcian blue-Periodic acid Schiff (AB-PAS) (pH 2.5) with and without diastase treatment; immunohistochemical LSAB method (DAKO Cytomation) and counterstaining with hematoxylin. The following methods were used: classical hematoxylin-eosin (H&E); histochemical Masson-trichrome, Alcian blue-Periodic acid Schiff (AB-PAS) (pH 2.5) with and without diastase treatment; immunohistochemical LSAB method (DAKO Cytomation) and counterstaining with hematoxylin was used. Formalin-fixed, paraffin-embedded tissue sections were cut at 5 μm and treated with 0.1 mol/L citrate, pH 6, in an 800-W microwave oven for 15 minutes for antigen retrieval before immunostaining. Monoclonal antibodies to Ki-67, c-kit (CD117), Vimentin, Estrogen receptor (ER), Progesterone receptor (PR) and to Her-2 (DAKO, Glostrup Denmark), 1:100 – 1:400 were used. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Nonspecific binding was reduced with a 10% normal goat serum block before incubation. Sections were scored by the three independent reviewers for the proportion and intensity of staining. Intensity was scored as follows: 3+, strong; 2+, moderate; 1+, weak; 0, no staining. The percentage of cells positive was scored by counting at least 50 cells in 10 high-power fields (HPFs, single field area of 0.20 mm²) and expressed in a 4-point scale (proportion score as follows: 0.4% or less; 1.5% to 33%; 2.34% to 66%; 3.66% or more). The combined immunoreactive score (CIS) was then calculated as the product of the intensity score and the proportion score, as described by Niezabitowski et al. Ki-67 was considered positive if nuclear staining of stromal cells was present. C-kit was considered positive if the cell membrane and cytoplasm of stromal cells were immunoreactive. For the detection ER and PR activity, American Society of Clinical Oncologists (ASCO) scoring system was used (from 0 to + 8) and for HER-2 oncoprotein expression, DAKO’s criteria were applied.

Kruskal-Wallis test was used in testing equality of expression in more than two groups. Wilcoxon test was used in...
comparison of marker expression between two groups. Spearman correlation coefficient and the test of independence were calculated to measure the correlation between different variables.

Results

We have shown the clinical and pathologic characteristics of MPT in 35 female patients: 20 (57%) benign, 6 (17%) borderline malignant and 19 (26%) malignant. Patients ranged in age from 30–62 years (mean 43.3 years, median 39 years). Twenty-two patients (62.8 %) underwent segmental mastectomy, while 13 (37.2%) had total mastectomies. Twenty-eight patients had negative surgical margins at original resection (57% benign, 17% borderline malignant, 26% malignant); there was surgical “clearance” on the distance to 2 cm. Seven patients had not data about margins. Follow-up information for seven patients was unavailable. Three patients experienced recurrent disease locally (at follow-up period), from 1 to 2 years from the borderline malignant group. The tumors were reexcised. Two patients (malignant group) had died after 2 years from the time of MPT diagnosis. Other patients are alive and they are followed-up.

Grossly, all of the tumors were well circumscribed, with a yellowish, lobulated, moist, sometimes mixoid nodules with characteristic leaf-like endings of different shape and with rare cystic areas filled with gelatineous-mucinous content (Figure 1). Some of them had areas of necrosis and punctuate hemorrhage. Malignant MPT were softer than benign tumors. The diameter of the smallest MPT was 1.5 cm but the largest involved the entire breast (18 cm in diameter). The mean size of malignant MPT (7.8 cm) was larger than that of benign MPT (4.5 cm). The histological hallmark of all phyllodes tumors was stromal hypercellularity and overgrowth. Benign MPT were predominantly of fibroadenomatous architecture with a mild cellular stroma and some distortion and elongation of glandular elements. Benign MPT were sometimes difficult to distinguish from fibroadenomas, with or without minimal atypia and up to two stromal mitotic figures per 10 HPF. Mammary phyllodes tumor contained numerous deformed ducts of unusual bizarre shapes in the depth (known like “animal menagerie” of ductal changes) covered with double-layered epithel (Figure 2). The stromal component was prominent and could show morphologic patterns that ranged from fibroadenoma-like to frankly sarcomatous. Metaplastic lipocytic and myxoid foci of the stroma were observed. Neither perineural nor vascular invasion was evident in any MPT. Significant features of the malignant MPT, for example stromal cellularity, stromal cellular atypism, high mitotic activity (in one case signet ring cell pattern) (Figure 3), atypical mitoses, stromal overgrowth, in-

Fig. 1 – Mammary phyllodes tumor: gross pattern

Fig. 2 – “Menagerie” of ductal changes in benign mammary phyllodes tumor (H&E, × 40)

Fig. 3 – Epithel of dillatated ductus in malignant mammary phyllodes tumor with “signet ring cell” pattern (H&E, × 200)

filtrative tumor contour and heterologous stromal elements, have been found. Apocrine and squamous metaplastic lesions were seen in 12 cases of MPT, in one case forming of real epidermal cyst (Figure 4). In three cases of malignant MPT we found a focal presence of moderate epithelial dysplasia (Figure 5).

Histochemical analysis of MPT showed the presence of acid mucopolysacharides in the stromal component of all types of these tumors (Figure 6). Benign MPT showed strong LAP activity in both epithelial and stromal components (Figure 7). Activity LAP was weak or absent, especially in the epithelial parts of the malignant tumors.

Immunohistochemical staining revealed nuclear localization of Ki-67 protein in all groups of the tumors in the epithelial and in the stromal cells. But, analysis of the three groups of MPT showed a significant difference between benign, borderline malignant and malignant ($p < 0.05$). Benign MPT had a lower Ki-67 than did borderline malignant MPT (4 versus 28) (Figure 8). Malignant MPT had a greater than 8-fold higher Ki-67 activity than did benign tumors (35 versus 4) (that Ki-67 expression correlates with the histological classification). C-kit stromal cell immunoreactivity was observed in 10% of benign MPT, 55% of borderline malignant MPT, and 69% of malignant MPT. Intracytoplasmatic c-kit expression was associated with the pathologic diagnosis of malignant MPT, correlating with increasing grade ($p < 0.05$) (Figure 9). Expression of epithelial ER was positive only in the ductal epithelium of the benign MPT, by the principle that every active Nul cell of the luminal layer was sharp demarcated from adjacent steroid receptor nonactive epithelial cells (“skip lesion”) (Figure 10). However, when considering borderline malignant MPT, the expression of hormonal PR proteins was high and in continual arrangement, along the whole ductal luminal layer. The stromal hypercellularity of the borderline malignant and malignant tumors have showed high ER activity in hyperplastic mast cells (Figure 11). Oncoprotein HER-2 activity has been observed in the epithelial component of this tumor and it was in correlation with pathologic grade of MPT ($p < 0.05$) (Figure 12).
Discussion

Mammary phyllodes tumor are biphasic neoplasms composed of a double-layered epithelial component arranged in cleftlike ducts surrounded by a hypercellular spindle-cell stroma. Currently, MPT are classified as benign, borderline malignant, or malignant based on histopathologic features. However, prognostic assessment of cellular periductal stromal tumors based solely on the above histological classification continues to be problematic. Histologically, benign MPT have been reported to have metastasized, whereas many histologically malignant tumors have neither recurred nor metastasized. Although local recurrences or metastases of seemingly benign tumors can be attributed to inadequate sampling for microscopical evaluation or to inadequate assessment of resection margins; these circumstances cannot account for all clinicopathologic inconsistencies in the literature. Attempts to correlate various gross and microscopical features of MPT with clinical behavior have not been uniformly successful in predicting the clinical outcome of these tumors. Recent studies have suggested that immunohistochemical analyzes of both Ki-67, a proliferation marker, and c-kit proto-oncogene (encodes the tyrosine-kinase receptors) may be implicated in predicting behavior of MPT. Our immunohistochemical staining revealed nuclear localization of Ki-67 protein in all groups of tumors in the epithelial and in the stromal cells. Our results support those of the others that Ki-67 protein expression correlates with the histological classification of MPT, but is not in correlation with clinical behavior.

The reported finding that both c-kit and Ki-67 proteins were predominantly expressed by the stromal cells immediate beneath the epithelium indicates that this layer contains the proliferative pool of cells resulting in the stromal hypercellularity characteristic of MPT and supports the hypothesis that MPT arise from periductal rather than intralobular stromal cells. We also have discovered the presence of the great quantities of acid mucopolysaccharides (Alcian blue +) in the stroma of the malignant groups of MPT, pointing out their intralobular stromal origin.

In order to overcome the disagreement between the histopathologic finding and clinical behavior, it is important to use “forgotten enzymohistochemical” and histochemical methods. The exopeptidases, especially LAP is capable to show the degree of organization or disorganization of MPT stroma. It is well known that the very strong activity is primarily the main characteristic of benign MPT group, while its activity disappears completely in the malignant group, especially in the parenchyme parts of the tumors; it seems, like that LAP activity moves itself from the parenchyme to the stromal part of the tumor. The other authors have reported that the stromal endothelin-1, inducing VEGF, is the significant factor in neoangiogenesis, and activating matrix-metalloproteinases it helps the invasiveness of MPT tumors. Additionally, the role of many hormones, especially the estrogen, on the stromal component of the breast tumors, is well known. Our results on discontinual ER positive expression in the epithelial ductal normal cells and in the atypical columnar cell lesion (ACCL) proliferations has not been described and explained in the literature that we used. This finding could be explained either by presence of less differen-
entiated ductal epithelial cells (like the stem cells of the ductulolobular segment, CAP cells and similar), or by mast cell numerous functions. Namely, mast cells produce and release various mediators, which in turn affect cell proliferation, desmoplasia and angiogenesis. Mast cell degranulation has been demonstrated to be a common feature in later stages of tumor proliferation. Once tumor-mediated degranulation occurs, mast cells lose their ability to regulate it. We found periductal rather than intralobular mast cell stromal localization which can be linked to the findings of other authors, that c-kit stromal expression is a mast cell phenomenon. Strletssova and Pavlenko-Mikhailov described the highest number of mast cells at the moment of benign tumor formation, a second most intensive rise during malignant transformation of the epithelium, and a decrease in the number of the mast cells following the moment of malignant transformation.

These findings are in line with our results that only ER+ stromal mast cells could be demonstrated, stimulating tumor growth and allowing the benign MPT to progress to malignant. In benign MPT variants we have not demonstrated the positive expression of HER-2, it has not been identified in stromal cells as well, which is in keeping with the above-mentioned data.

Conclusion

Positive surgical resection margins are the only parameter which can predict tumor recurrence. Besides well-known malignant features, additional parameters are: high Ki-67 and c-kit stromal expression, and weak LAP activity in epithelial part of malignant MPT, as well as mast cells with high expression of ER.

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