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Immunohistochemical study of Aquaporin-1, Cyclooxygenase-2 and Apoptosis Protease-Activating Factor-1 expression in breast cancers. Preliminary study

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ABSTRACT
The aim of the present study is to establish possible associations between Aquaporin-1, Cyclooxygenase-2 and Apoptosis Protease-Activating Factor-1 expression in breast cancers and pathological and immunohistochemical characteristics of the examined tumors.
For the purpose of this study we used paraffin embedded archived tumor material of 31 breast cancer patients from the Pathology Department of the Odorheiu Secuiesc Municipal Hospital. We performed immunohistochemistry reactions ER, PR, HER2, AQP1, COX2 and APAF1, and following independent evaluation by two pathologists the obtained data was statistically analyzed. The tumors were divided into three groups based on their histological properties, and correlations were made with the examined markers.
AQP1, COX2 and APAF1 immunostaining results produced significant correlations with HER2 status and histological groups. There were no statistical correlations between ER or PR status and the three examined markers.
Lobular carcinomas showed AQP1 and COX2 overexpression, and loss of APAF1 expression, which all correlated with HER2 negative status.
We concluded that AQP1 could be a useful marker for detecting more aggressive subtypes and also for evaluating tumor angiogenesis. COX2 and APAF1 immunoexpression, although somewhat specific to certain histological groups, needs to be further characterized in order to be a useful marker for the clinical setting.
Keywords: breast cancer, AQP1, COX2, APAF1

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Introduction

Breast cancer is a heterogeneous disease that can be classified using different clinical and pathological features. Classification can provide aid in establishing prognosis and it can also be used as a predictive marker for therapeutic success [1]. There are well established immunohistochemical marker panels [estrogen receptor (ER), progesterone receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2/Neu) and basal markers (eg. cytokeratins)] currently in use for patient stratification.

However, there are other promising markers for further refining the classification provided by the conventional ones.

Aquaporins (AQP) belong to the major intrinsic protein family of small, transmembrane, channel-forming glycoproteins that facilitate rapid water transport across biological membranes. AQP expression has been reported in a variety of human malignancies, e.g. tumors of the brain, prostate, breast, ovary, colon and lung [2]. Aquaporin water channels are expressed in high-grade tumor cells of different tissues. It has been suggested that AQP1 facilitates tumor cell migration and spread, thus increasing their metastatic potential [3].

Cyclooxygenase (COX) exists in two isoforms: COX1 and COX2, which are regulated independently. COX1 is expressed in normal tissue, whereas COX2 is expressed in various human malignancies, like colon and breast cancer [4]. COX catalyzes the conversion of arachidonic acid into prostaglandins and increased levels of prostaglandins are associated

with carcinogenesis [5]. COX isoenzymes are targeted by non-steroid anti-inflammatory drugs (NSAIDS), and it has been suggested that use of NSAIDS may be associated with a decrease in breast cancer risk [6].

The Apoptosis Protease-Activating Factor-1 (Apaf-1) is a central component of the mitochondrial apoptosis pathway [7]. In addition, APAF-1 is a key element of developmental programmed cell death [8]. The role of Apaf-1 as an initiator of apoptosis induced by several different anti-cancer agents suggests that the expression of this protein may play an amplifier role in the apoptotic response. The decreased expression of Apaf-1 has been correlated with melanoma progression [7]. In certain colorectal cancers, APAF-1 plays a role in tumor progression and survival. Loss of APAF-1 expression is a marker of adverse outcome in MutL homolog-1 (MLH1) negative colorectal cancer, and appears to affect survival time independently of known prognostic indicators [8].

The aim of the present study is to establish possible associations between AQP1, COX2 and APAF1 expression in breast cancers and pathological and immunohistochemical characteristics of the examined tumors.

Material and methods

For the purpose of this study we used paraffin embedded archived tumor material from the Pathology Department of the Odorheiu Secuiesc Municipal Hospital. The selected cases were diagnosed with different types of breast cancer between 2007 and 2010. The initial selection was aimed at describing the clinicopathological and immunohistochemical correlations of breast cancers in relatively young patients of ages under 50 years. This remained our main selection criterion, and eventually there were 31 breast cancer cases selected for the study, all females. In order to test and optimize the immunohistochemistry reactions we also used two cases of fibroadenoma and two specimens of normal breast tissue.

3 μm thick sections were mounted on silane coated microscope slides. After individually optimized antigen retrieval immunostaining was performed using commercially available antibodies. The antibodies we used are as follows: anti-ER [clone Ab11, Thermo Fisher Scientific (Waltham, USA), dilution 1:100, antigen retrieval 30 min 95°C water bath, citrate buffer, pH 6.0], anti-PR [clone SP-2, Thermo Fisher Scientific (Waltham, USA), dilution 1:100, antigen retrieval 30 min 95°C water bath, citrate buffer, pH 6.0], anti- HER2/neu [clone PN2A, DAKO (Glostrup, Denmark), dilution 1:400, antigen retrieval 60 min 95°C water bath, citrate buffer, pH 6.0], anti-AQP1 [clone 1/22, Histopathology Ltd. (Pécs, Hungary), dilution 1:600, antigen retrieval 50 min 95°C water bath, citrate buffer, pH 6.0], anti-COX2 [clone CX-294, DAKO (Glostrup, Denmark), dilution 1:100, antigen retrieval 50 min 95°C water bath, citrate buffer, pH 6.0] and anti-APAF1 [clone NCL-APAF1, Leica Biosystems Newcastle Ltd. (Newcastle, UK), dilution 1:600, antigen retrieval 50 min 95°C water bath, citrate buffer, pH 6.0]. The staining was performed manually according to standard protocols using the Ultravision Labeled Polymer system (LabVision, Fremont, CA, USA). Reaction products were developed using diamino-benzidine (DAB). Each reaction was tested against positive controls with tissue types specific for each antibody. Negative controls were performed by omitting the primary antibody.

Tumor type and grade were evaluated independently two pathologists, and discordant cases were discussed using a multiheaded microscope until agreement was established. The same pathologists performed the evaluation of the immunostained slides, and the same protocol was followed. In case of ER, PR, AQP1, COX2 and APAF1 the reaction was interpreted as positive if >10% of the cells displayed immunohistochemical staining. For the assessment of HER2 expression the DAKO score was used, meaning that a moderate or strong complete circumferential membranous staining of >10% of the tumor cells was considered as HER2 overexpression.

The staining results were classified as positive or negative based on the mentioned cut-off values. Statistical analysis was performed using Graph Pad In Stat 3 (v. 3.06) statistic calculation software

(GraphPad Software Inc., San Diego, U.S.A.). We considered the association significant when $p<0.05$, with 95% confidence interval.

Results

The studied tumors consisted of ductal carcinomas (G1 3 cases, G2 6 cases and G3 11 cases, and a combined ratio of 64.51%) and lobular carcinomas (11 cases, 35.49%). As there are major differences in the behavior of ductal carcinomas based on their grade, we stratified the population into low grade (G1+G2) ductal carcinomas (29%), high grade (G3) ductal carcinomas (35.5%) and lobular carcinomas (35.5%).

Immunoexpression of the studied markers

AQP1 staining was localized to the nuclear membrane of malignant cells (Figure 1A), while it did not show up in the normal components of the breast. Cytoplasmic staining was disregarded as false positive because the localization of the AQP proteins binds them to membranes. As expected, the marker also stained the cell membranes of endothelial cells. In case of the fibroadenoma control tissue, we noted the specific expression of AQP1 in the myoepithelial cells of the glands (Figure 1B).

The majority of the tumors (71%) displayed AQP positivity. In correlation with estrogen receptor/ progesterone receptor (E/P) expression, AQP1 expression was positive in 58% of the E/P+ cases, and it was negative in 19.3% of the E/P+ cases. When correlated with histological type groups, the AQP1+/E/P+ cases displayed a higher proportion in case of the high grade ductal carcinomas and lobular carcinomas, with 54.5% and 72.7% respectively.

When AQP1 expression was compared to HER2 expression, and correlated with histological

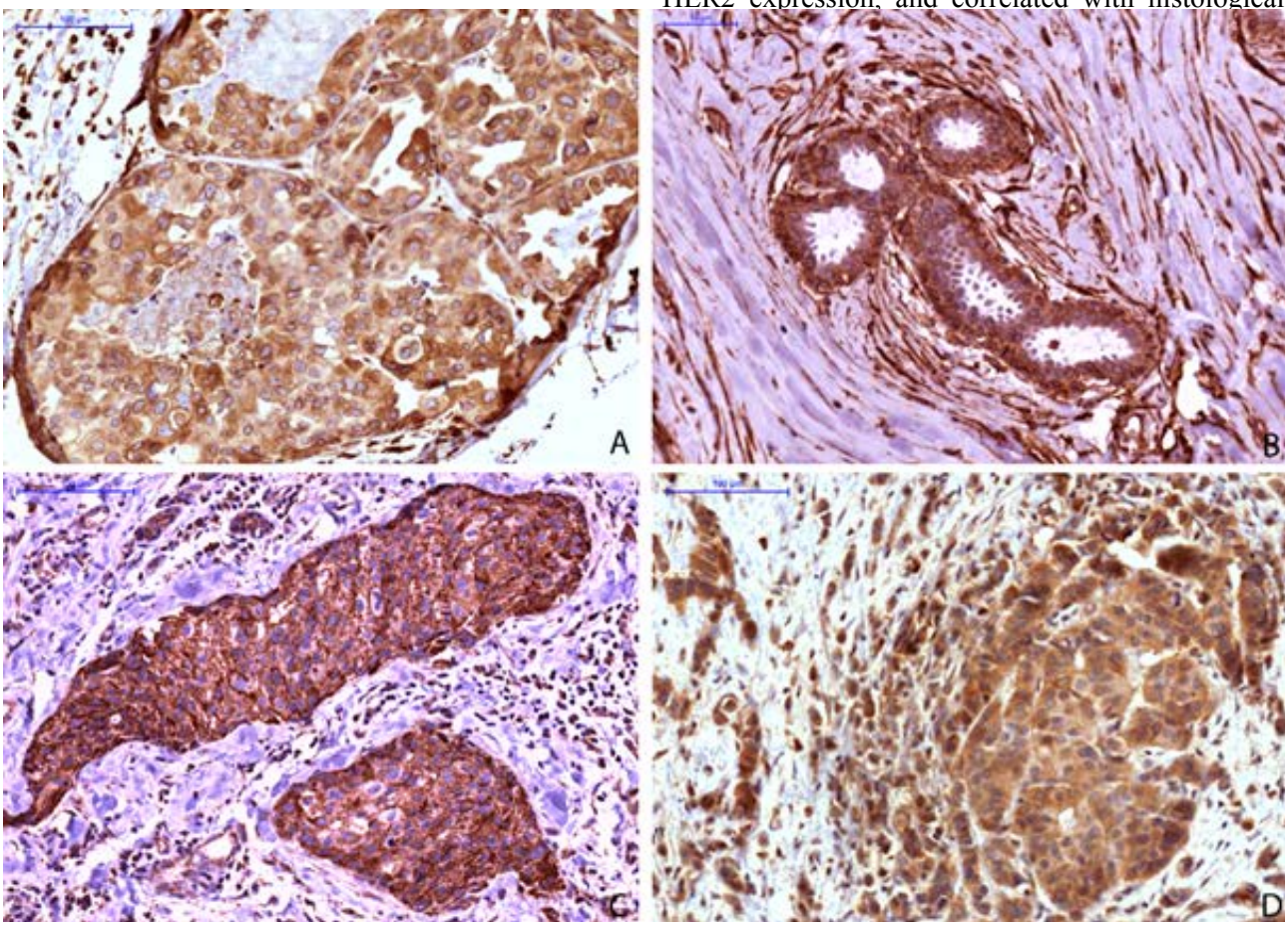

Figure 1 – A) AQP1+ reaction, 20x; B) AQP1+ reaction of myoepithelial cells, 30x; C – COX2+ reaction, 20x; D – APAF1+ reaction, 20x

groups, we noted that the majority of the lobular carcinomas were AQP+/HER2- (72%), and the AQP1+/HER+ immunephenotype was more characteristic to high grade ductal carcinomas (54%). There was a significant correlation towards negative HER2 expression in the lobular group (Figure 2).

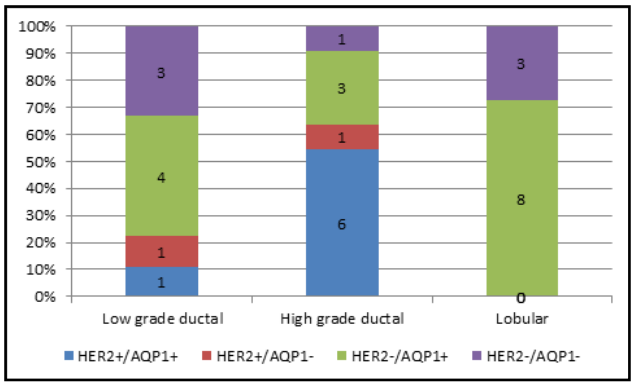

Figure 2 - Correlation of AQP1/HER2 expression with histological groups

COX2 staining appeared as expected as small cytoplasmic conglomerates throughout the malignant cells. It also stained the inflammatory cells seen throughout the tumor stroma. COX2 was positive in 71% of the E/P+ cases, and in 22.5% of the E/P- cases, resulting in a predominance of COX2 positivity for the majority of the studied population. This predominance was maintained in correlation with histological groups as the large majority of lobular carcinomas (81.80%) were COX2+/EP+, and the low and high grade ductal carcinomas presented a percent ratio of the same immunephenotype of 77.70%, and 54.50%, respectively.

Correlating COX2 expression with HER2 expression and histological groups, we noted a significant correlation in the distribution of staining patterns (p=0.002). The dominating immunephenotype was COX2+/HER2-, occurring in 91% of the lobular carcinomas, and in 88% of the low grade ductal carcinomas (Figure 3).

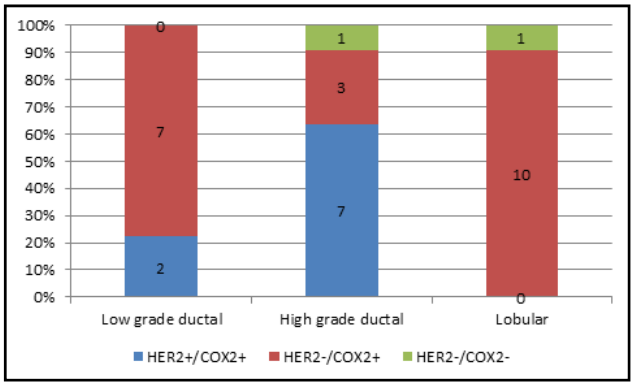

Figure 3 - Correlation of COX2/HER2 expression with histological groups

In case of APAF1 staining (Fig. 1D) the results were heterogeneous. The cytoplasmic staining pattern appeared consistently in the well differentiated and normal epithelial components of the breast. We observed consistent loss of expression in poorly differentiated areas of breast carcinomas. Comparing to EP expression, the distribution of APAF1+ and APAF1- cases was almost equal, with 38.7% APAF1+/EP+ cases, and similarly 38.7% APAF1-/EP+ cases. In correlation with the histological groups lobular carcinomas displayed mostly the APAF1-/EP+ (63%) immunephenotype, and the same appeared most frequently in the high grade ductal group (36%). The low grade ductal carcinomas comprised mostly APAF1+/EP+ cases (66%).

The distribution of APAF1 immunoexpression correlated significantly with HER2 immunoexpression and histological groups (p=0.015). Low grade ductal carcinomas displayed the APAF1+/HER2- immunephenotype in 55%, high grade ductal carcinomas were APAF1-/HER2+ in 36%, and in case of the lobular tumors the predominant immunephenotype was APAF1-/HER2- (72%) (Figure 4).

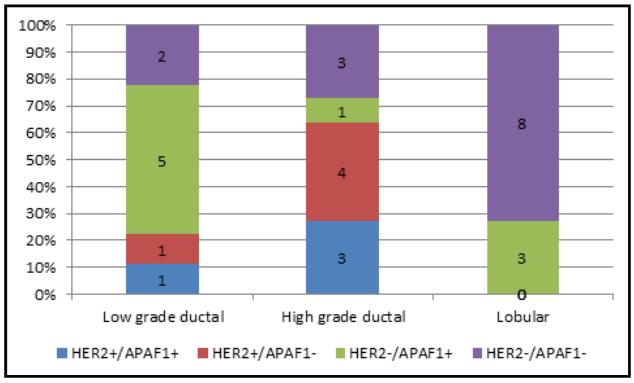

Figure 4 - Correlation of APAF1/HER2 expression with histological groups

The AQP1, COX2, APAF1 and HER2 results are presented according to histological group in correlation with E/P receptor (Table I) and HER2 (Table II) immunoexpression.

Table I - AQP1, COX2, APAF1 and HER2 distribution according to histological group and E/R receptor status

HER2 and E/P receptor status showed a significant correlation (p=0.02). The majority of the tumors were HER2-/EP+ (64.5%), and this immunephenotype made up 91% of the lobular carcinomas (Table I).

Discussions

Histological classification of breast cancers is used as one of the prognostic factors for determining the odds of short and long term overall survival and disease free survival. Lobular carcinomas and high grade ductal carcinomas are considered to be high risk conditions for metastasis and poor prognosis.

Table I - AQP1, COX2, APAF1 and HER2 distribution according to histological group and E/R receptor status

	LOW GRADE DUCTAL		HIGH GRADE DUCTAL		LOBULAR		p
	E/P+	E/P-	E/P+	E/P-	E/P+	E/P-	
AQP1+	4	1	6	3	8	0	P=0.5
AQP1-	3	1	1	1	2	1	
COX2+	7	2	6	4	9	1	P=0.5
COX2-	0	0	1	0	1	0	
APAF1+	6	0	3	1	3	0	P=0.1
APAF1-	1	2	4	3	7	1	
HER2+	1	1	3	4	0	0	*P=0.02*
HER2-	6	1	4	0	10	1	

Table II - AQP1, COX2 and APAF1 distribution according to histological group and HER2 receptor status

	LOW GRADE DUCTAL		HIGH GRADE DUCTAL		LOBULAR		p
	HER2+	HER2-	HER2+	HER2-	HER2+	HER2-	
AQP1+	1	4	6	3	0	8	*P=0.02*
AQP1-	1	3	1	1	0	3	
COX2+	2	7	7	3	0	10	*P=0.002*
COX2-	0	0	0	1	0	1	
APAF1+	1	5	3	1	0	3	*P=0.01*
APAF1-	1	2	4	3	0	8	

Positive E/P receptor status correlates with favorable prognosis, lower rate of cell proliferation and histologically evidenced tumor differentiation. Immunohistochemical characterization is also a predictive factor for establishing the applicability of hormonal or adjuvant therapies alongside surgery [9]. Our results showed that a significantly higher proportion of high grade ductal carcinomas and lobular carcinomas were E/P+. According to Henderson and Patek [10] there is an inverse relationship between E/P expression and tumor grade. This difference is probably caused by the selection of our population, regarding age as the primary criterion. At the same time, our E/P+ cases were HER2- in 64.5% of the cases, which would indicate a worse prognosis and no clinical benefit from adjuvant targeted therapy.

The above mentioned markers are currently used in the clinical setting, but there are still debates about the true value of these in the management of individual patients [10]. AQP1, COX2 and APAF1 expression of these tumors can provide further guidance in this matter.

AQP1 is expressed in a variety of cancers, including breast cancer, and it also appears in the vascular components of tumors, signaling its role in tumor angiogenesis [11]. Otterbach et al. demonstrated that AQP1 expression correlates significantly with high tumor grade and poor prognosis in ER- patients [2]. Our results also showed predominant AQP positivity in the high grade ductal and the lobular groups, but the majority of these cases were E/P+. This demonstrates that AQP1 expression is not limited to ER- cases, and it should be taken into consideration for the prognosis of these patients as well. This is further supported by our finding that there is a significant correlation towards negative HER2 expression in the lobular group.

Regarding COX2 expression our results showed that it was positive in the majority of the E/P+ cases, and the COX2+/EP+ immune phenotype appeared majority of lobular carcinomas (81.80%). In addition 91% of the same histological group displayed COX2+/HER2- characteristics. There is a general consensus about COX2 positivity of cancers in general. There are reports that COX2 overexpression associates with higher histological grade, and the intensity of the reaction correlates with tumor size, but no significant relationships could be established between other clinicopathological factors or immunohistochemical characteristics [12,13,14]. Their general conclusions are that the cyclooxygenase-2 isoenzyme may be a target for the prevention and treatment of breast cancer. Our results support the fact that COX2 expression is characteristic to breast carcinomas.

In our study APAF1 expression was distributed without any significant difference to all histological groups. Nevertheless, we obtained significant correlation between APAF1 and HER2 immunoexpression and histological groups, where the most prominent feature was the APAF1-/HER2- immune phenotype occurring in 72% of lobular carcinomas. In the past decade there were several reports suggesting decreased or absent APAF1 function in malignant diseases or cell lines, like metastatic malignant melanoma [15], leukemia [16] or ovarian cancer cells [17]. Leo C. et al. found significant correlation between loss of APAF1 expression and lymph node metastases in cervical cancer. However, there were no other data showing significant correlation [18]. We also demonstrated that APAF1 immunoexpression was characteristic to particularly aggressive HER2- lobular carcinomas.

Conclusions

Correlation of AQP1 expression with the classical immunohistochemical markers accepted as standards for classification of breast cancers may prove useful in detecting more aggressive subtypes and also for evaluating tumor angiogenesis. COX2 and APAF1 immunoexpression, although somewhat specific to certain histological groups, needs to be further characterized in order to be a useful marker for the clinical setting. Nevertheless, both of the latter hold the potential to become new targets for anticancer therapy.

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