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Correlation of carbonic anhydrase IX expression with clinico-morphological parameters, hormonal receptor status and HER-2 expression in breast cancer

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The hypoxia-inducible protein carbonic anhydrase IX is widely expressed in most human cancers, including breast carcinomas. CA IX attracts significant interest due to its strong association with neoplasms and its absence from corresponding normal tissues, suggesting its potential to serve as a promising diagnostic biomarker. This protein comes into the limelight also as a valuable prognostic and predictive parameter. Immunohistochemically, we examined the expression of this protein in 84 cases of invasive breast carcinoma to determinate the association with clinico-morphological and biological parameters such as age of patients, grade, stage and size of primary tumor, lymph node metastasis, vascular invasion as well as hormone receptor status and HER-2 expression. In each case, the subcellular localization of CA IX antigen, the intensity of staining and the percentage of labeled cells were assessed. Overall, CA IX was expressed in 34 cases (40.5%). The statistical analysis revealed a significant correlation between subcellular localization of CA IX and the age of patients. Furthermore, significant correlations were also found between the grade, estrogen and progesterone status and all immunohistochemical characteristics of CA IX expression (the subcellular localization of CA IX antigen, the intensity of staining and the percentage of labeled cells). We point out that mostly membrane or combined membrane and cytoplasmic positivity together with a higher intensity of CA IX immunoreactivity are associated with poor prognostic parameters, such as tumor grade 3 and also with negative estrogen and progesterone receptor status which may influence therapeutic approach. However, no significant correlations were shown with remaining clinico-morphological and biological parameters. We next investigate the relationship between CA IX expression in the group of invasive ductal carcinomas and the group of invasive lobular carcinomas and other less frequent types of breast carcinoma. There was, however, no significant difference. Our results suggest that moderate to strong membrane and combined membrane and cytoplasmic localization of CA IX may represent a valuable tumor biomarker as well as a promising prognostic and predictive parameter in invasive breast cancer.

Key words: breast carcinoma, carbonic anhydrase IX, immunohistochemistry, clinico-morphological and biological parameters

Breast cancer accounts for most frequent malignant tumors in female patients, and its incidence keeps increasing. Because of the multiplicity of oncogenic pathways, some carcinomas may behave extremely aggressive. Therefore, new markers detecting this aggressivity are urgently needed [1]. Rapid proliferation of tumour cells and angiogenic stimulus in tumors result in an abnormal and chaotic blood supply, which does not adequately or consistently supply the whole tumor with oxygen and nutrients [2]. Additionally, severe anaemia can lead to the development of tumor hypoxia [3,4]. Hypoxia is present in most of solid tumors and is associated with resistance to radiotherapy and chemotherapy as well as a more malignant phenotype [5]. This phenomenon correlates with poor prognosis [6]. Because of the important medical significance of hypoxia in tumors, there is an ongoing search for reliable biomarkers of this condition as they would represent both a valuable diagnostic marker and a potential therapeutic target. In this category, carbonic anhydrase IX (CA IX) has recently emerged as one of the most promising markers of cellular hypoxia [7]. This protein is a highly active member of the carbonic anhydrases family with an ability to catalyze efficiently the reversible hydration of carbon dioxide to carbonic

acid [8,9]. There are fifteen human CA isoforms that exhibit variable degrees of enzyme activity and differ by molecular characteristics, distribution in tissues, subcellular localization, expression levels and kinetic properties [10]. There are only two CAs that can be reasonably designated as cancer-related proteins: CA IX, which is almost exclusively associated with tumors, and CA XII, which is overexpressed only in some types of tumors. During the past decade, the protein CA IX has attracted a lot of attention, not only as a functional component of tumor physiology, but also as a tumor biomarker and potential therapeutic target [11]. CA IX attracts a significant interest due to its strong association with neoplasms and its absence from corresponding normal tissues, suggesting its potential to serve as a promising tumor biomarker [12]. The purpose of this study is to evaluate CA IX expression levels in breast carcinoma tissue by using immunohistochemistry and to determine the correlation between CA IX expression and histomorphological parameters, as well as expression of hormonal receptors (estrogen and progesterone) and HER-2 (human epidermal growth factor receptor 2, c-erbB2). Breast cancer is a heterogeneous disease with variable clinical presentation, histomorphological parameters and biological behavior. The particularity of this serious disease among other oncological pathologies is caused by specific genetic and epigenetic alterations, triggering initiation of carcinogenesis and progression of tumor growth. The traditional morphological (e.g. tumor size, grade, stage, vascular invasion, regional nodal status) and biological parameters (hormone receptors and HER-2 expression) are commonly used in assessment of the prognosis and therapeutic response. However, it was proved that these conventional features lack the power to reveal the true tumor heterogenity, individual malignant growth variability and appropriate therapeutic management [13,14]. The possibility to obtain the new prognostic and predictive information is very needed. Protein CA IX have been found to be representing a new promising prognostic as well as a predictive factor.

Materials and methods

The study was approved by the local Ethics Committee of Jessenius Faculty of Medicine in Martin, registered in Office for Human Research Protection, U.S., Department of Health and Human Services under No: EK 1384/2013.

Immunohistochemical staining. Each representative paraffin block was cut into 4 μm-thick sections subjected to immunohistochemical staining, where two sections from each were stained for detection of the CA IX protein. All of these sections were deparaffinized in xylene for 20 minutes, rehydrated at decreasing ethanol concentrations. The endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes. No antigen retrieval was necessary for CA IX staining. The mouse monoclonal antibody (M75) was used to detect the CA IX antigen. The primary antibody was incubated for 1 hour at room temperature followed by a 30-minute incubation with a peroxidase-conjugated secondary antibody. After incubation, slides were washed twice with Tris-buffered saline Tween for 10 minutes. The immunoreaction was visualized using 3,3´- diaminobenzidine (DAB) chromogen (Dako) and 3-amino-9-ethylcarbazole (AEC) chromogen (Dako). All sections were counterstained with hematoxylin (Dako). Negative controls were obtained by omitting the primary antibody and positive controls were realized on the epithelia of the stomach. CA IX antibody stained sections were examined under light microscopy. In each case, the following features were evaluated:

1) intensity of staining scored as weak (+), moderate (++), and strong (+++),
2) relative number of positively stained cells: less than 11%, 11-50%, and more than 50% per field of view,
3) subcellular localization of CA IX antigen: membrane (M), cytoplasmic (C), or both (MC).

To achieve good reproducibility, all assessed parameters were evaluated semi-quantitatively by two independent observers (MF, MA), who scored them using unified and clear cut-off criteria. The age of patients, stage, grade and size of the tumor, vascular invasion, lymph node metastasis, estrogen and progesterone receptors status were designated as clinicomorphological and biological parameters.

Statistical analysis. Statistical evaluation was performed with Microsoft Excel software package. Chi-square (χ^2) test was used to demonstrate the correlation between CA IX expression and clinicopathological parameters of breast cancer cases included into our study group. Fisher´s exact test (two tail) was used to evaluate the statistically significant difference between morphological types of breast carcinoma in CA IX expression. P value less than 0.05 was considered to indicate a statistical significance.

Results

Tissue and patient data. Formalin fixed paraffin embedded tissue samples of 84 invasive breast carcinomas were enrolled to our study. All tissue samples were taken from the patients during years 2013/2014. The clinical and pathologic parameters were collected from the surgical pathology reports. These included patient age, grade, stage and size of the tumor, lymph node metastasis, vascular invasion, estrogen receptor (ER) and progesterone receptor (PR) status, HER-2 expression. Histological type was revised according to the World Health Organization. Patients were female aged between 37 and 87 years at the time of tissue biopsy (mean age, 61.1 years). The size of the carcinomas varied from 0.1 to 9.5 cm (mean, 4.8 cm). Metastatic breast cancer was present in 34.8 % of the lymph nodes resected in these patients.

Evaluation of immunohistochemical staining. In our group of 84 carcinoma cases, carbonic anhydrase IX was expressed in 34 cases (40,5%). The positive cases showed a variable subcellular localization. We found three patterns

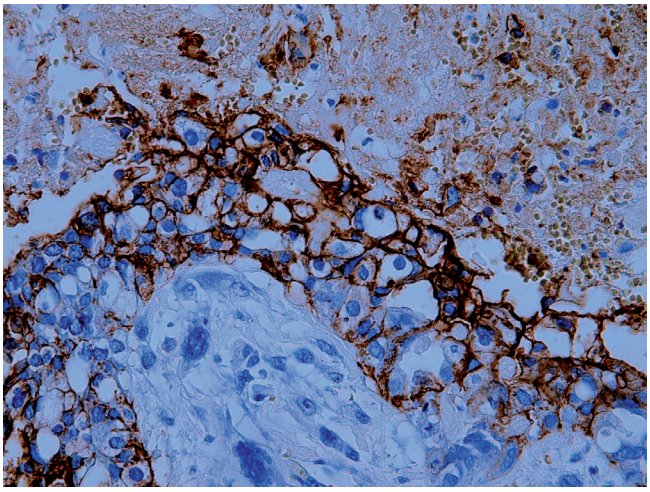


Figure 1. Strong membrane positivity in carcinoma cells (original magnification: x400)

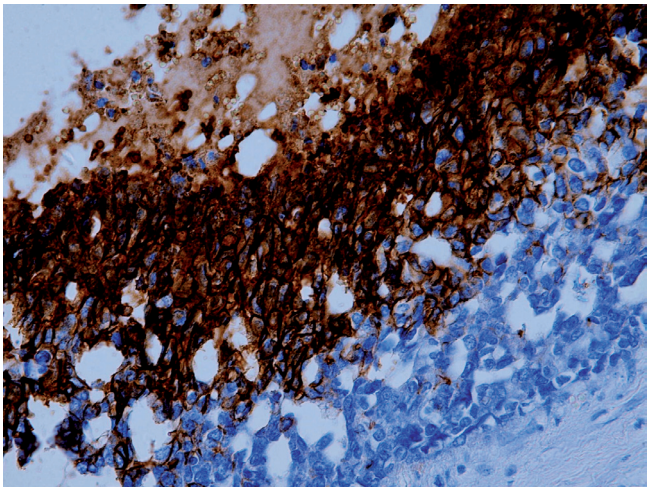


Figure 2. Strong combined membrane and cytoplasmic positivity in carcinoma cells (original magnification: x400)

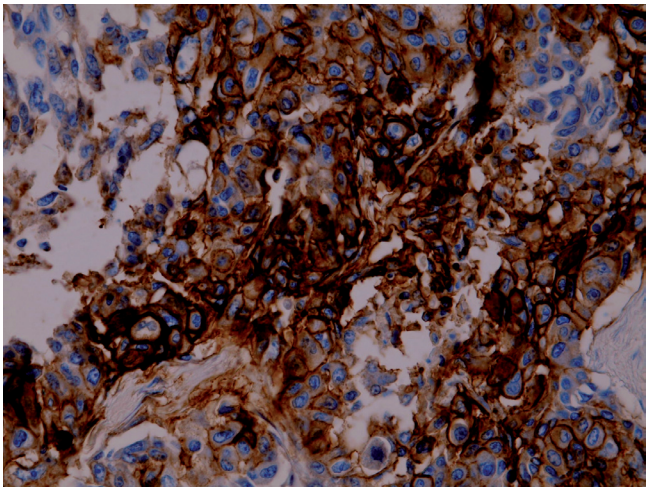


Figure 3. 79- year old woman (membrane localization of CA IX, original magnification: x400)

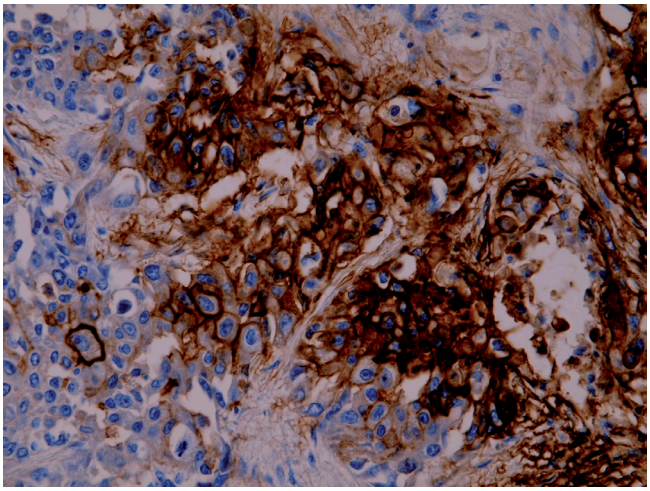


Figure 4. Grade 3 – strong membrane positivity (original magnification: x400)

of immunohistochemical positivity. Cytoplasmic staining was detected in 1 out of 84 cases (1.2%), while solely membrane positivity (Fig.1) was observed in 29 out of 84 cases (34.5%). Combined (membrane as well as cytoplasmic) expression (Fig.2) of carbonic anhydrase IX was demonstrated in 4 out of 84 cases (4.8%). In those cells with combined expression, the carbonic anhydrase IX localization was predominantly membrane. Furthermore, the majority of all positive cases enrolled in our study expressed membrane immunoreaction, which is typical for this zinc-dependent metalloenzyme. The intensity of immunoreactivity varied from weak to strong. Weak intensity of immunostaining was found in 3 out of 84 cases (3.6%) while moderate to strong intensity was found in 31 out of 84 cases (36.9%). The percentage of labeled cells in malignant lesions was less than 50% in 29 out of 84 cases (34.5%) and more than 50% in 5 out of 84 cases (6%). Due to the fact that carcinoma cells frequently expressed heterogenic carbonic anhydrase IX immunoreactivity, the dominant pattern was used for scoring.

Statistical analysis results. Clinicopathological findings in invasive breast cancer cases (age, histological grade, tumor stage and size, vascular invasion, lymph node metastasis, expression of estrogen and progesteron receptors and HER-2 expression) were confronted with the immunohistochemical characterization of CA IX expression – i.e. its subcellular localization, the intensity of immunostaining and the percentage of CA IX positive cells (Table 1). The cases with absent CA IX expression were also included in the statistical analysis of all immunohistochemical characteristics of carbonic anhydrase IX. Chi-square (χ^2) test was used to demonstrate the correla-

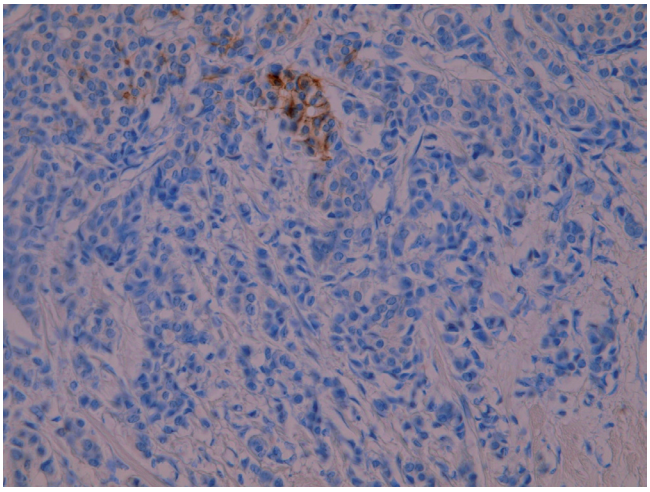


Figure 5. Grade 1 – weak membrane positivity (original magnification: x400)

tions between CA IX expression and clinicopathological and biological parameters.

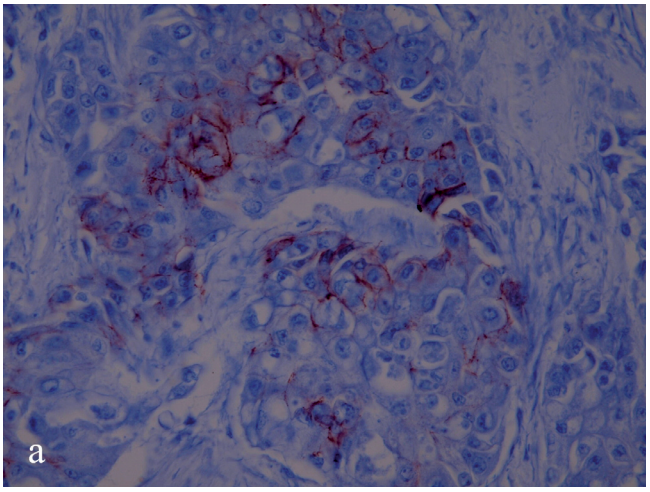
The statistical analyses revealed a significant correlation between age of cancer patients and the subcellular localization of CA IX ($p<0.05$) (Fig. 3). Moreover, another statistically significant correlation was confirmed between the tumor grade and the subcellular localization (Fig. 4, Fig. 5), the intensity of immunostaining and the percentage of CA IX positive cells ($p<0.05$). Grade 3 was associated with membrane or combined (membrane and cytoplasmic) localization of CA IX. It was demonstrated in only 6.3% of cases with grade 1 and 6.3% of cases with grade 2, while in 27.9% of cases with grade 3. Grade 3 was also associated with moderate to strong intensity of immunoreaction. It was present in only 6.3% of cases with grade 1 and 5.1% of cases with grade 2, while in 27.9% of cases with grade 3. In these malignant lesions, the percentage of labeled cells was less than 50% in 6.3% of cases associated with grade 1 and 7.6% of cases associated with grade 2 and in 22.8% of cases associated with grade 3. More than 50% of CA IX positive cells were detected in only 5.1% of cases associated with grade 3.

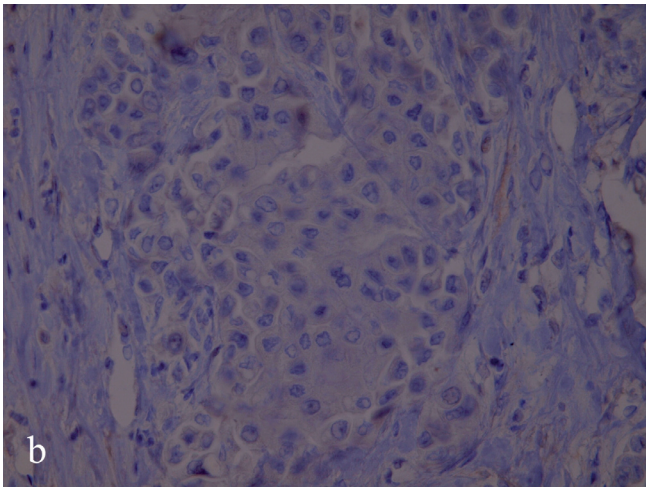
Furthermore, significant correlations were confirmed between the expression of estrogen receptors as well as the expression of progesterone receptors and all immunohistochemical characteristics of CA IX expression ($p<0.05$). In cases with positive ER was CA IX detected in 21 out of 81 cases (26%) and absent in 45 out of 81 cases (55.5%). On the other site, in cases with negative ER was CA IX detected in 12 out of 81 cases (14.8%) and absent in 3 out of 81 cases (3.7%). In cases with positive PR was CA IX detected in 19 out of 82 cases (23.2%) and absent in 45 out of 82 cases (54.9%). In contrast, in cases with negative PR was CA IX detected in 14 out of 82 cases (17%) and absent in 4 out of 82 cases (4.9%). Achieved results suggest the presence of CA IX expression is associated with ER and PR negative status (Fig. 6, Fig. 7).

Table 1. Correlation between CA IX expression and clinico-morphological and biological parameters in breast carcinoma

CA IX expression	A	Subcellular localization			Intensity of immunoreactivity			Percentage of labeled cells		
		C	MC	M	+	++	+++	<10%	11-50%	>50%
Age (n=79)										
<40	1	0	1	1	0	0	2	0	2	0
41-50	9	1	0	5	1	3	2	2	4	0
51-60	14	0	0	8	0	5	3	4	3	1
61-70	9	0	1	6	0	3	4	2	4	1
71-80	13	0	0	8	1	6	1	5	2	1
>80	0	0	1	1	0	1	1	0	1	1
p-value		**0.031**			0.481			0.231		
Grade (n=79)										
1	16	0	0	5	0	2	3	3	2	0
2	15	1	0	5	2	3	1	1	5	0
3	15	0	3	19	0	13	9	9	9	4
p-value		**0.029**			**0.008**			**0.041**		
Tumor size (n=81)										
<11mm	16	0	0	3	0	1	2	2	1	0
11-20mm	16	1	0	11	1	7	4	5	7	0
>20mm	18	0	3	13	1	8	7	5	7	4
p-value		0.108			0.399			0.104		
Stage (n=75)										
T1	31	1	0	14	1	8	6	6	9	0
T2	9	0	2	8	0	5	5	3	4	3
T3	1	0	0	1	0	1	0	0	1	0
T4	2	0	1	5	1	3	2	3	2	1
p-value		0.291			0.333			0.137		
LN metastasis (n=66)										
Positive	15	0	0	8	0	5	3	0	6	2
Negative	24	1	2	16	2	10	7	10	8	1
p-value		0.603			0.702			0.063		
Vascular invasion (n=82)										
Positive	25	0	0	14	1	6	7	5	7	2
Negative	24	1	3	15	1	12	6	8	9	2
p-value		0.276			0.592			0.857		
ER (n=81)										
Positive	45	1	0	20	2	13	6	9	12	0
Negative	3	0	3	9	0	5	7	4	4	4
p-value		**0.000094**			**0.000045**			**0.000024**		
PR (n=82)										
Positive	45	1	0	18	2	12	5	9	10	0
Negative	4	0	3	11	0	6	8	4	6	4
p-value		**0.00012**			**0.00024**			**0.000051**		
HER-2 (n=82)										
Positive	16	0	1	16	1	12	4	8	7	2
Negative	33	1	2	13	1	6	9	5	9	2
p-value		0.209			0.071			0.266		

A – absent, C – cytoplasmic, M – membrane, MC – combined membrane and cytoplasmic, LN – lymph node, ER – estrogen receptor, PR – progesterone receptor, HER-2 – human epidermal growth factor receptor 2





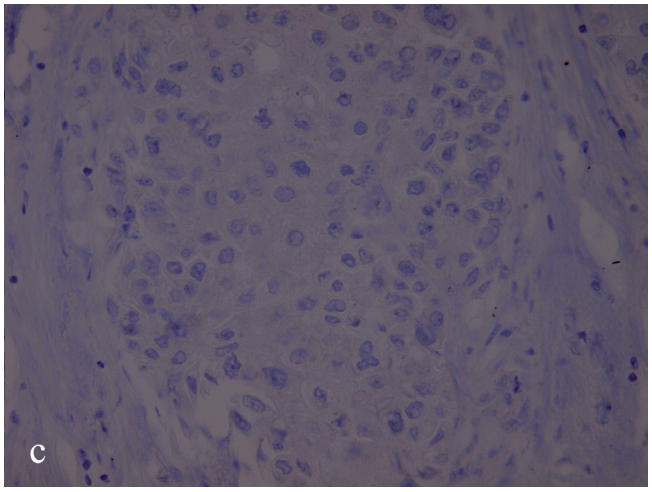
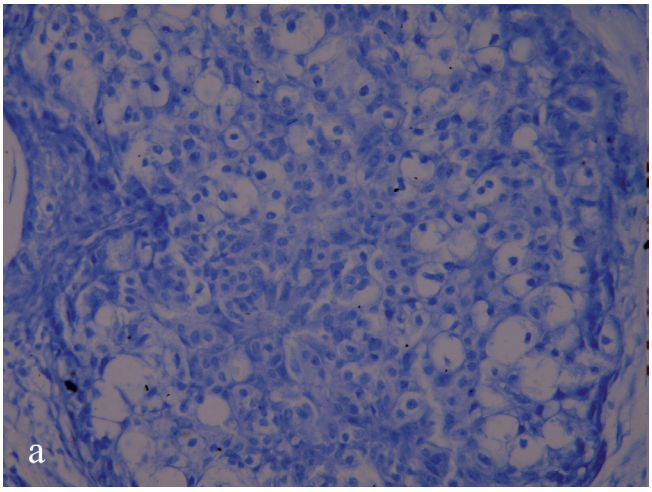
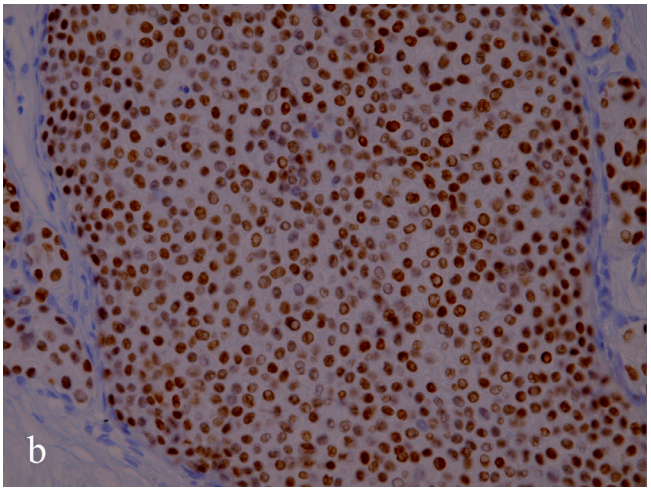


Figure 6. 69- years old woman a) CA IX – positive, b) ER – negative, c) PR – negative (original magnification: x400)





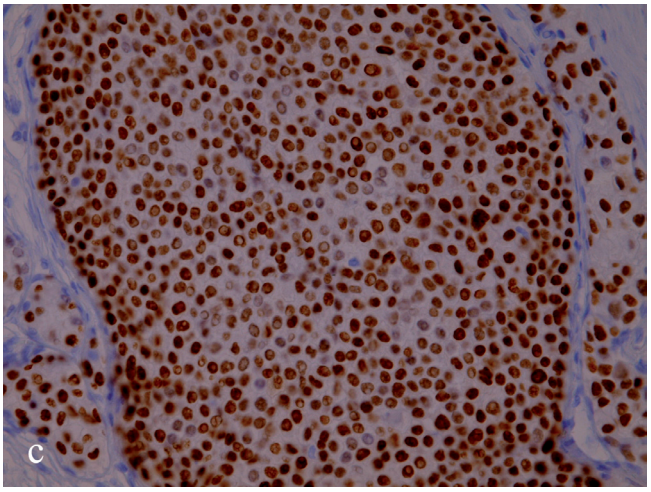


Figure 7. 34-years old woman a) CA IX – negative, b) ER – positive, c) PR – positive (original magnification: x400)

The remaining clinico-morphological and biological features (tumor size and stage, lymph node metastasis, vascular invasion and HER-2 expression) did not correlate with the immunohistochemical characteristics of CA IX expression (p>0.05) (Table 1).

Fisher´s exact test was used to evaluate the statistically significant difference of CA IX expression between invasive ductal carcinoma (IDC) and the group of invasive lobular carcinoma (ILC) and other less frequent types of breast carcinoma. There was however, no significant difference (p>0.05) (Table 2).

Table 2. CA IX expression in invasive ductal carcinomas (IDC) and in invasive lobular carcinomas (ILC) and other types of invasive breast carcinoma

CA IX (n = 83)	IDC	ILC + other
Positive	29	6
Negative	34	14
	p = 0,299	

Discussion

In this article, we focus on carbonic anhydrase IX, an interesting protein which was initially reported as a tumor antigen. CA IX was first detected with monoclonal antibody M75 in the human HeLa cell line derived from carcinoma of the cervix [15]. This protein consists of an N-terminal proteoglycan-like domain (PG), an extracellular CA catalytic domain, a transmembrane helical segment (TM) and a short cytoplasmic tail (IC). The first region represents a unique feature that appears to endow CA IX with the capacity to act in protein-mediated cell adhesion events, while the catalytic domain catalyzes the reversible hydration of CO_2 and so contributes to pH regulation [10].

Expression pattern of CA IX is characterized by limited expression in normal human tissues. Normal CA IX expression is predominantly linked with the epithelial tissues of gastrointestinal tract [16]. On the other hand, high ectopic expression of CA IX is detectable in a broad spectrum of human tumors, including carcinomas of the uterine cervix, kidney, brain, head and neck, esophagus and lung, colon, breast, ovaria, endometrium, vulva etc. [7, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28]. From an unknown reason, CA IX is generally absent in normal prostate tissues as well as in the prostate carcinomas [29].

It is now well and clearly documented that transcription of *CA9 gene* (the human gene coding CA IX) is responsive to hypoxia and high cell density, which are physiological stresses typical for microenvironment of solid tumors [30]. The CA9 promoter sequence contains a hypoxia response element (HRE) element recognized by hypoxia inducible factor 1 (HIF-1), which is the most important activator of CA9 trascription. Hypoxia-triggered architecture and phenotypic rearrangements of tumor tissue finally convert into development of necrotic areas surrounded by the zones of surviving hypoxic cells, which adapted to this stress and acquired agrressive behavior [31,32]. Hypoxia is a very crucial pathophysiologic consequence of a structurally and functionally disturbed microcirculation and the deterioration of oxygen diffusion conditions [33]. This phenomenon has long been known to be associated with poor prognosis and poor overall survival in cancer patients, since it may contribute to the development of more malignant tumor phenotypes and increased tumor invasiveness and metastatic potential [5]. Hypoxia has also the most important role in the development of resistance to chemotherapy and radiotherapy [34].

The rapid identification of hypoxic tumors remains a goal for the diagnostic and therapeutic management of solid tumors [35]. Detection of hypoxic regions within tumors is therefore very important for stratification of cancer patients for suitable treatment regimens and for prognosis of the disease development. Currently available approaches how to detect tumor hypoxia have invasive nature [36]. On the other hand, use of an intrinsic hypoxic marker would be highly advantageous, simple, reproducible, and can be performed in a clinical daily routine, using both prospective and retrospective archival samples. Within this context, CA IX has been shown to serve as a surrogate marker of hypoxia for many cancers with possible diagnostic, prognostic and therapeutic value. Therefore, immunohistochemical detection of CA IX in different types of tumors was performed with the aim to elucidate its clinical relevance. Characteristic expression pattern of CA IX related to hypoxia determines its relationship to clinical variables. Span et al. [37] concludate that CA IX staining intensity increased at increasing distance from vessels, suggesting an association of CA IX with diffusion limited hypoxia. Hypoxia occurs also in breast cancer [38,39].

Breast cancer is a heterogeneous disease showing marked morphological and clinical diversities as well as variability in prognosis and response to different therapeutic modalities [40]. Morphologically identical tumors can display divergent clinical outcomes and also responses to therapy. This can predominantly be attributed to molecular class differences that exist amongst histologically similar breast cancer types [41]. Therefore, several histomorphological and clinical parameters as well as tumor markers were reported to have prognostic significance in breast cancer patients [42,43,44]. From these, the histological grade of tumor, number of metastatic lymph nodes, tumor stage, vascular invasion and tumor size were found to be the most important histomorphological prognostic parameters widely accepted and used in daily practice. Hormone receptor (ER, PR) status and HER-2 should also belong to the routinely performed markers for the assessment of prognosis and therapeutic management of breast carcinoma patients [45]. Because all these parameters do not always lead to the exact prediction of prognosis and therapeutical response, new approaches are researched to allow a more precise stratification of patients [46]. The identification of new promising molecular biomarkers with the potential to predict treatment

outcome is essential for selecting patients to receive the most beneficial therapy. The tumor-associated protein CA IX was found to be representing an attractive biomarker as well as a novel prognostic and predictive factor and an anticancer therapy target [11,16].

Currently, there is a heated discussion within literature concerning the relationship of CA IX with several clinico-morphological and biological parameters in breast carcinomas.

Brennan et al. [47] found a significant correlation between CA IX expression and the tumor size, grade, ER-negative and PR-negative status. They concluded that CA IX is a marker of poor prognosis in premenopausal breast cancer patients and it is independent predictor of survival in patients with one to three positive lymph nodes. According to them CA IX may be associated with resistance to radiotherapy. Generali et al. [48] reported that CA IX expression was directly associated with c-erbB2 (HER-2), while it was inversely associated with steroid hormone receptor status. They confirmed this association suggesting that CA IX with its reversed correlation with ER status and positive correlation with HER-2 is associated with resistance to endocrine threrapy. Bartosova et al. [17] also reported a weak, but significant, correlation of CA IX in breast cancer with c-erbB2 (HER-2) expression.

Span et al. [37] also suggest that, either the reversed correlation between CA9 and ER status, or its positive correlation with HER-2, could explain the endocrine therapy resistance in tumors expressing high levels of CA9. They also obtained the results which indicate that patients with low CA9 levels benefit more from adjuvant treatment than do patients with high levels. In these studies, tissues specimens were analyzed using immohistochemistry and/or RT-PCR (reverse trancriptase-polymerase chain reaction). We also noticed that CA IX expression tended to correlate with the estrogene and progesterone recetor-negative phenotype.

In our study, no significant correlations between HER-2 expression and all immunohistochemical characteristics of CA IX expression were confirmed. Our results are not in line with above mentioned authors. Estrogen and progesterone receptors and HER-2 overexpression are routinely performed markers. This hormone receptor status is used to determine the appropriateness of adjuvant endocrine therapy. The loss of hormonal receptors is a characteristic for the most aggressive breast tumors. Low-grade tumors have positive ER and PR, but on the other side, the high-grade tumors are found to be negative for ER and PR and also have an overexpression or amplification of HER-2 [49]. The role of HER-2 overexpression in evaluating risk as well as determining optimal anticancer therapy is evolving. It is standard practice to use HER-2 status to identify these patients who are likely to benefit from trastuzumab therapy [50].

Chia et al. [51] studied CA IX expression and compared it with several established clinicopathological prognostic variables. Within the entire cohort, significant association were found between the presence and level of CA IX expression and the presence of necrosis, higher tumor grade and also negative ER status. There was no apparent relationship with other prognostic variables such as age, nodal status, tumor size, tumor type. Trastour et al. [1] showed no significant correlation between tumor size or nodal status and the expression of CA IX. On the other hand, they found statistically significant association between CA IX staining and the grade and also hormonal receptors loss. Furthermore, according to these authors, overexpression of CA IX correlates with poor outcome after conventional adjuvant therapy. Comparison of CA IX expression with clinicopathological parameters of the analyzed malignant breast tumors did not reveal any significant relationship with the age, nodal status, tumor size, tumor grade in the study of Bartosova et al. [17]. The study of Span et al. [37] reported that CA9 expression was mainly found in high-grade breast tumors and steroid receptor negative cancer tissues.

There are several studies showing positive correlation between CA IX expression and poor prognosis in different types of tumor. In general, CA IX significantly correlates with high tumor grade, necrosis, treatment outcome and poor prognosis in patients with the breast carcinomas [47, 48, 51, 52, 53].

In our group of 84 breast carcinomas, we also analyzed expression characteristics of CA IX and the possible correlation with the clinico-morphological and biological parameters. We demonstrated significant correlation between subcellular localization of CA IX and the age of cancer patients. Some of our significant findings are worth of attention. The subcellular localization of CA IX, intensity of immunoreaction, percentage of CA IX-positive cells were significantly correlated with the tumor grade. Findings within our panel of breast carcinomas showed that membrane and combined membrane and cytoplasmic CA IX expression as well as higher intensity of immunohistochemical reaction may be associated with histological grade 3. The age of breast cancer patients and tumor grade belong to the most important prognostic parameters used in daily practice. One of the absolute basic in the assessment of cancer is to grade the tumor according to the morphological differentiation. Malignant tumors are usually graded as either well (grade 1), moderately (grade 2) or poorly (grade 3) differentiated. In general, well differentiated tumors are less aggressive than the poorly differentiated tumors [55]. The number of metastatic lymph nodes, vascular invasion, tumor stage and tumor size were found to be important and widely accepted histomorphological prognostic parametres [42, 44, 45, 55]. We found no significant correlations between these above-mentioned parameters and characteristic of CA IX expression.

In our panel of 84 carcinoma cases, carbonic anhydrase IX was expressed in 34 cases (41%). Number of CA IX positive cases in our study is similar to number published in other papers. Generali et al. [48] detected CA IX immunopositivity in 41 out of 169 cases (24,2%). In the study of Wykoff et al. [52] CA IX was present in 34 out of 68 cases (50%). Bartosova et al. [17] revealed CA IX expression in 12 out of 26 cases (46%). Chia et al. [51] detected CA IX in 49 out of 103 cases (48%).

In contrast to other studies, we next investigate the relationship between CA IX expression in the group of invasive ductal carcinomas (IDC) and the group consists of invasive lobular carcinomas (ILC) and other less frequent types of breast carcinoma. There was however, no significant difference. IDC is the most common type of breast cancer affecting women today. ILC is less common than IDC, more difficult to detect mammographically, and usually diagnosed at a later stage [56]. The articles mentioned in this study did not discuss the relationship between IDC and ILC.

With a few exceptions, a wide variety of tumors showed only plasma membrane immunoreactivity for CA IX. Plasma membrane expression of this protein is the most typical expresion pattern of CA IX [57,58]. The significance of subcellular CA IX expression in breast carcinomas is still controversial. We found three patterns of immunohistochemical positivity (membrane, cytoplasmic, combined membrane and cytoplasmic). Studies focused on analysis of CA IX expression, described usually only focal membranous CA IX staining. However, only a small number of studies specify the subcellular localization of this protein in breast tumors [17]. Bartosova et al. [17] reported that the majority of weakly stained specimens showed a focal cytoplasmic pattern, while moderate to strong staining was mainly of focal membrane type. In their study, only one case of invasive lobular carcinoma showed weak cytoplasmic staining. Our research group found an analogical situation in CA IX expression within malignant breast lesions. In general, cytoplasmic CA IX location is very rare finding in cancer.

Based on our results, it seems to be necessary to score the subcellular localization of CA IX in a larger patient cohort. The reason for subcellular compartmentalization of CA IX in tumor cells is not fully understood.

In conclusion, we indicate that different subcellular CA IX expression as well as different intensity of immunoreaction are very important features of invasive breast carcinomas. In current study, we point out that mostly membrane or combined membrane and cytoplasmic positivity together with a higher intensity of CA IX immunoreactivity are associated with poor prognostic parameters, such as tumor grade 3 and also with negative estrogen and progesterone receptor status and so it may influence therapeutic approach. The CA IX expression may be used to aid in the selection of patients who would benefit most from hypoxia-modification therapies or bio-reductive drugs. Based on our findings, we can conclude that the hypoxia-inducible protein carbonic anhydrase IX may represent a poor prognostic marker for invasive breast carcinoma. Chia et al. [51] studied whether CA IX is an independent prognostic factor for breast cancer relapse and death. According to their findings, the presence of CA IX expression showed a significant association with a shorter relapse-free survival (RPS) and a poorer overall survival (OS). In general, this protein can serve as a clinically very useful biomarker of human breast cancer. Consequently, further research needs to be done in the field of the tumor-associated CA IX isoenzyme to understand better its exact role in cancer. Additional studies with larger cohort of patients are required to confirm our results and the clinical relevance and utility of the CA IX as a potential prognostic and predictive marker of this serious human oncology disease.

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