Gp96 and its different expression in breast carcinomas

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The aim of our work was to determine the expression of glycoprotein 96 (gp96; glucose-regulated protein 94 – GRP94) in 69 samples of breast carcinoma. Enzyme immunohistochemical method was chosen for the detection of gp96 protein and its expression was compared in breast cancer cells versus normal breast cells. We found higher expression of gp96 protein in breast carcinoma cells and low or no expression in normal breast cells. Furthermore, we demonstrate first time, that ductal invasive breast carcinomas showed higher expression of gp96 than lobular invasive breast carcinomas.

Key words: gp96, heat shock protein, breast carcinoma, immunohistochemistry.

Heat shock protein (HSP) gp96 is a member of the HSP90 family. This is a large family of proteins with different molecular weights and different intracellular localizations. HSPs are present in all living cells. They can exist in an unbound state or bound to specific client proteins. HSPs affect as molecular chaperones in numerous processes, such as protein folding, assembly and transport, peptide trafficking, and antigen processing under physiologic and stress conditions [1,2]. Expression of HSPs can be induced by several stressors, such as fever, oxidative stress, alcohol, inflammation and heavy metals, and by conditions causing injury and necrosis, such as infection, trauma and ischemic reperfusion. They serve to attenuate the damage and misfolding of proteins caused by these various stressors.

Human gp96 gene is located on chromosome 12. It encodes a protein of 803 amino acids. Mature gp96 consists of three distinct structural domains: an N-terminal ATPase domain, a highly charged intermediate domain and a C-terminal dimerization domain. Native gp96 assembles as a homodimer with an extended rod-like shape [3]. Gp96 was reported to have multiple enzymatic activities [4,5].

Chaperones participate in the folding of numerous protooncogene and oncogene products. Target proteins form stable complexes with chaperones which keep them in an activation-competent state. HSP90 is necessary for the folding of several protooncogene/oncogene protein kinases, such as members of the src and raf families [6]. Over-expression of chaperones in tumor cells is a rather general phenomenon caused by the increased demand of accelerated cell proliferation and the harmful environment. Chaperones protect malignant cells from many of the environmental stresses and render them more resistant against apoptosis, anticancer drugs and immune attacks. Chaperone-induction may also lead to an increase of the metastatic potential. Tumor chaperones may seem to be one of the devils of tumor therapy, thus various selective methods to impair their synthesis in tumor cells have high therapeutic potential [7].

The erbB2 gene encodes a 185-kDa receptor-like protein ($p185^{erbB2}$) with tyrosine kinase activity. This protein is overexpressed in many breast, ovarian and prostate carcinomas and is associated with poor prognosis. Chavany et al. [8] demonstrated that the $p185^{erbB2}$ receptor kinase forms a stable complex with the GRP94. Perhaps an association with GRP94 is required for both the proper intracellular trafficking and stability of a family of receptor proteins.

HSPs are also potent inducers of innate and antigen-specific immunity. Their role as „danger signals“ that prime multiple host defence pathways is being exploited in vaccine development for cancer and infections [9].

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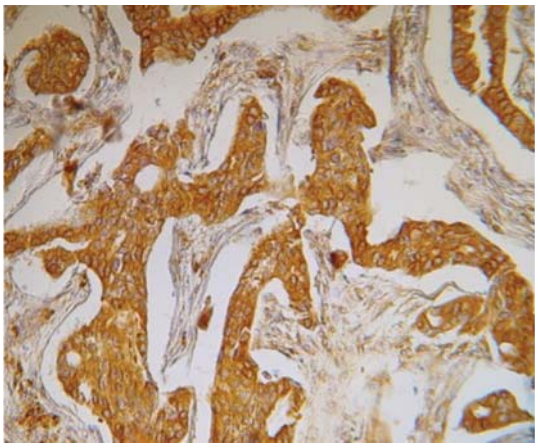


Figure 1. Expression of gp96 in ductal type of breast cancer was immunohistochemically detected by using of goat polyclonal antibody. To detect this protein we have used polyclonal antibody GRP94. Gp96 positive cells show brown staining.

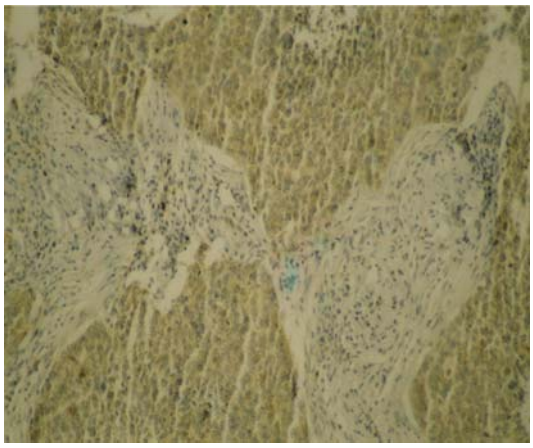


Figure 2. Gp96 positivity in other type (medullary carcinoma) of breast carcinoma.

Material and methods

Clinical samples. In this study we have used 69 samples of breast carcinoma and 5 samples of normal breast tissue. The samples were obtained from the Department of Pathological Anatomy, J. A. Comenius University, Jessenius Faculty of Medicine in Martin.

The breast carcinoma samples were according the histopathological type divided into 3 groups: 1. ductal carcinoma (45 samples), 2. lobular carcinoma (10 samples) and 3. other types (14 samples of papillary, cribriform, mucinous, medullary and tubular carcinoma). We have marked specimens of normal breast tissue as control group. All these samples were immunohistochemically analyzed for gp96. We have distinguished four categories of quantity of these proteins: 3+ = high level (91 – 100% of positive cells), 2+ = medium level (11 – 90% of positive cells), 1+ = low level (up to 10% of positive cells), – = negative cells (0% of positive cells). For statistical analysis as positive were considered only samples with high level (3+) and medium level (2+) protein expressions. Samples scored as 1+ or – were considered negative.

Immunohistochemical detection of gp96. We have used indirect enzymatic immunohistochemical method. Formalin fixed, paraffin embedded tissue blocks were cut (7 μm) and attached to the slides. The slides were processed for immunohistochemistry.

Tissue sections were deparaffinized with xylene and rehydrated in decreasing ethanols to water. Finally, the slides were washed in distilled water. Sections were pretreated in citrate buffer solution in microwave oven for 15 minutes. This was followed sequentially by washing in distilled water; endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol. Gp96 staining procedure continued by blocking nonspecific staining with milk buffer for 30 minutes at room temperature.

The next step was application of primary antibody. Goat polyclonal antibody GRP 94 (C-19, Santa Cruz Biotechnology, Inc.) was applied overnight in humidified chamber at 4°C.

After proper rinsing in 2% milk buffer (3x5 min.) the sections were subsequently incubated with secondary antibody, it was rabbit anti-goat antibody conjugated with horseradish peroxidase (Pierce Biotechnology, Inc.) for 30 minutes at room temperature. The procedure continued by washing with 2% milk buffer (2x5 min.) and Tris buffer (1x5 min.).

The sections stained for gp96 were then visualized with 3.3'-diaminobenzidine tetrachloride (DAB) at a concentration of 0.5 mg/ml in Tris buffer, pH 7.6 and 0.015% H_2O_2. Slides were stream-rinsed with tap water, counterstained with hematoxylin for 2 minutes, washed in tap water, dried, mounted and coverslipped. Sections processed with omission of primary antibody served as a negative control of immunohistochemical procedure.

Results

Sixty nine (69) samples of breast carcinoma and five (5) samples of normal breast tissue were immunohistochemically analyzed for gp96 expression. We have marked specimens of normal breast tissue as control group.

In the first group (ductal carcinoma), 32/71% tissue samples were gp96 positive (Fig.1), the rest showed no gp96 positivity.

In the second group (lobular carcinoma), gp96 immunopositivity was detected in one case of lobular carcinoma (Fig.3). The signal was low or not present in 9/90% samples and these samples were considered negative.

In third group (other type of breast carcinoma), gp96 expressed high and medium levels in 6/43% (Fig.2) and rest of specimens was negative.

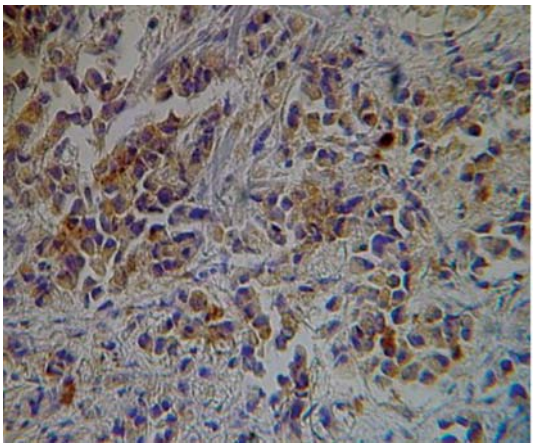


Figure 3. Demonstration of gp96 expression in lobular type of breast carcinoma as detected by immunostaining of paraffin tissue sections with GRP94. We can see medium level of gp96 positivity in tumor cells.

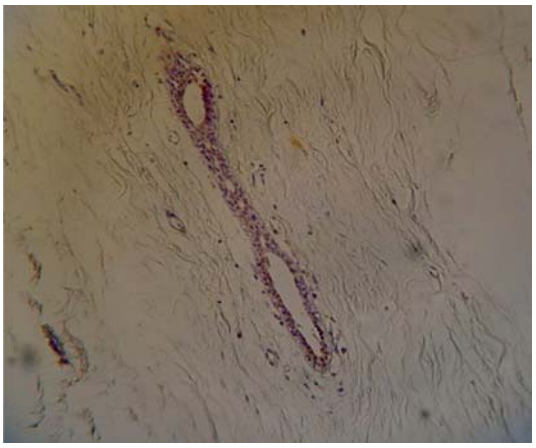


Figure 4. Normal breast tissue with no or low positivity of gp96.

Taken together, 39/57% samples of breast carcinoma were gp96 positive. For complete results with all tissue samples analyzed see also the Table 1 and Fig. 6.

In the control group (normal breast tissue), 3/60% samples showed low and 2/40% no expression of gp96. All these samples were considered as negative (Fig.4).

Discussion

The heat shock protein gp96 has been described as a classical ER-resident protein, but later studies show its surface expression on plasma membrane of some tumor cells [10,11]. The mechanism by which gp96 is directed and retained on the plasma membrane is still unknown. Another study showed that gp96 exerts also protective function during cellular stress and plays an important role in the maintenance of protein homeostasis. The housekeeping activity of this protein is ascribed to its ability to chaperone nascent or aberrantly folded proteins [12].

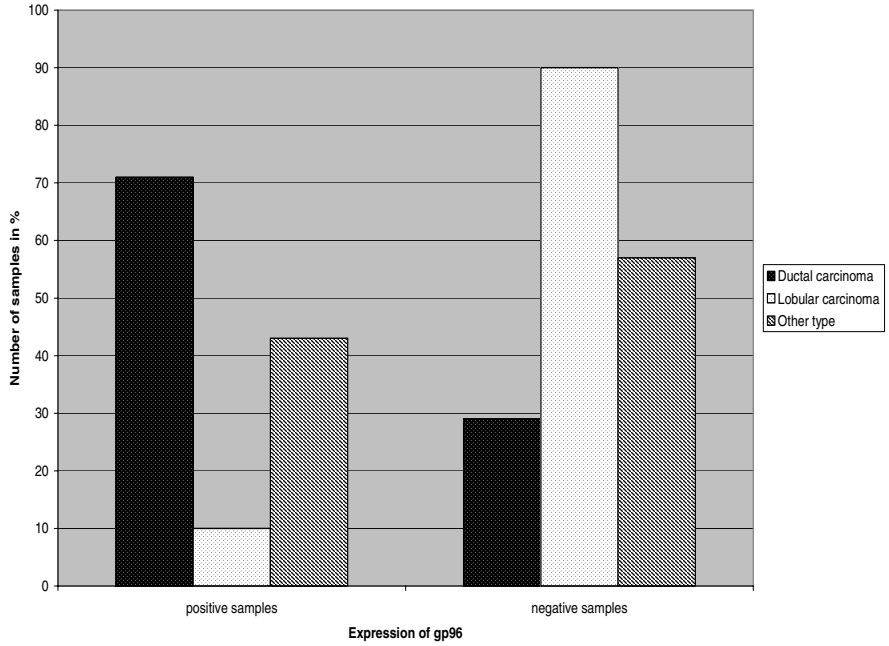
HSPs are thought to play important roles in the cell cycle and various processes of carcinogenesis. The expression of proliferating cell nuclear antigen and some members of the stress protein family were higher in breast carcinomas than in benign or normal breast tissue [13,14]. Our study confirmed the results of above mentioned authors about higher expression of HSP in breast carcinomas. In our study, GRP94 was detected in human breast carcinoma samples in significantly higher expression than in normal breast tissue.

Invasive ductal carcinoma and invasive lobular carcinoma are the two major types of breast cancer worldwide. Specific changes in gene expression distinguish ductal from lobular breast carcinoma. Immunohistochemical and cDNA microarray studies identified downregulation of E-cadherin [15,16,17] and upregulation of thrombospondin 4 [16,17] in lobular carcinoma versus ductal carcinoma. Both of E-cadherin and thrombospondin 4 are involved in cell adhesion, therefore the process of adhesion of lobular and ductal breast cancer cells seems to be different. Unlike invasive ductal carcinoma, invasive lobular carcinoma did not display ERBB2 overexpression and expression of the transcription factor E2F1 correlated inversely with tumor grade. The discrepancy in the pattern of the human oncogenes CCND1 and ERBB2, which are involved in the process of carcinogenesis of ductal tumors, appear to suggest a different molecular basis for development and progression of invasive lobular carcinoma [18]. Bini et al. [19] demonstrated the protein expression profiles in human breast ductal carcinoma and histologically normal breast tissue. In their study, GRP94 together with GRP78, GRP75, mitochondrial HSP60 and other proteins showed significantly higher expression in ductal breast carcinoma than in normal breast tissue. The same situation, at least with GRP94 was observed in our samples, where significantly higher level of gp96 protein in ductal versus lobular breast carcinoma was detected.

In the future we would like to show whether there is any correlation between the expression of gp96 and the expression of E-cadherin, ERBB2 or CCDN1 proteins in ductal breast carcinomas. The existence of gp96-associated tumor-specific peptide complexes in breast ductal carcinoma will also be studied.

Table 1. Various levels of expression of gp96 in 69 samples of breast carcinoma tissue.

Quantity of expression	Ductal carcinoma	Lobular carcinoma	Other type
+++	19 (42%)	0 (0%)	4 (29%)
++	13 (29%)	1 (10%)	2 (14%)
+	3 (7%)	3 (30%)	3 (21%)
–	10 (22%)	6 (60%)	5 (36%)
Number of positive samples	32 (71%)	1 (10%)	6 (43%)
Number of negative samples	13 (29%)	9 (90%)	8 (57%)



Graph 1. Comparison of gp96 expression in different types of breast carcinoma.

Breast cancer is an extremely expanded disease in industrial countries of the world. Over the last 30 to 40 years, substantial progress has been made in the diagnosis and treatment of breast cancer. For premenopausal women, ovarian function suppression with luteinizing hormone-releasing hormone analogs combined with tamoxifen has become the standard treatment, although aromatase inhibitors plus ovarian function suppression are under evaluation. In postmenopausal patients, aromatase inhibitors have proved to be superior to standard endocrine therapies in either first- or second-line treatment and novel antiestrogen compound, fulvestrant, has been introduced in clinical practice [20]. Chemotherapy remains the treatment of choice for hormone unresponsive or resistant patients. Anthracyclines and taxanes have been used either alone or in combination as first-line chemotherapy, but with the more frequent use of these agents in the adjuvant setting, new standards are needed for first-line chemotherapy, and new and more efficacious treatments are required. In the subgroup of patients with tumors that overexpress HER-2, the use of trastuzumab alone or in combination with chemotherapy is the most frequent [20].

The accumulation of HSPs is known to protect the organism from a great variety of pathological conditions. Conversely, lowering HSPs in cancer tissue can amplify the effectiveness of chemo- or radiotherapy [21]. The benzoquinone ansamycin – geldanamycin (GA) destabilized the complex of GRP94-p185^{erbB2} and prevents the translocation of the newly synthesized protein to the plasma membrane, instead trapping it in an intracellular vesicular compartment consistent with the endoplasmic reticulum/cis Golgi [16]. Exposure of SKBr3 cells to GA and other benzoquinoid ansamycins has been shown previously to result in rapid loss of p185^{erbB2} [22]. Furthermore, GA causes dissociation of heterocomplexes composed of HSP90, with which GRP94 shares 50% homology, and various signal transduction proteins, including v-Src, c-Raf, and progesterone receptor [23,24].

Recent studies have shown that gp96 can additionally exert potent stimulatory activity on both innate and adaptive immunity. For its immunological features, gp96 is deemed to be an appealing tool for immunization against tumor and infection. For example, oncophage (kind of autologous tumor derived gp96-peptide complex), has been currently in clinical trials for metastatic melanoma, kidney cancer, lymphoma, pancreatic and gastric cancer [25].

Our results demonstrate the cases of breast carcinomas where the new anti-gp96 and immunotherapy might be applied. The best candidate for anti-HSP and potentially vaccine therapy seems to be invasive ductal breast carcinoma.

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