The role of connexins and cell communication channels in breast cancer progression and prognosis

PhD thesis

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1. INTRODUCTION

Breast cancer is a major health care problem worldwide. It is one of the leading causes of cancer mortality among women in the economically developed countries including Hungary. The mortality rate has shown a slight tendency of reduction since 1990's due to the mammography screening, early diagnosis and personalized treatment.

The preoperative diagnosis is determined with fine needle aspiration biopsy (FNAB) or core biopsy. Further therapy is defined by a group of specialists including radiologists, surgeons, pathologists and oncologists, called the oncoteam.

A restricted number of predictive and prognostic markers are available to assess the disease outcome and response to neoadjuvant therapy. The age of patient and the size of the tumor are important at the time of diagnosis. One of the most relevant prognostic factor is the presence or absence of axillary lymph node metastasis at the time of diagnosis. In the routine diagnosis, besides histological grade, Nottingham prognostic index (NPI), vascular invasion, necrosis, expression of Ki67 and p53 can be applied. In many cases, however, these provide only limited advantage. Molecular subtyping (hormonreceptor status, HER2 expression) can also specifiy subtypes of breast cancer with different disease outcome.

The direct cell-cell communication through gap junctions allows a rapid and regulated transport of small regulatory molecules of <1.8 kDa between coupled cells. The cell membrane channels are formed by connexin (Cx) molecules which contribute to sharing ions, metabolites, secondary messengers and morphogenes within cells making up a functional tissue compartment, such as the breast gland. The importance of connexins are reflected by their high evolutionary conservation, ubiquitous presence at a large density in all solid tissues and by their occurrence as early as in 4-cell embryo. In the human, 21 connexin isotypes are cloned. The most common and –ancient is the Cx43. As hemichannels and gap junction channels, connexins play crucial roles in the maintanence of cell homeostasis, cell differentiation, survival and apoptosis, and in the control of the cell cycle. Gap junctions also mediate the propagation of action potential in the heart and brain. Moreover, connexins can interact with intracellular proteins (the connexin proteome) including oncogene products, protein kinases, citosceletal proteins and junctional proteins such as tigh junction structures (ZO1-2) resulting in the modifition of each others' funtions. Therefore, functional connexins can be localized both to the cell membrane and the cytoplasm (channel-independent function).

Available data of on connexin expression in normal mammary gland and breast cancer are controversial. Limitations of large scale screening of connexins are explained by scare

antibodies detecting their isotypes in archived tissues and difficulties of resolving the small (<1µm) connexin plaques.

So far, Cx43 and Cx26 have been detected *in vivo* in normal breast. Cx43, Cx26, Cx32 and Cx46 have been described in breast cancer. Most of published data are descriptive and only few studies analyzed the role of connexins in breast cancer progression and prognosis. In a study increased Cx43, Cx26 and Cx32 protein levels in the lymph node metasases were found compared to primary breast tumors. Another group examined the same connexins with same results but no significant correlation was detected between connexin expression and prognosis. Others found positive correlation between Cx26 expression and tumor size and worse grade. Recently a new connexin isotype Cx46 was decribed to assist MCF-7 breast cancer cells in adapting to hypoxia.

The role of connexins in breast cancer metastases is also controversial. It is not clear, if the connexins promote or inhibit the migration and intravasation of tumor cells. The expression of Cx is dinamically regulated in metastases. Some connexins can promote, others can inhibit the metastatization of tumors. Some studies confirmed tumor cell communication with the osteoblasts and endothelial cells, which can contribute to metastatic settling of breast cancer. It is known, breast cancer metastasis supressor 1 gene (BRMS 1) can reduce the breast cancer metastatic potential. Transfection with BRMS cDNS reduced the metastatic potential and restored the homotypic gap junction intracellular communication in MDA-MB-435 cell line. Activation of the *twist* metastasis gene increased Cx43 expression in brain metastasis during tumor colonization.

Recent data suggest the stage-dependent expression and function of connexins in tumor promotion and progression. Connexin expression can reduce in early cancer to disrupt the intercellular junction. After that, re-expression of connexin can help tumor cells to spread into the stroma and then to enter the vascular system or to colonize in another tissue.

2. OBJECTIVES

We examined:

1. The connexin expression of normal mammary gland besides the earlier confirmed Cx43 and Cx26.
2. The connexin expression of primary breast cancer beyond Cx43, Cx26, Cx32, Cx46.
3. The prognostic relevance of connexin mRNA and protein expression in adjuvant treated breast cancer.
4. The correlation between connexins and prognostic factors.
5. If the connexin mRNA or protein expression can refine the breast cancer molecular subgroups.
6. The effect of neoadjuvant chemotherapy in connexin expression.
7. If the connexin expression can help to predict the efficiency of neoadjuvant therapy using classification systems.
8. The prognostic value of connexin expression in neoadjuvant treated breast cancer.

3. METHODS

Patient cohort and datasets. Hungarian and swiss patient cohorts were used for protein examination. The hungarian tissue samples of 127 adjuvant treated primary breast cancer collected between 1999-2002 at Buda MAV Hospital, Budapest with 102 months follow-up time. The swiss 96 tumor samples selected between 1998-2002 from the archives of the Institute of Surgical Pathology, University Hospital, Zurich with 2-10 yerars follow-up time. Publicly available gene expression data and survival information of 1809 (Affymetrix array platform) and 1899 (Illumina array platform) breast cancer was performed. Normal mammary glands of 3 premenopausal women were examined for connexin expression.

Tissue microarray. The hungarian and swiss saples were collected in formalin fixed paraffin embeded tissue microarray (TMA) containing duplicate cores from each patient's samples. The diameter of hungarian cores were 2mm, while the swiss core were 0,6mm.

Immunohistochemistry. The subgroups of hungarian samples were determined in TMA , while the subgroups of swiss samples were detected during routine diagnosis, both in Ventana Benchmark immunostainer using iVIEW DAB detection kit. TMA slides of 4µm thick was used to investigate connexin and Ki67 proliferation marker expression. After overnight incubation with primary antibodies, fluorochrome-labelled secundary antibodies were applied.

Digitalization and scoring of the slides. Immunostained slides were digitalized using Pannoramic Scan. Multilayer and multichannel detection were applied. Immunoreaction for Cxs and Ki67 were evaluated by two independent assessors with TMA Modul software. The expression of connexins were examined using 4-scale system (0:<5%, 1+:5-20%, 2+:20-60%, 3+:>60%), while Ki67 expression were evaluated using 10-scale system (0: 0, 1: 0-1%, 2: 1-5%, 3: 5-10%, 4: 10-15%, 5: 15-20%, 6: 20-33%, 7: 33-50%, 8: 50-66%, 9: 66-80%, 10: 80-100%).

Assessing tumor response after neoadjuvant therapy. Five current pathological classification systems (NSABP, Sataloff, Miller-Payne, EWGBSP, CPS EG) were applied retrospectively to assess pathological response after neoadjuvant chemotherapy. These systems are based on the extent of residual tumor tissue or clinical and pathological parameters.

Statistical analysis. The gene chip mRNA expression results were analyzed within R statistical environment (R version 2.10.1). For statistical testing of protein expression was used SPSS 15.0 software.

4. RESULTS

Our investigations resulted in the following novel observations:

1. Normal mammary gland can expresses connexin (Cx) 30, Cx32 and Cx46 besides the earlier described Cx43 and Cx26.
2. Primary breast cancer can express Cx30 besides the earlier detected Cx43, Cx26, Cx32 and Cx46.
3. In line with the significant correlations found at the mRNA level, elevated Cx43 expression proved to be an independent, positive prognostic marker. On the contrary, Cx30 proved to be an independent, negative prognostic marker.
4. In neoadjuvant treated breast cancers:
   - Cx32 expression correlated positively with pre-chemotherapy HER2 level, negative correlation with pT and post-chemotherapy Ki67 status.
   - Cx43 leves showed positive correlation with hormonreceptor (HR) status pre- and post-chemotherapy and negative correlation with pre-chemotherapy HER2 level.
   - Cx46 levels showed positive correlation with pN but latter has no correlation witth overall survival.

   Correlation between connexin expression and prognostic markers:
   - Cx26 level had negative correlation with Nottingham Prognostic Index (NPI).
   - Cx32 expression had positive correlation with tumor grade, necrosis, NPI and negative correlation with HR status.
   - Cx43 expression had positive correlation with HR status and negative correlation with tumor grade.
   - Cx46 level had negative correlation with tumor grade.
5. In gene expression datasets, increased Cx43 mRNA level associated with better disease outcome in ER positive, luminal A and grade 2 tumors and showed significant worse prognosis in ER negative and triple negative subgroups.

   Elevated Cx30 mRNA level correlated with better survival in ER negative, HER2 positive and tripple negative subgroups and associated with poor outcome in ER positive and luminal A and B tumors. The regulation of Cx43 and Cx30 is probably inverse.
6. The expression of connexins dinamically changed after neoadjuvant chemotherapy. The level of Cx26 and Cx32 were significantly decreased, Cx46 expression showed increased tendency, while Cx43 levels did not change.

7. The increased Cx46 positivity either pre- and post-chemotherapy and reduced level of Cx26 post-chemptherapy may separate prognostically more favourable subgroups within the intermediate categories of the classification systems including Miller-Payne G2-3, Sataloff TB, EWGBSP TR2b and CPS EG 4.
8. In the intermediate prognostic categories (EWGBSP TR2b, Sataloff TB, Miller-Payne G3) only pre-chemo Cx46 levels correlated with the overall survival and proved to be the only potential prognostic factor.

5. CONCLUSION

The expression of connexins and their cell membrane channels dinamically change during the carcinogenesis, tumor progression and metastatic spread. The role of connexins in these sequences were unclear and controversial in breast cancer.

In our studies connexin expression showed prognostic correlations. Transcriptomic analysis showed prognostic value of Cx43 and Cx30, which have reciprocal regulation. Elevated Cx43 protein levels linked to better prognosis in ER positive, lymph node negative and grade 2 tumors and associated with worse disease outcome in ER negative subgroup. Elevated Cx30 levels showed good survival in ER negative, HER2 positive and tripple negative tumors and showed poor survival in ER positive cases. These results were validated by fluorescence immunohistchemistry in neoadjuvant and adjuvant treated breast carcinomas. Concerning Cx43, Cx30 and Cx46 good correlations were found between protein and mRNA data, which however were partly inconsitant for Cx26 and Cx32. This suggests that post-translational, epigenetic processes, differential degradation and treatment regimens may differentially influence the regulation of connexins expression.

In adjuvant treated breast cancer, Cx43 and Cx30, which respectively show positive and negative prognostic values, offer themselves as potential markers of breast cancer outcome. Moreover, Cx43 can stratify grade 2 tumors into good and poor relapse free survival. Cx30 can stratify grade3 cancers into poor and good overall survival subgroups.

During neoadjuvant chemotherapy reduced Cx26 and Cx32 levels and elevated Cx46 expression may also reflect the efficiency of chemotherapy. Decreased post-chemotherapy Cx26 expression statistically correlated with better overall survival. Elevated Cx46 expression pre- and post-chemotherapy and reduced Cx26 level post-chemotherapy correlated with significantly better survival in the intermediate subgroups of EWGBSP TR2b, Sataloff TB and Miller-Payne G2-3 and CPS EG classifications. Pre-chemotherapy Cx46 expression was the only marker that correlated with overall survival within this subgroup.

Moreover, for the first time we detected Cx30, Cx32 and Cx46 in normal breast and Cx30 in primary breast cancer.

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