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IMPACT OF BREAST CANCER MOLECULAR CLASSIFICATION ON PROGNOSIS AND THERAPY

ABSTRACT

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GENERAL PART

INTRODUCTION

Breast cancer is the most common cancer in women, and its incidence has increased significantly, due to the introduction of early detection methods or even systematic screening, especially in developed countries. In breast cancer, biomarkers analysis is a routine practice, which not only provides additional information in addition to the classic clinical factors, but also allows for personalized treatment with significant increase in the survival rate.

The molecular diagnosis of breast cancer allows the stratification of patients into risk groups with different prognosis and survival rates. The molecular profile of breast tumors may answer several major questions: whether tumor biology differs within the same subtype compared to normal breast tissue, whether it may have a predictive role in the clinical course of patients with histopathologically similar tumors, and whether it may have predictive character for the response to adjuvant therapy in individual cases. Initially, five distinct molecular types of breast cancer were identified by gene analysis: estrogen receptor positive, which includes the classic luminal A and luminal B types, and estrogen receptor negative, which include HER2, basal-like, and unclassifiable or normal-like types. Recently, each of the five molecular types has been divided into subgroups characterized in detail in terms of differences within each molecular type.

The main objectives of the present research were:

(1) Study of the literature regarding the evolution of the molecular profile in the normal human mammary gland from precursor cells to mature cells and the transition from normal development to local invasion and metastatic phenotype; (2) Identification of controversial markers of normal breast tissue, with potential impact on breast carcinogenesis; (3) Selection of molecular markers with a prognostic role in other types of tumors, but not included in the molecular classification of breast cancer, based on criteria of opportunity and novelty; (4) Carrying out the study itself; (5) Study the feasibility of including E-cadherin and P-cadherin as potential markers in the molecular classification of breast cancer; (6) Study of E- and P-cadherin coexpression with platelet-derived growth factors (PDGF) A and B in molecular forms of breast cancer; (7) Study of the role of Chloride intracellular channel 1 (CLIC1) in interrelation with E-cadherin and P-

cadherin in molecular forms of breast cancer; (8) Study the role of SOX2 correlated with the two cadherins as well as with CLIC1.

II. GENERAL PART

Recent studies have shown that normal breast tissue is composed of epithelial and nonepithelial cells with different profiles reflecting their maturation and differentiation. In addition, cells of normal mammary tissue are known to yield abnormal clones that may contribute to the development of both preneoplastic and tumor lesions. It appears that epithelial cells of the normal breast exhibit a heterogeneous profile depending on their differentiation stage. Considering these facts, several reports have referred to the existence of two luminal phenotypes and two basal phenotypes, based on differential immunohistochemical profiles. These cells are known to express CD24, CD49f, and the epithelial cell adhesion molecule (EpCAM). Luminal progenitor cells, of normal breast tissue, express both CD49f and EpCAM while their mature variants do not express CD49f. Myoepithelial cells, along with basal progenitor cells, lack EpCAM expression. Both mature luminal cells and progenitor cells express CD44 and CD24.

In addition to the expression of BRCA1 and c-myc, the epithelial cells of the normal mammary gland also express hypoxia inducible factor (HIF) 1 and 2, which are associated with a high metastatic rate and a poor prognosis. HIF-1α is expressed before epithelial cells gain functional polarity and HIF-2α is expressed in the latter stages of the mammary gland cycle. In addition, it appears that the behavior of epithelial cells, belonging to both normal and tumor breast tissue, is dependent on the expression of estrogen and progesterone receptors and on soluble factors derived from fat tissue. Microenvironment changes greatly influence the behavior and pheno-type of mammary epithelial cells. Upon exposure to conditioned media, cells are able to undergo various changes, altering motility and metalloprotease activity.

The "basal-like" type of breast cancer has been shown to be more common in patients with and early menarche, that were young age at first pregnancy, as well as in women that had a short breastfeeding period and obese women. Despite the relatively good response to chemotherapy, the "basal-like" type has a

poor prognosis due to frequent relapses associated with multiple visceral metastases. The results obtained by gene analysis overlap with those obtained by immunohistochemistry, based on the expression of hormone receptors (ER and PR), HER2, EGFR, monoclonal cytokeratins, p53 and Bcl-2. The recently identified molecular subtype "claudin-low" has been associated to a lesser extent with the conventional histopathological type of lobular carcinoma (most of which does not, in fact, have lobular architecture), and is a distinct entity of the "triple negative" type considering its low expression of claudins, but also its poorly differentiated appearance, high tumor grade and, most often, a rich inflammatory infiltrate. E-cadherin negative / Ki67 positive status in the case of triple negative tumors is considered an unfavorable prognostic factor. Adjuvant therapy has been shown to be effective in patients with triple-negative stage II tumors. The expression of receptors for androgen hormones in triple negative tumors, together with the differences in Bcl-2 expression observed in triple negative vs non-triple negative tumors are two aspects intensively studied as prognostic and therapeutic factors in breast tumors.

Prognostic markers for identifying cases with an increased potential for progression and metastasis are extremely limited and nonspecific in malignant tumors, also including breast tumors. There is currently no specific marker for tumor progression and metastasis, specific to tumors of different origins and, for this reason, the stratification of breast malignancies within the same molecular class is a stringent need. Studies in the literature describe a series of versatile markers that lose or change their expression depending on the tumor status, but at the moment, their role in tumor progression and metastasis is very little elucidated. Of these, the markers we chose specifically to study are extremely controversial. Moreover, the combination of E-cadherin, P-cadherin and CLIC1 for the stratification of malignant breast tumors within the same molecular class is not known and no data have been published to date.

E-cadherin is a calcium regulated adhesion molecule, expressed in most epithelial tissues. The gene for E-cadherin is located on chromosome 16q22. Selective loss of E-cadherin may cause differentiation and invasiveness in human carcinomas. In different cell lines, a reciprocal relationship has been demonstrated between E-cadherin expression levels and the degree of invasiveness. Decreased E-cadherin expression was observed in aggressive tumors of the esophagus, ovary and stomach.

P-cadherin, also known as placental cadherin, was originally found in the placental tissue of mice, and its coding gene, CDH3, is located on chromosome 16 16q22.1. In breast cancer, P-cadherin overexpression has been found to promote cell motility, cell migration, and invasion capability. A similar aggressive phenotype was observed in bladder and pancreatic cancer cell lines.

The platelet-derived growth factor receptor (PDGFR) pathway is a signaling network of importance for the normal development of cells of mesenchymal origin. The PDGF signaling network consists of two tyrosine kinase receptors, PDGFR alpha (PDGFRA) and PDGFR beta (PDGFRB) and five ligands PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD from four genetic products (PDGF A, B, C and D). Autocrine stimulation of the pathway is commonly seen in various neoplasms, such as gliomas, gastrointestinal stromal tumors (GIST), and chronic myelomonocytic leukemia. In addition, dysregulation of paracrine PDGFR signaling may cause extracellular matrix remodeling in a tumor-promoting manner to facilitate migration, invasion, angiogenesis, and possibly lymphangiogenesis.

CLIC1 (intracellular chlorine channel 1) is a protein belonging to the family of chlorine ion channels. This protein is naturally expressed in the human body and is involved in many cellular processes (regulation of cell volume, regulation of membrane potential, regulation of the cell cycle, cell proliferation and differentiation). A potential involvement of CLIC1 in tumor development is suspected both for its involvement in the cell cycle and for its functional expression during oxidative stress.

SRY (Y sex determination region) -box 2, also known as SOX2, is a necessary transcription factor for pluripotency during early embryogenesis and for maintaining embryonic stem cell identity. Expression studies suggest a pivotal role for the SOX2 gene in the developing central nervous system, especially in the pituitary, forearm and eyes. Scattered data on the involvement of the SOX2 gene in the normal development of other organs, such as the pancreas, liver, or gastrointestinal tract, have so far been published. Recently, the potential role of SOX2 in human carcinogenesis in squamous cell carcinoma of the gastrointestinal tract and in the basal phenotype, similar to breast cancer, has been highlighted.

III. SPECIAL PART

The special part includes 6 chapters: 2 introductory (motivation, materials and methods), and 4 immunohistochemical studies with distinct purposes: the study of E-cadherin and P-cadherin as potential markers in the molecular classification of breast cancer; study on the coexpression of E- and P-cadherin with platelet-derived growth factors (PDGF) A and B in the molecular forms of breast cancer; study on the role of protein 1 associated with chlorine channels (CLIC1) in interrelation with E-cadherin and P-cadherin in the molecular forms of breast cancer; study on SOX2 expression in molecular forms of breast cancer evaluated in correlation with E-cadherin, P-cadherin and CLIC1.

The research included 97 cases, represented by biopsy fragments taken during 2016-2018 from patients admitted to the Republican Clinical Hospital Chișinău and the Municipal Emergency Clinical Hospital Arad. The cases selected for the immunohistochemical technique were stained by simple immunohistochemical methods and double immunostaining. Molecular profile and immunohistochemical evaluation were performed using the following antibodies: ER, PR, Her2, Ki67, E-cadherin, P-cadherin, PDGFA, PDGFB, PDGFR beta, SOX2, CLIC, CD34. The automaton used for the immunohistochemical technique was Leica Bond-Max (Leica Biosystems, Newcastle upon Tyne, UK). The solutions used for unmasking were Bond Epitope Retrieval Solution 1 and 2, solutions with pH 6 and 9 (Leica Biosystems, Newcastle Ltd, Newcastle UponTyne NE 12 8EW, UK). 3% hydrogen peroxide was used to block endogenous peroxidase for 5 minutes. The next step was to incubate with the primary antibodies for 30 minutes. Secondary and tertiary antibodies were applied for 8 minutes each. The visualization was performed using the Bond Polymer Refine Detection System. Incubation with 3.3 diamino-benzidine chromogen was 10 minutes. The counter-staining was performed with hematoxylin, applied for 5 minutes. The double immunostaining used was SOX 2 / CLIC1.

From the immunohistochemical point of view, the cases that were included showed in normal structures cytoplasmic (CD34, PDGFA, PDGFB, CLIC 1), nuclear (Ki67, ER, PR, SOX 2) or membranous expressions (Her2, E-cahderin, P -cadherin, PDGFR beta). The analysis and processing of images was done using the Axiocam 506 color microscope, Zeiss, Jena, Germany.

The present research aimed to deepen the knowledge regarding the potential role of E-cadherin and P-cadherin as markers in the molecular

classification of breast cancer, but also the coexpression of E- and P-cadherin with platelet-derived growth factors (PDGF) A and B. Moreover, we wanted to study the role of Protein 1 associated with chlorine channels (CLIC1) in interrelation with E-cadherin and P-cadherin in molecular forms of breast cancer, but also the importance of newly emerged markers, such as SOX2, as potential therapeutic targets for treatment of breast cancer.

In tumor conditions, E- and P-cadherins are intensively studied in the processes of invasion and metastasis, at the same time also responsible for the epithelial-mesenchymal transition, which supports tumor aggression. The use of these markers suggests the review of molecular forms of breast cancer with the introduction of new factors to better explain molecular heterogeneity, different response to therapy and unpredictable long-term prognosis of cases that were initially included in the same molecular subgroup. Our results reveal the differentiated expression and coexpression of the two types of cadherins in breast cancer molecular forms.

The interrelationship of E- and P-cadherin with growth factors is relatively less studied, so that the interaction of E- and P-cadherin with PDGFA and B in breast cancer is related only to the classical forms and to a very small extent to the molecular forms of breast cancer. Our results reveal the interaction of E- and P-cadherins with differentiated expression of PDGFA and PDGFB in molecular forms of breast cancer.

We found it useful to associate the expression of P-cadherin with CLIC1 to define a class of tumors in which a percentage contain stem cells in the molecular forms of breast cancer. The quadruple association between E-cadherin, P-cadherin and CLIC1 in vessels and tumor cells defined 16 phenotypes that were analyzed for the molecular forms of breast cancer included in the study to observe the existence of particular phenotypes, specific to each molecular type. The triple association of cytoplasmic E-cadherin with CLIC1 expression in tumor cells and tumor vessels has defined, as in previous cases, 8 phenotypes for each molecular form of breast cancer.

The present research has identified in the molecular groups of breast cancer distinct subclasses stratified on the basis of certified markers with a role in progression, metastasis and development of resistance to therapy. These subclasses represent clear directions for new larger studies that may lead to a change in the current paradigm in the molecular diagnosis of breast cancer.

CONCLUSIONS AND PERSONAL CONTRIBUTIONS

1. The study of E- and P-cadherin in molecular forms of breast cancer revealed significant differences between their expressions in different molecular classes.

2. Heterogeneity of E- and P-cadherin expression was noted in the same tumor, which suggests the existence of unstable tumor areas, at risk of spreading tumor cells with a particular phenotype and increased potential for invasion and metastasis. In the case of P-cadherin, the positive areas should be identified, being potential sources of cells with stem role and adaptive mechanism to conventional and targeted therapy.

3. E- and P-cadherin expressions correlated with tumor grade for Luminal A type, aspect that was not found in mixed-type cases. In the HER2 type, cytoplasmic expression of E-cadherin had a statistically significant correlation with that of P-cadherin. The TNBC type was characterized by the expression of P-cadherin, as well as by the statistically significant correlation between its expression and G. Cases of Luminal B type showed the highest variability of expression of the two cadherins.

4. Analysis of PDGF and cadherin expression in breast cancer without stratification into well-known molecular forms demonstrated only one correlation between PDGFB and membranous and cytoplasmic expression of E-cadherin, with P-cadherin having no statistically significant correlations with the studied factors.

5. Broken down analysis into molecular forms of breast cancer and correlated with differentiated, membrane, cytoplasmic or mixed expression of E- and P-cahderins, PDGF family members revealed significant differences between different molecular classes.

6. HER2 and TNBC types showed a statistically significant correlation between PDGFA and B expressions in the stromal, tumor and vascular compartments and differentiated expression of E-cadherin and P-cadherin.

7. For Luminal type A, PDGFA and B in tumor cells were correlated with E-cadherin expression, while stromal PDGFB was statistically significantly correlated with P-cadherin.

8. Mixed-type cases were characterized by significant coexpression of vascular PDGFA with cytoplasmic E-cadherin.

9. HER2 type was governed by the coexpression of P-cadherin with PDGFA and stromal B, as well as with vascular-expressed PDGFA.

10. For triple negative cancers, PDGFB and P-cadherin characterized a subgroup of risk.

11. Regardless of the combination used between E-cadherin, P-cadherin and CLIC1 (T and V), we defined a major impact of their combinations on the molecular classes of Luminal B, HER2 + and TNBC types.

12. The global evaluation of the co-expression of E-cadherin / CLIC1T showed that in TNBC we had a percentage of 100% Ecadh + / CLIC1T + cases. Differentiated quantification of cytoplasmic E-cadherin coexpression with CLIC1T stratified TNBC-type cases into two subgroups in which CLIC1T was positive, but E-cadherin varied. Between the two groups, we consider that the subgroup with cytoplasmic expression of E-cadherin and CLIC1 positive in tumor cells (Ecadh.C + / CLIC1T +) is a subgroup at risk for TNBC, with high metastatic potential and possibly increased resistance to therapy.

13. The association of CLIC1V expression (in tumor vessels) with the Ecadh + / CLIC1T + phenotype defined the Ecadh + / CLIC1T + / CLIC1V + phenotype. In this subtype, 89.90% of cases showed cytoplasmic expression of E-cadherin, so that EcadC + / CLIC1T + / CLIC1V + can be considered a risk subgroup.

14. Evaluation of Pcad / CLIC1T demonstrated a similar percentage to that of the Ecad / CLIC1T association for the phenotype in which both markers are positive in the molecular forms HER2 + and TNBC. In the case of P-cahderin, the subgroup Pcad + / CLIC1T + was of interest. The lowest percentage of this phenotype was recorded in Luminal A type, while the TNBC type had the highest percentage of such cases. In contrast, the Pcad- / CLIC1T + phenotype was present in over 55% of Luminal A cases, while in TNBC and HER2 types this phenotype was absent.

15. The Ecad / Pcad / CLIC1T association defined a phenotype specific only to Luminal A type: Ecad- / Pcad + / CLIC1T + in a low percentage of cases (7.69%).

This group can be considered a high-risk group in Luminal A-type breast carcinomas.

16. The Ecad / Pcad / CLIC1T / CLIC1V phenotype certified the usefulness of this panel in stratifying breast tumors within the same molecular group.

17. E-cadherin, P-cadherin, SOX2 and CLIC1 stratify each molecular group of breast cancer into risk subclasses depending on the presence or absence of one of these markers.

18. SOX2 is frequently encountered in aggressive molecular forms such as HER2 and TNBC, its inclusion in the molecular evaluation of breast cancer is mandatory, with a predictive role on the response to therapy.

19. The development of particular phenotypes based on the combination of cadherins with SOX2 and CLIC1 will lead to a differentiated therapeutic approach even within the same molecular group.

20. The E-cadC + / P-Cadh + / SOX2 + phenotype is predominantly observed in HER2 and TNBC cases and characterizes subgroups of tumors with an intense epithelio-mesenchymal transition, doubled by the existence of cells with stem potential.

21. In the SOX2 + / CLIC1 + group, P-cadherin coexpression is also specific to HER2 and TNBC types, this phenotype being absent in Luminal A and Luminal B type cases.

LIST OF PUBLISHED ARTICLES

1. **Madalin Marius Margan**, Anca Maria Cimpean, Amalia Raluca Ceausu, Marius Raica. *Differential expression of e-cadherin and p-cadherin in breast cancer molecular subtypes*. Anticancer Research. October 2020 vol. 40 no.10 5557-5566. (IF=1.994 *-2019*)

2. **Madalin Marius Margan**, Andreea Adriana Jitariu, Anca Maria Cimpean, Cristian Nica, Marius Raica. *Molecular portrait of the normal human breast tissue and its influence on breast carcinogenesis.* Journal of Breast Cancer. 2016 Jun;19(2):99-111. (IF=2.204 *-2016*)

3. Veaceslav Fulga, Lucian Rudico, Amalia Raluca Balica, Anca Maria Cimpean, Lilian Saptefrati, **Madalin-Marius Margan**, Marius Raica. *Differential expression of e-cadherin in primary breast cancer and corresponding lymph node metastases.* Anticancer Research. February 2015 vol. 35 no. 2 759-765. (IF=1.895 *-2015*)

4. **Margan Mădălin-Marius**. *Molecular markers in breast cancer and their clinical significance.* Fiziologia (Physiology), 2015, VOL. 25, No.3 (87), p. 29 – 35. (BDI)