CELL ADHESION AND CELL POLARITY MOLECULES IN INVASIVE MICROPAPILLARY BREAST CARCINOMAS COMPARED WITH INVASIVE BREAST CARCINOMAS OF NO SPECIAL TYPE

PhD thesis

Zsófia Kramer

Semmelweis University Doctoral School

Pathology and Oncology Division

Supervisor: Anna Mária Tőkés, Ph.D

Official reviewers: Baghy Kornélia, Ph.D

József Tóvari, D.Sc

Head of the Complex Examination Committee: Anna Sebestyén, Ph.D

Members of the Complex Examination Committee:

András Rókusz, M.D. Ph.D

Andrea Ladányi, Ph.D

Budapest

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1. Introduction

Breast cancer is the most prevalent malignant tumor in women and the leading cause of mortality in females worldwide. The most common subtype of breast cancer is the hormone receptor positive subtype. Histologically, the majority of breast carcinomas are invasive breast carcinomas of no special type (IBC-NST). Invasive micropapillary breast carcinoma (IMPC), a special subtype, comprise 1-8.4% of all breast carcinoma cases. Distinct histopathological features of IMPC tumors include tumor cell clusters or morules which are situated in empty stromal spaces. The tumor cells in these clusters show a reversed polarity: the apical side faces the stroma, while the basal part of the cells looks toward the center of the cell groups. Histological visualization of this reversed polarity is performed by immunohistochemistry using Epithelial Membrane Antigen (EMA), which shows a typical inside-out staining pattern with linear positivity at the periphery of the morule-like clusters. IMPC tumors are described as showing higher rate of locoregional recurrence, lymphovascular invasion and axillary lymph node involvement. Interestingly, despite these findings, differing IMPC survival rates were reported in various studies.

The histological features of IMPCs are well described, but the underlying mechanisms forming this special appearance and the background pathomechanisms causing the high locoregional aggressiveness are not entirely understood. Several studies examined the genetic alterations in IMPC tumors, and concluded that this special histological subtype comprises a heterogenous group of tumors with genetic alterations different from IBC-NST tumors. Better understanding of the processes behind the behavior of IMPCs may open new therapeutic opportunities for patients presenting with this special tumor subtype.

Considering the inverted polarity in IMPCs, it is reasonable to assume that the reversed polarity plays a significant role in the high locoregional aggressiveness of these tumors.

Cell polarity is regulated by three main protein complexes, i.e. Crumbs-, Par-, and Scribble complexes. These protein complexes play a crucial role in normal cellular and structural development, epithelial apico-basal polarity and directed cell migration. Alterations in these protein complexes are widely

observed in cancer development and progression. According to Gruel et al, LIN7A, a cell polarity gene, plays a significant role in polarity defects seen in breast carcinomas, especially in IMPCs. More and more research studies focus on how alterations in cell polarity impact the regulation of tumor growth, cell survival and apoptosis via signalling pathways. It has been described that polarity proteins are involved in several signalling pathways such as the mTOR, Hippo, Hedgehog, JAK/STAT or MAPK pathways, which all influence cell proliferation.

The gain of migratory properties is another crucial step in carcinogenesis. Cell migration requires cell polarity changes and changes in cell adhesion molecules, which is called the epithelial-mesenchymal transition (EMT) that allows tumor cells to migrate individually. It has been described that cancer cells do not necessarily undergo a complete epithelial-mesenchymal transition during the invasion but can migrate collectively, requiring a transition to a hybrid epithelial/mesenchymal state. During EMT, the apico-basal polarity is disrupted, and the intracellular actin dynamics and microtubule network undergo significant alterations. These changes impact the extracellular matrix properties as well.

Polarity proteins also play role in the cell adaptation to metabolic stresses. Scrib and Lgl2, proteins which are part of the Scrib polarity complex, are involved in recruiting and stabilizing amino acid transporters which help increase the leucine uptake required for cell proliferation in nutrient stress in estrogen-receptor positive breast cancer. These proteins are also involved in the development of tamoxifen resistance in these tumor types.

Cell adhesion molecules and tight junction proteins are crucial in tissue morphogenesis, in cell-cell, and cell-extracellular matrix signalling. The main tight junction proteins include claudins, occludins, PALS1 (Proteins Associated with Lin Seven 1), MUPP1 (multi-PDZ domain protein 1) and the zonula occludens proteins ZO-1, ZO-2, ZO-3 (27), which are all framework forming proteins connecting transmembrane proteins with cytoskeletal actin. The localization pattern and expression profile of these proteins have been studied by various groups, which found differences in normal and tumorous tissues, as well as in different cancer types. These molecules play a critical role in both tumor progression and suppression via distinct mechanisms. One

pathway is the above mentioned EMT, activated by the WNT/B-CATENIN, JAK/STAT3 and PI3K/AKT pathways. Tight junctions are mainly formed by claudin proteins, first described by Furuse et al. Claudins form continuous strands in the apical region of epithelial cells. Still, they are also present along the lateral cell membrane as free strand ends. Altered claudin expression was described in numerous cancer types, as they highly contribute to tumor progression in a tissue-specific manner. Up to date 27 human claudins have been identified, with claudin-1, -3, -4 and -7 being the most studied in breast carcinomas. Higher recurrence rate and metastatic potential and also poor prognosis are suggested to be associated with a decrease in or loss of claudin-1 expression. Claudin „low" breast carcinomas are a subset of breast tumors that are defined by decreased gene-expression of claudins-1, -3, -4, -7 and -8, or by decreased protein expression of claudin-3, -4, -7, E-cadherin and calcium-dependent cell-cell adhesion glycoprotein. Claudin „low" breast carcinomas are histologically mostly triple negative, high grade tumors, with an intermediate response rate to standard chemotherapy.

2. Objectives

1. To compare the invasive micropapillary carcinoma (IMPC) with the invasive breast carcinoma of no special type (IBC-NST) based on clinicopathological characteristics.
2. To perform mRNA analysis on the cohort to identify the genes involved in forming the distinct structure of IMPC tumors.
3. To investigate whether the genes differently expressed on the mRNA level also exhibit altered protein expression in IMPC and IBC-NST tumors.
4. To assess the correlation between mRNA expression and protein expression levels in these tumor types.
5. To analyse the relationship between gene and protein expression patterns and clinical outcomes, including survival, tumor grade, and lymph node involvement.
6. To identify distinct prognostic groups to facilitate the development of tailored therapeutic approaches.
7. To evaluate claudin expression patterns across various molecular subtypes of breast cancer.
8. To determine the prevalence of IMPC tumors exhibiting a “claudin-low” phenotype.
9. To identify proteins or protein groups specifically expressed in IMPC tumors that could serve as potential therapeutic targets.

3. Materials and Methods

a. Patient cohort

The cohort comprised of 36 cases of IMPC, 36 age- and stage-matched IBC-NST tumors and 8 mixed (IMPC/IBC-NST) tumors. All samples were selected from the archive of the Department of Pathology, Forensic and Insurance Medicine (Semmelweis University, Budapest), from the time period between 2000 and 2018. For the immunohistochemical analyses we used a largely identical cohort with the addition of 2 cases from the time period of 2019 to 2021 (37 IMPC, 36 age- and stage-matched IBC-NST and 9 mixed IMPC/IBC-NST cases). IMPC subtype was confirmed by the specific inside-out staining pattern of EMA immunohistochemical staining. Patient data, tumor characteristics, and patient follow-up information were collected from the Semmelweis University Health Care Database and the National Cancer Registry. The study was reviewed and approved by the Semmelweis University Research Ethics Committee (permission number: 240/2016).

b. Assembling the gene panel for the study

Gene expression analyses were performed using the NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA) with a custom designed codeset for all samples. Genes, involved in cell-adhesion, tight junction, cell polarity and cancer signalling pathways including epithelial-mesenchymal transition associated with breast carcinomas were reviewed in the literature. 43 genes of interest and five housekeeping genes were selected altogether.

c. mRNA isolation

Three to five, 5 μm thick sections were cut from FFPE tissue blocks and set in sterile Eppendorf tubes. In the cases of mixed IMPC/IBC-NST (8 cases altogether), based on the H&E morphology, the two components were separately macrodissected and further analyzed. mRNA was extracted from the tissue samples using the QIAGEN® RNeasy® FFPE Kit according to the

manufacturer's instructions. mRNA concentrations were measured by Quantus Fluorometer (Promega), and the samples were diluted to 30 ng/µl.

d. NanoString nCounter mRNA analysis

mRNA hybridization was set up using the 12-tube PCR hybridization strips, Reporter CodeSet and Capture ProbeSet provided by NanoString following the manufacturer's guide. T samples were then placed into the nCounter Prep station, and analyzed in the Digital Analyser (nCounter FLEX Analysis System).

e. Immunohistochemical analysis of claudin-1, -3, -4 and -7

FFPE tissues were used for immunohistochemical analyses. Immunohistochemical reactions on claudin-1, claudin-3, claudin-4, and claudin-7 were performed on 3-5 µm thick sections using the Ventana BenchMark Ultra system and according to the Universal UltraView DAB manufacturer's protocol.

f. Quantification of claudin expression

Slides were scanned with a 3D HISTECH Pannoramic® 1000 digital slide scanner. One expert histopathologist (ZK) analyzed all immunohistochemical slides on digitized slides. A second expert (AT) analyzed 20% of the cases, and the agreement of the results was evaluated. The two components were separately evaluated in cases of mixed IMPC/IBC-NST tumors. Correspondingly, 91 samples, 46 IMPC and 45 IBC-NST were analyzed. To date, no standardized methods have been available to quantify the expression of claudin proteins. In our study two different methods were used to quantify the IHC results:

a. A 4-tier immunohistochemical score system was applied on the cohort. No evidence of membranous or cytoplasmic staining was evaluated as a score of 0; increasing staining intensities were scored from 1+ to 3+. Samples showing a score of 0 were declared as negative, and scores 1+, 2+, and 3+ were grouped as positive samples.

b. The H-score was determined by adding the results of the multiplication of the percentage of cells with staining intensity ordinal value (scored from 0

for “no signal” to 3 for “strong signal”) with 301 possible values. High and low expression was determined by calculating median values. H-score values below the median were considered as low expression and those above the median were considered as high expression.

g. Statistical analysis

The median of mRNA expression values of examined genes was set as the threshold. mRNA expression values below median were defined as „low expression” and above median as „high expression”. Kaplan–Meier analysis was performed using distant metastasis free survival (DMFS) as the endpoint in the mRNA analysis. DMFS intervals were determined as the time period from the initial diagnosis to the time of diagnosing distant organ metastasis. Mixed IMPC/IBC-NST cases were excluded from the survival analysis due to the low patient number. Statistical significance was confirmed when p-values were <0.05. To compare our results of the prognostic impact of selected genes (based on DMFS) with a large database, the KM Plotter Online Tool, a publicly available database, was used.

4. Results

a. Patients characteristics

In the mRNA expression study, samples of 80 breast cancer patients were examined (36 IMPC, 36 IBC-NST and 8 mixed IMPC/IBC-NST cases). For the immunohistochemical analyses, a largely identical cohort was used as for the mRNA study, with a few extra cases added: 36 IBC-NST, 37 IMPC and nine mixed IMPC/IBC-NST tumors were examined. Mixed tumor components were analyzed separately for protein expression and were included to the IMPC (46 samples) and IBC-NST (45 samples) groups respectively (91 samples in total). All cases were categorized into surrogate subtypes according to the 2011 St. Gallen International Expert Consensus. The data of patients' tumor characteristics selected for our studies are presented in **Table 1.,** showing merged data of the mRNA and immunohistochemical analysis. All three patient groups showed similar distribution regarding age and prognostic factors.

Table 2. Patients' and tumors' characteristics

	IBC-NST	**IMPC**	**Mixed IMPC/IBC-NST**	**p-value***
Total patient number	36	37	9	
Number of samples examined	45	46		
Median years of age (range)	63 (34-83)	63 (33-85)	61 (34-69)	
Median of Ki67 LI (range)	15 (1-100)	15 (1-90)	16 (5-90)	0.22[1]
Grade				0.90[2]
I	3 (8.3%)	3 (8.1%)	1 (11.1%)	
II	20 (55.5%)	23 (62.2%)	4 (44.45%)	
III	13 (35.2%)	11 (29.7%)	4 (44.45%)	
T				0.85[2]
1	14 (38.9%)	18 (48.6%)	3 (33.3%)	
2	11 (30.6%)	8 (21.6%)	4 (44.5%)	
3	8 (22.2%)	8 (21.6%)	1 (11.1%)	
4	3 (8.3%)	3 (8.1%)	1 (11.1%)	

N				0.73[2]
0	17 (47.2%)	20 (54.1%)	3 (33.3%)	
1	8 (22.2%)	8 (21.6%)	4 (44.5%)	
2	6 (16.7%)	3 (8.1%)	1 (11.1%)	
3	5 (13.9%)	6 (16.2%)	1 (11.1%)	
ER				0.05[2]
+	27 (75%)	35 (94.6%)	8 (88.9%)	
-	9 (25%)	2 (5.4%)	1 (11.1%)	
PR				**0.005[2]**
+	17 (47.2%)	29 (78.4%)	8 (88.9%)	
-	19 (52.8%)	8 (21.6%)	1 (11.1%)	
HER2				0.59[2]
+	5 (13.9%)	8 (21.6%)	1 (11.1%)	
-	31 (86.1%)	29 (78.4%)	8 (88.9%)	
Distant metastasis				0.27[2]
absent	22 (61.1%)	29 (78.4%)	6 (66.7%)	
present	14 (38.9%)	8 (21.6%)	3 (33.3%)	
Surrogate molecular subtypes				0.93[2]
LUM-A	10 (27.8%)	20 (54.1%)	4 (44.5%)	
LUM-B1	14 (38.9%)	7 (18.9%)	3 (33.3%)	
LUM-B2	3 (8.3%)	8 (21.6%)	1 (11.1%)	
HER2 positive	2 (5.6%)	0	0	
TNBC	7 (19.4%)	2 (5.4%)	1 (11.1%)	

[1] Kruskal-Wallis test, [2] Chi-square test

b. Gene expression pattern difference between IMPC and IBC-NST groups

The distribution of patient characteristics was similar between the IMPC and the mixed groups, therefore IMPC component of mixed tumors was included to the IMPC group for gene expression pattern comparison of the two groups. mRNA expression levels were significantly different in 12 genes out of the examined 43 genes. In IMPCs, the expression levels of CLDN1 (p=0.004), DLG1 (p= 0.002), ITGA1 (p= 0.04), SLUG/SNAI2 (p=0.007), ZEB1 (p=0.04) were significantly lower, while those of AF6 (p=0.000005), CLDN3 (p=0.000005), CLDN4 (p=0.002), CLDN7 (p=0.0001), LIN7A (p=0.00008), CDH1 (p=0.01), OCLN (p=0.0002) were significantly higher (**Figure 1.**).

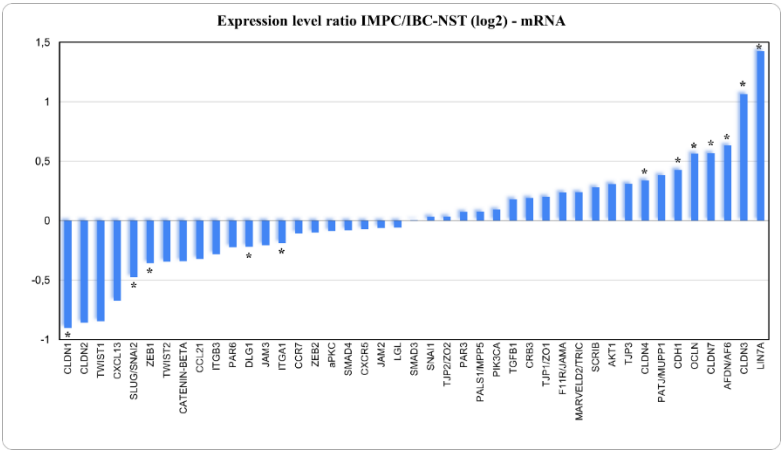

Figure 1. Gene expression ratio in IMPC/IBC-NST tumors.
Diagram showing the gene expression ratio of IMPC/IBC-NST tumors in the examined 43 genes. X axis: 43 examined genes, Y axis: logarithm of ratio of IMPC/IBC-NST mRNA expression values. Asterix (*) marks genes showing significantly different expression values between the two tumor groups

c. Protein expression analysis

Among other findings, the mRNA expression study revealed differences in gene expression of CLDN1, CLDN3, CLDN4, and CLDN7 between the two histological subtypes. Our next aim was to examine these expression differences at the protein level.

Immunohistochemical protein expression of claudin-1 was generally weak membranous and/or cytoplasmic or negative. Claudins-3, -4 and -7 showed variable intensity, mainly circumferential or partial membrane positivity.

d. Protein expression pattern difference between IMPC and IBC-NST groups

While mRNA expression of CLDN1, CLDN3, CLDN4, and CLDN7 showed significant differences between the two histological subtypes, only claudin-7 protein expression exhibited significantly higher H-score values in the IMPC group (p=0.01). No significant differences in protein expression were observed for claudin-3 or claudin-4 between the two histological subtypes (p-values: 0.15 and 0.28, respectively). The median H-score values for claudin-1 protein expression were 0.5 in the IMPC group and 0 in the IBC-NST group.

e. Comparison of protein and mRNA expression levels

mRNA expression level results and H-score values were compared to see the potential correlation between the two expression values. Very low median values were found for both CLDN1 mRNA (184.74) and claudin-1 protein expression (0.5). Interestingly, while the median value of CLDN4 mRNA expression was high (3683.8), claudin-4 protein expression showed very low (10) median value. Both mRNA and protein expression was high in approximately two-thirds of the samples for claudin-3 (68%), claudin-4 (61%), and claudin-7 (70%), whereas samples exhibiting both low mRNA and low protein expression were seen in 36% (claudin-3), 49% (claudin-4) and 34% (claudin-7) of cases. For claudin-1, the correlation was 52% (high expression) and 47% (low expression).

f. The prognostic impact of the analyzed genes and proteins

<u>Impact of mRNA and protein expression on survival</u>

DMFS intervals of the 36 pure IMPC and 36 IBC-NST patients, as well as of the 8 mixed IMPC/IBC-NST patients were compared. No significant differences in DMFS between IMPC, IBC-NST and mixed IMPC/IBC-NST patients were found by statistical analyses (p= 0.92), or when comparing only pure IMPC and IBC-NST cases (p=0.71). No differences were seen in DMFS between the IMPC and IBC-NST tumors in the extended cohort of the immunoexpression analysis (p=0.63).

Levels of mRNA expression of all examined genes in the entire cohort were correlated with DMFS times. Micropapillary component of mixed IMPC/IBC-NST tumors was added to the IMPC group for survival analysis. Low expression levels of PAR6 and high levels of CLDN3, and PALS1 were associated with shorter DMFS intervals (p=0.04, p=0.01 and p=0.01, respectively) (**Figure 2A., 2B. and 2C.**). The expression level of the other examined genes showed no statistically significant association with DMFS.

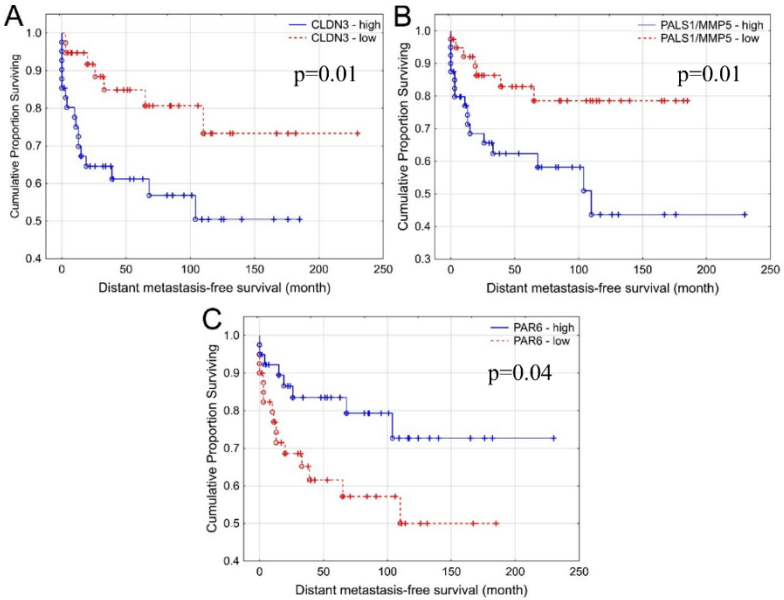

Figure 2. Correlation of gene expression data with distant metastasis free survival

mRNA expression levels of CLDN3, PALS1 and PAR6 showed significant association with DMFS (A, B and C)

Immunoexpression levels of the IMPC and IBC-NST cases were used for statistical. The H-score evaluation resulted in very low median value for claudin-1 expression (0.5), so we did not perform further statistical analysis on claudin-1 protein expression results. Claudin-3 and -7 showed no correlation with DMFS (p=0.74 and 0.96, respectively). Low claudin-4

expression correlated with significantly longer DMFS (p=0.002) (**Figure 3A-C.**). According to the evaluation of the 4-tier system mostly similar results were obtained. Claudin-4 positivity was associated with significantly shorter DMFS (p=0.006). Claudin-3 and claudin-7 protein expression were not associated with DMFS (p=0.20 and p=0.45, respectively) **(Figure 4A-C.)**.

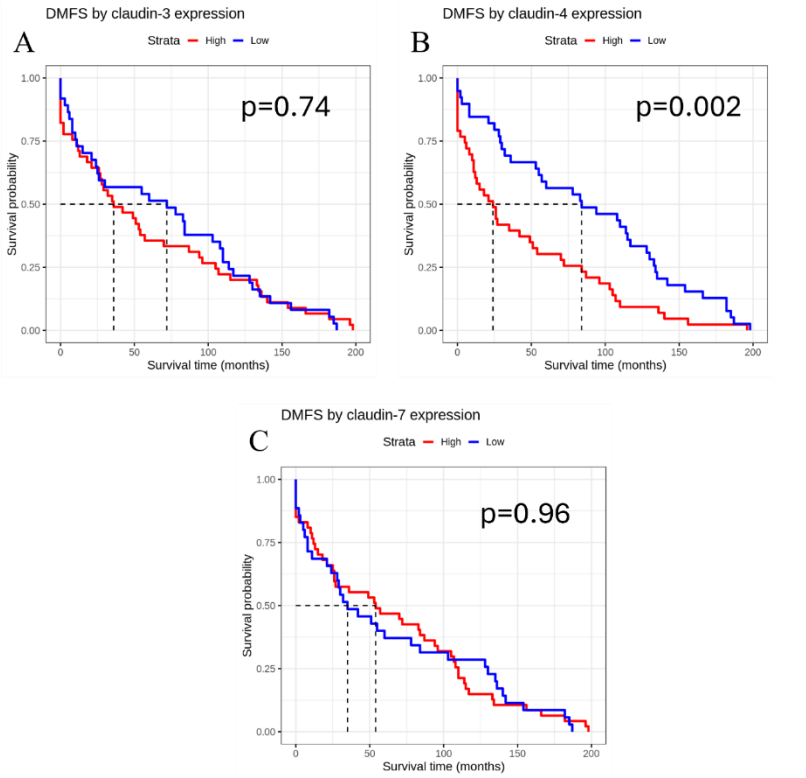

Figure 3. Claudin expression effect on DMFS after evaluation of immunoexpression according to the H-score. Effect of Claudin expression on DMFS based on H-scoring evaluation of the immunohistochemical staining. High claudin-4 protein expression was associated with significantly shorter DMFS (B), while claudin-3 and claudin-7 protein expression showed no correlation with DMFS (A and C).

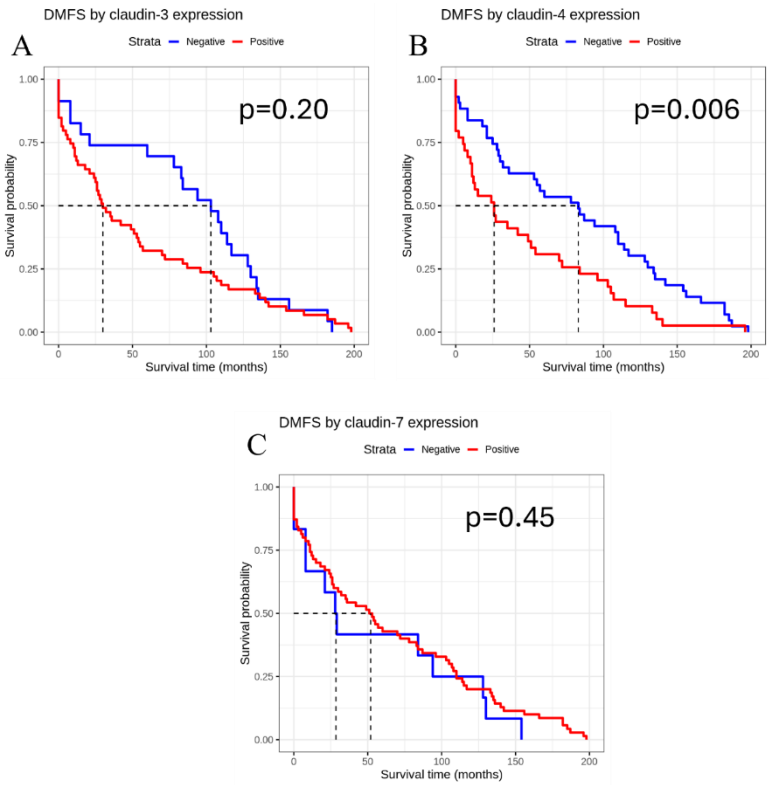

Figure 4. Effect of Claudin expression on DMFS after evaluating the immunohistochemical reactions based on the 4-tier system. High claudin-4 protein expression was associated with significantly shorter DMFS (B), while claudin-3 and claudin-7 protein expression showed no correlation with DMFS (A and C).

Relationship between gene and protein expression and tumor histological grade

We also analyzed whether CLDN3, PALS1 and PAR6 mRNA expression levels were associated with tumor grade (grade 1 and 2 tumors were grouped

together, while grade 3 tumors were in a separate group). High CLDN3 expression levels were associated with high grade tumors (p=0.0005).
In univariate analysis, no significant correlation with tumor grade was found with PALS1 and PAR6 expression levels, suggesting that they might be grade independent prognostic factors (p=0.80 and p=0.90 respectively). Multivariate analysis confirmed only PALS1 as a grade independent prognostic factor (p=0.007). Similarly to the mRNA expression results, claudin-3 protein expression was associated with tumor grade (p=0.03). At the same time, claudin-4 and -7 did not show a correlation with grade (p=0.15 and 0.37, respectively).

Gene expression data and its association with axillary lymph node involvement

A potential association between gene expression levels and lymph node status (pN0 vs. positive cases) was also analyzed. High expression levels of AKT1 were associated with lymph node metastasis (p=0.03). The analyzed chemokines and their receptors did not show any association with lymph node involvement in the cohort.

g. Prognostic analysis of CLDN3, PALS3 and PAR6 mRNA in breast cancer: a comparison with the KM Plotter Database

High mRNA levels of CLDN3, PALS1 and low levels of PAR6 correlated with shorter DMFS in our cohort. Additionally, in accordance with the online KM Plotter database, which presents data from their own large cohort of breast carcinomas (regardless of their histological type), high CLDN3 level is associated with shorter DMFS (p=0.003), while in the KM Plotter database, PALS1 and PAR6 showed no significant correlation with DMFS.

h. Claudins' distribution among breast cancer subtypes

In the extended cohort of the immunohistochemical analysis, hormone receptor positivity (HR+) was seen in 94.6% of the IMPCs and 75% of IBC-NST samples. Examining the HR+ and HR- samples separately, we have seen that HR+ samples showed positivity in 56.71% of the samples for claudin-3,

46.1% for claudin-4 and 89% for claudin-7. LUM-A-like subtype was the most representative subtype in the cohort (38/91, 41.7%) showing claudin-3 and claudin-7 positivity and claudin-4 negativity in 76%, 92% and 58% of the samples, respectively. Evaluation according to the 4-tier method showed an association of claudin-7 expression with the surrogate subtype (p=0.001). In our cohort, 8 samples were considered negative for claudin-3, -4, and -7 immunoexpression (claudin all low group), 6 IBC-NST, and 2 IMPC tumors. Four samples were LUM-A, 2 samples LUM-B1 and 2 samples TNBC surrogate subtype. Due to low sample numbers, further statistical analyses were not performed on the claudin all low group.

5. Conclusion

In our study we have compared IMPCs, a special histological subtype of breast carcinomas with IBC-NST tumors in an age-, stage-, and grade matched cohort. Twelve genes associated with cell adhesion, cell polarity, and EMT exhibited significant mRNA expression differences in IMPC compared to IBC-NST. Increased mRNA expression of LIN7A, CDH1 and OCLN along with decreased CLDN1 and DLG1 expression may be associated with the unique histological appearance of IMPC tumors. However, changes in epithelial polarity do not appear to be associated with claudin-1, -3 and -4 protein expression, as these proteins showed mostly similar expression in IMPC and IBC-NST tumors. In contrast, high claudin-7 protein expression was significantly more prevalent in IMPCs than in IBC-NST tumors and associated with LUM-A-like subtype. Claudin-low phenotype was only observed in 8 samples in our immunohistochemical study. Similarly to recent literature data, we have not shown differences in DMFS between the two histological groups.

Interestingly, high PALS1 and low PAR6 mRNA expression were linked to shorter DMFS, with PALS1 emerging as a grade independent prognostic factor across the entire cohort. Additionally, gene expression alterations in the mTOR signalling pathway highlight the potential benefit of AKT/mTOR inhibitors in IMPCs, similarly to IBC-NSTs.

Survival data based on protein expression revealed that claudin-4 positive tumors were associated with significantly shorter DMFS, suggesting the potential importance of claudin-4 in cancer progression. If inverted polarity is a feature seen only in cancer cells, further investigation into its development may uncover critical therapeutic targets.

6. Bibliography of the candidate's publications

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