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

PhD thesis

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Content

List of abbreviations:

IMPC: Invasive micropapillary carcinoma

IBC-NST: Invasive breast carcinoma of no special type

EMA: Epithelial Membrane Antigen

DMFS: Distant metastasis free survival

OS: Overall survival

JAM-A: Junctional adhesion molecule A

ZO-1, ZO-2, ZO-3: Zonula occludens-1, -2 and -3

PALS1: Proteins Associated with Lin Seven 1

PATJ: Pals1-associated tight junction

MUPP1: multi-PDZ domain protein 1

DCIS: Ductal carcinoma in situ

CLDN1, -3, -4, -7: Claudin-1, -3, -4 and -7 genes

LUM-A: Luminal-A

LUM-B1: Luminal B-HER2 negative

LUM-B2: Luminal B-HER2 positive

TNBC: Triple negative breast carcinoma

HR+: Hormone receptor positive

HR-: Hormone receptor negative

FFPE: Formalin fixed paraffin embedded

TJ: Tight junction

TGF-ß: Transforming growth factor-ß

ER: Estrogen receptor

PR: Progesterone receptor

LI: Labeling index (%)

CDH-1: E-cadherin gene

AF-6: Afadin gene

OCLN: Occludin gene

CAFs: Carcinoma-associated fibroblasts

MARVEL: MAL and related proteins for vesicle trafficking and membrane link

mTOR: Mammalian target of rapamycin

EMT: Epithelial-mesenchymal transition

1. Introduction

Breast cancer is the most prevalent malignant tumor in women and the leading cause of mortality in females worldwide (1). 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) (1). Invasive micropapillary breast carcinoma (IMPC), a special subtype, comprise 1-8.4% of all breast carcinoma cases (2, 3).

Distinct histopathological features of IMPC tumors include tumor cell clusters or morules which are situated in empty stromal spaces (1). 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 (**Figure 1.**). IMPC tumors are described as showing higher rate of locoregional recurrence, lymphovascular invasion and axillary lymph node involvement (2-9). Interestingly, despite these findings, differing IMPC survival rates were reported in various studies. Some research groups described poor prognosis, while other studies suggested that IMPCs have better long-term survival (5, 9). However, recent studies found no differences in the outcome of IMPCs when compared to IBC-NST tumors (2-9). IMPCs have been described as a special subtype not only of breast carcinomas, but also of tumors of several other organs such as the urinary bladder, stomach, colon, pancreas and lung (10-13).

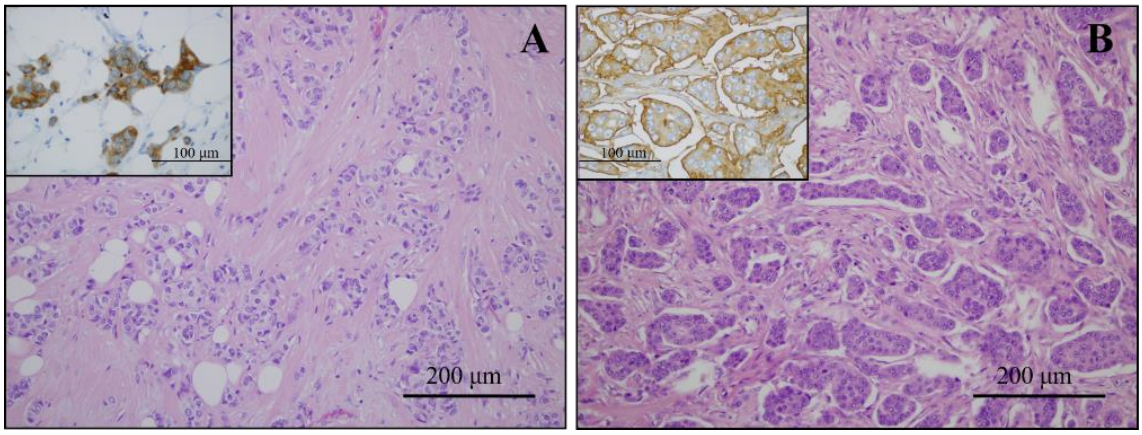

Figure 1. Histopathological appearance of IBC-NST and IMPC tumors
A: H&E picture of an IBC-NST tumor, insert - EMA immunohistochemical stain
B: H&E picture of an IMPC tumor, insert - EMA immunohistochemical stain showing the typical inside-out staining pattern.

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 (14, 15). 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 (16-20). Loss of cell polarity is one of the hallmarks of cancer, which allows cancer cells to gain new functions such as migration and invasion. However, studies

showed that these changes were tumor permissive without acting as direct tumor promoters (17). In breast cancer, genes responsible for coding cell polarity proteins show altered copy numbers in 40% of cases (19). According to Gruel et al, LIN7A, a cell polarity gene, plays a significant role in polarity defects seen in breast carcinomas, especially in IMPCs (21). 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 (20).

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 (18, 22). 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. These interactions between extracellular matrix remodeling and polarity signalling influence cancer progression (23). Xue et al. showed that lower expression of Par3, a key element of the Par cell polarity complex, is seen in metastases of breast cancers, compared with primary tumors (24). Par3 deletion in breast carcinomas also activates aPKC and the downstream JAK/Stat3 pathway, which promotes matrix-metalloproteinase enzyme production, and induces the Par3 depleted tumor cell detachment and metastasis (25).

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 (26).

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 (28-31). Tight junctions are mainly formed by claudin proteins, first described by Furuse et al. (32). 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. Continuous turnover of claudins is seen along the lateral membrane, providing stability to the tight junctions (33). Altered claudin expression was described in numerous cancer types (29), as they highly contribute to tumor progression in a tissue-specific manner (34). 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 (35). In „basal-like", triple negative breast carcinomas, high claudin-1 and -4 expression was observed in most of the cases (36-38), and high cytoplasmic claudin-3 expression correlated with poor survival (39). According to several studies, claudin-3 and -4 expression correlated with tumor grade (40, 41). However, other studies showed that increased claudin-4 expression was associated with higher tumor grade and with basal-like phenotype (37, 42). Furthermore, histological grade in ductal carcinoma in situ (DCIS) and in invasive carcinoma was previously found to be correlated with decrease in or loss of claudin-7 expression (43, 44). 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 (38), or by decreased protein expression of claudin-3, -4, -7, E-cadherin and calcium-dependent cell-cell adhesion glycoprotein (45, 46). Claudin „low" breast carcinomas are histologically mostly triple negative, high grade tumors, with an intermediate response rate to standard chemotherapy (38, 45, 46).

Directional migration of cells is crucial in tumor biology, leading to tumor cell dissemination, leukocyte migration, and angiogenesis. Chemokines, chemotactic cytokines are the primary cell movers. The main function of chemokines is leukocyte recruitment, but they also regulate cell differentiation, proliferation, survival and senescence. Oncogenes, such as beta-catenin, Ras, and mutant p53 have been found to target chemokines and chemokine receptors. Many studies focus on chemokines and chemokine receptors in breast cancer tumorigenesis and metastasis (47-53). Tumor cells show a chemokine receptor expression repertoire unrelated to the tissue of origin (50). Biswas et al. showed a significant association of co-expression of CXC chemokine ligand 13 (CXCL13) and its receptor (CXCR5) with lymph node metastasis (47, 48). Muller et al. confirmed that breast cancer cells show increased expression of CXCR4 and CCR7. Neutralization of the interactions of CXCL12/CXCR4 decreases the metastatic ability a breast cancer cells to regional lymph nodes. (51). Invasive micropapillary carcinomas of the breast, however, have not been in focus of research regarding chemokines and their receptors.

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). All cases were reviewed by board certified pathologists and classified according to the World Health Organization (WHO) criteria. IMPC subtype was confirmed by the specific inside-out staining pattern of EMA immunohistochemical staining (performed with automated Ventana BenchMark ULTRA system using Cell Marque Mouse Monoclonal antibody, 1:200). 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 (14-16, 21, 30, 47, 54-59). Gene selection for the mRNA analyses was based on the results of our previous research studies about the role of cell-adhesion- and tight junction molecules in breast cancer (42, 60-62), other published results in the field, and on cell polarity (14, 16, 21, 29), but also considered the role of these genes in chemoresistance and as potential therapeutic targets according to the literature (63-65). Additionally, chemokine-chemokine receptor molecules described

in previous publications (47-49, 51-53, 55) and thought to play a role in the development of lymph node metastases, were also included (**Table 1.**). 43 genes of interest and five housekeeping genes were selected altogether.

Table 1. Evaluated genes arranged in groups by their main function (functions may overlap between groups).

	Gene name	**Encoded protein**
Cell polarity genes	CRB3	Crumbs Cell Polarity Complex Component 3
	PALS1/MPP5	Protein-Associated with Lin7
	PATJ/MUPP1	Pals1-Associated Tight Junction Protein
	PAR3	Partitioning-Defective Protein 3
	PAR6	Partitioning-Defective Protein 6
	aPKC	Atypical protein Kinase C
	SCRIB	Protein Scribble Homolog
	LGL	Drosophila Lethal Giant Larvae Protein Homolog-1
	DLG1	Discs Large MAGUK Scaffold Protein 1
	LIN7A	Lin7A Homolog Protein
Tight junction and cell adhesion genes	CLDN1	Claudin-1
	CLDN3	Claudin-3
	CLDN4	Claudin-4
	CLDN7	Claudin-7
	CLDN2	Claudin-2
	TJP1/ZO1	Tight Junction protein-1/Zonula occludens-1
	TJP2/ZO2	Tight Junction protein-2/Zonula occludens-2
	TJP3	Tight Junction protein-3
	MARVELD2/TRIC	Tricellulin
	F11R/JAMA	Junctional Adhesion Molecule-1
	JAM2	Junctional Adhesion Molecule-2
	JAM3	Junctional Adhesion Molecule-3
	CDH1	E-cadherin
	OCLN	Occludin
	ITGA1	Integrin-alpha-1
	ITGB3	Integrin-beta-3
Genes in cancer signalling pathways	SNAI1	Snail Family Transcriptional Repressor 1
	SLUG/SNAI2	Snail Family Transcriptional Repressor 2
	ZEB1	Zinc Finger E-Box Binding Homeobox 1
	ZEB2	Zinc Finger E-Box Binding Homeobox 2
	SMAD3	SMAD Family Member Related Protein 3
	SMAD4	SMAD Family Member Related Protein 4
	TGFB1	Tumor Growth Factor Beta 1
	TWIST1	Twist Related Protein 1
	TWIST2	Twist Related Protein 2
	CATENIN-BETA	Catenin-beta
	AFDN/AF6	Afadin
	PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3Kinase Catalytic Subunit Alpha
	AKT1	AKT Serine/Threonine Kinase 1
Chemokines and	CCR7	C-C Motif Chemokine Receptor 7
	CCL21	C-C Motif Chemokine Ligand 21

their receptors	CXCR5	C-X-C Motif Chemokine Receptor 5
	CXCL13	C-X-C Motif Chemokine Ligand 13
Housekeeping genes	AMMECR1L	AMMECR1L-like protein
	CC2D1B	Coiled-Coil and C2 Domain Containing 1B
	SAP130	Sin3A Associated Protein 130
	ZNF143	Zinc Finger Protein 143
	NUBP1	Nucleotide Binding Protein 1

c. mRNA isolation

Before mRNA isolation, tumor cell content was defined on hematoxylin-eosin (H&E) stained slides. The proportion of tumor cells was greater than 50% in all analyzed tumors. 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. After deparaffinisation, 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, 8 µl of Master Mix (Mixture of Reporter CodeSet and Hybridization Buffer) was added to 5 µl of sample mRNA (altogether 150 ng extracted mRNA) in a tube. After adding 2 µl of Capture ProbeSet to each tube, the solution was gently mixed, briefly spun, and immediately placed in a pre-heated 65°C thermal cycler for 24-26 hours. After incubation, the samples were immediately placed into the nCounter Prep station and then analyzed in the Digital Analyser (nCounter FLEX Analysis System). Measurements were taken at high sensitivity with 555 FOV.

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

FFPE tissues were used for immunohistochemical analyses. Immunohistochemical reactions were performed on 3-5 µm thick sections using the Ventana BenchMark Ultra system and according to the Universal UltraView DAB manufacturer's protocol. The following primary antibodies and dilutions were used: claudin-1 (Cell Marque, Rabbit polyclonal antibody, 1: 100), claudin-3 (Invitrogen, Rabbit polyclonal antibody, 1:100), claudin-4 (Invitrogen, Rabbit polyclonal antibody, 1:100), claudin-7 (Invitrogen, Rabbit polyclonal antibody, 1:100). All primary antibodies were incubated for 32 minutes on 42°C. Hematoxylin was used for counterstaining after antibody visualisation. All immunohistochemical reactions were performed using external positive control tissue.

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. In case of discrepancies between the evaluations by the two experts, the slides were reviewed jointly by both experts (ZK and AT) to reach a consensus. If necessary, additional reviews were conducted to ensure accurate interpretation (JK). 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 (41, 66, 67).
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 raw mRNA expression data from the Digital Analyzer were normalized using nSolver version 4.0 (NanoString Technologies, Seattle USA). The mRNA expression data were background corrected by using the geometric mean of the negative controls. The data then were normalised with the geometric mean of the five housekeeping genes. 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”. Categorical data were compared using Chi-square or Fisher’s exact tests. For statistical analysis of immunoexpresssion, the score values were processed using JupyterLab with R language (v 4.2.0). Homogeneity test of data was performed by Kolmogorov-Smirnov test. Asymmetrical numeric data (IMPC vs. IBC-NST) were analyzed by Mann-Whitney test. 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. The comparison of survival functions for different strata was assessed with the log-rank statistics. Multivariate analysis of prognostic factors was performed using Cox's regression model. In the immunohistochemical analysis, Cox proportional hazard model calculation was performed to evaluate the predictive and hazard value of variables, both regarding DMFS and overall survival (OS). P-values presented in the tables represent measurements of the entire cohort (compared to the reference variable or between positive and negative expression) and are not subgroup (IBC-NST, IMPC or mixed IMPC/IBC-NST) related due to statistical adequacy. 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. Statistical analysis for mRNA expression was performed using Statistica 13.5 software (TIBCO Software Inc,

Palo Alto, CA). In the immunohistochemical study, survival curves calculated by Cox proportional hazard model were created with ggsurvplot function of ggplot2 R package. 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 (68, 69).

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). The mean age of the patients was 62 years in the IBC-NST group, 63 years in the IMPC, and 65.5 years in the mixed IMPC/IBC-NST group, respectively. Most tumors were grade 2 and stage pT1-2, and most of the patients presented with lymph node metastasis. Median follow-up time was 48 months (range: 0-230 months). In 25 out of 80 cases (15/36 in IBC-NST, 8/36 in IMPC and 2/8 in mixed IMPC/IBC-NST cases), distant metastases developed.

For the immunohistochemical analyses, a largely identical cohort was used as for the mRNA study, with a few extra cases added (**Table 2.**): 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). Median age of the patients was 61 years in the IBC-NST group, 62 years in the IMPC and 64 in the mixed IMPC/IBC-NST group. About half of the patients presented with lymph node metastasis (42/82), and most of the tumors were grade 2, stage pT1-2. Median follow up time was 49 months (range: 0-230 months). Distant metastases occurred in 25 out of 82 cases (14/36 in IBC-NST, 8/37 in IMPC and 3/9 in mixed IMPC/IBC-NST cases).
All cases were categorized into surrogate subtypes according to the 2011 St. Gallen International Expert Consensus (70). All Estrogen and Progesterone receptor positive, Her2 negative tumors showing low proliferation rate (Ki-67 below 20%) were grouped into the Luminal-A surrogate subtype. ER positive, PR negative or high proliferating (Ki-67 ≥20%), Her2 negative tumors were considered Luminal-B1 surrogate subtype, while ER positive and or PR negative, HER2 positive tumors were categorized into the Luminal-B2 surrogate subtype. ER, PR negative or hormone receptor-negative, HER2 positive tumors were defined in the HER2 positive-, and ER, PR, HER2 negative tumors were grouped in the triple negative (TNBC) surrogate subtype.

The data of patients' tumor characteristics selected for our studies are presented in **Table 2.,** 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)	61 (34-83)	62 (33-85)	64 (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 (**Table 3.** and respective heatmap shown on **Figure 2.**). 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 3.**).

Table 3. Difference in gene expression pattern compared between IMPC and IBC-NST groups.

Gene name	**p-value**	**Gene name**	**p-value**
AFDN/AF6	**0.000005**	MARVELD2/TRIC	0.05
AKT1	0.12	OCLN	**0.0002**
CATENIN-BETA	0.09	PALS1/MPP5	0.47
CCL21	0.50	PAR3	0.80
CCR7	0.52	PAR6	0.55
CDH1	**0.01**	PATJ/MUPP1	0.07
CLDN1	**0.004**	PIK3CA	0.30
CLDN2	0.48	SCRIB	0.22
CLDN3	**0.000005**	SLUG/SNAI2	**0.007**
CLDN4	**0.002**	SMAD3	0.91
CLDN7	**0.0001**	SMAD4	0.47
CRB3	0.18	SNAI1	0.48
CXCL13	0.94	TGFB1	0.48
CXCR5	0.83	TJP1/ZO1	0.05
DLG1	**0.002**	TJP2/ZO2	0.63
F11R/JAMA	0.17	TJP3	0.05
ITGA1	**0.04**	TWIST1	0.34
ITGB3	0.16	TWIST2	0.23
JAM2	0.66	ZEB1	**0.04**
JAM3	0.23	ZEB2	0.37
LGL	0.05	aPKC	0.76
LIN7A	**0.00008**		

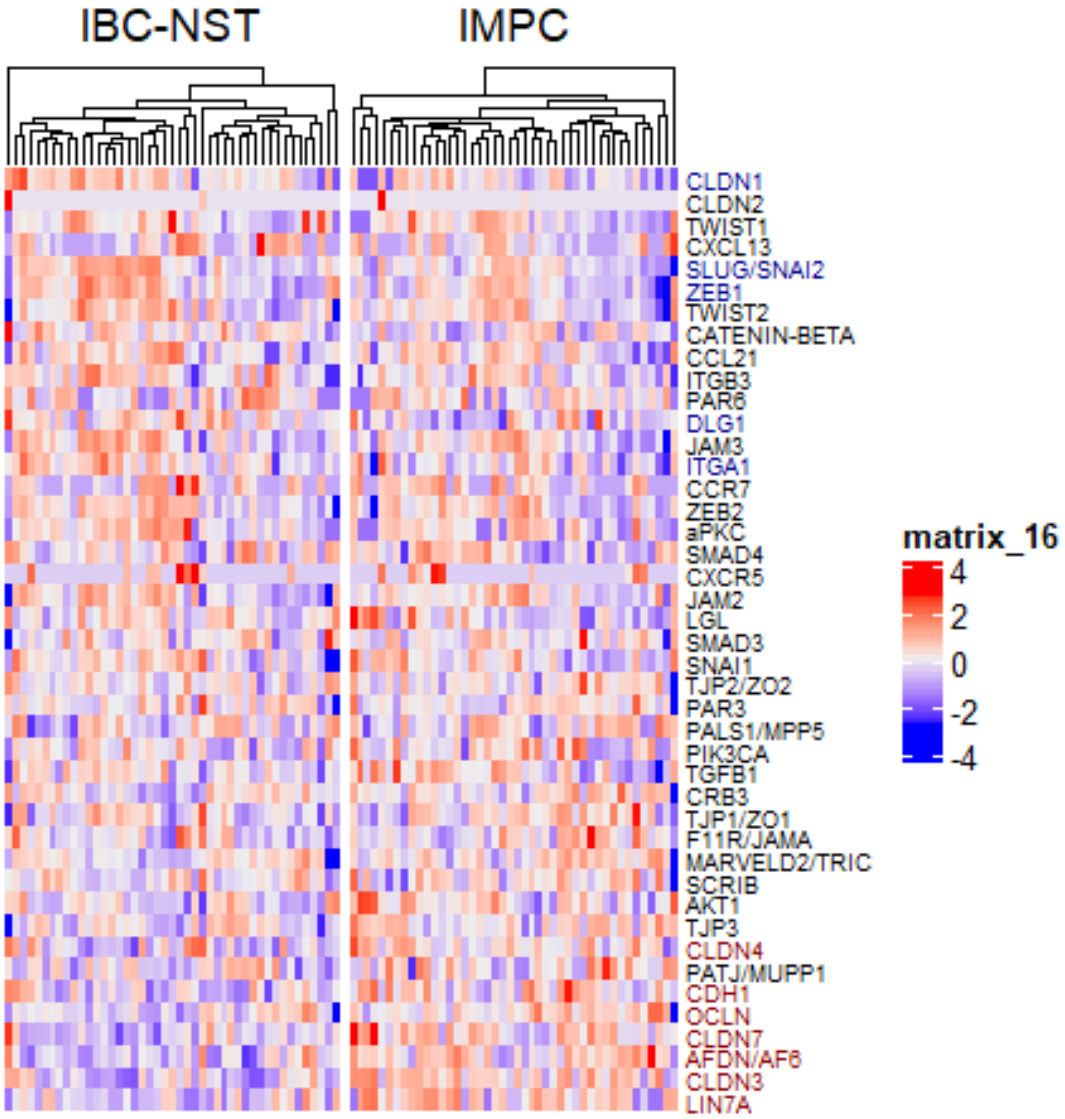

Figure 2. Heatmap of gene expression levels in the IBC-NST and IMPC group. Genes highlighted (right column) in blue showed significantly lower mRNA expression levels in the IMPC group, whereas those highlighted in red exhibited higher expression levels compared to the NST group (71).

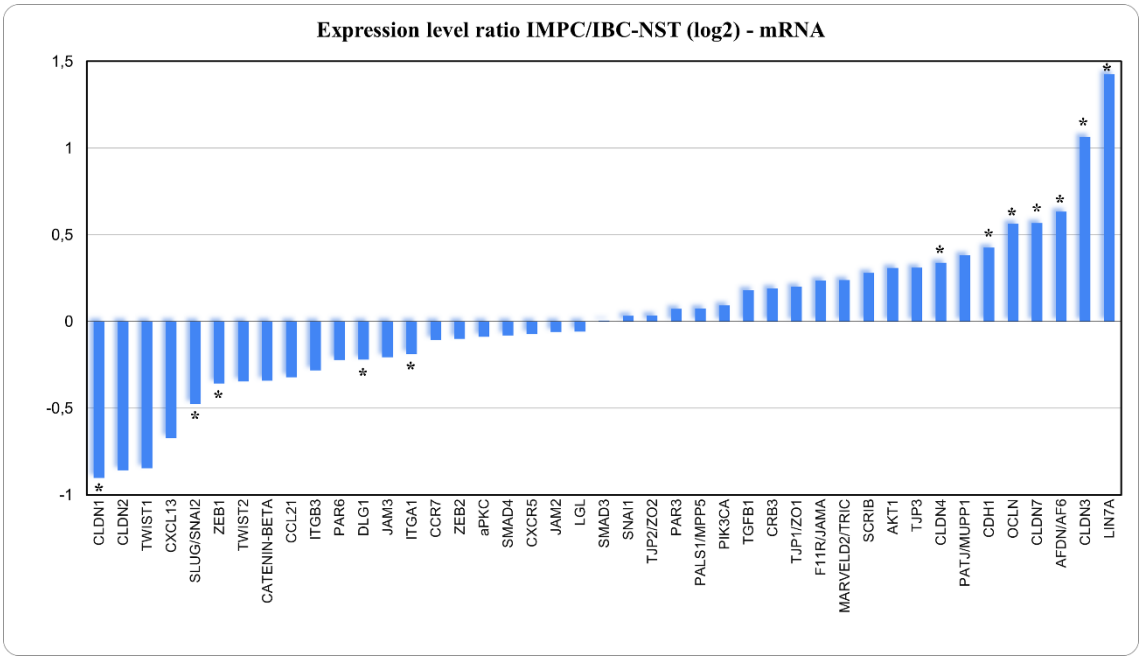

Figure 3. Gene expression ratio in IMPC/IBC-NST tumors.

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 (71).

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 (**Figures 4-6.**).

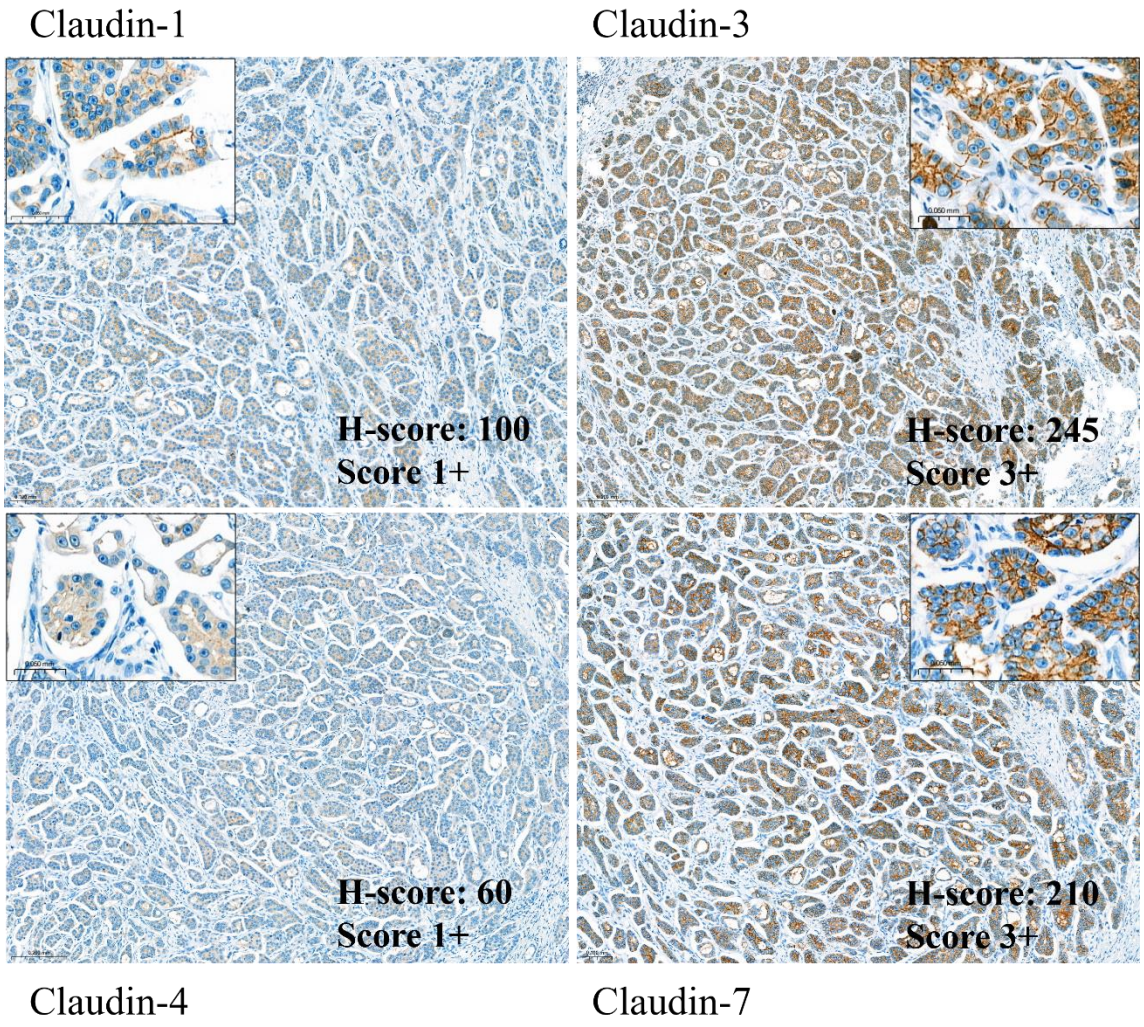

Figure 4. Immunohistochemical analysis of claudin proteins - a case of IMPC LUM-A, grade II tumor categorized as high expression (H-score) and positive (4-tier method) for all four examined claudins.

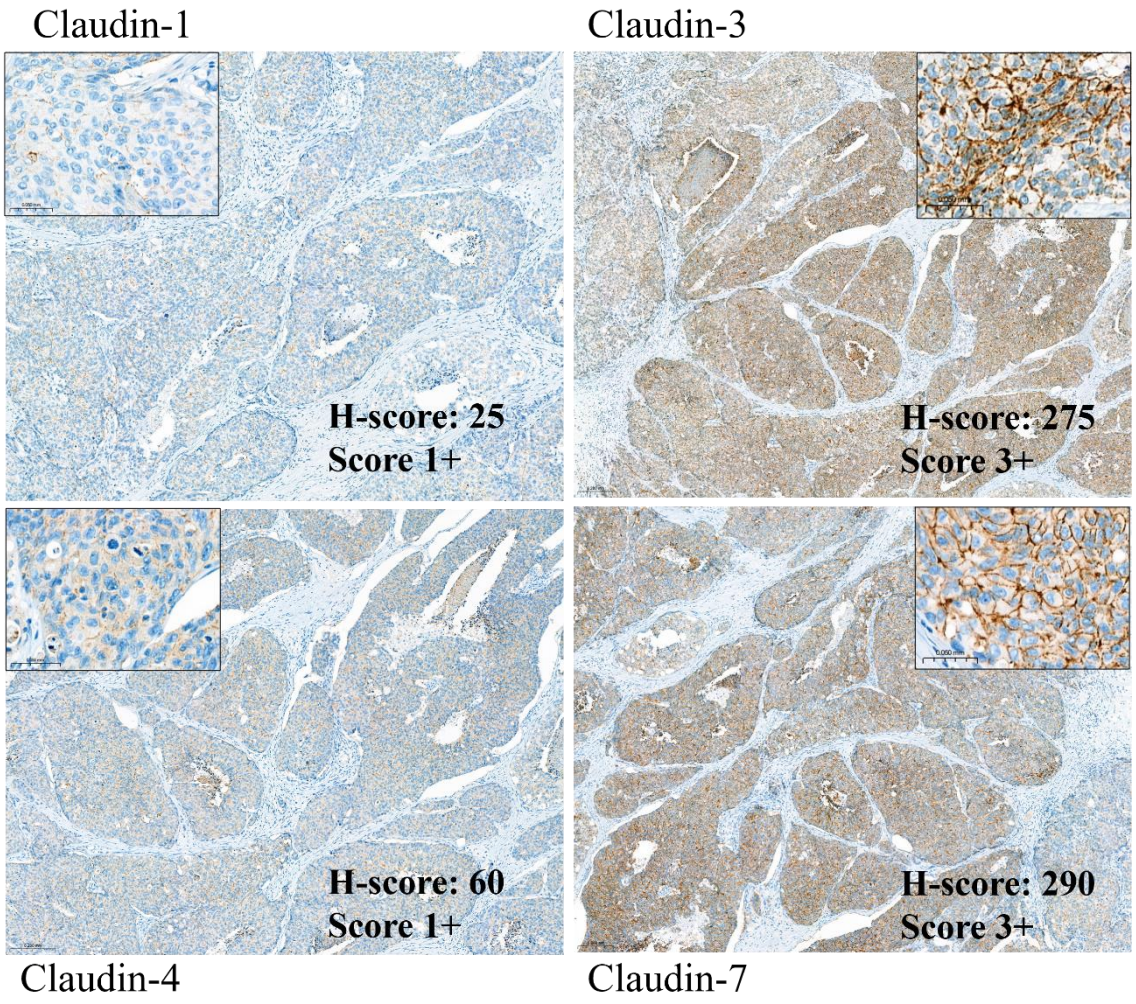

Figure 5. Immunohistochemical analysis of claudin proteins - a case of IBC-NST LUM-B2, grade III tumor categorized as high expression (H-score) and positive (4-tier method) for all four examined claudins.

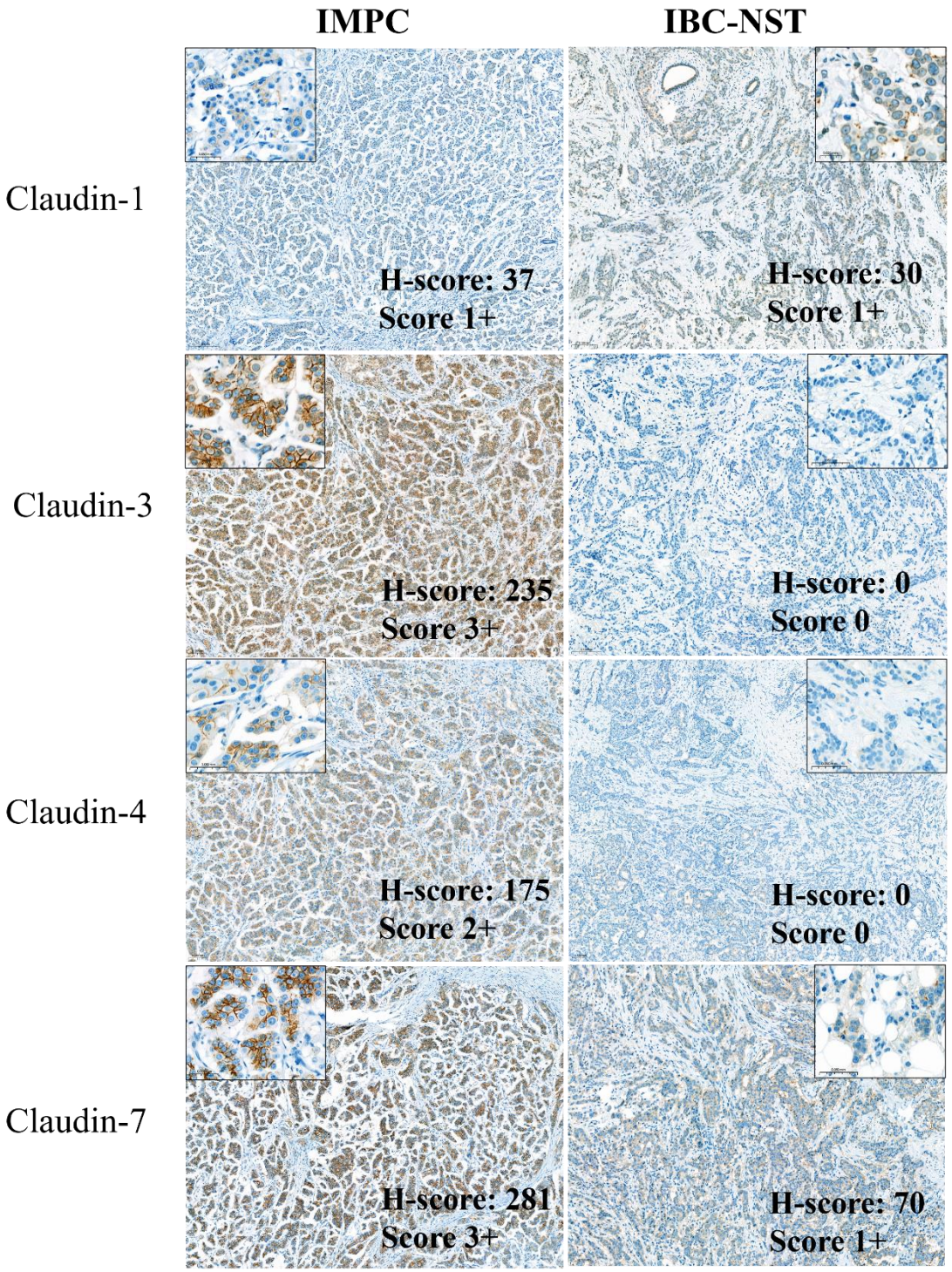

Figure 6. Immunohistochemical analysis of claudin proteins - a case of a mixed IMPC/IBC-NST LUM-B2, grade III tumor showing variable protein expression in both components.

Results of the semiquantitative scoring method

Initially, we scored our cohort according to the traditional 0, 1+, 2+ and 3+ system. We have found no significant difference between claudin-1, -3, -4 and -7 protein expression levels between the IMPC and IBC-NST groups (p=0.17, 0.31, 0.34, 0.22, respectively). None of the samples (0/91) showed high claudin-1 expression (score 3+). High claudin-3 expression (score 3+) was detected in 5/91 samples (2 IBC-NST, 3 IMPC), high claudin-4 in 1/91 sample (IBC-NST) and high claudin-7 expression was found in 13/91 samples (5 IBC-NST and 8 IMPC).

36/91 samples (15 IBC-NST, 21 IMPC) showed positivity for all of the above mentioned three claudins, while 8/91 samples (6 IBC-NST, 2 IMPC) were negative for claudin-3, -4 and -7.

Cytoplasmic positivity was seen in a small percentage of samples (8.8%, 32/364) in both the IMPC and IBC-NST groups (11 and 21 respectively): 9.9% (9/91) of claudin-1, 8.8% (8/91) of claudin-3, 12% (11/91) of claudin-4, and 4.4% (4/91) of claudin-7.

Results of the H-score analysis

The results of H-score evaluation were used to correlate protein expression with mRNA expression examined in our previous study. Variable staining intensity of claudins-1, -3, -4 and -7 was detected. Median H-score values of claudin-3 and claudin-7 expression were 115 and 150, respectively. Most of our samples showed no staining with claudin-1 (median value 0.5). Claudin-1 H-score above 100 was found in 5 samples, showing no correlation with tumor subtype, grade, or receptor status. Claudin-4 expression was low, with values below 50 to no expression in 60 out of 91 samples, yielding a median value of 10. Intermediate claudin-4 expression values (50-100) were detected in 14 samples, while H-scores above 100 were observed in 17 samples. However, claudin-4 expression showed no association with tumor characteristics.

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, **Figure 7A.**), or when comparing only pure IMPC and IBC-NST cases (p=0.71, **Figure 7B.**). No differences were seen in DMFS between the IMPC and IBC-NST tumors in the extended cohort of the immunoexpression analysis (p=0.63).

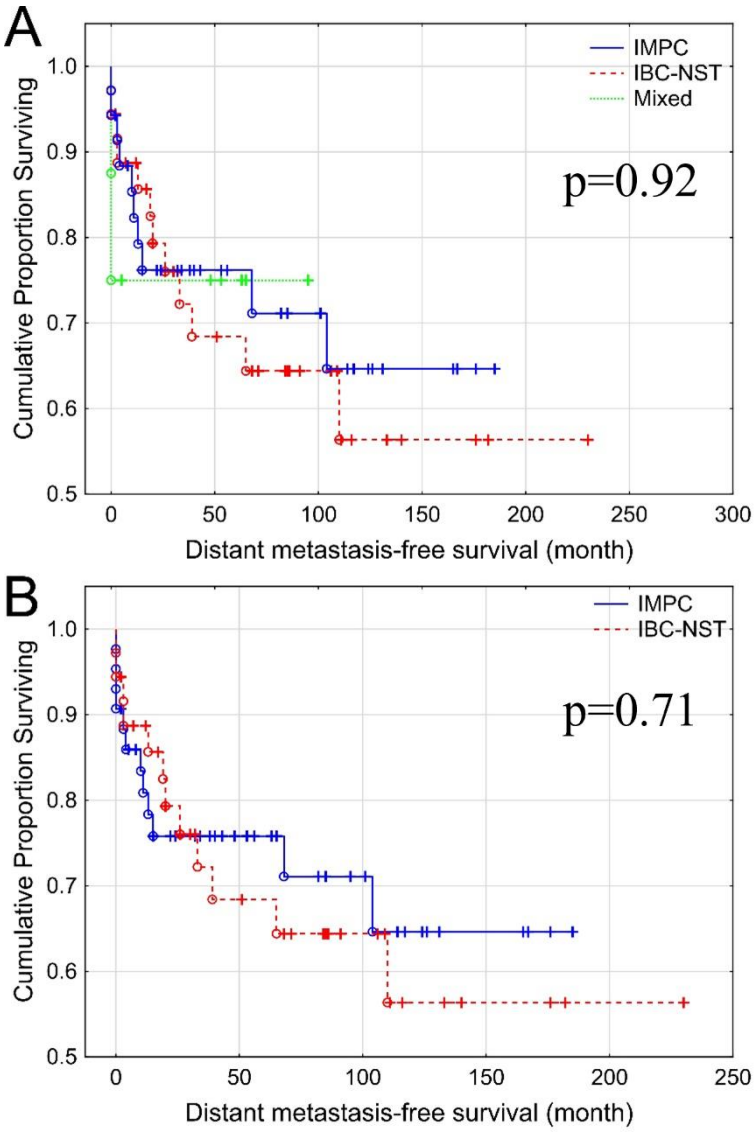

Figure 7. Correlation of histological tumor type with distant metastasis free survival
No significant differences were detected in DMFS between the IMPC, IBC-NST and mixed IMPC/IBC-NST groups (A) and between pure IMPC and IBC-NST cases (B) (71).

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 8A., 8B.** and **C.**). The expression level of the other examined genes showed no statistically significant association with DMFS.

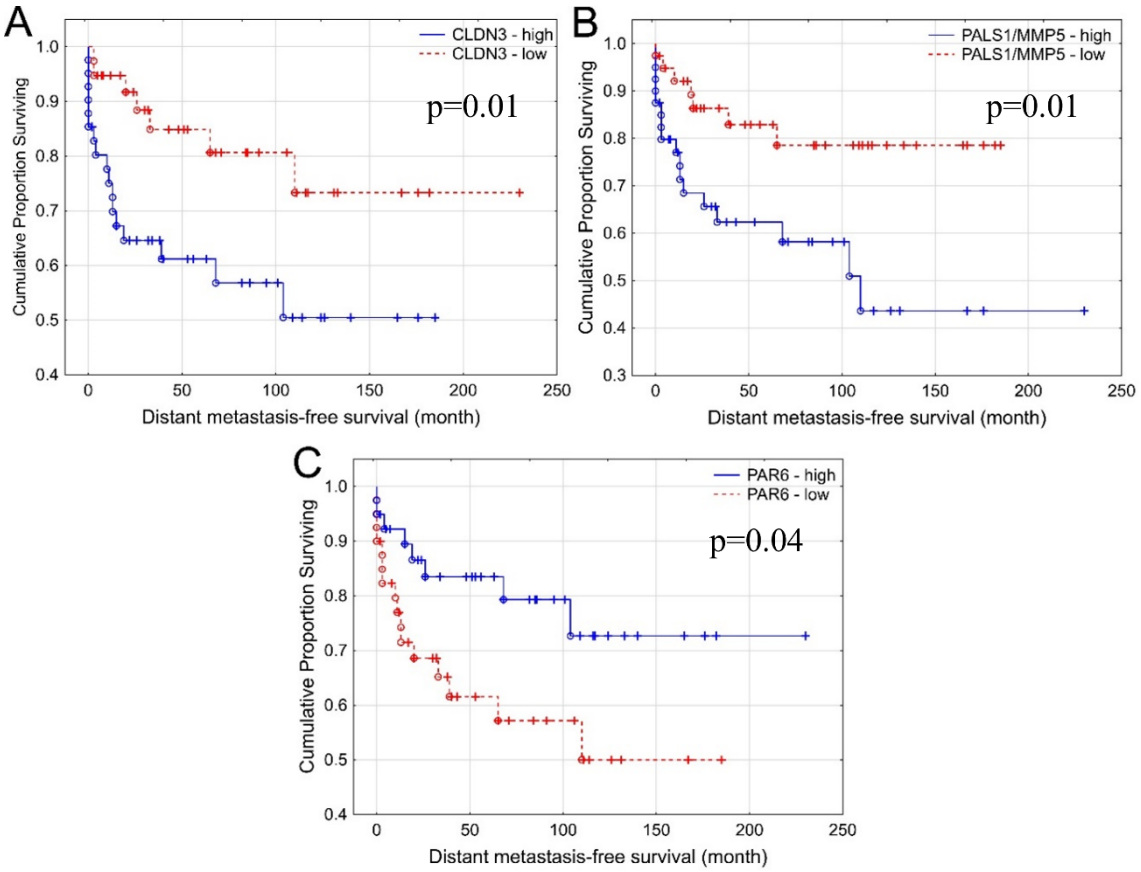

Figure 8. Correlation of gene expression data with distant metastasis free survival mRNA expression levels of CLDN3, PALS1 and PAR6 showed significant association with DMFS irrespective of histological subtype (A, B and C) (71).

Immunoexpression levels of the IMPC and IBC-NST cases were used for statistical analysis (similarly to the mRNA analysis, micropapillary component of the mixed IMPC/IBC-NST cases were added to the IMPC cohort). 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 9A-C.**).

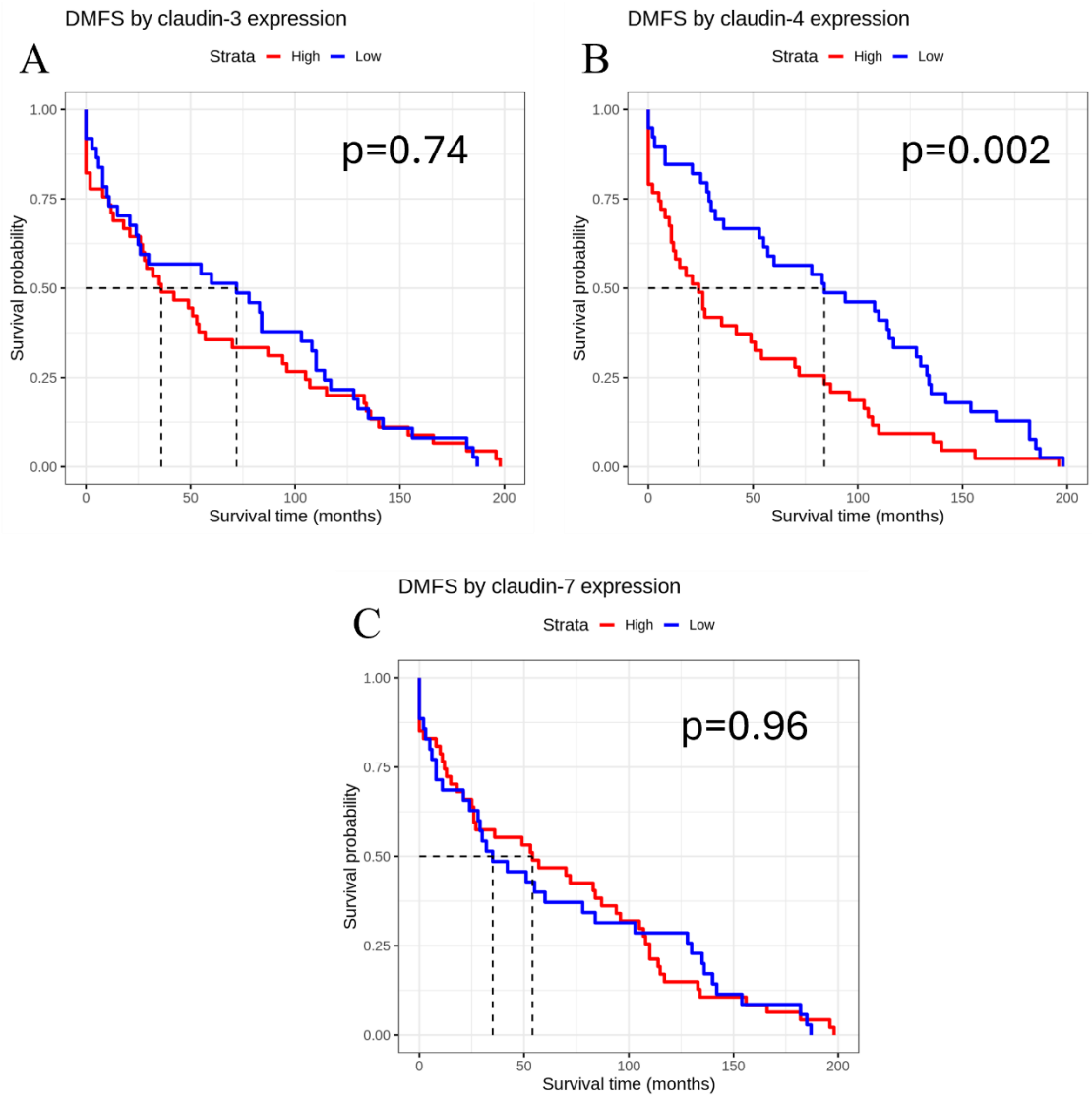

Figure 9. Claudin expression effect on DMFS after evaluation of immunoexpression according to the H-score, irrespective of histological subtype. High claudin-4 protein expression was associated with significantly shorter DMFS (B), while claudin-3 and claudin-7 protein expression showed no correlation with DMFS (A and C).

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 10A-C.**).

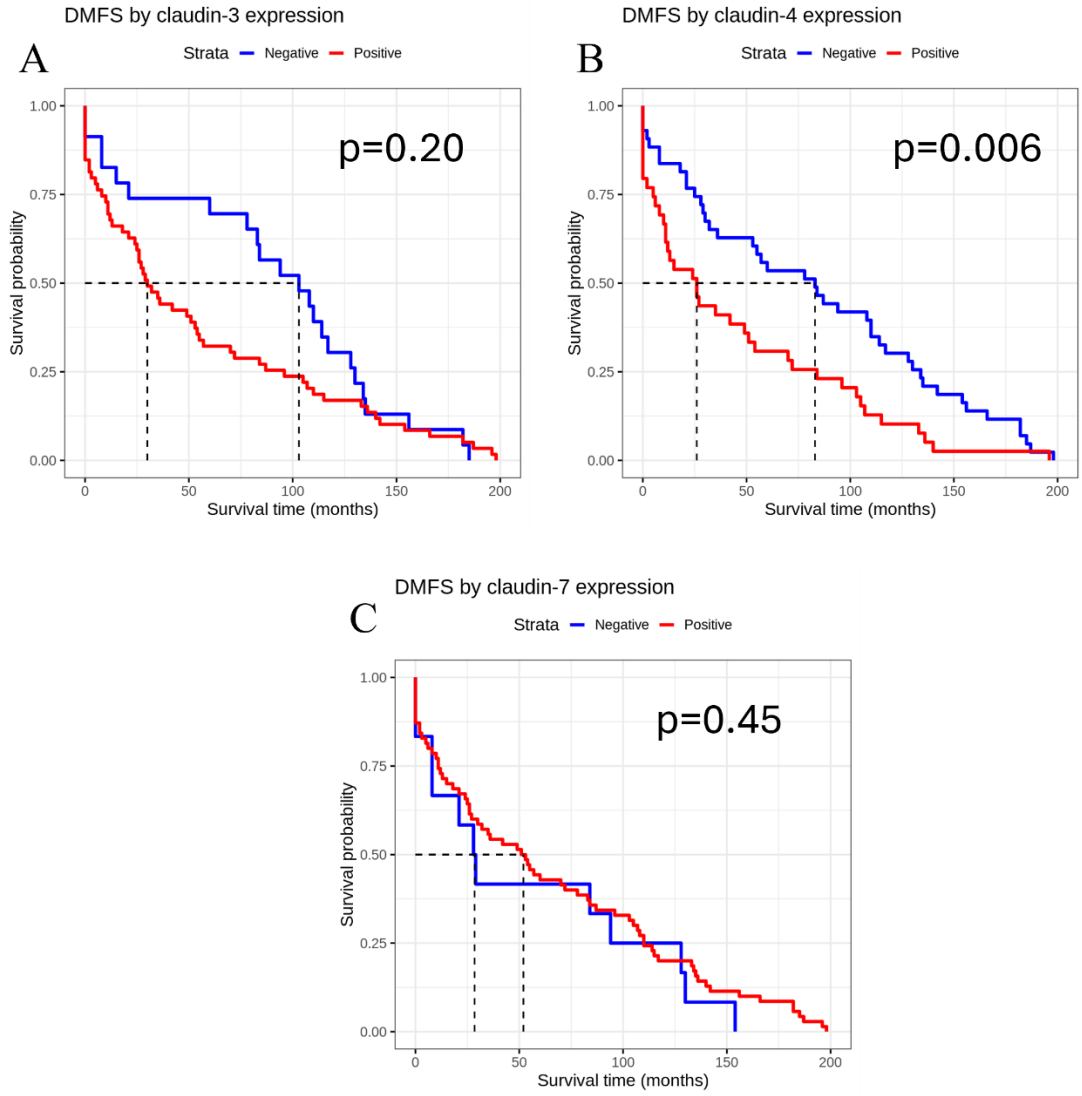

Figure 10. Effect of Claudin expression on DMFS after evaluating the immunohistochemical reactions based on the 4-tier system, irrespective of histological subtype. High claudin-4 protein expression was associated with significantly shorter DMFS (B), while claudin-3 and claudin-7 protein expression showed 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, **Figure 11A.**).

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) (**Figure 11B. and 11C.**). Multivariate analysis confirmed only PALS1 as a grade independent prognostic factor (p=0.007, **Table 4.**). 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).

Table 4. Correlation of PAR6 and PALS1 with DMFS in multivariate analysis

	RR (95% CI)	**p-value**
Age	1.016 (0.974-1.06)	0.45
Grade (3 vs. 1-2)	4.96 (1.614-185.236)	0.005
Histology (IMPC vs. IBC-NST)	0.771 (0.332-1.793)	0.54
PAR6 (high vs. low)	0.508 (0.211-1.223)	0.13
PALS1 (high vs. low)	3.797 (1.44-10.01)	0.007

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) (**Figure 11D**). The analyzed chemokines and their receptors did not show any association with lymph node involvement in the cohort.

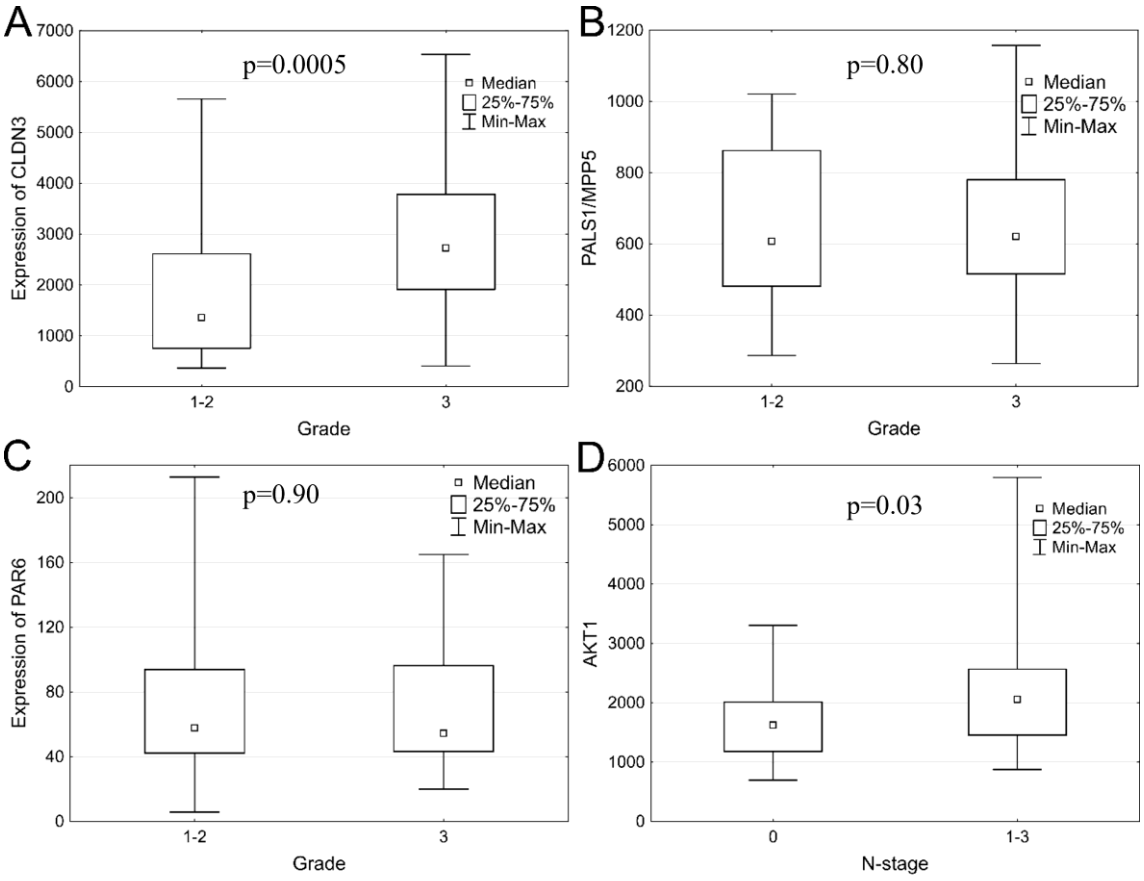

Figure 11. Association of gene expression with histological tumor grade and axillary lymph node metastasis. High CLDN3 mRNA expression levels were associated with high-grade tumors (A), while PALS1 (B) and PAR6 (C) expression revealed no correlation with tumor grade. High AKT1 expression exhibited an association with the presence of lymph node metastases (D) (71).

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 (68, 69), 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 (**Figure 12.**)

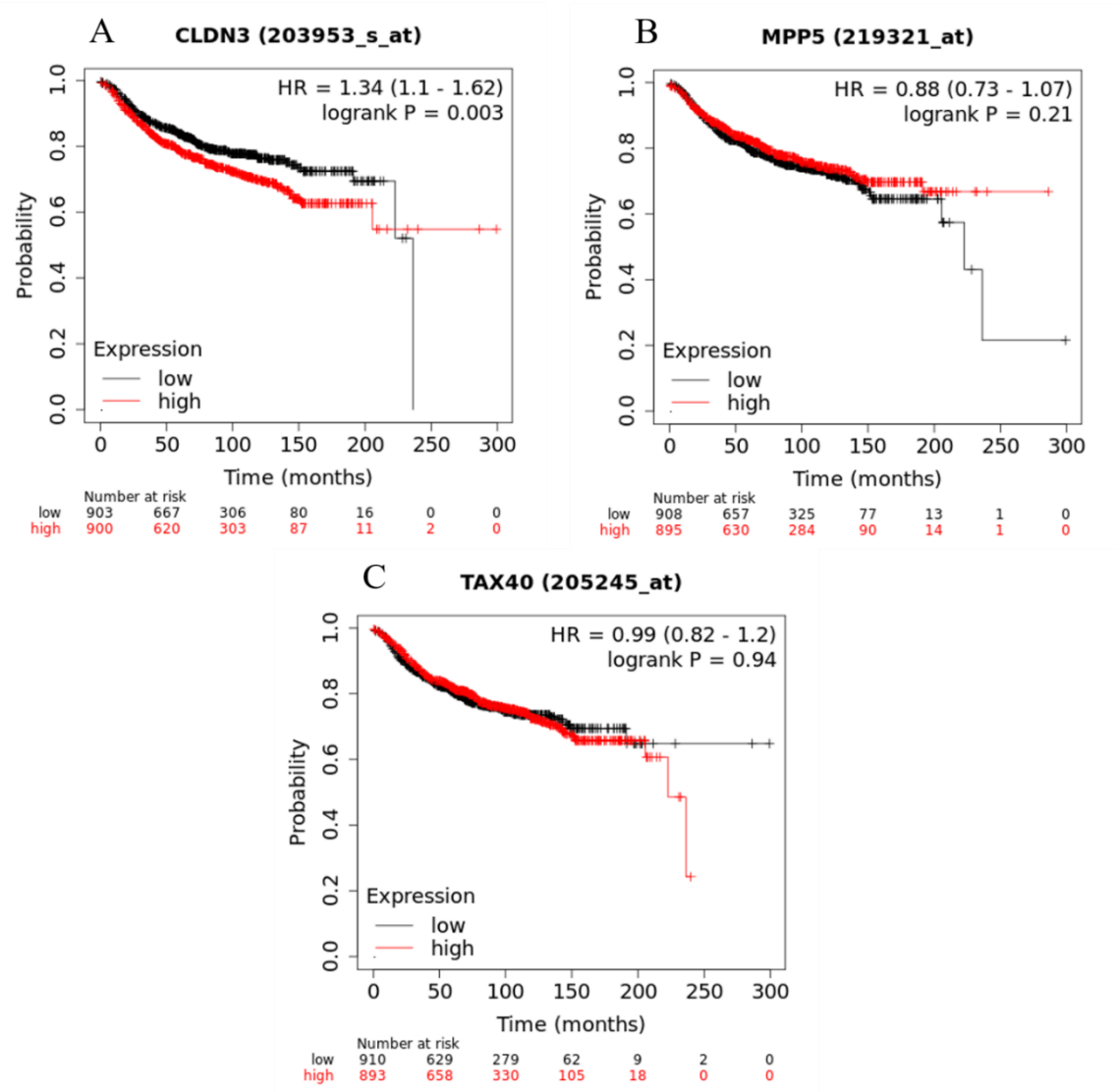

Figure 12. KM Plotter DMFS intervals in an independent large cohort of breast carcinomas (regardless of histological type) from KM Plotter online database (68, 69, 72). High CLDN3 mRNA expression was associated with shorter DMFS (A), while PALS1 (MPP5) (B) and PAR6 (TAX40) (C) expression levels showed no correlation with DMFS (71).

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. The HR- samples showed a ratio of 69%, 53% and 53% for claudin-3, -4 and -7 positivity, respectively.

Claudin distribution between the molecular and histological tumor subtypes is shown in **Table 5.** (after evaluation according to the 4-tier method) and **Table 6.** (after evaluation to the H-score).

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.

Table 5. Claudin expression distribution between surrogate subtypes in the 91 samples using the 4-tier method

	claudin -1 neg.	claudin -1 pos.	p-value	claudin -3 neg.	claudin -3 pos.	p-value	claudin -4 neg.	claudin -4 pos.	p-value	claudin -7 neg.	claudin -7 pos.	p-value
All	76	15		26	65		48	43		14	77	
IBC-NST	40	5	0.17[1]	15	30	0.31[1]	26	19	0.34[1]	9	36	0.22[1]
IMPC	36	10		11	35		22	24		5	41	
LUM-A	29	9	0.76[1]	9	29	0.59[1]	22	16	0.67[1]	3	35	0.001[1]
LUM-B1	27	0		10	17		12	15		4	23	
LUM-B2	9	4		3	10		8	5		1	12	
HER2	2	0		0	2		0	2		0	2	
TNBC	9	2		4	7		6	5		6	5	

[1] Chi square test

Table 6. Claudin expression distribution between molecular subtypes in the 91 samples using the H-score method

	claudin-3 low	claudin-3 high	p-value	claudin-4 low	claudin-4 high	p-value	claudin-7 low	claudin-7 high	p-value
All	44	47		41	50		44	47	
IBC-NST	24	21	0.34[1]	20	25	0.90[1]	27	18	0.02[1]
IMPC	20	26		21	25		17	29	
LUM-A	22	16	0.47[1]	19	19	0.69[1]	18	20	0.76[1]
LUM-B1	13	14		10	17		11	16	
LUM-B2	5	8		7	6		7	6	
HER2	0	2		0	2		1	1	
TNBC	4	7		5	6		7	4	

[1] Chi square test

5. Discussion

Our research focused on IMPCs, a distinct subtype of breast cancer. Despite significant findings, many questions remain unanswered. Although several studies suggest that IMPCs exhibit aggressive clinical behavior with a high incidence of early lymph node metastases, recent research indicates that overall survival rates do not significantly differ between IMPCs and IBC-NSTs (2, 6-8). Histologically, IMPCs are characterized by clusters of tumor cells with reversed polarity, a hallmark feature. The molecular mechanisms underlying the development of this unique structure are not yet fully understood. Studies examining cohorts of IMPCs with case numbers comparable to ours have revealed that IMPCs constitute a heterogeneous group of tumors with distinct genetic alterations compared to IBC-NSTs (14, 15, 72, 73).

In our research study, we examined the mRNA expression of selected genes associated with cell adhesion and tight junction molecules as well as cell polarity complexes. No other comprehensive studies were found in the literature examining this broad extent of related genes at the mRNA level. We have found 12 genes with significant differences in mRNA expression levels between IMPC and IBC-NST tumors.

In epithelial cells, the formation and maintenance of polarity require the complex regulation of tight junctions and the involvement of actin and microtubule cytoskeleton. Various proteins aid this process, but three main complexes are highlighted in maintaining apicobasal polarity: the apically located Crumbs and Par complexes and the basolateral Scribble complex (74-76).

It is well understood how tight junction proteins modulate different signalling cascades which play key role in cell growth, proliferation, migration and cellular differentiation (17-20, 27, 29, 34, 76-78). By polarity switching, which is a critical step in metastasis formation, tumor cell clusters switch from apical-in to apical-out polarity during vascular invasion. Furthermore, integrins, part of the tight junction, are key molecules interacting between the cells and the extracellular matrix, and integrin signalling may activate intracellular cytoskeletal and regulatory proteins which have been described to be activated in cancer cells (79). Certain signalling pathways are found to be related to tight junction proteins: TGF-ß-dependent pathway, Ras-Raf-MEK-ERK and PI3K/Akt pathways, Wnt/ß-catenin and STAT signalling, the Hedgehog and the Notch pathways (78).

Tetsuhisa et al. examined the role of tight junctions in epithelial polarity by systematically knocking out its components and showed that epithelial polarity was disrupted in ZO-1/ZO-2–deficient cells, but not in claudin-deficient cells. They concluded that claudins and JAM-A regulated tight junction formation and epithelial polarity (77). Our study did not reveal any differences in the mRNA expression levels of JAM-A in the two histological tumor types.

Crumbs complex is another key member of the apical polarity complexes which play a significant role in apico-basal polarity and cell migration. The complex comprises various proteins including PALS (Proteins associated with LIN7) and PATJ (Pals1-associated tight junction) as main components. Loss of cell polarity and tissue organization is caused by the dysregulation of any components of the complex (59, 80). Gruel et al. found that the polarity protein LIN7A is upregulated in IMPCs, and other polarity proteins show abnormal expression and localization as well (21). Although we did not find differences in the mRNA expression levels of PALS1 and PATJ in the IMPC and IBC-NST groups, we found significantly higher mRNA levels of LIN7A in the IMPC group suggesting that the specific histological presentation of IMPCs may be related to LIN7A expression. Examining the prognostic value of Crumbs complex members, our study highlighted that high mRNA expression of PALS1 is associated with shorter DMFS. PALS1 was also shown to be a grade independent prognostic factor in the entire cohort.

PAR protein complex, composed of PAR3/PAR6/aPKC proteins, is associated with the regulation of epithelial cell polarity (81). The asymmetric distribution of the cytoskeleton in epithelial cells is induced by PAR6, which is often located at the apical part of the cells. PAR6 may act as a signalling molecule in tumorigenesis and cancer development (82). Nolan et al. described that PAR6 was overexpressed in breast carcinomas and might induce cell proliferation (83). In our study we showed that low mRNA expression of PAR6 was significantly associated with shorter DMFS in the univariate analysis.

The Scribble polarity complex, together with the PAR-based and Crumbs-based complexes, is a significant contributor to the regulation of epithelial cell polarity, and is comprised of the products of Drosophila tumor suppressor genes: SCRIBBLE, LGL and DLG proteins, (84) (85). Studies described that loss of DLG1 dismantled tight junctions and was observed in poorly differentiated ductal breast carcinomas (86, 87). Our research

has showed lower mRNA expression levels of DLG1 in IMPC tumors compared to IBC-NSTs.

Epithelial tumor cells undergo epithelial-mesenchymal transition when gaining metastatic ability. EMT is activated by various transcription factor families (ZEB, SNAIL, TWIST etc.) and intracellular signalling networks act together with these transcription factors. It has been described that proteins such as TGF-β and the loss of E-cadherin also participate in the process of EMT (88, 89). Matsumura et al. reported that carcinoma-associated fibroblasts (CAFs) in the tumor stroma induce the formation of tumor cell clusters composed of two distinct cancer cell populations: one highly epithelial state (characterized by high E-cadherin and low/negative ZEB1 expression), and one in a hybrid epithelial/mesenchymal state (showing low E-cadherin and high ZEB1 expression). These CAFs promote invasive and metastatic tumor cell clusters via epithelial-mesenchymal plasticity (90). We demonstrated that higher mRNA expression level of CDH1 (E-cadherin) and lower levels of ZEB1, SNAI2 and ITGA1 were found in the IMPC group compared to the IBC-NST tumors. These findings indicate that the IMPC tumor cell clusters are in a highly epithelial state and that these clusters form during the stromal invasion and metastasis. In contrast, IBC-NST tumors utilize the traditional EMT pathway (91).

OCLN is a member of the tight junction molecules containing the tetra-spanning MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain (92). We have found that significantly higher expression levels of OCLN are seen in the IMPC group. This result may partly indicate that tumor cell clusters are stabilized during the invasion with the assistance of this tight junction molecule.

AF6 (Afadin) is recognized as an adherens junction protein. Tabariés et al. described that high protein levels of CLDN2 and AF6 in breast cancers were associated with poor prognosis (93). Elloul et al. found that phosphorylation of AF6 relocated the protein to the nucleus, promoting increased breast cancer cell migration (94). Our study revealed higher AF6 mRNA expression in the IMPC group. However, we did not observe any correlation between DMFS and AF6 expression levels when examining the entire cohort.

In the mRNA expression study, CLDNs -1, -3, -4 and -7 all showed differences in expression levels in the two groups, with CLDN1 showing significantly higher expression

levels in IBC-NST. In comparison, the other three CLDNs presented higher expression levels in the IMPC group.

Claudins are membrane proteins and key components of the tight junction complex, known to interact with various signalling pathways (58). Different subtypes of breast cancer exhibit distinct patterns of claudin expression (34, 36, 38, 39, 60, 61, 95-98). We have demonstrated that high mRNA expression levels of CLDN3 were associated with higher tumor grade and shorter DMFS across the entire cohort. After evaluating the mRNA expression levels, we investigated the claudin-1, -3, -4 and -7 protein expression to explore the possible role of these proteins in the development of inverted polarity. We compared the mRNA expression data with protein expression patterns between the two tumor subtypes and also assessed protein expression as potential prognostic factor.

Currently, there are no standardized methods for quantifying claudin protein expression. Different research groups employ various evaluation systems, including semiquantitative scales, the H-score, or custom scoring methods that combine staining intensity with the percentage of positive cells. In addition, the cut-off values used to distinguish positive and negative cases may differ across studies. For this reason, we evaluated protein expression using two distinct methods: a 4-tier system and the H-score.

Although we found significant differences in the mRNA expression of CLDNs 1, 3, 4 and 7 between IMPC and IBC-NST tumors, no differences in the expression of claudin-1, -3, and -4 proteins between the two histological groups were detected, suggesting that these proteins are not strictly related to the inverted polarity in IMPCs. On the other hand, high claudin-7 expression was observed in significantly more IMPC tumors than IBC-NST cases and claudin-7 showed significantly higher protein expression in IMPC tumors.

When examining the entire cohort for claudin protein expression, we showed that claudin-4 positive/high expression was associated with shorter DMFS values.

An important question is whether mRNA and protein expression levels are in any concordance. We could not show any obvious correlation between mRNA and protein expression, which is in accordance with the literature data. Li et al. studied the correlation between gene and protein expression of claudins (99). Their research concluded that the expression levels do not necessarily correlate. These differences may be due to epigenetic alterations, transcription factors, RNA alternative- or mis-splicing, posttranslational modifications, or signalling pathway effects. Post-transcriptional regulation of the mRNA

also may contribute to the difference in mRNA and protein expression levels. RNA binding proteins and processing factors act on the fate of the produced mRNA strands. Only a minority of these proteins are expressed in a tissue-specific manner, the majority are equally expressed throughout the different tissues (100).

Several study groups investigated the prognostic role of different claudins. Different cancer types show different associations with claudin expression. In breast cancer, overexpression of claudin-4 was associated with progression, migration and poor prognosis, which is in concordance with our findings (40, 41, 101, 102). Jaaskelainen et al. found that high cytoplasmic but not membranous claudin-3 and claudin-7 expression was predictive of poor outcome in TNBC (39). At the same time, another group described that membranous claudin-3 overexpression was associated with poor survival in TNBCs (40). In high grade breast carcinomas, lower claudin-7 expression was found and ER- tumors also showed decreased claudin-7 expression (43, 103).

The so called claudin low phenotype of breast carcinoma is defined by low expression of cell adhesion genes, high expression of epithelial-mesenchymal transition genes, and stem cell-like expression pattern (46). These tumors are characterized by intensive immune- and stromal cell infiltration, lower levels of proliferation, and low levels of genomic instability. Fougner et al. re-evaluated the nature of claudin low breast carcinomas and described that these tumors were not a recognizable, sixth subtype of breast carcinomas. Claudin low tumors are present in all breast cancer subtypes, showing features closer to their intrinsic subtype rather than to claudin low tumors (104). Claudin-low tumors are characterized by low gene expression levels of CLDN3, -4 and -7.

Future research may evaluate whether the loss of claudin expression is associated with inversed cell polarity. In our cohort, only a low number of samples showed negativity for all three claudins (claudins -3, -4 and -7) by IHC (6 IBC-NST and 2 IMPC samples) so, further analysis was not possible to be performed on this group. Pan et al. have recently described that the decrease or loss of claudin expression is associated with cell-cell adhesion- and polarity damage (105).

Several molecular targets against claudin-4 are being developed. Multiple research studies confirmed, including our cohort, that high claudin-4 expression was associated with worse prognosis (40, 41, 98, 101, 102). Luo Yi et al. found that anti-claudin-4 extracellular domain antibody, 4D3 increased the chemotherapeutic antitumor effect of

paclitaxel in two human breast cancer cell lines (98). Patients suffering from breast cancer showing high claudin-4 protein expression may benefit from anti-claudin-4 antibody treatment as part of the treatment protocol. The specific antibody connection enhancing the chemotherapeutic effect in these tumors may prolong patient survival.

Comparing the survival data of our mRNA expression analysis with the Kaplan Meier (KM) plotter, we have also shown that high CLDN3 expression is associated with shorter DMFS. Based on our results and contrary to the KM Plotter data, PALS1 and PAR6 expression were associated with distant metastasis free survival.

Lymph node metastasis is frequently seen in patients with IMPC. The underlying mechanisms leading to the lymphotrophy of this cancer subtype are not fully understood. Several studies investigated the role of certain chemokines and their receptors and other molecules in forming lymph node metastases in IMPCs on the protein expression level and found significant differences compared with IBC-NSTs (49, 106, 107). We did not find any association of the mRNA expression levels of genes playing any role in chemotaxis (chemokines and their receptors) between the two histological subtypes. Examining the entire cohort, the mRNA expression levels in all cases with and without lymph node metastasis showed, that high levels of AKT1 were associated with lymph node metastasis. AKT1, a protein kinase that plays an important role in carcinogenesis by inducing tumor progression via the mammalian target of rapamycin (mTOR) signalling pathway (108). AKT activation is also related in the development of drug resistance in breast cancer and is a potential target to overcome chemoresistance (109).

To date no specific, personalized therapy protocol exists specifically for patients with IMPC tumors. According to the European Society For Medical Oncology (ESMO) Guidelines, endocrine therapy remains the mainstay for hormone-receptor positive tumors regardless of histological subtype. Anti-HER2 therapy is offered to patients with HER2 positive tumors. PIK3CA and PD-L1 status assessment is also an important tool in certain patient groups (110). Further examination of AKT1 and other molecules of the mTOR pathway could potentially open new therapeutic targets for breast carcinoma patients especially those harbouring IMPC tumors. Overall, management of IMPC should combine guideline-concordant systemic therapy with heightened attention to its unique biology and integration of molecular testing to enable personalized therapy.

Considering the low frequency of IMPC tumors, a key limitation of our study, similar to other studies is the relatively low sample size. We performed our analyses focusing on selected genes that have crucial functions in cell adhesion, tight junction, cell polarity and major cancer signalling pathways.

While most of our findings align with previously reported data, it remains possible that genes showing no significant changes or differences in expression levels within our cohort may exhibit alterations when analyzed in a substantially larger patient population.

6. 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.

7. Summary

Invasive micropapillary carcinoma of the breast, a special subtype of breast carcinomas, is characterized by a unique histomorphology. The biological alterations leading to the inside-out pattern observed in IMPCs remain mostly unknown. IMPCs are also known to show locoregional aggressiveness but their overall prognosis does not differ significantly from IBC-NST tumors. Our extended cohort comprised 37 IMPCs, 36 IBC-NST and 9 mixed IMPC/IBC-NST tumors. We analyzed the mRNA expression profile of IMPCs comprised of 43 genes playing role in cell polarity, cell adhesion and EMT signalling pathways using the NanoString nCounter analysis system. We compared them to expression data of IBC-NST tumors. We have found 12 genes differently expressed in the two histological subtypes.

Based on the mRNA expression results, we have examined whether there are similar differences in claudin-1, -3, -4 and -7 expression on the protein level. Two scoring systems were used to quantify protein expression for immunohistochemical analyses: a 4-tier scoring system and the H-score method. Of the examined proteins, only claudin-7 showed significantly higher expression levels in the IMPC group.

Distant metastasis free survival (DMFS) intervals were used as endpoints for prognosis evaluation. DMFS values did not differ between IMPC and IBC-NST tumors. Examining the entire cohort, high CLDN3, PALS1 and low PAR6 mRNA expression levels correlated with shorter DMFS; and PALS1 was proven to be grade independent prognostic factor. On the protein level, we found that negative/low claudin-4 expression was associated with shorter DMFS. Lymph node metastasis was associated with higher levels of AKT1 mRNA expression.

The differences in gene expression between IMPC and IBC-NST tumors may play a role in the special morphology of IMPCs. However, on the protein level only claudin-7 showed association with tumor subtype. We did not find differences in DMFS between the two histological groups, corresponding to the literature data. Examining the entire cohort claudin-4 expression was associated with shorter DMFS suggesting its role in breast cancer progression. Correlation of AKT1 mRNA expression with lymph node metastasis formation may highlight the benefit of AKT/mTOR inhibitors in these patients.

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9. Bibliography of the candidate's publications

Publications related to the thesis:

1. Kramer Z, Kenessey I, Gángó A, Lendvai G, Kulka J, Tőkés AM.
   Cell polarity and cell adhesion associated gene expression differences between invasive micropapillary and no special type breast carcinomas and their prognostic significance.
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2. Kramer Z, Budai A, Pesti A, Kulka J, Tőkés AM.
   Invasive micropapillary carcinoma of the breast and invasive breast carcinoma of no special type: a comparison of claudin proteins' expression and its impact on survival.
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Other publications:

1. Pataki BÁ, Olar A, Ribli D, Pesti A, Kontsek E, Gyöngyösi B, Bilecz Á, Kovács T, Kovács KA, Kramer Z, Kiss A, Szócska M, Pollner P, Csabai I.
   HunCRC: annotated pathological slides to enhance deep learning applications in colorectal cancer screening.
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Inhibition of c-Met with the specific small molecule tyrosine kinase inhibitor SU11274 decreases growth and metastasis formation of experimental human melanoma.
Curr Cancer Drug Targets. 2010 May;10(3):332-42. doi: 10.2174/156800910791190184. PMID: 20370683. Impact factor: 4.771

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