Original paper

Invasive ductal carcinoma of no special type and its corresponding lymph node metastasis: do they have the same immunophenotypic profile?

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In the present study we compared the immunophenotypic subtypes of breast ductal invasive carcinomas with their ipsilateral, axillary lymph node metastasis. The ER (estrogen receptor), PR (progesterone receptor), Her2 (human epidermal growth factor receptor 2), and CK5 (cytokeratin 5) status and the proliferation marker Ki-67 were determined by immunohistochemistry on specimens from 43 women. All selected cases were diagnosed as invasive breast carcinomas, of no special type (NST), G2 grade of differentiation. The most frequently encountered subtype at both sites was luminal B. We determined that tumor profile evaluated by surrogate markers is not stable during the metastatic process. The total rate of shifted cases was 23.26% (10 cases), and the highest rate of shifting (6.98%) was encountered from luminal B/Ki-67 to luminal A subtype. In five cases, the subtype shifted to a poorer one according to prognosis. These data support the hypothesis that breast cancer is a heterogeneous disease, with substantial variability of cellular components within each category, a statement applicable to invasive breast carcinomas of NST type too. The receptor profile of this carcinoma, indicated by surrogate markers, is not stable throughout the metastatic process.

Key words: breast cancer, metastasis, surrogate markers.

Introduction

Breast cancer is the most common cancer in women worldwide and one of the malignant diseases most studied by scientific communities [1]. It is described as a heterogeneous disease, variable in its clinical course, pathological aspects, therapy and prognosis [2, 3]. Breast tumors with similar histology may express various clinical and pathological features. Because the previous classifications focusing only on morphology could not fully capture the diversity of the disease, another classification system, based on hormone receptor status and gene expression profile of breast cancers, has been developed [4, 5]. Nowadays, estrogen receptor (ER), progesterone receptor (PR) and Her2 receptor status of breast tumors can be determined by using immunohistochemical markers as surrogates, and the breast cancer cases can be classified into at least four molecular subtypes including luminal A, luminal B, basal-like and Her2/neu.

An important decisional factor of therapeutic strategy, which determines the patients' future outcome, is the presence of metastasis, especially in the regional

lymph nodes. Traditionally, the presence of metastasis and the number and level of involved lymph nodes were described. Nowadays, it seems to be important to investigate the molecular profile of metastasis. Some data reveal the instability of tumor cell receptors throughout the metastatic process [6-10]. But few results concerning this field are published and existing data are quite sparse. One reason could be the inhomogeneous groups for analysis. Because invasive ductal cancer is one of the most frequent diagnoses in histopathological practice, we considered it opportune to describe this type of cancer in relation to its lymph node metastasis from the molecular classification position. Based on Goldhirsch's recommendations for oncological practice, the intrinsic subtypes have been defined by five surrogate markers [11]. In the present work we found that the molecular profile of invasive breast cancer of no special type is not homogeneous and not stable during tumorigenesis.

Material and methods

Patient data

In this retrospective study, we used the specimens from 43 women who underwent a radical mastectomy and lymph nodes dissection in the Oncological Institute, Republic of Moldova, between 2012-2013. Patients did not receive radio- and/or chemotherapy before surgery. The ages of the women ranged from 37 to 85 years old. Three independent pathologists confirmed histopathology of the tumors. Histological grade was scored by the Scarff-Bloom-Richardson grading system, with Ellston-Ellis modification. All 43 selected cases were diagnosed as invasive ductal carcinoma of NST type and G2 grade of differentiation (as the most commonly encountered cases in the studied group).

Tissue processing

The specimens were fixed in 10% phosphate buffered formalin and paraffin embedded. Sections were sectioned and stained with hematoxylin (Mayer) and eosin (HE) for routine histopathological assessment. To avoid any misunderstandings about tissue processing, primary tumor and lymph node from the same patient were embedded in a single paraffin block and sections were stained on the same slide (Figs. 1, 2). The lymph node metastases were confirmed by immunohistochemistry with an AE1/AE3 cytokeratin cocktail. In addition, sections were also stained with 5 monoclonal antibodies (Table I). All stages of immunohistochemistry, from dewax to

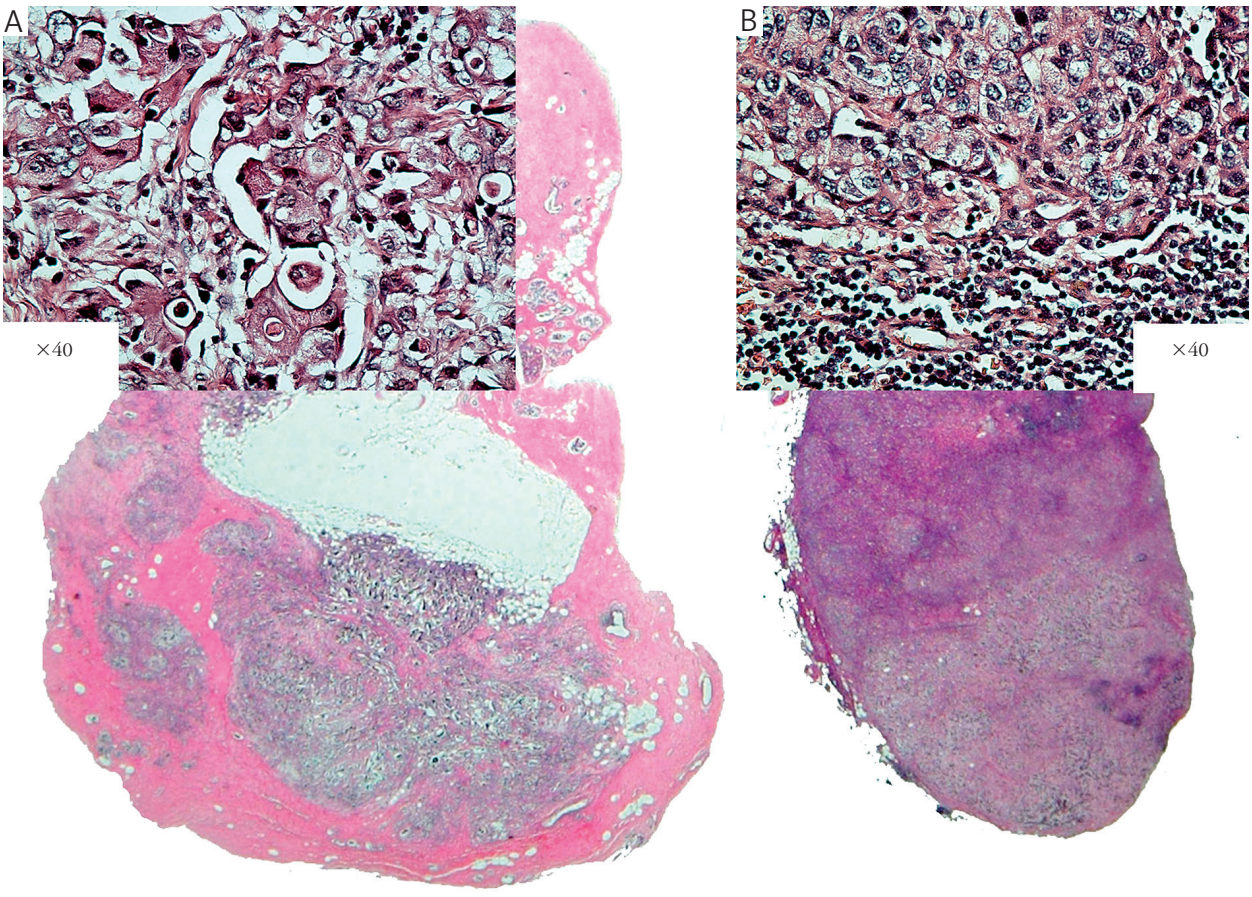

Fig. 1. Breast carcinoma NST type. Primary tumor (A) and its LNM (B) processed on the same slide. HE, magnification 10×

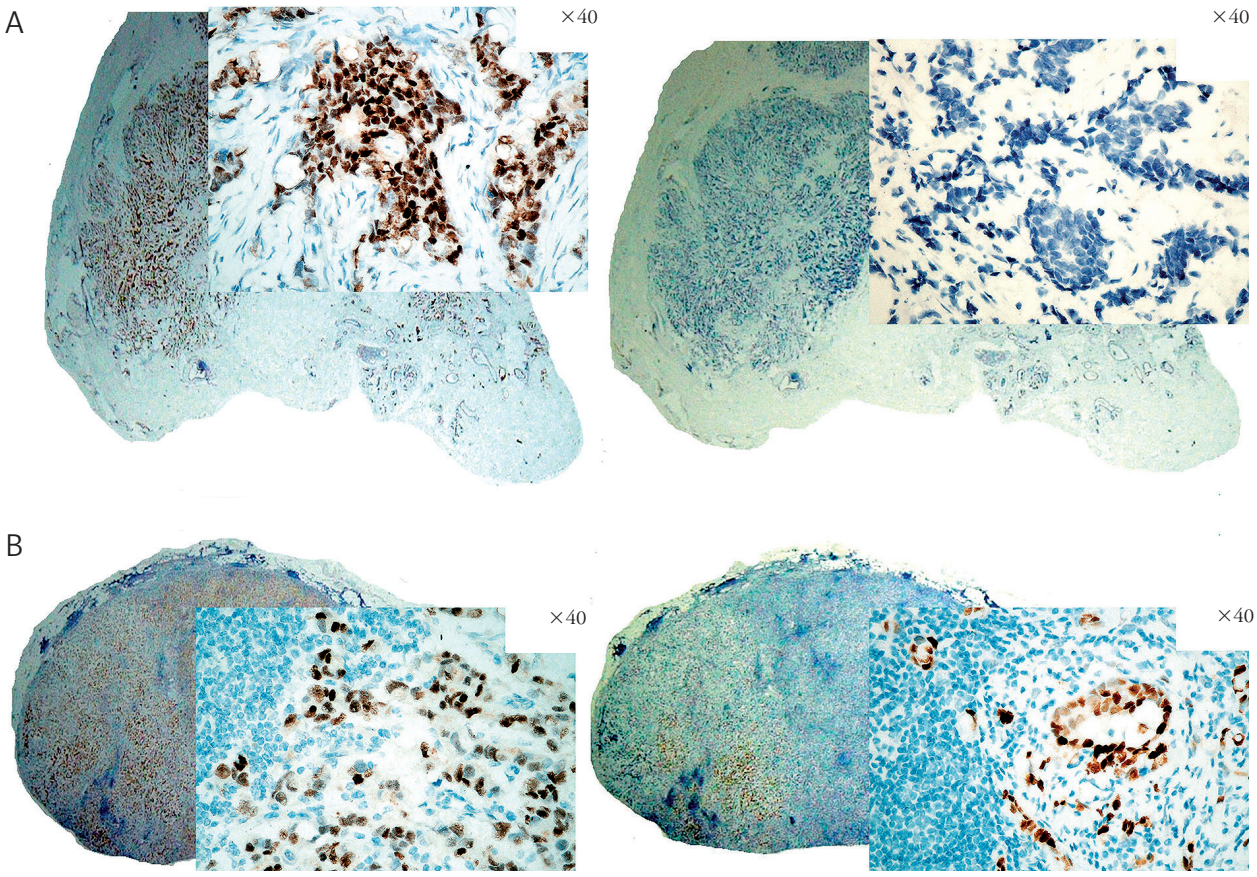

Fig. 2. Breast carcinoma NST type. Primary tumor (A) and its LNM (B) stained with anti-ER (I) and anti-PR (II) markers, magnification 10×

Table I. Antibodies and conditions used for immunohistochemical analysis

Antibody/clone	Source/incubation time/ dilution	Retrieval system/time	Detection/time
ER/6F11	Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK/15 min/RTU	Bond Epitope Retrieval Solution 1, (Leica Biosystems, Newcastle Upon Tyne, UK)/20 min	Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK), 15 min
PR/16			
Multi-cytokeratin/(AE1/AE3)			
Her2 /policlonal	Dako Glostrup Denmark/30 min/RTU	Dako Target Retrieval Solution, pH6/20 min	EnVision-HER/30 min
Ki-67/K2	Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK/15 min/RTU	Bond Epitope Retrieval Solution 2, (Leica Biosystems, Newcastle Upon Tyne, UK)/20 min	Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK), 15 min
CK5/ XM26	Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK/15 min/RTU		

counterstaining, were performed automatically on a Leica Bond-Max autostainer (Leica Biosystems, Newcastle Upon Tyne, UK) in accordance with the manufacturer's recommendations. The Her2 protocol was performed on Leica Bond Oracle Her2 IHC System. Modified Lille hematoxylin was used for nuclear counterstain.

Microscopic evaluation

The hormone receptor status was evaluated by using Allred score [12]. We combined the percentage of positive cells with intensity of nuclear staining from 10 microscopic fields. Cases with +1-+3 were considered ER, PR positive.

The Her2 status was assessed according to ASCO recommendations [13]: "0" – no staining observed or weak, barely perceptible membrane staining until 10% of cells; "+1" – weak membrane staining of > 10%; "+2" – incomplete, weak/moderate circumferential membrane staining > 10% of tumor cells or complete circumferential intense staining <10% of cells; "+3" – intense, circumferential staining of > 10% of tumor cells. Cases with Her2 grade of +2 and +3 were considered as positive.

The CK5 expression was interpreted as Azoulay previously defined [14]: 0 – no tumor cells stained; +1 – less than 10% of tumor cells stained; +2 – 10-50% of positive tumor cells; +3 – more than 50% of tumor cells stained. Expression was scored positive (> 0) if any cytoplasmic and/or membranous staining tumor cells were observed.

For the Ki-67 marker we used a 14% threshold as the limit to define positive/negative cases [11]. The results were grouped in 7 subgroups:

1. ER^-, PR^-, $Her2^-$, $CK5^-$ as 5NP (five negative phenotype),
2. ER^-, PR^-, $Her2^-$, $CK5^+$ as basal-like,
3. ER^-, PR^-, $Her2^+$, $CK5^-$ as $Her2^+$ (Her2 overexpressed),
4. ER^+ and/or PR^+, $Her2^+$, $CK5^-$, Ki-67 < 14 as luminal B/Her2,
5. ER^+ and/or PR^+, $Her2^+$, $CK5^-$, Ki-67 > 14 as luminal B/Her2/Ki-67,
6. ER^+ and/or PR^+, $Her2^-$, $CK5^-$, Ki-67 > 14 as luminal B/Ki-67,
7. ER^+ and/or PR^+, $Her2^-$, $CK5^-$, Ki-67 < 14 as luminal A.

Table II. The frequency of subtypes in primary tumors

Subtype	No. of cases	%		
5NP	2	4.65	11.63	11.63
Her2	3	6.98		
Luminal A	14	32.56	32.56	88.37
Luminal B/Her2	2	4.65	55.81	
Luminal B/Her2/Ki-67	5	11.63		
Luminal B/Ki-67	17	39.53		
Total	43	100	100	100

Table III. The frequency of subtypes at lymph node metastasis level (LNM)

Subtype	No. of cases	%		
5NP	3	6.98	18.6	18.6
Basal-like	1	2.33		
Her2	4	9.30		
Luminal A	16	37.21	37.21	81.4
Luminal B/Her2	3	6.98	44.19	
Luminal B/Her2/Ki-67	1	2.33		
Luminal B/Ki-67	15	34.88		
Total	43	100	100	100

Image acquisition and data processing

Three pathologists scored immunohistochemical results independently. The number of ER, PR, Ki-67 positive cells was evaluated on a Nikon Eclipse 80i instrument using a Nikon DS-Fi1 installed camera and Nis-elements 2.30 imaging software in accordance with Suciu's method [15]. The average from ten microscope fields (40×) with the greatest number of positive cells was determined. A MS Access 2003 database was used to store and group the data.

Statistical analysis

The WINSTAT 2012.1 software was used for descriptive statistics. In order to determine the shifting direction (from basal to luminal and vice versa) and Pearson's correlation coefficient evaluation, 5NP subtype was assigned as "1", basal-like as "2", $Her2^+$ as "3", luminal B/Her2 as "4", luminal B/Ki-67 as "5", luminal B/Her2/Ki-67 as "6" and luminal A as "7". A p value of less than 0.05 was considered significant.

Ethics

The study has been approved by the Ethics Committee of the "Nicolae Testemitanu" University of Medicine and Pharmacy, Chisinau, Republic of Moldova, based on patients' informed consent.

Results

The luminal group was observed in the most of the cases (38 cases or 88.37%) in comparison to the basal one (5 cases, 11.63%) at the primary tumor level. Luminal B/Ki-67 was the most common subtype (39.53%) followed by luminal A with 32.56% (Table II). The basal group consisted of 5NP and $Her2^+$ subtypes. None of the selected cases fulfilled the criteria of basal-like subtype.

In the lymph node metastasis the luminal group was also the most common – 35 cases/81.4% (Table III). Luminal B was considered as a leader of this group too (44.19%), followed by luminal A with 37.21%. In the structure of the basal group (18.6%) one case of basal-like subtype was determined.

By comparing the molecular subtype of primary tumors and their metastases, we realized that in 10 cases (23.26%) the subtype from lymph node metastasis shifted to another one (Table IV).

The statistical analysis reveals that the hormonal receptors' grade from tumor (ERtm, PRtm) correlates

Table IV. Molecular subtypes defined by surrogate markers in primary tumors compared to their metastasis in axillary lymph nodes

Primary tumor subtype	LNM subtype	No.	%	
5NP	5NP	2	4.65	76.74
Her2	Her2	3	6.98	
Luminal A	Luminal A	11	25.58	
Luminal B/Her2	Luminal B/Her2	1	2.33	
Luminal B/Her2/Ki-67	Luminal B/Her2	1	2.33	
Luminal B/Her2/Ki-67	Luminal B/Her2/Ki-67	1	2.33	
Luminal B/Her2/Ki-67	Luminal B/Ki-67	1	2.33	
Luminal B/Ki-67	Luminal B/Ki-67	13	30.23	
Luminal A	5NP	1	2.33	23.26
Luminal A	Luminal B/Her2	1	2.33	
Luminal A	Luminal B/Ki-67	1	2.33	
Luminal B/Her2	Luminal A	1	2.33	
Luminal B/Her2/Ki-67	Her2	1	2.33	
Luminal B/Her2/Ki-67	Luminal A	1	2.33	
Luminal B/Ki-67	BasalLike	1	2.33	
Luminal B/Ki-67	Luminal A	3	6.98	

positively with homologous markers from metastasis and molecular subtypes from both sites (Table V). Increase of hormonal grade correlates negatively with Her2 and CK5 status. The level of proliferation marker Ki-67 from the primary tumor is linked with Ki-67 and CK5 grade from metastasis. A negative correlation was determined between Ki-67 value and tumor subtype.

Discussion

Using gene expression profiling, Weigelt *et al.* confirmed that human primary breast tumors are similar to distant metastases (in lung, ovary, skin, lymph node) of the same patient [16]. Van der Vijver also considers that the metastatic proficiency of a tumor is pre-programmed from its beginning and supports Bernards' results indicating that metastatic outcome is determined by events occurring early in the development of a tumor, rather than being dictated exclusively by events that occur many years later at the culmination of tumor progression [17, 18].

On the other hand, a series of data revealed differences in the expression signatures of tumors derived from cloned weakly/non-metastatic human cell lines and from their isogenic metastatic counterparts of the same patient [19, 20]. This is in line with previous studies, which provided direct proof that individual malignant cells, co-existing within a given tumor, differ in metastatic capability [21]. Plus, as the metastatic ability of the cell population increases, the receptor profile changes concomitantly. This conclusively demonstrates that the molecular signature of breast carcinoma is not pre-determined and static, but continues to evolve in a tumor throughout its life history [7].

Nowadays, gene expression analysis has resulted in the definition of several different subtypes of breast cancer [4, 5]. Because obtaining gene expression array information is quite laborious and expensive, Cheang *et al*. proposed a useful shorthand [22]. According to Goldhirsch A. this approach uses the immunohistochemical definition of estrogen and progesterone receptor to define the hormonal-dependent (or luminal) and hormonal-independent (or non-luminal) group [11]. Detection of human epidermal growth factor receptor-2 overexpression was proposed as routine practice to uncover the $Her2^{+}$ subtype. The Ki-67 proliferation marker and Her2 stratify luminal B, and CK5 reveals the basal-like subtype. In our research approach we tried to act from a usual clinical laboratory position and used all five surrogate markers to define the intrinsic subtypes of breast cancer.

As stated previously, the most common subtypes in breast carcinoma classification belong to the luminal group [23]. In our results as well, the luminal one was the richest, and luminal B had the majority part. As Ki-67 has a crucial role in defining the patients' future, we considered it useful to describe its activity in luminal B/Her2 cases and subdivided it

Table V. Spearman rank correlation between surrogate markers, Her2 status, molecular subtypes from primary tumor and lymph node metastasis (LNM)

	ER	PR	Her2	Ki-67	CK5	Primary tumor subtype	ER	PR	Her2	Ki-67	CK5	LNM subtype
ERtm		primary tumor							LNM			
r		0.41	–0.33	–0.01	–0.18	0.54	0.80	0.42	–0.31	0.01	–0.41	0.54
p		**0.003**	**0.015**	0.474	0.121	**0.000**	**0.000**	**0.003**	**0.021**	0.465	**0.004**	**0.000**
PRtm												
r	0.41		–0.27	0.22	–0.23	0.21	0.45	0.58	–0.23	–0.01	–0.25	0.40
p	**0.003**		**0.038**	0.080	0.073	0.094	**0.001**	**0.000**	0.065	0.478	0.057	0.004
Her2tm												
r	–0.33	–0.27		–0.03	0.18	–0.32	–0.25	–0.29	0.73	0.19	0.13	–0.32
p	**0.015**	**0.038**		0.413	0.121	**0.017**	**0.050**	**0.028**	**0.000**	0.108	0.211	**0.018**
Ki-67tm												
r	–0.01	0.22	–0.03		0.13	–0.36	–0.01	0.00	–0.03	0.65	0.34	–0.16
p	0.474	0.080	0.413		0.201	**0.009**	0.487	0.494	0.424	**0.000**	**0.014**	0.147
CK5tm												
r	–0.18	–0.23	0.18	0.13		–0.02	–0.29	–0.30	0.18	0.14	0.67	–0.45
p	0.121	0.073	0.121	0.201		0.438	**0.032**	**0.028**	0.128	0.186	**0.000**	**0.001**
Primary tumor subtype												
r	0.54	0.21	–0.32	–0.36	–0.02		0.32	0.28	–0.25	–0.27	–0.23	0.55
p	**0.000**	0.094	**0.017**	**0.009**	0.438		**0.019**	**0.036**	**0.050**	**0.038**	0.076	**0.000**
ER(LNM)												
r	0.80	0.45	–0.25	–0.01	–0.29	0.32		0.50	–0.33	0.03	–0.33	0.56
p	**0.000**	**0.001**	**0.050**	0.487	**0.032**	**0.019**		**0.000**	**0.015**	0.427	**0.018**	**0.000**
PR(LNM)												
r	0.42	0.58	–0.29	0.00	–0.30	0.28	0.50		–0.22	–0.01	–0.28	0.50
p	**0.003**	**0.000**	**0.028**	0.494	**0.028**	**0.036**	**0.000**		0.075	0.469	**0.036**	**0.000**
Her2(LNM)												
r	–0.31	–0.23	0.73	–0.03	0.18	–0.25	–0.33	–0.22		0.21	0.12	–0.46
p	**0.021**	0.065	**0.000**	0.424	0.128	**0.050**	**0.015**	0.075		0.090	0.217	**0.001**
Ki-67(LNM)												
r	0.01	–0.01	0.19	0.65	0.14	–0.27	0.03	–0.01	0.21		0.35	–0.32
p	0.465	0.478	0.108	**0.000**	0.186	**0.038**	0.427	0.469	0.090		**0.013**	**0.018**
CK5(LNM)												
r	–0.41	–0.25	0.13	0.34	0.67	–0.23	–0.33	–0.28	0.12	0.35		–0.32
p	**0.004**	0.057	0.211	**0.014**	**0.000**	0.076	**0.018**	**0.036**	0.217	**0.013**		**0.021**
LNM subtype												
r	0.54	0.40	–0.32	–0.16	–0.45	0.55	0.56	0.50	–0.46	–0.32	–0.32	
p	**0.000**	**0.004**	**0.018**	0.147	**0.001**	**0.000**	**0.000**	**0.000**	**0.001**	**0.018**	**0.021**	

ER – estrogen receptor; PR – progesterone receptor; Her2 – Her2 marker; CK5 – cytokeratin 5; Ki-67 – marker of proliferation; r – Spearman rank correlation coefficient; "tm" – primary tumor level; LNM – lymph node metastasis level. Significant values are given in bold.

into 2 subgroups, with low and high activity of proliferation (luminal B/Her2/Ki-67). This division allowed us to determine that these cases "like" to have a high proliferative activity (5 from 7 cases). Such results correlate with Cheang's data concerning high aggressiveness of luminal B/Her2 subtype [22]. But, 2 cases had a low Ki-67 index, so for other groups it is suggested to evaluate the prognostic value of luminal B/Her2 subclassification by Ki-67 level.

In our results, both hormone receptors (ER and PR) showed a strong similarity of correlations at both sites, the primary tumor and its metastasis. It is natural that both of them correlated positively with intrinsic subtype, as we associated the luminal group with the highest marks during statistical analysis. These markers look quite stable during metastasis. The single difference between hormone receptors was that only the level of the tumor's ER (without PR) correlated negatively with the basal marker CK5 from lymph node metastasis (LNM) (Table V).

All surrogate markers from our study correlated positively with their level from LNM. Such results could drive us to a hasty conclusion that cell profiles from both sites are homogeneous.

The most common determined "intrinsic" subtype at both sites in our assays was luminal B. But in 7 cases this subtype shifted to another one (Table IV). The highest frequency of changes was encountered from luminal B/Ki-67 to luminal A by diminishing the rate of proliferation. As we counted the Ki-67 positive cells thoroughly, as the primary tumor and metastasis were on the same slide and technical risks were reduced to a minimum, we have to recognize that the panel of five enables one to describe the reason for this switch. These results can be explained partly by Zhou's data, where luminal A and B subtypes had the highest risk of metastasis in non-sentinel lymph nodes [24]. These 7 cases are in contradiction with Park's data, which affirm significant up-regulation of Ki-67 protein in the metastatic site compared to the primary tumor [25].

The total number of switched cases was 10 from 43 (23.26%). In five of them the subtype changed to one with a poor prognosis. The possible reasons could be different. Falck *et al.* also concluded that molecular profiles are not stable throughout tumor progression in breast cancer [10]. This supports the hypothesis that the malignant phenotype and its molecular signature are not pre-determined and static, but continue to evolve in a tumor throughout its life history [7].

On the other hand, Prat *et al.* noted that up to 10% of basal-like tumors are also positive for hormone receptors [5]. This genetic instability, when luminal receptors are determined immunohistochemically (IHC) in the basal one and vice versa, restricts the practical utility of surrogate markers. In our results one luminal B/Ki-67 subtype shifted to basal-like because of +3 grade of CK5, which in Nielsen's opinion is driving the case in a group with significantly poorer outcome [26]. Similar results to ours, about migration to a poor prognosis subtype and increased aggressiveness of luminal and triple-negative subtypes throughout tumor progression, were reported by Castaneda [27].

There are some limitations of our study. We classified the tumors according to their ER, PR, Ki-67, CK5 and Her2 status based on IHC surrogates, which is only an approximation of the genotype-based breast cancer subtype. But, nowadays IHC assays are cost-effective and have been accepted as useful clinical tests by many scientific communities.

According to Carey *et al.* the immunohistochemical definition of basal-like subtype is "ER negative, PR negative, Her2 negative, cytokeratin 5/6 positive and/or HER-1 positive" [28]. As we did not use the HER-1 marker, it is possible that some 5NP cases (2 in the primary tumor and 3 in LNM) in fact could be basal-like. However, the total amount of cases in the basal group did not change.

In the structure of the non-luminal basal group, oncologists recognized another molecular subtype, 5 negative phenotype (negative for all five markers, 5NP), which is proven to be histologically less aggressive than basal-like and more aggressive than luminal A tumors [28, 29]. We encountered such a phenotype in 2 cases at the primary level and 3 in LNM. Could it be a "claudin-low" some of them we can't say, but a dangerous switch of luminal A to 5NP was determined once.

Her2 marker grade was increased in 4 from 10 switched cases. One has to recognize the fact that the lack of hybridization techniques in our tests could misclassify these cases. If to omit Her2 involved cases, the rate of switched cases (4 from 43) remains high anyway and possibilities of shifting of one molecular subtype to another during the metastatic process remain a source for future debates.

In conclusion, our data support the hypothesis that breast cancer is a heterogeneous disease, with substantial variability of cellular components within each category, a statement applicable in invasive breast carcinomas of NST type too. The receptor profile of this carcinoma, indicated by surrogate markers, is not stable throughout the metastatic process.

The authors declare no conflict of interest.

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