

EXPRESSION OF CK5 BASAL CYTOKERATIN DURING METASTATIC DEVELOPMENT OF BREAST CARCINOMA

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ABSTRACT

Objective. Breast cancer is a one of the most common cancers in females worldwide. Basal cytokeratin CK5 represent the marker of progenitors for glandular and myoepithelial lineages of mammary epithelium. During epithelial differentiation there is a gradual decrease of CK5 expression. The purpose of this study was to compare the expression of basal cytokeratin CK5 vs hormone receptors, HER2, Ki67 and molecular subtypes immunohistochemically defined in the primary breast carcinoma of NST type and axillar lymph node metastasis. **Material and Methods.** We processed immunohistochemically 91 invasive breast carcinomas of NST type and their ipsilateral axillar lymph node metastasis (LNM). **Results.** The majority of primary tumors were evaluated as CK5 negative (78 cases/85.7%). The majority of cases were evaluated as Luminal B (50 cases/54.9%) and Luminal A (28 cases/30.8%) tumors. The HER2 subtype was confirmed in 8 cases/8.8%, 5NP in 3 cases/3.3% and Basal-like in 2 cases/2.2%. The parallel comparison of CK5 expression at both sites, primary and metastatic, revealed that this marker is not stable during metastatic progression. The molecular subtypes were not stable during metastatic process in 21 cases/23.1%. **Conclusions.** The majority of NST invasive ductal breast carcinomas are CK5 negative. The molecular subtypes and CK5 are not stable during metastatic process. Cancerous cells prefer to lose this marker in the lymph node environment. The presence of cases with simultaneous expression of CK5 and hormone receptors is an open field to debate the existence of other, transient molecular subtypes. We expect a further confirmation in larger study groups.

Key Words: molecular subtypes, invasive carcinoma NST type, basal cytokeratin.

INTRODUCTION

Breast cancer is a one of the most common cancers in women worldwide. The new concept purpose to identify at least five molecular subtypes: two hormone positive types (Luminal A and Luminal B) and three hormone negative types (HER-2 overexpressed, Basal-like, and Normal breast-like). Every molecular subtype has distinct clinical features, and different prognosis [1]. Nielsen et al. (2004) purposed to differentiate immunohistochemically these subtypes by a panel of four antibodies (ER, HER1, HER2, and cytokeratin 5), point of view sustained later also by Goldhirsch et al. [2, 3].

Actually, the secretory portion of the breast is considered to be made of five distinct cell populations: committed stem (progenitor) cells CK5 positive, glandular precursor cells which express all spectrum of cytokeratins (CK5+/CK8/18/19+), glandular end cells, positive for luminal cytokeratins (CK8/18/19+), myoepithelial precursor cells positive for CK 5/6+ and SMA+ (smooth muscle actin), and myoepithelial mature cells, SMA positive. By Böcker et al. (2005) the CK5 positive cells represent progenitors for both glandular

and myoepithelial lineages of mammary epithelium [4]. During epithelial differentiation there is a gradual decrease of CK5 expression.

After spreading from its primary situs, metastases arise as solid tumors in distant organs. But the microenvironment of the tissue in which they have landed it's quite different from which they have originated. Weigelt et al. (2003) affirmed that primary breast tumors are similar to the distant metastases in lung, ovary, skin, lymph nodes [5]. Authors consider that metastatic capacity of breast cancer is an inherent feature and the metastatic proficiency of a tumor is pre-programmed from its beginning [6].

By the other side a series of publications showed intriguing results about different immunohistochemically defined subtypes at primary site and LNM [7-10]. Authors concluded that molecular profiles of breast cancer are not stable throughout tumor progression.

The aim of this study was to compare the expression of basal cytokeratin CK5 vs hormone receptors, HER2, Ki67 and molecular subtypes immunohistochemically defined in the primary breast carcinoma of NST type and axillar LNM.

In the present work we found, that basal cytokeratin CK5 and molecular subtypes of invasive breast carcinoma of no special type are not stable during metastatic process.

MATERIAL AND METHODS

Patients

Ninety one cases of breast carcinomas and their LNM collected during 2013-2014 from the Oncological Institute, Republic of Moldova. No drug therapy preceded and all of patients (33-86 years old) underwent radical mastectomy and lymph nodes dissection.

Tissue processing and immunohistochemistry

The specimens were fixed in 10% phosphate buffered formalin for 24-48 h and paraffin (Paraplast High Melt, Leica Biosystems) embedded as traditionally. To avoid any misunderstandings about tissue processing, the specimens (primary tumor and lymph node) of the same patient have been embedded in a single paraffin block and sections were processed on the same slide (Fig.1). For histopathological assessment 3-5 μ m sections were cut (Shandon HM355S Automatic Microtome, Thermo Scientific, USA) and stained with hematoxylin Harris (HHS32, SigmaAldrich) and eosin CS701 (Dako, Denmark). Histological grade was scored by the Scarff-Bloom-Richardson grading system. All cases were morphologically confirmed as invasive ductal breast carcinomas of NST type with developed axillar LNM. The immunohistochemical assessment included 5 markers: for estrogen (ER), progesterone (PR), human epidermal growth factor receptor 2 (HER2), marker of proliferation Ki67 and basal cytokeratin CK5 (Table 1).

Specimens were processed automatically on Leica Bond-Max autostainer (Leica Microsystems GmbH, Wetzlar, Germany). The hematoxylin Mayer, Lille modified (HMM500, ScyTek Laboratories, Inc.) was used for counterstaining.

Microscopic evaluation. Hormone receptors, as well Ki67 marker were counted by using a semi-quantitative method Suciú et al. (2014) purposed [11]. In accordance with Goldhirsch et al. (2011) recommendations for Ki67 marker we used a 14% threshold as a limit to distinguish positive/negative cases [12]. For estrogen and progesterone evaluation we followed the guidelines purposed by Allred et al. [13]. The cases scored as +1 – +3 were considered positive. The threshold of positivity was 10% (Fig. 2).

The HER2 status was interpreted in accordance with ASCO (American Society of Clinical Oncology) recommendations [14]: HER2+0 – if no staining observed or weak, barely perceptible membrane staining until 10% of cells; HER2+1 – in case of a weak membrane staining of >10%; HER2+2 – in case of incomplete, weak/moderate circumferential membrane staining of more than 10% of tumor cells or complete circumferential intense staining less than 10% of cells; HER2+3 – in case of intense, circumferential staining of more than 10% of tumor cells. Cases with HER2 scored as +2 and +3 were considered positive. The positive cells of normal ducts served as internal control.

The CK5 expression was interpreted in accordance with Azoulay et al. recommendations: 0 – no tumor cells stained; +1 – less of 10% of tumor cells stained; +2 – 10-50% of positive tumor cells; +3 – more than 50% of tumor cells stained [15]. Expression was scored as

Fig.1. Invasive breast carcinoma of NST type, primary tumor and LNM processed on the same slide, hematoxylin-eosin, x6.

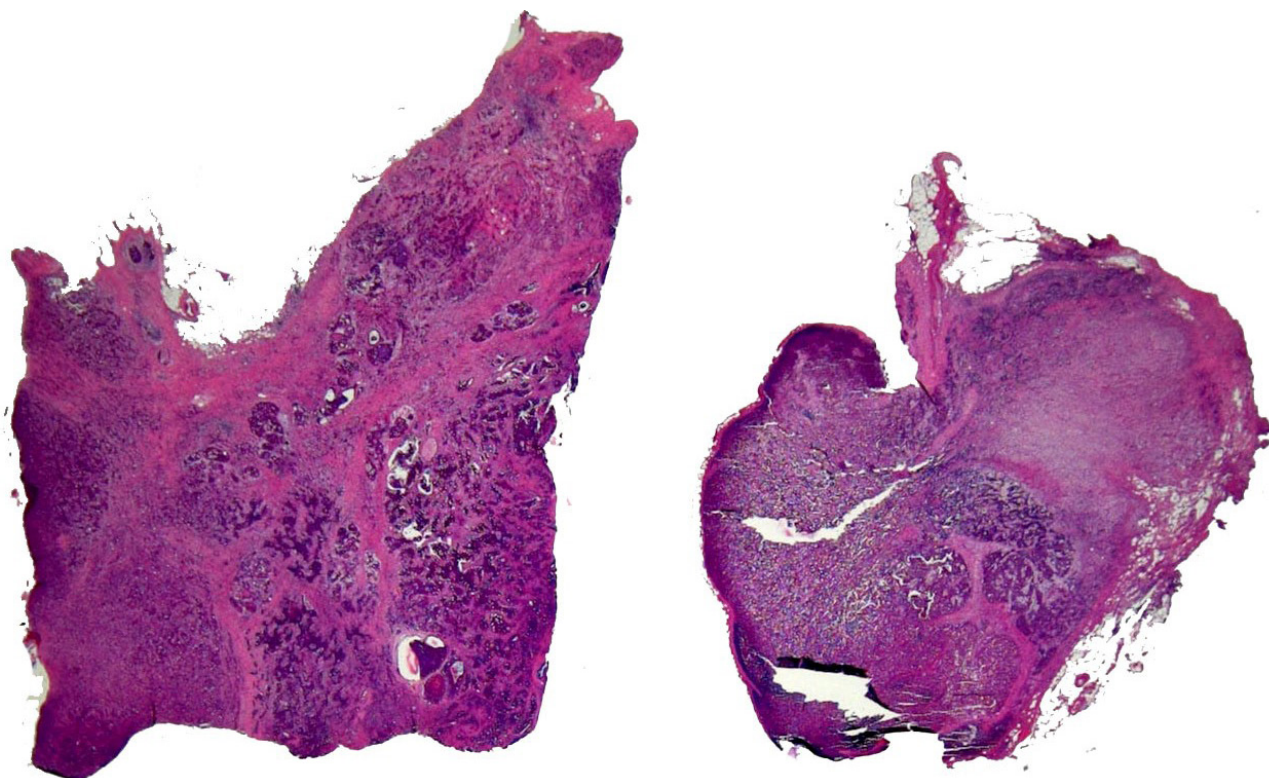
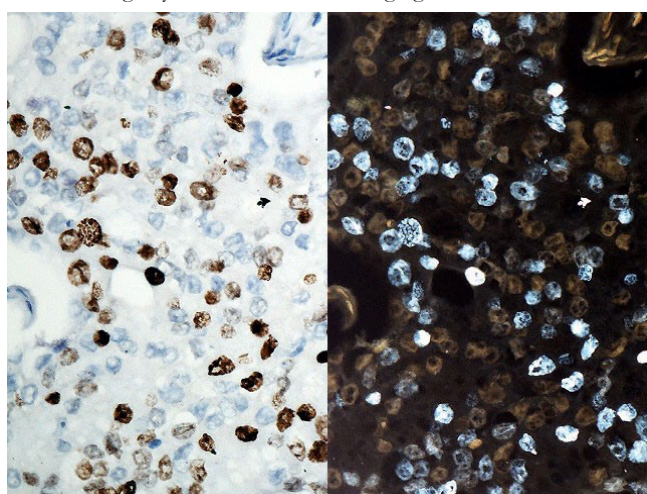


Table 1. Immunohistochemical supplies: source and dilution of antibodies, systems of detection and retrieval, time of incubation.

Antibody/Clone	Source/incubation time /dilution	Detection/time	Retrieval system/time
ER/6F11	Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK/15 min/RTU	Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK), 15 min	Bond Epitope Retrieval Solution 1, (Leica Biosystems, Newcastle Upon Tyne, UK)/20 min
PR/16			
HER2 /polyclonal	Dako Glostrup Denmark/30 min/RTU	EnVision-HER/30 min	Dako Target Retrieval Solution, pH6/20 min
Ki67/K2	Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK/15 min/RTU	Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK), 15 min	Bond Epitope Retrieval Solution2, (Leica Biosystems, Newcastle Upon Tyne, UK)/20 min
CK5/ XM26			

RTU: ready to use

Fig. 2. Invasive ductal breast carcinoma NST type, G3 stained for Ki67. The tumor was appreciated as Ki67 positive (61%). Counterstaining with Lille modified hematoxylin. On the right side is represented the inverted image by Nis-elements 2.30 imaging software, x400.



positive (>0) if any cytoplasmic and/or membranous staining tumor cells were observed.

The results were grouped in 7 subtypes: ER-, PR-, HER2-, CK5- as 5NP (five negative phenotype); ER-, PR-, HER2-, CK5+ as Basal-like; ER-, PR-, HER2+, CK5- as HER2+ (HER2 overexpressed); ER+, PR+, Her2+, CK5-, Ki67<14 as Luminal B/Her2; ER+, PR+, Her2+, CK5-, Ki67>14 as Luminal B/Her2/Ki67; ER+, PR+, Her2-, CK5-, Ki67>14 as Luminal B/Ki67; ER+, PR+, Her2-, CK5-, Ki67<14 as Luminal A.

The Luminal B subtype can be defined by ER, PR expressions simultaneously with HER2 and/or Ki67 positivity threshold over than 14%. As the Luminal A and B subtypes are different by prognosis, as well as the tendency of modern medicine is to personalize the treatment for every patient apart we considered opportune to highlight in the end of the name of Luminal B subtype the marker(s) which switched Luminal A case to Luminal B.

Image acquisition and data processing

Slides were examined on Nikon Eclipse 80i microscope with Nikon DS-Fi1 installed camera by using Nis-elements 2.30 imaging software (Nikon Instruments Europe BV). By this software we inverted images,

possibility which facilitates the ER, PR and Ki67 numeric evaluation. A MS Access 2007 database was used to store and group the data.

Statistical analysis

The WINSTAT 2012.1 (R. Fitch Software, Bad Krozingen, Germany) software was used for a descriptive statistics: the mean value, standard error of mean and median were determined for Ki67. For all the tests a $p \leq 0.05$ was considered significant. A Pearson's correlation (r) was used to determine the relationship between different variables. The CK5 expression from primary tumor and LNM was compared by t-Student and linear regression tests.

Ethics

This study has been approved by the Ethics Committee of the "Nicolae Testemitanu" University of Medicine and Pharmacy from Chisinau, Republic of Moldova (approval number 21/13/31.03.2014).

RESULTS

The G1 grade of differentiation was determined in 4 cases/4.4%, G2 in 51 cases/56% and G3 in 36 cases/39.6%. The majority of primary tumors were evaluated as CK5 negative (78 cases/85.7%), of which 3 cases/3.3% had G1 grade of differentiation, 45 cases/49.5% were G2 and 30 cases/33% were evaluated as G3. The CK5 positive tumors had predominantly G2 (6 cases/6.6%) and G3 (6 cases/6.6%) grades. Only one case evaluated with +2 for CK5 had a G1 grade.

The majority of cases were evaluated as Luminal B (50 cases/54.9%) and Luminal A (28 cases/30.8%) tumors. The Luminal B structure was as follow: Luminal B/Ki67 in 38 cases/41.8%, Luminal B/HER2/Ki67 in 9 cases/9.9% and Luminal B/HER2 in 3 cases/3.3%.

The HER2 subtype was confirmed in 8 cases/8.8%, 5NP in 3 cases/3.3% and Basal-like in 2 cases/2.2%.

By comparing CK5 expression with molecular subtypes, we determined that 70 cases/76.9% evaluated as CK5 negative were diagnosed as Luminal one (A or B), 5 cases/5.5% as HER2 and 3 cases/3.3% as 5NP.

Table 2. CK5 expression in primary tumor and LNM vs molecular subtype.

CK5 primary tumor	CK5 metastasis	Primary tumor subtype	Metastasis subtype	n	%
0	0	5NP	5NP	3	3,3
0	0	HER2	HER2	5	5,5
0	0	Luminal A	5NP	1	1,1
0	0	Luminal A	Luminal A	22	24,2
0	0	Luminal A	Luminal B/HER2	1	1,1
0	0	Luminal A	Luminal B/Ki67	2	2,2
0	0	Luminal B/HER2	Luminal A	1	1,1
0	0	Luminal B/HER2	Luminal B/HER2	2	2,2
0	0	Luminal B/HER2/Ki67	HER2	1	1,1
0	0	Luminal B/HER2/Ki67	Luminal A	1	1,1
0	0	Luminal B/HER2/Ki67	Luminal B/HER2/Ki67	2	2,2
0	0	Luminal B/HER2/Ki67	Luminal B/Ki67	2	2,2
0	0	Luminal B/Ki67	Luminal A	8	8,8
0	0	Luminal B/Ki67	Luminal B/Ki67	25	27,5
0	1	Luminal A	Luminal A	1	1,1
0	2	Luminal B/Ki67	Luminal B/Ki67	1	1,1
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1	0	Basal-like	5NP	1	1,1
1	0	Luminal B/Her2/Ki67	Luminal B/HER2	1	1,1
1	0	Luminal B/Ki67	Luminal A	1	1,1
1	1	HER2	HER2	1	1,1
1	3	Luminal B/Ki67	Basal-like	1	1,1
2	0	HER2	HER2	1	1,1
2	0	Luminal A	5NP	1	1,1
2	0	Luminal B/Ki67	Luminal B/Ki67	1	1,1
2	1	Luminal B/HER2/Ki67	Luminal B/HER2/Ki67	1	1,1
2	2	Luminal B/Ki67	Luminal B/Ki67	1	1,1
3	1	Basal-like	Basal-like	1	1,1
3	3	HER2	Luminal B/HER2/Ki67	1	1,1
3	3	Luminal B/HER2/Ki67	Basal-like	1	1,1
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Total				91	100

The CK5 positive cases (13 cases/14.3%) could enface features of any molecular subtype, except 5NP (Table 2).

The statistical assay demonstrated a positive correlation between CK5 expression from primary tumor and CK5 from metastatic site, HER2 and Ki67 from both sites. A negative correlation was determined when CK5 was associated with ER and PR expression in primary tumor and LNM.

More, CK5 value from metastatic side depends of CK5 expression from primary tumor, statement proved by linear regression and t-Student ($t=1.30, p<0.20$) tests (Fig.3).

The parallel comparison of CK5 expression at both sites, primary and metastatic, revealed that this marker is not stable during metastatic progression: 2 cases/2.2% (both G3) evolved with CK5 acquiring in metastatic

place and 6 cases/6.6% (2 cases were G2 and 4 cases as G3) with loosing CK5 in LNM.

Fig. 3. The relationship between CK5 expression from primary tumor and lymph node metastasis: the simple linear regression test.

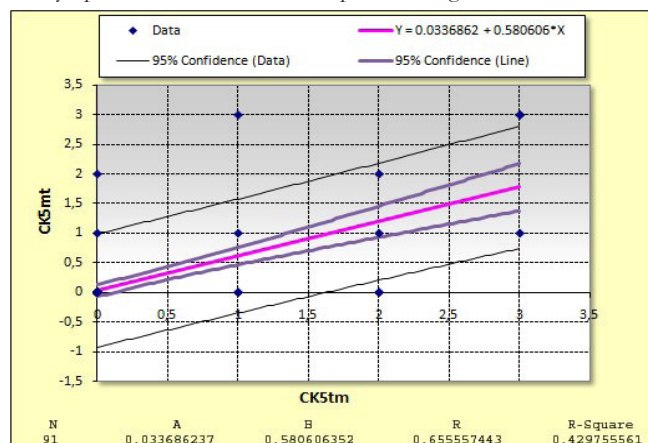


Table 3. CK5 expression vs ER, PR, HER2, Ki67, patients age and tumor grade of differentiation: Pearson correlation.

	CK5 tm	CK5 mt	ER tm	ER mt	PR tm	PR mt	HER2 tm	HER2 mt	Ki67 tm	Ki67 mt	Age	Grade
CK5tm												
r		0,66	-0,38	-0,40	-0,20	-0,28	0,21	0,20	0,40	0,37	-0,09	0,04
p		0,00	0,00	0,00	0,03	0,00	0,02	0,03	0,00	0,00	0,19	0,34
CK5mt												
r	0,66		-0,32	-0,30	-0,04	-0,13	0,15	0,13	0,37	0,35	-0,11	0,00
p	0,00		0,00	0,00	0,37	0,11	0,08	0,11	0,00	0,00	0,14	0,50

Endings: **tm** - primary tumor, **mt** - metastasis. **r** - Pearson correlation coefficient. **Grade** - means grade of differentiation, **age** - patients' age. Statistical significant results ($p \leq 0.05$) are given with **bold**.

The molecular subtypes were not stable during metastatic process in 21 cases/23.1%: Basal-like change to 5 NP in 1 case/1.1%, HER2 to Luminal B/HER2/Ki67 in 1 case/1.1%, Luminal A to 5 NP in 2 cases/2.2%, Luminal A to Luminal B/HER2 in 1 case/1.1%, Luminal A to Luminal B/Ki67 in 2 cases/2.2%, Luminal B/HER2 to Luminal A in 1 case/1.1%, Luminal B/HERses/3.3% to Basal-like, HER2 and Luminal A, Luminal B/Ki67 resulted in 1 case/1.1% with Basal-like and in 9 cases/9.9% evolved in Luminal A. The 15 cases of subtypes transfers were CK5 negative tumors and 6 cases were CK5 positive at primary site. Have to mention, that 2 cases, where CK5 changed from negative status in primary tumor to positive in LNM had a stable molecular subtype (Luminal A and Luminal B/Ki67). All other shifted cases had a stable CK5 negative status at both sites.

From 6 CK5 positive cases, 3 loosed this marker at LNM site and changed molecular subtype as follow: Basal-like to 5NP, Luminal B/Ki67 to Luminal A and Luminal A to 5 NP.

DISCUSSION

Cancer is a leading cause of death worldwide and the breast cancer is the most common reason of cancer death in women. Classical pathology has segregated this carcinoma into multiple groups, based on their overall morphology. The most common reported is invasive ductal carcinoma NST type. Beside of well known classical histological features, as tumor size, histological type and grade, vascular invasion, identifying of new molecular factors has become the objective of many research studies [1]. A common feature of malignant tumors is the ability to invade the nearby tissues and spread to more distant parts of the body through the bloodstream or lymphatic system. After spreading from its primary site, metastases arise as solid tumors in distant organs [16]. But the microenvironment of the tissue in which they have landed it's quite different from which they have originated and possibly creates a pressure on these cells requiring them to develop an adaptive responses which allow them to grow as a tumor in these nodes. Plus, was demonstrated the role of extracellular

matrix components in breast cancer progression and metastasis [17].

In the present study we analyzed the evolution of another basic marker, CK5. By contemporary opinion this marker is expressed by basal/myoepithelial cells and together with CK14, CK8/18, p63 is used to distinct the basal group of breast carcinomas. CK5 positive cells in fact represent progenitors for both glandular and myoepithelial lineages of mammary epithelium. During epithelial differentiation there is a gradual decrease of CK5 expression, associated with an increase in expression of CK8/18/19 in the glandular cells, and smooth muscle actin in the myoepithelial cells along the pathways of differentiation. By the Bocker W et al. (2005) data, in the lactating breast, there is a segregation of epithelial structures into CK8/18 expressing secretory zone and the proliferative zone which harbors cells of both glandular (CK8/18+) and basal /myoepithelial (CK 5/6+) type [4].

In case of benign lesions the luminal tumors present a high number of CK5/6 positive cells due to a high proliferation of both glandular and basal cells. In Heatley M et al. (1995) opinion, the majority of malignancies which are derived from differentiated glandular cells line do not reveal immunohistochemical staining with CK5/6 leading, by this explaining and CK5 negativity in most lesions of atypical hyperplasia and ductal carcinoma in situ [18].

In Laakso et al. (2005) opinion CK5/14 positive tumors encounter about 9% and majority of them are of histological grade 3 [19]. In our study this subtype was slightly higher, 14,3% and CK5 positive cases were equally G2 and G3.

By this article we confirmed the Laakso et al. (2005) results that primary tumor and LNM are statistically concordant for CK5 expression. But in our study, the parallel comparison of CK5 expression revealed that this marker is not stable during metastatic progression: 2 cases/2.2% (both G3) evolved with CK5 acquiring in metastatic place and 6 cases/6.6% (2 cases were G2 and 4 cases as G3) with loosing of CK5 in LNM.

The parallel comparison of CK5 with expression of surrogate markers included in study, confirmed its basal localization: inverse correlation with ER, PR and positive

association with Ki67, HER2. The last correlation value, with HER2 is contradictory to Laakso et al. (2005) which reported a negative value of CK5 expression with HER2 amplification. Plus, CK5 basal origin was disturbed by the fact, that CK5 positive cases (13 cases/14.3%) could enface features of any molecular subtype, inclusive Luminal and except 5NP.

By the present moment, are few data which compared the molecular profiles of primary tumor and its metastasis. Falck et al. (2010) initially have not determined a statistically significant skewness by comparing the fraction of ER, PR, HER2 and Ki67 positive cells from both sites, but a fraction of 7% (ER), 16% (PR), 3% (HER2) discordant pairs were reported [7]. The same authors, showed also discordance between primary tumors and lymph node metastasis in breast cancer patients according to a multiple molecular phenotype [8]. The discordant 16% of cases were shifted from Luminal A to a subtype where survival analysis showed an impaired prognosis compared to this subgroup. Moreover authors determined that when a shift in subtype between primary tumor and metastatic lymph node was happened, the prognosis (for 10 years) seemed to follow the subtype of the lymph node [9]. It means that the prognostic information for individual patients appears to be available only after synchronous analysis of biomarker expression in metastatic lymph nodes.

In the present study the molecular subtypes were not stable during metastatic process in 21 cases/23.1%. Have to mention that majority of shifted cases had a stable CK5 negative status at both sites.

Montel et al. (2005, 2006) demonstrated several 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 [20, 21]. Urquidi et al. (2002) have provided direct proof that individual cancer cells, co-exist within a given tumor, but differ greatly in metastatic capability, moreover some of them are non-metastatic [22]. It seems that primary cancers contain many types of tumor cells which can differ by expression profiles and their metastatic activity. Plus, as the metastatic ability of the cell population increases, the receptors profiles changes concomitantly. These are demonstrating conclusively 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 [23]. Raica et al. (2014) reported about 20% of cases with switch of molecular subtypes during metastatic development, especially because of ER, which seems to be the most unstable parameter during invasive process [10]. This evidence supported the most frequent switching from the luminal A subtype in the primary tumor to the luminal B subtype in the matched LNM. Authors concluded that primary cancer probably contain many types of tumor cells, which can differ by their expression profiles and their metastatic potential.

The Basal-like breast carcinoma was reported to have the worst prognosis [24]. These tumors are described as negative for hormone receptors and HER2, and positive for CK5 and/or HER1 [25]. Basal-like tumors are also the most common type of tumors in patients with germline BRCA1 mutations. Such tumors are considered as high proliferative one, with a low cellular differentiation [26, 27]. Such results are confirmed by the present study, where majority of tumors were confirmed as G2 and G3. In accordance to Sood et al. (2014) the CK5 did not show statistically significant correlation with age, tumor size and stage, histological type, the state of tumor margins, presence of lymphoid infiltrate and necrosis, lymph node status, and Ki67 positivity [27]. Authors reported a single significant correlation, CK5 vs tumors' grade, result debated by Rao et al. (2013) and confirmed by us: CK5 correlated significantly with all studied markers, except grade of differentiation [26].

Some authors debate in the literature whether triple-negative tumors (negative for hormone receptors and HER2, CK5 positive) are synonym with Basal-like carcinoma [28]. Cheang et al. (2008) by using additional markers identified patients with a significantly worse outcome in the group of triple-negative tumors, which it means that breast carcinoma is not homogenous even inside of the molecular subtypes [29].

With present study we entwine a series of our previous publications concerning breast cancer stability throughout during metastatic development. We tested the surrogate markers which are commonly accepted by the oncologist to stratify the molecular subtypes of this tumor. We entitled the studies from stability position and didn't answer from tumors cells homogeneity. By this we raise the question to research communities: another profile of metastatic cells is due to inhomogeneous cellular structure of primary tumor or these cells are changing receptors kit in metastatic ambiance?

CONCLUSION

The majority of NST invasive ductal breast carcinomas are CK5 negative. The molecular subtypes and CK5 are not stable during metastatic process. Cancerous cells prefer to lose this marker in the lymph node environment. The presence of cases with simultaneous expression of CK5 and hormone receptors is an open field to debate the existence of other, transient molecular subtypes. We expect a further confirmation in larger study groups.

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AUTHOR CONTRIBUTION

MR and AMC participated in developing the study design, data interpretation and manuscript editing. VF collected and processed the data, wrote the primary version of manuscript. LS participated in manuscript editing. ARB processed immunohistochemically all cases and interpreted results. The final version of manuscript was read and approved by all authors.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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