ORIGINAL ARTICLE

P53 IS UNSTABLE DURING METASTATIC DEVELOPMENT OF THE HUMAN BREAST CANCER: A COMPARISON BETWEEN THE PRIMARY TUMOR AND LYMPH NODE METASTASIS

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ABSTRACT

Objective. The aim of this study was to highlight p53 expression during metastatic progression of invasive breast carcinoma of no special type (NST) and to evaluate its role in stratifying patients based on molecular classification. **Material and Methods.** The specimens, primary tumors and corresponding lymph node metastases (LNM) from 84 patients were immunohistochemically stained for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER)-2, basal cytokeratin CK5 and nuclear proteins Ki67, p53. **Results.** No statistical significant differences of p53 expression were found between the two compared sites, but the p53 instability was found in 11 cases (13.2%). Switch of molecular subtype was noticed in 22.62% of cases. Only 5 cases of p53 transitions, from positive to negative status, were involved in molecular subtypes switch, from Luminal B to Luminal A. **Conclusions.** The p53 marker and molecular subtypes are not stable during tumor progression. Breast cancer during its metastatic development can gain or lose the p53 expression and cases developed with p53 vanishing in metastasis prevailed. A link between p53 instability and molecular subtypes switch was found only between p53 loss and Luminal B to Luminal A transition.

Key Words: p53, molecular subtypes, breast cancer, metastases.

INTRODUCTION

Breast cancer is a heterogeneous disease with different clinical outcomes. It is one of the most common cancers in females worldwide. Over- or under-expression of apoptotic genes can result with the lack of cell death, mechanism that has been also demonstrated in breast cancer. Beside of well-known prognostic factors, as histological type, tumor size, grade and vascular invasion, identification of new molecular markers has become the objective of many research studies. Such a potential marker is p53 protein.

The p53 is one of the most intriguing potential factors involved in the metastatic process, which directly controls the transcription of genes responsible for cell adhesion, motility, invasion and anoikis. The wild-type p53 tumor suppressor protein acts in G1 cell cycle and in response to DNA damage promotes repairing or cell death. This protein is not expressed in normal breast based on immunohistochemical evaluation, due to its short time of expression (action). Loss of its function was associated with poor outcome in breast cancer [1]. Intriguing, but there are no data until now regarding the simultaneous expression of p53, ER, PR, HER2, CK5, of molecular subtypes in primary tumor and corresponding metastatic axillary lymph nodes.

The purpose of the current study was to highlight p53 expression in the primary tumor and corresponding lymph node metastases (LNM) in association with ER, PR, HER2, basal cytokeratin CK5 status and molecular subtypes assessed by above mentioned surrogate markers. As a result we found that p53 marker and molecular subtypes are not stable during metastatic process.

MATERIAL AND METHODS

Patients. There were analyzed specimens of breast carcinoma and corresponding axillary lymph node metastases from 84 patients of 33-86 years old (57.7±1.3, with a median of 58.5). All patients underwent a Madden modified radical mastectomy with lymph nodes dissection, without prior chemo- and radiotherapy. Histopathological diagnosis was assessed by two pathologists and cases suitable for immunohistochemistry were carefully selected: all 84 cases were diagnosed as NST (no special type) of breast carcinoma, lobular and special types of cancer, as well equivocal results of staining/interpretation were excluded.

Specimens processing and immunohistochemistry. The specimens were fixed in 10% buffered formalin and paraffin embedded (Paraplast High Melt, Leica Biosystems). Primary tumor and its LNM were placed in one block. The 5-μm thick sections were immunohistochemically assessed with 6 markers: ER (clone Er/6F11, Leica Biosystems), PR (clone Pr16, Leica Biosystems), human epidermal growth factor receptor 2 (HER2/polyclonal, DakoCytomation), marker of proliferation Ki67 (clone K2, Leica Biosystems), basal cytokeratin CK5 (clone XM26, Leica Biosystems) and pro-apoptotic protein p53 (p53/DO-7, Leica Biosystems). Incubation with primary antibodies was followed by the use of HercepTest PharmDx Kit (DakoCytomation) and Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK). All cases were evaluated also by FISH

as international rules recommend (PathVysion HER-2 DNA Probe Kit II, Abbott)". Slides were processed automatically on Leica Bond-Max autostainer (Leica Microsystems GmbH, Wetzlar, Germany). The Harris modified hematoxylin solution (HHS32, SigmaAldrich) was used for counterstaining.

Microscopic evaluation. The hormone receptors were scored in according to Allred et al. guideline of ER and PR assessment [2]. Cases scored as +1 to +3 were considered positive. The threshold of positivity was 10%. The HER2 expression was evaluated by LeicaBond Oracle HER2 IHC System (LeicaBiosystem). The HER2 status was interpreted according to American Society of Clinical Oncology recommendations [3]. Cases interpreted as +2 and +3 were considered positive. Leica HER2 control slides ensured the control and accuracy of our decisions.

For Ki67 a 14% threshold was used. This marker, as well hormone receptors were counted using a semi-quantitative method performed by Suciu et al. [4]. The basal cytokeratin CK5 was interpreted in accordance to Azoulay et al. recommendations [5]. Cases evaluated as +1 to +3 were considered positive. The p53 marker was assessed as Yamashita et al. purposed: 0 – no specific staining; +1 – less of 10% tumor cells are p53 positive; +2 – 10-30% of cells express p53; 3 – more than 30% exhibit a specific nuclear pattern [6]. Breast stroma and lymph node lymphocytes served as positive control.

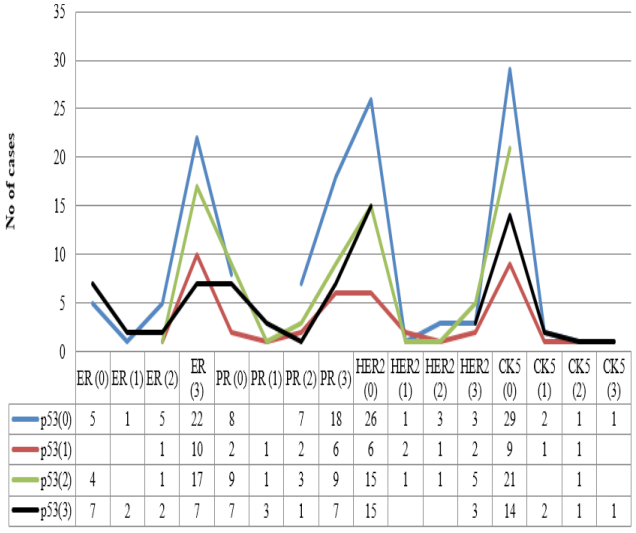
Based on Goldhirsch et al. recommendation the molecular subtypes were clustered as follow: ER+ and/or PR+, HER2−, CK5-, Ki67<14% as Luminal A; ER+ and/or PR+, HER2+, CK5− as Luminal B/HER2; ER+ and/or PR+, HER2−, CK5−, Ki67>14% as Luminal B/Ki67; ER+ and/or PR+, HER2+, CK5−, Ki67>14% as Luminal B/HER2/Ki67; ER−, PR−, HER2+, CK5− as HER2-overexpressed; ER−, PR−, HER2− and CK5+ as Basal-like; ER−, PR−, HER2− and CK5- as 5NP (5-negative phenotype) [7].

Statistical analysis. For descriptive statistics (the mean and standard error of the mean, the median) and Pearson's correlation was used WinStat 2012.1 software (R. Fitch Software, Bad Krozingen, Germany). The p53 values from 2 sites were compared by t-dependent test. For all tests a value of $p<0.05$ was considered significant.

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

RESULTS

The most frequent histological grade was G2, found in 45 cases (53.6%), G3 in 40.5% or 34 cases and G1 constitute 6%/5 cases. The p53 positive expression vs tumors' histological grade was clustered as 1case or 1.2% with G1, 29 cases/34.5% with G2, 21 cases/25% with G3. The hormone receptors (ER, PR) had the highest rate of positivity compared to other analyzed markers in primary tumor. The p53 was determined as positive in majority of NST tumors – 51 cases/60.7%. Its expression vs ER, PR, HER2 and CK5 is given in details in Figure 1.



	ER (0)	ER (1)	ER (2)	ER (3)	PR (0)	PR (1)	PR (2)	PR (3)	HER2 (0)	HER2 (1)	HER2 (2)	HER2 (3)	CK5 (0)	CK5 (1)	CK5 (2)	CK5 (3)
p53(0)	5	1	5	22	8		7	18	26	1	3	3	29	2	1	1
p53(1)			1	10	2	1	2	6	6	2	1	2	9	1	1	
p53(2)	4		1	17	9	1	3	9	15	1	1	5	21		1	
p53(3)	7	2	2	7	7	3	1	7	15			3	14	2	1	1

Fig.1. *The p53 expression in relation to ER, PR, HER2 and CK5 scores at primary site. The numbers from table below indicate the number of cases (from 84).*

The Ki67 marker was considered positive (≥14) in 50 cases/59.52% of primary tumors. The negative p53 expression was supported in 12 cases/14.3% by a high proliferation rate and in 21 cases/25% by a Ki67 level less than 14. The positive p53 cases were a highly proliferating in 45.2% (38 cases) and low proliferating in 15.5% (13 cases).

The most often subtype at primary site was Luminal B (45 cases/53.6%), followed by Luminal A (26 cases/31%) and hormone-negative group (13 cases/15.5%). The positive p53 marker had the highest expression in Luminal B/Ki67 group (27 cases/32.1%), followed in by Luminal A (9 cases/10.7%), Luminal B/HER2/Ki67 (6 cases/8.3%) and HER2 (5 cases/6%) (Table 1). By comparing the p53 scores from both sites, t-test revealed no significant statistical differences ($t=1.83$, $p<0.07$). The comparison of molecular profile of primary tumor and its LNM revealed a transition of subtype to another one in 19 cases/22.62% (Table 1).

Table 1. Molecular subtypes, Ki67 activity and p53 evolution during breast cancer progression: a comparative study of primary tumor and corresponding metastases.

Molecular subtype		p53		Ki67		No	%
Tm	Mt	Tm	Mt	Tm	Mt		
5NP	5NP	3	3	>14	>14	1	1,2
5NP	5NP	3	3	>14	<14	1	1,2
5NP	5NP	0	0	<14	<14	1	1,2
BasalLike	**5NP**	0	0	>14	<14	1	1,2
BasalLike	BasalLike	3	3	>14	>14	1	1,2
HER2	HER2	0	0	>14	>14	1	1,2
HER2	HER2	***0***	***1***	<14	>14	1	1,2
HER2	HER2	2	3	>14	>14	1	1,2
HER2	HER2	2	2	<14	>14	1	1,2
HER2	HER2	2	2	<14	<14	1	1,2
HER2	HER2	2	1	>14	>14	1	1,2
HER2	HER2	3	3	<14	>14	1	1,2
HER2	**Luminal B/HER2/Ki67**	0	0	>14	>14	1	1,2
Luminal A	**5NP**	0	0	<14	<14	1	1,2
Luminal A	Luminal A	0	0	<14	<14	14	16,7
Luminal A	Luminal A	***2***	***0***	<14	<14	1	1,2
Luminal A	Luminal A	***0***	***1***	<14	<14	1	1,2
Luminal A	Luminal A	3	2	<14	<14	1	1,2
Luminal A	Luminal A	2	3	<14	<14	1	1,2
Luminal A	Luminal A	2	1	<14	<14	1	1,2
Luminal A	Luminal A	2	2	<14	<14	2	2,4
Luminal A	Luminal A	3	3	<14	<14	1	1,2
Luminal A	**Luminal B/HER2**	2	2	<14	<14	1	1,2
Luminal A	**Luminal B/Ki67**	0	0	<14	>14	1	1,2
Luminal A	**Luminal B/Ki67**	2	3	<14	>14	1	1,2
Luminal B/HER2	**Luminal A**	0	0	<14	<14	1	1,2
Luminal B/HER2	Luminal B/HER2	1	1	<14	<14	1	1,2
Luminal B/HER2	Luminal B/HER2	0	0	<14	<14	1	1,2
Luminal B/HER2/Ki67	**HER2**	3	3	>14	>14	1	1,2
Luminal B/HER2/Ki67	**Luminal A**	***2***	***0***	>14	<14	1	1,2
Luminal B/HER2/Ki67	Luminal B/HER2	1	1	>14	<14	1	1,2
Luminal B/HER2/Ki67	Luminal B/HER2/Ki67	2	1	>14	>14	1	1,2
Luminal B/HER2/Ki67	Luminal B/HER2/Ki67	3	3	>14	>14	1	1,2
Luminal B/HER2/Ki67	Luminal B/HER2/Ki67	***1***	***0***	>14	>14	1	1,2
Luminal B/HER2/Ki67	Luminal B/Ki67	0	0	>14	>14	1	1,2
Luminal B/Ki67	**BasalLike**	3	3	>14	>14	1	1,2
Luminal B/Ki67	**Luminal A**	***2***	***0***	>14	<14	1	1,2
Luminal B/Ki67	**Luminal A**	3	3	>14	<14	1	1,2
Luminal B/Ki67	**Luminal A**	0	0	>14	<14	2	2,4
Luminal B/Ki67	**Luminal A**	2	2	>14	<14	1	1,2
Luminal B/Ki67	**Luminal A**	***3***	***0***	>14	<14	2	2,4
Luminal B/Ki67	**Luminal A**	***1***	***0***	>14	<14	1	1,2
Luminal B/Ki67	**Luminal A**	1	1	>14	<14	1	1,2
Luminal B/Ki67	Luminal B/Ki67	2	1	>14	>14	3	3,6
Luminal B/Ki67	Luminal B/Ki67	1	1	>14	>14	3	3,6
Luminal B/Ki67	Luminal B/Ki67	0	0	>14	>14	5	6,0
Luminal B/Ki67	Luminal B/Ki67	1	3	>14	>14	1	1,2
Luminal B/Ki67	Luminal B/Ki67	2	2	>14	>14	4	4,8
Luminal B/Ki67	Luminal B/Ki67	3	3	>14	>14	5	6,0
Luminal B/Ki67	Luminal B/Ki67	***0***	***1***	>14	>14	1	1,2
Luminal B/Ki67	Luminal B/Ki67	1	2	>14	>14	2	2,4
Luminal B/Ki67	Luminal B/Ki67	***3***	***0***	>14	>14	1	1,2
Total						**84**	**100,0**

Note: Molecular subtypes shifted cases are selected with Bold and primed with gray color.
The p53 transitions are selected with Bold, Italics and Underlined. Tm – primary tumor, Mt – metastases.

The p53 shifted in 11 cases/13.1% (Fig.2). In 8 cases/9.5% metastases lost p53 and in 3 cases/3.6% the tumor begins to express this marker in the metastatic site. A particular mention of 5 cases of p53 transition, from positive to negative status, that were involved in molecular subtypes switch, from Luminal B to Luminal A. The statistical assays revealed a significant, positive correlation of p53 expression with Ki67 activity (r=0.28, p=0.006) and negative correlations with ER (r=-0.22, p=0.002), PR (r=-0.20, p=0.003) and molecular subtype (r=-0.19, p=0.04).

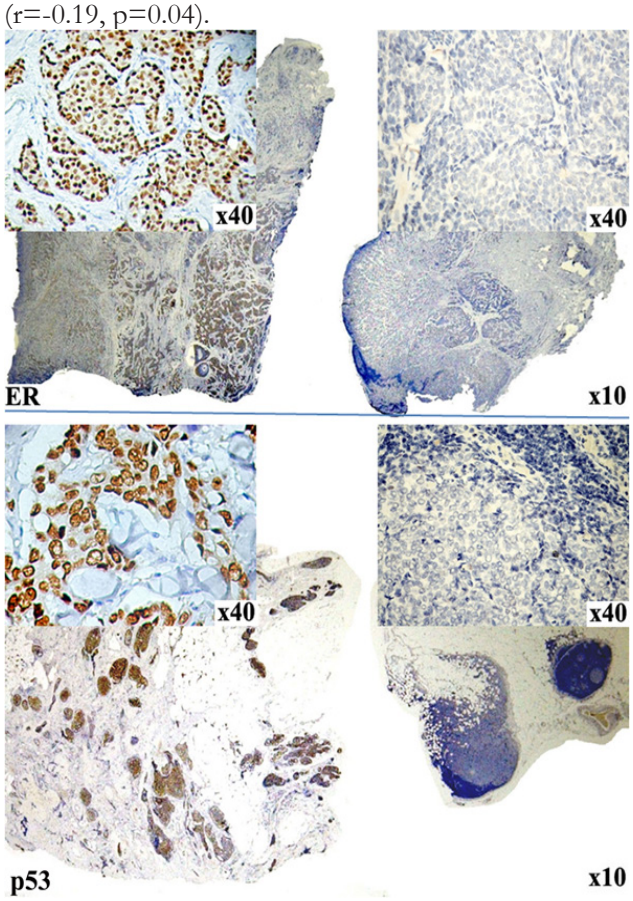


Fig.2. *The representative images of ER (assessed with Er/6F11 marker) and p53 (clone p53/DO-7) transitions during metastatic progression, from positive status at primary site to negative in LNM. Note that primary tumor and its lymph node metastasis were processed on the same slide.*

DISCUSSIONS

In previous studies we described in details that molecular subtypes and anti-apoptotic receptor BCL2 are unstable during metastatic development of breast cancer [8,9]. Now we focused the research on the pro-apoptotic marker p53. The p53 regulates cell proliferation and is considered the "guardian" of genome stability. By several transcription-regulating functions, including the induction of G1 cell cycle arrest by activation of p21 and by down-regulation of BCL2 it induces the apoptosis in response to irreparable DNA damage. Due to its short life, the p53 protein is not usually detected immunohistochemically in normal tissues. The TP53 gene, which encodes p53 protein, is considered as the most frequent (more than 50%) mutated gene in human cancers. In breast cancers about one-third of cases have mutations in the TP53 gene, which are associated with high histological grade and clinical aggressiveness. Most TP53 alterations found in breast carcinomas are point mutations leading to the synthesis of a stable, mal-functional, and non-degradable protein that accumulates in tumor cells. The result of these mutations is detected by immunohistochemical assays as a nuclear accumulation of the protein. Yamashita et al. found that the expression of p53 is associated with poor prognosis in breast cancer [6]. The authors noticed a significant correlation of p53 with HER2 and Ki67 level, in detriment of overall survival. Our study confirms the positive association of p53 expression with the proliferation activity.

Silwal-Pandit et al. demonstrated that TP53 mutations have a different clinical relevance in molecular subtypes of breast cancer and suggest diverse roles for TP53 in the biology of breast cancer development [10]. TP53 mutations were associated with increased mortality in patients with Luminal B, HER2-enriched and normal-like tumors, but not in cases with Luminal A and Basal-like tumors. In addition, the presence of p53 was found to be an independent marker of poor prognosis in estrogen receptor-positive cases. In our cases predominated a negative correlation of p53 to ER and PR scores.

The present study emphasized p53 instability during breast cancer metastatic development. In 11 cases p53 changed its expression, 8 of which evolved with p53 vanishing at metastatic site. A question arises then: does the malignant cell could "repair" p53 itself at metastatic site or primary tumor is heterogeneous by cellular components?

In 2005 Weigelt et al. summarized the possible models of metastatic process development [11]. The prevailing model suggests that metastatic capacity is a late, acquired event in tumorigenesis. Other ideas consider that metastatic ability might be an inherent feature of tumors or breast cancer is intrinsically a systemic disease. The switch of markers expression in LNM, especially of p53 in our case pushes on us to debate the most appropriate metastatic model.

The traditional and spontaneous models could be taken in consideration in case that tumor has the same markers expression at both sites (77.4% of cases). We correlated the markers expression from primary site with homonym receptors from LNM and in majority of cases received the same grade of association, which support the idea that in metastatic site is the same type of tumor. But what about the 22.6% of subtypes transitions, as well p53 and other receptor switches mentioned by Raica et al. [8]? In these cases probably the most applicable theories are of "clonal dominance" and "genometastasis hypothesis". Anyway, it remains unclear which factors are triggering the acquiring or losing of tumors features in metastatic site.

In summary, p53 marker and molecular subtypes are

not stable during tumor metastatic progression. Breast cancer can gain or lose the p53 expression and cases developed with p53 vanishing in metastasis, prevailed. A linkage of p53 instability and molecular subtypes switch was determined only between p53 loss and Luminal B to Luminal A transitions.

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