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ZFP36 expression profiles in breast tumors of different stages and hormonal receptor status

A.O. Hubiernatorova[1], L.A. Syvak[2], N.O. Verovkina[3], S.V. Kropyvko[1]

[1] Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03143
[2] National Research Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine
53, Yuriia Illienka Str., Kyiv, Ukraine, 04050
[3] Brown University. Department of Pathology and Laboratory Medicine
02906, Providence RI, USA
april.scorpionen@gmail.com

Abstract. *ZFP36* is a gene which is downregulated in various malignancies, and its expression level is often considered as a potential prognostic and diagnostic marker. **Aim.** In current study we investigated how the *ZFP36* expression varies in breast tumors of different histological types, grades, metastatic status, as well as in tumors with different progesterone and human EGFR receptor 2 (HER2) status to assess its use as a potential breast cancer biomarker. **Methods.** RT-qPCR. **Results.** We show that the *ZFP36* expression is elevated in T1–2 and N0–N1 tumors compared to adjacent tissues and T3–4 and N2 tumors, respectively. We also show that the *ZFP36* expression does not vary in samples with different PR status, but significantly increases with HER2 amplification. **Conclusions.** The *ZFP36* gene is a promising candidate to the HER2-enriched breast cancer biomarker, although further studies are necessary for verification.

Keywords: gene expression, carcinogenesis, breast cancer, ZFP36, TTP.

Introduction

Tristetraprolin (TTP, ZFP36, encoded by the *ZFP36* gene) is an RNA-binding protein, which negatively regulates malignancy-associated mRNAs and is extensively studied as a positive prognostic marker in various malignancies, since decrease of its expression, as well as its functional defects, are often associated with tumor progression and poor survival prognosis [1–3]. It is known that *ZFP36* is significantly downregulated in various tumors, including breast cancer (BC), and its expression level negatively correlates with tumor aggressiveness and metastatic potential [4, 5].

Breast tumors are usually divided into 4 main groups depending on clinical characteristics and specific markers. Molecular subtypes are usually diagnosed using information on the presence or absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2/neu) expression, and further prognosis and therapeutic strategy are based on this classification. Another commonly used classification is TNM classification (tumor, nodulus, metastasis) which relies on clinical and pathological characteristics of the tumor and describes not only the features of tumor itself (size and spread, T1–T4, with progressive enlargement and invasiveness), but also gives information on presence or absence of metastasis to lymph nodes (N0–N3, where N0 — no node metastases, and N1–N3 — the abundance of node metastases), distant metastasis (M0 where there are no distant metastases, and M1 when there are), as well as tumor differentiation grade (G1–4, with progressive dedifferentiation from 1 to 4). The detailed information can be found in [9].

Decades of research indicate the existence of many more subtypes even within the main molecular groups, indicating that the same tumor subtype can be more or less aggressive in different patients, depending on additional factors, and may consequently change the prognosis and therapeutic strategy for each individual patient. Since the patients with low *ZFP36* demonstrated poorer survival rates and more aggressive tumors, elevated *ZFP36* expression is considered as a potential positive prognostic marker for BC [6]. However, in our previous study we showed that if we divide BC tumor samples by subtype, high *ZFP36* expression may be associated with both positive and negative prognosis, as well is it was exclusively elevated in HER2 enriched subtype tumors [7]. Moreover, *ZFP36* expression changes under the doxorubicin treatment, which may also limit its use as a marker [8]. Here we investigate the *ZFP36* expression levels in tumors of different TNM status, as well as of different grades and hormone receptor status to assess the possibility of *ZFP36* use as a prognostic or diagnostic marker in BC.

Table1. **Clinical information on the samples used in the study**

Histological type of tumor	Sample number
Invasive carcinoma	53
Adjacent tissues	13
Normal tissues	1
Stage	
I	11
II	33
III	7
IV	2
TNM classification (tumor, nodulus and metastasis)	
T1	14
T2	36
T3	1
T4	2
N0	37
N1	10
N2	6
M0	52
M1	1
Receptor status	
ER^+PR^+HER2/neu^-	13
ER^-PR^-HER2/neu^-	12
ER^+PR^+HER2/neu^+	11
ER^-PR^-HER2/neu^+	17

Materials and Methods

Information on the samples used in current study: The data on samples are presented in Table 1. All the patients gave their informed consent. The same pool of samples (total number of tumor specimens 53) was classified by different characteristics, such as TNM grade, receptor status or G-grade.

Total RNA isolation from breast tumor samples*:* Breast tumor samples were collected from the National Cancer Institute, rapidly frozen in liquid nitrogen post-surgery, and stored at –80 °C. Total RNA was extracted from 0.2–1.5 g of tissue using the guanidinium isothiocyanate method. The extraction was performed with innuSOLV reagent (Analytik Jena) or RNA Go (BioLabTech), following the manufacturer's protocols, with subsequent electrophoresis to ensure the RNA integrity and yield.

cDNA synthesis. 5–8 μg of isolated total RNA was treated with DNase I (ThermoFisher) to eliminate any genomic DNA contamination, following the manufacturer's instructions. Subsequently, cDNA was synthesized using reverse transcriptase (ThermoFisher) in 20 μl reaction volume, adhering to the manufacturer's protocol. The resulting cDNAs were stored at –20 °C for later use.

RT-qPCR with fluorescence labeled probes. PCR was performed in 25 μl of mixture containing 0.2 μM of each specific primer and 0.1 μM Taq-Man probe, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 units DreamTaq DNA polymerase (ThermoFisher) and the corresponding buffer. Each sample was analyzed as duplicates. *TBP* gene was selected as a reference based on the analysis of literature sources that showed its reliability as a control gene regardless of breast tumor type [10–12]. Primers and probes used for PCR were as follows:

For. *TBP* 5'GTGCCCGAAACGCCGAATA TA3',

Taq-Man probe *TBP* 5'(BHQ1) ATCCCA AGCGGTTTGCTGCGGT (FAM)3',

Rev. *TBP* 5'CCGTGGTTCGTGGCTCTCT TA3';

For. *ZFP36* 5'CATGGATCTGACTGCCAT CTAC3',

Taq-Man probe *ZFP36* 5'(FAM) AGCCCT GACGTGCCCGTGCC (BHQ1)3',

Rev.*ZFP36* 5'CTGGAGTCGGAGGGG CTCA3'.

RT-qPCR results calculation. Relative gene expression was calculated using the following equation:

$$RelExp = (E_{GOI})^{\Delta Ct_{(GOI)}} / (E_{HKG})^{\Delta Ct_{(HKG)}}$$

where RelExp — relative expression, E_{GOI} — primers efficiency for gene of interest, $\Delta Ct_{(GOI)}$ — ΔCt for the gene of interest, E_{HKG} — primers efficiency for the reference gene, $\Delta Ct_{(HKG)}$ — ΔCt for the reference gene.

Statistical analysis*:* The analysis was performed using Prism GraphPad 9.5 software. The data were checked for normality. Since all the data showed non-gaussian distribution, they were analyzed using Kruskal-Wallis ANOVA with Tukey's correction. All the data show median ± 95% confidence interval (CI).

Results and Discussion

Previously, we showed that the *ZFP36* expression level was significantly elevated in all tumor types compared to the adjacent tis-

sues [7]. Although the literature suggests that both *ZFP36* mRNA and its protein product are often significantly reduced in tumor compared to normal tissues, both here and previously we used adjacent, conditionally healthy tissues as controls that represent a unique tissue type, distinct from both tumor and healthy donor tissues [13, 14].

In current study we analyzed the transcriptional profile of *ZFP36* depending on tumor histological type, malignancy stage, as well as metastatic and hormone receptor status in the same pool of specimens (Fig. 1). The analysis showed the *ZFP36* mRNA level was significantly increased in T1 and T2 tumor samples compared to the adjacent tissues. However, no significant differences in expression were found between tumors of different types relative to each other.

A similar trend was observed when categorizing the samples by metastatic stage (Fig. 1, b). Interestingly, in non-invasive N0 samples, *ZFP36* level was significantly higher compared to the adjacent tissues. The same effect was observed in more invasive N1 samples, whereas in highly invasive N2 samples it was not found. Moreover, the *ZFP36* expression did not significantly differ between tumors of varying invasiveness, similarly to tumors of different histological types. All tumors (G1–G4) showed significant differences in the *ZFP36* expression levels compared to the adjacent tissues, with the greatest difference observed in G2 tumors (Fig. 1, c). Interestingly, *ZFP36* levels in T2-type tumors were more variable than in other samples, and most of these samples belonged to G2-stage tumors (43 out of 48). G2 stage is considered as an intermediate stage between differentiated (G1) and poorly differentiated (G3) tumors [15]. Nottingham system used here for the tumor grading in part reflects the EMT (epithelial-mesenchymal transition) since this process significantly affects cellular architecture, which means G2 tumors are to some extent undergoing EMT [16]. Given this, we hypothesize that such a variability in the *ZFP36* expression level might be due to some pathophysiological processes possibly related to EMT, and might reflect a peak in dysregulated cellular metabolism during malignant transformation. Although based on our data we cannot say whether the observed phenotype indeed correlates with EMT in every particular specimen, we suggest it might be of high practical use to conduct more research on the relationship between the *ZFP36* expression, EMT and tumor grading.

Since our previous study revealed that the *ZFP36* expression is significantly elevated in HER2-enriched tumor samples compared to other tumor types, we analyzed the *ZFP36* expression levels in samples with and without HER2 receptor expression, as well as based on HER2 amplification levels (Fig. 1, f, g). The analysis showed that the *ZFP36* expression was significantly lower in HER2-negative samples compared to HER2-positive ones. Among HER2-positive tumors, those with normal HER2 expression (HER2+) differed significantly from those with high levels (HER2+++), but not from tumors with moderately elevated HER2 levels (HER2++). Interestingly, there was no difference in *ZFP36* between ER positive and ER negative tumors (data not shown).

The presence or absence of steroid hormone receptors can affect transcriptional landscapes and subsequent cellular processes, potentially

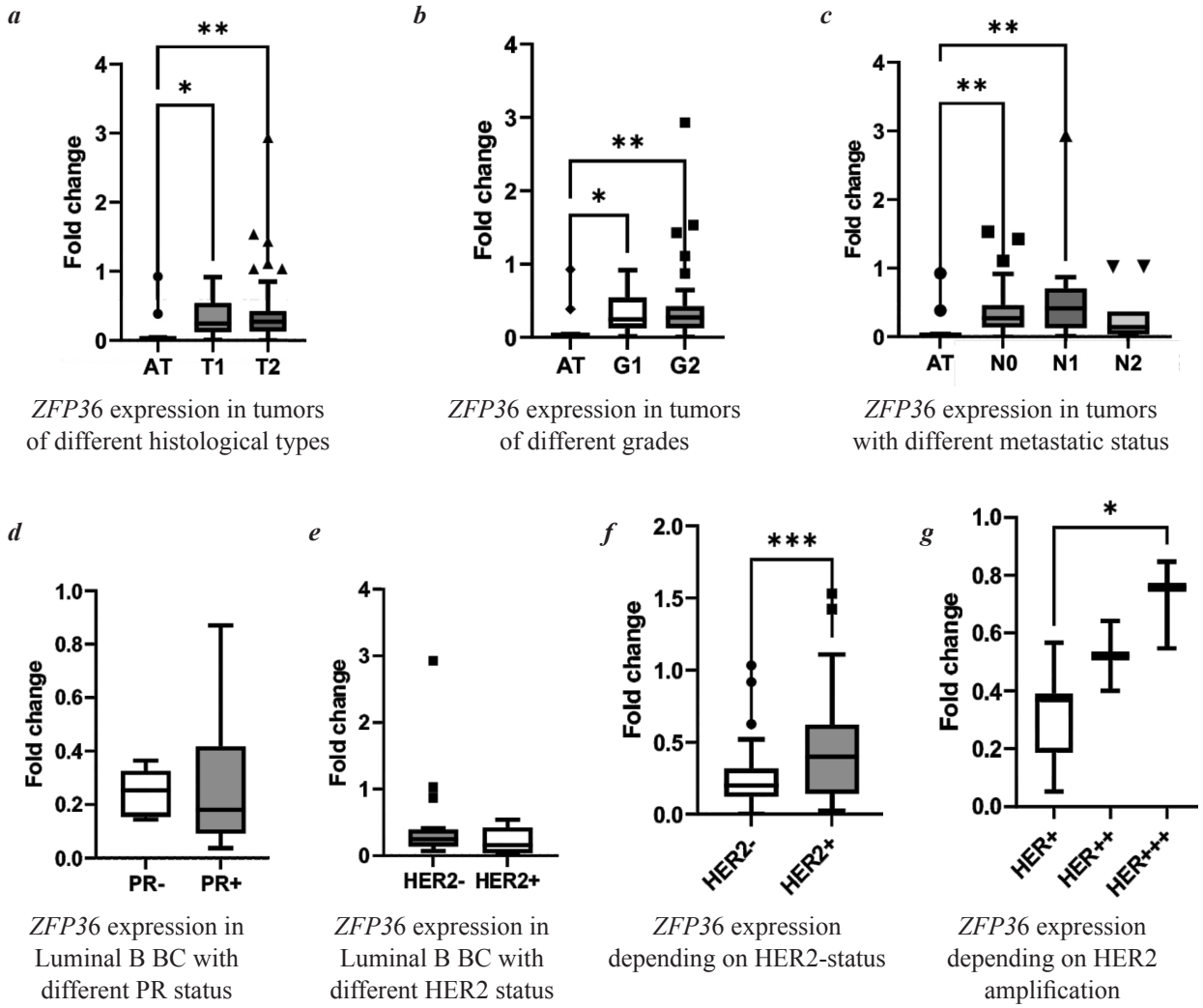

Fig. 1. *ZFP36* expression levels in tumors of different characteristics. *a, b, c* — expression levels in tumors of different histological types, grades and proximal metastasis status, respectively; *d* — expression levels in luminal B tumors with different PR status; *e* — expression levels in luminal B tumors with different HER2 status; *f* — expression levels in all tumor samples with present or absent HER2 expression; *g* — expression levels in HER2-enriched tumors depending on HER2 amplification level. All data represent median and CI 95% with Tukey's correction. T (tumor) — histological type of tumor, G — tumor grade, N (nodulus) — metastasis to lymph nodes. PR — progesterone receptor, HER2 – human EGFR receptor 2. * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$.

affecting biomarker levels. In this study, the luminal B subtype was represented by samples with and without HER2 receptor expression, as well as with and without progesterone receptor (PR) expression. We further analyzed whether the *ZFP36* expression varied based on the pre-

sence or absence of these receptors in luminal B tumors (Fig. 1 d, e). As shown in the figure, no significant changes in the *ZFP36* expression were detected in luminal B tumors depending on HER2 or PR status. Given that we observed a correlation between the *ZFP36* expression and HER2 status across all tumor types, we suggest that luminal B tumors might exhibit specific pathophysiological processes that differ from tumors of other types, which results in observed phenotypes, via distinct mechanism of the *ZFP36* expression regulation. For example, unlike triple-negative and HER2-enriched subtypes, both luminal subtypes are characterized with high incidence of non-silent mutations in *GATA3* gene, which encodes a transcriptional factor GATA3, important for the maintenance of luminal identity and is downregulated in luminal A and B tumors. This might potentially affect also the *ZFP36* expression [17, 18]. Nevertheless, more research should be conducted to investigate the mechanisms of changes observed in current study.

It is known from the literature that binding of HER2 to a ligand leads to the activation of multiple signaling cascades, one of which is the activation of NF-κB [19]. NF-κB is also known to activate the *ZFP36* expression in lipopolysaccharide-activated macrophages by directly binding to its promoter [20]. Given this, we hypothesize that an increased HER2 activity (whether amplification or increased receptor activity) could lead to an NF-κB-dependent increase in the *ZFP36* expression, and could not only explain our results, but also contribute to further study on the possibility of using a high level of the *ZFP36* expression as a potential marker of the HER2-enriched subtype of BC.

Conclusion

Our data show that the expression level of *ZFP36* is elevated in BC samples of T1 and T2 types, as well as in N0 and N1 types compared to the adjacent tissues. It also positively correlates with HER2 levels: the higher the expression HER2, the higher the expression of *ZFP36*, indicating a certain relationship between HER2-dependent signaling and the *ZFP36* expression, which was shown in our study for the first time. However, in luminal B breast cancer there was no significant difference between HER2+ and HER2– samples. Moreover, the *ZFP36* level did not correlate with PR status. Therefore, we consider high *ZFP36* as a promising prognostic marker for the HER2-enriched breast tumors. However, further studies utilizing larger sample sizes are necessary to verify this consideration.

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Профілі експресії *ZFP36* в пухлинах раку молочної залози різних стадій та статусу гормональних рецепторів

А.О. Губєрнаторова, Л.А. Сивак , Н.О. Верьовкіна, С.В. Кропивко

ZFP36 — ген, експресія якого знижується в злоякісних пухлинах різних типів, а рівні його експресії часто розглядаються як потенційні прогностичні та діагностичні маркери. Мета: у поточному дослідженні ми дослідили, як експресія *ZFP36* змінюється в пухлинах раку молочної залози різних гістологічних типів, ступенів, метастатичного статусу, а також у пухлинах з різним статусом прогестерону (PR) та рецептора 2 епідермального фактора росту (HER2), щоб оцінити його використання як потенційного біомаркера для раку молочної залози людини. Методи: RT-qPCR. Результати: нами виявлено, що експресія *ZFP36* підвищена в пухлинах типів T1–2 і N0–N1 порівняно з прилеглими тканинами та пухлинами типів T3–4 і N2 відповідно (за класифікацією TNM). Ми також показали, що експресія *ZFP36* не змінюється у зразках з різним статусом PR, але значно підвищується з ампліфікацією HER2. Висновки: ми вважаємо, що *ZFP36* є багатообіцяючим кандидатом для біомаркера HER2-збагаченого раку молочної залози, проте для верифікації необхідні подальші дослідження із більшою вибіркою.

Ключові слова: експресія генів, канцерогенез, рак молочної залози, TTP/

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