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Expression of *ITSN2* and *TKS5* in different subtypes of breast cancer tumors

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Aim. Regardless a great progress in treatment, 15 % of breast cancer cases remain lethal. One of the main problems in diagnosis and cure of this cancer type is its high clinical and genetic heterogeneity, and the identification of markers for personalized treatment is still a topical issue.

Methods. Collection and characterization of clinical material, RNA isolation and analysis of the *ITSN2* and *TKS5* isoforms expression using real-time quantitative PCR with fluorescence labeled probes. **Results.** The reduced expression of *ITSN2-S* has been found in the HER2/neu-positive tumors with poor prognosis. There was no significant difference in the expression of *ITSN2-L* and *TKS5-L* in the analyzed samples. **Conclusions.** Our study has shown a potential usage of the *ITSN2* short isoform (*ITSN2-S*) as a breast cancer prognostic marker.

Key words: breast cancer, *ITSN2*, *TKS5*, mRNA expression analysis.

Introduction

Mammary gland malignant tumor is one of the most widespread among female cancer patients. According to the data of the GLOBOCAN project of the International Center for Cancer Research, 6.23 million of new breast cancer cases (36 % of all tumors in women) were discovered in the world over five years (from 2008 to 2012), and 15 % of these cases were lethal. Despite many years of

research and extensive experience in the treatment of this cancer type, one of the major problems in diagnosis and cure is its high clinical and genetic heterogeneity.

To date, from the clinical and therapeutic point of view, and taking into account the specificity of the marker genes expression, this group of diseases is divided into 4 main types, according to the expression of estrogen receptors (ER), progesterone (PR), and forms of the epidermal growth factor receptor (HER2/neu):

Table 1. Breast cancer classification: histology, molecular markers and survival prognosis [2–9]

Types	Subtypes	Additional gene markers		Prognosis
Luminal A ER ⁺ PR ⁺ HER2 ⁻ /neu ⁻ ≈40–60 %*		Low Ki-67	CK8/18 ⁺ , FOXA1 ⁺ , ESR1, GATA3, KRT8, KRT18, XBP1, FOXA1, TFF3, CCND1, LIV1, CK5/6 ⁻ , EGFR ⁻	Favorable
Luminal B ER ⁺ PR ⁺ HER2 ⁻ /neu ^{+/-} ≈6–20 %*	Luminal B HER2⁻ ER ⁺ PR ⁺ HER2 ⁻ /neu ⁻ ≈15–20 %*	High Ki-67	ESR1, GATA3, KRT8, KRT18, XBP1, FOXA1, TFF3, SQLE, LAPTM4B, CK5/6 ⁻ , EGFR ⁻ , TP53 ⁻	Intermediate
	Luminal B HER2⁺ ER ⁺ PR ⁺ HER2 ⁻ /neu ⁺ ≈6 %*			Poor/ Intermediate
HER2 enriched ER ⁻ PR ⁻ HER2 ⁺ /neu ⁺ ≈10–20 %*			CK5/6 ⁺ , GRB7 ⁺ , ERBB2, EGFR ^{+/-} TP53 ⁻	Poor
Triple negative ER ⁻ PR ⁻ HER2 ⁻ /neu ⁻ ≈5–25 %*	Adenoid cystic carcinoma ≈1 %*	MYB-NFIB gene fusion	EGFR ⁺ , CK5/6 ⁺ , CK14 ⁺ , CK17 ⁺ HER1 ⁺ , Cyclin E ⁺ , CDKN2A ⁺ , KRT5, CDH3, ID4, FABP7, KRT17, TRIM29, LAMC2, ITGB4	Favorable
	Medullar carcinoma ≈2 %*			Favorable
	BRCA1-associated	ID4 ⁺		Poor
	Basal ≈10–25 %*	BRCA1 ⁻ , TP53 ⁻ , CDKN2A ⁺ , RB1, FGFR2, stem cell markers ⁺ , CK5/6 ⁺ , EGFR ⁺		Poor
	With low claudin ≈7–14 %*	GATA3 regulators ⁻ , cell adhesion genes, CDH1 ⁻ , claudin ⁻ , CK5/6 ^{+/-} , EGFR ^{+/-} CD44 ⁺ , SNAI3 ⁺		Poor
	Metaplastic carcinoma ≈1 %*	GATA3 regulators ⁻ , cell adhesion genes, PIK3CA ⁻ , AKT ⁻ or KRAS ⁻ , EMT ⁺ , stem cell markers ⁺		Intermediate
	Apocrine carcinoma			Intermediate
	Interferon enriched ≈10 %*	STAT1 ⁺ , SP110 ⁺ , interferon-regulating genes ⁺		Intermediate

* — percentage according to [2–9].

luminal A, luminal B, HER2-enriched and triple negative (ER-PR-HER2/neu⁻) [1].

Table 1 summarizes the classification of breast cancer types and subtypes, depending on the molecular markers expression discovered in recent years [2–9].

The definition of tumor aggressiveness and the choice of strategy for the further patients treatment are based on this classification. As can be seen from Table 1, even within the same type there is a certain tumor heterogeneity with its own prognosis and sensitivity to a particular treatment. Thus, for example, the triple negative type has at least eight subtypes according to the latest research; among them three with poor prognosis, three — with intermediate, and two — with favorable [4, 7]. Similarly, the tumors of luminal B type are divided into two subtypes: luminal B HER2/neu⁺ and luminal B HER2/neu⁻, which have their own treatment specificity. Each of the subtypes, besides three to four main markers, has a set of markers that more specifically characterize individual tumors and are useful for the personalized choice of treatment strategy, but it is still a generalization of data from a large number of patients. In practice, almost every single tumor has a specific expression pattern of the markers listed in Table 1. Thus, the idea of personalized treatment of each case, depending on the individual markers becomes more and more actual. As stated above, about 15 % of breast cancer cases are lethal, including those of incorrectly chosen treatment strategy. Significant progress has been made over the past few years thanks to the development of gene expression profiling technologies. Tens of new marker genes have been identified to characterize breast tumors. Thus, in experiments on gene expression anal-

ysis using microchips, it has been found that the *ITSN2*, *CXCL9* and *GNAI2* genes are among the few, the expression levels of which are significantly different in breast cancer tumor samples from patients with recurrences and in patients remained healthy after tumor removal and CMF (cyclophosphamide, methotrexate and fluorouracil) chemotherapy. The high *ITSN2*, *CXCL9* and *GNAI2* expression levels correlated with the absence of distant metastases for a long period of time. Besides, it has been found that the expression ratio of only two genes, *CXCL9/ITSN2* may be an independent prognostic factor to predict the absence of recurrences [10].

ITSN2 is an adaptor/scaffold protein [11], which is involved in many cellular functions, including clathrin-mediated endocytosis [12], actin cytoskeleton reorganization [13], cell signaling, and others [14]. Two main *ITSN2* isoforms are expressed in tissues: short (*ITSN2-S*) and long (*ITSN2-L*), the latter has three additional domains. Recently, it has been shown that *ITSN2* interacts with WIP, the marker protein of the invadopodia [15, 16] invasive cancer cells structures responsible for their mobility and metastasis [17].

The TKS family is another recently discovered family of scaffold proteins, which includes TKS4 and TKS5 that play a key role in the invadopodia formation [18, 19]. Likewise *ITSN2*, TKS4 and TKS5 are characterized by multiple alternative splicing. The *TKS5* gene has three additional promoters which give rise to three short isoforms without PX domain that is responsible for membrane binding. The elevated mRNA level of the long *TKS5* isoform (*TKS5-L*) containing all the domains, together with the reduced mRNA level of short isoform,

correlates with the progression of metastasis and poor prognosis for lung adenocarcinoma patients [20].

These data indicate a high potential of the *ITSN2* and *TKS5* genes and their isoforms as cancer prognostic markers, particularly for breast cancer. Therefore, we decided to investigate the mRNA expression levels of the *ITSN2* short and long isoforms and the *TKS5* long isoform in the breast tumor samples using quantitative real-time PCR to evaluate the possibility of their use as prognostic markers for the breast cancer.

Materials and Methods

Total RNA isolation from breast tumor samples. Breast tumor samples were obtained from the National Cancer Institute, frozen in liquid nitrogen immediately after surgery and stored at -80°C . Total RNA was isolated from 0.2–0.9 g tissue by guanidinium isothiocyanate method using the innuSOLV reagent (Analytik Jena) in accordance with the manufacturer's recommendations. Clinical information on the obtained samples is presented in Table 2.

cDNA synthesis. 5 μg of total RNA were pre-treated with DNase I (Fermentas), according to the manufacturer's recommendations, to remove residues of genomic DNA. After that, cDNA synthesis was performed in 20 μl according to the manufacturer's recommendations. The cDNAs were stored at -20°C .

PCR 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 Taq DNA polymerase (Fermentas) and the corresponding buffer. Amplification was performed under the fol-

lowing conditions: denaturation — $+94^{\circ}\text{C}$, 15 s (in the first cycle — 2 min); the time and temperature of the primers reassociation and synthesis were combined: $+60^{\circ}\text{C}$ 1 min, for 50 cycles. Each sample was analyzed in triplicate. PCR was performed on iQ5 BioRad. The *TBP* gene was selected as a reference based on the analysis of literature sources that showed its appropriateness as a control for the genes expression analysis in breast cancer [21–23]. Primers and probes used for PCR: (For. *TBP* 634-654 5'gtgccgaaacgccgaatata3',

Table 2. Clinical information of tumor samples included in the study

Histological type of tumor	Sample number
Invasive lobular carcinoma	44
Invasive tubular carcinoma	4
Invasive tubulolobular carcinoma	8
Invasive undifferentiated carcinoma	1
TNM classification (tumor, nodus and metastasis)	
T1	17
T2	40
N0	40
N1	9
N2	8
M0	56
M1	1
Differentiation level (G)	
G1	13
G2	35
G3	8
G4	1
Receptor status	
ER ⁺ PR ⁺ HER2/neu ⁻	31
ER ⁻ PR ⁻ HER2/neu ⁻	11
ER ⁺ PR ⁺ HER2/neu ⁺	8
ER ⁻ PR ⁻ HER2/neu ⁺	7

Taq-Man probe *TBP* 655-676 5' (FAM) atcccaagecggg (BHQ1)ttgctgcggg3', Rev.*TBP* 708-688 5'ccgtggttcgtggctctctta3'); (For. *ITSN2-S* 3798-3821 5'cgtaagatgacgacagactcaga3', Taq-Man probe *ITSN2-S* 3820-3844 5'(FAM)cagcacaccact (BHQ1)ggtgacttggatc3', Rev.*ITSN2-S* 3866-3845 5'attgtgtccagggtttgcagat3'); For.*ITSN2-L* 4324-4345 5'cgc-taccactgctcatcagaa3', Taq-Man probe *ITSN2-L* 4362-4385 5'(FAM)cccggagagccat (BHQ1)gcagaccattc3', Rev.*ITSN2-L* 4382-4403 5'agggccagctttagggaggaaat3'; *TKS5* For. *TKS5-L* 239-262 5'tgactccacctcccagactatcta3', Taq-Man probe *TKS5-L* 354-379 5' (BHQ2)atcccctctcccaggcaagatcct(ROX)3', Rev. *TKS5-L* 407-431 5'gatgggcttcagctcttcacagct3'. The primers nucleotide positions correspond to the *TBP* cDNA with the GenBank accession number NM_006277, the human *ITSN2* long and short isoforms — NM_147152 and NM_001172085.1 respectively, *TKS5* — NM_014631.

Real time PCR results calculation. The following formula was used to calculate PCR results: $Exp = E_{target}^{-Ct(target)}/E_{ref}^{-Ct(ref)}$, where E_{target} — the PCR efficacy for the target gene, E_{ref} — the PCR efficacy for the reference gene, $Ct(target)$ — mean cycle value for the target gene, $Ct(ref)$ — mean cycle value for the reference gene. The PCR efficacy was determined using the "R" software. Fluorescence values in the interval between 10 and 35–40 PCR cycles were entered to the program, resulting in an indicator of the efficacy of each individual PCR reaction. The cycle values for the target and reference genes were determined as the point of intersection of the fluorescence curves with the threshold, above which the fluorescence value is considered significant. The

threshold level was set the same for all experiments at 100 RFU. Statistical processing of the PCR data was carried out with Statistica v.8.0 software using the ANOVA method with Fisher's criterion.

Results and Discussion

ITSN2 mRNA expression analysis in breast cancer samples by quantitative RT-PCR. Specht and co-workers [10] have demonstrated with quantitative RT-PCR that high *ITSN2* mRNA levels in the surgically removed breast tumor samples correlated with the absence of distant metastases in patients for a long period of time, but they did not verify possible variations in the mRNA expression of *ITSN2* isoforms.

We have conducted such analysis on the tumor samples of various stages obtained from the National Cancer Institute. Table 2 shows the tumor data of patients whose samples were analyzed in this study. These samples were divided into three groups. The first group included [the] tumors with favorable/intermediate prognosis that had the receptor status ER⁺PR⁺HER2/neu⁻, or luminal A type. The second group included the samples with triple negative status (ER⁻PR⁻HER2/neu⁻). This group was the most heterogeneous, however, the majority of tumors with this diagnosis have a poor survival prognosis (Table 1) [4]. The third group united the tumors of luminal B type and HER2/neu-enriched type, the receptor status of which was respectively ER^{+/-}PR^{+/-}HER2/neu⁺. Patients with these tumors had a poor survival prognosis.

The total *ITSN2-L* and *ITSN2-S* mRNA content in tumor samples with receptor status ER^{+/-}PR^{+/-}HER2/neu⁺ was significantly lower ($p = 0.043$) than in tumors with triple negative

receptor status, and counted 1.17–5.11 a.u. (median — 2.16 a.u.) against 1.62–7.7 a.u. (median — 2.89 a.u.). The expression level of two *ITSN2* isoforms in the ER⁺-PR⁺-HER2/neu⁺ tumors was also lower than in tumors of patients with a favorable prognosis ER⁺PR⁺HER2/neu⁻: 0.97–7.39 a.u. (median — 3.69 a.u.) (Fig. 1A).

Significant difference in the expression levels of the individual *ITSN2* isoforms in different analyzed tumor groups were detected only for *ITSN2-S*. The lowest *ITSN2-S* mRNA content was detected in HER2/neu-positive

tumors (0.38–2.0 a.u., median — 0.69 a.u.); the highest — in tumors with triple negative receptor status (0.57–5.17 a.u., median — 2.21 a.u.) (Fig. 1B). There were no differences in the *ITSN2-L* amount in the studied tumor groups (Fig. 1C). The *ITSN2-S*/*ITSN2-L* ratio reflects the overall picture of the study. The lowest ratio was observed in HER2/neu-positive tumors compared to tumors with triple negative receptor status (Fig. 1D).

We have also compared the levels of *ITSN2* isoforms mRNA expression in the tumors of patients younger or older than 55 years with

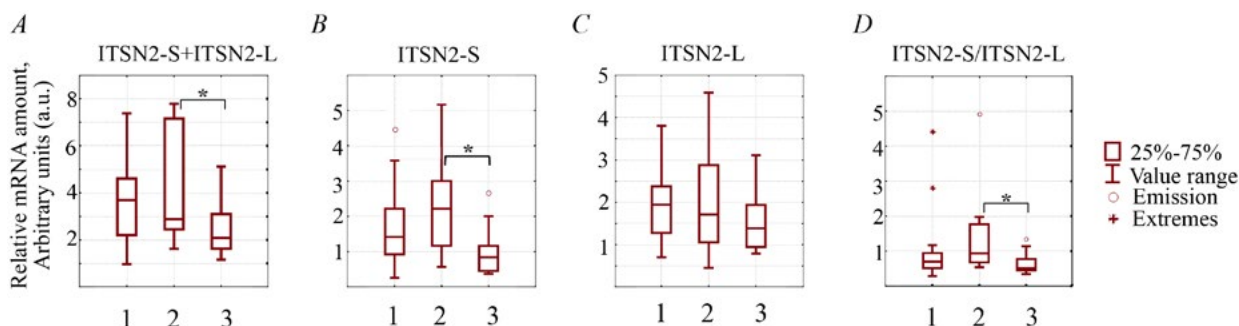


Fig. 1. The relative *ITSN2-S* and *ITSN2-L* mRNA content in breast tumor samples with different receptor status. 1 — ER⁺PR⁺HER2/neu⁻; 2 — ER⁻PR⁻HER2/neu⁻; 3 — ER⁺-PR⁺-HER2/neu⁺; A — *ITSN2-S* and *ITSN2-L* together; B — *ITSN2-S*; C — *ITSN2-L*; D — *ITSN2-S*/*ITSN2-L* ratio; * — $p < 0.05$.

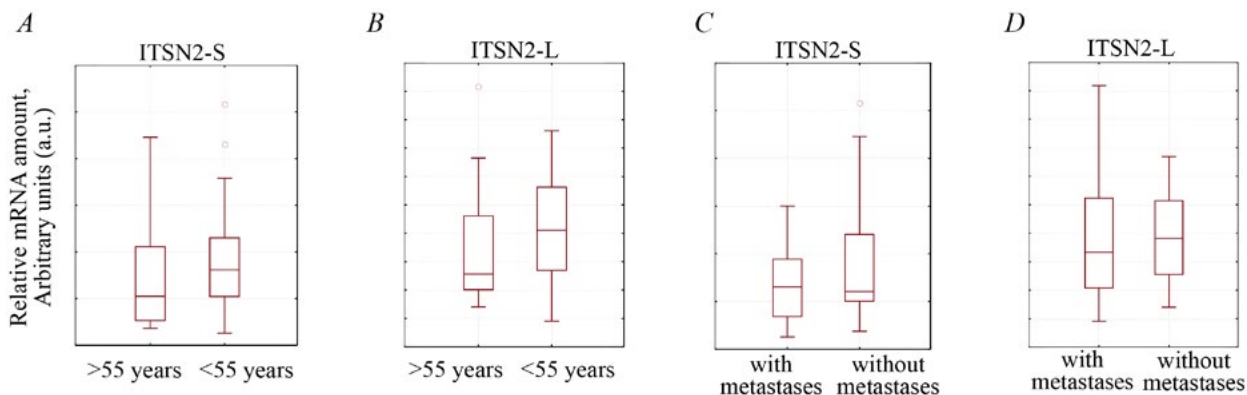


Fig. 2. The relative *ITSN2-S* (A, C) and *ITSN2-L* (B, D) mRNA content in breast tumor samples from patients of different age (A, B) with or without metastases in lymph nodes (C, D).

the presence or absence of metastases in the lymph nodes, which is also a criterion for tumor aggressiveness, but there was no correlation of the *ITSN2* isoforms mRNA content with the patient age or the presence of metastases in the lymph nodes (Fig. 2). We have also confirmed the *ITSN2*-S and *ITSN2*-L expression on the protein level (Fig. 3). The Western blot results reflect the general trend that every individual tumor has its own expression pattern.

Summarizing all data, we presume that the short *ITSN2* isoform can be considered as one of the potential prognostic markers for patients with breast cancer. Our results have demonstrated that it is the short *ITSN2* isoform that reduces its expression in tumor samples with

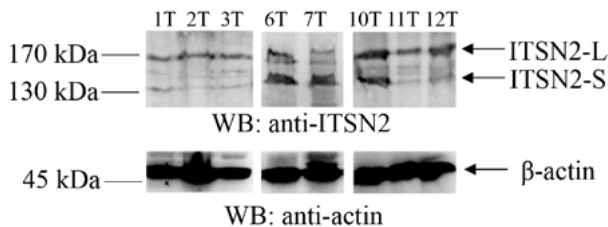


Fig. 3. Western blot analysis of *ITSN2* protein expression in breast tumor samples. Actin was used as a loading control.

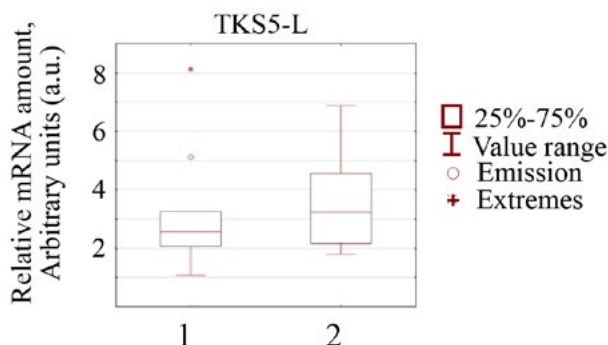


Fig. 4. The relative *TKS5*-L mRNA content in breast tumor samples. 1 — ER⁺PR⁺HER2/neu⁻; 2 — ER^{+/-}PR^{+/-}HER2/neu⁺.

poor prognosis, which in turn leads to a decrease in total *ITSN2* levels as found by Specht and co-workers [10]. These results were expected, since it is the long *ITSN2* isoform that has the guanosine-metabolic activity and takes part in the actin cytoskeleton reorganization, which in turn is an important condition for cancer metastasis [14, 18].

TKS5 long isoform mRNA expression analysis in breast cancer samples by quantitative RT-PCR. *TKS5*, a member of the TKS family, is a necessary component for the formation of invasive cancer cell structures - invadopodia [18]. The mRNA of the *TKS5* gene undergoes a number of alternative splicing events that affect the structure of the final protein molecule. Expression of the longest of these isoforms (*TKS5*-L), which has a membrane phosphatidylinositol phosphates-binding domain, correlates with lung adenocarcinoma metastasis [20]. Therefore, we have chosen to analyze its expression in the breast tumor samples. To compare the *TKS5*-L mRNA expression, we have divided breast tumor samples into two groups: the first contained 10 tumor samples with ER⁺PR⁺HER2/neu⁻ receptor status and had a favorable prognosis; the second group contained 10 tumor samples with ER^{+/-}PR^{+/-}HER2/neu⁺ receptor status and had a poor prognosis. The real-time PCR results have shown that *TKS5*-L did not significantly alter its expression in breast cancer tumors either with favorable (ER⁺PR⁺HER2/neu⁻) or with unfavorable (ER^{+/-}PR^{+/-}HER2/neu⁺) prognosis (Fig. 4).

Conclusions

We have demonstrated a decrease in the *ITSN2* mRNA expression in breast tumor

samples with poor survival prognosis (ER^{+/-}PR^{+/-}HER2/neu⁺) which is due to a decrease of the short ITSN2 isoform (ITSN2-S) amount. Thus, the *ITSN2-S* mRNA expression can be used as a one of the prognostic markers for breast cancer. There were no significant differences in the *ITSN2-L* and *TKS5-L* expression in the analyzed tumor samples.

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Експресія *ITSN2* та *TKS5* у різних підтипах раку молочної залози

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Мета. Незважаючи на значний прогрес у лікуванні, 15 % випадків захворювання на рак молочної залози залишаються летальними. Однією з головних проблем у діагностиці та лікуванні цього типу раку є його висока клінічна та генетична гетерогенність, тому ідентифікація маркерів для персоналізованої терапії є ак-

туальною проблемою. **Методи.** Збір та характеристика клінічного матеріалу, ізоляція РНК та аналіз експресії ізоформ *ITSN2* та *TKS5*, використовуючи кількісну ПЛР в реальному часі з флуоресцентно міченими зондами. **Результати.** Нами було виявлено, що *ITSN2-S* знижує свою експресію в HER2/неу-позитивних пухлинах з поганим прогнозом. Не було відмічено істотної різниці в експресії *ITSN2-L* і *TKS5-L* в проаналізованих зразках. **Висновки.** В даній роботі продемонстровано потенційну можливість використання короткої ізоформи *ITSN2* (*ITSN2-S*) як прогностичного маркера раку молочної залози.

Ключові слова: рак молочної залози, *ITSN2*, *TKS5*, аналіз експресії мРНК.

Экспрессия *ITSN2* и *TKS5* в различных подтипах рака молочной железы

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Цель. Несмотря на значительный прогресс в лечении, 15 % случаев рака молочной железы остаются летальными. Несмотря на многолетние исследования, одной из главных проблем в диагностике и лечении этого типа рака является его высокая клиническая и генетическая гетерогенность, поэтому идентификация маркеров для персонализированной терапии является актуальной проблемой. **Методы.** Сбор и характеристика клинического материала, изоляция РНК и анализ экспрессии изоформ *ITSN2* и *TKS5*, используя количественную ПЦР в реальном времени с флуоресцентно мечеными зондами. **Результаты.** Нами было выявлено, что *ITSN2-S* снижает свою экспрессию в HER2/неу-позитивных опухолях с плохим прогнозом. Не было отмечено существенной разницы в экспрессии *ITSN2-L* и *TKS5-L* в проанализированных образцах. **Выводы.** В данной работе продемонстрировано потенциальную возможность использования короткой изоформы *ITSN2* (*ITSN2-S*) как прогностического маркера рака молочной железы.

Ключевые слова: рак молочной железы, *ITSN2*, *TKS5*, анализ экспрессии мРНК.

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