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RELATIONSHIP BETWEEN NF-KB, ER, PR, HER2/NEU, KI67, P53 EXPRESSION IN HUMAN BREAST CANCER

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***Aim*: The aim of the present study was to investigate expression patterns of transcription factor NF-κB (p50 and p65), ER, PR, Her2/neu, Ki-67 and p53 in tumor tissue of patients with breast cancer (BC) and analyze correlation between these markers. *Patients and Methods*: 62 BC patients previously not treated with chemo- or radiotherapy were included in the study. All tumors belong to invasive ductal carcinoma of different grade. Expression of molecular markers was determined by immunohistochemical analysis on paraffin-embedded tissue sections. *Results*: The correlation between tumor grade and expression of ER, PR, Ki-67 and p53 was defined. NF-κB expression was found to be changed dependent on expression of ER, PR and p53 and also on molecular subtype (luminal, Her2-positive, hybrid, basal-like). The highest levels of NF-κB, Ki-67 and p53 were found in Her2/neu+ and basal-like tumor subtypes. *Conclusion*: The increase of nuclear expression of NF-κB correlates with a decrease of expression of steroid hormone receptors (ER and PR), increase of p53 accumulation, and is associated with Her2-positive and basal-like tumor types.**

***Key Words*: breast cancer, receptors of steroid hormones, Her2/neu, Ki-67, p53, NF-κB, molecular subtype.**

Breast cancer (BC) is by far the most frequent cancer in women, and the main cause of death in 35–55 years old women. Despite the improvement of diagnostic methods and chemotherapeutic regimens overall 5-year survival of patients significantly depends on the stage of disease and for the period 2000–2005 was 56.2% in Ukraine and 88.0% in the USA [1].

Such clinical characteristics as age, menstrual status, tumor size, lymph node status and morphological characteristics of the tumor (histological type, grade, lymphatic/vascular invasion) traditionally are the most important prognostic factors. However, in the last decades understanding of tumor nature has been greatly improved by molecular biology researches that allowed application of tumor's molecular features for prognosis of the disease course. Most molecular markers that are studied today, determine the ability of cells to malignant growth [2]. Estrogen and progesterone receptors (ER, PR) were the first predictive molecular markers for BC. Patients with positive status of these steroid hormone receptors generally have high sensitivity to hormone therapy. Next marker that was included in clinical practice is HER2/neu, which positive status in patients with BC correlates with high sensitivity to targeted therapy with trastuzumab. The use of these markers in clinical practice contributed to individualization of treatment and choice of adequate chemotherapeutic schemes for patients. However, 25–50% of ER and PR positive tumors are resistant to hormone therapy [3], and tumors with Her2/neu overexpression not always respond to trastuzumab therapy. There are also about 20% of patients with BC negative by all three markers mentioned above, and these tumors are more resistant to traditional therapy schemes.

Transcription factors may be considered as promising therapeutical targets because they affect the transcription of oncogenes that could play an important role in the formation of chemo-and radioresistance. One of the transcription factors dysfunction of which often occurs in malignant tumors, is a nuclear transcription factor NF-κB. NF-κB family is composed of polypeptides c-rel, p50, p52, p65 (RelA), p68 (RelB), p100 and p105, that belong to the rel-family proteins homologous to oncogene v-rel. The best studied of them are proteins p50 and p65, which form the classic NF-kappaB heterodimer (p50/p65). In most cells, NF-κB is present as a latent, inactive, IkB-bound complex in the cytoplasm. Under external stimuli, I-κB proteins are degraded via the ubiquitin-proteasome pathway, leading to release of the active form of NF-κB that translocates to the nucleus where it regulates the expression of target genes. NF-κB was discovered as a protein that specifically binds to a sequence of positive regulator of immunoglobulin's κ light chain gene [4–6].

Further studies have established the crucial role of NF-κB in the formation of immune response as well in processes of tumor development and growth. In many tumors (including BC, colon cancer, prostate cancer, lymphoid tumors, and probably many others) NF-kB is constitutively active and located in cancer cell nucleus [7]. NF-κB activation is affected by a variety of inducers such as TNFα [8, 9], LPS [4, 6], PMA [4], interleukin-1 [10] and 2 [11], many viruses [12, 13], hydrogen peroxide [14], ionizing radiation [15, 16] and others. The time of NF-κB activation varies from 10 min for TNFα [4], up to 6 h for PMA [17] and is highly dependent on cell type. For re-inactivation NF-κB requires synthesis of I-κB *de novo* that takes some time, so the action of activated NF-κB can last up to 8 h [4]. One of the most important effects of NF-κB activation is the blockade

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Abbreviations used: BC – breast cancer; ER – estrogen receptor; HIF-1 – hypoxia inducible factor-1; NF-κB – nuclear factor kappa B; PR – progesterone receptor; TNFα – tumor necrosis factor alpha.

of apoptosis. Classic inducer of NF-κB activation is a tumor necrosis factor (TNFα). TNFα may cause two opposite effects: on the one hand, it activates cytotoxicity mechanisms that lead to cell death, on the other hand, indirectly activates the transcription of genes which products can block apoptosis. NF-κB activation plays a key role in prevention of TNF-induced apoptosis. TNFα is not the only agent capable to induce apoptosis and activation of NF-κB. Similar processes can be induced by a majority of NF-κB activators. It should be noted that the effect of NF-κB on apoptosis is highly dependent on many factors such as the type of inducer, cell type and functional status of the cell at a given time, the presence of external influences on the cell and many others [18]. In many malignancies constitutive level of NF-κB [19–21] is increased, at the same time, dysregulation of NF-κB in initially normal cell may lead to its malignant transformation [22, 23]. These and other data suggest an important role of NF-κB in cell proliferation and maintaining the tumor cell viability. This role has at least two important aspects. The first is that some oncogenes (c-myc), oncoproteins (p53) and oncoviruses are under the influence of NF-κB, or activate it during malignant transformation (eg, ras). Another important point is the influence of NF-κB on tumor sensitivity to chemotherapy and immune reactions [24]. Action of many anticancer drugs, as well as most defense reactions is to induce apoptosis in cancer cells [25, 26]. At the same time, the activation of NF-κB by these apoptosis inducers (TNFα, ionizing radiation, etc.) can protect cells from death. Montagut et al. [27] have shown that activation of NF-κB was significantly correlated with resistance to chemotherapy in BC patients. Furthermore, in some cases NF-κB activation increased after chemotherapy exposure, which could cause the formation of drug resistance [27].

Also, correlation of NF-κB expression with other markers has been studied. The need for such studies is that the effect exerted by NF-κB on cell depends on the expression and functional status of many proteins, for example, very complex and varied interactions between NF-κB and p53, which can lead to opposite effects in different systems. One of the most important functions of p53 is induction of apoptosis in response to some signals, such as viral infection or DNA damage. Due to these properties p53 is considered a tumor suppressor what is shown in different models [28, 29]. Many studies also have shown that both p53 and NF-kB inhibit each other's ability to stimulate gene expression and that this process is controlled by the relative levels of each transcription factor. Expression of either wild-type p53 or NF-kB suppresses stimulation of transcription by the other factor. However, mutations in the p53 gene lead to loss of its ability to regulate the transcription and activity of the targets (including NF-kB), which causes the loss of apoptotic properties. So, it is important to compare NF-κB expression with expression of other markers that may have a significant effect on the functional activity of NF-κB. There are also many contradictions in the results of studies of NF-κB expression due to the absence of a standard method of determining the NF-κB activation, and the differences in interpretation of results obtained by the same method. However, in BC patients most results suggest that NF-κB is activated mainly in ER-negative and Her2/neu-positive tumors [27, 30–32]. These tumors usually possess high proliferative index, are of high grade and drug resistance. These data allow to propose that the activity of NF-κB may affect the drug sensitivity of tumors, and that the possibility of regulation the NF-κB activity open a new features in therapy of tumors [33].

MATERIALS AND METHODS

62 patients with BC previously not treated with chemo- or radiotherapy and cured in Ivano-Frankivsk Regional Oncology Dispensary (Ivano-Frankivsk, Ukraine) were included in the study. All tumors belong to invasive ductal carcinoma of different grade. Clinical data of patients and tumor characteristics are shown in Table 1.

Table 1. Clinical data of patients and morphological characteristics of tumors

Parameter	Number of cases (%)
Stage	
I	4 (6.4)
II	40 (64.5)
III	7 (11.3)
IV	2 (3.2)
«X»	9 (14.5)
Axillary nodal status	
+	33 (53.2
-	29 (46.8
Tumor grade	
1	8 (12.9)
2	32 (51.6)
3	16 (25.8)
"X"	6 (9.7)

Expression of molecular markers was determined by immunohistochemical approach on formalin-fixed paraffin-embedded 4 μm tissue sections. Antigen retrieval was performed at temperature 98 °C for 30 min, endogenous peroxidase was blocked with 0.03% hydrogen peroxide for 5 min. Slides were then washed with Tris-buffered saline solution at pH 7.6 and incubated for 30 min with the following primary antibodies: Estrogen Receptor α (Monoclonal Rabbit Anti-Human, RTU, clone SP1, Dako, Denmark), Progesterone Receptor (Monoclonal Mouse Anti-Human, RTU, clone PgR 636, Dako, Denmark), c-erbB2 (Polyclonal Rabbit Anti-Human, 1:1000, Dako, Denmark), NF-κB p50 (NLS) (Polyclonal Rabbit Anti-Human, 1:200, sc114, Santa Cruz Biotechnology, USA), p-NF-κB p65 (Ser 536) (Polyclonal Rabbit Anti-Human, sc-33020, Santa Cruz Biotechnology, USA), Ki-67 (Monoclonal Mouse Anti-Human, RTU, clone MIB-1, Dako, Denmark), p53 (Polyclonal Rabbit Anti-Human, 1:100, RP 106, Diagnostic BioSystems, USA), Cytokeratin 5/6 (Monoclonal Mouse Anti-Human, RTU, clone D5/16 B4, Dako, Denmark). After rinsing, slides were incubated with HRP from FLEX detection system (Dako, Denmark) for 20 min, treated with DAB for 3 min and counterstained with haematoxylin. Slides were washed in tap water, dehydrated, and mounted with glass coverslips.

For interpretation the reaction with antibodies against ER, PR, p50 and p65 the H-score method was used. The score is obtained by the formula:

H= 3 x % of strongly stained nuclei + 2 x % of moderately stained nuclei + % of weakly stained nuclei.

Expression level of marker with H>100 was classified as high, H=50-99 — moderate and H<50 — low.

Her2/neu expression level was determined by reaction with antibodies against HER2/neu. For interpretation the reaction such criteria were applied: 0 (negative): no staining is observed or membranous staining is observed in less than 10% of the tumor cells. 1+ (negative): A faint/barely perceptible staining is detected in more than 10% of the tumor cells. The cells are stained in part of their membrane. 2+ (equivocal): A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells. 3+ (positive): A strong complete membrane staining is observed in more than 30% of the tumor cells. Tumors with expression level 0 and 1+ considered HER2/neu-negative, and with 2+ and 3+ — positive.

Proliferation level was determined by reaction with antibodies against Ki-67: 0 — no nuclear staining is observed in tumor cells; 1 — nuclear staining is detected in 1–10% of the tumor cells; 2 — 11–20%; 3 — 21–50%; 4 — >50%. In tumors with level of Ki-67 expression "0", "1", "2" proliferation level was considered as low; and with "3" and "4" — high.

P53-status of tumors was determined by the level of accumulation in the nuclei of tumor cells. Negative (0) — nuclear staining is detected in less than 10% of the tumor cells; positive: 1 — 11–20; 2 — 21–50%; 3 — 51–75%; 4 — > 75%.

To check basal cell origin the expression of CK 5/6 in tumors with three-negative phenotype (ER-/PR-/Her2-) was determined. The tumors with cytoplasmic staining of more than 10% of tumor cells were considered as three-negative basal; otherwise — three-negative non-basal.

For statistical analysis of the data the SPSS program was used. To identify the correlation, the Pearson coefficient of correlation was defined, and to verify its validity χ^2 test was used.

RESULTS

In the 62 tumors of BC patients, expression levels of the following molecular markers were investigated: ER, PR, Her2/neu, Ki-67, p53 and NF-κB subunits (p50 and p65 proteins). 47 tumors (76%) were ER-positive (H > 10) and 15 (24%) — ER-negative (H⩽10). PR-status was positive (H>10) in 43 cases (68%), and negative — in 20 (32%). The Her2/neu overexpression was identified in 25 tumors (40%), other 37 patients had negative Her2/neu status. The study of NF-κB (p50 та p65) expression have shown high levels of p50 (H > 100) and p65 (H > 100) in tumors, respectively, in 39 (63%) and 7 (11%) patients. It should be noted that all tumors with high expression of p65 had high levels of p50.

Proliferative activity of tumors was high (Ki-67 > 20%) in 29 (47%) and low (Ki-67<20%) in 33 (53%) patients. p53 status in tumors was positive in 22 (35%) and negative in 40 (65%) patients.

According to molecular profile, tumors of BC patients were divided according to the classification of David J. Dabbs [34] (Table 2). Tumors with high expression of ER (H > 100) and negative Her2/neu status (0, 1+), regardless of PR expression were attributed to luminal A subtype. Tumors with negative HER2/neu status and low-intermediate expression of ER (H = 11–99) or negative ER status (H ⩽ 10) with positive PR expression (H > 10) were attributed to luminal B subtype. Basal-like subtype tumors had negative ER- (H⩽ 10), PR- (H ⩽ 10) and Her2/neu-status (0, 1+). To verify the basal origin, samples of triple-negative tumors were stained for cytokeratin 5/6. CK5/6+ tumors were attributed to three negative basal and CK5/6- — to triple-negative non-basal. Her2/neu+ subtype is characterized by negative ER/PR status and Her2/neu hyperexpression. The remaining tumors with positive status of ER, PR and Her2/neu were referred to the hybrid luminal A/Her2/neu or luminal B/Her2/neu subtype according to the level of ER/PR expression. Due to limited number of patients (n = 62), for statistical analysis such subtypes of tumors were included: luminal A and B — to luminal; triple-negative basal and non-basal — to basal-like; hybrid luminal A and B/Her2/neu+ — to hybrid luminal/Her2/neu+ subtype [35]. So, after integration, the distribution of tumors by subtypes was as follows: tumors of 25 patients (40%) belonged to luminal subtype, 12 (20%) — to basal-like, 7 (11%) — to Her2/neu+, 18 (29%) — to hybrid (luminal/Her2/neu+) subtypes (Table 3).

Table 2. Expression of molecular markers in BC

Experession of marker	Number of cases (%)
ER	
-	15 (24)
+	47 (76)
PR	
-	20 (32)
+	42 (68)
Her2/neu	
-	37 (60)
+	25 (40)
p65 expression	
Low	55 (89)
High	7 (11)
p50 expression	
Low	23 (37)
High	39 (63)
Ki-67 expression	
Low (< 20%)	33 (53)
High (> 20%)	29 (47)
p53	
-	40 (65)
+	22 (35)

The next relationships between clinical and morphological characteristics of tumors and molecular markers expression were found: 1) the direct correlation between tumor grade and proliferation index (Ki-67: r=1.0; p < 0.01); also, significantly increased accumulation of p53 was found in tumors with grade 2 and 3 compared to grade 1 tumors (Fig. 1); 2) the inverse correlation was between tumor grade and ER (significant correlation: r = -0.999; p < 0.05), PR (un-

Table 3. Molecular subtypes of breast cancer

Molecular subtype		Criteria used for proposed categories	Number of patients (%)	
Luminal [15]	Luminal A [14]	ER 3+; Her2 0, 1+	20 (32)	25 (40)
	Luminal B [14]	ER 1+, 2+; Her2 0, 1+; або ER 0; PR ⩾ 1+; Her2 0, 1+	5 (8)	
Basal-like [15]	Triple-negative basal [14]	ER (H<30); PR (H<30); Her2 0, 1+; CK5/6 +	8 (13)	12 (20)
	Triple-negative non-basal [14]2	ER (H<30); PR (H<30); Her2 0, 1+; CK5/6 -	4 (7)	
Her2/neu+ [14, 15]		ER (H<30); PR (H<30); Her2 2+, 3+	7 (11)	
Luminal-Her2/neu+ hybrid [15]	Luminal A-Her2/neu+ hybrid [14]	ER 3+; Her2 2+, 3+	12 (19)	18 (29)
	Luminal B-Her2/neu+ hybrid [14]	ER 1+, 2+; Her2 2+,3+; або ER 0; PR ⩾1+; Her2 2+,3+	6 (10)	

reliable correlation: r = -0.964; p = 0.086) (Fig. 1).

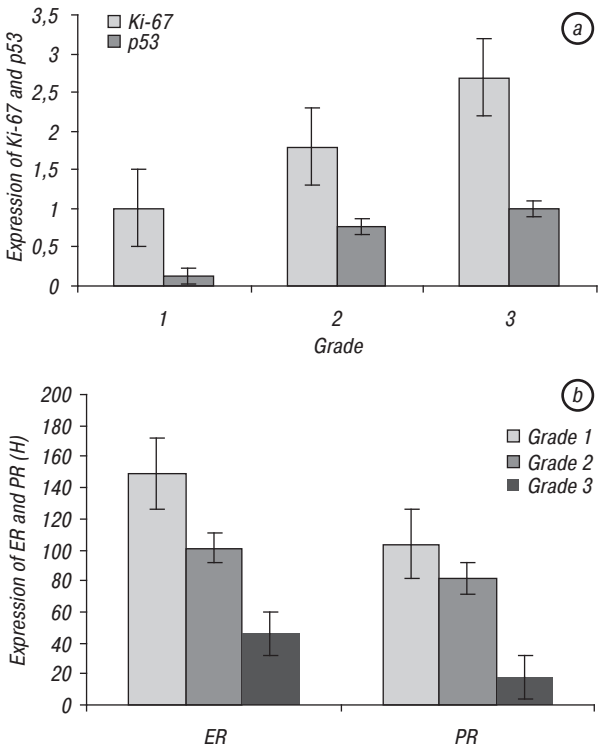

Fig. 1. Correlation between tumor grade and expression of Ki-67 and p53 (*a*), ER and PR (*b*)

No relationship between expression of studied markers and clinical characteristics (age, stage, axillary lymph node status) was found.

The inverse correlation between expression of steroid hormone receptors and: p50 expression (ER: p=-0.991, r<0.05; PR: p=-0.998, r<0.05); p53 accumulation (ER: p=-0.986, r=0.053; PR: p=-0.998, r<0.05) was found. The correlation between p65 and these markers was not significant.

The vast majority of tumors with high expression of p50 and p65 were found to be ER (5 of 7) and PR (6 of 7) negative, while the percentage of ER- and PR- tumors was respectively 24% and 32%. To study the relationship of markers expression and expression of NF-κB, we divided patients into 3 groups: I — with high levels of p50 and p65 nuclear expression; II — with high p50 and low p65 levels; III — with low levels of p50 and p65. The inverse correlation between expression of NF-κB and receptors of steroid hormones (ER: r=-1.0, p<0.01; PR: r=-0.999, p<0.05); and direct correlation between p53 accumulation and NF-κB expression (r=1.0, p<0.01) were found (Fig. 2).

Also the relation between NF-κB, p53, Ki67 expression and molecular profile of tumors was analyzed. Increase of NF-κB expression (p50: r=0.917, p<0.05; p65: r=0.974, p<0.05) in the direction of: Hybrid → Luminal → Basal-like → Her2/neu+ subtype was found (Fig. 3). These results are in contradiction to literature data [27, 30–32], according to which the lowest expression of NF-kB was observed in luminal subtype, but this could be explained by a small cohort studied.

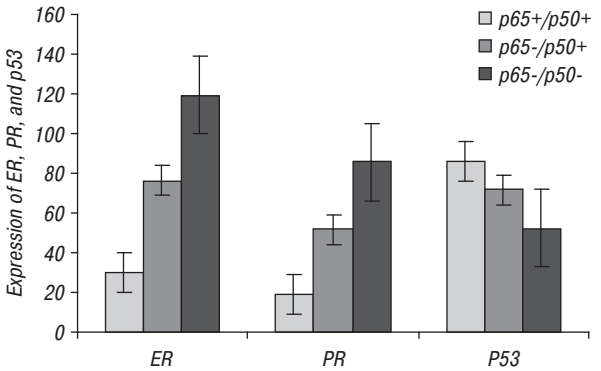

Fig. 2. Correlation between expression of NF-kB and ER, PR and p53 in BC

Ki-67 proliferation index and p53 accumulation increased in this direction: Luminal → Hybrid → Basal-like → Her2/neu+ subtype (Ki-67: r=0.928, p<0.05; p53: r=0.956, p<0.05) (Fig. 3).

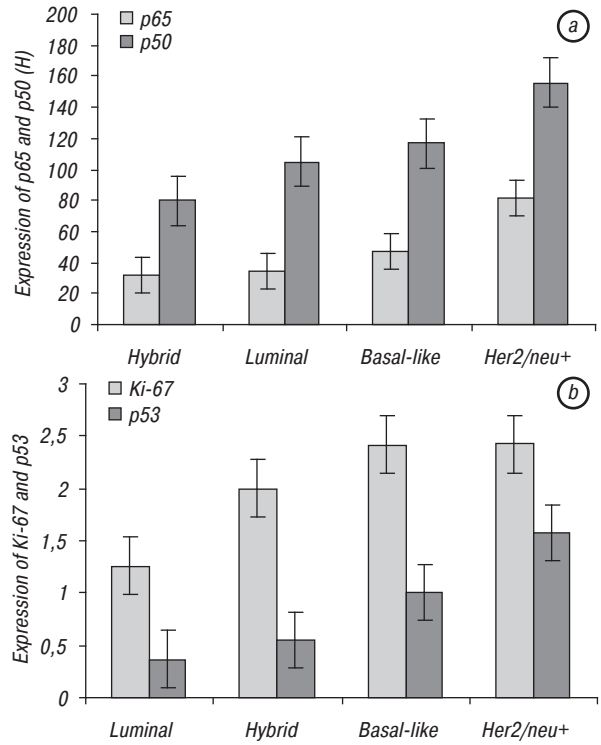

Fig. 3. Correlation between molecular subtype of BC and expression of p50 and p65 (*a*), and Ki67 and p53 (*b*).

So, summing up the results, we can say that NF-kB expression is correlated with expression of markers

(ER, PR, p53, molecular profile of tumors) that predict the poor BC prognosis.

DISCUSSION

In this study we have investigated the expression level of NF-κB subunits, and its relationship with clinical and morphological parameters and the expression of other molecular markers. Being initially described as a crucial element in the formation of immune response, presently NF-κB is considered also as a potential target for cancer treatment. The possibility of this application of NF-κB is based on the fact that this transcription factor can inhibit apoptosis, stimulate cell proliferation, promote drug and radioresistance of cells. However, the effects of NF-κB activation depend on many factors, including the expression of other proteins that can regulate its functional activity.

Another important point to consider in the study of NF-κB is that high expression of this factor is not always indicating its activation. This comes up from the fact that normally NF-κB is located in the cytoplasm in an inactive, associated with the I-κB state, and only after activation moves to the nucleus, where affects the transcription of target genes. In this work the expression level of NF-κB was determined by immunohistochemical method, which allows taking into account location of protein in the cell with a high probability to speak about his activation. Immunohistochemical study is also quite reliable method for detection the mutations in the p53 gene. According to the literature, positive p53 nuclear reaction in 90–100% of cases corresponds to missense-mutations in the p53 gene, which determines mutant immunophenotype [36]. The half-life of "wild type" p53 is up to 30 min due to rapid utilization in proteosome system, whereas mutant p53 has a lower affinity for proteins of this system, what increases its half-life to several hours, and concentration to a level that can be determined by immunohistochemistry.

Summarizing the results we can say that the higher tumor grade correlates with a decrease of ER and PR expression, increase of proliferation index (Ki-67) and p53 accumulation. As for relationship between expression of NF-κB and other molecular, clinical and morphological features of BC, several reported data are contradictory. According to our results, the highest levels of NF-κB, Ki-67 and p53 were found in Her2/neu+ and basal-like subtype of tumors that are associated with poor prognosis. The increase of nuclear expression of NF-κB correlates with a decrease of ER and PR expression and increase of p53 accumulation, that also worsens BC prognosis. These results are in agreement with literature data about the drug resistance of tumors with positive p65 status [37].

Increased NF-κB expression is associated with molecular and physiological changes that contribute to its activation. So, Her2/neu + and basal-like tumor subtypes generally have a solid growth pattern and are associated with an increased inflammatory response, which leads to increased levels of hypoxia. The normal cellular response to hypoxia is governed by two dimeric transcription factors, hypoxia inducible factor-1 (HIF-1) and NF-kB. One of the effects of hypoxia is the degradation of I-kB and NF-kB activation. NF-kB activates signaling pathways that promote cell survival and angiogenesis. Regulator of these processes is p53, which inhibits the activation of HIF-1 and NF-kB. Mutations in the p53 gene leads to the loss of their ability to regulate the activity of these factors that promote angiogenesis and tumor progression [38]. Using this logic, the inhibition of HIF-1 and/or NF-kB can inhibit the angiogenic activity of tumors, thereby curtailing their growth and metastases.

In conclusion, we have reveale correlation between expression levels of ER, PR, Ki-67, p53 and BC grade, correlation between expression profile of NF-κB and expression level of ER, PR and p53. The highest levels of NF-κB, Ki-67 and p53 were detected in Her2/neu+ and basal-like subtype of tumors. Our data allow to propose that activated NF-κB in BC may indicate poor prognosis and development of drug resistance, however, further research and retrospective analysis performed on larger number of patients is required.

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