The Expression and DNA-Binding Activity of NF-κB Nuclear Transcription Factor in the Tumors of Patients with Breast Cancer

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The content and DNA-binding activity of NF-κB nuclear transcription factor subunit p65 (NF-κBp65) were evaluated by quantitative enzyme immunoassay in tumors and histologically intact tissues of 119 patients with breast cancer. DNA-binding activity of NF-κBp65 in the tumors was higher than in adjacent tissue in 97% cases. This elevation was paralleled by an increase in total protein content in the majority of cases. No significant relationship of the parameter with the disease stage, tumor size and histology, and degree of lymph node involvement was detected. However, the content of NF-κBp65 in tumors of malignancy degree III was significantly higher than in tumors of malignancy degree II. An increase in total expression of NF-κBp65 protein was found in HER-2$^+$ tumors. This increase was not related to steroid hormone receptor status and was not paralleled by elevation of DNA-binding activity, which was maximum in tumors with the "triple negative" receptor status (RE$^-$RP$^-$HER-2$^-$).

Key Words: *NF-κB nuclear transcription factor; NF-κBp65; steroid hormone receptors; HER-2/neu; breast cancer*

Nuclear transcription factor NF-κB initially identified as a protein binding to specific decamer DNA sequence (GGG ACT TTC C) in the intron kappa-immunoglobulin light chain gene in mature B cells and plasma cells, plays an important role in cell proliferation, apoptosis, inflammatory and autoimmune reactions, as it regulates the expression of genes involved in these processes [6]. This factor (NF-κB) is a heterodimer complex of Rel family proteins, inert in the majority of silent cells, and located in the cytoplasm in complex with specific inhibitors (IκB). Five NF-κB family proteins were identified. They contain common DNA-binding domain NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (NF-κBp65), RelB, and c-Rel. In most cells, these proteins are present in the form p50/RelA(p65) heterodimer. NF-κB DNA-binding activity is stimulated in response to many exogenous stimuli and this process does not depend on *de novo* protein synthesis.

In the majority of normal cells, NF-κB/IκB complexes are located in the cytoplasm in transcription-inert state and are activated only in response to the appropriate regulatory stimulus perceived by the cell. The regulation of NF-κB signal pathway is impaired in many human tumors, including breast cancer (BC); NF-κB is permanently active in the majority of tumor cells and is located in the nucleus [1,4]. The significance of this factor is particularly high in estrogen receptor (ER)-negative breast tumors carrying epidermal growth factor or HER-2/neu receptors [5]. Hyperactivation of NF-κB is a cause of BC resistance to antiestrogens, drugs, and radiotherapy [2,3,8,10,13]. According to some data, evaluation of NF-κB activation in ER-positive BC helps to detect a subgroup of

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hormone-resistant patients [14,15]. One more clinically significant feature of NF-κB is its capacity to stimulate the formation of osteolytic BC metastases in bones [9]. It is expected that creation of antitumor agents blocking the NF-κB signal pathway will improve cell sensitivity to therapies used at present and may have a therapeutic significance of its own, including, among other things, prevention of BC metastases to the bones [4,12]. However, the majority of findings proving the key role of the NF-κB signal cascade in BC were obtained in experimental systems, while the data on the expression and activity of this factor in human tumors are scanty, obtained by different methods, and require further confirmation and development [1,7,11,15].

Here we compared the expression (total content) and DNA-binding activity of one of the key proteins of the NF-κB family, p65, in tumors and adjacent histologically intact tissues in BC patients and analyzed the relationships of these parameters with the receptor status of tumors and the main clinical morphological features of the disease.

MATERIALS AND METHODS

The study was carried out in 119 patients with BC aging 23-84 years (median 54 years); 37 patients were at reproductive age, 18 were in premenopausal, and 64 in menopause of different length. The distribution by disease stages was as follows: 37 patients with stage IIA, 28 with IIB, 20 with stage I, 20 with stage IIIA, and 14 with disseminated process of IIIB-IIIC stages.

The tumor histology was as follows: ductal infiltrative cancer in 95 cases, lobular infiltrative in 14, and solitary cases of other BC types. The majority of tumors (n=84) were of second degree malignancy, 28 of third, and 7 of first degree malignancy. ER, progesterone (PG), and HER-2/neu receptor status were evaluated by immunohistochemical methods in all tumors.

Specimens of tumor and histologically intact mammary tissue (200-500 mg) for analysis of NF-κBp65 were collected during surgery and stored at -70°C. The specimens were then fragmented (powdered) in liquid nitrogen and lyzed in 1:3 proportion in buffer of the following composition: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1 μg/ml leupeptine. The lysates were centrifugated at 20,000 rpm (4°C, 30 min; Optima TLX centrifuge, Beckman).

The summary content of NF-κBp65 in the resultant nucleoytoplasmatic tissue extracts was evaluated using NF-κBp65 (Total) standard direct EIA kits (Invitrogen) according to the instruction after preliminary 1:150 dilution. The content of NF-κBp65 was expressed in ng/mg total protein, measured by Lowry's method.

NF-κBp65 DNA-binding activity was measured using TransAM NF-κB p65 kits (Active Motif). The method consists in specific binding of stimulated NF-κBp65 to oligonucleotide (immobilized on a 96-well plate) containing the 5'-GGGACTTTCC-3' consensus sequence, after which standard direct enzyme immunoassay of bound NF-κBp65 is carried out with colorimetric detection at 450 nm. The calibration curve was plotted using DNA-binding activity of the nuclear extract of tetraphorbolacetate-stimulated Jurkat cells, which was taken for 100 units (U). The NF-κBp65 DNA-binding activity in analyzed samples was expressed in U/mg total protein.

Measurements were carried out on an EL_x800 automated universal reader for microplates (Biotek Instruments Inc.).

The data were processed using Statistica 7.0 software. The parameters and relationships between them were analyzed using nonparametric methods: Mann–Whitney, Kruskal–Wallis, and Spearman rank correlation test (R). The differences and correlations were considered significant at $p<0.05$.

RESULTS

Measurable levels of total NF-κBp65 protein (0.66-67.2; median 11.1 ng/mg protein) were detected in 118 (99%) tumor specimens and 96 (81%) specimens of histologically intact mammary tissue (0.05-42.50; median 2.9 ng/mg protein; Fig. 1, *a*). The content of NF-κBp65 in tumors was significantly higher than in adjacent tissue in 86% patients with BC ($p<0.0001$). Measurable DNA-binding activity of NF-κBp65 was detected in 99% patients in the tumor (1-711; median 193 U/mg protein) and in 81% patients in adjacent tissue (0.14-143; median 28.7 U/mg protein; Fig. 1, *b*). This parameter was significantly higher in the tumor compared to adjacent tissue in 97% cases ($p<0.0001$). The content and DNA-binding activity of NF-κBp65 in the tumor did not correlate with the corresponding values in intact mammary gland. On the other hand, a slight but significant positive correlation between total NF-κBp65 level and its DNA-binding activity was detected in the tumor and in normal tissue (R=0.31, $p<0.01$ and R=0.26, $p<0.05$, respectively). Hence, an increase of DNA-binding activity of NF-κBp65 was found in virtually all tumors of patients with BC. In the majority of cases, this increase was paralleled by an increase in the content of this transcription factor protein. However, the increase of NF-κBp65 activity was more pronounced than the increase in protein content. DNA-binding activity surpassed the upper 95% threshold of normal (118 U/mg protein) in 81% cases,

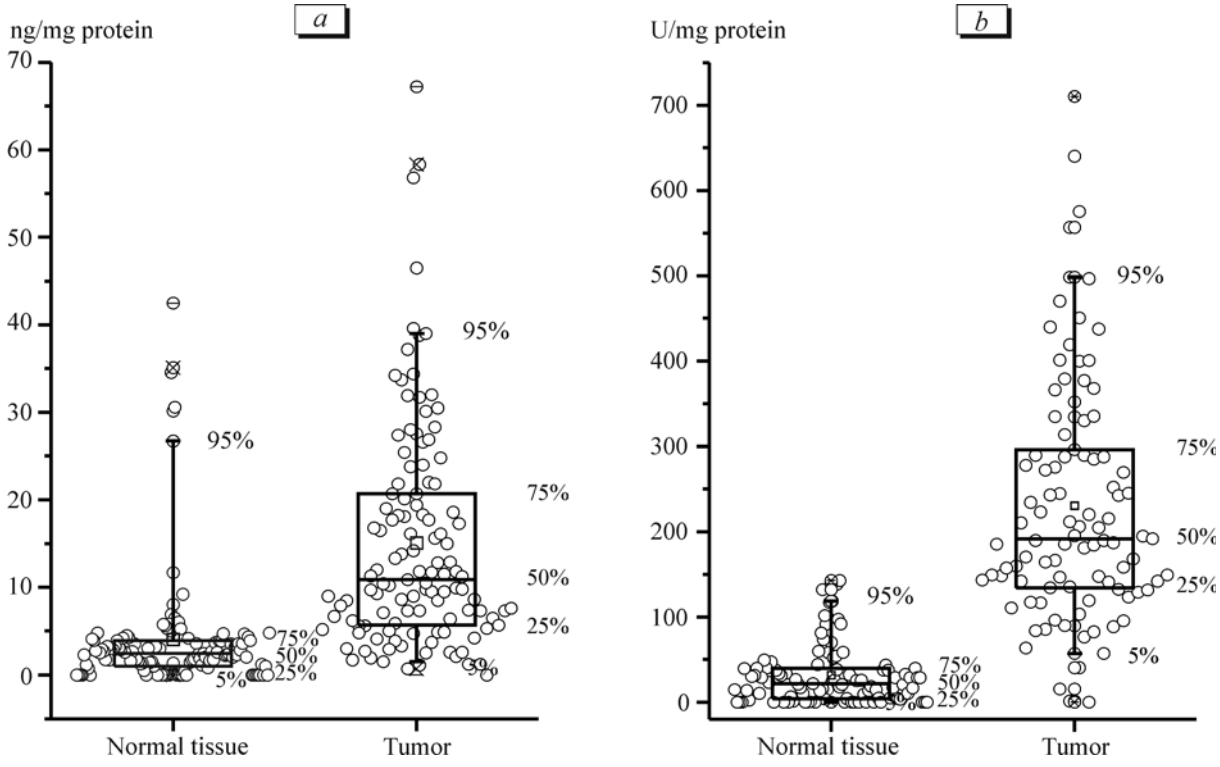

Fig. 1. Content (*a*) and DNA-binding activity (*b*) of NF-κBp65 in nucleocytoplasmic extracts of tumors and adjacent histologically intact (normal) tissues from 119 patients with BC

while protein content surpassed the threshold value (95% threshold normal value 26.7 ng/mg protein) in just 18% cases. This result is in line with the concept on independent NF-κBp65 activation in tumors, irrespective of *de novo* synthesis of this protein.

For evaluation of the relationship between NF-κBp65 expression and DNA-binding activity, on the one hand, and clinical picture and morphology of BC, on the other, three estimated parameters were studied, in addition to the main ones: increment of p65 content and of its activity in the tumor in comparison with the adjacent tissue (T-N), and the ratio of activity to protein content (specific activity) of NF-κBp65 in tumor tissue. No significant relationship with such clinical morphological factors as disease stage, tumor size (T index) and histological structure, lymph node involvement (N index) was detected for any of the parameters. The content of total NF-κBp65 protein in poorly differentiated tumors of malignancy degree III (1.7-67.2; median 17.7 ng/mg protein) was significantly higher than in moderately differentiated tumors of malignancy degree II (0-58.3; median 9.5 ng/mg protein; $p<0.05$). Other parameters did not depend on BC malignancy.

The important role of NF-κB signal pathway activation in the regulation of hormone and drug sensitivity of BC suggested evaluation of the relationship between the expression and DNA-binding activity of one of its subunits with the receptor status of the tumor. The data for clinically most significant groups of tumors with different receptor status are presented in Table 1. No significant differences depending on the steroid hormone receptor status were detected for any of the parameters. A trend to an increase of NF-κBp65 content and DNA-binding activity in ER^- compared to ER^+ tumors was seen. On the other hand, a significant increase of total NF-κBp65 protein content ($p<0.01$) and its shifts in comparison with the normal level ($p<0.01$) and reduction of NF-κBp65 specific activity ($p<0.05$) were found in $HER\text{-}2^+$ compared to $HER\text{-}2^-$ tumors.

Analysis of combinations of three receptors showed the following regularities: significant decrease in NF-κBp65 specific activity in $ER^-RP^-HER\text{-}2^+$ tumors, highly significant in comparison with $ER^-RP^-HER\text{-}2^-$ ones ($p<0.01$); the greatest increment of NF-κBp65 DNA-binding activity in comparison with the adjacent tissue in tumors negative by three receptor types ($p<0.05$ compared to $ER^+PR^+HER\text{-}2^-$ tumors); and comparison of tumors with the classical receptor combinations ($ER^-RP^-HER\text{-}2^+$ and $ER^+PR^+HER\text{-}2^-$) showed a significant increment of the summary level and total NF-κBp65 in $ER^-RP^-HER\text{-}2^+$ tumors ($p<0.05$).

Thus, total expression of NF-κBp65 protein in mammary gland $HER\text{-}2^+$ tumors increases irrespective of steroid hormone receptor status and is not paralleled by an increase of DNA-binding activity. On the

TABLE 1. Content and DNA-Binding Activity of NF-κBp65 in Nucleocytoplasmic Extracts in BC with Different Receptor Status (Median and Range)

Receptor status	*n*	Total NF-κBp65, ng/mg protein		NF-κBp65 DNA-binding activity, U/mg protein		Specific DNA-binding activity, U/ng NF-κBp65
		content in tumor	difference compared to intact tissue (T-N)	activity in tumor	difference compared to intact tissue (T-N)	
ER^+	81	8.95 (0-67.2)	6.83 (-28.1-67.1)	187 (0-640)	158 (-28.7-557)	15.7 (0-182)
ER^-	34	13.7 (2.1-46.5)	9.49 (-18.7-46.5)	223 (63.5-497)	196 (63.5-447)	15.8 (3.42-77.8)
ER^+PR^+	65	9.47 (0-58.3)	7.17 (-20.4-55.8)	193 (0.98-640)	162 (-28.7-557)	19.3 (0.34-182)
ER^-RP^-	31	13.3 (2.08-46.5)	9.12 (-18.7-46.5)	212 (63.5-497)	193 (63.5-447)	16.0 (3.42-77.8)
$HER\text{-}2^+$	18	18.4 (4.25-67.2)	15.4 (2.21-67.2)	166 (63.5-497)	160 (63.5-402)	9.29 (3.42-45.3)
$HER\text{-}2^-$	97	9.73** (0-58.3)	6.96** (-28.1-55.8)	191 (0-640)	171 (-28.7-557)	16.7* (0-182)
$ER^+PR^+HER\text{-}2^-$	62	9.38 (0-58.3)	7.0 (-20.4-55.8)	193 (0.98-640)	162 (-28.7-557)	19.3 (0.34-182)
$ER^+PR^+HER\text{-}2^+$	3	21.8 (7.41-33.7)	20.2 (5.08-33.7)	202 (131-272)	185 (101-268)	20.3 (3.90-36.7)
$ER^-RP^-HER\text{-}2^+$	13	18.3^+ (4.25-46.5)	14.8^+ (2.21-46.5)	158 (63.5-497)	149 (63.5-358)	9.29^{xx} (3.42-37.2)
$ER^-RP^-HER\text{-}2^-$	18	10.2 (2.08-34.4)	7.12 (-18.7-34.4)	282 (123-450)	231 (118-447)	27.5 (8.35-77.8)

Note. *$p<0.05$, **$p<0.01$ compared to $HER\text{-}2^+$; $^+p<0.05$ compared to $ER^+PR^+HER\text{-}2^-$; $^{xx}p<0.01$ compared to $ER^-RP^-HER\text{-}2^-$.

other hand, total and specific DNA binding activities of NF-κBp65 are maximum in tumors with the so-called "triple negative" receptor status resistant to endocrine and molecular targeted therapies. Presumably, this subgroup of BC patients will receive some advantages from the use of NF-κB selective inhibitors.

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