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GENOTOXIC FACTORS ASSOCIATED WITH THE DEVELOPMENT OF RECEPTOR-NEGATIVE BREAST CANCER: POTENTIAL ROLE OF THE PHENOMENON OF SWITCHING OF ESTROGEN EFFECTS

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Aim: **About 30–40% of breast cancers lack steroid receptors (ER and/or PR) at diagnosis that worsen prognosis and limit the usage of hormone therapy. The aim of this paper has been to study the role of DNA-damaging factors as the potential modifiers of the receptor-negative tumors incidence.** ***Materials and Methods:*** **The investigation consisted of two principal parts. In one of them ER and PR content was measured in breast cancer samples from 2284 primary patients (350 of them — current or previous smokers). In separately studied subgroup of 1010 patients 95 suffered with diabetes mellitus type II.** ***Results:*** **As it was shown, smokers and diabetics carry more frequently ($p \leq 0.05$) tumors with phenotypes ER+PR- and PR- only in the group of women with conserved menstrual cycle that is in case of relatively higher estrogenic stimulation. In another part of the investigation immunohistochemical study of DNA damage marker — 8-hydroxy-2-deoxyguanosine (8-OH-dG) in 16 R(-) and 18 R(+) breast cancer specimens demonstrated more frequent positive staining in the former group of samples ($p = 0.05$). Besides, as it was revealed in breast cancer cell line MCF-7 the combination of estradiol with aryl hydrocarbonic receptors agonist beta-naphtoflavone induced pronounced genotoxic damage (by 8-OH-dG content) in association with the loss of ER.** ***Conclusion:*** **Thus, pro-genotoxic status (smoking, diabetes) and direct signs of genotoxic injury, in accordance with regularities of the phenomenon of switching of estrogen effects.**
can be reckoned among the factors promoting the development of receptor-negative breast cancer.
Key Words: **breast cancer, steroid receptors, smoking, diabetes, genotoxicity, 8-hydroxy-2-deoxyguanosine (8-OH-dG).**

Estrogen and progesterone help regulate growth and differentiation of normal breast tissue, and they are considered important in the development and progression of breast cancer [12, 24]. Estrogen receptors (ER) and progesterone receptors (PR) are nuclear receptors. The estrogen-ER complex binds directly to DNA and influences the expression of estrogen-responsive genes, including the gene for PR. In other words, since PR is an estrogen-dependent protein, its expression depends on the both: the existence of ER and transfer of estrogen signal as well [12, 29].

The expression of steroid receptors has important implications for biology of breast tumors and their treatment. About 30–40% of breast cancers lack steroid receptors (ER and/or PR) at diagnosis that worsens prognosis and limits the usage of hormone therapy. Opinions differ as to whether those cancers which lack expression of receptors arise from R(-) compartment within the mammary epithelium or represent evolution from R(+) to R(-) state. Recently evidence in support of the idea on distinct etiologic pathways rather than different stages in the natural history of breast cancer has been growing [13, 32]. Contemporary concept says that receptor-positive and receptor-negative breast cancer (BC) subtypes may have associations with distinctive risk factors and mechanistic heterogeneity by hormone-receptor status related to initial existence of the two separate types of cancer (R+ and R-) is rather possible [28].

The mechanisms leading to the development of receptor-negative BC warrant further studies. Existing interpretations are not abundant and can be reduced to the role of several genetic (including BRCA1 and BRCA2) and epigenetic factors, interrelations with the presence of EGF and erbB2/HER-2/neu receptors in tumor tissue and certain features of endocrine (reproductive) system embracing level of estrogenemia and intratumoral aromatase/estrogen synthetase activity [3, 10, 15, 16, 25]. Taking into account principal characteristics of the phenomenon of switching of estrogen effects (PSEE) described by us earlier [1, 4], the assumption has been made that peculiar to this phenomenon weakening of hormonal and strengthening of genotoxic activity of estrogens may be of importance in predisposing to the disturbances in estrogen signal transduction and formation of receptor-negative BC, that needs further consideration.

One of the PSEE inductors is displayed by tobacco smoke. Chemical substances that its contains have rather often the direct DNA-damaging capacity and, in the same time, are able to modify the metabolism of estrogens into direction of the increased formation of estrogenic catechol derivatives, which give in their turn an impulse for the generation of different free-radical compounds [1, 27, 30]. Pro-genotoxic oxidative stress is characteristic also for diabetes mellitus type II [7], the disease, which through variety of mechanisms (including increase in free estradiol fraction) is related to the risk of breast cancer [18]. As a consequence, the first task of this investigation consisted in the evaluation of the effect of smoking and diabetes on the

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Abbreviations used: AhR – aryl hydrocarbonic receptors; ER – estrogen receptors; 8-OH-dG – 8-hydroxy-2'-deoxyguanosine; PR–progesterone receptors; PSEE– phenomenon of switching of estrogen effects.

receptor phenotype of BC patients in the conditions of variable estrogenic stimulation, i.e. in females of reproductive and postmenopausal age.

As it was mentioned above, PSEE is characterized with the decrease in hormonal and the increase in genotoxic effects of natural estrogens [1, 4]. Similarly act certain xenoestrogens, which action is realized through aryl hydrocarbonic receptors, or AhR [23]. Altogether, it stimulated conducting of the another part of our research which included the comparison of the content of specific marker of oxidative DNA damage 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in human receptor-positive and receptor-negative BC samples as well as the evaluation of 8-OH-dG, ER and PR in breast cancer cell line MCF-7 treated with estradiol, AhR agonist beta-naphtoflavone and their combination.

MATERIALS AND METHODS

The content of ER and PR has been determined in tumor tissue of totally 2284 primary (without neoadjuvant therapy) breast cancer patients, which were in clinical stages I-IIIa ($T_{0-1}N_0M_0$-$T_{1-3}N_2M_0$) and in age varying from 27 to 84 yrs. Among these patients 815 were characterized with preserved menstrual cycle and remaining 1469 women were in menopause with the duration not less than 1 year. 350 of these patients (197 — in reproductive and 153 — in postmenopausal period) were current or ever smokers consuming daily from 4–5 to 20–25 cigarettes. In subgroup of 1010 patients (361 with conserved menstrual cycle and 649 postmenopausal), in which the analysis of tumor steroid receptor content was performed, 95 women suffering with diabetes mellitus type II were discovered. Technically, BC tissue samples collected during operation were immediately transferred into laboratory and were placed into liquid nitrogen for further processing. Estrogen and progesterone receptor contents in tumor tissue were evaluated by the dextrane-charcoal radioligand assay according to Saez et al. [22] with small modifications [3] and with the usage of labeled steroids (2,4,6,7-^{3}H-Oestradiol и 1,2,6,7-^{3}H-Progesterone) from “Amersham”, UK. The receptor activity was expressed as fM/mg protein. Protein content was determined by the Lowry method. Statistical analysis of the data was performed by methods allowing for means and standard errors. The significance of the differences between the groups was tested using hi-square approach by computerized programs (SigmaPlot, Statistica 6 and MyStat). The differences with $p \leq 0.05$ were considered as significant.

In the second part of the study the content of ER and PR in human breast tumors and in MCF-7 cell line was determined with the same method. Immunohistochemical or immunocytochemical evaluation of 8-OH-dG in the sections of paraffin tumor blocks or in MCF-7 cells was done according to Bianco et al. [6] with diluted 1 : 100 antibodies 1F7 from “Trevigen”, USA. Immunostaining was developed by the peroxidase-antiperoxidase procedure with the usage of Vecstain ABC kit (Vector Laboratories, USA) and DAB (3,3′-diaminobenzidine) reaction. As a negative control, tissue sections or cells were incubated without the primary antibody. In clinical part of this study material was received from 34 postmenopausal breast cancer patients with $T_{1-3}N_{0-1}M_0$ stages of the disease. 18 tumors were receptor-positive and 16 — receptor-negative (with ER and PR values less than 10 fM/mg protein). Results of 8-OH-dG immunohistochemical staining were evaluated independently by two investigators and were presented as + (positive staining), ± (moderate positive staining) and – (negative staining). During the processing of the data results in groups + and ± were combined. In the experiments with cell line MCF-7, cells were grown in the MEM medium until they were 70–80% confluent. Then cells were seeded (5×10^4/site) into culture dishes and treated with estradiol (10^{-7} M), beta-naphtoflavone (4×10^{-7} M) and their combination in the duration of 1, 24 and 48 h from the start of incubation. Smears of the cells collected by the end of the experiment were fixed 10 min in 70% ethanol, air-dried and kept in hermetic containers at –20 °C until immunocytochemical reaction performance. Results of the latter were evaluated on the basis of stepping procedure by two investigators and presented in the interval from 0 (no staining) to 1.5 (maximal staining) with the step equal to 0.25. Statistical analysis of the data was the same as described above.

RESULTS AND DISCUSSION

Data on distribution of steroid receptor phenotypes in 2284 breast tumors and in the subgroup of 1010 cancers are presented in Table 1. Statistically significant difference between groups of interest was revealed only in reproductive period and only in regard of ER + PR- tumors which were overrepresented in smokers vs. non-smokers ($t = 2.18$, $p < 0.05$; $\chi^2 = 5.01$, $p = 0.025$) as well as in patients suffering with diabetes mellitus type II vs. patients without diabetes, $t = 2.01$, $p = 0.05$; $\chi^2 = 6.38$, $p = 0.012$ (see Table 1). These results deserve special assessment at least in the two directions. First of all, smoking and diabetes as a rule worsen prognosis *quo ad vitam* in patients with breast cancer [17, 31]. Since the survival rate is usually worse in younger patients in comparison with postmenopausal females [11], it may be assumed that tumor receptor phenotypes discovered in premenopausal smokers and diabetics additionally predispose to the more aggressive course of the disease. Secondly and more importantly in the context of this paper, an inclination to the predominant formation of the tumors with phenotype ER+PR- (that evidently reflects impossibility or failure of estrogenic signal transduction and insufficient induction of estrogen-dependent proteins including PR [12, 13]) in smoking and suffering with diabetes females was observed in reproductive period and not in menopause, suggesting that smoking and diabetes realize mentioned effect predominantly in the case of excessive or non-deficient estrogenic stimulation. Thus, it may be concluded that combined

Table 1. The phenotypes of tumor steroid receptors in smoking and suffering from diabetes mellitus breast cancer patients

Patients	Steroid receptor phenotypes								Totally	
	ER+PR+		ER+PR-		ER-PR+		ER-PR-			
	n	%	n	%	n	%	n	%	n	%
Smokers										
All	144	41.1 ± 2.6	92	26.3 ± 3.9	34	9.7 ± 1.6	80	22.9 ± 2.2	350	100
RP	70	35.5 ± 3.4	53	27.0 ± 3.1	23	11.7 ± 2.3	51	26.0 ± 3.1	197	100
MP	74	48.4 ± 4.0	39	25.5 ± 3.5	11	7.2 ± 2.1	29	19.0 ± 3.2	153	100
Non-smokers										
All	799	41.3 ± 1.1	441	22.8 ± 0.9	210	10.8 ± 0.7	484	25.0 ± 1.0	1934	100
RP	266	43.0 ± 2.0	120	19.4 ± 1.6	76	12.3 ± 1.3	156	25.2 ± 1.7	618	100
MP	533	40.5 ± 1.3	321	24.4 ± 1.2	134	10.2 ± 0.8	328	25.0 ± 1.2	1316	100
With diabetes mellitus type II										
All	39	41.0 ± 5.0	26	27.4 ± 4.6	9	9.5 ± 3.0	21	22.1 ± 4.2	95	100
RP	3	23.0 ± 17.3	6	46.0 ± 13.8	1	8.0 ± 7.5	3	23.0 ± 11.7	13	100
MP	36	44.0 ± 5.5	20	24.0 ± 4.7	8	10.0 ± 3.3	18	22.0 ± 4.6	82	100
Without diabetes mellitus										
All	398	43.5 ± 1.6	185	20.0 ± 1.3	100	11.0 ± 1.0	232	25.5 ± 1.4	915	100
RP	159	46.0 ± 2.7	63	18.0 ± 2.0	46	13.0 ± 1.8	80	23.0 ± 2.2	348	100
MP	239	42.0 ± 2.1	122	21.5 ± 1.7	54	9.5 ± 1.2	152	27.0 ± 1.9	567	100

Notes: RP – reproductive period; MP – postmenopausal period. For statistical significance values – see text.

action of estrogens and genotoxic shifts peculiar to the diabetes and tobacco smoke influence may create conditions favoring the development of receptor-negative breast cancer.

As it was mentioned above, in another part of the study the staining of breast tumors and MCF-7 cell line was performed with antibodies to 8-OH-dG aiming to demonstrate, whether DNA damage signs are associated with receptor phenotype. In studied material the content of ER and PR in R+ breast cancers (n = 18) varied correspondingly from 20 to 250 fM/mg protein and from 19 to 470 fM/mg protein while in R(-) tumors (n = 16) it varied from 0 to 4 fM/mg protein (ER) and from 0 to 8 fM/mg protein (PR). Specific positive immunohistochemical 8-OH-dG staining (Fig. 1) was revealed in 9 of 18 cases (50.0 ± 11.7%) of R+ cancers and in 13 of 16 (81.3 ± 9.8%) of R(-) tumors; difference is statistically significant (t = 2.05, p = 0.05). Thus, although 8-OH-dG can be found also in receptor-positive BC, the incidence of its discovery in R(-) tumors is higher presenting the evidence of the greater significance of genotoxic factors just for the formation of receptor-deficient tumors.

Immunocytochemical detection of 8-OH-dG in MCF 7 cells (Fig. 2) demonstrated that whereas in the cells treated only with estradiol or beta-naphtoflavone content of 8-OH-dG increased steadily and significantly by 48 h (Table 2), the genotoxic damage induced by the joint action of these two agents have reached its peak already by 1 h, when simultaneous decrease in ER content and low level of PR were discovered (data not shown). So, it can not be excluded, that the accumulation of DNA damage can be accelerated in the situation when hormonal effect of estrogens is modified by additional pro-genotoxic factor (tobacco smoke, diabetes-associated pro-oxidant metabolic shifts, activation of AhR pathway etc) and this, in combination, leads to transition of the cells from R+ to the R(-) state. Although preceding data on the 8-OH-dG content in receptor-positive and receptor-negative BC are the matter of some controversy [6, 20], the firm evidence on the contribution of oxidative stress to an increasing incidence of ER+PR- tumors with aging are presented recently [21].

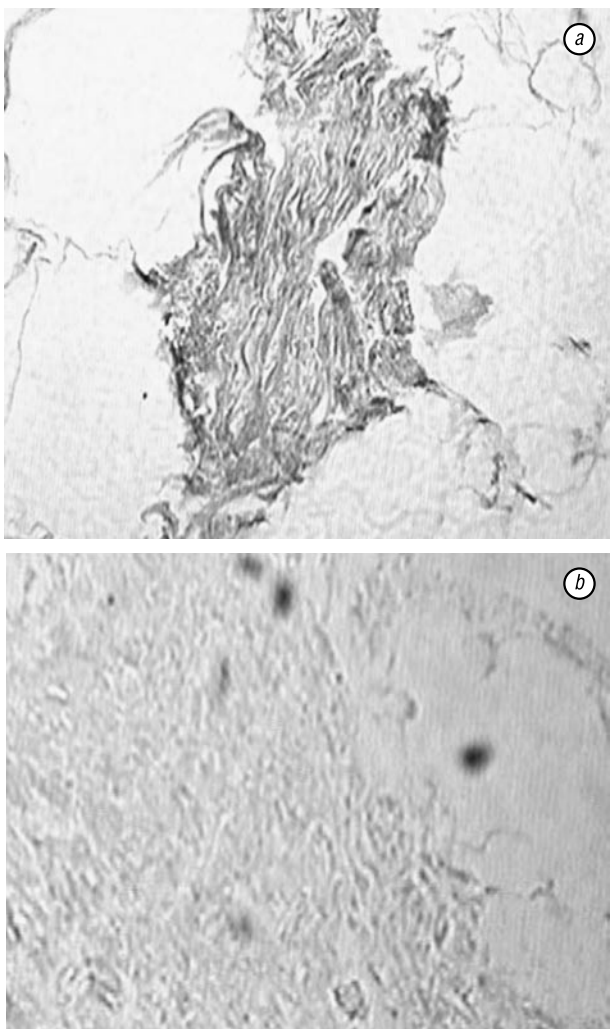

Fig. 1. Immunohistochemical detection of 8-hydroxy-2-deoxyguanosine (8-OH-dG) in human breast cancer sections: *a* — receptor-negative tumor, positive 8-OH-dG staining; *b* — receptor-positive tumor, negative 8-OH-dG staining

Table 2. The dynamic of the accumulation of 8-hydroxy-2-deoxyguanosine (8-OH-dG) in the MCF-7 cells under the influence of estradiol (E2), beta-naphtoflavone (B) and their combination

Group/Time	1 h	24 h	48 h
E2	0.625 ± 0.075	1.00 ± 0.05	1.25 ± 0.05*
B	0.25 ± 0.075	0.50 ± 0.10	1.25 ± 0.05*
E2 + B	1.125 ± 0.025**	1.075 ± 0.025	1.00 ± 0.012

Note: Data of immunocytochemical analysis are presented in conditional units (see section "Material and methods").

* The difference with 1 h value is significant ($p < 0.02$).

** The difference with the data in E2 and B groups is significant ($p < 0.05$).

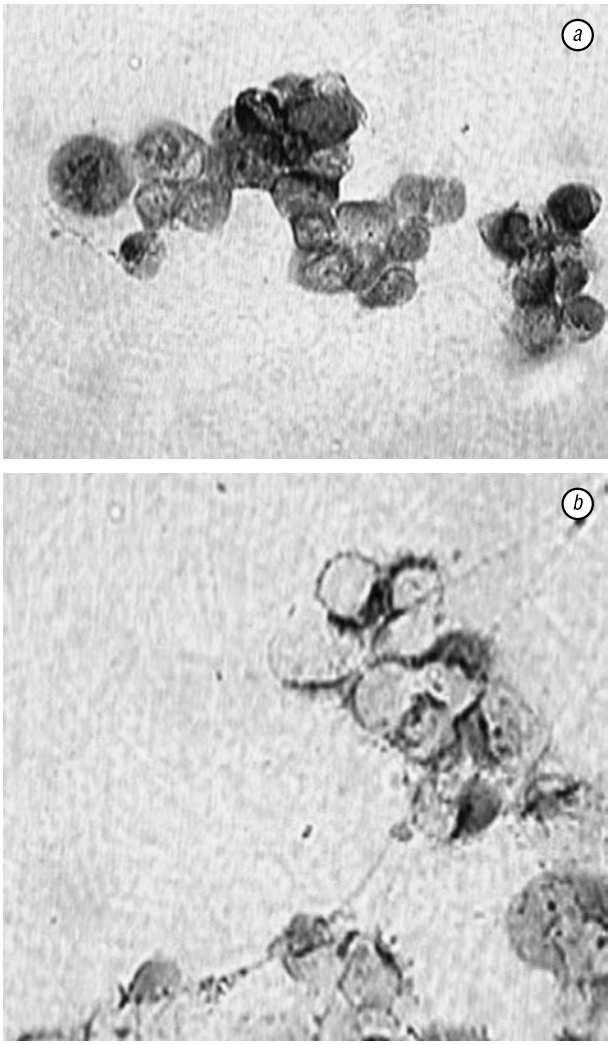

Fig. 2. Immunocytochemical detection of 8-hydroxy-2-deoxyguanosine (8-OH-dG) in MCF-7 cell line: *a* — positive staining; *b* — negative staining

Of note, in normal breast tissue ER's and markers of proliferation (e.g. Ki-67) are detected in separate cell populations, indicating that ER+ cells (which mostly originate from the luminal type of epithelium) normally are not dividing, or that the receptor is down-regulated as cells enter division. In contrast to the normal human breast, the separation between steroid receptor expression and proliferation is disrupted starting at an early stage of breast carcinogenesis, and co-expression of these two markers (ER and Ki-67) occurs frequently in tumor cells. Therefore, suggestion is made that ER+ cells are quiescent stem cells acting as "steroid hormone sensors" which might secrete paracrine factors to influence the proliferative activity of adjacent ER- or progesterone receptor (PR)-negative cells originating predominantly from the basal/myoepithelial lineage. According to this assumption, after R+ cells are transformed, they acquire the ability to proliferate in breast lesions [8, 25]. On the other hand, transformed mammary stem or progenitor cells may undergo aberrant differentiation processes that result in generation of the phenotypic heterogeneity [9], which might serve as a basis for the variability observed in the steroid hormone receptor status of breast cancer. This corresponds with a notion that receptor-negativity is not only a result of cancer progression from a receptor-positive state and that, in contrast, a distinction in the content of receptors may reflect different pathogenesis of the disease [28, 32]. As a result, R+ and R(-) breast tumors differ in clinical aggressiveness and proliferative activity, which frequently is higher just in R(-) carcinomas [11, 28]; the latter observation is in the agreement with the basic patterns of the phenomenon of switching of estrogen effects, or PSEE [1, 4].

What are the practical consequences which can be deduced from the said above? Since along with the endocrine and genetic mechanisms genotoxic damage is related to the development of receptor-negative breast cancer, existing and future prevention measures should include in a degree greater than before not only anti-hormonal agents but antigenotoxicants also. The list of the latter recently somewhat widened and includes f.e. N-acetylcysteine, melatonin, resveratrol etc [5, 14]. Additionally, some hypolipidemic and antidiabetic drugs can be used with the same aim and as a supplement to neoadjuvant and adjuvant treatment especially because some of them (like statins) were rather efficient in the experiments with receptor-negative cell lines [2, 19]. Finally, the possibility to improve situations due to the inductors of steroid receptors (interferons etc) should not be ignored also, since their usage had been rather effective in tamoxifen-resistant receptor-negative breast cancer [26]. It is suggested that further studies might be based on proteomic approach and on the taking into account the ratio in hormonal/genotoxic properties of mammary adipose tissue surrounding correspondingly receptor-positive and receptor-negative breast cancers.

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ГЕНОТОКСИЧЕСКИЕ ФАКТОРЫ, АССОЦИИРОВАННЫЕ С ВОЗНИКНОВЕНИЕМ РЕЦЕПТОР-НЕГАТИВНОГО РАКА МОЛОЧНОЙ ЖЕЛЕЗЫ: ПОТЕНЦИАЛЬНАЯ РОЛЬ ФЕНОМЕНА ПЕРЕКЛЮЧЕНИЯ ЭСТРОГЕННОГО ЭФФЕКТА

***Цель исследования*: примерно в 30–40% случаев рака молочной железы (РМЖ) рецепторы стероидных гормонов (ЭР и/или ПР) не выявлены, что оказывает неблагоприятное влияние на прогноз заболевания и ограничивает применение гормонотерапии. Задачей настоящей работы было изучение роли факторов, ассоциированных с повреждением ДНК, как потенциальных модификаторов частоты возникновения рецепторнегативных новообразований. *Материалы и методы*: исследование состояло из двух основных частей. У 2284 первичных больных РМЖ (350 из них — курящие или курившие ранее) содержание ЭР и ПР в опухолевой ткани определяли рецепторным методом. В отдельно проанализированной подгруппе из 1010 больных у 95 пациенток диагностировали сахарный диабет II типа. *Результаты*: установлено, что в отличие от пациенток в постменопаузальный период с сохраненной менструальной функцией (то есть в условиях более высокой эстрогенной стимуляции) отмечается достоверное преобладание опухолей фенотипа ЭР + ПР — у курящих по сравнению с некурящими ($t = 2{,}18$, $p < 0{,}05$), а также у больных сахарным диабетом по сравнению с больными без него ($t = 2{,}01$, $p = 0{,}05$). Кроме того, в репродуктивный период у больных сахарным диабетом доля ПР(-)-опухолей достоверно превосходила таковую у пациенток без него ($t = 2{,}17$, $p < 0{,}05$). Во второй части исследования с помощью иммуногистохимического метода изучено содержание показателя повреждения ДНК 8-гидрокси-2-дезоксигуанозина (8-OH-dG) в 16 рецепторнегативных и 18 рецепторпозитивных опухолях молочной железы. Выявлено, что в первой группе позитивное окрашивание наблюдали в $81{,}3 \pm 9{,}8\%$, а во второй — в $50{,}0 \pm 11{,}7\%$ случаев, $p = 0{,}05$. Помимо этого, результаты эксперимента на клеточной линии MCF-7 рака молочной железы свидетельствуют, что при комбинации эстрадиола с агонистом арилгидрокарбоновых рецепторов бета-нафтофлавоном значительное усиление генотоксического повреждения (по данным определения 8-OH-dG) отмечается уже к 60 мин эксперимента, когда выявляют и снижение концентрации ЭР. *Выводы*: прегенотоксический статус (курение, сахарный диабет), равно как и признаки прямого генотоксического повреждения, в соответствии с представлениями о феномене переключения эстрогенного эффекта могут быть причислены к факторам, способствующим развитию рецепторнегативного РМЖ.**

***Ключевые слова:* рак молочной железы, рецепторы эстрогенов, рецепторы прогестерона, рецепторнегативные опухоли, курение, диабет, генотоксичность, 8-гидрокси-2-дезоксигуанозин (8-OH-dG).**