

UDC 577.17 + 616-006.6 + 615.277.3

Effect of anticancer drugs on breast cancer cells sensitive and resistant to doxorubicin: expression of mRNAs of TGF- β and its receptors

I. V. Chorna¹, O. V. Fedorenko², R. S. Stoika²

¹Sumy State University, Ukraine
2, Rimskogo-Korsakova Str., Sumy, Ukraine, 40007

²Institute of Cell Biology, NAS of Ukraine
14/16, Drahomanov Str., Lviv, Ukraine, 79005

stoika@cellbiol.lviv.ua

Aim. Comparative study of the effect of chemotherapeutic drugs (doxorubicin, methotrexate and cisplatin) and TGF- β on the human breast carcinoma MCF-7 cells, sensitive (wt) and resistant (DOX/R) to the doxorubicin action. **Methods.** Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used for the estimation of expression of mRNAs coding for the TGF- β isoforms (TGF- β_1 and TGF- β_2) and the TGF- β type I and II receptors (T β RI and T β RII). Trypan blue exclusion method was used for measuring cell number and cell viability. **Results.** The MCF-7(DOX/R) cells were more refractory to the TGF- β -dependent growth inhibition than the MCF-7(wt) cells. The level of mRNAs coding for TGF- β and its receptors was higher in the untreated MCF-7(DOX/R) cells comparing to the MCF-7(wt) cells. The expression of mRNA coding for T β RII was decreased in both cell lines treated with doxorubicin, methotrexate and cisplatin, while the down-regulation of mRNA coding for T β RI was revealed only in the MCF-7(DOX/R) cells upon the treatment with doxorubicin and methotrexate. **Conclusions.** The differential effects of studied anticancer drugs and TGF- β on the doxorubicin-sensitive and -resistant cells have been demonstrated. The elucidation of the molecular mechanisms of escape of the MCF-7(DOX/R) cells from the growth inhibition by TGF- β requires further investigation.

Keywords: MCF-7, TGF- β , doxorubicin, methotrexate, cisplatin, resistance.

Introduction. Chemotherapy is a major tool in the treatment of breast cancer. However, the main problem hindering the success of chemotherapy is the development of acquired tumor drug resistance during the treatment. Although the most common chemotherapeutic drugs used for the breast cancer treatment cause DNA damage and induce apoptosis, they exert the cell killing effects through different molecular mechanisms. The biological effects displayed by doxorubicin that may be responsible for the inhibition of tumor cell growth involve the covalent DNA binding and DNA cross-linking, topoisomerase II inhibition, arrest of tumor cell cycle progression in G2 phase, induction of apoptosis

and free radicals formation [1, 2]. The alkylating agent cisplatin binds to DNA bases causing intra- and inter-strand cross-links and breaks in DNA strands, and thus, interfering with the DNA replication [3]. The antimetabolite methotrexate inhibits the enzyme dihydrofolate reductase preventing normal synthesis of the nucleoside triphosphates [4].

The tumor cells, resistant to a single chemotherapeutic agent, can also become cross-resistant to several other anti-cancer drugs due to the development of their multi-drug resistance. Such resistance was shown to be associated with numerous mechanisms: 1) decreased drug accumulation and/or altered intracellular distribution of drugs; 2) increased activity of the cell detoxification systems; 3) alterations in the cell cycle and signal

Institute of Molecular Biology and Genetics, NAS of Ukraine, 2014

transduction pathways; 4) increased repair of DNA damage and high anti-apoptotic potential of the tumor cells [5–7].

The multi-drug resistance in the MCF-7(DOX/R) cells was shown to be associated with various mechanisms including the over-expression of P-glycoprotein, glutathione S-transferase and glutathione peroxidase, the loss of estrogen responsiveness, the DNA hypomethylation (loss of cytosine methylation of the *MDR1*, *GST* and *MGMT* chemoresistance related genes) [6, 8], and the changes in the expression of several cell cycle regulators (decreased Ki-67, cyclin D1 and pRb and increased p21 expression) [9]. The elucidation of novel mechanisms of drug resistance and further development of the ways of overcoming such resistance or preventing its occurrence might increase the efficiency of treatment of cancer patients.

TGF- β belongs to the most perspective molecular markers which have both prognostic and predictive significance in the breast cancer [10, 11]. This cytokine is involved either in the regulation of cell proliferation, differentiation, apoptosis or in the intracellular and extracellular processes which contribute to the tumor progression (*e. g.* pro-tumorigenic effects on the vascular, immune and fibroblastic cells) [10]. However, TGF- β may induce the entirely different cellular responses, depending on the cell type and the stimulation context, under both physiological and pathological conditions [12]. An alteration in the expression of the TGF- β receptors and the post-receptor signal transduction proteins may play a critical role in the rendering malignant cells resistant to TGF- β [13–15].

Thus, there are multiple etiologies of the development of drug resistance, and the role of TGF- β regulatory system in the refractoriness of cancer cells to chemotherapeutic agents is not fully understood. In the present study, we have compared the effect of some of the most common drugs used for breast cancer treatment (doxorubicin, cisplatin and methotrexate) on the expression of mRNAs coding for TGF- β and its receptors in the doxorubicin-sensitive and the doxorubicin-resistant human breast cancer cells. Besides, we have compared the inhibiting effect of TGF- β on cell growth in the tested cell sub-lines in order to check if the tumor cross-refractoriness to both doxorubicin and TGF- β may exist.

Materials and methods. *Cell lines and culture conditions.* Human breast carcinoma MCF-7 cells differing in their sensitivity to doxorubicin (sensitive MCF-7 (wt) and resistant (MCF-7(DOX/R) cells) were obtained from Cell Collection of the Institute of Oncology («Gliwice», Poland). The cells were cultured in the Dulbecco's modified Eagle's medium (DMEM, «Sigma Chemical Co.», USA) supplemented with 10 % heat-inactivated fetal calf serum (FCS, «Sigma Chemical Co.») and 50 g/ml gentamycin («Sigma Chemical Co.»). The cultured cells were maintained at 37 °C in a humidified (100 %) incubator with 5 % CO₂.

Drug treatment. Doxorubicin and cisplatin were purchased from «Ebewe» (Austria), and methotrexate – from «Leberle» (USA). Each drug was added 24 h after cell plating and each sample was incubated for 24 h with the chemotherapeutic drug of appropriate concentration. Transforming growth factor β_1 was purchased from «R & D Systems, Inc.» (USA).

Measuring cell number and viability. The effect of TGF- β_1 and doxorubicin on cell growth and viability was determined using trypan blue (0.1 % (w/v)) exclusion method. The numbers of dead (stained) cells and alive (unstained) cells were counted in the hemocytometer chamber under the light microscope.

Isolation of total RNA and RT-PCR assay. The amount of mRNAs coding for TGF- β_1 , TGF- β_2 , T RI and T RII was determined by the reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from the cells by Trizol Reagent («Life Technologies, Inc.», USA) following the standard protocol, and quantified spectrophotometrically. The first-strand cDNA synthesis and amplification of the specific DNA sequence were performed according to the manufacturer's instructions («RevertAid™ First Strand cDNA Synthesis Kit», «Fermentas», Lithuania). Briefly, 5 g of total RNA was used for cDNA synthesis using 1 l of oligo(dT) primer (0.5 g/l) in the presence of 1 l (200 U) of Moloney murine leukemia virus reverse transcriptase. The obtained cDNAs were used in RT-PCR analysis as described previously [16]. The cDNA encoding TGF- β_1 was amplified for 30 cycles (94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min). The cDNA encoding TGF- β_2 was amplified for 33 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min). The cDNAs encoding T RI and T RII were amplified for 30 cycles (94 °C for

The effect of TGF- β_1 (10 ng/ml) and doxorubicin (5 μ g/ml) treatment on growth and viability of MCF-7(wt) and MCF-7 (DOX/R) cells

Factor	MCF-7(wt)		MCF-7(DOX/R)	
	Cell number, % of control	Cell viability, % of dead cells	Cell number, % of control	Cell viability, % of dead cells
<i>Time of incubation, 24 h</i>				
Control	100.0 \pm 2.4	2.0 \pm 0.3	100.0 \pm 3.2	3.0 \pm 0.5
Doxorubicin	24.0 \pm 2.5**	3.0 \pm 0.6	95.0 \pm 2.8	2.0 \pm 0.5
TGF- β_1	83.0 \pm 2.2**	7.0 \pm 0.5**	89.2 \pm 4.3	2.0 \pm 0.3
<i>Time of incubation, 48 h</i>				
Control	100.0 \pm 1.5	4.0 \pm 0.8	100.0 \pm 1.6	3.0 \pm 0.9
Doxorubicin	13.0 \pm 1.5**	7.0 \pm 0.9*	90.0 \pm 4.5*	5.0 \pm 0.5
TGF- β_1	75.0 \pm 3.3**	16.5 \pm 2.3**	95.0 \pm 4.4	5.1 \pm 0.7

*p < 0.05; **p < 0.001 (in treated versus the respective control (untreated) cells).

1 min, 55 °C for 1 min, and 72 °C for 1 min). The cDNA encoding β -actin was amplified for 25 cycles (94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min). The expected sizes of the PCR products and the primer sequences used were as follows:

forward primer 5'-TGAACCGGCCTTTCCTGCT TCTCATG-3' and reverse primer 5'-GCGGAAGTC AATGTACAGCTGCCGC-3', 152 bp; TGF- β_1 ;

forward primer 5'-ACAGGAACCTGGGATTAA AATAAG-3' and reverse primer 5'-TTTCTGATCA CCACTGGTATATGTG-3', 152 bp; TGF- β_2 ;

forward primer 5'-TGAACAGAAGTTAAGGCC AAATATC-3' and reverse primer 5'-CAGGCAAAGC TGTAGAATTACATTT-3', 192 bp; T RI;

forward primer 5'-CGGTAAATAACGACATGA TAGTCAC-3' and reverse primer 5'-TCATGGCAA CTGTCTCTAGTGTTA-3', 217 bp; T RII;

forward primer 5'-TCACCCACACTGTGCCCAT CTA-3' and reverse primer 5'-CAGCGGAACCGCTC ATTGCCAA-3', 295 bp; β -actin.

-Actin was used as an internal control for normalizing the amount of sample loading.

The reaction products were analyzed by electrophoresis in 2 % agarose gels and visualized by ethidium bromide staining of DNA. Gels were examined under UV-light using the transilluminator Macro Vue UV-20 («Hoeffer Pharmacia Biotech Inc.», USA) and photographed using the Olympus C-4000 digital camera. Semi-quantitation of the ethidium bromide stained DNA bands was performed using the Gel-Pro Analyzer v3.1.00.00 program («Media Cybernetics, L. P.», USA).

Statistical analysis. The experiments were repeated 3 times and significance of difference was assessed by Student's t-test. The level of significance was set at 0.05.

Results and discussion. Our previous study has shown that the MCF-7(DOX/R) cells, comparing to the MCF-7(wt) cells, are more refractory to the cytotoxic and cytostatic effects of the studied anticancer drugs [17] and ionizing radiation [18]. We also have revealed a higher DNA repair potential in the MCF-7 (DOX/R) cells than in the MCF-7(wt) cells [19]. The treatment of the MCF-7 and T47D cell lines of human breast carcinoma with doxorubicin (1–100 μ g/ml) induced a secretion of TGF- β_1 by both cell lines in a dose-dependent manner [20]. An elevated level of TGF- β_1 was detected in the blood plasma of breast [21], lung [22], colorectal [23, 24] cancer patients and metastatic patients with prostate cancer [25].

In order to compare the sensitivity of studied cells to TGF- β_1 , the cells were incubated in the absence (control) or presence of TGF- β_1 (10 ng/ml) for 24 and 48 h. We found that the MCF-7(wt) cells were more sensitive to the growth inhibition by the exogenous TGF- β_1 action than the MCF-7(DOX/R) cells (Table). The TGF- β_1 inhibited growth and caused cell death (16.5 \pm 2.3 %) of the MCF-7(wt) cells (p < 0.001) after a 48 h incubation, whereas in the similarly treated MCF-7 (DOX/R) cells no statistically significant changes were observed in the growth inhibition comparing to the control (untreated) cells of that sub-line. Doxorubicin (5 μ g/ml) was used as a positive control of the drug effect on the cell growth and viability of the sensitive and resistant sub-lines of

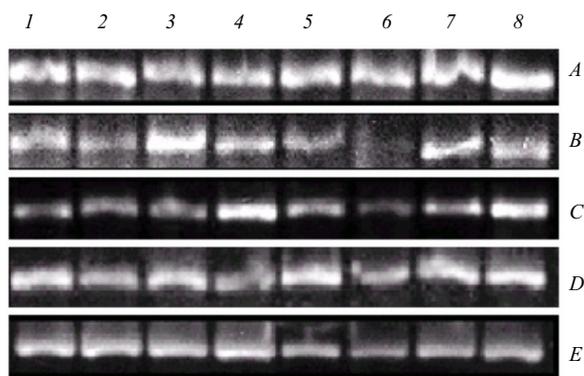


Fig. 1. Representative RT-PCR analysis of cDNA corresponding mRNA coding for TGF- α_1 (A), TGF- α_2 (B), T RI (C), T RII (D) and β -actin (E) in MCF-7(wt) cells (1–4) and MCF-7(DOX/R) cells (5–8): 1, 5 – untreated cells; 2, 6 – doxorubicin (5 μ g/ml); 3, 7 – methotrexate (25 μ g/ml); 4, 8 – cisplatin (10 μ g/ml)

the MCF-7 cells (Table). It is known that during the early phase of epithelial tumorigenesis, TGF- α inhibits the primary tumor development and growth by inducing the cell cycle arrest and apoptosis. At the late stages of tumor progression when the tumor cells become resistant to the growth inhibition by TGF- α due to inactivation of the TGF- α signaling pathway or aberrant regulation of the cell cycle, TGF- α exhibits a pro-metastatic role [26]. Earlier it has been found that the cisplatin-resistant sub-line of murine leukemia L1210 cells possesses a cross-resistance to TGF- α_1 and the mechanism of drug resistance, based on the involvement of TGF- α_1 , has been proposed [27].

We also studied whether the above mentioned anticancer drugs can alter the expression of mRNAs for TGF- α and its receptors in the MCF-7(wt) and the MCF-7(DOX/R) cells. The cells of both sub-lines were exposed to different doses of the anticancer drugs, doxorubicin (5 μ g/ml), methotrexate (25 μ g/ml) and cisplatin (10 μ g/ml) at 24 h exposure. When using β -actin as an internal control for normalizing the amount of sample loading, we found that the levels of mRNAs coding for TGF- α_1 , TGF- α_2 , T RI and T RII, were higher in the untreated MCF-7(DOX/R) cells comparing to the untreated MCF-7(wt) cells (Fig. 1 and Fig. 2). It was reported that the expression of mRNAs coding for TGF- α_2 and TGF- α receptors (T RI and T RII) was higher in most malignant tumor cells, for example, in the breast cancer cells of Hs578T line [28]. Thus, we hypothesize that the genes of the TGF- α regulatory system, the ex-

pression of which is higher in the doxorubicin-resistant cancer cell line (MCF-7(DOX/R)), may be responsible for the observed chemoresistance via their protein products.

The results presented in Fig. 1 and Fig. 2, A, show that under the doxorubicin action there was no statistically significant increase in the expression of the TGF- α_1 mRNA in both cell lines tested, whereas a decrease in the expression of the TGF- α_2 mRNA was revealed in the MCF-7(wt) and MCF-7(DOX/R) cells (1.4 and 2.3 fold, correspondingly) (Fig. 2, B). Methotrexate and cisplatin did not affect the expression of mRNAs coding for TGF- α_1 ligand in both studied cancer cell lines (Fig. 2, A). We found an increase in the TGF- α_2 mRNA expression in the MCF-7(wt) cells treated with methotrexate and in the MCF-7(DOX/R) cells treated with methotrexate and cisplatin (Fig. 2, B).

TGF- α s elicit their effects via binding with receptors on the cellular surface [10]. T RI and T RII form a heteromeric receptor complex. The direct involvement of both T RI and T RII in conferring TGF- α s effects indicates that the loss of either of the functional receptors would contribute to the failure to respond to autocrine and exogenous TGF- α [13, 14, 29]. It has been shown that the mutational inactivation of T RII occurs frequently in a subset of the microsatellite unstable colon cancers [30]. The inactivation of T RII has also been described in the non-small cell lung adenocarcinomas and the small cell lung carcinomas [31]. Several studies describe the mutations in T RI and T RII in the adenomas and gliomas [32–34] as well as a correlation between higher expression of T RI and T RII and more aggressive glioma cell lines and tumors [35, 36].

The treatment of cells with doxorubicin and methotrexate decreased the production of mRNA coding for T RI (2.0 and 1.4 fold, correspondingly) in the doxorubicin-resistant sub-line, while no statistically significant changes in the level of T RI mRNA were found in the doxorubicin-sensitive sub-line under the same conditions (Fig. 2, C). A two-fold increase of mRNA coding for T RI was detected in the MCF-7(wt) cells treated with cisplatin. At the moment, the reasons for such dynamics in the T RI expression in MCF-7(wt) cells under cisplatin treatment are unknown, and additional studies are needed to get a support for such explanation of the detected differences.

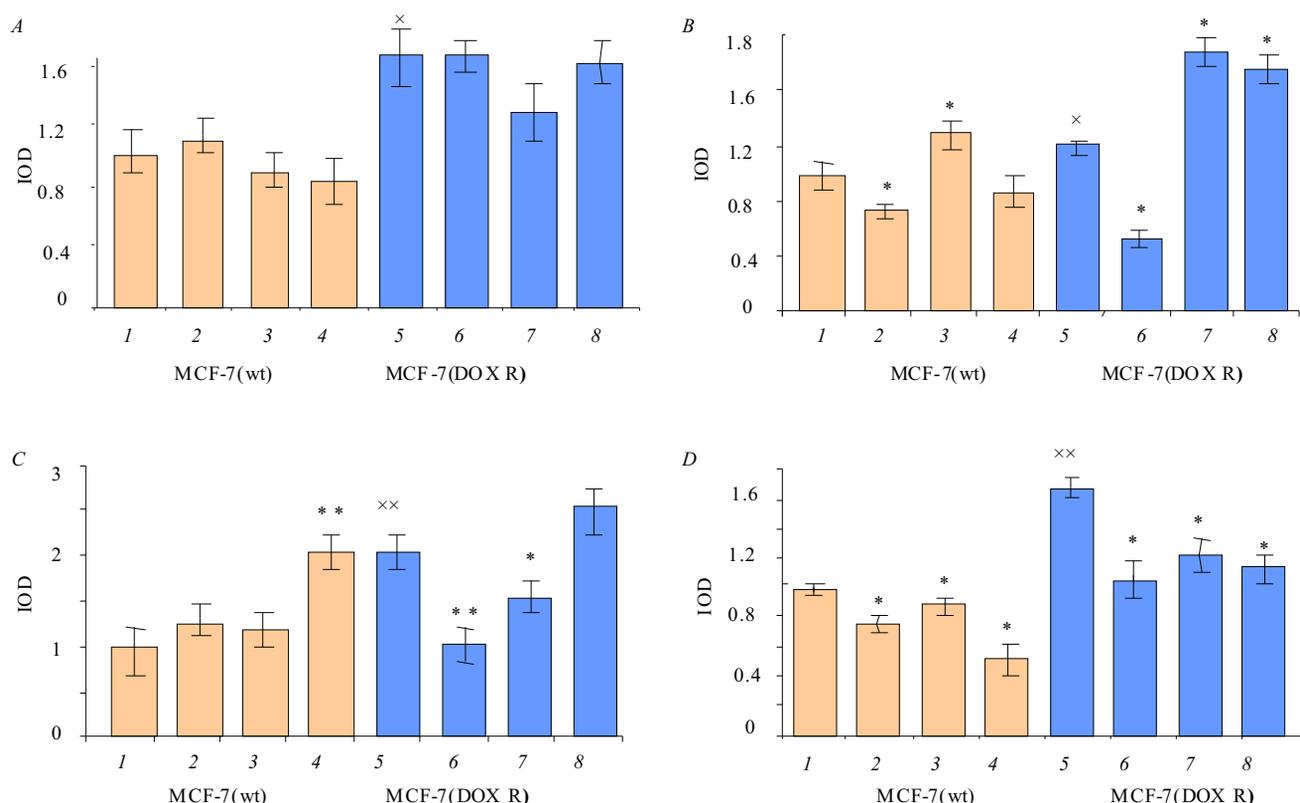


Fig. 2. Densitometric measurement of cDNA corresponding mRNA coding for TGF- β_1 (A), TGF- β_2 (B), T RI (C) and T RII (D) mRNAs normalized against β -actin mRNA levels in MCF-7(wt -yellow) (1–4) and MCF-7(DOX/R - blue) (5–8) cells: 1, 5 – untreated cells; 2, 6 – doxorubicin (5 μ g/ml); 3, 7 – methotrexate (25 μ g/ml); 4, 8 – cisplatin (10 μ g/ml); $p < 0.05$; $p < 0.01$ (in MCF-7(DOX/R) versus MCF-7(wt) cells); * $p < 0.05$; ** $p < 0.01$ (in cells treated by anticancer drugs versus the respective control (untreated) cells)

We also revealed a decreased expression of the mRNA coding for T RII in both studied cell sub-lines under the action of anticancer drugs used. The most prominent effect on the MCF-7(wt) cells was found for the cisplatin action, while in MCF-7(DOX/R) cells – for the doxorubicin action (Fig. 2, D). Earlier, it has been established that anticancer drugs doxorubicin and cisplatin decrease the expression of the mRNA coding for T RII in the human lung adenocarcinoma A549 cells, and cause the changes in the post-receptor Smad-dependent TGF- β_1 signaling pathway [37].

In summary, we have got the evidence that the resistance of the human breast carcinoma cells to doxorubicin is a multi-factorial phenomenon. The multi-factorial resistance to doxorubicin might explain the cross-resistance of MCF-7(DOX/R) cells to other compounds (methotrexate and cisplatin) which are even not structurally related.

The following conclusions can be suggested taking into account the results obtained: 1) tumor cell refracto-

ness of the MCF-7(DOX/R) cells to both doxorubicin and TGF- β_1 was found; 2) the studied anticancer drugs (doxorubicin, methotrexate and cisplatin) can inhibit the transduction of TGF- β regulatory signals in both cell sub-lines tested owing to the violation of the corresponding signal pathway at different levels, particularly by decreasing the expression of the mRNA coding for T RII; 3) TGF- β might affect the response of the breast cancer cells to the anticancer drugs, thereby, allowing for a possibility of chemotherapeutic modulation.

Вплив протипухлинних препаратів на клітини карциноми
молочної залози, чутливі та стійкі до доксорубіцину:
експресія мРНК ТФР- β і його рецепторів

І. В. Чорна, О. В. Федоренко, Р. С. Стойка

Резюме

Мета. Порівняльний аналіз впливу протипухлинних препаратів (доксорубіцину, метотрексату і цисплатину) та ТФР- β на клітини карциноми молочної залози людини лінії MCF-7, чутливі (wt) і стійкі (DOX/R) до дії доксорубіцину. **Методи.** Напівкількісну полімеразну ланцюгову реакцію (ПЛР) використовували для визна-

чення експресії мРНК ізоформ ТФР-₁ і ТФР-₂ та рецепторів ТФР-₁ і II типу (T RI і T RII). Метод видуження трипанового синього застосовано для виявлення кількості і життєздатності клітин. **Результати.** Клітинам лінії MCF-7(DOX/R) притаманна більша стійкість до рістінгібувальної дії ТФР-₁, ніж клітинам лінії MCF-7(wt). Рівень мРНК ТФР-₁ його рецепторів виявився вищим у необроблених клітинах MCF-7(DOX/R) порівняно з клітинами MCF-7(wt). Експресія мРНК T RII знижувалася в обох клітинних лініях, оброблених доксорубіцином, метотрексатом і цисплатином, тоді як падіння рівня мРНК T RI спостерігали лише в клітинах MCF-7(DOX/R), підданих впливу доксорубіцину і метотрексату. **Висновки.** Встановлено відмінності в дії досліджених протипухлинних препаратів і ТФР-₁ на доксорубіцин-чутливі і -стійкі клітини. Подальші дослідження необхідні для з'ясування молекулярних механізмів уникнення рістінгібувальної дії ТФР-₁ клітинами лінії MCF-7(DOX/R).

Ключові слова: MCF-7, ТФР-₁, доксорубіцин, метотрексат, цисплатин, резистентність.

Влияние противоопухолевых препаратов на клетки карциномы молочной железы, чувствительные и устойчивые к доксорубицину: экспрессия мРНК ТФР-₁ и его рецепторов

И. В. Черная, А. В. Федоренко, Р. С. Стойка

Резюме

Цель. Сравнительный анализ влияния противоопухолевых препаратов (доксорубицин, метотрексат и цисплатин), а также ТФР-₁ на клетки карциномы молочной железы человека линии MCF-7, чувствительные (wt) и устойчивые (DOX/R) к действию доксорубицина. **Методы.** Полуколичественную полимеразную цепную реакцию (ПЦР) использовали для определения экспрессии мРНК изоформ ТФР-₁ (ТФР-₁) и рецепторов ТФР-₁ I и II типа (T RI и T RII). Метод исключения трипанового синего применен для установления количества и жизнеспособности клеток. **Результаты.** Клеткам линии MCF-7(DOX/R) свойственна большая устойчивость к ростингибирующему действию ТФР-₁, чем клеткам линии MCF-7(wt). Уровень мРНК ТФР-₁ и его рецепторов оказался выше в необработанных клетках MCF-7(DOX/R) по сравнению с клетками MCF-7(wt). Экспрессия мРНК T RII снижалась в обеих клеточных линиях, обработанных доксорубицином, метотрексатом и цисплатином, тогда как падение уровня мРНК T RI выявлено только в клетках MCF-7(DOX/R), подвергшихся влиянию доксорубицина и метотрексата. **Выводы.** Установлены различия в действии изученных противоопухолевых препаратов и ТФР-₁ на доксорубицин-чувствительные и -устойчивые клетки. Дальнейшие исследования необходимы для выяснения молекулярных механизмов избежания ростингибирующего действия ТФР-₁ клетками линии MCF-7(DOX/R).

Ключевые слова: MCF-7, ТФР-₁, доксорубицин, метотрексат, цисплатин, резистентность.

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Received 14.05.13