PREFERENTIAL COEXPRESSION OF THE FUNCTIONALLY ACTIVE RECEPTORS FOR EGF AND TRANSFERRIN IN SOME HUMAN TUMOR CELL LINES OF THE EPITHELIAL ORIGIN

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КОЭКСПРЕССИЯ ФУНКЦИОНАЛЬНО АКТИВНЫХ РЕЦЕПТОРОВ ЭФР И ТРАНСФЕРРИНА В ЧЕЛОВЕЧЕСКИХ ОПУХОЛЕВЫХ КЛЕТКАХ ЭПИТЕЛИАЛЬНОГО ПРОИСХОЖДЕНИЯ

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Expression of epidermal growth factor receptor (EGF-R) and transferrin receptor (Trf-R) have been studied by flow cytometry in 14 human tumor cell lines. A positive and highly significant correlation between the levels of the EGF-R and Trf-R has been observed in tumor cell lines of epithelial origin. No such relationship was found in tumor cells of other histogenesis, except of HT-1080 fibrosarcoma cell line. It was also shown that EGF-R and Trf-R determined by flow cytometry are functionally active because exogenously added EGF and transferrin induced DNA synthesis in cells under study. We conclude that coexpression of EGF-R and Trf-R is specific for tumor epithelial cells and may serve as one of the mechanisms for maintenance of the neoplastic state. In addition, reported findings are extensively discussed as an experimental basis for clinical settings.

***Key Words*: EGF receptor, transferrin receptor, human tumor cells, flow cytometry, DNA synthesis.**

С помощью метода проточной цитофлуориметрии определен уровень экспрессии рецептора эпидермального фактора роста (ЭФР) и рецептора трансферрина на поверхности опухолевых клеток человека 14 перевиваемых линий. В опухолевых клетках эпителиального происхождения выявлена положительная и статистически достоверная корреляция между экспрессией рецепторов ЭФР и трансферрина. В опухолевых клетках иного гистогенеза (кроме клеток фибросаркомы линии НТ-1080) подобная корреляция не выявлена. Кроме того, экзогенно добавляемые ЭФР и трансферрин стимулируют пролиферацию в анализируемых клетках, что свидетельствует о функциональной активности рецепторов ЭФР и трансферрина, определяемых с помощью метода проточной цитофлуориметрии. Полученные результаты позволяют сделать вывод о том, что для опухолевых клеток эпителиального происхождения характерна коэкспрессия рецепторов ЭФР и трансферрина. На основании полученных данных обсуждаются возможности использования антител против рецепторов ЭФР и трансферрина в терапии пациентов с онкологическими заболеваниями.

***Ключевые слова*: рецептор ЭФР, рецептор трансферрина, опухолевые клетки человека, проточная цитофлуориметрия, синтез ДНК.**

The loss of growth control is one of the important properties common to all transformed cells. At least 3 mechanisms of growth control disorders were delineated: disturbed balance of stimulatory *versus* inhibitory extracellular signals; abnormal cellular "interpretation" of such signals; and short–circuiting of the steps of growth–regulated pathways [1]. In this context the balance of growth–stimulatory and growth–inhibitory factors could be disarranged in two ways: (i) hyperproduction of growth–stimulatory factors and/or overexpression of their receptors; and (ii) abnormalities in the production of growth–inhibitory factors and/or expression of their receptors. There is a great deal of evidence that an increase in the number or apparent affinity (or both) of growth factor receptors on the cell surface might promote an increased responsiveness to ligand and aberrant stimulation of the growth.

Overexpression of epidermal growth factor receptor (EGF–R) was found in a wide variety of human malignancies [2]. It was shown that the presence and overexpression of c–erbB oncoprotein (EGF–R) has been often associated with the enhanced tumor aggressiveness [3–5]. Epidermal growth factor (EGF), TGF–α, amphiregulin, schwannoma–derived growth factor, heparin–binding EGF, betacellulin, and epiregulin are the members of superfamily ligands for the EGF–R. In certain cells the overexpression of these EGF–related cytokines can lead to malignant transformation (reviewed in [6]).

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Abbreviations used: EGF–R — epidermal growth factor receptor; FCA — flow cytometric analysis; TNF — tumor necrosis factor; Trf–R — transferrin receptor.

Transferrin (Trf) is a major iron–binding serum glicoprotein interacting with specific receptors on cell surface. A direct relationship between expression of the Trf receptor (Trf–R) and proliferative capacity of cells was established [7, 8]. The levels of Trf–R in urinary bladder carcinoma [9], colorectal [10], and brain [11] cancers were significantly higher than in normal tissues of similar origin. Moreover, the presence Trf–R in tumor tissue may be an indicator of more unfavorable prognosis in patients with non–small cell lung cancer and oral tumors [12, 13]. These data indicate that both Trf–R and EGF–R can be involved in aberrant regulation of tumor cell proliferation. However, their role in maintaining the malignant phenotype remains to be elucidated.

The aim of the present investigation was to study EGF–R and Trf–R expression using large panel of human tumor cell lines of different histogenesis. All tumor cells of epithelial origin used in the study turned out to coexpress functionally active EGF–R and Trf–R.

MATERIALS AND METHODS

Cytokines and antibodies. EGF from mouse submandibular glands (receptor grade) was purified by hydrophobic chromatography as described earlier [14]. Highly purified human recombinant tumor necrosis factor α (TNF–α, $5 \cdot 10^7$ U/mg) was gifted by Dr. V. Korobko (Institute of Bioorganic Chemistry, Moscow, Russia). Human Trf was purchased from Sigma (USA). Monoclonal anti–EGF–R antibody R1 was kindly provided by Dr. I. Gout (Ludwig Institute for Cancer Research, UK). Fluorescein 5–isothiocyanate (FITC)–conjugated monoclonal anti–human Trf–R antibody was purchased from Becton Dickinson (USA).

Cell cultures. Fourteen human tumor cell lines were used: A–431 (squamous vulvar carcinoma), A–549 (lung adenocarcinoma), CaOv (ovary carcinoma), COLO–320 HSR (colon adenocarcinoma), EwS (Ewing's sarcoma; established by Yu.I. Kudryavets from Ewing's sarcoma tumor, transplanted into the nude mice), HeLa (cervical epithelioid carcinoma), HEp–2 (laryngeal carcinoma), HT–1080 (fibrosarcoma), IMR–32 (neuroblastoma), K–562 (chronic myelogenous leukaemia), KB (epider–moid carcinoma), Mg–63 (osteosarcoma), PA–1 (ovary teratocarcinoma), and U–937 (histiocytic lymphoma) cells. Cell lines were purchased from Cell Line Collection of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). All cells were cultured in RPMI 1640 medium supplemented with 10% FCS at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in 50 cm^2 tissue culture flasks or 24–well plates (Nunclon, Denmark).

Flow cytometric analysis. In flow cytometric analysis (FCA) all cell lines were studied in the late phase of logarithmic growth. Single–cell suspensions of tumor cells were washed twice with phosphate–buffered saline (PBS) and 10^5 tumor cells in 50 μl BSA–PBS were incubated with 10 μl of FITC–labeled anti–Trf–R antibody. Incubation was carried out at 4°C to prevent inter–nalization of the antibody–antigen complex. Cells were then washed twice with 2 ml of BSA–PBS at 4°C and propidium iodide was added to each sample to a final concentration 2 μg/ml to allow the exclusion of dead cells from analysis. The cell fluorescence was analyzed using FACScan flow cytometer (Becton Dickinson, USA) equipped with 15 mW argon laser set at 488 nm. No less than 10^4 living cells in each experiment were analyzed. Relative amounts of surface Trf–R were quantitated by calculating the difference in fluorescence intensities between cells exposed and not exposed to the antibody. For indirect FCA tumor cells incubated with 10 μl of monoclonal anti–human EGF–R antibody R1 and then washed twice and resuspended in BSA–PBS containing 5 μl of FITC–labeled goat anti–mouse IgG (Becton Dickinson, USA). The results were analyzed by the LYSYS software (Becton Dickinson, USA).

^{3}[H]–Thymidine incorporation. $3–4 \cdot 10^4$ cells were plated in each well of 24–well plates. The medium was replaced by serum free medium (SFM) for 26–30 h, and then either single cytokine (EGF, Trf, TNF–α) or their combinations in fresh SFM were added for up to 20 h. EGF, Trf, and TNF–α were tested in doses 6 nM, 10 nM, and 1000 U/ml, respectively. Upon incubation, methyl–^{3}H–thymidine (0.5 μCi per well) was added in SFM for at least 4 h. The medium in each well was aspirated and cells were resuspended in trypsin/versene before the addition of 20% TCA. The cells were harvested 1.5 h later on the filter mats (Wallac Oy, Finland) by washing the wells two times in 10% TCA and water and then drying the filter mats at 60°C for 30 min. Each mat was then counted in Betaplate 1205 counter (Wallac Oy, Finland).

Data analysis. For estimating the levels of EGF–R and Trf–R expression on the surface of tested cells we used the Kolmogorov—Smirnov statistics, allowing us to estimate the level of differences between histograms of specifically labeled and unlabeled cells [15]. Student's *t* test was used to assess statistical significance in the cell growth experiments. A statistical difference was considered significant when $P < 0.05$. The *r* correlation coefficient between EGF–R and Trf–R status was computed using least squared regression analysis. *P* values reported are two–sided.

RESULTS

The representative histograms of human tumor cell lines studied by FCA are shown in Fig. 1. To measure relative amounts of the cell surface EGF–R and Trf–R in different cell lines we used D/s(n) value indicative of the similarity between the compared curves [15]. The closer to zero D/s(n) is the more alike two curves are. The null hypothesis was rejected with probability lower than $P < 0.001$ (given D/s(n) = 14.1). In accordance with this D/s(n) > 14.1 was used as a cutoff value for EGF–R or Tfr–R positivity.

As seen in Fig. 1 and Table 1, high amounts of EGF–R (D/s(n) ranged from 20 to 35) were found in A–431, HEp–2, HeLa and KB cell lines. A–549, CaOv, HT–1080 and COLO–320 HSR cells expressed EGF–R, but to a lesser degree than above mentioned cell lines. No significant difference was observed between control and R1 histograms for IMR–32, MG–63, PA–1, EwS, K–562 and U–937 cells (D/s(n) is ≤14.1). At the same time, U937,

Table 1. Comparative analysis of EGF–R and Trf–R expression levels in human tumor cells under study according to the D/s(n) value

EGF–R		Trf–R	
> 14.1	≤ 14	> 14.1	≤ 14.1
Cell lines			
A–431	IMR–32	U–937	PA–1
U–373 MG	Mg–63	K–562	Mg–63
HEp–2	EwS	EwS	
HeLa	PA–1	HEp–2	
KB	U–937	A–431	
CaOv	K–562	KB	
HT–1080		HeLa	
COLO–320 HSR		A–549	
		CaOv	
		HT–1080	
		COLO–320 HSR	
		IMR–32	

K562, EwS, HEp–2 and A–431 cells expressed high number of Trf–R (D/s(n) ranged from 34 to 40). Significantly lower amounts of Trf–R were found in A–549, KB, HeLa, CaOv, HT–1080, COLO–320 HSR and IMR–32 cell lines. PA–1 and MG–63 cells did not demonstrate any specific reactivity with anti–Trf–R antibody.

EGF–R and Trf–R status of human tumor cell lines under study is summarized in Table 2. As shown, all eight EGF–R–positive cell lines were Trf–R–positive while only two of 6 EGF–R–negative cell lines were Trf–R–negative. The findings of FCA were generally in good agreement with the results of radioreceptor assays (data not shown). In addition, comparison of the EGF–R and Trf–R status shows (Fig. 2) that these two endpoints for cells of epithelial origin have a positive correlation (r = 0.86). In contrast, in tested cells of another origin such a relationship is not fulfilled.

In order to demonstrate a functional activity of EGF–R and Trf–R, we have examined the mitogenic response of tested cells to exogenous EGF and Trf.

Table 2. EGF–R and Trf–R status in human tumor cell lines under study

Receptor status	EGF–R–positive	EGF–R–negative	Total
Trf–R–positive	8	4	12
Trf–R–negative	0	2	2
Total	8	6	14

Three tumor cell lines were chosen for this purpose: A–431 cells (with high level of both EGF–R and Trf–R expression), HT–1080 cells (with low level of both EGF–R and Trf–R expression), and PA–1 cells which did not show any EGF–R or Trf–R expression.

As shown in Fig. 3, in A–431 carcinoma cells EGF was very effective in promoting a decrease in ^{3}[H]–thymidine incorporation over basal values. In contrast, treatment of serum–free A–431 cells with Trf enhanced DNA synthesis in these cells. A–431 cell growth was also accelerated by combination of both EGF and Trf. Interestingly, A–431 cells do not respond to EGF, Trf, and TNF–α when these factors were added together (Fig. 3).

EGF by itself, stimulated growth of HT–1080 fibrosarcoma cells (Fig. 3). Similarly, Trf increased the proliferation of HT–1080 cells, although to a lesser degree than in A–431 cells. EGF and Trf synergistically enhanced DNA synthesis in HT1080 cells. Although TNF–α alone inhibited growth of these cells, EGF and Trf diminished this effect and weakly increased cell proliferation. EGF was incapable to stimulate serum–free growth of PA–1 teratocarcinoma cells either alone or in combination with Trf or TNF–α (Fig. 3).

DISCUSSION

According to the data of the comparative analysis of EGF–R and Trf–R in the human tumor cell lines under study could be classified into three major groups. All 8 EGF–R–positive tumor cell lines turned out to share Trf–R, being therefore EGF–R/Trf–R–positive. Four of

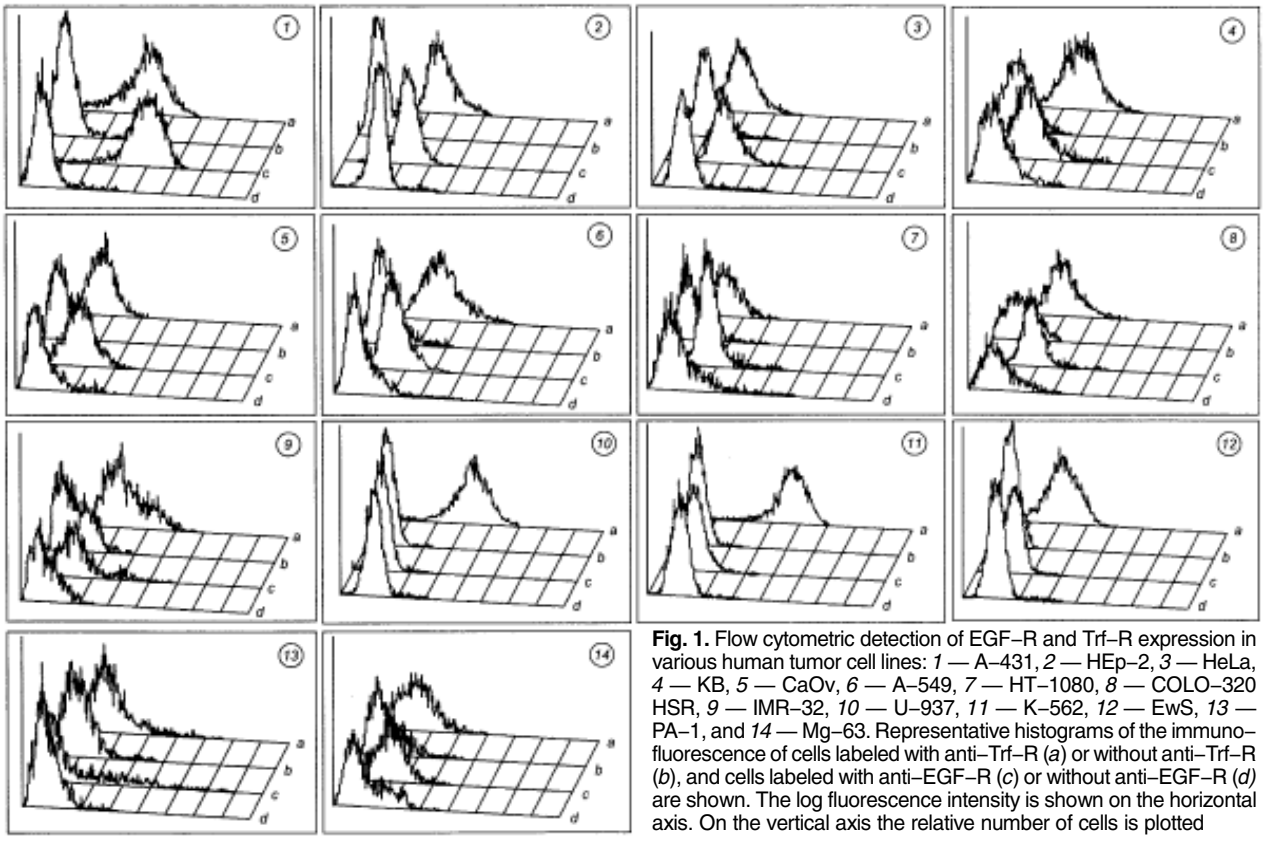

Fig. 1. Flow cytometric detection of EGF–R and Trf–R expression in various human tumor cell lines: *1* — A–431, *2* — HEp–2, *3* — HeLa, *4* — KB, *5* — CaOv, *6* — A–549, *7* — HT–1080, *8* — COLO–320 HSR, *9* — IMR–32, *10* — U–937, *11* — K–562, *12* — EwS, *13* — PA–1, and *14* — Mg–63. Representative histograms of the immunofluorescence of cells labeled with anti–Trf–R (*a*) or without anti–Trf–R (*b*), and cells labeled with anti–EGF–R (*c*) or without anti–EGF–R (*d*) are shown. The log fluorescence intensity is shown on the horizontal axis. On the vertical axis the relative number of cells is plotted

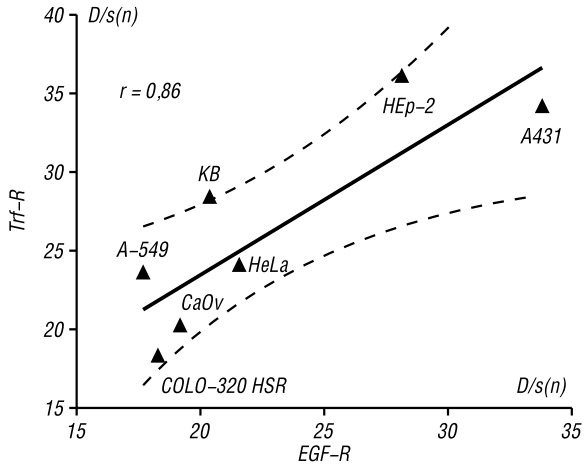

Fig. 2. Correlation between EGF–R and Trf–R status in human tumor cells of epithelial origin

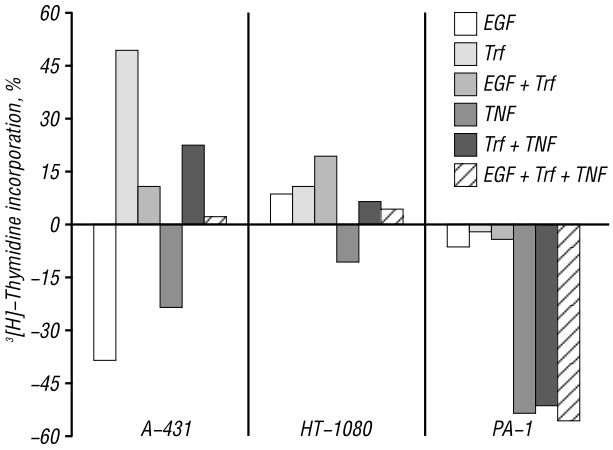

Fig. 3. Effect of EGF, Trf, and TNF–α on proliferation of A–431, HT1080 and PA–1 cells measured by ^{3}[H]–Thymidine incorporation (0.5 μCi/well added) into quiescent cells after addition of exogenous cytokines. Data are expressed as percentage of incorporation relative to control

12 Trf–R–positive cell lines (EwS, IMR–32, K–562, U–937) did not express EGF–R (EGF–R–negative/Trf–R–positive cells). MG–63 and PA–1 cells were EGF–R/Trf–R–negative. The evidence obtained are therefore in favour of coexpression of EGF–R and Trf–R characteristic of all lines of epithelial origin, except for HT–1080 fibrosarcoma cells. The findings have been further corroborated by the correlation analysis (Fig. 2). In fact, this phenomenon is quite consistent taking into account the maximum proliferative capacity pertaining to the epithelium. Such a capacity should be maintained by both specialized and versatile growth stimulation agents such as EGF and Trf respectively.

The coexpression of EGF–R and Trf–R has also been reported in several *in vivo* studies. C. Limas [16] found that four of six patients with invasive urotelial neoplasms whose biopsies were positive for Tfr–R also demonstrated strong EGF–R staining. Other authors have obtained similar results with adenocarcinoma of the prostate [17]. Interestingly, EGF–R and Trf–R tended to be coexpressive in malignant epithelial cells.

The response of A–431 and HT–1080 cell lines to EGF or Trf demonstrates that EGF–R and Trf–R are capable of transducing regulatory signals initiated by these factors. The data on HT–1080 cells suggest that Trf could potentiate mitogenic effect of EGF. This result is consistent with the data obtained in both normal and tumor cells [18, 19]. Several reports demonstrating that EGF binding to EGF–R have resulted in rapid (within 5 min) mobilization of Trf–R to the cell surface of normal fibroblasts and NIH 3T3 cells [20, 21]. In epidermoid carcinoma KB cells, formation of prodigious ruffles in the plasma membrane was observed after treatment with EGF and correlated with up–regulation of cell surface Trf–R [22]. One might speculate that the synergy between EGF and Trf in our study is a consequence of Trf–R transmodulation by EGF.

The data reported here may have additional importance. Simultaneous detection of EGF–R and Trf–R expression by FCA may be a valuable method for a precise evaluation of the tissue origin of the tumor under study. More important, our findings provide an experimental basis for clinical settings. Several cancer treatment modalities targeted at EGF–R or Trf–R are under development, including the anti–receptor antibodies coupled to the toxic agents or radionuclides [23, 24]. In particular, a few bifunctional single–chain immunotoxin fusion proteins with the ability to bind specifically to EGF–R or Trf–R and other tumor–associated antigens have been recently engineered [25, 26]. Moreover, C. Somasundaram *et al*. [27] reported trispecific $F(ab')_3$ antibody conjugate (TAC) with specificities for the Fc gamma receptor I (Fc gamma RI/CD64), EGF–R and HER2/neu receptor. The TAC was cytotoxic to cancer cells expressing the targeted tumor–associated receptors. Compared with conventional EGF–R monoclonals bi– and trispecific antibodies induced significantly enhanced cytotoxicity. The results described herein suggest that EGF–R– and Trf–R–directed immunotoxins or radioimmunoconjugates may offer an perspective approach for targeting epithelial malignancies refractory to conventional chemotherapeutic strategies.

Finally, from the screening assay by FCA performed in 14 human tumor cell lines it was concluded that all human tumor cells of epithelial origin studied in present work coexpressed EGF–R and Trf–R. Moreover, EGF–R and Trf–R detected by FCA are functionally active as reflected by increased DNA synthesis in response to exogenous EGF and Trf. In HT–1080 cells, EGF and Trf act synergistically when present together. It is possible that transmodulation of Trf–R by EGF is directly involved in a stimulatory effect of EGF on Trf–induced cell proliferation.

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