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CLINICAL SIGNIFICANCE OF ONCOGENES AND GROWTH FACTORS IN OVARIAN CARCINOMAS

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Summary—The expression of epidermal growth factor receptor (EGF-R), transforming growth factor alpha (TGFα) and the c-*myc* oncogene was investigated in different specimens of gynecologic carcinomas. EGF specific binding sites were detected in about 50% of adenocarcinomas (ovarian, endometrial, breast) and in over 90% of squamous carcinomas (cervical). There is a positive correlation between the EGF-R binding assay, immunohistochemistry and the relative amounts of mRNA by Northern blotting. TGFα was investigated by immunohistochemistry and Northern blotting. TGFα immunoreactivity was detected exclusively in the epithelial cells of nonmalignant tissues (skin, cervix, endometrium, large bowel, lung) as well as different ovarian carcinomas. The TGFα immunostaining score correlates with the TGFα mRNA amounts. The c-*myc* expression was analyzed by Northern blotting in the specimens of ovarian carcinomas. Whereas, a positive correlation between the c-*myc* and TGFα expression was noticed, no correlation existed between EGF-R and c-*myc* expression. Progressive disease (PD) of ovarian carcinomas after chemotherapy was mainly noticed in the group of EGF-R$^-$ tumors and those with high amounts of c-*myc* mRNA. EGF-R$^+$ ovarian carcinomas responded significantly better to chemotherapy. However, similar survival times existed between the EGF-R$^+$ and EGF-R$^-$ group and the survival times of patients having responded to the treatment was reduced in the EGF-R$^+$ group. This indicates that EGF-R$^+$ and those carcinomas expressing high amounts of c-*myc* constitute a more aggressive group of ovarian carcinomas.

INTRODUCTION

Ovarian cancer, with a 5 yr survival rate of about 20–30%, is the most fatal gynecological cancer [1]. The prognosis of patients with advanced ovarian carcinoma depends on the operability, the histological subtype, the tumor differentiation and the response to chemotherapy, which demonstrates that there are different malignant phenotypes [2]. In the last few years, much attention has been focused on the role of the activation of oncogenes and growth factors (GF) in the development of human malignancies. It has been assumed that inappropriate functions of these genes influence the malignant phenotype and may be used as prognostic markers. Apparently in breast cancer the amplification of the oncogene ergB-2 [3] and the increased expression of EGF receptors (EGF-R) are indicators of early recurrence and reduced survival probability [4]. Similar associations may also exist for ovarian carcinomas [3, 5–7] and in cervical carcinomas the c-*myc* expression [8] has prognostic implications.

Our investigations focused on the analysis of the parameters of the EGF system in ovarian carcinomas, especially the transforming growth factor alpha (TGFα), EGF-R and c-*myc* as one of the early responses of the EGF signal transduction pathway [9]. TGFα is a single-chain polypeptide that stimulates the growth of various mammalian epithelial cells [10]. The biological actions of TGFα are mediated through binding to the cell membrane-bound EGF-R [11], which increases its tyrosine kinase activity [12] and triggers the mitogenic signal transduction [12]. TGFα is synthesized by many solid tumors [13] and by the embryo itself [14], modifying tumor growth [15] through autcrine or paracrine mechanisms. However, studies with TGFα cDNAs and with antibodies were able to detect the expression of this gene and the factor production, not only in tumor tissues, but also in normal adult cells [16, 17]. Analysis of rat and human cDNAs has indicated that TGFα is synthesized as part of a large precursor [18]. Transformed cells that express TGFα accumulate substantial amounts of bioactive TGFα high molecular

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weight forms having mitogenic properties similar to the mature low 6 kDa form [19].

The expression of EGF-R, TGFα and c-*myc* in different ovarian carcinomas were investigated by means of molecular–chemical, biochemical and immunohistochemical methods. The results were compared with each other and the relation to clinical prognostic factors and the survival rate of the patients was analyzed.

MATERIALS AND METHODS

^{125}I-Labeled EGF was obtained from Amersham/Buchler (Braunschweig, F.R.G). A Eco RI 1.8 kb fragment of EGF-R cDNA corresponding to the extracellular domain of the receptor protein was a gift from M. Waterfield (Ludwig Institute for Cancer Research, London), the 1.4 kb Cla I/Eco RI fragment of a c-*myc* oncogene cDNA (exon III) was obtained from G. Bornkamm (Virology, University of Freiburg) and an Eco RI 1.4 kb fragment of human TGFα cDNA was obtained from R. Walker (Triton Biosciences, San Francisco, U.S.A.). The cDNA probes were labeled by random primer extension to a specific activity of about 5×10^8 dpm/μg. The TGFα monoclonal antibody was kindly provided from Triton Biosciences (San Francisco U.S.A.) and the secondary biotinylated horse anti-mouse IgG antibody was obtained from Vector Laboratories-Atlanta (Heidelberg, F.R.G.).

The surgical specimens obtained during operations were immediately frozen in liquid nitrogen and stored at -70°C for subsequent biochemical immunohistochemical and RNA analysis. The EGF-R binding assay was performed on a crude membrane preparation from the tumor specimens by a single-point assay with 0.2 ng ^{125}I-EGF as described previously [5, 6, 23].

The RNA analysis was done on total cellular RNA isolated from frozen tissues by the quanidinum–isothiocyanate cesium chloride method, electrophoresed in a 1% agarose 2.2 M formaldehyde gel, hybridized and washed under stringent conditions as described elsewhere [7, 21].

The EGF-R and TGFα immunohistochemistry was performed on cryostat sections of tumors with a monoclonal EGF-R antibody (Amersham Buchler, F.R.G.) and a monoclonal TGFα antibody (Triton Biosciences) followed by a sandwich technique with the avidin biotin staining reaction as reported previously [7, 20, 21].

RESULTS

Figure 1 shows the frequencies of specific EGF binding at ovarian, endometrial, breast and cervical carcinomas. It can be seen that about 50% of the gynecologic adenocarcinomas (ovarian, endometrial, breast) and over 90% of the squamous cell carcinomas of the cervix uteri are EGF-R$^+$. Between both carcinoma types there are also considerable differences in the number of EGF-R binding sites: about 10% of the adenocarcinomas and over 20% of the cervical carcinomas had binding capacities >20 fmol/mg.

The EGF-R gene structure analysis by Southern blotting did not show any modifications or gene amplification (figure not shown). However,

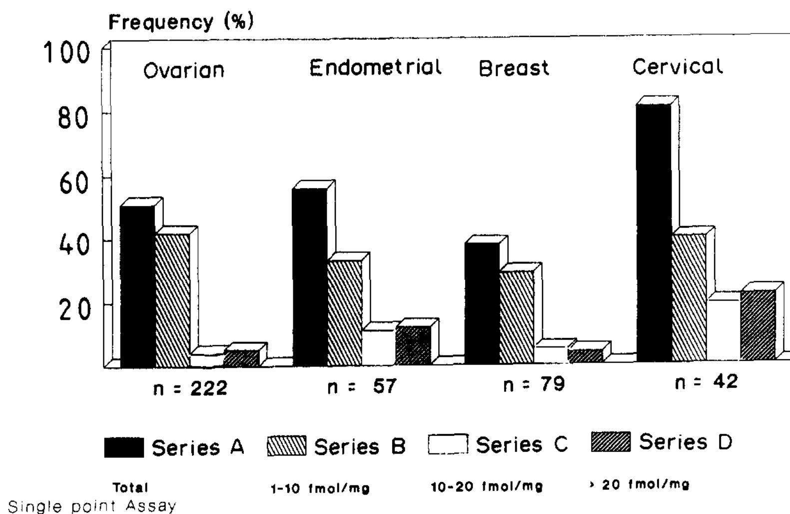


Fig. 1. EGF-R binding capacities in gynecologic carcinomas.

the expression analysis by Northern blotting detected tumors with high, low or negative amounts of EGF-R specific mRNA (Fig. 2). When the same specimens were investigated by EGF-R immunohistochemistry again tumors with positive or negative immunostaining were found as has been described elsewhere [20]. Whereas the squamous cell carcinomas usually exhibited a homogeneous membrane-staining, both EGF-R$^+$ and EGF-R$^-$ cell clones were found in the adenocarcinomas. Stromal and inflammation cells stained EGF-R$^-$. The comparison of the molecular, biochemical and immunohistochemical results showed a clear correlation [21]. The EGF-R mRNA expression rate is similar in many EGF-R$^+$ adenocarcinomas and squamous cell carcinomas. However, in squamous cell carcinomas all tumor cells are EGF-R$^+$ and in adenocarcinomas EGF-R$^+$ and EGF-R$^-$ clones usually coexist, which explains the biochemically detected differences in the receptor number.

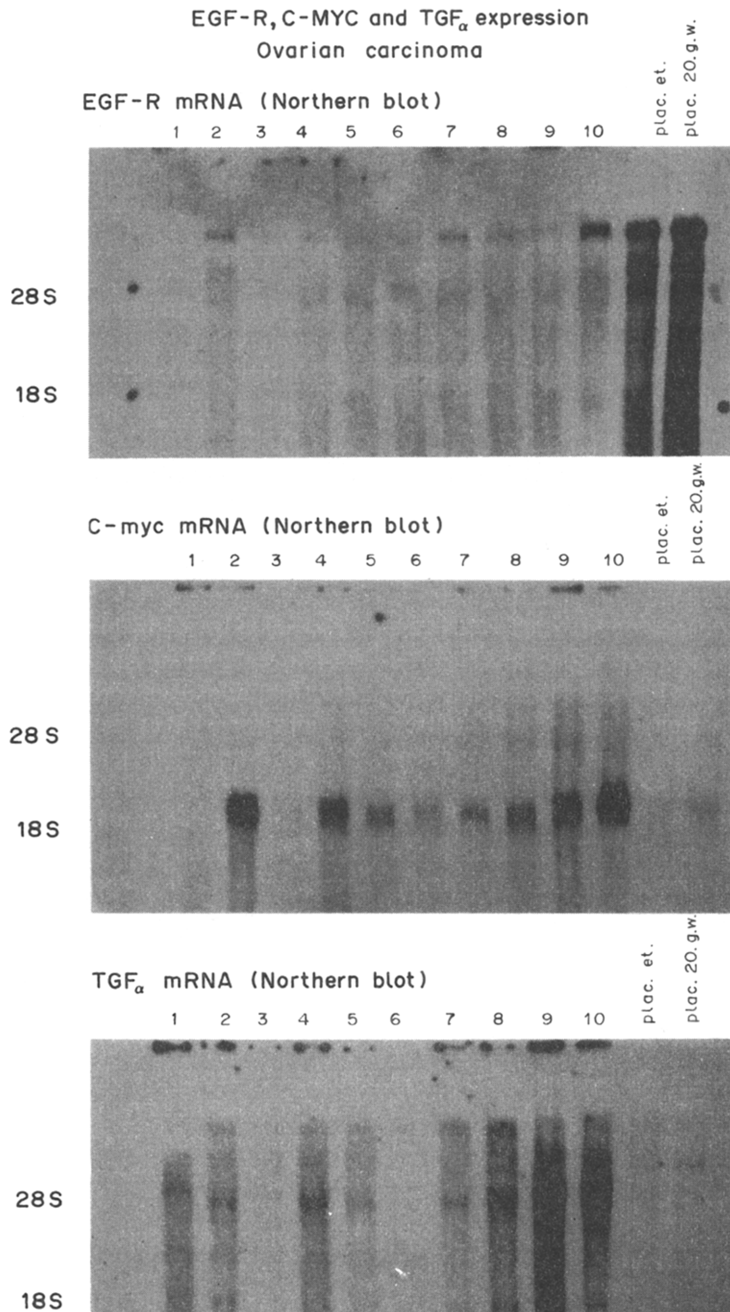


Fig. 2. EGF-R, c-*myc* and TGFα expression in ovarian carcinomas.

The expression of TGFα in malignant as well as nonmalignant tissues was then analyzed. Figure 2 shows different TGFα Northern blots from ovarian carcinoma specimens with different amounts of TGFα specific mRNA. To investigate the localization of the TGFα product and find out TGFα producing cells, ovarian carcinomas and nonmalignant tissues (skin, cervix, endometrium, large bowel, lung) were assayed by TGFα immunohistochemistry (Fig. 3). TGFα immunoreactivity was present exclusively in the epithelial cells of the investigated tissues, in both the malignant as well as nonmalignant specimens. The stromal cells and the inflammatory cells were completely TGFα negative. Coffey *et al.* [16] describe the TGFα expression in the stratified epidermis of the normal skin using *in situ* hybridization and immunohistochemistry. Our results demonstrate a similar distribution of specific TGFα immunostaining in the normal skin. TGFα immunoreactivity was also found in endometrial cells, mucosa of large bowel, in the bronchial system of the lung, as shown in Fig. 3, and in the mammary ductal cells and the tubuli of the kidney [21]. In the investigated ovarian carcinomas only the tumor cells stained TGFα$^+$. Three out of 20 cases had the highest possible staining index, two cases were TGFα$^-$ and the remaining cases were weak to middle-strong TGFα$^+$. There was a good correlation between the semiquantified immunoreactivity and the relative amounts of mRNA (Fig. 4). In earlier experiments the levels of EGF-like factors (EGF-F) were measured in tissue extracts by means of an EGF-R assay [22, 23]. This assay recognizes TGFα and EGF. The comparison of the TGFα immunostaining index, the TGFα mRNA amounts, and the EGF-F, all measured in the same specimens, shows that these values correlate, as is shown in Fig. 4.

Table 1. Ovarian carcinomas. Comparison of TGFα, EGF-R and c-*myc* mRNA

TGFα low expression	TGFα high expression
No. of cases ($n = 9$)	No. of cases ($n = 8$)
5 × low expression EGF-R 4 × high expression	5 × low expression EGF-R 3 × high expression
4 × low expression c-*myc* 5 × high expression	1 × low expression c-*myc* 7 × high expression

Northern blot.

c-*myc* Expression was analyzed in ovarian carcinomas. As can be seen in Fig. 2, there are large differences in the amounts of c-*myc* mRNA. No positive correlation exists between the c-*myc* and EGF-R expression [7]. Tumors with a high TGFα expression, however, in most cases had an increased c-*myc* expression and vice versa (Table 1).

The clinical relevance of the EGF-R, c-*myc* and TGFα was proven for ovarian carcinomas. The EGF-R status was measured in 222 ovarian carcinomas (Fig. 1) and the receptor state statistically correlated with the tumor stage, the histological subtype, the steroid receptor state, the results of *cis*-platinum combination chemotherapy, and the survival rate. No significant correlation existed to the tumor stage including recurrent disease, the tumor differentiation and the histological subtype. Three mesotheliomas ($n = 3$) and three extra-ovarian multifocal carcinomas were EGF-R$^+$ ($n = 3$) with the highest binding capacities, but only 35% of the clear cell carcinomas were EGF-R$^+$. There is a weak significant inverse correlation (<0.05) between

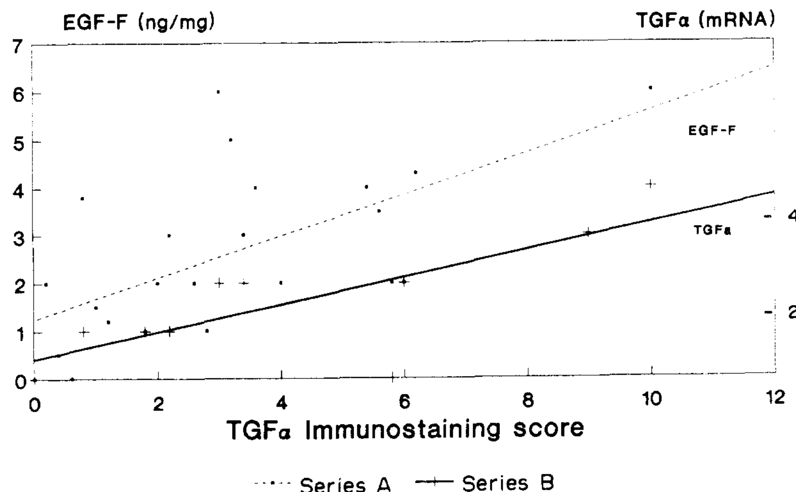
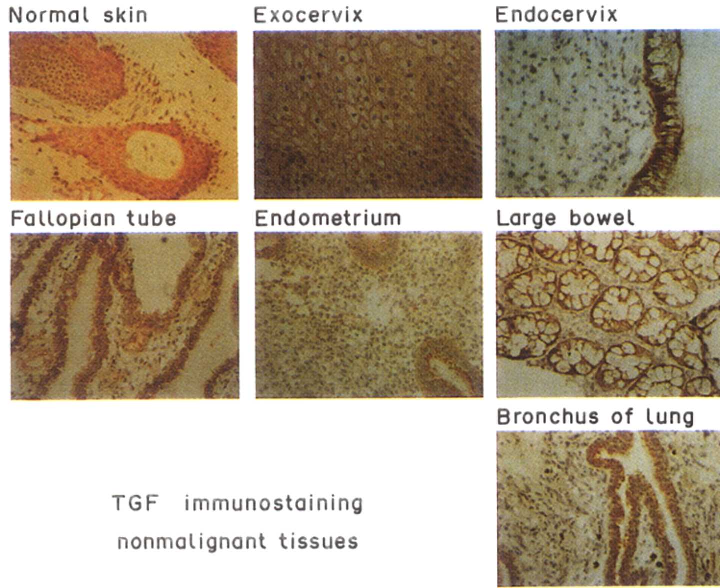


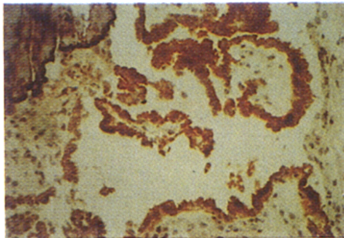
Fig. 4. Correlation of EGF-F and TGFα in ovarian carcinomas.

TGF immunohistochemistry

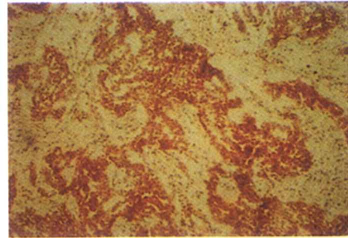
Nonmalignant tissues and ovarian carcinomas



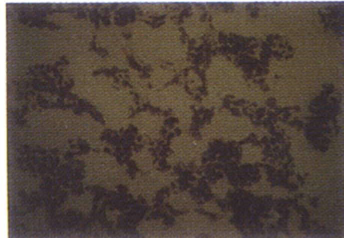
(a) Ovarian carcinoma
Strong TGF$_{\alpha}$ immunostaining



(b) Ovarian carcinoma
Medium TGF$_{\alpha}$ immunostaining



(c) Ovarian carcinoma
Negative TGF$_{\alpha}$ immunostaining



TGF$_{\alpha}$ Northern blotting
Ovarian carcinomas a,b and c

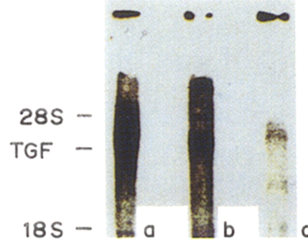
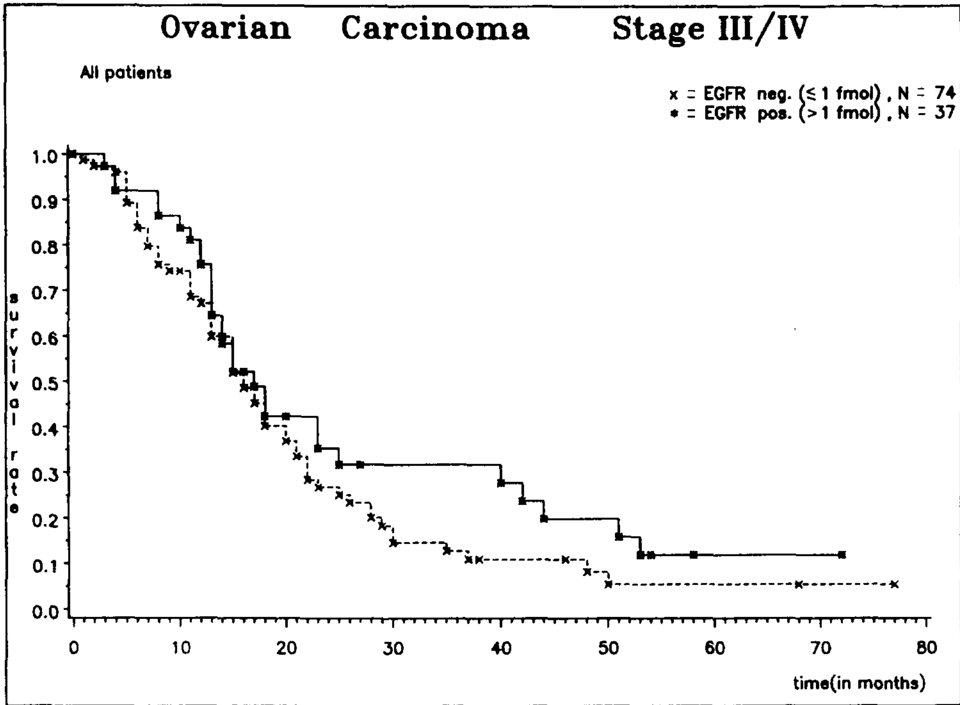


Fig. 3. TGFα immunostaining and TGFα Northern blots for the ovarian carcinomas a, b and c.



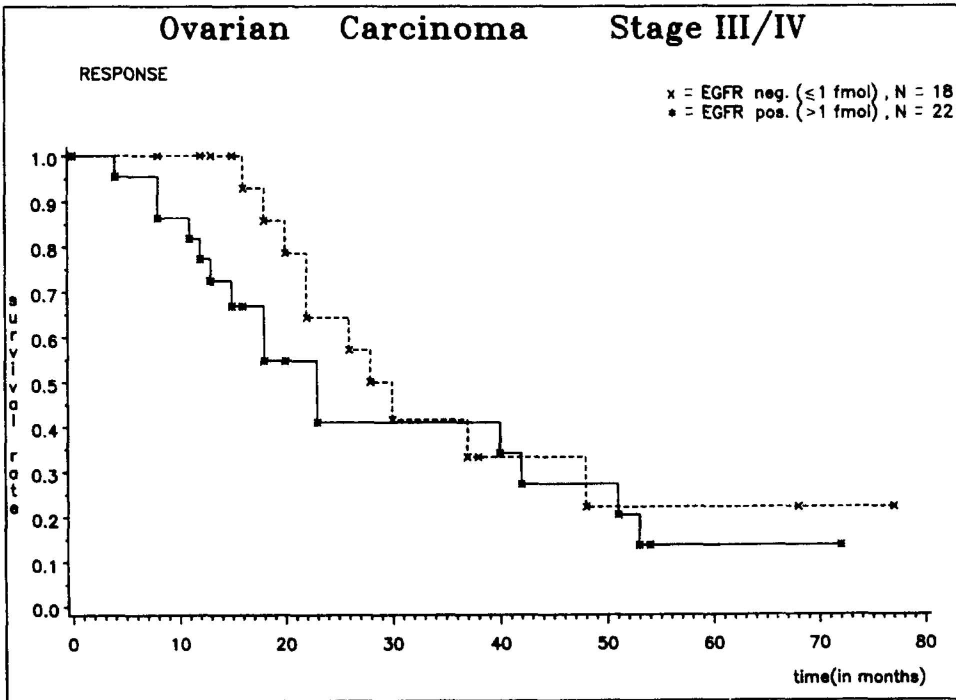


Fig. 5. EGF-R status and survival times.

Table 2. Ovarian carcinomas stage III/IV. EGF-R and results of chemotherapy[a]

	EGF-R status (%)			
Results	EGF-R(+)	EGF-R(−)	ND	Total
CR	15 (22)	7 (10)	1	23 (16)
PR	25 (36)	9 (13)	1	35 (25)
NED	6 (9)	5 (7)	1	12 (8)
NC	4 (6)	11 (16)	0	15 (10)
PD	12 (17)	38 (54)	2	52 (36)
ND	7 (10)	0 (0)	0	7 (5)
Total	69 (100)	70 (100)	5	144 (100)

[a]*cis*-platinum combination.
CR = complete remission, PR = partial remission, NED = no evidence of disease, NC = no change, PD = progression, ND = not done.

the EGF-R and the steroid receptor state (data not shown). The closest correlation was noticed between the EGF-R state and the response to *cis*-platinum chemotherapy. From 144 controlled cases with advanced ovarian carcinomas the receptor state was known in 139 cases. 40/69 (58%) EGF-R$^+$ responded to treatment, whereas only 16/70 (23%) EGF-R$^+$ cases were in remission after treatment (Table 2). However, a comparison of the survival times of both groups showed no differences. Furthermore, the survival rate of patients having responded to the treatment was reduced in the EGF-R$^+$ group. (Fig. 5). The correlation of c-*myc* expression or the TGFα data with the clinical parameters are not useful due to the small number of cases. However, progression was observed in five out of six tumors with large amounts of c-*myc*, which can be considered a tendency (Table 3).

DISCUSSION

The present study demonstrates the expression of EGF-R, TGFα as an EGF-like factor, and c-*myc* in different ovarian carcinomas. The EGF-Rs were either quantified biochemically by the EGF-R assay or semiquantitatively analyzed by immunohistochemistry or the amounts of EGF-R mRNA were checked by Northern blotting. By means of a TGFα monoclonal antibody (Triton Corp.) TGFα-specific immunostaining could also be performed and the TGFα expression could be analyzed with a TGFα probe. We also tried to measure the tissue levels of TGFα, however, for technical reasons we used an EGF receptor assay on placental membranes, which does not discriminate between TGFα or EGF [22]. We are presently developing a TGFα Elisa for the biochemical TGFα quantification in tissue extracts. Finally, the c-*myc* expression was tested by Northern blotting. In an earlier report we described c-*myc* immunohistochemistry on ovarian carcinomas [20]. However, the c-*myc* immunostain quantification did not show differences similar to those found by Northern blotting. We hope that by using different c-*myc* antibodies we will be able to find a better correlation in c-*myc* expression analysis.

Table 3. Ovarian carcinomas stage III/IV. Results of chemotherapy[a] and relative amounts of EGF-R and c-*myc* mRNA, (No. of cases)

	c-*myc*		EGF-R		
Results	High	low	High	low	Total
Progression	5	1	1	5	6
No Change	0	3	0	3	3
Remission	1	18	5	14	19
Total	6	22	6	22	28

[a]*cis*-platinum combination chemotherapy.

A comparison of the results of expression analysis on the mRNA and protein level showed a good correlation for the EGF-R and TGFα in the tested ovarian carcinoma specimens. That no gene modification was detected by Southern blotting (data not shown) indicates that tumors such as ovarian carcinomas possess variable potential to express oncogenes and GF or to activate GF pathways as is described here for the EGF system. It might be speculated that transacting factors as described for the EGF-R [24, 25] might be overproduced in some tumors and that they may be involved in pathway activation. The detection of TGFα in many different nonmalignant tissues exclusively expressed by the epithelial components and the assumption that probably only epithelial malignant tumors are able to produce TGFα support the hypothesis that the EGF/TGFα-dependent control for proliferation and differentiation of epithelial cells are mainly modified in carcinomas influencing the biological behaviour of carcinomas. The differences in c-*myc* expression in the investigated ovarian carcinoma specimens in some cases might be the result of an increased TGFα transduction signal, which would explain the coexpression of TGFα and c-*myc*. However, carcinomas without this correlation were also detected. Slamon *et al.* [3] described an overexpression of the c-*erbB*-2 oncogene in ovarian carcinomas. It might therefore be assumed that additional mitogenic pathways are involved in the activation of c-*myc*.

This study also determined to what extent these parameters might be used for a better clinical characterization of ovarian carcinomas. The EGF-R state is described as a prognostic factor in breast carcinoma for example [4]. Our study on ovarian carcinomas shows a high response rate of EGF-R$^+$ tumors to chemo-

therapy. However, the similar survival rates of both groups and the reduced survival probability in the groups which had responded to treatment suggest that EGF-R$^+$ carcinomas behave more aggressively than their EGF-R$^+$ counterparts [6]. The number of cases in this study is too low to allow a definitive conclusion. Nevertheless, tumors expressing larger amounts of c-*myc* activated by an increased TGFα signal seem to constitute a tumor subgroup that behaves aggressively. Further studies are being planned which will investigate the interaction of GF and oncogenes in a larger number of ovarian carcinomas. The goal of these studies will be to detect an aggressive subgroup of ovarian carcinomas which have not been found with conventional methods.

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