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ORIGINAL ARTICLE

The value of expression of EGFR, telomerase and topoisomerase IIα in malignant effusion smears before and after chemotherapy

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Summary

Purpose: *To evaluate the significance of expression of epidermal growth factor receptor (EGFR), telomerase and topoisomerase IIα (topo IIα) in cells of malignant effusions of patients under chemotherapy.*

Methods: *We studied the expression of EGFR, telomerase and topo IIα in malignant effusion smears of 95 cancer patients before and after chemotherapy. Immunocytochemical and in situ hybridization techniques were applied.*

Results: *Positive expression before chemotherapy of telomerase, topo IIα and EGFR was found in 64.2, 63.2 and 69.5% of the cases, respectively; the expression of these markers following chemotherapy was 43.6, 28.2 and 53.8%, respectively. The stronger prognostic factor affecting survival before chemotherapy was telomerase (p=0.0002), whereas after chemotherapy the strongest factor was EGFR (p<0.0001). A positivity for all three markers following chemotherapy was associated with shorter survival compared with positivity for only 1 or 2 markers (p<0.0001) or with a negative expression.*

Conclusion: *It seems that expression of EGFR, telomerase and topo IIα in malignant effusion smears is adversely affecting prognosis and survival.*

Key words: EGFR, malignant effusion, telomerase, topoisomerase IIα

Introduction

Resistance of tumor cells to cytotoxic drugs is a major cause of failure in cancer chemotherapy. Intracellular mechanisms exerted by these cells in achieving resistance are usually multifactorial and include activation of drug transport from the intracellular to the extracellular environment, modulation of the redox state and regulation of cell signaling-cell repair process [1,2].

EGFR, a 170-kd protein, also known as HER-1 or c-erbB-1, belongs to a family of receptors with a role preventing apoptosis; inhibition of EGFR activity induces apoptosis [3]. EGFR activation contributes to the release of vascular endothelial growth factor (VEGF), a promotor of angiogenesis. Transforming growth factor-α (TGF-α) is closely related to EGF; it can also bind to EGFR with similar effects. TGF-α is strongly correlated with micro vessels density in invasive breast cancer [4-7].

Telomerase, a ribonucleoprotein enzyme complex, is referred to as cellular immortalizing enzyme; it is responsible for the maintenance of length of chromosomal telomeres by adding hexameric (TTAGGG) repeats into the telomeric ends. Possible roles of telomeres include the prevention of end-to-end fusions of chromosomes, degradation of distal ends, rearrangements, and the eventual loss of chromosomes. Telomeres in human somatic cells undergo progressive shortening during cell division by replication-dependent loss of sequence at DNA termini. Thus, telomerase reactivation is thought to be essential for the stabilization of telomeres for attainment of cellular immortality in human carcinogenesis. Telomerase activity is mostly associated with malignant human tumors; it is only exceptionally detected in normal somatic cells. In cultured cells, 98% of immortal and none of mortal cell populations express telomerase activity [8-10]. *In vivo*, telomerase activity appears to

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be repressed in somatic cells except some reproductive somatic cells such as epidermal cells, intestinal mucosal cells, peripheral blood monocytes, and hematopoietic progenitor cells. In tumor cells telomerase activity was detected in 94% of neuroblastoma, 93% of colorectal cancer, 85% of gastric cancer, and 85% of hepatocellular carcinoma specimens. Telomerase activity was detected in almost all small cell lung cancers and in some 80% of non-small cell lung cancer specimens [11-18].

Topo IIα is a 170 KD homodimer that induces transient double-strand breaks to the DNA molecule [19]. It plays an essential role in many events of DNA metabolism, namely replication, transcription, recombination and segregation. It is overexpressed in several cancers like cervix, lung, and colon. This nuclear isozyme constitutes a target for antibiotics [20], anti-tumor drugs [21,22] or auto antibodies [23,24]. It is also involved in the pathogenesis of genetic disorders. It was shown that overexpression of topo IIα correlates with resistance of cancer cells to chemotherapy, being a target of anti-tumor strategy [25,26].

The aim of this study was to determine whether the expression of EGFR, telomerase and topo IIα in malignant pleural and peritoneal effusions obtained before and after chemotherapy are related with clinical response to chemotherapy.

Methods

We studied 95 patients with malignant effusions. Table 1 depicts the distribution of their clinicopathological characteristics; there were 35 pleural effusions from carcinomas of the lung (n=15), breast (n=18), esophagus (n=1) and malignant mesothelioma (n=1). Sixty peritoneal effusions from patients with ovarian (n=30) renal (n=2), gastric (n=8) bile duct (n=2) colorectal (n=5) and pancreatic (n=13) carcinomas were also studied. All effusions were studied before and after 14 days of chemotherapy. Sixty-four patients were male and 36 female with average age 65.1 years (±SD 10.3). The average follow up time was 9.65 ± 7.95 months (range 1-34). During the follow up period, 66/95 (69%) patients died. Follow up evaluation included physical examination, chest X-rays, MRI/CT scan and ultrasonography in relevance with each case.

All patients were initially subjected to surgical treatment of the primary tumor followed by chemotherapy.

Smears were prepared from each specimen using Papanicolaou and Giemsa stains to confirm the presence of cancer cells. After air drying, additional cytologic smears were fixed with 5% buffered formalin for 10 min and stored until the immunocytochemical study.

Immunostaining was performed by the Avidin-Biotin Complex (ABC) immunoperoxidase method using anti EGFR (clone DAK-H1-1197) and topo IIα (Clone Ki-S1) (Dako, Denmark) at dilution of 1:50 and 1:100, respectively. Smears were incubated for 45 min with a normal rabbit serum diluted 1:40 in PBS. Then, the smears were rinsed in 3 changes of PBS for 5 min each and incubated overnight with primary antibodies. After washing in PBS the smears were incubated with rabbit anti mouse biotinylated immunoglobulins diluted 1:200, followed by ABC-horseradish peroxidase treatment. Visualization was achieved by a final incubation in 3,3'-diaminoleucidine tetrahydrochloride. The smears were counted after being stained with Mayer's haematoxylin (Figures 1 and 2).

Table 1. Data of 95 studied patients with malignant effusions

Histological diagnosis	*No*
Pleural effusion	
Lung adeno Ca	15
Breast adeno Ca	18
Esophageal Ca (squamous cell Ca)	1
Malignant mesothelioma	1
Total	35
Peritoneal effusion	
Ovarian adeno Ca	30
Renal adeno Ca	2
Gastric adeno Ca	8
Bile duct adeno Ca	2
Colorectal adeno Ca	5
Pancreatic adeno Ca	13
Total	60

Males 65, females 36; Age, years (mean) 65.1 (±10.3); Median follow 9.65 ±7.95 months (range 1-34); During follow up 66/95 (69.5%) patients died

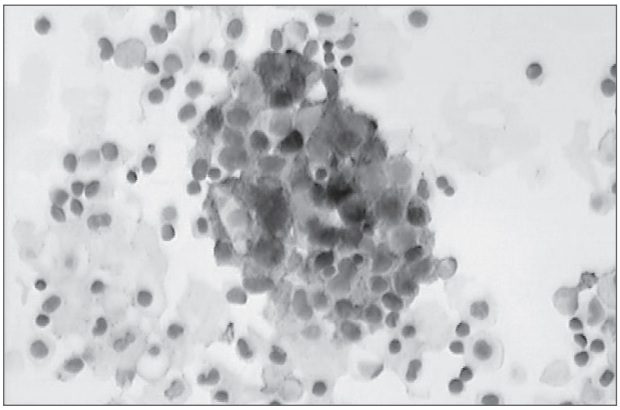

Figure 1. Pleural fluid smear; lung adenocarcinoma cells with positive cytoplasmic reaction for EGFR (×500).

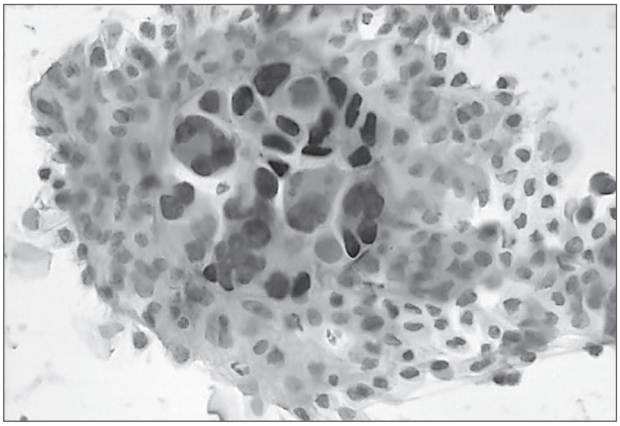

Figure 2. Peritoneal fluid smear; ovarian serous cystadenocarcinoma cells with positive nuclear reaction for topo IIα (×500).

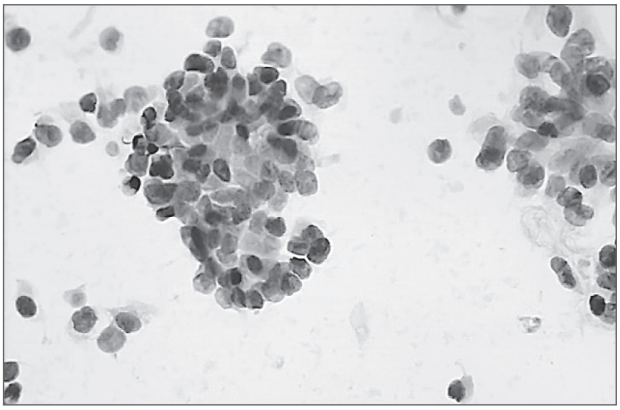

Figure 3. Pleural fluid smear; breast adenocarcinoma cells with positive nuclear reaction for telomerase (×500).

Smears of known positive reactivity for the EGFR and topo IIα were included as positive controls; as negative controls we stained specimens by omitting incubation with primary antibodies.

Human telomerase RNA expression on smear cells was detected by a standard *in situ* hybridization method as previously described [25,26]. A protocol according to the manufacturer's instructions (BioGenex San Ramon, CA USA) was followed. Results were interpreted by two independent cytologists (Figure 3). In cases in which the staining was heterogeneous in the slide examined we included those with the highest and those with the lowest percentage of stained cells. In all smears the percentage of positivity for EGFR, telomerase and topo IIα was determined by counting at least 500 cells within randomly selected areas on each slide and the percentage of immunostained cells was determined. Cytoplasmic and membranous staining for EGFR and nuclear staining for telomerase and topo IIα was positive if >10% and negative if <10% of malignant cells were stained (Figures 1-3).

Statistical considerations

The Pearson's x^2 test was applied for statistical evaluation of the expression of factors before and after chemotherapy. We used the log-rank test to evaluate patients' survival for various prognostic factors; the Kaplan-Meier method was utilized for graphic presentation. The most important parameters affecting overall survival were identified with Cox regression analysis.

Results

The immunoreaction of telomerase, topo IIα and EGFR in cytology effusions before and after chemotherapy are shown in Table 2. Positivity for telomerase, topo IIα and EGFR was observed in 64.2, 63.2 and 69.5% of the smears with positive cytology before chemotherapy, respectively. After chemotherapy the results were 17.9, 21.1 and 23.2%, respectively with respective p values of 0.99, 0.89 and 0.99. No effusion was observed in 56 cases after chemotherapy. Fourteen out of 61 smears positive for telomerase before chemotherapy were negative after chemotherapy (p=0.999), 10/60 positive for topo IIα before chemotherapy were negative after chemotherapy (p=0.08973) and 14/66 smears positive for EGFR before chemotherapy were negative after chemotherapy (p=0.999).

The Cox regression analysis showed that among all three markers studied after chemotherapy the most important marker linked with survival was EGFR (p<0.0001). Patients with a positive expression for EGFR had a 10.62-fold worse survival compared with patients with smears negative for EGFR expression. The significance of the other parameters was p=0.567 for topo IIα and p=0.094 for telomerase, respectively (Table 3).

Figure 4 shows the survival in patients with malignant effusion according to the positive expression of all three markers (EGFR, topo IIα, telomerase), 2 or only 1 of them.

Table 2. Telomerase, topo IIα and EGFR before and after chemotherapy and cases without effusion after chemotherapy

		Before chemotherapy			*After chemotherapy*			*No effusion after chemotherapy*		
		N	*%*	–	*%*	+	*%*	*N*	*%*	*p-value*
Telomerase	–	34	35.8	8	8.4	–	–	26	27.3	0.00036
	+	61	64.2	14	14.8	17	17.9	30	31.6	0.999
topo IIα	–	35	36.9	8	8.4	1	1.1	26	27.3	0.00023
	+	60	63.1	10	10.6	20	21.1	30	31.6	0.08973
EGFR	–	29	30.5	3	3.1	–	–	26	27.3	0.00030
	+	66	69.5	14	14.8	22	23.2	30	31.6	0.999

Table 3. Cox regression analysis for survival of the studied variables

		B	*SE*	*wald*	*df*	*p-value*	*Exp (B)*	*95% CI for Exp (B)*
EGFR	–							
EGFR	+	2.362	0.523	20.394	1	<0.0001	10.697	3.81-26.57

B: coefficient, SE: standard error, df: degrees of freedom, CI: confidence interval.
The significance of the other markers was for topo IIα p=0.567 and for telomerase p=0.094

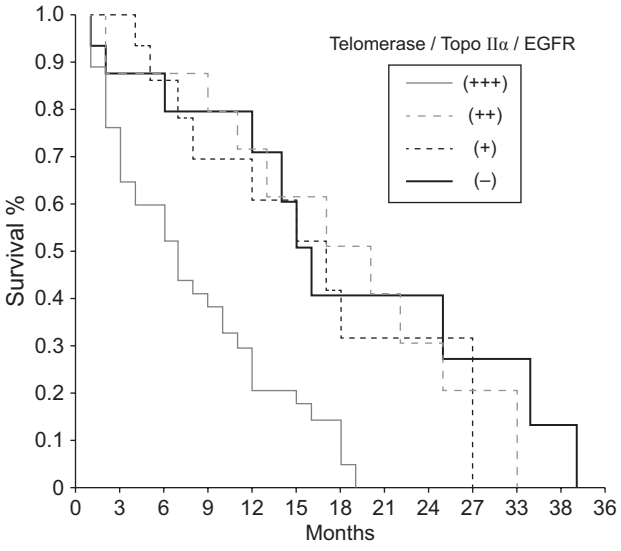

Figure 4. Patients with 3 positive markers had statistically significant worse survival compared with the other groups (p=0.0001).

Patients with all three positive markers had a worse survival compared with the other groups (p=0.0001).

Discussion

In recent years attention is being paid to several biological markers predicting response to chemotherapy [1,27].

A high telomerase activity and its subunit hTR (human telomerase RNA) has been shown to correlate with the survival of patients with various cancers [25]. More than 80% of human malignancies express telomerase activity while normal somatic cells lack this characteristic [11-18]. Telomerase activity can be detected in cells of malignant effusions, offering a potentially diagnostic adjunct in cancer detection and also a valuable prognostic indicator of response to chemotherapy [28,29].

According to Mokbel, differential expression of telomerase in cancer cells may be an attractive target in tumor management. Antisense oligonucleotides directed against the RNA template of hTR and small molecules than can interact and stabilize the G-quadruplex may represent promising therapeutic strategies [30].

In cancer cell lines, telomerase activity was found to be linked with chemosensitivity to anticancer drugs [31]. In gynecologic neoplasms the activity of telomerase was higher in neoplastic than non neoplastic conditions. Also, telomerase activity in response to cisplatin-based chemotherapy was low in cases where the drugs were effective, contrary to cases resistant to platinum where telomerase remained high [32].

This can interpret our findings associating the decreased expression of telomerase after chemotherapy with prolonged survival.

Recently, the activity of telomerase was used in the differentiation of malignant from benign tumors [33,34]. It was also noted that telomerase activity was high in advanced stages, while there was a link between the interval before relapse and also with survival.

Finally the effectiveness of telomerase on peritoneal washings was an important factor in examining residual disease in patients who were subjected to additional chemotherapy [34].

Topo IIα is a nuclear enzyme with a significant role in DNA integrity. Its overexpression is linked with resistance of cancer cells to chemotherapy. In addition topo IIα has a very important role in the separation of chromosomes during mitosis [35-37].

Topo IIα was functionally linked to oncogene p53; in order to examine the resistance or not of cancer cells to chemotherapy, p53 was found to reduce topo IIα with the use of a transcriptional mechanism; as a result the cells were rendered sensitive to cytotoxic drugs. In addition, the effectiveness of topo IIα was found to be high in cases with mutated p53, a fact that could explain the worse prognosis [38].

The effectiveness of topo IIα was also linked to the malignancy or not in various gynecological neoplasms. The expression of topo IIα was higher in ovarian carcinomas in contrast with a low expression in cystadenomas [39].

Regarding the effectiveness of anticancer drugs, topo IIα was related to chemosensitivity in gynecological tumors. This can explain the results of our study in negative samples after successful chemotherapy [39].

Similar findings regarding the prognosis in tumors expressing topo IIα were found in studies where the level of effectiveness of topo IIα was connected with histology, mitotic activity and prognosis of ovarian and endometrial cancer [40,41].

Smears with negative topo IIα expression before chemotherapy in our study showed an average survival of 17 months in contrast to 8 months of patients with positive topo IIα. Also patients with negative expression of this marker after chemotherapy had an average survival of 12 months in contrast to 4 months of patients whose topo IIα expression remained positive.

EGFR is an important factor regulating cell proliferation, differentiation and survival. Its downstream signaling pathways are involved in multiple aspects of cancer cell biology. In addition, EGFR has been identified as an important target for cancer therapy with various kinds of EGFR inhibitors currently tried in several cancers [42].

Studies have shown that EGFR overexpression

ranges from 40 to 80% in many malignant tumors; this expression is linked with poor prognosis and short survival [42,43].

In our study the results of EGFR overexpression before and after chemotherapy were connected with poor prognosis and short survival which is in accordance with several relevant studies [44-46]. The negativity of EGFR in effusion smears after chemotherapy was linked with longer survival (16 vs. 4 months) of patients with positive expression of EGFR after chemotherapy.

Studies have also shown that in ovarian carcinomas EGFR overexpression was linked with patient outcome and overall survival [45]. Contrary to this, in a paper by Elie et al. no prognostic role of EGFR in ovarian cancer and no relationship between EGFR status and clinical parameters were demonstrated [46].

A study by Bo et al. indicated that high expression of EGFR was correlated with development, invasion and metastasis of breast cancer. The high expression of EGFR is a sign of high malignant behavior and poor prognosis [42].

The identification of EGFR mutations is important for patients with primary and recurrent non-small cell lung cancer since it is valuable in predicting responsiveness to EGFR-targeted drugs [47].

In our study expression of telomerase before chemotherapy was the strongest prognostic factor for favorable response, while expression of EGFR was the strongest factor for survival after chemotherapy.

The results of the present study have demonstrated that patients with a combined positive reactivity in effusion smears for 3 or 2 of the studied markers show poor survival compared to those with only one marker positive or with negative expression.

In conclusion, the expression of telomerase, topo IIα and EGFR in malignant effusion smears of patients before and after chemotherapy is a prognostic indicator for the final disease outcome. Furthermore, the immunocytochemistry study with specific antibodies detecting EGFR, topo IIα and telomerase expression may be valuable in predicting response to targeted chemotherapy.

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