Telomerase activity is not a key determinant of sensitivity to standard cytotoxic drugs in human esophageal carcinoma cell lines

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The aim of the present study was to investigate if basal telomerase activity levels may predict sensitivity to cytotoxic drugs in a panel of human esophageal carcinoma cell lines. The TRAPeze telomerase detection assay was used to investigate telomerase activity in the cell lines. Cytotoxic drug sensitivity for 20 standard cytotoxic agents was assessed using the fluorometric microculture cytotoxicity assay (FMCA). Telomerase activity was detected in all cell lines with a broad range of activity levels. Drug sensitivity also varied considerably between the cell lines. Except for a *P* value towards a correlation between mitoxantrone and telomerase activity ($P=0.054$), no statistically significant correlation was found between telomerase activity levels and sensitivity to investigated drugs, including key drugs such as cisplatin ($P=0.9$), 5-fluorouracil ($P=0.8$) and doxorubicin ($P=0.54$). We therefore conclude that basal telomerase activity level is not a key determinant of sensitivity to standard cytotoxic drugs in esophageal carcinoma cell lines. *Anti-Cancer Drugs* **17:503–509**

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Introduction

Esophageal carcinoma has poor prognosis and is the seventh most common cause of cancer-related death in the western world [1]. Standard treatment for patients with advanced disease is chemotherapy, and the combination of cisplatin and 5-fluorouracil (5-FU) is most commonly used. Probably due to variations in drug target expression, DNA repair and apoptosis [2], a diversity of responses against chemotherapeutics are seen amongst these patients.

Telomerase is a reverse transcriptase involved in the unlimited growth of tumor cells. It synthesizes telomeric repeats onto chromosomal ends, thus compensating for telomere shortening during the DNA replication cycle [3]. Telomerase is repressed in the majority of somatic cells, but activated in approximately 85% of all human cancer types [4].

Telomerase activity has been shown to distinguish Barret's metasplasia from adenocarcinoma [5] and pre-cancerous lesions from gastric adenocarcinoma [6]. Furthermore, in patients with non-small cell lung cancer [7], colorectal cancer [8] and gastric cancer [9], telomerase activity is up-regulated during cancer progression, making it a potential marker of prognosis.

Higher telomerase activity levels are generally seen in esophageal carcinoma cell lines compared with clinical samples [10,11] and clinical samples from esophageal carcinoma patients show a wide range of telomerase activity levels [9,10]. Telomerase activity might affect apoptosis and several studies have investigated if inhibition of telomerase increases sensitivity to cytotoxic drugs [12]. Inhibition of telomerase activity has been shown to sensitize breast cancer cells to topoisomerase inhibitors [13] and malignant glioma cells to temozolomide [14]. However, in melanoma cell lines, inhibition of telomerase activity caused increased resistance towards temozolomide [15]. Previous studies in patients with esophageal carcinoma have shown that telomerase activity is a possible determinant of drug sensitivity [16,17].

In the present study, 10 human esophageal carcinoma cell lines were characterized according to the telomerase

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Table 1 Description of the investigated cell lines: histology, origin and known mutations (data provided by Deutsche Sammlung von Mikroorganismen und Zellkulturen)

Cell line	Origin	Described mutations
Kyse-140	moderately differentiated invasive esophageal squamous cell carcinoma resected from middle intra-thoracic esophagus of a 54-year-old Japanese man prior to treatment (depth of invasion was a1)	p53 mutation [40]
Kyse-450	well-differentiated invasive esophageal squamous cell carcinoma resected from middle intra-thoracic esophagus of a 59-year-old Japanese man prior to treatment (depth of invasion was not beyond the submucosa)	p53 mutation [40] and amplification of the oncogene c-*myc* [55]
Kyse-180	well-differentiated esophageal squamous cell carcinoma resected from middle intra-thoracic esophagus of a 53-year-old Japanese man prior to treatment (tumor was invading contiguous structures)	silent p53 mutation [40]
Kyse-510	well-differentiated esophageal squamous cell carcinoma with moderate invasion of the adventitia resected from cervical esophagus of a 67-year-old Japanese woman after cisplatin and radiotherapy	p53 mutation [40] and amplification of c-*myc*, *hst*-1 and cyclin D1 [55]
Kyse-520	moderately differentiated invasive esophageal squamous cell carcinoma resected from lower intra-thoracic esophagus of a 58-year-old Japanese woman prior to treatment (tumor had invaded the adventitia)	p53 mutation [40] and amplification of the oncogene c-*myc* [55]
Kyse-270	well-differentiated invasive esophageal squamous cell carcinoma resected from middle intra-thoracic esophagus of a 79-year-old Japanese man prior to treatment (depth of invasion was not beyond the muscularis propria)	p53 mutation [40]
Kyse-30	well-differentiated invasive esophageal squamous cell carcinoma resected from middle intra-thoracic esophagus of a 64-year-old Japanese man prior to treatment; cell line established from tumor cells heterotransplanted into athymic mice	p53 mutation [40] and amplification of c-*erb*B, c-*myc* and cyclin D1 [55]
Kyse-410	poorly differentiated invasive esophageal squamous cell carcinoma resected from the cervical esophagus of a 51-year-old Japanese man prior to treatment (tumor invasion into the adventitia was obvious)	Overexpressed *hst*-1 (=heparin-binding growth factor) and cyclin D1 [55]
Kyse-70	poorly differentiated invasive esophageal squamous cell carcinoma resected from middle intra-thoracic esophagus of a 77-year-old Japanese man prior to treatment (depth of invasion was not beyond the muscularis propria)	p53 mutation [40]
Kyse-150	poorly differentiated esophageal squamous cell carcinoma resected from upper (cervical) esophagus of a 49-year-old Japanese woman after receiving radiotherapy (tumor was invading contiguous structures)	Amplified oncogenes, c-*erb*B (8-fold) and cyclin D1 (4-fold) [55]

activity assay as well as sensitivity against different chemotherapeutics according to the fluorometric microculture cytotoxicity assay (FMCA) – a short-term semi-automatic method based on dye inclusion of surviving cells [18]. The aim of the present study was to assess the relationship between telomerase activity and sensitivity to standard cytotoxic drugs in human esophageal carcinoma cell lines.

Material and methods

Cell lines and culture conditions

A total of 10 human esophageal carcinoma cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and descriptive data concerning the cell lines are presented in Table 1. The cell lines were generated from tumors with varying differentiation grades spanning from poorly to well differentiated [19]. A majority of the cell lines harbored p53 mutations, and in addition mutations in c-Erb, c-Myc, cyclin D1 and Hst-1 were found in some of the cell lines. The cells were cultured in RPMI 1640 with 5% FCS, 5% new-born calf serum (NCS) and 1% glutamine at 37°C in 95% air/5% CO_2. To maintain exponential growth the cell lines were cultivated every third day. Cell counting was performed using a Coulter counter (particle count Zeiss analyzer) on days 1, 3, 6, 9 and 13.

Reference cell lines in the telomerase assay were KTC-1 [20], HTh 7 [21], LP-1[22], Karpas 707 [23] and human diploid neonatal foreskin fibroblasts AG-1523 purchased from the Human Genetic Mutant Cell Repository (Camden, New Jersey, USA).

Table 2 Presentation of drugs used in the test panel, and the minimum, median and maximum cytotoxic drug IC_{50} (μg/ml) values for all the esophageal cell lines

Mechanistic classes	Drugs	Minimum IC_{50}	Median IC_{50}	Maximum IC_{50}
Topoisomerase inhibitors	aclarubicin	0.01	0.07	2.60
	idarubicin	0.05	0.43	5.00
	doxorubicin	0.001	0.72	40.00
	teniposide	0.84	4.40	12.3
	mitoxantrone	0.30	0.84	40.40
	daunorubicin	0.25	0.58	100
	amsacrine	2.20	24.75	42.80
	etoposide	7.40	39.40	59.10
	topotecan	0.01	1.50	2.70
Tubulin active agents	docetaxel	0.01	8.04	16.30
	vinorelbine	0.22	10.20	27.10
	vinblastine	0.86	16.85	33.70
	paclitaxel	0.03	18.10	30.00
	vincristine	0.53	21.75	70.70
Alkylating agents	mitomycin C	0.40	2.60	7.60
	cisplatin	1.30	2.45	10.30
	melphalan	3.00	20.05	42.70
	chlorambucil	23.50	100	100
Anti-metabolites	fluorouracil	0.92	58.60	100
	cytarabine	0.001	100	100

Drug preparation

The drugs used were topoisomerase inhibitors ($n = 9$), alkylating agents ($n = 4$), tubulin active agents ($n = 5$) and anti-metabolites ($n = 2$) as shown in Table 2. The compounds were dissolved in PBS and sterile water according to the instructions from the manufacturers. Stock solutions at 1 mg/ml were prepared and serially diluted to obtain 96-well microtiter plates of concentrations ranging from 10 to 0.001 μg/ml. These plates were used as templates for the preparation of 384-well microtiter plates (Nunc, Roskilde, Denmark) using an

automated Biomek 2000 robotic system (Beckman Coulter, Fullerton, California, USA). An aliquot of 5 µl of each drug was dispensed per well in the 384-well plate. The plates were sealed and kept frozen at −70°C until further use.

Measurement of drug sensitivity using the FMCA is based on measurement of florescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes [24]. An aliquot of 45 µl of cell suspension containing of $2.5–5 \times 10^3$ cells was seeded into each well of the drug-prepared plates using an automated Precision 2000 robot (Bio-Tek Instruments, Winooski, Vermont, USA). Two columns with cells without drugs served as controls and two columns containing culture medium only served as blanks. The plates were incubated in 5% CO_2 at 37°C for 72 h, and at the end of the incubation period medium was aspirated and cells were washed in PBS. Then, 25 µl 0.1% FDA was added to each well followed by incubation for 40 min in 5% CO_2 at 37°C and the fluorescence in each well was measured using a Fluostar fluoromatic (Beckman Coulter, Fullerton, California, USA) at wavelengths of 520 and 590 nm. Cell survival was expressed as survival index (SI), defined as the fluorescence in experimental wells as a percentage of that in control wells, with blank values subtracted. For correlation with telomerase activity, the drug concentration producing a SI of 50% (IC_{50}) was used.

Quality criteria for a successful assay was a fluorescence signal in the control wells of more than 10 times mean blank values, and a coefficient of variation in the control wells and blanks wells of less than 30%.

Telomerase assay

Telomerase activity was analyzed using the TRAPeze telomerase detection kit (Chemicon, Temecula, California, USA), which is an extension of the original TRAP (telomeric repeat amplification protocol) method [25]. Cells (2×10^5) were washed once in PBS. Cell extracts were prepared according to the manufacturer's instruction. Protein content was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). Cell extracts were assayed for telomerase activity in 50-µl reactions provided with the TRAPeze telomerase detection kit with the exception of AmpliTaq DNA polymerase (Perkin-Elmer LifeScience, Boston, Massachusetts, USA). A combined elongation and amplification reaction was performed in a PTC-225 Peltier thermal cycler. After 30 min of incubation at 30°C the reaction mixture was heated to 94°C for 5 min, and then subjected to 33 cycles of 94°C for 30 s and 55°C for 30 s. The reaction mixtures were size-fractionated by electrophoresis in a 10% non-denaturing polyacrylamide gel and stained with SYBR Green 1 dye (BioWhittaker Molecular Applications, Rockland, Maine, USA). The gels were photographed using the GelFotosystemGFS1000 (Techtum, Umeå, Sweden) and analyzed with the Gel-Pro-Analyzer software (MediaCybernetics, Silver Spring, Maryland, USA). In order to be in the linear range in the PCR reaction, dilution series were made for each cell line to determine the correct amount of protein to be used in each assay. The relative telomerase activity of each extract was determined by taking the ratio of the entire telomerase ladder to that of the internal control. To be able to compare telomerase activity between different cell lines, relative telomerase activity was correlated to the total amount of protein run in each assay.

Statistics

Telomerase activity is shown as mean ± SEM, and three independent experiments were analyzed with ANOVA and Fishers PLSD using the StatView program. Spearman's rank-order correlation was utilized to test the associations between telomerase activity and drug sensitivity.

Results

High basal telomerase activity levels in esophageal carcinoma cells

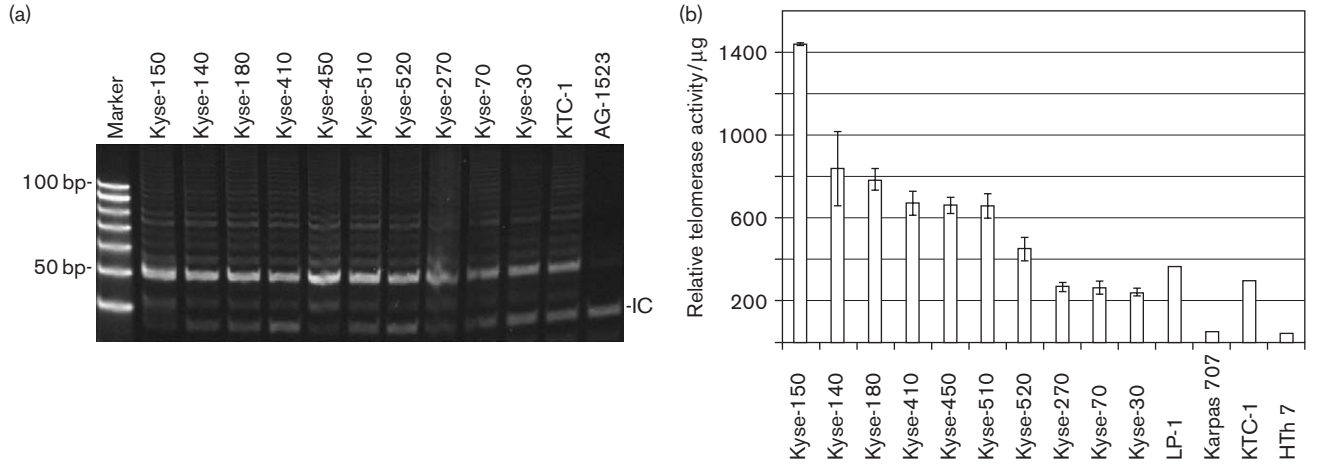
The present study used a panel of 10 human esophageal carcinoma cell lines with varying degrees of differentiation. As shown in Fig. 1, all of the esophageal carcinoma cells used expressed telomerase activity at varying levels. The telomerase activity varied approximately 7-fold in the cell panel. The highest telomerase activity was found in Kyse-150, followed by Kyse-140, -180, -410, -450, -510 and -520. The lowest telomerase activities were detected in Kyse-270, -70 and -30.

It should be noted, however, that overall the telomerase activity levels were very high in all of the esophageal carcinoma cells analyzed in this study compared with reference cell lines of multiple myeloma (LP-1 and Karpas 707) and anaplastic thyroid carcinoma (KTC-1 and HTh 7) (Fig. 1b).

Telomerase activity levels and cell proliferation

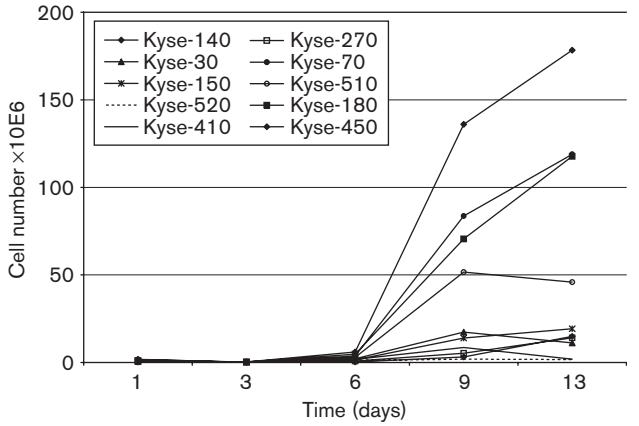
It has been suggested that malignant transformation and tumor aggressiveness are associated with increased telomerase activity [8,25]. Calculation of cell proliferation rate is commonly included in evaluation of tumor aggressiveness. We therefore aimed to investigate if telomerase activity levels correlated with growth potential in the human esophageal carcinoma cell lines. Growth curve experiments were performed for the 10 cell lines and the doubling time was calculated from the exponential growth (Fig. 2). The cell doubling time was calculated as 16 h for Kyse-70 and -450; for Kyse-180 and -510 the doubling times were 20 h. The cell doubling time for Kyse-30 and -150 was estimated as 25 h, and for Kyse-410 and -140 the doubling time was 32 h. Kyse-270 was calculated to have a doubling time of 39 h and for Kyse-520

Fig. 1



Relative telomerase activity in esophageal carcinoma cell lines. (a) Cells were assayed for telomerase activity using the TRAP assay. In order to be in the linear range in the assay the following protein amounts were used in each reaction: Kyse-150, 0.005 μg; Kyse-180 and Kyse-410, 0.009 μg; Kyse-450, 0.01 μg; Kyse-140 and -510, 0.016 μg; Kyse-270, 0.018 μg; Kyse-520, 0.022 μg; Kyse-30 and -70, 0.03 μg. KTC-1: positive control cell line; AG-1523: negative control cell line. (b) Plot of telomerase activity presented as telomerase activity/μg protein, quantified as described in Materials and methods.

Fig. 2



Cell proliferation measured over 13 days in the esophageal carcinoma cell lines.

the doubling time was 59 h. According to these data we could not find any relationship between cell proliferation doubling time and telomerase activity.

Telomerase activity and drug sensitivity

Telomerase overexpression has been suggested to be associated with resistance towards apoptosis [26]. We were therefore interested in analyzing whether the basal telomerase activity levels correlate with sensitivity to cytotoxic drugs. The drugs used were topoisomerase inhibitors ($n = 9$), alkylating agents ($n = 4$), tubulin active agents ($n = 5$) and anti-metabolites ($n = 2$) (Table 2). The cell lines exhibited varying degrees of activity towards the set of drugs used, and the minimum, median and maximum cytotoxic drug IC_{50} values for the 10 cell lines are shown in Table 2.

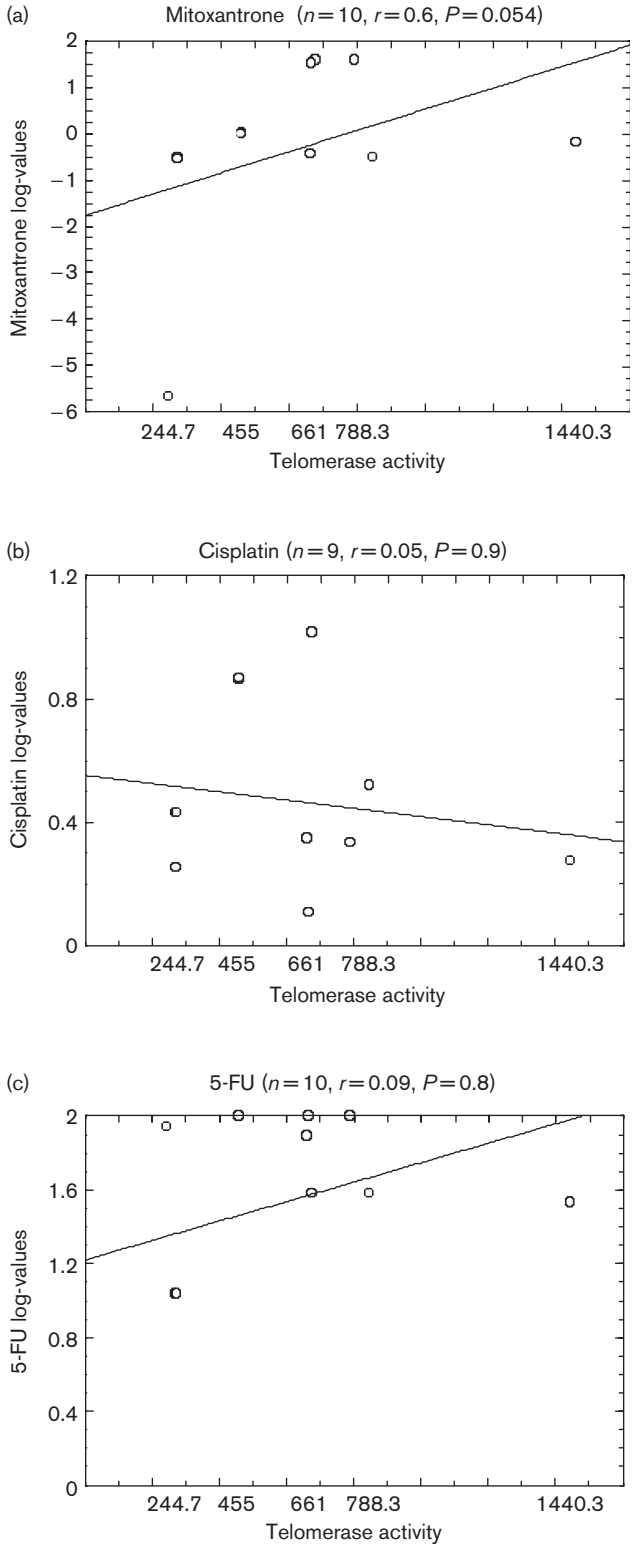
There was a P value towards a statistical correlation between mitoxantrone (topoisomerase inhibitor) sensitivity and telomerase activity levels ($P = 0.054$, $r = 0.62$) (Fig. 3a and Table 3). No significant correlations were found between telomerase activity and sensitivity to cisplatin (an alkylating agent; $P = 0.90$, $r = 0.05$) (Fig. 3b) or 5-FU (an anti-metabolite; $P = 0.8$, $r = 0.09$) (Fig. 3c). None of the other investigated drugs showed any significant correlation between telomerase activity and drug sensitivity (Table 3).

Discussion

Chemotherapeutic treatment in patients with esophageal carcinoma often results in divergent responses, which have been related to heterogeneity in chemosensitivity. In the present study, the observed IC_{50} values for the investigated standard drugs covered a wide range, indicating a considerable heterogeneity in drug sensitivity. The IC_{50} values were close to those obtained in a cell line panel composed to cover clinically very sensitive to very resistant tumor types [27]. Since esophageal carcinoma in general is considered to be chemoresistant, our findings might indicate that the esophageal carcinoma cell line panel used in the present study did not reflect clinical drug sensitivity.

Clinical studies performed in patients with esophageal carcinoma investigating the role of chemosensitivity

Fig. 3



Correlation and regression coefficients between telomerase activity and cytotoxic drug IC_{50} values in the esophageal carcinoma cell lines: (a) mitoxantrone (topoisomerase inhibitor), (b) cisplatin (alkylating agent) and (c) 5-FU (anti-metabolite).

Table 3 Correlation and regression coefficients for all investigated drugs between telomerase activity and cytotoxic drug IC_{50} values in the esophageal carcinoma cell lines calculated with Spearman's rank-order correlation

Mechanistic classes	Drugs	Correlation coefficient
Topoisomerase inhibitors	aclarubicin	–
	idarubicin	$r=0.52$, $P=0.15$
	doxorubicin	$r=0.23$, $P=0.55$
	teniposide	$r=-0.15$, $P=0.68$
	mitoxantrone	$r=0.6$, $P=0.054$
	daunorubicin	$r=0.03$, $P=0.93$
	amsacrine	$r=0.22$, $P=0.53$
	etoposide	$r=0.32$, $P=0.41$
	topotecan	$r=0.08$, $P=0.83$
Tubulin active agents	docetaxel	–
	vinorelbine	$r=0.04$, $P=0.91$
	vinblastine	$r=-0.03$, $P=0.93$
	paclitaxel	$r=0.18$, $P=0.63$
	vincristine	$r=0.05$, $P=0.90$
Alkylating agents	mitomycin C	$r=0.14$, $P=0.70$
	cisplatin	$r=0.05$, $P=0.90$
	melphalan	$r=-0.10$, $P=0.78$
	chlorambucil	–
Anti-metabolites	fluorouracil	$r=0.09$, $P=0.80$
	cytarabine	$r=-0.17$, $P=0.63$

assays, however, have found that the MTT assay [28] as well as the ATP-based chemosensitivity assay showed considerable heterogeneity in terms of sensitivity to chemotherapeutics [29]. Our results in human esophageal carcinoma cell lines thus support previously reported studies suggesting heterogeneity in sensitivity against chemotherapeutics for these tumors. Further studies in patient samples from esophageal carcinoma are needed to elucidate this issue.

Malignant transformation is associated with increased telomerase activity levels and aggressive tumors express higher telomerase activity levels than slowly progressing tumors [8,30]. Further, in a study by Li *et al.*, esophageal carcinoma telomerase activity levels have been reported to correlate with nodal metastasis [31]. To investigate if telomerase activity was associated with malignant potential in these cell lines we performed proliferation studies and also compared the provided differentiation data (Table 1). In agreement with Asai *et al.* [16], no correlation between telomerase activity levels and doubling time was found. Furthermore, the cell lines expressing the lowest telomerase activity levels did not consistently correspond to cell lines derived from well-differentiated tumors. These results might suggest that telomerase activity is not associated with malignant potential. Nevertheless, these data should be interpreted with caution since the number of investigated cell lines was limited and the investigated in-vitro system might not be representative of the clinical situation [32].

Further, a known activator of telomerase activity is the transcription factor c-Myc [33,34]. In that regard it is interesting to notice the lack of correlation between high

telomerase activity and previously described amplification of c-Myc in the cell lines (Table 1). It has been reported that wild-type p53 downregulates telomerase activity and increased telomerase activity is associated with p53 mutations [26,35–39]. Eight of the analyzed esophageal carcinoma cell lines have been found to harbor p53 mutations according to a previous investigation by Tanaka *et al.* [40]. In the present study, no association between the reported p53 mutations and telomerase activity levels could be observed.

High telomerase activity has been suggested to be associated with resistance to apoptosis and telomerase overexpression rescued tumor cells from apoptosis induced by agents causing double-strand breaks [41,42]. Thus, a possible link between sensitivity to cytotoxic drugs and telomerase activity levels may exist. Previously performed drug sensitivity studies have focused on investigating telomerase levels after exposure to cytotoxic drugs, but the relationship between the basal telomerase activity and cytotoxic drug sensitivity is still unclear [17,43–50]. Since tumors express varying degrees of telomerase activity levels, we aimed to elucidate if the initial basal telomerase level may predict sensitivity to cytotoxic drugs. In the present study, no general correlation between the basal levels of telomerase activity and sensitivity to cytotoxic drugs was found. However, correlation analysis showed a P value towards a statistical correlation between mitoxantrone and telomerase activity ($P = 0.054$). Mitoxantrone (Novantrone) is an anthracycline derivative used in the treatment of acute lymphocytic leukemia and acute myeloid leukemia [51]. Mitoxantrone has been investigated as a telomerase inhibitor in cell lines with diverging results [52,53]. In patients treated with a chemotherapeutic combination including mitoxantrone for acute myelogenous leukemia, high levels of telomerase activity in the bone marrow after chemotherapy were associated with treatment failure due to resistant disease [54]. If there is a true correlation between telomerase activity levels and sensitivity to mitoxantrone or if this was a chance finding needs to be confirmed in future studies.

Taken together, the result from the present study does not give support for a causal relationship between basal telomerase activity and cytotoxic drug sensitivity in human esophageal carcinoma cell lines. This finding does not exclude a role for telomerase activity for sensitivity to cytotoxic drugs in human cells as reflected by the above-cited findings of changes in drug sensitivity when telomerase activity was manipulated. The particular cell drug sensitivity is probably multifactorial and the present findings indicate that measurement of telomerase activity alone is not informative for cytotoxic drug sensitivity. Further studies on the prognostic and predictive role of telomerase activity are needed, however, and should preferably include experiments in tumor cells from patients.

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