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Telomerase catalytic subunit gene expression does not influence survival of patients with squamous cell carcinoma of the larynx and hypopharynx: a case-control study

Boštjan Luzar, MD, PhD, Mario Poljak, MD, PhD*, Janez Fischinger, MD, PhD†, Ulrika Klopčič, MD‡, Nina Gale, MD, PhD

Abstract

Aims: to determine correlations between relative quantities of telomerase catalytic subunit m-ribonucleic acid (hTERT mRNA) and conventional clinicopathological parameters (such as site, size and grade of tumour, the presence of regional lymph node metastases, and, in particular, survival) in patients with laryngeal and hypopharyngeal squamous cell carcinomas (SCCs).

Material and methods: The relative quantity of hTERT mRNA was analysed by a commercially available LightCycler *T*elo *TAGGG* hTERT Quantification Kit in 56 cases of SCC (40 laryngeal and 16 hypopharyngeal). The association with cancer-specific survival was evaluated by univariate and multivariate analysis.

Results: Location of the tumour in the hypopharynx was the only significant negative predictive factor for survival, as determined by univariate analysis ($p = 0.028$). Although a tendency towards a better overall survival was observed for female patients younger than 50 years, for lower tumour grades and sizes, and for the absence of regional lymph node metastases, the prognostic significance of these factors could not be confirmed. No differences existed in hTERT mRNA expression between laryngeal and hypopharyngeal SCCs. Furthermore, no correlation was found between the relative quantities of hTERT mRNA and the tumour size, regional lymph node metastases or survival of patients with laryngeal or hypopharyngeal SCCs.

Conclusions: The results of the present study suggest that genetic abnormalities other than telomerase reactivation are responsible for progression of laryngeal and hypopharyngeal SCCs.

Key words: Telomerase; Carcinoma; Squamous Cell; Larynx; Hypopharynx; Survival

Introduction

The potential clinical prognostic factors for head and neck squamous cell carcinomas (SCCs) can be categorized according to patient, tumour and treatment variables.[1] Suggested patient-based factors influencing survival are performance status and gender, with a poor prognosis in many patients being related to their poor overall health.[2] Tumour-based factors consistently found to be independent predictors of survival are tumour size, extent of local tumour spread, and the presence and extent of lymph node metastases.[1,2] A better understanding of the molecular biology of cancer has indicated the possible adverse prognostic significance of certain molecular biomarkers, especially p53, epidermal growth factor receptor, transforming growth factor α and cyclin D1.[1]

Telomerase, a peculiar ribonucleoprotein enzyme that maintains the length of telomeres through the addition of tandem repeat nucleotide sequences (TTAGGG), has been the subject of intensive research in the last decade due to its major implications in human carcinogenesis.[3,4] While telomerase is generally undetectable in normal human somatic cells, with the exception of germ and stem cells, telomerase reactivation is associated with cellular immortality and can be detected in nearly 90 per cent of human malignancies, including head and neck SCCs.[4]

The main aims of the present study were to analyse whether the relative quantity of telomerase catalytic subunit m-ribonucleic acid (hTERT mRNA) (an estimate for telomerase activity) had any influence on various biological parameters of laryngeal and hypopharyngeal SCCs, such as tumour size and grade, as well as on the presence of regional lymph node metastases. To the best of our knowledge, the present study is the first to analyse the correlation between the relative quantity of hTERT mRNA and the survival of patients with SCCs of the larynx and hypopharynx.

Material and methods

A retrospective analysis was performed on 56 frozen-tissue specimens containing invasive SCCs of the larynx and hypopharynx, obtained from 56 patients (51 men, five women, aged between 36 and 76 years, mean age 57 years) treated with pharyngectomy, laryngectomy or hemilaryngectomy over the period 1998–2001 at the Department of Otorhinolaryngology and Cervicofacial

From the Institute of Pathology, and the *Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, the †Department of Otorhinolaryngology and Cervicofacial Surgery, Clinical Center, Ljubljana, and the ‡Department of Cytopathology, Institute of Oncology, Ljubljana, Slovenia.
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Surgery at the Ljubljana Clinical Center, Slovenia. Immediately after the surgical procedure, tumour samples from a resected specimen were snap-frozen in liquid nitrogen and subsequently stored at −80°C for later analysis. Prior to the surgery, patients received no chemotherapy or radiation therapy. There were no differences among the patients regarding post-operative treatment.

The SCCs of the larynx and hypopharynx were staged according to the International Union Against Cancer tumour-node-metastasis (TNM) classification and graded according to the World Health Organization (WHO) classification of tumours of the upper respiratory tract as well differentiated (G1), moderately differentiated (G2) and poorly differentiated tumours (G3).[5,6]

Frozen-tissue analysis

Frozen-tissue blocks containing SCCs of the larynx and hypopharynx were analysed as follows. Initially, one 4 μm thick section was cut and stained with haematoxylin and eosin (H&E) in order to verify that the specimen contained SCC. By combining the use of a low-power field lens (×2.5) of a Nikon Eclipse 600 microscope (Nikon, Kanagawa, Japan), a thin surgical blade and the H&E-stained slide as a reference, the necrotic part of the tumour as well as the normal tissue were cut from the tissue block in order to obtain the maximum amount of tumour in the sample (>90 per cent of the slide). Thereafter, serial sectioning of 10 μm slices was performed to obtain approximately 2.5 mm^3 of tissue for subsequent total RNA isolation. The last section was again stained with H&E to check whether it still contained a sufficient amount of SCC.

Total RNA isolation

Total RNA was isolated from laryngeal and hypopharyngeal tissue specimens by using a High Pure RNA Tissue Kit (Roche Diagnostics, Mannheim, Germany), strictly following the manufacturer's recommendations. Eluted total RNA was stored at −80°C for later analysis.

Quantification of hTERT mRNA

A commercially available LightCycler *T*elo *TAGGG* hTERT Quantification Kit (Roche Diagnostics) was used for real-time polymerase chain reaction (PCR) quantification of hTERT mRNA. In brief, the hTERT mRNA was reverse-transcribed and a 198 bp fragment of generated c-deoxyribonucleic acid (cDNA) was amplified in a one-step reverse transcription (RT)-PCR reaction. The amplicons were detected by fluorescence using a specific pair of hybridization probes, consisting of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of the amplification cycle. In a separate one-step RT-PCR process, the quantity of mRNA for the house-keeping gene porphobilinogen deaminase (PBGD) was determined. The PBGD mRNA served as an internal control for RT-PCR performance as well as a reference standard for relative quantification.

For each sample tested, 2 μl of the eluted total RNA was used. Quantification was performed by real-time monitoring for identification of the exact time point at which the logarithmic linear amplification phase could be distinguished from the background. External standards containing 10^6, 10^5, 10^4, 10^3 and 10^2 copies of hTERT mRNA per 2 μl were used in each run. The cycle numbers of the logarithmic linear phase were plotted against the logarithm of concentration of hTERT mRNA. By comparing the crossing line intercept of an unknown sample with the standard curve, a quantitative estimate of the starting copy number of hTERT mRNA (as well as PBGD mRNA) was calculated. The normalized hTERT mRNA value (hTERT index), a measure of hTERT mRNA relative quantity, was calculated by dividing the amount of hTERT transcript by the amount of endogenous house-keeping gene PBGD mRNA of the same sample, multiplied by 100.

Statistical analysis

Statistical analysis was performed by a nonparametrical Mann–Whitney U test with Bonferroni correction (nonparametrical Wilcoxon test) using SPSS 10.1 for Windows.

Cancer-specific survival was calculated from the time of the diagnosis of SCC to the date of death due to SCC. Where patients were alive at the end of the study (January 1 2003), this date was used for calculation. Data on patients' survival were obtained from the Cancer Registry of Slovenia at the Institute of Oncology, Ljubljana, Slovenia. Patients were followed from one to 67 months (mean 30 months, standard deviation (SD) ± 16). Survival analysis was performed by using the Kaplan and Meier method. Analyses of differences between curves were done by the log rank test and Cox's multivariate proportional hazard model. A p value of less than 0.05 was considered statistically significant.

Results

The relative quantity of hTERT mRNA was determined in 56 SCCs obtained from 56 patients with SCC of the larynx

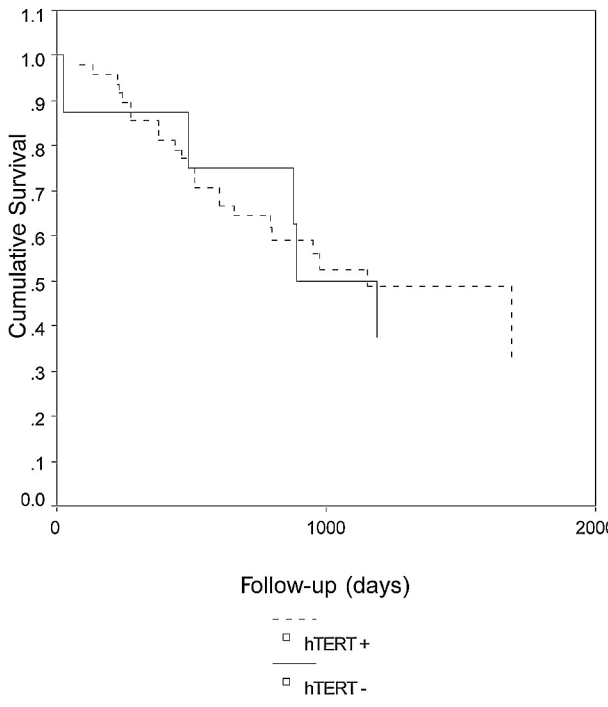

FIG. 1

Cumulative survival of patients with laryngeal and hypopharyngeal squamous cell carcinomas, according to the expression of human telomerase catalytic subunit m-ribonucleic acid (hTERT mRNA) in the tumour. hTERT+ = hTERT mRNA-positive squamous cell carcinomas; hTERT- = hTERT mRNA-negative squamous cell carcinomas.

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TABLE I

hTERT mRNA IN SCCs OF THE LARYNX AND HYPOPHARYNX

SCC	*n*	hTERT mRNA positivity		hTERT index		
		n	%	Mean	Range	SD
Size						
T_1	3	3	100	1.38	0.96–1.67	0.37
T_2	19	18	94.7	2.66	0–7.5	2.09
T_3	27	22	81.5	2.69	0–11.55	3.11
T_4	7	5	71.4	1.16	0–3.46	1.18
Grade						
G1	6	4	66.7	0.81	0–1.93	0.80
G2	25	20	80	2.03	0–7.16	2.03
G3	25	24	96	3.18	0–11.55	3.03
Regional lymph nodes						
N_0	24	21	87.5	2.59	0–11.55	2.90
N_1	8	6	75	1.81	0–5.07	2.10
N_2	24	21	87.5	2.44	0–8.84	2.37

SCC = squamous cell carcinoma; SD = standard deviation; hTERT mRNA = telomerase catalytic subunit m-ribonucleic acid; T = tumour; G = grade; N = node

(40 patients) and hypopharynx (16 patients). The presence of hTERT mRNA and hTERT index in SCCs, grouped according to the size (T status) and grade of tumours as well as the status of the regional lymph nodes (N status), are summarized in Table I. All patients lacked distant metastases at the time of the operation and were designated as M_0 according to the TNM classification.

As shown in Table I, a progressive increase in the presence and relative quantity of hTERT mRNA was observed with increasing histological grade of SCC. Grade 3 SCCs had significantly higher relative quantities of hTERT mRNA than did grade 1 SCCs ($p = 0.046$). However, no statistical difference was found between the presence and relative quantities of hTERT mRNA and the size of the SCCs ($p = 0.464$) or the regional lymph node status ($p = 0.862$). In addition, there was no difference in the frequency of hTERT mRNA expression between laryngeal and hypopharyngeal SCCs ($p = 0.11$).

Of the various clinicopathological parameters tested (such as patients' sex, age at the time of diagnosis, tumour size and grade, and regional lymph node status), only location of the tumour in the hypopharynx constituted a negative prognostic factor for survival, as determined by univariate analysis ($p = 0.028$) (Table II). Although there was a tendency towards better survival for female patients younger than 50 years, for lower tumour grades and sizes, for absence of regional lymph node metastases and for absence of hTERT mRNA (Figure 1), the prognostic significance of these factors could not be confirmed statistically ($p > 0.1$).

Discussion

Head and neck SCC is the sixth most frequent cancer, accounting for 6 per cent of all cancers worldwide.[7] In general, laryngeal and hypopharyngeal SCCs form the largest subset of this group, usually occurring in older men.[8] The relationship between cigarette smoking and laryngeal/hypopharyngeal carcinoma risk has been consistently demonstrated in many epidemiological studies, as has been the independent effect of alcohol consumption and lowered intake of a variety of essential nutrients.[9] The multiplicative effect of smoking and alcohol intake is well recognized.[9] Despite improvements in diagnosis and treatment, the mortality rate for this condition has remained relatively stable over the last two decades, with an overall death risk as high as 40 per cent.[10] Among head and neck cancers, SCCs of the hypopharynx have the worst prognosis, which is usually related to their presentation at a late stage, multisite involvement within the hypopharynx, unrestricted soft-tissue tumour growth and extensive mucosal lymphatic network, promoting metastases, as well as to restricted surgical possibilities for complete resection.[2]

TABLE II

OVERALL SURVIVAL OF PATIENTS WITH LARYNGEAL AND HYPOPHARYNGEAL SCC, TUMOUR CHARACTERISTICS AND hTERT mRNA EXPRESSION

Prognostic factor	Type	Patients (*n*)	Overall survival (% (SD))	*p**
Sex	Male	51	32.8 (±11.1)	0.2
	Female	5	66.7 (±27.2)	
Age (years)	≤50	16	60.2 (±12.9)	0.18
	>50	40	26.3 (±12.3)	
Location	Larynx	40	39.9 (±13.3)	0.028
	Hypopharynx	16	28.1 (±12.2)	
Tumour grade	G1	6	66.7 (±19.2)	0.31
	G2	25	44.9 (±11.2)	
	G3	25	22.0 (±16.5)	
Tumour size	Small (T_1, T_2)	22	51.4 (±11.2)	0.39
	Large (T_3, T_4)	34	20.7 (±15.5)	
Node stage	N_0	24	41.2 (±18.4)	0.11
	N_1, N_2	32	35.5 (±9.2)	
hTERT mRNA	Absent	8	37.5 (±17.1)	0.86
	Present	48	32.5 (±14.3)	

*Univariate analysis. SCC = squamous cell carcinoma; hTERT mRNA = telomerase catalytic subunit m-ribonucleic acid; SD = standard deviation; T = tumour; G = grade; N = node

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Traditional clinical prognostic factors consistently found to be independent predictors of survival for patients with head and neck SCCs are tumour size, extent of local tumour spread and the presence of lymph node metastases.[1,2] However, a better understanding of tumour cell biology has focussed on exploring the role of additional molecular biomarkers thought to be involved in tumour development, in order to predict more precisely the biological potential of the tumour, to increase the sensitivity of standard histological diagnostics and to optimize treatment possibilities accordingly. Reactivation of the enzyme telomerase has been shown to be associated with cellular immortality, a crucial event in human carcinogenesis.[4]

Conflicting data are available on the correlation between telomerase activity or reactivation and survival of patients with head and neck SCCs. Koscielny *et al.* analysed telomerase activity in 80 cases of SCC of the head and neck and failed to demonstrate any prognostic significance of telomerase reactivation.[11] In contrast, Liao *et al.*, using multivariate analysis on 217 cases of SCC, mostly of the oral cavity, showed telomerase activity to be an independent prognostic factor for survival in these patients.[12] However, Patel *et al.* could not demonstrate any correlation between telomerase activity and disease-free survival in 110 cases of head and neck SCC.[13] However, they did find that disease-free survival correlates well with telomere length.[13]

Only a few studies have been performed correlating telomerase activity with various clinicopathological parameters in a limited number of patients with head and neck SCCs, but these have produced inconclusive results.[14–16] No correlation was found between telomerase activity and age,[14,15] smoking and drinking habit,[14,15] gender,[14] or size[14,16] and grade of laryngeal and/or hypopharyngeal SCCs.[14–16] Nevertheless, Hohaus *et al.* found a positive correlation between telomerase activity and the stage of disease; stage 1 tumours were either negative or weakly positive, whereas stage 4 tumours showed the strongest telomerase activity.[15] In addition, these authors showed that patients with little or no telomerase activity were less likely to have lymph node metastases than patients with higher telomerase activity.[15] Similarly, Thurhner *et al.* detected significantly lower telomerase activity in tumours without regional lymph node metastases and hypothesized that telomerase activity may in fact facilitate lymph node mestastases.[14] Most recently, Koscielny *et al*. observed a tendency for head and neck SCC patients with telomerase activity to develop lymph node metastases more frequently, but noted that a higher number of cases should be evaluated to ascertain any prognostic clinical relevance of this association.[11]

Several studies have shown that the expression of hTERT is the rate-limiting step for telomerase activity and that the quantity of hTERT mRNA can be used as a good estimate of telomerase activity.[4,17,18] To the best of our knowledge, our study is the first to explore the prognostic significance of hTERT mRNA relative quantities in patients with laryngeal and hypopharyngeal SCCs.

In the present study, hTERT mRNA was detectable in 85 per cent of the examined SCCs. There were no differences in the frequency of hTERT mRNA expression or its relative quantities, comparing laryngeal and hypopharyngeal SCCs. Among traditional prognostic factors, only a laryngeal origin of SCC has been found by univariate analysis to be an independent prognostic factor associated with better survival. Although female patients younger than 50 years, lower tumour grade and size (T parameter), and the absence of regional lymph node metastases (N parameter) were associated with a better overall survival, these parameters lacked statistical significance. Despite a better survival of T_1/T_2 patients compared with T_3/T_4 patients, the difference between these two groups was not statistically significant, possibly due to a relatively small number of patients. Although a progressive increase in the relative quantities of hTERT mRNA has been observed with increasing grade of SCC, the only differences between well and poorly differentiated laryngeal and hypopharyngeal SCCs were statistically significant. Furthermore, the overall survival of patients without hTERT mRNA (37 per cent) was slightly better than the overall survival of patients with hTERT mRNA (32 per cent), although the differences were not statistically significant.

- **Telomerase, a ribonucleoprotein enzyme, has been the subject of intense research due to its role in human carcinogenesis**
- **This study looks at the relationship between telomerase activity and various parameters in laryngeal and hypopharyngeal squamous cell carcinoma**
- **No correlation between telomerase activity and tumour size, regional nodal metastasis or survival was found in patients with this condition**

We have recently shown that telomerase reactivation is an early event in laryngeal carcinogenesis, already detectable at the stage of precancerous laryngeal epithelial changes.[19] While low levels of hTERT mRNA, occasionally found in normal and reactively hyperplastic laryngeal epithelium, reflect the regenerative capacities of squamous epithelium, significantly higher relative quantities of hTERT mRNA in nearly 90 per cent of precancerous lesions and in up to 85 per cent of laryngeal SCCs were consistent with telomerase reactivation.[15,16,19–21] Considering the published data on molecular events in head and neck carcinogenesis, it can be summarized that telomerase reactivation is the most frequent and the most consistently present genetic aberration. Namely, tumour suppressor inactivation of p53 and p16 was found in 50 per cent of laryngeal SCCs and amplification of the oncogene cyclin D1 in one-third of cases.[22,23] While alterations in p53 and p16 genes and telomerase reactivation occur in the early stages of neoplastic transformation, amplification of cyclin D1 is believed to represent a late event and has been linked to progression of intraepithelial carcinoma into invasive SCC.[22,23] It appears that telomerase reactivation in laryngeal/hypopharyngeal carcinogenesis conveys cellular immortality, with unlimited growth potential, to such cells, enabling other genetic abnormalities to accumulate. Genetic abnormalities other than telomerase reactivation are therefore responsible for tumour progression, and it is not surprising that we have failed to demonstrate correlations between the levels of telomerase activity (measured indirectly as the relative quantities of hTERT mRNA) and clinicopathologic parameters such as the site, size and stage of the tumour, the presence of lymph node metastases and the survival of patients with laryngeal or hypopharyngeal SCC.

In conclusion, to the best of our knowledge, this is the first study to analyse the prognostic significance of relative quantities of hTERT mRNA, an estimate of telomerase activity, in patients with laryngeal and hypopharyngeal SCCs. Although patients without detectable hTERT mRNA in their laryngeal/hypopharyngeal SCC had slightly better overall survival than did patients with detectable

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SCC hTERT mRNA, the differences were not statistically significant. In addition, we found no correlation between the relative quantities of hTERT mRNA in laryngeal and hypopharyngeal SCCs and already well established, traditional clinicopathological prognostic factors (such as site, size and stage of the tumour, and the presence of regional lymph node metastases). The results of our study suggest that genetic abnormalities other than telomerase reactivation are necessary for the progression of SCCs of the larynx and hypopharynx.

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Address for correspondence:
Assistant Prof Boštjan Luzar, MD, PhD,
Institute of Pathology,
Faculty of Medicine,
University of Ljubljana
Korytkova 2,
1000 Ljubljana, Slovenia.

Fax: + 386 1 543 7101
E-mail: bostjan.luzar@mf.uni-lj.si

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