Telomerase Activity in Head and Neck Cancer

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Abstract. *Background: Telomerase activity is associated with many malignancies, including head and neck cancer. The use of telomerase activity as a diagnostic and prognostic marker of head and neck cancer development was examined and compared with standard histological analysis. Patients and Methods: Telomerase activity was determined using quantitative dual-colour real-time TRAP (telomeric repeat amplification protocol). In each of 58 patients, a sample of tumour tissue, adjacent mucosa and normal muscle was collected. Results: Telomerase activation was observed in 88% of tumour tissues and 34% of tumour-adjacent mucosa samples. No telomerase activity was detected in normal muscle tissues. Telomerase activity correlated with tumour grade, with an average of 4.6 telomerase units (T.U.) in well-differentiated, 8.3 T.U. in moderately-differentiated and 20 T.U. in poorly differentiated tumours. Relapse occurred in 13 patients and no telomerase activity was detected in 3 recurrent tumours. Conclusion: Telomerase activity may be used as an objective parameter inversely related to tumour differentiation. Prognosis in telomerase-negative tumours is worse than that of the telomerase-positive group.*

Telomeres are functional elements at the ends of linear eukaryotic chromosomes. Human telomeres consist of a 5-20 kb long tandemly-repeated DNA sequence TTAGGG and telomere-associated proteins, which together form a complex protecting the chromosome ends against exonucleases and inappropriate action of DNA repair mechanisms. Their major characteristic is connected with the DNA end-replication problem: incomplete replication of the lagging DNA strand causes telomere shortening in each cell cycle and their loss below a critical length stops cell division because at this stage repair mechanisms become unable to distinguish chromosome ends from unrepaired chromosome breaks. Cells then enter the state known as senescence which is characterized by changes in protein expression patterns and growth arrest. Some cells can overcome this state by reactivation of the specific enzyme telomerase or by a telomerase-independent telomere lengthening process (see (1) for a review).

Telomerase is a reverse transcriptase which contains two essential subunits: the protein subunit telomerase reverse transcriptase (TERT) and the RNA subunit which provides a template for telomere DNA synthesis. In humans, telomerase activity is present in embryonic, germline and tissue stem cells. It also becomes activated in tumour tissues and immortalized cell lines (2-4). Telomerase activity has been observed in a variety of malignancies including head and neck cancer (5), which makes telomerase a potentially important target for cancer diagnostics and potential anticancer therapy (6).

Head and neck squamous cell carcinoma (HNSCC) is one of the most common neoplasms worldwide and is associated with high mortality; 5-year survival does not exceed 55% (7). The recurrence of disease varies between 20-30% in early-stage cancer (stages 1 and 2) and 50-60% in advanced stages (stages 3 and 4). The aetiology of HNSCC is closely connected with risk factors including smoking, alcohol drinking, viral infection and nutrition. The incidence of disease is country-specific and differs with the kinds of risk factors and also with genetic predispositions in the population (8, 9). Genetic factors play an important role in the mechanism of HNSCC progression(10-12). Reactivation of telomerase in HNSCC has been observed in many reports, however its clinical and prognostic relevance in these studies varied (8, 9, 13-15). One of the possible reasons for this variation was the use of different telomerase activity assays by different groups. The recent introduction of the real-time version of measurement of telomerase activity has made it possible to increase the precision of telomerase activity determination (16, 17), thus increasing the potential interest of this tumour marker. The aim of this study was to evaluate the possible use of quantification

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of telomerase activity in tumours and tumour-adjacent tissues as a prognostic marker of head and neck cancer progression in a sufficiently large collection of patients.

Patients and Methods

Patients and tissue samples. Biopsies of tumours were obtained from St. Ann's University Hospital, Brno, Czech Republic. Samples of tumour and tumour-adjacent tissue were taken from patients undergoing surgical intervention for a histologically verified tumour and, where indicated, samples of normal muscle tissue were also collected. The samples of tumour-adjacent tissue were taken from the mucous membrane at the edge of the dissected tissue at a safe distance from the tumour and their status (the absence of tumour cells) was checked by histological analysis. All the participants gave their informed consent. In each tumour sample, the following parameters were assessed: gender and age of the patient, tumour localisation, histopathological classification and grade, N- and T-stage, and telomerase activity.

Telomerase extract preparation. The tissue samples were homogenized by freezing in liquid nitrogen and grinding. The collected material was then extracted with CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate) lysis solution from the TRAPeze®XL Telomerase Detection Kit (Chemicon International Inc., Temecula, CA, USA) following the manufacturer's recommendations. The supernatant was transfered to a fresh tube and the total protein concentration was determined by the Bradford assay (Bio-Rad Protein Assay Kit; Bio-Rad, Hercules, CA, USA).

Telomerase assay. Telomerase activity assays were performed in dual-colour real-time mode using the Rotorgene 3000 (Corbett Research, Sydney, Australia) as described previously (16). Briefly, the TRAPeze®XL Telomerase Detection Kit contains fluorescently labelled Amplifluor® primers for amplification of both telomerase products and an internal control template in the same tube. For the determination telomerase activity absolute quantification was used. A standard curve was obtained using dilutions (0.01-0.1 μmol) of the TSR8 control template (substrate oligonucleotide extended by 8 telomeric repeats) included in the TRAPeze®XL Telomerase Detection Kit. To each reaction mix, 1 μl of sample extract containing 1 μg of total protein was added. Telomerase activity was expressed in relative telomerase units (T. U.; 100 T.U. corresponds to 0.1 μmol of TSR8 control template).

Analysis of telomere lengths. Telomere lengths were analysed using a technique of terminal restriction fragments (3). Briefly, genomic DNA (2 μg) was digested with 20 units of restriction enzyme *Taq*I (New England Biolabs, Ipswich, USA), separated by agarose electrophoresis (5 V/cm) and blotted onto Hybond XL membrane (GE Healthcare, Vienna, Austria). After Southern hybridisation with a probe for human telomeric sequence (CTAACCCTAACCCTAACCCTAACCCTAA), terminal restriction fragments were detected using Phospho-fluoroimager STORM 860 (GE Healthcare).

Statistical analysis. Statistical evaluation was performed using Statistica 7.1 software (StatSoft CR, Praha, Czech Republic). The Wilcoxon rank test was used to identify significant differences in telomerase activity between tumour tissue and tumour-adjacent tissue. To analyze the correlation between tumour grade and telomerase activity the Spearman test and one-way ANOVA analysis were used.

Results

Telomerase activity in tumour and tumour-adjacent tissues. Fifty-eight patients, 50 men and 8 women, all of them smokers were tested. The patient characteristics, tumour type and localisation are shown in Table I.

Telomerase activity was detected in 88 % of the tumours and in 34% of the tumour-adjacent tissues. The activity in the tumours was significantly higher than in the mucosa; the average activity in the tumours was 10.7 T.U (Figure 1). Histologically negative mucosa was telomerase positive in 34% of cases, the average activity was 1.4 T.U.

Seven tumours were telomerase negative and three of these (43%) recurred. Considering the low frequency of telomerase-negative tumours, the incidence of three of them in a group of recurrent tumours was markedly high. Overall, 13 patients (22 %) had a verified relapse, with an average telomerase activity of 6.9 T.U. Five patients had neoadjuvant chemotherapy or chemoradiotherapy, and one of these relapsed. The telomerase activity in the mucosa of the patients with relapse was 0.8 T.U, and in the mucosa of the patients with neoadjuvant chemotherapy 0.3 T.U. The muscle samples were telomerase negative in 100% of cases.

Correlation of telomerase activity with histological characteristics. Telomerase activity in the tumour tissue was statistically higher in comparison with that in the histologically normal tissue (Figure 1). Moreover, the activity correlated with tumour grading (Figure 2), the average activity was 4.6 T. U. in the well-differentiated, 8.3 T. U. in the moderately-differentiated and 20 T.U. in the poorly differentiated tumours, and the differences between the values were statistically significant (p=0.025) using Spearman correlation and of marginal significance (p=0.1) by ANOVA. Telomerase activity became higher with N stage, but the difference was not statistically significant. The distribution of the tumour samples according to grade of differentiation is shown in Figure 3 and was asymmetrical in favour of the intermediate grade.

Telomere lengths in telomerase-negative tumours. The telomeres in telomerase-negative tumours (detected as terminal restriction fragments) showed patterns similar to those of normal cells, ranging between 3 and 7 kb in length, while in the telomerase-positive tumours, the lower limit of telomere size was shifted below 2 kb (Figure 4).

Discussion

The results demonstrated the significant clinical usefulness of measuring telomerase activity and suggested that this is a more accurate method of predicting the biological behaviour of a tumour than standard grading, which is a much more subjective method. The unequal division of tumours at each

Table I. *Patient characteristics*.

PN	Localisation	Diagnosis	Gender/Age	Tumour classification			Telomerase activity (T.U.)			Relapse
				Grade	N stage	T stage	Tumour	Adjacent tissue	Muscle	
1	larynx	SpinoCa	M/62	1	1	3	8	0	–	N
2	oropharynx	SpinoCa	M/63	2	0	4	3	1	–	Y
3	oropharynx	SpinoCa	M/55	1	2	1	3	0	–	N
4	larynx	SpinoCa	M/62	3	2	3	3	0	–	Y
5	oropharynx	SpinoCa	M/61	2	1	2	1	0	–	N
6	larynx	SpinoCa	M/69	3	0	2	12	2	–	N
7	oropharynx	SpinoCa	M/64	3	1	2	27	0	–	N
8	larynx	SpinoCa	M/75	2	0	3	0	0	–	Y
9	larynx	SpinoCa	M/66	2	2	4	9	0	–	N
10	oral cavity	SpinoCa	F/45	2	1	2	36	0	–	N
11	oral cavity	SpinoCa	M/64	1	0	2	0	0	–	N
12	oropharynx	SarcoCa	M/61	3	1	1	6	0	–	N
13	oropharynx	SpinoCa	M/50	1	0	2	14	0	–	Y
14	oropharynx	ACC	M/64	1	1	4	3	0	–	N
15	larynx	SpinoCa	M/58	3	3	4	16	0	–	N
16	oropharynx	SpinoCa	M/62	2	0	4	0	1	–	Y
17	hypopharynx	SpinoCa	M/66	3	2	3	3	0	–	N
18	oropharynx	SpinoCa	M/52	2	2	3	1	0	–	N
19	oral cavity	SpinoCa	F/63	3	2	4	3	0	–	N
20	oropharynx	SpinoCa	M/54	3	0	1	8	1	–	N
21	oropharynx	SpinoCa	M/56	1	1	2	3	0	–	N
22	oropharynx	SpinoCa	M/66	2	2	3	11	0	–	N
23	oral cavity	SpinoCa	M/50	2	0	2	0	0	–	Y
24	larynx	SpinoCa	M/60	2	0	3	17	7	–	N
25	oral cavity	SpinoCa	M/61	2	1	2	13	5	–	N
26	oral cavity	SpinoCa	M/51	2	1	2	1	0	–	Y
27	larynx	SpinoCa	M/68	3	1	4	9	0	0	N
28	larynx	SpinoCa	M/61	2	2	2	17	3	0	Y
29	oral cavity	SpinoCa	M/54	2	2	3	3	0	0	N
30	oral cavity	SpinoCa	M/58	2	2	1	20	1	0	N
31	oropharynx	SpinoCa	M/40	2	1	4	7	5	0	Y
32	oral cavity	SpinoCa	M/56	2	1	2	5	1	0	N
33	oral cavity	SpinoCa	F/63	2	2	3	13	0	0	Y
34	larynx	SpinoCa	M/52	3	0	4	9	0	0	N
35	oropharynx	SpinoCa	M/78	3	1	2	12	1	–	N
36	larynx	SpinoCa	M/64	1	0	3	5	1	0	N
37	oral cavity	SpinoCa	M/60	2	2	3	1	0	0	N
38	larynx	SpinoCa	M/63	3	0	2	34	11	0	N
39	larynx	SpinoCa	M/62	2	2	4	6	1	0	N
40	oropharynx	SpinoCa	F/73	2	2	4	18	0	0	Y
41	oropharynx	SpinoCa	M/60	2	1	1	12	0	0	Y
42	larynx	SpinoCa	F/74	3	3	2	9	0	–	N
43	oropharynx	SpinoCa	M/57	1	1	4	0	0	0	N
44	larynx	SpinoCa	M/60	2	0	2	0	0	0	N
45	hypopharynx	SpinoCa	M/64	2	1	3	2	0	0	Y
46	larynx	SpinoCa	M/62	1	0	3	5	0	0	N
47	larynx	SpinoCa	M/65	2	1	3	2	0	–	N
48	oropharynx	SpinoCa	F/62	2	1	4	7	0	0	N
49	larynx	SpinoCa	M/65	2	0	2	0	1	0	N
50	oral cavity	SpinoCa	F/47	2	0	2	1	0	0	N
51	oropharynx	SpinoCa	M/63	2	2	4	6	1	0	N
52	larynx	SpinoCa	M/78	2	0	4	6	1	–	N
53	larynx	SpinoCa	M/77	2	0	4	50	32	–	N
54	larynx	SpinoCa	M/54	2	0	4	2	2	–	N
55	hypopharynx	SpinoCa	F/47	3	2	2	8	2	–	N
56	oral cavity	SpinoCa	M/47	2	0	4	11	0	–	N
57	hypopharynx	SpinoCa	M/54	2	2	4	11	0	–	N
58	larynx	SpinoCa	M/59	3	2	3	141	0	–	N

PN, Patient number; SpinoCa, spinocellular carcinoma; ACC, adenoid cystic carcinoma; sarcoCa, Sarcomatoid carcinoma; M, male; F, female.

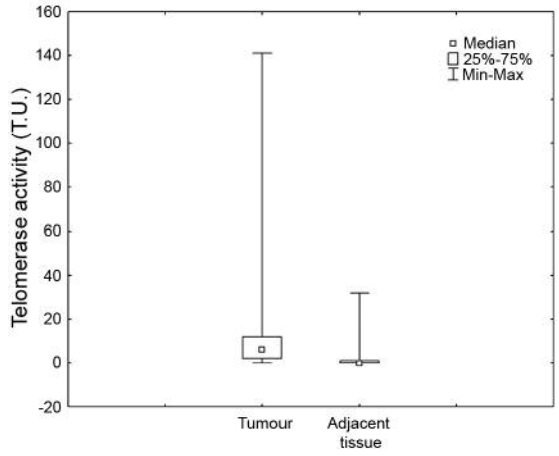

Figure 1. *Median and range of telomerase activity (T.U.) in tumour and in histologically normal tissue samples.*

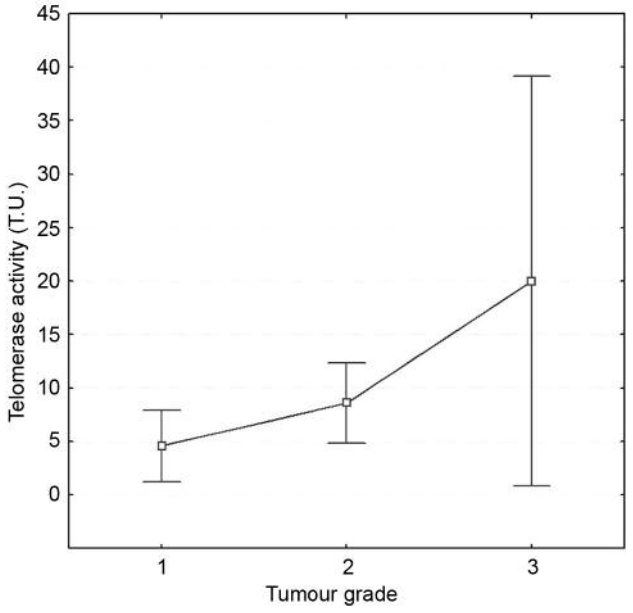

Figure 2. *Average values of telomerase activity and confidence intervals for individual grades of tumour differentiation.*

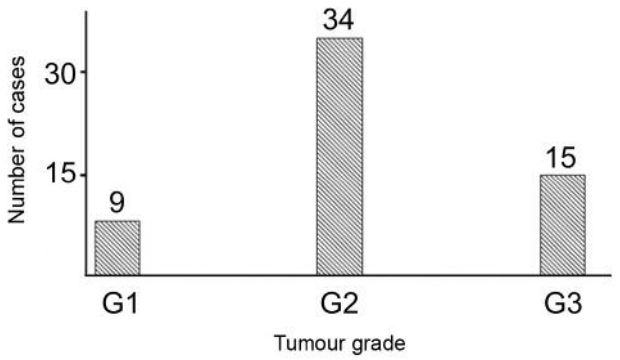

Figure 3. *Distribution of tumour samples according to their grade of differentiation (G1, G2 and G3).*

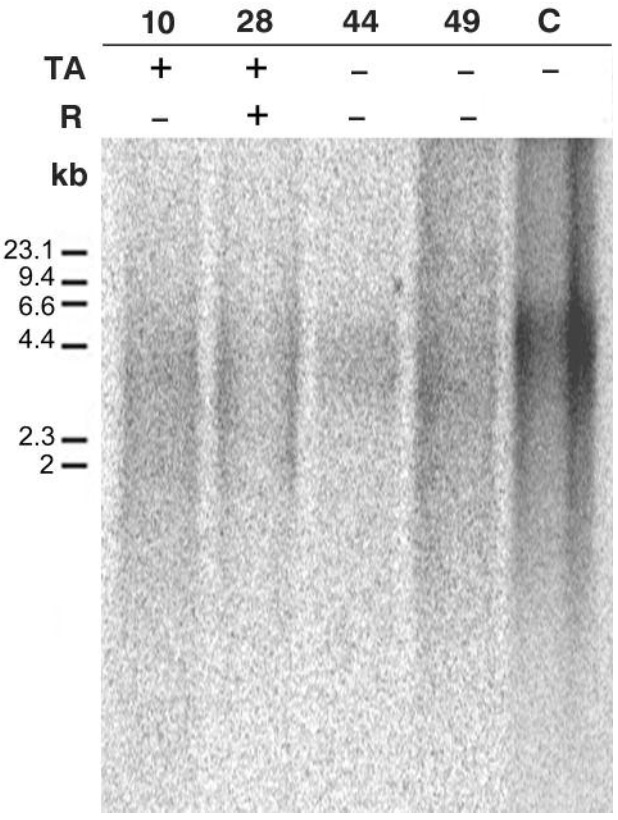

Figure 4. *Hybridisation of terminal restriction fragments with telomeric probe. Lanes 10, 28, 44, 49 correspond to patient numbers (see Table I), lane C: control (telomerase negative healthy tissue); TA (+/–): telomerase activity (positive/negative); R (+/–): occurrence of relapse (yes/no).*

stage (Figure 3) probably reflected this subjective factor; tumours at the boundary of the criteria for well-differentiated or poorly differentiated grade are nowadays often staged as intermediately differentiated.

Surprisingly, the prognosis of the patients with a telomerase-negative tumour was much worse than that of the telomerase-positive group and it is quite interesting that these patients were at a higher risk of local relapse. This group was not detected by standard histological testing, because these patients had various stages of grading. Possibly telomerase-negative tumours maintain their telomeres *via* alternative lengthening of telomeres, the mechanism based on homologous recombination (18). The standard oncological protocols of therapy (in this group surgery and radiotherapy) are probably less suitable for this group.

In addition to the absence of telomerase, the detection of another common feature of alternative lengthening of

telomeres in telomerase-negative tumours, the presence of extremely expanded and heterogeneous telomeres (18, 19), was attempted. However, the telomeres in these tumours showed patterns similar to those of normal cells. This would mean that the function of alternative lenthening of telomeres in these tumours is able to maintain telomere lengths within a normal range, thus protecting these cells from progressive increase in genome instability and promoting their survival.

It is well known that telomerase activity is expressed in 80-90% of most types of malignancies (20) including in head and neck squamous cell cancer, with some minor differences. Liao *et al*. detected telomerase activity in only 63.3% of cases (14), but usually results are within the range of 75-90% (8, 15, 20). Lee *et al*. found telomerase activation in 73% of samples from peripheral blood mononuclear cells in head and neck cancer patients; the difference in telomerase expression between healthy individuals and cancer patients was significant and was associated with poor survival of the patients (21).

The data obtained in the present study support the previous findings that telomerase may be a suitable molecular target for clinical use in prognosis and tailor-made therapy of tumour diseases (6, 22) as well as for identifying patients in need of a close follow-up and vigorous adjuvant treatment.

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