Telomerase activity levels in the surgical margin and tumour distant tissue of the squamous cell carcinoma of the head-and-neck

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Abstract. The survival of patients with a head-and-neck squamous cell carcinoma is determined by loco-regional recurrence and second primary carcinomas. As a complement to histopathology, molecular changes of tumour marginal and tumour distant tissue may confirm curative surgical tumour extirpation. We tested telomerase activity with PCR-ELISA kits.

20 tumour margin biopsies were chosen by the surgeon from 20 patients. In addition, 3 tissue samples were taken from each of 20 additional patients, one from the carcinoma centre, the tumour margin and one distant from the tumour. 50% of the carcinoma centres were telomerase-positive. Thirteen of the 40 tumour margin samples showed increased telomerase levels, and in 3 of these residual carcinoma was histopathologically detected. Six of the 20 tumour distant tissues revealed increased telomerase levels. Telomerase positivity in carcinoma-free tumour margins correlated with a good prognosis. Confirmation of the results in a larger patient group is needed.

Keywords: Head-and-neck cancer, squamous cell carcinoma, surgical tumour margin, telomerase, TRAP assay, ELISA

1. Introduction

Despite much concerted effort, successes in the therapy of head-and-neck cancers in the last 30 years have not brought any significant prolongation of the 5-year survival of these patients [2,4,29,66]. The incidence of these tumours is increasing, and despite curative therapy there is a high risk of recurrence and second primary tumours (SPTs) for patients with squamous cell carcinoma in this region [2,47,92]. Loco-regional recurrences could be caused by undetected individual tumour cells, i.e., minimal residual cancers [24,67,89]. Immunohistochemical examination, for example with monoclonal antibodies against epithelial epitopes ([25, 62,88], cf. [24]), makes the demonstration of these tumour cells easier.

According to the concept of field cancerization, to establish molecular changes as the cause of SPTs [47, 67,84], molecular-biological and genetic investigation in tumour adjacent and tumour distant tissues must be carried out and the findings compared with those of the original tumour [6,8,9,39,47,58,67,77,80, 81,84]. The clinical significance of molecular-genetic changes is, however, as yet unclear [94]. Tumour markers are being sought which can identify molecular lesions in carcinoma-free tumour marginal and distant normal tissue [6–9]. Particular attention must be given to the molecular characterization of p53 mutants [8,9, 67]: 13 of 25 patients (52%) showed p53-mutations. Only 5/13 patients (38%) suffered recurrence. Partridge et al. [67] demonstrated p53 mutants in 6 of 18 patients (28%). All 6 patients with and 3 patients without p53 gene mutations (25%) suffered recurrence or metastases.
Our results with wild type p53 and p53 mutants [44], corresponding with those of van der Toorn et al. [87], showed that the sensitivity of the immunohistochemical p53 detection is too low for the identification of patients who suffer recurrence. In the same patients we examined tissue for human papilloma viruses (HPV) in PCR. In 5 tissues from 2 of the 20 patients, we found high risk HPV (types 16 and 18) in the tumour centre, adjacent tumour margin, and in the case of one patient also in tumour distant tissue [40]. In an extensive nordic case control study, Mork et al. [55] demonstrated infection with HPV-16 as a risk factor for head-and-neck tumours. Since both p53 and HPV might be involved in the activation of telomerase [38,56,91], we used detection of telomerase activity in our study.

Since 1994 [37] the activation of telomerase in tumours, including head-and-neck tumours, has been studied and used for the diagnostic and prognostic assessment of the course of disease [46,51,68,82]. Telomerase reactivation stabilizes telomere length. It is also associated with their elongation and acquisition of an infinite proliferative capacity for cellular immortalization [31]. Increased telomerase levels have also been demonstrated in carcinoma-free tissue adjacent to tumours [19,20,51,68], cf. [52]. Proof of telomerase has demonstrated sensitivity for single carcinoma cells in several studies [26,79,91,95].

In this study, we wanted to investigate whether the expression of telomerase can be used for the characterization of carcinoma-free tumour marginal and tumour distant sections, and we sought to compare its significance in these sections with its significance in the carcinoma centre. We also compared the further course of disease in patients with telomerase-positive and telomerase-negative tissues.

2. Material and methods

2.1. Tissue samples and cell lines

To determine the ELISA cut-off value under our laboratory conditions we examined the telomerase levels in head-and-neck tissue samples from 40 tumour-free patients in our clinic (Table 1). The biopsies were harvested with the previous consent of the patients who were undergoing an unrelated surgical intervention. These were cryo-conserved and kept at −80°C.

We also investigated the telomerase activity of 4 cell lines in the logarithmic growth phase. The cell line XF 354 (kindly made available to us by the DKFZ Heidelberg) had been established from a strongly keratinized primary squamous cell carcinoma in the floor of the mouth of a 51-year-old male patient. In addition, we acquired the cell lines UM-SCC 14A to 14C (made available with thanks to Prof. T.E. Carey, University of Michigan, USA). They originate from three different local recurrences of a squamous cell carcinoma of the floor of the mouth (T1 N0 M0; G2-G3) from a 58-year-old female patient after surgical therapy and radiation [30]. In every PCR, we used the cell line UM-SCC 14C as positive control, and, after 25 minute inactivation at 85°C, as negative control.

We examined 80 tissue samples from 40 patients of our clinic between 1994 and 1997 with squamous cell carcinoma of the head-and-neck region. The samples had to be cryo-conserved for the telomerase PCR. They were harvested by the surgeon from tumours which had been completely removed but had not yet been formalinized. Three tissue samples – one each from the tumour centre, the surgical margin and from a site approximately 2 cm from the tumour – were made available to us from each of 20 patients (Table 2). All 3 samples from each patient originated from the same surgical treatment. From 20 other patients we examined only the tissue adjacent to the tumour: Table 3. All samples were cryo-conserved and archived at −80°C.

In all cases, the histopathological diagnosis of the tissues was known. Parallel to a molecular-biological examination of telomerase we subjected at least two sections of ca. 4 µm per cryo-conserved tissue sample to histopathological examination with hemalaun-eosin staining to determine whether they contained carcinoma.

2.2. Detection of telomerase

The telomerase levels were determined with the TRAP assay (telomeric repeat amplification protocol) as described by Kim et al. [37] with the easily reproducible TeloTAGGG telomerase PCR-ELISA kit (Roche Diagnostics/Mannheim, Cat. no. 1 854 666 [1, 28,96]) and a biotinylated primer. With this kit the products of amplification can be demonstrated after hybridization with a labelled probe in ELISA. As a rule, we used 3 and 0.5 µg protein for the tissue and examined 0.01 to 0.5 µg for the 4 cell lines per PCR. The protein concentration in the cell lysates was determined in accordance with Lowry et al. [49] with the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, USA).
Table 1
Telomerase levels in normal tissues from 40 patients without tumours for determination of cut-off value: tissues and patients’ data

| Control-No. | Tissue sample from | Sex/age | Nicotine/alcohol abuse | Telomerase activity* | Histo-pathological evaluation** |
|-------------|-------------------|---------|------------------------|----------------------|---------------------------------|
| Co1         | normal buccal mucosa | F/18    | none                   | 0                    | 0                               |
| Co2         | normal buccal mucosa | M/20    | unk                    | 0                    | 0                               |
| Co3         | normal buccal mucosa | M/20    | none                   | 0                    | 1                               |
| Co4         | normal buccal mucosa | M/23    | unk                    | 0                    | 0                               |
| Co5         | normal buccal mucosa | M/36    | unk                    | 0                    | 1                               |
| Co6         | normal buccal mucosa | F/50    | unk                    | 0                    | 1                               |
| Co7         | normal mucosa (nose) | F/46    | unk                    | 1                    | 0                               |
| Co8         | gingiva           | F/55    | none                   | 0                    | 0                               |
| Co9         | normal mucosa (tongue) | M/30    | none                   | 0                    | 0                               |
| Co10        | normal mucosa OPh | F/30    | unk                    | 0                    | 1                               |
| Co11        | OPh (scar)        | M/12    | none                   | 0                    | 1                               |
| Co12        | OPh              | M/58    | none                   | 0                    | 0                               |
| Co13        | OPh              | M/58    | unk                    | 0                    | 0                               |
| Co14        | lip (scar)        | M/1     | none                   | 0                    | 1                               |
| Co15        | lip (scar)        | M/3     | none                   | 0                    | 0                               |
| Co16        | lip (scar)        | F/15    | none                   | 0                    | 2                               |
| Co17        | normal skin (scalp) | F/7     | none                   | 0                    | 0                               |
| Co18        | normal skin (scalp) | F/10    | none                   | 0                    | 0                               |
| Co19        | normal skin (scalp) | M/18    | A                      | 0                    | 0                               |
| Co20        | normal skin (scalp) | F/30    | none                   | 0                    | 0                               |
| Co21        | normal skin (scalp) | M/54    | none                   | 0                    | 0                               |
| Co22        | normal skin (ear) | M/4     | none                   | 0                    | 0                               |
| Co23        | normal skin (ear) | M/5     | none                   | 0                    | 0                               |
| Co24        | normal skin (ear) | F/7     | none                   | 0                    | 0                               |
| Co25        | normal skin (ear) | M/8     | none                   | 0                    | 0                               |
| Co26        | normal skin (ear) | M/15    | none                   | 0                    | 0                               |
| Co27        | normal skin (ear) | F/24    | none                   | 0                    | 0                               |
| Co28        | normal skin (ear) | M/31    | none                   | 0                    | 0                               |
| Co29        | normal skin (ear) | F/35    | N/A                    | 0                    | 0                               |
| Co30        | normal skin (ear) | F/44    | none                   | 0                    | 0                               |
| Co31        | normal skin (nose) | M/10    | none                   | 0                    | 0                               |
| Co32        | normal skin (scar) | M/12    | none                   | 0                    | 1                               |
| Co33        | normal skin (scar) | M/34    | unk                    | 0                    | 0                               |
| Co34        | normal skin (face) | M/20    | none                   | 0                    | 0                               |
| Co35        | normal skin (face) | F/78    | none                   | 0                    | 0                               |
| Co36        | atheroma eye      | F/41    | none                   | 0                    | 0                               |
| Co37        | normal skin (eye) | M/11    | none                   | 0                    | 0                               |
| Co38        | normal skin (eye; scar) | M/46    | none                   | 0                    | 0                               |
| Co39        | normal skin (eyelid) | F/57    | none                   | 0                    | 0                               |

M = male; F = female; OPh = oropharynx; none = no or minimal nicotine and alcohol; unk = unknown; A = alcohol abuse (> 70 g/day); N/A = nicotine (>20 cigarettes/day) and alcohol abuse;

* Value of telomerase activity: 0 = negative = 0–150 mOD; 1+ = 150–450 mOD; 2+ = 450–750 mOD; 3+ = 750–1050 mOD; 4+ = above 1050 mOD;

** Evaluation of normal tissues: 0 = no lymphocytic and/or granulocytic infiltration; 1 = sparse to low infiltration; 2 = moderate lymphocytic infiltration.
Table 2
Telomerase activity (TA) of carcinoma tissue and carcinoma-free samples from 20 patients with head-and-neck squamous cell carcinoma and the clinical and histopathological details

| No. | Tumour site | Sex/age | TNM classification* | Tumour stage grading | Tumour nicotine/ alcohol abuse | TA-TC** | TA-TM** | Histopathological evaluation of tumour margin*** | TA-TD*** | Histopathological evaluation of tumour distant tissue*** |
|-----|-------------|---------|---------------------|----------------------|-------------------------------|---------|---------|---------------------------------|----------|-------------------------------------------------|
| 1   | buccal mucosa | F/76    | pT3pN1              | III                  | G2                            | none    | 0       | 1 1 and 4                             | 0        | 1                                               |
| 2   | gingiva      | M/59    | pT4pN2              | IVA                  | G2                            | N/A     | 2       | 0 2 1                                 | 1        | 3                                               |
| 3   | gingiva      | M/66    | pT1pN0              | I                    | G3                            | A       | 1       | 0 2 0 1–2                             | 0 1–2    | 1                                               |
| 4   | FoM          | M/39    | pT3pN2              | IVA                  | G3                            | N/A     | 0 0     | 0 1 4                                 | 3        |                                                 |
| 5   | FoM          | M/50    | pT4pN1              | IVA                  | G3                            | none    | 1 0     | 0 1 1–2                               | 0 1      |                                                 |
| 6   | FoM          | M/61    | pT2pN2              | IVA                  | G3                            | A       | 1 0     | 1 0 0                                 | 0 0      |                                                 |
| 7   | FoM          | M/63    | pT2pN0              | II                   | G2                            | N/A     | 1 1     | 1 1 symptoms                            | 1        | 1                                               |
| 8   | FoM          | F/65    | pT2pN0              | II                   | G2                            | none    | 1       | 0 0 1 1–2                             | 0 1      | 1                                               |
| 9   | FoM/ tongue  | M/50    | pT2pN2              | IVA                  | G2                            | N/A     | 0 1     | 2 0 0                                 | 1 0      |                                                 |
| 10  | FoM/ tongue  | M/52    | pT4pN0              | IVA                  | G3                            | none    | 0 0 1   | 0 1                                   | 0 1      |                                                 |
| 11  | FoM/ tongue  | M/52    | pT1pN0              | I                    | G2                            | A       | 0 1     | 1 1–2                                 | 0 1      |                                                 |
| 12  | FoM/ tongue  | M/52    | pT4pN0              | IVA                  | G2                            | none    | 0 0 1   | 0 1 2 3 and 4                         | 4 3 and 4 |                                                 |
| 13  | FoM/ tongue  | F/72    | pT2pN2              | IVA                  | G2                            | none    | 1 1     | 2 1–2                                 | 1 0      | 1                                               |
| 14  | tongue       | M/45    | pT3pN2              | IVA                  | G3                            | N/A     | 0 0 1   | 1 0 1                                 | 0 1      |                                                 |
| 15  | tongue       | M/58    | pT2pN1              | III                  | G2                            | none    | 0 0 2 3 | 3 3                                 | 3 3      |                                                 |
| 16  | tonsil       | M/55    | pT2pN1              | III                  | G3                            | none    | 4 1     | 1 1 0                                 | 0 0      |                                                 |
| 17  | alveolus     | F/61    | pT4pN0              | IVA                  | G2                            | none    | 4 4 1   | 2 0 1                                 | 1 1      |                                                 |
| 18  | ear          | M/58    | pT4pN1              | IVA                  | G3                            | none    | 3 1 1–2 | 0 0 1                                 | 0 1      |                                                 |
| 19  | ear          | M/75    | pT4pN1              | IVA                  | G2                            | none    | 0 0 0 0 | 0 0 1                                 | 0 1      |                                                 |
| 20  | relapse under M. sternocl. | M/58    | pT3pN2              | IVA                  | G3                            | N/A     | 0 1 1   | 0 0 2                                 | 0 1      |                                                 |

FoM = floor of mouth; M. sternocl. = Musculus sternocleidomastoideus; OPh = oropharynx; F = female; M = male; none = no or minimal nicotine and alcohol; N = nicotine abuse (>20 cigarettes/day); A = alcohol abuse (>70 g/day); N/A = nicotine and alcohol abuse; TC = tissues from tumour centre; TM = tissues from tumour margin; TD = carcinoma-free tumour distant tissues.

*All patients were M0 (without distant metastases).

**Value of telomerase activity: 0 = negative = 0–150 mOD; 1+ = 150–450 mOD; 2+ = 450–750 mOD; 3+ = 750–1050 mOD; 4+ = above 1050 mOD.

***Evaluation of adjacent tumour margin as well as tumour distant tissue: 0 = no lymphocytic and/or granulocytic infiltration; 1 = sparse to low infiltration; 2 = moderate lymphocytic infiltration; 3 = strong lymphocytic and granulocytic infiltration; 4 = pre-cancerous lesion (dysplasia 1 with acanthosis).

****Tumour margin with residual cancer.
Table 3
Telomerase activity (TA) of carcinoma-free samples from tumour margin of 20 patients with head-and-neck squamous cell carcinoma and the clinical and histopathological details

| No. | Tumour site      | Sex/age | TNM classification* | Tumour stage | Tumour grading | Nicotine/alcohol abuse | TA-TM** | Histopathological evaluation of tumour margin*** |
|-----|------------------|---------|---------------------|--------------|----------------|------------------------|----------|-----------------------------------------------|
| 21  | buccal mucosa    | M/58    | pT4pN3              | IVB          | G2             | none                   | 1        | 1–2 and 4                                     |
| 22  | buccal mucosa    | M/58    | pT1pN0              | I            | G2             | none                   | 0        | 1                                             |
| 23  | cheek            | M/55    | pT1pN1              | III          | G3             | none                   | 0        | 1                                             |
| 24  | cheek            | F/71    | pT3pN0              | III          | G1             | none                   | 0        | 2                                             |
| 25  | FoM              | F/43    | pT1pN0              | I            | G2             | N                      | 0        | 1                                             |
| 26  | FoM              | M/49    | pT2pN1              | III          | G2             | N/A                    | 0        | 1                                             |
| 27  | FoM              | M/52    | pT1pN0              | I            | G2             | none                   | 0        | 1                                             |
| 28  | FoM              | M/63    | pT1pN0              | I            | G2             | A                      | 1        | 2–3                                           |
| 29  | FoM/tongue       | M/42    | pT4pN2              | IVA          | G2             | A                      | 0        | 1–2                                           |
| 30  | FoM/tongue       | F/48    | pT4pN2              | IVA          | G2             | A                      | 0        | 2                                             |
| 31  | FoM/tongue       | M/53    | pT2pN0              | II           | G3             | A                      | 0        | 1                                             |
| 32  | FoM/tongue       | M/54    | pT1pN0              | I            | G3             | N/A                    | 0        | 0                                             |
| 33  | tongue           | M/50    | pT1pN0              | I            | G3             | A                      | 0        | 2                                             |
| 34  | tongue           | M/52    | pT1pN0              | I            | G1             | A                      | 1        | 1 and 4                                       |
| 35  | tongue           | F/64    | pT2pN2              | IVA          | G3             | none                   | 0        | 1                                             |
| 36  | tongue/OPh       | M/57    | pT1pN0              | I            | G2             | N/A                    | 0        | 1                                             |
| 37  | OPh              | M/52    | pT2pN2              | IVA          | G3             | A                      | 0        | 1                                             |
| 38  | lip               | F/59    | pT2pN0              | II           | G2             | A                      | 0        | 1                                             |
| 39  | lip               | M/74    | pT2pN0              | II           | G2             | N                      | 2        | 1                                             |
| 40  | nose              | F/89    | pT2pN0              | II           | G2             | none                   | 0        | 0                                             |

FoM = floor of mouth; OPh = oropharynx; F = female; M = male.

*All patients were M0 (without distant metastases); none = no or minimal nicotine and alcohol; N = nicotine abuse (>20 cigarettes/day); A = alcohol abuse (>70 g/day); N/A = nicotine and alcohol abuse; TA = telomerase activity; TM = tissues from tumour margin.

**Value of telomerase activity: 0 = negative = 0–150 mOD; 1+ = 150–450 mOD; 2+ = 450–750 mOD; 3+ = 750–1050 mOD; 4+ = above 1050 mOD.

***Evaluation of adjacent tumour-margin tissue: 0 = no lymphocytic and/or granulocytic infiltration; 1 = sparse to low infiltration; 2 = moderate lymphocytic infiltration; 3 = strong lymphocytic and granulocytic infiltration; 4 = pre-cancerous lesion (dysplasia 1 with acanthosis).

2.3. Verification of negative results

2.3.1. Checking taq polymerase inhibition
In all telomerase-negative tissues we examined whether the false negative results were attributable to a taq polymerase inhibition, and we used an internal 216 bp homologous amplification standard from a Telomerase PCR-ELISAPlus kit (Roche Diagnostics/Mannheim, Cat. no. 2 013 789). The standard must always show a positive result with a regular PCR in PCR-ELISA. We defined the ELISA extinction value for the internal standard as at least 500 mOD. If the result with the standard in the telomerase-negative tissue at 3 and 0.5 µg protein per PCR was negative, we reduced the quantity of protein to 0.1 and 0.01 µg protein per PCR until the PCR result with the standard was positive. We then repeated the telomerase PCR with this protein quantity and the same lysate.

2.3.2. Checking for telomerase inhibition
We tested all negative lysates for telomerase inhibition. In each PCR we mixed the corresponding negative lysate with the lysate from our positive control (0.063 µg protein from the lysate of cell line UM-SCC 14C, cf. Fig. 1) and compared the telomerase levels with and without negative tissue lysate [42]. As a rule, we used 3 µg protein of the negative lysate from our tissue samples. Since some lysates showed an inhibition of the taq polymerase (for an examination of this inhibition cf. 2.3.1.), we used protein dilutions from the lysates which were only uninhibited in the PCR with the internal standard.

2.4. Statistics
For the statistic calculations [70] we worked with SPSS 10.0 for Windows and used the Contingency Ta-
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Fig. 1. Telomerase activity in the tumour cell lines XF-354 and UM-SCC 14A to 14C.

bles, including the calculation of the Chi² value und
the Fisher Exact Test from the J.C. Pezzullo Webpage
(University of Georgetown, USA). All statistical com-
parisons are 2-sided.

3. Results

3.1. Telomerase findings in normal tissue samples to
determine the PCR-ELISA cut-off

From the telomerase levels of the 40 tumour-free
 tissue samples (Table 1) the mean plus the double
value of the standard deviation yielded a cut-off value
rounded up to 150 mOD extinction. The telomerase
level of one of the 40 normal tissue samples (3%) was
above the cut-off and was thus categorized posi-
tive (Sample 7 with a telomerase extinction value of
187 mOD, cf. Table 1). We set a quantitative extinc-
tion value of the telomerase activity as follows: nega-
tive up to 150 mOD; 1+ = 150–450 mOD; 2+ = 450–
750 mOD; 3+ = 750–1050 mOD; 4+ = above 1050
mOD.

3.2. Telomerase activation in cell lines

All four cell lines showed high telomerase levels.
There was no significant difference between the telom-
erase activity of the cell line from a primary tumour
(XF 354) and the three cell lines of various different
recurrences (UM-SCC 14A to 14C): Fig. 1 (Wilcoxon
test: p > 0.05).

3.3. Telomerase activity in samples from tumour
patients

Table 2 (cf. Fig. 2) lists the telomerase levels of
the 3 tissue samples taken from each patient together
with the patients’ data. Ten of 20 tumour centre tissues
were telomerase-positive (50%) with values between
1+ and 4+. This result is comparable with the telom-
erase activation in 3 of the 7 tumour margin samples
with residual carcinoma (43%) and values between 1+
to 4+ (U-test by Mann and Whitney: p = 0.868; ex-

Fig. 2. Telomerase activity in the tissue samples examined. Values of telomerase activity: negative = 0–150 mOD; 1+ = 150–450 mOD; 2+ =
450–750 mOD; 3+ = 750–1050 mOD; 4+ = above 1050 mOD.
tinction value – not listed – \( p = 0.561 \). In these 7 patients we compared the values of the tumour centres and tumour margin tissues with residual carcinoma. Two patients revealed different values for telomerase activation (No. 8 and 13).

Ten of the 33 carcinoma-free tumour marginal tissue samples (30%) were telomerase-positive, with lower telomerase levels of between 1+ and 2+ (Fig. 2, Tables 2 and 3) and 6 of 20 carcinoma-free tumour distant tissues (30%) showed levels between 1+ and 4+ (\( U \)-test by Mann and Whitney: \( p = 0.971 \); extinction values – not listed – \( p = 0.369 \)). In the three patients with very high telomerase levels in tumour distant tissues (patient no. 4, 12 and 15) the samples from the tumour centres and the tumour margin were telomerase-negative (Table 2). The rate of telomerase-positive samples from the tumour patients did not differ significantly among the individual tissue sections (Fisher Exact test: \( p > 0.05 \)). The rate of telomerase-positive samples from the tumour patients was significantly higher than the normal tissue of the tumour-free patients (Table 1) (Fisher Exact test: normal tissue to tumour centre tissue: \( p < 0.001 \), tumour margins with residual carcinoma: \( p = 0.008 \), carcinoma-free tumour margins: \( p = 0.001 \) and tumour distant tissue: \( p = 0.004 \)). In a statistical comparison of the extinction values of the telomerase activity in the individual tissue samples of tumour patients, the values of the tumour marginal sections were significantly lower than in the tumour centre tissue: \( U \)-test by Mann and Whitney: \( p = 0.00374 \).

### 3.4. Histopathological evaluation of the carcinoma-free tumour margins and tumour distant tissues

To interpret the telomerase levels in the carcinoma-free tumour margins and tumour distant tissues, a histopathological evaluation of the lymphocytic or granulocytic infiltration and proof of dysplastic changes were needed: Tables 2 and 3. Statistically we found no significance between the rate of telomerase activity and the rate of infiltration of mononuclear cells in the carcinoma-free tumour margin tissue (Contingency 2 \( \times \) 2 Tables – \( \chi^2 \)-test: \( p = 0.161 \)) and no significance in the tumour distant tissue (\( p = 0.057 \)). Likewise, we compared telomerase activity of tissue samples from carcinoma-free patients (Table 1) with the rate of mononuclear cell infiltrations: \( p = 0.897 \).

### 3.5. Proof of PCR inhibition in the negative samples to identify false negative results

#### 3.5.1. Proof of inhibition of taq polymerase in the negative samples

We tested the 39 telomerase-negative tissue lysates from normal tissue of the tumour-free patients (Table 1) and 51 telomerase-negative lysates from tumour patients’ tissue (Tables 2 and 3) with the internal amplification standard (compare Material and methods, 2.3.1.) to determine whether an inhibition of the PCR was attributable to false negative telomerase results (Table 4).

Five of the 39 normal tissues showed an inhibition with the internal amplification standard at 3 \( \mu \)g protein (samples 2, 12, 14, 22 and 24, cf. Table 1) and became positive with the internal standard at 0.5 \( \mu \)g protein/PCR. Only one specimen (sample 6, Table 1) reacted with the internal standard positive at 0.1 \( \mu \)g protein per assay. A repeat PCR of the 39 samples with 0.1 \( \mu \)g protein per assay was once again negative in the telomerase PCR.

All of the 51 lysates from the tumour patients with telomerase-negative tissue showed an inhibition of the taq polymerase: Table 3. In 21 inhibited specimens the internal standard was negative only at 3 \( \mu \)g protein/PCR. Thirteen negative samples were inhibited at 3 and at 0.5 \( \mu \)g and one negative sample was inhibited at 3, at 0.5 as well as at 0.1 \( \mu \)g protein/PCR. When using reduced protein dilutions which yielded a positive PCR with the internal standard, all 51 samples were once again telomerase-negative.

#### 3.5.2. Proof of telomerase inhibition in negative samples

We tested inhibition of telomerase in the telomerase-negative lysates. We mixed lysates with our positive control (cf. 2.3.2.) [42]. Only one tissue lysate (No. 33, Table 3) revealed a moderate telomerase inhibition along with a moderate taq polymerase inhibition (Table 4, No. 33) and a moderate lymphocytic infiltration (Table 3).

### 3.6. Correlation of telomerase findings with clinical data and tumour follow-up

A comparison of our telomerase results and tumour staging and grading showed no correlation with tumour size, lymph node status or with tumour grading (\( U \)-test by Mann and Whitney: \( p > 0.05 \)).

In the context of our study, we also examined whether the patients’ further course of disease can be
Table 4
Examination of telomerase-negative tissue samples with the internal standard from patients with head-and-neck squamous cell carcinoma (cf. 2.3.1. Material and methods as well as Tables 2 and 3)

| No. | Inhibitory limit of internal standard with 3, 0.5 or 0.1 µg protein/PCR |
|-----|---------------------------------------------------------------|
| 1   | none                                                                 |
| 2   | none                                                                 |
| 3   | none                                                                 |
| 4   | 3 and 0.5 µg                                                    |
| 5   | 3 µg                                                                |
| 6   | 3 and 0.5 µg                                                    |
| 7   | 3 µg                                                                |
| 8   | none                                                                 |
| 9   | none 3 and 0.5 µg                                               |
| 10  | none 3 µg                                                       |
| 11  | none 3 and 0.5 µg                                               |
| 12  | none 3 and 0.5 µg                                               |
| 13  | none 3 µg                                                       |
| 14  | 3, 0.5 and 0.1 µg                                               |
| 15  | 3 and 0.5 µg                                                    |
| 16  | 3 µg                                                                |
| 17  | none                                                                 |
| 18  | none                                                                 |
| 19  | 3 and 0.5 µg                                                    |
| 20  | 3 and 0.5 µg                                                    |
| 21  | none                                                                 |
| 22  | none                                                                 |
| 23  | none                                                                 |
| 24  | none                                                                 |
| 25  | 3 and 0.5 µg                                                    |
| 26  | 3 and 0.5 µg                                                    |
| 27  | none                                                                 |
| 28  | none                                                                 |
| 29  | none                                                                 |
| 30  | none                                                                 |
| 31  | 3 µg                                                                |
| 32  | none                                                                 |
| 33  | 3 µg                                                                |
| 34  | none                                                                 |
| 35  | none                                                                 |
| 36  | none                                                                 |
| 37  | none                                                                 |
| 38  | none                                                                 |
| 39  | none                                                                 |
| 40  | none                                                                 |

None = PCR non-inhibited; TC = tissues from tumour centre; TM-C = tissues from tumour margin with residual carcinoma; TM-F = tissues from carcinoma-free tumour margin; TD = carcinoma-free tumour distant tissues.

predicted by using the telomerase activation measured in the tumour margin tissue samples as a marker. Figure 3 shows that patients with a telomerase activation suffered recurrent disease or death less frequently, although the difference is not significant (Fisher Exact test: \( p > 0.05 \)). This is supported by a Kaplan–Meier curve of the 33 patients with carcinoma-free tumour marginal tissue (Fig. 4): Log Rank 0.1744 (mean relapse time of telomerase-positive patients 52 months, of telomerase-negative patients 35 months). The Cox’s proportional hazard regression model with recurrence as event revealed for covariates telomerase activity in the tumour margin samples (\( p = 0.202 \)), tumour stage (\( p = 0.049 \)), tumour grading (\( p = 0.639 \)) and nicotine and/or alcohol abuse (\( p = 0.637 \)) significance only for the tumour stage.

4. Discussion

The high rate of loco-regional recurrence and second primary tumours, which are decisive for the 5-year survival of patients [4,24,29,66,92], has been the object of intense investigation. Histopathologically undetectable molecular changes in a field cancerization process [78] are thought to be involved in the high rate of second primary tumours [11,15,50,65,90]. Bedi et al. [3] and Scholes et al. [73] have explored the possibility of a common clonal origin of multiple tumours [23].

Single tumour cells – “minimal residual disease” – in surgical tumour margins [8,9,24,62,87], lymph nodes near the tumour [67,88], bone marrow [7,24,25,62] cf. [63] or peripheral blood [7,88] could also be responsible for recurrence or metastasis.

Since 1996 researchers have been investigating the role of telomerase reactivation in head-and-neck tumours in clinical application [51]. The aim of our study was to examine telomerase activation as a complement to histopathology to identify molecular changes in normal tumour marginal tissue and in normal tumour distant tissue. For comparison we examined carcinoma centres.

4.1. Telomerase activity in tumour cell lines

The PCR-ELISA kits from Roche Diagnostics offer a convenient method of demonstrating telomerase, as seen in the reproducible results achieved over a longer time period using the cell line UM-SCC 14C in all PCRs as positive control (cf. Fig. 1). Mao et al. [51] also demonstrated significant telomerase activation in the cell lines UM-SCC 14A and 14B, and Sumida et al. [83] showed that other oral SCC cell lines also have high telomerase levels.
4.2. Telomerase levels in carcinoma centres

Ries et al. [69] found an increase in telomerase levels by 46% in oral carcinomas, a finding in accordance with our study (50%, cf. Fig. 2). There is a clear difference in the results reported by Sumida et al. [83] and Thurnher et al. [85]: 100%; Mao et al. [51] and Curran et al. [19]: 90%; Mutirangura et al. [57]: 88%; Chang et al. [13]: 86%; Liao et al. [46]: 82%; Kannan et al. [36]: 75%; Califano et al. [10]: 74%; and Patel et al. [68]: 78%. Dhaene et al. [20] also determined an overall increase of 94%.

In comparison to the strong telomerase activity found by most investigators, our results with lower telomerase activation may have various causes. We know from the work of Carey et al. [12] on mammal carcinoma tissue, that a carefully controlled histopathological selection of tissue specimens can raise the
4.3. Telomerase activation in carcinoma-free tumour had removed heparin from their culture-medium. It is plausible that by washing the cells previous to lysis they might have been present in the tissues we examined. Noga et al. [61] revealed that the heparin secreted in vivo from the mast cells of human lungs has a higher molecular weight. In our study the internal standard was amplified after reduction of the protein quantity/PCR in all telomerase-negative lysates. In further studies we will investigate whether our results can be improved by washing the tissue sections previous to lysis and optimizing tissue selection for the telomerase identification [12]. By doing so, Chaves-Dias et al. [14] were able to demonstrate a temporary telomerase activation in normal proliferating mast cells in cultures from peripheral blood and permanent non-inhibited telomerase levels in malignant mast cell lines. It is possible that by washing the cells previous to lysis they had removed heparin from their culture-medium.

4.3. Telomerase activation in carcinoma-free tumour margin and tumour distant tissues

As a main objective in our study we examined telomerase levels in normal tumour marginal and normal tumour distant tissues: Fig. 2. Contrary to the control patients, 3% of whom (1/40, cf. Table 1) showed increased telomerase level in tissues without carcinoma, 30% of the tumour patients had increased telomerase levels in all carcinoma-free tissue samples: Tables 2 and 3. Our findings are comparable with the telomerase-positive tumour adjacent tissues found by Mao et al. [51] – 11/29 specimens (38%), by Curran et al. [19] – 7/20 (35%), by Ries et al. [69] – 1/3 (33%), and in the review compiled by Dhaene et al. [20] – 7/20 (35%). In the study by Patel et al. [68] using the same Roche Diagnostics test kit used in our study, the telomerase activity was significantly higher (20/27 = 74%).

It is conceivable that the increased telomerase values of our study in 30% of the carcinoma-free tumour marginal tissue samples and in 30% of the tumour distant tissues are primarily or partially associated with infiltration by lymphatic or inflammatory cells. In the tumour margins and the tumour distant tissues we demonstrated lymphocytic and granulocytic infiltrates of varying intensity (cf. 3.4.): Tables 2 and 3. After in vitro stimulation of normal peripheral mononuclear blood cells, above all T and B lymphocytes, a telomerase activation can be demonstrated which varies with the age of the test person [35] and depends on the nature of the stimulation [5,32,35,48,86,97]. Telomerase is activated after contact with antigens [35]. Thus, all three tumour distant samples with high telomerase levels (patients No. 4, 12 and 15, Table 2) contained lymphatic tissue elements and might be interpreted in this direction.

The stimulation of infiltration of mononuclear cells could also be the explanation for the fact that in one of the tonsillar carcinomas examined, the telomerase levels were very high, both in the carcinoma centre and in the carcinoma-free marginal tissue (Table 2, sample 16). The activity level of telomerase in the carcinoma could have added to the activity in the lymphatic tissue. In an earlier investigation using the PCR, we detected high risk HPV (human papilloma virus) type 16 and 18 both in the tumour centre and in the carcinoma-free tumour marginal and tumour distant tissues [40]. The tumour distant tissue infected by HPV, which was antigen-stimulated, showed no telomerase activity. Despite the high telomerase levels in the tumour centre and tumour margin, the prognosis of this patient was good. In 88 months of tumour post-operative follow-up, the patient suffered no recurrence or metastasis. This one case could correspond to the findings reached in 253 patients by Gillison et al. [27]. 62 patients in whom they demonstrated high risk HPV Type 16 had a
better prognosis than HPV-negative patients. The HPV patients however also had lower tobacco and alcohol consumption than the HPV-negative patients. Mellin et al. [53] explained these unexpected findings with an antigenic heterogenization of the HPV infected tumour and an increased immunoresistance. Mork et al. [55] point to HPV infection as being a patient risk factor independent of tobacco consumption.

Further investigation is necessary to determine telomerase activation through in situ detection of hTERT expression (a human protein catalytic subunit of telomerase). Immunohistochemistry or in situ hybridization should clarify which cells participate in telomerase activation, above all in carcinoma-free tumour adjacent and tumour distant tissues [22,33,43,45,93,98].

4.4. Telomerase activation, cancer risk factors and course of disease

Some researchers are looking to the demonstration of molecular or genetic lesions as a means of assessing and predicting patients at high risk of a second primary tumour [6,16,17,71,72,80,84]. According to Patel et al. [68] immortal cells in various sites and a field cancerization process [78] may be the reason for the high telomerase activation in carcinoma-free adjacent and distant tissues. These telomerase levels are explained by the multiple mutational steps of carcinogenesis [75]. Risk factors, e.g., tobacco and alcohol abuse, are believed to be potential causative factors [15,17,65,90]. We compared our telomerase results in the tumour marginal and tumour distant tissue with the data on patient nicotine and alcohol abuse. Statistically, neither nicotine nor alcohol abuse correlated significantly with the rate of telomerase activity in the carcinoma-free tumour marginal or tumour distant tissues (Fisher Exact test: tumour marginal tissues \( p = 0.440 \), tumour distant tissues \( p = 0.574 \)). We found a comparable frequency for telomerase-negative tissue in patients with alcohol and/or nicotine abuse: Tables 2 and 3.

In an 8-year study by Papadimitrakopoulou et al. [64] the mean risk of an oral leukoplakia becoming tumorous was 36%. Sudbø et al. [80,81], in a follow-up study lasting approximately 8 years, reported a mean carcinoma risk of 24% (36/150 patients) depending upon the ploidy status: 3% (diploid lesions) and 84% (aneuploid lesions). Of the total of 163 pre-cancerous lesions investigated, increased levels of telomerase activity were recorded in 103 tissue samples (63%) [10, 13,46,57,68,69,74,83] ranging between the extremes of 39% [57] and 100% [68]. This common result from retrospective studies suggests that telomerase activation in pre-cancerous oral lesions may be a useful marker for cancer risk assessment. Prospective studies warrant investigation into telomerase activation in early carcinogenesis and comparison with genomic and proliferation markers [41].

In this study we tested the relation of telomerase reactivation in tumour marginal and distant regions with the postoperative recurrence rate of our patients. Although statistically not significant, the incidence of telomerase activation was associated with a better course of disease (Figs 3 and 4). This is contradictory to our expectations and seems controversial. In the 88 months since surgery, the tonsillar carcinoma patient (No. 16, Table 2) has had no recurrence, despite high levels of telomerase activity in the carcinoma centre and moderate levels in the tumour margin.

The telomerase activation in tumour marginal and distant tissue might be partially due to the presence of telomerase-competent infiltrating mononuclear cells. On the other hand, telomerase activity was found only in one of 40 cases with histopathologically normal epithelium of the oral mucosa in the non-tumour patients (Table 1). It must yet be clarified whether these lymphocytes are evidence of a specific activation of a local immunodefence against genetically damaged mucosa of oral carcinogenesis.

5. Conclusion

One object of this investigation into telomerase-positive tumour adjacent normal tissue was the question of whether an adjuvant anti-telomerase therapy could be a specific approach to head-and-neck cancer therapy. The individual cells in carcinoma-free margins and tumour distant sites which are affected by telomerase activation must be identified using an in situ method. In this way it should be possible to distinguish between telomerase-involved field cancerization with immortal cell clones and telomerase activation which rather indicates interfering cell-mediated immunoreaction.

Once this question has been clarified, telomerase detection might become a useful biomarker for cancer risk assessment when patients with head-and-neck carcinoma are being considered for an adjuvant therapy based on specific telomerase inhibition [34,54,76]. The unexpectedly improved prognosis of the patients with telomerase-positive tumour-adjacent tissues could be an argument against systemic anti-telomerase therapy. This result must be confirmed with an extensive follow-up in a larger patient group.
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