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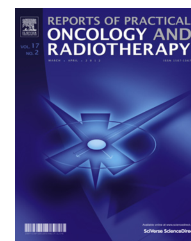
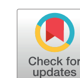


Original research article

Change of telomerase activity in peripheral blood of patients with head and neck squamous cell carcinoma pre and post curative treatment



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ABSTRACT

Background: There is no clinically applicable tumor marker for head and neck cancers. Telomerase is detected in approximately 90% of all malignant tumors, it may predict poor or favorable outcomes, thus being both a highly attractive biomarker and a target for the development of molecular-based cancer diagnostics, prognostics, and therapeutics

Aim: Primary aim was to detect a change of telomerase activity before and after curative treatment.

Materials and Methods: Patients with biopsy proven head and neck squamous cell carcinoma, stage I-IVB treated with a curative intent, performance status 0–2 and malignancy at one primary site were included in the study. Telomerase levels were tested in tissue biopsy. Plasma telomerase levels were tested at baseline, 5 days and at 3 months after treatment using ELISA.

Results: Raised plasma telomerase activity was seen in all the patients with cancer at baseline. The mean plasma telomerase level at baseline was 861.4522 ng/ml, at 5 days after completion of curative treatment was 928.92 ng/ml and at 3 months of follow up was 898.87 ng/ml. The mean tissue biopsy telomerase level was 19768.53 ng/mg. There was a significant increase in baseline telomerase levels in cancer patients compared to normals (volunteers) ($t = -3.52$, $p = 0.001$).There was a significant increase in plasma levels of telomerase at 3 months compared to baseline values ($z = -1.98$, $p = 0.04$). The increase in telomerase level did not correlate with the response of the treatment.

Conclusion: In patients with head and neck squamous cell carcinomas treated with a curative intent, the change in levels of telomerase correlates neither with the disease status nor with prognostic factors.

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Currently the study has not been presented in part or in any other form.

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1. Background

Head and neck squamous cell carcinomas (HNSCC) are the most common malignancy in India[1] and the sixth most common malignancy in the world. HNSCCs commonly present with advanced stage, with majority of the patients being treated with multi-modality therapy consisting of surgery followed by adjuvant radiation therapy with or without chemotherapy or definitive chemo-radiotherapy with salvage surgery in non- or poor responders.[2,3] The 5 year survival rates are only 50% with recurrence of disease varying between 20–30% in early stage cancer (stages I and II) and 50–60% in advanced stages (stages III and IIV).[4] Approximately 35 % of the patients develop distant metastasis and 30 % develop second primary tumors of the upper aero digestive tract, lung, or esophagus in the later period.[5,6]

Traditional prognostic factors such as overall stage and neck metastases at presentation seem to have limited prediction accuracy and reproducibility.[7] In an era where personalized cancer therapy is the need of the hour, biological markers have their own clinical significance. Several investigators are currently examining new biological markers. like chemokine receptors, Human papilloma virus, methylation markers, etc. as prognostic and predictive factors in HNSCC [8]. Biomarkers that are thus able to stratify the risk and enable clinicians to tailor treatment plans and to personalize post-therapeutic surveillance strategies are highly desirable. Till date there is no clinically applicable tumor marker for head and neck cancer. Recent studies suggest that telomere attrition, which may be an early event in human carcinogenesis, and telomerase activation, which is detected in up to 90% of malignancies, could be a potential marker of cancer risk and disease outcome.[9]

The telomere/telomerase interplay is an important element in determining genomic stability and cellular replicative potential,[10] and both telomerase expression/activity and telomere dysfunction have been extensively investigated in human cancer[11–13] with most studies indicating that they are crucial, early events in tumorigenesis often detectable at the precursor lesion stage.[14–18] Telomerase reverse transcriptase (TERT) promoter mutations resulting in increased telomerase expression have also been detected in a significant proportion of HNSCC patients.[19–21] However, obtaining samples from most tumors is invasive and not amenable to serial analyses. For this reason, investigators have begun to assay telomerase activity in body fluids that might contain tumor cells that have been released from the tumor site and have begun to compare the results with such variables as tumor progression and tumor stage.[22,23]

Several authors have reported higher TERT mRNA plasma levels in cancer patients with respect to those in controls, and a correlation has been found between circulating TERT levels and more severe clinical-pathological features and disease outcomes[9,24,25]; thus, circulating TERT could be considered a noninvasive tool for detecting cancers and monitoring the course of treatment. To our knowledge, the only study that has investigated the significance of TERT mRNA plasma levels in HNSCC patients reported that the values were indeed significantly elevated before surgery and that they decreased significantly two days after surgery.[26] Telomerase activity alone has also been studied in the past, but majority of the studies were done on populations different from those found in India. The etiology of the disease in India is completely different from other countries. Tobacco consumption is more prevalent in India and the mode of tobacco consumption (e.g. chewing, snuffing, bidi smoking, reverse smoking) is also different. Also, majority of studies carried out until now have attempted to photograph telomerase activity at a single point in time and have been unable to capture the change in telomerase activity after a curative treatment. To the best of our knowledge there is no study that quantifies the change in telomerase levels from baseline to end of the treatment to follow up.

Therefore, the present study was undertaken to assess change of telomerase activity by measuring its levels in plasma of patients with head and neck squamous cell carcinoma treated with curative treatment at timely intervals.

2. Aims and objectives

Primary objective was to detect a change of telomerase levels before and after curative treatment in patients diagnosed with head and neck squamous cell carcinomas. Secondary objectives were to evaluate the corelation between tissue telomerase and blood telomerase activity in the same patient and to correlate telomerase activity with response to treatment.

3. Materials and methods

Sample size: 101 (calculated with prevalence of peripheral blood telomerase positive patients being 73%, power 90% with a precision error of 10% using the study done by LEE BJ et al.[27]

The patients presenting to the outpatient department of Vydehi Cancer Centre were enrolled into the study. Inclusion criteria were biopsy proven HNSCC stage I, II, III, IVA and IVB treated with a curative intent, performance status 0–2 and malignancy at one primary site. Patients treated with palliative intent, patients with metastatic disease and patients treated for any other synchronous malignancy were excluded from the study.

All the patients underwent thorough history and physical examination. Biopsy was taken from the primary site. Biopsy sample was sent for histopathological confirmation of malignancy and then for tissue telomerase testing. Complete metastatic work-up was done according to the NCCN 2015 guidelines. Informed consent was taken from all the patients. The plasma telomerase testing was performed as shown in Table 1.

For every patient, three samples were collected. Response assessment was done with clinical examination, Head and neck CT scan with contrast or direct laryngoscopy or both at the end of three months using RECIST criteria based on the requirement.

Table 1 – Testing timeline.

Only surgery	Baseline (plasma)	Post op day 5 (plasma)	At 3 months post-surgery (plasma)	Tissue telomerase at the time of diagnosis
Definitive chemoradiation/radiation	Baseline (plasma)	Post 5 days of treatment (plasma)	At 3 months post radiation/chemoradiation (plasma)	Tissue telomerase at the time of diagnosis
Neoadjuvant chemoradiation followed by surgery	Baseline (plasma)	Post op day 5 (plasma)	At 3 months post-surgery (plasma)	Tissue telomerase at the time of diagnosis
Surgery followed by adjuvant radiation/chemoradiation	Baseline (plasma)	Post op day 5 (plasma)	At 3 months post radiation/chemoradiation (plasma)	Tissue telomerase at the time of diagnosis

4. Telomerase testing

Plasma from 20 controls (volunteers) was used for the assay. Biopsy from HNSCC patients were collected before curative treatment. Plasma from HNSCC patients was collected before curative treatment and after 5 days (half life of telomerase 20–24 hours) and 3 months after curative treatment. These plasma samples were used to test the Telomerase activity using ELISA.

Human TE (Telomerase) ELISA kit from Elabioscience (Catalog#E-EL-H0164) was used. This kit is based on the sandwich method of ELISA. The microplate provided in this kit has been pre-coated with an antibody specific to TE. The standard solution provided in the kit was serially diluted to obtain the kit recommended concentrations (0 ng/ml, 0.16 ng/ml, 0.31 ng/ml, 0.63 ng/ml, 1.25 ng/ml, 2.5 ng/ml, 5 ng/ml and 10 ng/ml). ELISA was performed on these standard solutions to obtain the standard curve as per manufacturer's instructions.

The biopsy samples were weighed and washed with 1X phosphate buffered saline. The tissue was then frozen using liquid nitrogen and crushed. It was then transferred to a tube and 200 μl of modified RIPA buffer containing protease and phosphatase inhibitors was added. The sample was then sonicated for 5 pluses at 40% amplitude. The samples were then stored in −80 °C overnight. The samples were then spun down at 10,000 rpm for 10 min. The protein was estimated using the BCA method. This lysate was used to perform ELISA as mentioned below.

The biopsy and the plasma samples from the control and the patients were centrifuged at 1000*g* for 15 min at 4 °C. The biopsy samples were then diluted to 1:50 using sample diluent buffer provided in the kit. The plasma samples were then diluted to 1:250 using the sample diluent buffer provided in the kit. 100 μl of the sample was added to each well in duplicates and incubated for 90 min at 37 °C. The sample was then aspirated. 100 μl of biotinylated detection antibody was added and incubated for 1 h at 37 °C. After 1 h, the antibody was aspirated and the wells were washed thrice using a wash buffer. After washing, 100 μl of HRP conjugate was added and the plate was incubated for 30 min at 37 °C. The wells were then washed five times with a wash buffer and 90 μl of substrate was added to each well and incubated for 15 min. 50 μl of stop solution was added after 15 min and then the plate was read at 450 nm using a plate reader. The optical density of the samples was analyzed using the equation obtained from the standard curve. The results obtained were represented as ng/mg for biopsy and ng/ml for plasma.

5. Statistical analysis

Data was analyzed using SPSS 16 for windows. Frequency distribution was determined for categorical variables. Kolmogorov smirnov tests for normality showed a significant deviation from normal distribution for telomerase levels. Hence non-parametric tests were applied. Friedman's rank test was used to correlate the levels of telomerase across various time points. Wilcoxon's Sign rank test was used to ascertain group differences at two time points or between tissue and blood telomerase levels. Unpaired T test was used to compare baseline telomerase levels in cancer patients with controls.

6. Results

The patient characteristics, site, staging and treatment details are shown in Table 2.

Male to female ratio was almost same and there was a significant increase in the mean telomerase of the biopsy specimen between males and females (p-value = .004), Table 6 for more details. Almost three fourth of the total patients used tobacco in some form and the mean telomerase in the biopsy specimen was more for patients who used tobacco but this increase was not statistically significant (p-value < .02). Majority of the patients had oral cavity as a primary cancer. Lymph node positivity was seen in 42 patients but the telomerase levels in the biopsy specimen were statistically insignificant between node positive and node negative patients. Patients were more in T2 and T4 stages and there were more patients with locally advanced cancers (n = 64) and less early cancers(n = 37). There was no statistical difference between the mean telomerase levels in the biopsy of early and advanced stage cancers (p < 0.8) Thirteen patients received surgery as the sole modality. Eighty eight patients received radiation therapy either in the form of definitive or adjuvant.

Response evaluation at 3 months is shown in Table 3. Eight patients did not come back for three month follow up.

Table 2 – The patient characteristics, site, staging and treatment details are shown in Table 2, along with Mean Telomerase in tissue.

Gender		Number of patients (percentage)	Mean telomerase in biopsy (ng/mg)
	Male	52 (51.5)	21481.8
	Female	49 (48.5)	19768.5
Tobacco	Yes	76 (75.2)	21173.3
	No	25 (24.8)	19300.1
Site	Oral cavity	82 (81.2)	20770.5
	Oropharynx	12 (11.9)	18958.7
	Larynx	2 (2)	
	Hypopharynx	4 (4)	
	Nasopharynx	1 (1)	
T stage	T1	11 (10.9)	
	T2	39 (38.6)	
	T3	19 (18.8)	
	T4	31 (30.7)	
N stage	Negative	59 (58.4)	20061.4
	Positive	42 (41.6)	19269.3
Group stage	Stage I	9 (8)	22014.35
	Stage II	29 (28.7)	20012.04
	Stage III	20 (19.8)	19280.26
	Stage IV	42 (41.5)	19509.28
Treatment modality	Surgery	13 (12.9%)	
	Surgery + Adjuvant RT	60 (59.4%)	
	Definitive chemo radiation	28 (27.7%)	

Table 3 – Response evaluation for patients who underwent radiation with or without chemotherapy.

Clinical/radiological response	Complete response	Partial response	Progressive disease	Local recurrence	Not available
Primary	85 (84.2)	5 (5)	1 (1)	2 (2)	8 (7.9)
Neck nodes	90 (89.1)	3 (3)			8 (7.9)

Table 4 – Baseline blood telomerase comparison between head and neck cancer patients and controls.

	Group	N	Mean	Std. deviation	Std. error mean
Baseline normal	Normal group (Volunteers)	20	587.49	203.27	45.45383
	Experimental group (Cancer patients)	20	807.95	192.28	42.99608

Table 5 – Telomerase levels in blood and tumor tissue.

		Telomerase levels (Baseline)	Telomerase levels (Day 5)	Telomerase levels (3rd Month)	Telomerase levels baseline biopsy
Total	Mean	861.45	928.92	898.87	19768.53
	Std. Deviation	198.352	487.60	145.89	15711.31
	N	101	101	101	101

7. Telomerase levels

The raised plasma telomerase activity was seen in all the patients with cancer at baseline. The mean plasma telomerase level at baseline was 861.4 ng/ml, at 5 days after completion of curative treatment it was 928.92 ng/ml and at 3 months of follow up was 898.87 ng/ml. The mean tissue biopsy telomerase level was 19768.53 ng/mg.

There was a significant increase in baseline blood telomerase levels in cancer patients compared to controls (volunteers) ($t = -3.52$, $p = 0.001$) (See Table 4)

There was a significant increase in plasma levels of telomerase at 3 months compared to baseline values on Wilcoxon's sign rank test ($z = -1.98$, $p = 0.04$). However, there was no significant change in telomerase levels after day 5 of radiotherapy compared to baseline (See Table 7)

The increase in telomerase level did not correlate with the response of the treatment. Use of tobacco, T stage, and N stage did not influence the telomerase levels in any way (Table 2). All patients with high blood telomerase levels at baseline also had high telomerase levels in the tumor tissue (Table 5).

8. Discussion

In the process of transformation of a cell from normal state to malignant, one of the key steps is the immortalization of cells. Recent evidence indicates that telomerase plays an important role in cell immortalization. Telomerase is a highly stable ribonucleoprotein complex, half-life of which is over 20 h in vivo. Loss of telomerase activity(TA) has been correlated with cellular senescence whereas its reactivation may be a prerequisite for the development of malignant tumor cells from

Table 6 – Change in telomerase level in relation to gender.

Gender		Telomerase levels (Baseline) blood	Telomerase levels (Day 5)	Telomerase levels (3rd Month)	Telomerase levels baseline biopsy
Female	Mean	804.2796	940.7605	910.8210	17951.1807
	Std. deviation	168.94648	652.91485	168.37815	12893.35695
	N	49	49	49	49
Male	Mean	915.33	917.7543	887.5998	21481.0220
	Std. deviation	210.18150	254.34779	121.58290	17927.26954
	N	52	52	52	52
Total	Mean	861.4522	928.9157	898.8655	19768.53
	Std. deviation	198.34672	487.59594	145.88912	15711.31
	N	101	101	101	101

Table 7 – Change in Telomerase levels from baseline to the 3rd month post curative treatment.

		Telomerase levels (Baseline)	Telomerase levels (Day 5)	Telomerase levels (3rd month)	Telomerase levels baseline biopsy
Total	Mean	861.45	928.92	898.87*	19768.53***
	Std. deviation	198.352	487.60	145.89	15711.31
	N	101	101	101	101
Friedmans rank test Wilcoxons Sign rank test	P value		Baseline vs DAY 5, p = 0.2	Baseline vs 3rd month p = **0.48**	Baseline biopsy vs baseline blood, p < .00001

somatic cells. More than 85% of human cancer cells are found to have TA.

Reactivation of telomerase in HNSCC has been observed in many reports, however its clinical and prognostic relevance in these studies has varied.[28–31] 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,[32,33] thus increasing the potential interest of this tumour marker. When real-time PCR or TRAP has been utilized, weak telomerase expression and activity have frequently been found in normal upper aero digestive tract epithelium.[34,35] High levels of telomerase expression have been detected in as much as 75–100 % of HNSCC.[36,37] In our study, all the patients have shown raised telomerase levels in plasma at baseline. The levels were also detected in volunteers, who were free from cancers. But the rise in cancer patients was higher and was statistically significant. ($t = -3.52$, $p = 0.001$). Similar results were found by Lee B.J. et al.[27] Peripheral blood samples were collected from 100 head and neck squamous cell cancer patients and 20 normal control group. The difference to the telomerase expression of peripheral blood mononuclear cells (PBMC) in the normal and cancer patients was significant. ($P < 0.001$).

The correlation of telomerase levels with clinical and histopathological parameters showed conflicting results. In the same study by Lee B.J. et al.,[27] the expression of telomerase in PBMCs of patients was significantly correlated with T classification ($P = 0.005$), N classification ($P = 0.002$), and AJCC stages ($P < 0.001$). On multivariate analysis, N classification ($P = 0.007$), AJCC stages ($P = 0.02$), and telomerase expression ($P = 0.017$) showed independent factors associated with poor survival. Where, as in an Indian study by Rai A. et al.,[38] telomerase levels showed no correlation with clinical parameters, such as sex of the patient, history of adverse habits, site of the lesion, size of the lesion, lymph node involvement, or overall stage of the patient. However, significant correlation with age of the patient and histopathological grades of malignancy was observed. In the present study, there was no correlation between telomerase levels with T stage, N stage and tobacco use, although at baseline male patients had statistically significantly increased telomerase levels. Similar results have also been reported by Fabricius and Koscielny et al.[29,39]

So far, only one study has answered whether telomerase can serve as an indicator of disease status in follow up.[26] The study evaluated telomerase activity in peripheral whole blood from HNSCC patients as a biomarker for diagnosis or detection of recurrence during follow-up. Among 41 HNSCC patients who showed positive telomerase activity before surgery, 32 (78.1%) showed a conversion of telomerase activity to negative after surgery. In follow-up, 6 out of 8 (75%) showed conversion of telomerase activity from negative to positive after recurrence. Telomerase activity was changed to negative in 4 out of 6 (66%) recurred patients with positive telomerase activity after second surgery. The rate of telomerase activity in PBMCs was found to be significantly higher in HNSCC patients with respect to that in healthy controls and associated with advanced stage, lymph node metastases, and poor overall survival (OS).[27,40] Two mechanisms have been hypothesized to explain this finding.[26] First, PBMC could be activated by soluble factors, secreted either by cancer or by the tumor microenvironment. Mean serum vascular endothelial growth factor levels have, indeed, been significantly linked with telomerase activity in PBMCs in HNSCC patients.[41] Alternatively, PBMC could be activated after tumor antigen processing and cross-presentation has taken place in draining lymph nodes. In our study, we did not find any fall in telomerase levels after treatment. On the contrary, we found a rise in 3rd month samples which was statistically significant and which did not correlate with the residual disease status. According to the theory of field cancerization, carcinogenic insults (e.g. tobacco) may

result in multiple malignant foci. This fact may be one of the reasons for persistent high telomerase levels, as three fourth of the patients in our study were tobacco users with majority of them being tobacco chewers. Also the telomerase levels were significantly very high in biopsy tissue samples and plasma in our patients. Hence, the plasma levels of telomerase measured quantitatively in patients with HNSCC is a complex issue that might warrant further studies. Based on our study we cannot recommend testing blood telomerase as a marker for response and prognosis in head and neck squamous cell carcinomas. The role of telomeres and telomerase in head and neck squamous cell carcinoma from pathogenesis to clinical implications has been discussed in detail by Rizzo et al.[9] They felt that our understanding of the complicated telomere/telomerase interplay in human cancer remains, in fact, for the time being incomplete. The negative results of our study show that telomerase testing in head and neck squamous cell carcinoma patients is not reliable.

9. Conclusions

In patients with head and neck squamous cell carcinomas treated with curative intent, the change in levels of telomerase in peripheral blood correlates neither with the disease status nor with burden of disease post curative treatment. Hence, at present quantifying the change in telomerase from blood samples cannot be recommended as a biomarker for response or prognosis in head and neck cancers.

Conflict of interest

None declared.

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