

Immunohistochemical and mutational status of telomerase reverse transcriptase in conjunctival squamous cell carcinoma

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Purpose: Mutations in human telomerase reverse transcriptase (TERT) are associated with increased telomerase activity in cutaneous melanomas. Conjunctival squamous cell carcinoma, also referred to as ocular surface squamous cell carcinoma, is cancer on the surface of the eye. Recent studies have identified UV signature mutations in TERT promoters in ocular melanoma and ocular surface squamous neoplasia. However, its immunohistochemical status has not been reported in ocular surface squamous cell carcinoma. This study aimed to explore the immunohistochemical and mutational status of TERT in ocular surface SCC. **Methods:** The immunohistochemical expression of TERT and mutational status of TERT promoter was evaluated in 19 ocular surface squamous cell carcinoma cases. Conjunctival melanoma tissue was used as a positive control. **Results:** The cytoplasmic overexpression of TERT was detected in 11/19 (57%), and TERT promoter mutations were identified in 6/19 (31%) of ocular surface squamous cell carcinoma. Out of these, 66% had a C228T mutation, and 33% had a C250T mutation. The TERT expression was found to be associated with a high ($\geq T3$) AJCC category ($P = 0.023$), and TERT immunorexpression was significantly correlated with reduced disease-free survival ($P = 0.024$, log-rank analysis) in ocular surface squamous cell carcinoma patients. **Conclusion:** The present study demonstrates that TERT promoter mutations with UV signatures are frequent in ocular surface squamous cell carcinoma. The increased expression of TERT could be of biological significance in aggressive ocular surface squamous cell carcinoma.

Key words: Conjunctival, immunohistochemistry, ocular surface squamous cell carcinoma, TERT, UV signature mutations

Conjunctival squamous cell carcinoma is less frequent but highly aggressive and metastatic ocular surface tumors.^[1,2] The yearly occurrence of ocular surface squamous cell carcinoma is reported to range from 0.02 to 3.5 per 100,000 people.^[3] The tumor suppressor p53 is an important mediator of cellular responses to DNA damage in mammalian cells. It attributes to the regulation of the cell cycle and apoptosis.^[4,5] The ultraviolet (UV) signature in the form of tandem mutations (CC to TT) in TP53 is common in SCC, indicating the crucial role of chronic exposure to UV light in the pathogenesis of these tumors. Additional predisposing factors of ocular SCC include human papillomavirus 18 or 16, human immunodeficiency virus, and hepatitis B and C infection.^[6,7]

Telomerase is an RNA-dependent DNA polymerase that synthesizes nucleotides in a TTAGGG sequence at the end of the chromosome, which makes the cell immortal by stabilizing its length. TERT is the catalytic subunit of the telomerase enzyme and is linked with the telomerase RNA component (TER).^[8,9]

The TERT gene, located on chromosome 5, encodes the rate-limiting catalytic reverse transcriptase subunit of the telomerase ribonucleoprotein (RNA) complex. The UV signature mutations in the TERT promoter region have been associated with up to 71% of cutaneous melanoma. The mutations in the TERT promoter regions enhance the expression of TERT by creating *de novo* CCGGAA/T (a general binding motif for E-twenty-six (ETS)/ternary complex transcription (TCF) factor), which differs from the preexisting CGAA/T ETS binding sites present within the TERT promoter region.^[10,11]

In addition, TERT promoter mutations are common in conjunctival melanoma and had been reported first in cutaneous melanoma.^[12] However, the association between TERT mutation and its immunohistochemical expression has not been analyzed in ocular surface SCC. Here we investigate the relation between mutational status of TERT promoter region and TERT immunorexpression in ocular surface squamous cell carcinoma and its association with poor prognostic features of ocular surface SCC.

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Methods

Tissue and DNA isolation

A total of 19 samples of histopathologically confirmed cases of ocular surface SCC were acquired from the department of ocular pathology. The clinicopathological characteristics, gross appearance, and radiological features of the selected patients were noted. Ocular surface SCC was classified as poorly differentiated (PD), moderately differentiated (MD), or well-differentiated (WD) based on the extent of keratinization. AJCC cancer staging criteria (8th edition) was used to determine the tumor stage. All cases included in this study were CIN (corneal intraepithelial neoplasia).^[13]

Fresh tumor tissues of 19 ocular surface SCC cases were available for DNA extraction, light microscopy, and immunohistochemistry (IHC). The DNA was extracted using a DNeasy blood and tissue kit (Qiagen Dusseldorf, Germany).

The ocular surface SCC patients were followed up at 6 months (after surgery) interval for a mean period of 43 months (range: 14–55 months). Inclusion criteria for this study: histopathologically proven cases of ocular surface SCC. Patients who had received radiotherapy/chemotherapy were excluded from the study.

DNA sequencing and mutational analysis of TERT promoter

Polymerase chain reaction was performed on the extracted DNA from the fresh ocular surface SCC tissues to screen for the mutation in TERT promoter by using forward primer 5'-CAGCGCTGCCTGAAACTC-3' and reverse primer 5'-GTCCTGCCCTTACCTT-3' (product size: 163 bp) as previously described [Fig. 1a].^[14] The PCR products were sequenced using Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and loaded onto the ABI 310 automated DNA sequencer (Applied Biosystems, Foster City, CA) [Fig. 1b]. The chromatograms were downloaded and the sequences were compared with reference sequence NC_000005.10 obtained from the NCBI database using the BioEdit software [Fig. 1c].

Immunohistochemistry

The unstained tumor sections were taken on Poly Lysine microscope adhesion slides (Thermo Scientific, Fermont CA, USA) from paraffin-embedded formalin-fixed blocks. These slides were then deparaffinized in xylene, followed by rehydration through graded alcohols. Antigen retrieval was performed by heating the slides in citrate buffer (pH 6.0) for 20 min by using the LabVision PT module. After cooling, the slides were washed using TBS (pH 7.5) and then incubated for 20 min with 0.3%-v/v H₂O₂, followed by incubation with primary monoclonal antibodies against human TERT (A-6, SCBT, Santa Cruz, CA, USA) at a dilution of 1:50 and were processed using the UltravisionQuanto detection system (Thermo Scientific, Fermont CA, USA). The immunoreactivity was detected using the DAB peroxidase substrate. Counterstaining was performed with hematoxylin, and the sections were dehydrated and mounted with DPX (BDH, Poole, UK) and examined under a light microscope.

The semi-quantitative staining results were analyzed based on percentage positivity and staining intensity, where the cut-off value was observed to be 2 [Table 1].^[12,15] TERT

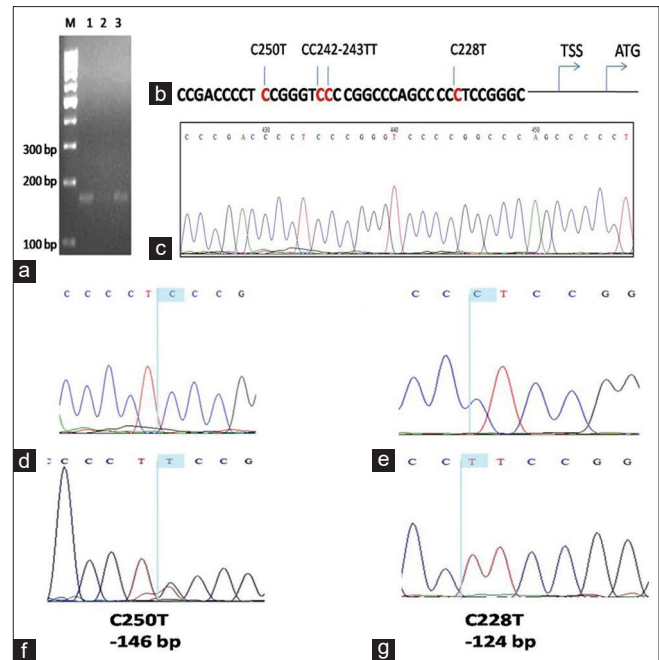


Figure 1: (a) PCR-amplified products of TERT promoter from conjunctival SCC cases (M, DNA size marker). (b) Schematic representation showing the translation (ATG) start site, transcription start site, and known hotspot mutations in TERT promoter (the region highlighted in red) (c) Wild-type chromatogram of the promoter region of TERT from normal skin obtained by Sanger sequencing. (d and e) Sequence chromatograms showing the wild-type sequence. (f and g) The mutations c.-146C>T (C250T; Chr.5: 1295250C>T) and c.-124C>T (C228T; Chr.5:1295228C>T) identified in TERT promoter locus in conjunctival SCC cases

staining was appraised by one pathologist and two observers simultaneously by a multihead light microscope and a consensus was reached for each IHC score.

Statistical analysis

The Chi-square test was performed to analyze the relation between the immunoreactivity of TERT and its mutational status with clinicopathological parameters. Survival analysis was performed using the Kaplan-Meier analysis using the log-rank test. All statistical analysis was carried out using SPSS 19.0 for Windows (SPSS Inc., IBM Company, IL, USA). $P < 0.05$ was considered to be statistically significant.

Results

Association of TERT immunoreactivity with clinicopathological parameters and survival in patients with ocular surface SCC

Cytoplasmic overexpression of TERT was observed in 11/19 (57%) ocular surface SCC cases [Fig. 2a]. Conjunctival melanoma tissue was used as a positive control [Fig. 2b]. For negative control, the primary antibody was replaced by TBS [Fig. 2c]. TERT immunoreactivity was found to be less or negligible in normal eyelid skin [Fig. 2d]. In our study, the prognostic parameters, namely size, TNM, staging, invasion, surgical intervention, and recurrence, were compared. TERT positivity was significantly associated with higher ($\geq T3$) AJCC category in 9/11 (81%) ocular surface SCC cases ($P = 0.023$) [Table 2]. However, no significant

association of TERT expression was observed with other poor prognostic features [Table 2]. The prognostic potential of TERT expression was determined using Kaplan–Meier survival analysis. TERT overexpression was associated with lower disease-free survival in ocular surface SCC ($P = 0.024$, log-rank analysis) [Fig. 3].

Table 1: Immunohistochemistry scoring

| Antibody | Score | Criteria | | Result |
|----------|-------|------------------|------------------------|---------------------------|
| | | % Positivity (A) | Staining intensity (B) | AxB=IHC SCORE |
| TERT | 0 | - | Negative | Negative (0) |
| | 1 | <10% | Weak | Negative (1-2) |
| | 2 | 10-50% | Moderate | Moderately positive (4-6) |
| | 3 | >50% | Strong | Strongly positive (6-9) |

Table 2: TERT immunohistochemical expression and association with clinicopathologic features and TERT promoter mutation in ocular surface squamous cell carcinoma

| Parameter | TERT (+ve) (n=11) | TERT (-ve) (n=8) | P |
|-----------------------------------|-------------------|------------------|---------|
| Age (Years) | | | |
| ≥60 (n=7) | 4 (57.14%) | 3 (42.86%) | 1.000 |
| <60 (n=12) | 7 (58.33%) | 5 (41.67%) | |
| Gender | | | |
| Females (n=6) | 2 (33.33%) | 4 (66.67%) | 0.31 |
| Males (n=13) | 9 (69.23%) | 4 (30.77%) | |
| Location | | | |
| Upper lid (n=16) | 9 (56.25%) | 7 (43.75%) | 1.000 |
| Lower lid (n=3) | 2 (66.67%) | 1 (33.33%) | |
| Largest tumor diameter (mm) | | | |
| ≥20 (n=12) | 6 (50%) | 6 (50%) | 0.633 |
| <20 (n=7) | 5 (71.43%) | 2 (28.57%) | |
| Histopathological differentiation | | | |
| Moderately differentiated (n=10) | 8 (80%) | 2 (20%) | 0.069 |
| Well differentiated (n=9) | 3 (33%) | 6 (66%) | |
| Sunlight exposure (h) | | | |
| Less (n=10) | 5 (50%) | 5 (50%) | 0.6499 |
| More (n=9) | 6 (66.67%) | 3 (33.33%) | |
| Surgical intervention | | | |
| Excision (n=8) | 5 (62.5%) | 3 (37.5%) | 1.0000 |
| Exenteration (n=11) | 6 (54.55%) | 5 (45.45%) | |
| Stage | | | |
| T1 + T2 (n=8) | 2 (25%) | 6 (75%) | 0.0237* |
| T3 + T4 (n=11) | 9 (81%) | 2 (18%) | |
| Recurrence | | | |
| Present (n=7) | 6 (85.71%) | 1 (14.29%) | 0.1473 |
| Absent (n=12) | 5 (41.67%) | 7 (58.33%) | |
| Tumor related death (n=1) | 0 | 1 | |
| TERT promoter status | | | |
| Wild-type (n=13) | 8 (61%) | 5 (38%) | 1.00 |
| Mutant (n=6) | 3 (50%) | 3 (50%) | |

*Significant

TERT promoter mutation status in ocular surface squamous cell carcinoma

Direct sequencing analysis revealed mutations in 6/19 (31%) of ocular surface squamous cell carcinoma cases. Of these, four cases (66%) had C250T mutation and two cases (33%) had C228T mutation [Fig. 1d-g]. Both the mutations generated a *de novo* ETS (E-26 transformation-specific) binding motif (5'-tcc-3').

Correlation of mutational status of TERT promoter region with immunohistochemistry, clinicopathological parameters, and clinical outcomes in ocular surface SCC

TERT overexpression was found to be in 3/6 (50%) cases with TERT promoter mutation. TERT immunopositivity was also observed in 8/13 (61%) cases without mutation in the TERT promoter region. No significant association was observed between mutational status in TERT and TERT immunopositivity ($P = 1.000$). No significant association was observed between TERT promoter mutation and reduced disease-free survival in patients of ocular surface SCC ($P = 0.098$, log-rank test) and with any of the poor prognostic features of ocular surface SCC [Table S1].

Discussion

Mutations in the TERT promoter region were first observed in cutaneous melanoma and were later reported in different cancers, including SCC of the skin and ocular surface origin.^[10,11,16]

Our study revealed that (6/19) 31% of our ocular surface squamous cell carcinoma cases harbored TERT promoter mutations, creating binding sites for the ETS transcription

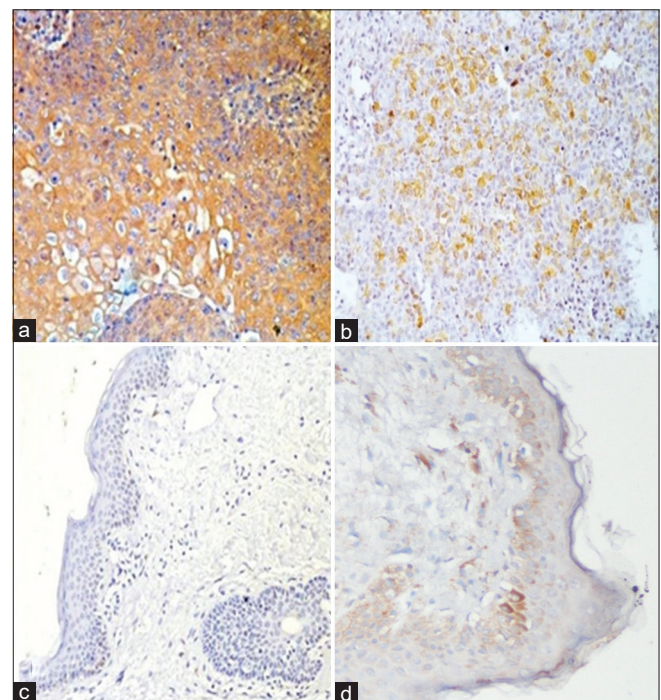


Figure 2: (a) Strong cytoplasmic positivity of TERT in a case of conjunctival squamous cell carcinoma. (b) Positive control showing cytoplasmic positivity of TERT in a case of conjunctival melanoma tissue (c) Negative control; primary antibody replaced by TBS. (d) low expression of TERT in skin eyelid

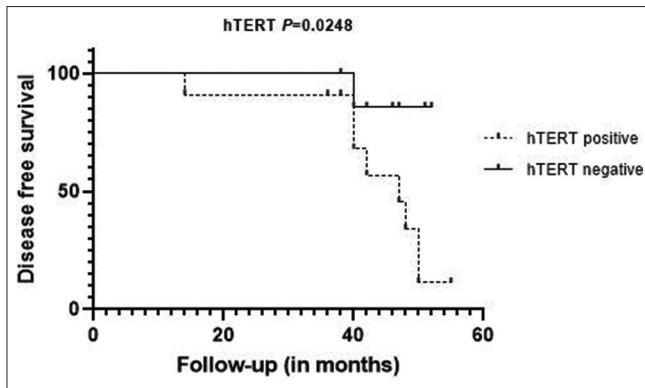


Figure 3: Kaplan–Meier analysis showing reduced disease-free survival rates in conjunctival squamous cell carcinoma in patients with positive TERT expression

factor. The mutations detected in ocular surface SCC were at previously reported regions with a UV signature (C > T).^[10,11] The results presented in our study are in concurrence with studies previously performed by Scholz *et al.*^[17] in ocular surface neoplasia where (40.4%) invasive SCC revealed the presence of UV signature mutations in TERT promoter.

Various carcinomas with this type of promoter mutation in TERT are shown to be associated with increased TERT mRNA expression, indicating an increased telomerase activity.^[11,18,19] However, the expression pattern of TERT has not been investigated in ocular surface SCC. We observed strong immunostaining of TERT protein in 57% of ocular surface SCC. TERT immunopositivity was also observed in cases without mutations in the TERT promoter region, which could be the reason for the alternative mechanism of telomerase activity in these cases. A similar observation of TERT overexpression in the absence of mutation in the TERT promoter region has also been observed in cutaneous melanoma and follicular thyroid carcinoma. The lack of association between 146C>T mutation and overexpression of TERT in melanoma infers different mechanisms such as promoter hypermethylation rather than point mutations.^[12,20,21] The reason for this discordance may be attributed to alternative mechanisms of telomerase activation, such as alternative splicing of TERT mRNA and DNA methylation. The overexpression could be a reason for the cooperation of signaling pathways such as non-canonical NF- κ B with ETS factor inducing the reactivation of telomerase.^[22-24] Recent findings have uncovered that the methylation of TERT hypermethylated oncological region (THOR) increases the expression of TERT in the absence of mutations in the TERT promoter by preventing the repressive elements to bind at the repressive region thereby, allowing the promoter to be activated by appropriate transcription factors.^[25]

It has been observed in lung cancer that high expression of hTERT is associated with the TNM stage, lymphatic metastasis, and poor prognosis. In our study, the overexpression of hTERT was associated with poor overall survival and disease-free survival of human cancer patients. Thus, hTERT could be a potential target gene for cancer.^[26] A significant association was observed between cytoplasmic overexpression of TERT in tumors with a higher AJCC category (≥ 3) and in patients with reduced disease-free survival in ocular surface SCC. However, we observed no relationship between TERT promoter

mutations with overall disease-free survival in ocular surface SCC. It must be said that the number of mutated TERT promoter ocular surface SCC cases was low in our cohort, and a more extensive study with more patients is needed to assess survival credibly.

Many therapeutic strategies, such as the development of oligonucleotide inhibitors of telomerase, are in their early phase of a clinical trial, but no clinical efficacy has been demonstrated yet. Telomerase-directed immunotherapies have been developed as endogenous TERT peptides produced by cancer cells can be recognized by MHC Class I and II and trigger adaptive immune responses. Responses to TERT immunotherapy can be boosted by enrolling more patients with TERT promoter mutation and high expression of TERT as it may enhance the TERT antigen presentation and can produce better results.^[27]

Conclusion

In summary, we have reported the immunohistochemical staining pattern of TERT expression in ocular surface SCC, in addition to attempting to correlate the immunostaining to the mutational status of the TERT promoter region. The TERT promoter mutations having UV signatures in our study were found to be more common in ocular surface SCC, suggesting the possible role of UV-induced genetic alteration in the pathogenesis of ocular surface SCC, which is in concurrence with the study by Lin *et al.*^[28] The immunohistochemical overexpression of TERT along with TERT promoter mutation in ocular surface SCC provides further evidence of UV-induced pathogenesis kinship with cutaneous and ocular melanoma.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Table S1: TERT promoter mutation and association with clinicopathological features in ocular surface squamous cell carcinoma

| Parameter | Mutant TERT (n=6) | Wild-type TERT (n=13) | P |
|-----------------------------------|-------------------|-----------------------|-------|
| Age (Years) | | | |
| ≥60 (n=7) | 2 (28.57%) | 5 (71.43%) | 1.000 |
| <60 (n=12) | 4 (33.33%) | 8 (66.67%) | |
| Gender | | | |
| Females (n=6) | 1 (16.66%) | 5 (83.34%) | 0.604 |
| Males (n=13) | 5 (38.46%) | 8 (61.54%) | |
| Location | | | |
| Upper lid (n=16) | 5 (31.25%) | 11 (68.75%) | 1.000 |
| Lower lid (n=3) | 1 (33.33%) | 2 (66.67%) | |
| Tumor growth pattern | | | |
| Nodular (n=5) | 2 (40%) | 3 (60%) | 1.000 |
| Diffuse (n=14) | 4 (28%) | 10 (71%) | |
| Largest tumor diameter (mm) | | | |
| ≥20 (n=12) | 4 (33%) | 8 (66%) | 1.000 |
| <20 (n=7) | 2 (28%) | 5 (71%) | |
| Histopathological differentiation | | | |
| Moderately differentiated (n=11) | 5 (45%) | 6 (54%) | 0.177 |
| Well differentiated (n=8) | 1 (12%) | 7 (87%) | |
| Sunlight exposure (h) | | | |
| Less (n=10) | 4 (40%) | 6 (60%) | 0.628 |
| More (n=9) | 2 (22%) | 7 (77%) | |
| Surgical intervention | | | |
| Excision (n=8) | 2 (25%) | 6 (75%) | 1.000 |
| Exenteration (n=11) | 4 (36%) | 7 (63%) | |
| Stage | | | |
| T1 + T2 (n=8) | 2 (25%) | 6 (75%) | 1.000 |
| T3 + T4 (n=11) | 4 (36%) | 7 (63%) | |
| Recurrence | | | |
| Present (n=7) | 4 (57%) | 3 (42%) | 0.128 |
| Absent (n=12) | 2 (16%) | 10 (83%) | |
| Tumor-related death (n=1) | | 1 | |