Expression of Glycosyltransferases; ST3GAL1, FUT3, FUT5, and FUT6 Transcripts in Oral Cancer

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ABSTRACT: Oral carcinogenesis process is frequently accompanied by alterations in glycosylation, regulated by sialyltransferase (ST) and fucosyltransferase (FUT) enzymes. The study aimed to assess ST3GAL1, FUT3, FUT5, and FUT6 mRNA expression by semi-quantitative reverse transcriptase PCR in 50 oral cancer and 50 adjacent normal tissues. The results indicated increased ST3GAL1 mRNA levels in malignant tissues as compared to adjacent normal tissues. A significant decrease in FUT3 and FUT5 transcripts was observed in malignant tissues as compared to adjacent normal tissues. Survival analysis of FUT3 transcript levels depicted significant lower survival with values above cutoff. The levels of ST3GAL1 and FUT6 were found to be higher in metastatic tissues as compared to the non-metastatic tissues and were also higher in advanced disease as compared to the early disease. The results indicated potential clinical utility of ST3GAL1, FUT3, FUT5, and FUT6 transcript levels in oral cancer pathogenesis.

KEYWORDS: fucosyltransferase, glycosyltransferase, glycosylation, oral cancer, sialyltransferase

Introduction

The Global Cancer Statistics has reported 263,900 new oral cancer cases and 128,000 deaths worldwide because of oral cancer.1 The Indian subcontinent accounts for one-third of the world’s oral cancer burden, which is mainly attributed to different forms of tobacco consumption.2,3 The increasing incidence and late presentation of disease have generated a need for the development of newer markers for early diagnosis, prognosis, and disease monitoring, along with development of newer drug targets for future interventions. Oral carcinogenesis is a multistep process and is frequently accompanied by drastic alterations in cell surface oligosaccharide expression. Carbohydrate moieties, which are expressed on cancer cells, mostly contain sialylated and/or fucosylated structures. The increased expression of sialylated and fucosylated glycans has been associated with tumor progression. This has been suggested to stem from the altered expression of sialyltransferase (ST) and fucosyltransferase (FUT) genes encoding enzymes that are responsible for the biosynthesis of tumor antigens.4–6 Previous studies from our laboratory have indicated elevated serum and tissue ST and FUT enzyme activities in oral cancer patients.7–9 Elevations in serum and salivary sialic acid have been reported in oral cancer, which might be because of increase in ST or sialidase activities.7 Various STs (ST3GAL, ST6GAL, ST6GALNAC, and ST8) are named according to the sialyl linkages they form.10 ST families are further sub-divided into 20 sub-families in mammals; each of them has conserved amino acid positions.11 ST3GAL family contains α-2,3 STs, which catalyze the transfer of sialic acid residues via an α-2,3 linkage to galactose residue of terminal Galbeta1,3GalNAc
structure on O-linked oligosaccharide of glycoproteins or on glycolipids. It has been predicted that ST3GAL1 expression was mainly involved in biosynthesis of O-linked oligosaccharides of glycoproteins. The mRNA expression of ST3GAL1 responsible for sialylation of O-glycans has been observed to be increased in colorectal cancer, breast carcinoma, and bladder cancer. Wang et al have observed significant down-regulation of ST3GAL1 with enhanced ST6GAL1 mRNA expression in cervical cancer. ST3GAL3 and ST6GAL1 have been shown to be associated with poor prognosis of human breast cancer and colorectal cancer. Elevated levels of α-2,3 ST enzyme activity have been observed earlier in oral cancer patients. However, the studies on its transcript levels (ST3GAL1) are lacking in oral cancer patients.

FUT genes from human genome are divided in three sub-families, α-1,2 FUT, α-1,3/4 FUT, and α-1,6 FUT. FUT3–FUT8 and FUT9–FUT11 belong to the group of α-1,3/4 FUTs. The altered expression of FUT enzyme activities has been reported in oral cancer patients; however, expression of different types of FUT transcripts has not been studied earlier in oral cancer. Earlier reports have indicated no significant alterations of FUT3 and FUT5 and a moderate increase in FUT6 in colon cancer, while studies by Hiraiwa et al have shown increase in FUT3, FUT6, and FUT8 transcripts in colon cancer tissues. An increase in FUT4 has been observed in colorectal adenomas and carcinomas. An increase in FUT6 expression has been observed in breast cancer and in colon cancer cells.

Earlier reports have documented elevations in ST and FUT enzyme activities in oral cancer patients. However, the expressions of ST and FUT transcripts have not been explored in oral cancer patients. Therefore, present investigation aimed to evaluate clinical significance of mRNA expressions of ST3GAL1, FUT3, FUT5, and FUT6 in malignant and adjacent normal tissues obtained from oral cancer patients.

### Subjects and Methods

**Study subjects.** The study was approved by Institutional Review Board of the Gujarat Cancer & Research Institute, Ahmedabad, India. We enrolled 50 untreated oral cancer patients with no major disease in recent past. Patients gave their written, informed consent to participate in the research. Pathological tumor, node, and metastasis (pTNM) staging of oral cancer patients was determined as per American Joint Committee on Cancer (AJCC) norms. The details of the oral cancer patients are mentioned in Table 1. In all, 18% of the patients were tobacco non-habituates, whereas 82% were tobacco habituates. The patients were followed for a period of 45 months, and correlation of the molecular markers under study with overall survival was analyzed.

**Sample collection.** Tissue samples from oral cancer patients were collected on ice from operation theater immediately after surgical resection of the tumors. Adjacent normal tissue samples were selected from the tumor free margins at least 2–3 cm away from the tumor as defined by the histopathologist. The tissue specimens were washed with ice-cold phosphate buffer saline (PBS: pH 7.4) and “RNA later” (Qiagen, Valencia, CA, USA; Cat No.: 1017980) is RNA stabilizing agent was added, and were stored at −80°C until analyzed.

### Methodology

**RNA.** RNA was isolated from all the tissue samples (paired adjacent normal and malignant, n = 50) collected from oral cancer patients using RNA isolation kit (Qiagen, Valencia, CA, USA) and stored at −80°C. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for transcripts ST3GAL1, FUT3, FUT5, and FUT6 was carried out using specific primer sequences as depicted in Table 2. BACTIN was used as internal control in all the reactions, and RT-PCR was carried out using one-step RT-PCR kit (Qiagen, Valencia, CA, USA). The amplifications were performed using thermal cycler (Eppendorf Mastercycler gradient, Eppendorf, CA, USA). The amplifications were performed using ther...
Hamburg Germany). Reactions contained 500 ng of RNA, 0.6 μM of primers for the target genes, and 0.3 μM of primers for the house-keeping gene (βACTIN) in 50-μL RT-PCR reaction volume. The reaction conditions are shown in Table 3. The reaction products were electrophoresed on 1.5% agarose gels containing ethidium bromide, and gels were analyzed densitometrically using gel documentation system (Alpha Innotech, USA). For semi-quantitative analysis of ST3GAL1, FUT3, FUT5, and FUT6 transcripts, the integrated density value (IDV) of each sample was compared with the IDV of βACTIN coamplified in the same tube, and relative expression (IDV of the glycosyltransferase transcripts/IDV of βACTIN) was measured. Reproducibility of the samples was checked by running the samples in the same batch as well as in different batches.

**Statistical analysis.** Statistical analysis of 50 paired adjacent normal and tumor tissues was carried out using SPSS statistical software version 15.0. Student’s paired t-test was used to compare the levels between adjacent normal and malignant tissues of the oral cancer patients. Student’s independent t-test was performed to assess the levels of significance of markers with various clinicopathological parameters (Table 1). As receiver operating characteristic (ROC) curve analysis is used to analyze diagnostic utility, we have taken into account ROC cutoff to analyze its prognosis utility as well for survival analysis. ROC curves were constructed using MedCalc statistical software (Supplementary file 1) to obtain optimal cutoff point (which showed greatest sensitivity and specificity i.e the uppermost left part of the curve) for survival analysis. Kaplan-Meier survival analysis was used to analyze correlation of the markers with overall survival, and significance of differences in survival rates was analyzed by log-rank test. Multivariate analysis was performed to correlate the markers with various clinicopathological parameters. For multivariate analysis, all the markers were correlated with various clinicopathological parameters. The values were expressed as the mean ± standard error of mean (SEM). “P” values less than 0.05 was considered to be statistically significant.

**Results**

Expression of ST3GAL1, FUT3, FUT5, and FUT6 in malignant and adjacent normal tissues. Figure 1 shows the representative pattern of ST3GAL1 expression, and Figure 2 shows the graphical representation of the levels of ST3GAL1 mRNA expression in adjacent normal and malignant tissues. It depicts that ST3GAL1 mRNA expression was higher in malignant tissues (ratio: 0.364) as compared to adjacent normal tissues (ratio: 0.334). Figure 3 is the representative pattern of FUT3 and FUT5 mRNA expression, and Figure 4 is the representative pattern of FUT6 mRNA expression. FUT3 and FUT5 transcripts levels were found to be significantly lower (P = 0.008 and P = 0.0021, respectively) in malignant tissues (ratio: 0.189 and 0.170, respectively) as compared to the adjacent normal tissues (ratio: 0.381 and 0.363, respectively) (Fig. 5). The mRNA expression of FUT6 was comparable between malignant (ratio: 0.279) and adjacent normal tissues (ratio: 0.236).

Correlation of ST3GAL1, FUT3, FUT5, and FUT6 transcript levels with overall survival of oral cancer patients. The optimal cutoff point of the transcripts was determined using ROC curve analysis (Supplementary file 1) with maximum sensitivity and specificity, which can distinguish

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### Table 2. Primer sequence and amplicon size of genes.

<table>
<thead>
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<th>GENES</th>
<th>PRIMER SEQUENCE</th>
<th>AMPILICON SIZE</th>
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<tr>
<td>ST3GAL1</td>
<td>F5′-ATGAGGGTGGACCTTGTCGCGG-3′ R5′-AACGGCTCAGCAAGATG-3′</td>
<td>253 bp</td>
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<tr>
<td>FUT6</td>
<td>F5′-CTCAAGACGATCCAGTGTAGC-3′ R5′-CAGGTCGCTAGGCTGATG-3′</td>
<td>404 bp</td>
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<td>FUT3 and FUT5</td>
<td>F5′-CTGCTGCTGCGGTGTTTCTTCTTAC-3′ R5′-CAGGTCGCTAGGCTGATG-3′</td>
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<tr>
<td>FUT6</td>
<td>F5′-GGTACCACCATGCGCCCAT-3′ R5′-GGATGCGCACAGGACTCCATG-3′</td>
<td>320 bp</td>
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### Table 3. Reaction conditions for RT-PCR analysis.

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<th>GENES</th>
<th>REVERSE TRANSCRIPTION AT 50°C FOR 30 MINUTES, INITIAL PCR ACTIVATION AT 95°C FOR 15 MINUTES</th>
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<td></td>
<td>CYCLING CONDITIONS</td>
</tr>
<tr>
<td></td>
<td>DENATURATION</td>
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<tr>
<td>ST3GAL1</td>
<td>94°C for 1 min.</td>
</tr>
<tr>
<td>FUT3 and FUT5</td>
<td>94°C for 1 min.</td>
</tr>
<tr>
<td>FUT6</td>
<td>94°C for 1 min.</td>
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</tbody>
</table>
adjacent normal and malignant tissues. The levels below cutoff and above cutoff were analyzed for overall survival analysis. Survival analysis depicted significant lower survival (log-rank chi² = 4.76, P = 0.029) in patients with expression above ROC cutoff (cutoff = 0.164, sensitivity = 63.04, specificity = 65.91, AUC = 0.675, P = 0.002) of FUT3 transcripts (Fig. 6) in malignant tissues. Also the results of ROC curve analysis depicted that FUT3 expression could significantly (P = 0.002) distinguish malignant and adjacent normal tissues. The optimal ROC cutoff of FUT5, FUT6, and ST3GAL1 is as mentioned in Table 4. The Kaplan–Meir’s survival analysis depicted no significant association of FUT5, FUT6, and ST3GAL1 transcript levels with overall survival.

Correlation of ST3GAL1, FUT3, FUT5, and FUT6 expression with various clinicopathological parameters. The expression levels of ST3GAL1, FUT3, FUT5, and FUT6 transcripts were compared between lymph-node negative (non-metastatic) (n = 29) and lymph-node positive (metastatic) tumors (n = 18) of the patients. Figure 7 documents the graphical representation of the mRNA levels of ST3GAL1, FUT3, FUT5, and FUT6 in non-metastatic and metastatic tumors of oral cancer patients. It was observed that the mean levels of ST3GAL1 and FUT6 were higher in metastatic tumors (ratio: 0.433 and 0.348, respectively) as compared to non-metastatic tumors (ratio: 0.315 and 0.228, respectively). The levels of FUT3 and FUT5 were comparable between non-metastatic (ratio: 0.189 and 0.138, respectively) and metastatic tumors of the patients (ratio: 0.220 and 0.149, respectively).

The expression of ST3GAL1, FUT3, FUT5, and FUT6 transcripts was compared between early (n = 17) and advanced (n = 31) stages of the disease. As depicted in Figure 8, the bar chart represents ST3GAL1, FUT3, FUT5 and FUT6 mRNA levels in early and advanced disease in oral cancer patients. It was observed that ST3GAL1 and FUT6 transcript levels were higher in advanced disease (ratio: 0.380 and 0.315, respectively) as compared to the early stage of the disease.
(ratio: 0.309 and 0.179), whereas the mean mRNA levels of FUT3 and FUT5 were comparable between early (ratio: 0.193 and 0.120, respectively) and advanced (ratio: 0.203 and 0.151, respectively) stages of the disease.

Moreover, multivariate analysis revealed significant association of ST3GAL1 expression with tumor infiltration ($F = 4.321, P = 0.054$) and FUT6 expression with differentiation ($F = 5.778, P = 0.016$) (Table 5). Further, pairwise analysis revealed that FUT6 expression was significantly different when compared between well and moderately differentiated tumors ($P = 0.021$) and also between well and poorly differentiated tumors ($P = 0.005$).

**Discussion**

Malignant transformation is frequently accompanied by alterations in surface glycosylation. Carbohydrate determinants expressed preferentially on cancer cells contain sialylated and/or fucosylated structures. Synthesis of these sialylated and/or fucosylated carbohydrate determinants in cancer is regulated by a set of ST and FUT.5,6 STs with altered mRNA expression in carcinoma tissue have been reported to be important as prognostic factors and potential targets for therapeutic approaches.6,13,25-28 However, the study of the relevance of STs in cancer is a complex task, because of overlapping substrate specificities, tissue-restricted patterns of expression, etc.13,27 Alterations in enzyme activity of α-2,3 and α-2,6 STs, and FUT have been reported in serum of oral cancer patients.7,8 However, there are no earlier reports on mRNA levels of ST and FUT in oral cancer.

The present study demonstrated an increase in ST3GAL1 mRNA levels in malignant oral cancer tissues as compared to adjacent normal tissues. Enhanced ST3GAL1 expression has been observed in various malignancies including carcinoma of lung, breast, colon, bladder, and ovary.5,13,15,25-28 Elevated levels of α-2,3 ST enzyme activity have been reported in oral cancer patients.7 It was hypothesized that the elevation of α-2,3 ST enzyme activity observed in oral cancer patients might be because of increase in ST3GAL1 expression as observed in the present study. ST3GAL1 and ST3GAL2 transcript levels along with enzyme activity of α-2,3 ST have been reported to be significantly increased in colorectal carcinoma tissues.14 In breast cancer, ST3GAL1 expression has been found to be increased in malignant tissues as compared to normal tissues, and its expression was reported to be related to the grade of the tumors.15 Earlier reports have also documented that ST3GAL1 and ST3GAL2 transcripts were increased in invasive cervical carcinomas.29 On the other hand, Wang et al have found downregulation of ST3GAL1 expression along with increased ST6GAL1 expression in squamous cell carcinoma of the cervix.17

Our results indicated that ST3GAL1 mRNA levels were higher in metastatic tumors as compared to the non-metastatic tumors of oral cancer patients. Schneider et al have shown that ST3GAL1 mRNA expression was significantly increased in cases showing invasion of lymph vessels.14 In the present study, the ST3GAL1 mRNA expression was found to be elevated in advanced disease as compared to that in the early disease. Moreover, multivariate analysis showed significant association of ST3GAL1 mRNA levels with tumor infiltration. Earlier reports have indicated increase in α-2,3 ST enzyme activities in advanced disease as compared to those in early disease in oral cancer;7 however, studies on mRNA expression of ST3GAL1 have not been reported. Wang et al have observed no correlation of ST3GAL1 and ST6GAL1.
with stage, differentiation, amount of ascites, and serum levels of CA125 in ovarian cancer.²⁷

FUT3 predominantly exhibits α-1,4 FUT activity synthesizing Leα, sialyl Lewis (SLε), and Leβ, and a minor α-1,3 FUT activity, synthesizing Leα, SLε, and Leβ. FUT5 and FUT6 synthesize Leα and SLε; moreover, FUT5 has been reported to produce Leα, Leβ, and SLε.³⁰ In the present study, the expressions of FUT3 and FUT5 transcripts were found to be significantly decreased in malignant oral cancer tissues as compared to those in adjacent normal tissue and levels. Hanski et al have reported that human colon carcinomas showed equal or even lower expression of FUT3 mRNA than normal mucosa.³⁰ Previous reports have indicated elevated FUT enzyme activities in oral cancer; however, the transcripts levels of FUT were not analyzed. There are mixed reports on alterations of FUT genes in various neoplastic diseases. Earlier reports have indicated that FUT3 and FUT6 transcripts were not significantly altered, whereas FUT6 showed moderate increase in cancer tissues when compared to adjacent non-malignant colonic epithelia.⁴ In the present study, the levels of FUT6 transcripts were comparable between oral cancer tissues and adjacent normal tissues. Moreover, FUT6 expression depicted moderate increase in metastatic tumors as compared to non-metastatic tumors and was also higher in advanced stage of disease as compared to early stage of disease. We predict that a significant decrease in FUT3 and FUT5 transcripts observed in the present study might be causing upregulation of other FUT transcripts that are further involved in increasing FUT enzyme activity. Moreover, survival analysis depicted that levels above cutoff of FUT3 transcripts were associated with significantly lower survival of patients. It is expected that, in patients with values above cutoff of FUT3 transcripts, there is increased production of SLε expression which is known to be involved in metastasis and aggressive behavior of disease. Earlier increased expression of SLε has been observed in metastatic breast cancer.³³ Increased FUT7 expression has been reported to be associated with survival of patients in lung carcinoma.³⁴ Hiraïwa et al have shown increase in FUT3, FUT6, and FUT7 in colon cancer tissues along with increase in SLε antigen.²⁰ Earlier studies have shown increase in FUT4 in colorectal adenomas and carcinomas.¹⁹,²¹ Increased expression of FUT5 and FUT6, and decreased expression of FUT4 expression was earlier observed in gastrointestinal carcinoma cells.¹⁰ FUT6 expression has been shown to be involved in SLε expression in breast cancer cells.²² The present study depicted higher expression of FUT6 in metastatic and advanced tumors, which is known to be involved in upregulation of SLε in metastatic disease. Our earlier studies have indicated increased SLε in oral cancer and a significant positive correlation with advanced stage of disease and metastasis.³⁵ Earlier reports have indicated increase in FUT6 in colon cancer and have observed that its knockdown caused decrease in FUT6 mRNA and inhibition of SLε expression.²³ The present study indicated that FUT3 and FUT5 transcripts were comparable between metastatic and non-metastatic tumors. Petretti et al showed that FUT3 was less expressed in carcinomas exhibiting distant metastasis and in highly invasive tumors.⁷⁹

Table 4. ROC curve analysis of the markers showing cutoff, sensitivity, specificity, and AUC.

<table>
<thead>
<tr>
<th>MARKERS</th>
<th>ROC CUTOFF</th>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
<th>AUC</th>
<th>P VALUE</th>
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<tr>
<td>FUT3</td>
<td>0.164</td>
<td>63.04</td>
<td>65.91</td>
<td>0.675</td>
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<td>FUT5</td>
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<td>73.47</td>
<td>47.43</td>
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<td>FUT6</td>
<td>0.307</td>
<td>38.1</td>
<td>85.4</td>
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<td>ST3GAL1</td>
<td>0.281</td>
<td>65.0</td>
<td>61.1</td>
<td>0.572</td>
<td>0.2893</td>
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</table>

Figure 7. Comparison of ST3GAL1, FUT3, FUT5, and FUT6 mRNA expression levels in non-metastatic (n = 29) and metastatic (n = 18) oral cancer tissues. The levels are expressed as ratio of IDV of the glycosyltransferase and βACTIN.

Abbreviations: FUT, fucosyltransferase; ST, sialyltransferase.

Figure 8. Comparison of ST3GAL1, FUT3, FUT5, and FUT6 mRNA expression levels in oral cancer tissues with early (n = 17) and advanced diseases (n = 31). The levels are expressed as ratio of IDV of the glycosyltransferase transcripts and βACTIN.

Abbreviations: FUT, fucosyltransferase; ST, sialyltransferase.
Recent developments in this field have focused on designing the carbohydrate mimetics and the structure–activity relationships of substrate-based ST inhibitors. This may prove useful for inhibition of ST in elucidating the biological functions of sialylation. Also recent advancement has led into development of various glycan antagonists and inhibitors of STs and FUTs.

In conclusion, increase in ST3GAL1 transcript levels in malignant tissues as compared to adjacent normal tissues and higher expression in advanced stage and metastatic tumors highlights its role in aggressive behavior of the disease. A significant decrease in FUT3 and FUT5 mRNA expressions in oral cancer tissues and significant association of increased FUT3 expression with lower survival of oral cancer patients indicates its potential utility in prognostication and disease monitoring. In furtherance, the correlation of FUT3, FUT5, and FUT6 transcripts and its associated molecules like SLeX/SLeα might elaborate the involvement of specific subtypes in oral carcinogenesis. The results strongly warrant evaluation of other sub-families of FUTs and STs genes with a larger sample size, which might give deeper insights into involvement of specific subtype of FUT3 and STs in oral cancer pathogenesis.

Abbreviations
AUC, area under curve; FUT, fucosyltransferase; IDV, integrated density value; ROC, receiver’s operating characteristic; RT-PCR, reverse transcriptase polymerase chain reaction; SLe, sialyl Lewis; ST, sialyltransferase.

Acknowledgments
PSP, RB and BNV conceived and designed the experiments. BNV, KRP, and PSP analyzed the data. BNV wrote the first draft of the manuscript. KRP contributed to the writing of the manuscript. FDS, JBP and RB agreed with manuscript results and conclusions. PSP, RB, FDS, and JBP jointly developed the structure and arguments for the paper. PSP, RB and GMJ made critical revisions and approved the final version. All the authors reviewed and approved the final manuscript.

Supplementary Data
Supplementary file 1. ROC curve analysis and cut-off determination.

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