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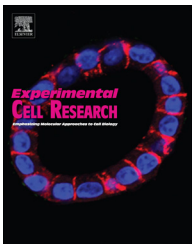

Research Article

The early manifestation, tumor-specific occurrence and prognostic significance of TGFBR2 aberrant splicing in oral carcinoma

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ABSTRACT

Alternative splicing is an important mechanism that can disrupt cell cycle control resulting in tumorigenesis. Although many alterations of Transforming Growth Factor Beta (TGFβ) signaling are reported in cancers, the role of splice aberrations in destabilizing this signaling is the least understood mechanism. In this study, we compared TGFBR2 alternative splicing events in potentially malignant oral disorders (PMDs) and oral squamous cell carcinoma (OSCC) samples with those in normal samples. Interestingly, there were five alternatively spliced forms of TGFBR2 with a deficient kinase domain in OSCCs. The TGFBR2 aberrant splicing was tumor-specific, suggesting that selective splicing out of TGFBR2 kinase domain could be a mechanism misused by cancer cells for evading TGFβ signaling–mediated anti-tumor activities. Moreover, these aberrant transcripts were present in PMDs as well, suggesting an early occurrence of these events during oral carcinogenesis and offering the possibility of early diagnosis of malignancy. Furthermore, OSCC patients who harbored these aberrantly spliced transcripts exhibited poor disease free survival ($p=0.028$) and poor overall survival ($p=0.013$). Thus, assessing the presence of these TGFBR2 transcripts can serve as a prognostic marker for oral cancer.

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Introduction

Oral cancer is a major health problem worldwide, accounting for about 274,000 new cases and 145,000 deaths per year, of which two-thirds occur in developing countries. Despite advances in treatment, the five-year survival rate of individuals with oral carcinomas has remained at less than 50% over the past two decades [1,2]. Though a complete response has been achieved once, local or loco-regional treatment failures often happen [3]. Hence, it is necessary to identify molecular markers that can identify the aggressive OSCCs so that appropriate treatment can be implemented. Transforming Growth Factor Beta (TGFβ) signaling is a commonly altered pathway that exhibits a dual role in cancers [4]. It has a potent anti-tumor role during early stages of

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carcinogenesis. But the genetically unstable cancer cells often selectively disable its tumor suppressive arm and misuses other features of TGFβ signaling for tumor progression [5,6].

Each regulatory point in the control of gene expression is subject to profound alterations during the development of cancers. Among gene expression mechanisms, alternative splicing (AS) has the potential for more diverse outcomes than any other mechanism [7]. Several studies suggest that alternative splicing can disrupt the control mechanisms over cell cycle and result in tumorigenesis. Furthermore, splice variants can be used as diagnostic as well as prognostic markers in different cancers such as breast, colon, bladder, prostate, and ovarian cancers [8–11]. In one of our previous studies, we have shown the influence of Cyclin D1 A870G polymorphism on the survival of oral cancer patients [12]. This polymorphism can act as a determining factor in alternative splicing of Cyclin D1, resulting in with the variant Cyclin D1 protein with increased half-life.

Even though six alternatively spliced protein coding forms of TGFBR2 are known (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&l=TGFBR2), their differential role in cancer is unknown. In the present study, we analyzed TGFBR2 alternative splicing events in OSCCs and its association with various clinico-pathological factors. This study identified novel alternatively spliced forms of TGFBR2 that were found have an influence on the prognosis of oral cancer. These splice variants were also present in PMDs, suggesting the early occurrence of these events during oral carcinogenesis, and pointing towards the possibility of early detection and prevention.

Materials and methods

Sample collection, ethics statement, and follow-up

OSCC cases for the present study were collected from the Head & Neck Cancer Clinic of the Regional Cancer Centre, Thiruvananthapuram, India; normal oral mucosa samples and PMDs were collected from the Department of Oral Medicine and Radiology, Government Dental College, Calicut, after obtaining each patient's written informed consent. The present study was approved by the Institutional Review Board and the Human Ethics Committee. In total, 126 samples were collected for the present study. The clinical staging of the patients was carried out according to WHO criteria. Patients then received standard treatment based on their tumor stage and individual clinical status, as we detail elsewhere [13]. All of the patients were actively followed-up, at respective clinics, at an interval of six to eight weeks for a minimum period of 36 months.

Cell culture

The oral cancer cell lines UPCC:SCC29B, UPCC:SCC40, and UPCC:SCC66 were kindly provided by Professor Susanne M. Gollin of the University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania, USA [14,15], while UM-SCC83B was kindly provided by Professor Thomas E Carey, Department of Otolaryngology/Head & Neck Surgery, University of Michigan, Ann Arbor, Michigan, USA [16]. Other cell lines, such as K562, KB, HepG2, A375 and A549, were obtained from National Centre for Cell Sciences (NCCS), Pune, India (the National Cell Repository, Government of India). All of these cell lines except K562 were maintained in 10% DMEM (PAN Biotech GmbH, Am Gewerbepark, Aidenbach, Germany). For K562, 10% RPMI was used.

RNA isolation

Total RNA from tissue samples and 80% confluent cell lines was extracted using TRI Reagent (Sigma Aldrich, St. Louis, Missouri, USA) as per the manufacturer's protocols. The isolated RNAs were quantified spectrophotometrically and quality was checked by agarose gel electrophoresis.

3′Rapid Amplification of cDNA Ends (3′RACE)

3′RACE was used for analyzing the splice events at the 3′ side of the TGFBR2 [17]. The primer sequences and PCR conditions are given in Supplementary Table S1. The PCR products were resolved on 1.5% agarose gel, and bands differentially expressed in tumor samples as compared to normal mucosa were gel eluted using HiPurA Agarose Gel DNA Purification Spin Kit (HiMedia, L.B.S. Marg, Mumbai, India) and used for direct sequencing.

DNA isolation and differential PCR

DNA was extracted by using the standard phenol:chloroform extraction method [18]. Differential PCR was carried out on 35 samples—DNA from 15 tumor samples that showed TGFBR2 kinase domain deficient transcripts, 10 tumor samples with no TGFBR2 transcript alterations, and 10 samples of blood DNA from 10 healthy donors (used as controls). The primer sequences and PCR conditions are given in the Supplementary Table S2. TGFBR2 was co-amplified with β-Actin as an internal control [19]. The ratio of the intensity of the target gene versus that of the control was measured using Quantity One 1-D Image Analysis Software (Bio-Rad Laboratories, Hercules, California, USA). Samples with values $<50\%$ of the control signal were considered as having that particular region deleted.

Sequencing

The 3′RACE products were gel eluted using HiPurA™ Agarose Gel DNA Purification Spin Kit (HiMedia, L.B.S. Marg, Mumbai, India), and sequencing PCR was performed using the ABI PRISM Big Dye Terminator Kit v3.1 (Life Technologies, Foster City, California, USA). Sequencing was done in both sense and anti-sense directions using an ABI 3500Dx genetic analyzer (Life Technologies, Foster City, California, USA) to confirm the results.

Bioinformatics

The mRNA sequences obtained were aligned using the NCBI-BLASTN search program and were used for deducing the possible protein sequences using the ExPaSY translate tool (http://web.expasy.org/translate/). The interacting partners of the kinase domain deficient splice variants of TGFBR2 as compared to the full length TGFBR2 were identified using Motif Scan software available from the Scansite website (http://scansite.mit.edu/motifscan_seq.phtml). The possible alternative splice sites were identified using Human Splicing Finder software Version 2.4.1 (http://www.umd.be/HSF/) and Alternative Splice Site Predictor (ASSP) software (http://wangcomputing.com/assp/index.html).

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