Canonical Wnt Pathway Gene Expression and Their Clinical Correlation in Oral Squamous Cell Carcinoma

Abstract

Aim: The aim of this study is to explore the prognostic significance and clinicopathological correlations of the Wnt pathway genes in a cohort of surgically treated patients with oral squamous cell carcinoma (OSCC) patients. Settings and Design: A prospective genetic study on patients with OSCC was carried out during the period from July 2014 to January 2016. Informed consent from patients and institutional ethical approval for the study was obtained and the guidelines were strictly followed for collection of samples. Subjects and Methods: Clinical data and mRNA expression analysis of ten genes in the canonical Wnt pathway were evaluated and their relationships with clinical and demographic variables were studied in 58 tissue samples. Wnt-3A, β-catenin, secreted frizzled-related proteins sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wnt inhibitory factor 1, dickkopf-1, c-MYC, and cyclin-D1 from cancer (n = 29) and normal (n = 29) tissue samples were investigated using quantitative reverse transcription-polymerase chain reaction. Statistical Analysis: Descriptive statistics were used to summarize the sample characteristics and clinical variables. If the data were normal, then parametric tests were used; otherwise, nonparametric alternatives were used. All the analyses were carried out using SPSS version 23.0 (IBM SPSS Inc., USA). Results: Expression of sFRP-1, sFRP-2, and sFRP-5 in control samples and expression of c-MYC and cyclin D1 in cancer samples showed statistical significance. Significant expression of Wnt3A was observed among patients who had recurrence and were deceased. Conclusion: Wnt3A, β-catenin, and cyclin D1 are recognized as key components of Wnt/β-catenin signaling. However, in this study, there was no significant expression of all the three genes in OSCC. The proto-oncogene c-MYC showed statistically significant upregulation in cancer tissue samples suggesting that the OSCC among South Indian population is primarily not mediated by the canonical Wnt signaling pathway.

Keywords: Canonical Wnt pathway, gene analysis, oral squamous cell carcinoma, quantitative reverse transcription-polymerase chain reaction

Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent oral malignancy. Although it has been suggested that OSCC arises through the accumulation of multiple genetic changes, the precise molecular mechanism and their relevance to clinicopathological variables are still unclear.[1] This expression analysis study aims to identify the role of canonical Wnt signaling pathway in OSCC and their implication in clinical variables and prognosis. The rationale behind choosing the Wnt signaling pathway was their wide evidence in various other cancers,[2,3] yet less studied among South Indian population.

The canonical Wnt pathway (Wnt/β-catenin pathway) plays an important role in regulating cell proliferation, migration, and apoptosis and is commonly deregulated in several types of cancer, such as colon, liver, stomach, breast, childhood T-cell acute lymphoblastic leukemia, and head and neck squamous cell carcinoma (HNSCC). Many small molecule inhibitors are in preclinical and clinical phase of studies to specifically target Wnt signaling proteins, such as Frizzled, Disheveled, Porcupine, or Tankyrase. However, their potential application in oral cancer therapy has not been investigated.[4]

For these reasons, further studies should be carried out to better understand the role of Wnt pathway in oral carcinogenesis process. Therefore, the present study was designed to screen the expression pattern of selected canonical Wnt pathway genes including the ligand, inhibitors and target genes-Wnt-3a, dickkopf-1 (DDK1), Wnt

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inhibitory factor 1 (WIF1), secreted frizzled-related protein 1 (sFRP1), sFRP2, sFRP4, sFRP5, c-MYC, cyclin D1, and β-catenin in South Indian oral cancer patients and to compare the expression pattern with its clinicopathological features to understand the molecular pathology of oral cancer for better clinical management.

Subjects and Methods

Sample selection

Patients diagnosed with primary OSCC were included in the study. The inclusion criteria were that they had to be South Indian origin with primary OSCC, not having undergone any radiation and chemotherapy or any other form of treatment for primary cancer. The informed consent was obtained from all the patients to collect demographic details, be treated surgically, be available for follow-up, and to use their tissues for genetic studies. Cancer tissue samples were carefully collected along with their control samples from the unaffected contralateral side of the same patients. Ethical approval for this study was obtained from the Institutional Ethical Committee.

Sample size calculation

The power calculation for this study was carried out based on the t-test for comparing the mean relative gene expression between patient and control samples. Assuming an effect size of 0.7, a study with 80% power will require a total sample of 52 (26 patients and 26 controls) to detect the difference between the two groups using t-test at 5% level of significance. The power calculation was carried out using G power version 3.1.5. A total of 58 tissue samples were collected from 29 patients diagnosed with OSCC (cancer tissue n = 29 vs. control tissue n = 29) and were included in the study.

Clinical data

The demographic and clinical details such as name, age, gender, habits, occupation, chief complaint, duration of symptoms, history of illness, and presence of any comorbidities were collected from all the patients. A detailed description of tumor site, size, nodal involvement, radiographic interpretation, tumor, node, and metastasis (TNM) staging, and histopathology were noted. Surgery with or without neck dissection and reconstruction were planned to the need of the patient. Immediate postoperative and long follow-up were undertaken.

Tissue samples

Tissue samples were collected in sample collection tubes containing 3 mL of RNA stabilization reagent RNA later (Sigma, USA) for RNA analysis. The samples were transported to the laboratory on gel ice pack and stored appropriately.

RNA extraction

Total RNA was extracted from 29 pairs of tissue specimens (cancer and control) using RNAzol RT by manual method according to the manufacturer’s instructions. RNA samples were quantified by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Germany). Extracted total RNA was reverse transcribed using Thermo Scientific Revertaid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The cDNA was stored at -20°C until required for use in real-time reverse transcription-polymerase chain reaction (RT-PCR). Quantitative PCR (qPCR) was carried out in Bio-Rad CFX96 PCR system (Bio-Rad, USA). Reaction was performed using Kapa SYBR® Green Master Mix kit (containing all the PCR components along with SYBR green dye).

RNA quantification

RNA samples were quantified by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Germany). The instrument was blanked with 1X TE or water, and the concentration was estimated by measuring absorbance at 260 nm (A260). An optical density value of 1 at 260 nm corresponds to 40 μg/mL for RNA. The ratio between the absorbance values measured at 260 and 280 nm (A260/A280) gave the purity of the sample. A ratio of 2.0 of RNA is considered pure without protein/phenol residues. (Ratios got were less 1.8–2 to be considered). Primers and their annealing temperatures are shown in Table 1.[5-7] Cyc-A was used as the internal control.

Statistical methods

Descriptive statistics were used to summarize the sample characteristics and clinical variables. The relative gene expressions were calculated based on the methods suggested by Schmittgen and Livak.[8] The relative gene expression for different genes was expressed as mean and standard deviation for patients and controls separately. Bar graphs were used to present the relative gene expression for different genes along with 95% confidence intervals. The normality of the data was checked using Shapiro-Wilk test, histogram, and box plot. If the data were normal, then parametric tests were used; otherwise, nonparametric alternatives were used. The mean relative gene expression between patients and controls were compared using Mann–Whitney test. All the analyses were carried out using IBM SPSS Statistics Version 23.0 (IBM Cooperation, Newyork, USA).

Results

Out of the sample of 29 patients included in this study, 18 (62.1%) were males and the remaining 11 were females. The mean age of the patients was 47.97 years with a standard deviation of 9.81 years. Regarding the habits, majority of the patients (86.2%) had chewing habits while only 34.5 and 37.9% had alcohol and smoking habits,
Table 1: List of primer sequence and their annealing temperature

| Name of the Gene | Primer Sequence (5'→3') | Annealing temperature °C |
|------------------|-------------------------|--------------------------|
| sFRP-15          | F- CGAGTTTGCACTGAGGATGA | 55                       |
|                  | R- CAGCACAAGCTTCTTACAGT |                         |
| sFRP-25          | F- CGAGGAAGCTCCAAAGGTAT | 57                       |
|                  | R- CTCCTTCATTTATTTACGTA |                         |
| sFRP-45          | F- TCTTGGCAGTTGACACATC  | 55                       |
|                  | R- CTCCTTCCTCCACTGATGGA |                         |
| sFRP-55          | F- CGCCTCGATGACCAAGAT   | 58                       |
|                  | R- GATGCGCATTTGGACCACAA |                         |
| WNT-3a           | F- CCTTCCTCACTCCACGCTCA | 60                       |
|                  | R- GACCTCTTCACACCCTTCCCA|                         |
| c-MYC            | F- TCAAGAGGCAAGACAACAC  | 56                       |
|                  | R- GGGCTTTTCATGTTTCCA   |                         |
| β-Catenin6       | F- CTGCTGTTTGGTTCGAGATG | 53                       |
|                  | R- CCATGGGCTCCTGTGAAAGA |                         |
| Cyclin D17       | F- ACAAACAGATCGCCACACAC | 53                       |
|                  | R- TGGTGCCGCTCCTCAGGTC  |                         |
| WIF-13           | F- TCTTGTCACAGCTCCTGTG | 57                       |
|                  | R- CCTTTTTATGGCAGTGTCTCA|                         |
| DKK-15           | F- ACAGGCCACAGCTGTTGTA  | 58                       |
|                  | R- CCTTCATGAGCTGGCACC   |                         |
| Cyc-A            | F- GTGTTTGGCAGAAAGTGA   | 60                       |
|                  | R- TCGAGTTTGCCACAGTCAG  |                         |

Table 2: The analysis results of Clinicopathological features and mRNA expression

| Study samples     | Sample size | n=58 | Genetic Expression | mRNA   |
|-------------------|-------------|------|--------------------|--------|
|                   |             |      | WNT3+ βcat sFRP1 sFRP2 sFRP4 sFRP5 DKK1 WiFi cMYC CyclinD1 |
| Cancer            | 29          | ++   | +                  | ++     |
| Control           | 29          | +    | ++                 | ++     |
| Clinical variables|             |      |                    |        |
| Gender            |             |      |                    |        |
| Male              | 18          | +    | +                  | ++     |
| Female            | 11          | +    | +                  | +      |
| Age               |             |      |                    |        |
| <50 years         | 14          | +    | ++                 | +      |
| ≥50 years         | 15          | +    | +                  |        |
| Habits            |             |      |                    |        |
| Chewer            | 25          | +    | ++                 | +      |
| Non               | 4           | +    | +                  | +      |
| Smoker            | 18          | +    | +                  | +      |
| Non               | 11          | +    | +                  | +      |
| cTNM staging      |             |      |                    |        |
| I, II             | 6           | +    | ++                 |        |
| III, IV           | 23          | ++   | +                  | +      |
| Tumour size       |             |      |                    |        |
| T1=4, T2=13, T3=3, T4=9 |        |      |                    |        |
| Nodes             |             |      |                    |        |
| N0=7, N1=9, N2=13, N3=0 |        |      |                    |        |
| Prognosis         |             |      |                    |        |
| Survivors         | 23          | ++   | ++                 |        |
| Deceased          | 6           | ++   | +                  | ++     |

+ increased expression but not statistically significant. ++ statistically significant increased expression.

respectively. The mRNA expression of various Wnt pathway genes are compared to control versus cancer tissue samples and then correlated to the clinicopathological features. These are depicted as bar chart graphs in Figures 1 and 2. The details of the statistical analyses that showed significant expressions are summarized in Table 2.
Figures 1 and 3 showed mean relative gene expression for Wnt-3a [Figure 1]; sFRP1, sFRP2, sFRP4, sFRP5, WIF1, and DKK1 [Figure 4]; and β-catenin, c-MYC, and cyclin D1 [Figure 3] with 95% confidence intervals for normal and patients, $n = 58$ (29 normal noncancerous tissues as controls vs. 29 cancer tissues). Figures 5 and 2 showed mean relative gene expression for Wnt pathway genes with 95% confidence intervals (Figure 5 – gender-wise mean, Figure 6 – age-group wise, Figure 7 – Chewer and nonchewer, Figure 8 – Smoker and nonsmoker, Figure 9 – clinical stage, and Figure 2 – recurrence and mortality).

The expression of mRNA analysis of Wnt-3a, β-catenin, sFRP1,2,4,5, DKK1, WIF1, c-MYC, and cyclin D1 genes between control and cancer tissue samples and among different clinical variables in OSCC patient samples was studied. The expression analysis of Wnt-3a [Figure 1] and β-catenin [Figure 3] using qRT-PCR did not show any significant upregulation in cancer tissues when compared to the control samples. The proto-oncogene c-MYC showed statistically significant upregulation in cancer tissue samples whereas cyclin D1 showed statistically significant upregulation in normal tissue samples [Figure 3]. The Wnt antagonists sFRP1, sFRP2, and sFRP5 [Figure 4] showed statistically significant upregulation in control tissue samples. c-MYC was elevated in male, young patients, tobacco chewers, with high-grade tumors, and in those who were deceased [Figures 5-7, 9 and 2]; Wnt-3a was expressed significantly in patients who had recurrence and deceased [Figure 2]. The secreted frizzled receptors sFRP1, sFRP2 and sFRP5 showed a significantly upregulated expression in low-grade tumors and patients who survived throughout the study period (>2 years’ follow-up) [Figures 9 and 2]. The other interesting findings of this study are the increased expression of the Wnt pathway inhibitors (sFRP1, sFRP2, sFRP5, DKK1, WIF1), target genes (c-MYC and cyclin D1), and decreased expression of Wnt-3a, β-catenin among tobacco chewers. This pattern was in exact reversal among the smokers [Table 2].
**Discussion**

OSCC remains a major health problem and understanding the molecular basis of this malignancy is of great importance. Hardly, few studies have been carried out in the past to study the correlations of clinically relevant variables and the expression changes of possible oncogenic genes. The goal of the scientific research is to find the new biological markers which should be able to define the “tumor biological fingerprint” and to identify the molecular key players that are involved in oropharyngeal carcinogenesis.[9] Cancer cells exhibit a wide range of genetic alterations that include gene rearrangements, point mutations, and gene amplifications, leading to disturbances in molecular pathways modifying cell growth, survival, and metastasis. Several research teams have focused on the analysis of gene expression patterns in normal oral mucosa and in HNSCC. Among the various signaling systems that control cell proliferation, cell death, motility, migration, and stemness, the Wnt pathway is identified as one of the master developmental pathways that contributes to cancer initiation and development.[10] The function of the canonical Wnt pathway is to regulate the transcription of β-catenin, which regulates transcription factors involved in cancer-promoting activities such as cell proliferation and survival.[11] A study by Uraguchi et al. has shown amplified expression levels of 11 of the 19 members of the family, Wnt-3, 3a, 4, 5a, 5b, 6, 7a, 7b, 10b, 11, and 14 in OSCC using RT-PCR.[12] Among the genes expressed, Wnt-3 has been identified as a typical and powerful member of the family activating the β-catenin-mediated signaling pathway to initiate malignant transformation and enhance cellular proliferation, invasion, and metastasis.[8] Wnt-3a expression has been found to be higher in OSCC compared with control nonmalignant tissues.[13] β-catenin is a key component in the canonical Wnt pathway. The elevated cytoplasmic β-catenin activates T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription and enhances the expression of oncogenic proteins (c-MYC, cyclin D1, MMP-7).[14]

The expression patterns of the studied ten Wnt pathway genes are correlated to various demographic and clinical variables.
such as gender, age, habits, tumor site, nodal metastasis, tumor staging, and prognosis. Histopathological correlation could not be calculated as all the cancer samples included in the study were well differentiated squamous cell carcinoma.

Genetic expression between normal and tumor samples grouped under various clinical variables showed that among the ten genes studied, interesting genetic expression patterns were observed for c-MYC, Wnt-3a, and sFRP 1,2,5 between samples of patients with good prognosis versus the samples of deceased patients. sFRPs are antagonists that directly bind to Wnts. sFRPs frequently act as negative regulators of the Wnt pathway and are downregulated during carcinogenesis, mainly through hypermethylation. On the basis of sequence homology, sFRP1, sFRP2, and sFRP5 form a subgroup, as do sFRP3 and sFRP4, which are quite distantly related to the other sFRPs.

Conventional Wnt signaling causes β-catenin accumulation in a complex with the transcription factor TCF/LEF that regulates target gene, such as c-MYC and cyclin D1 expression. In the absence of Wnt signaling, the level of β-catenin is kept low through its ubiquitination and degradation. The significant expression of the oncogenes c-MYC and cyclin D1 along with the inhibitors in low-grade tumors with no significant expression of Wnt-3a and β-catenin questions the initial role of Wnt signaling pathway in OSCC among the South Indian population. However, significant expression of Wnt-3a was observed in high-grade tumors and deceased samples. A plausible explanation could be that the Wnt activity at the initial stages is being suppressed by the sFRPs which are silenced in late stages of OSCC. Downregulation of the sFRP genes has been shown to be an alternative mechanism of stabilization and activation of β-catenin. sFRP1 is a natural blocker of the Wnt signaling pathway in normal adult cells but is epigenetically silenced in cancer cells leading to aberrant proliferation. Moreover, the consistently significant expression of the proto-oncogene c-MYC, believed to contribute to oral cancer development, suggests an active carcinogenesis possibly by other linked mechanisms, rather than the canonical Wnt signaling pathway. Cyclin D1 is significantly upregulated in control samples and c-MYC in cancer samples. This further emphasizes that the oncogenesis is c-MYC mediated initially.

The expression of Wnt-3a and c-MYC is suggestive that the Wnt pathway is activated to trigger the downstream target gene c-MYC. However, data released by the cancer genome atlas (TCGA) wherein a study was done on 564 head and neck cancers showed that Wnt-3a and MYC are negatively correlated (r = −0.1065 and P = 0.0114). In the set of OSCC samples in this study, 12 out of 29 samples (42%) showed an upregulation of the c-MYC.

The downregulation of cyclin D1 is an interesting observation that needs further probing. Studies have shown that Wnt does not require cyclin D1 because tumors can develop in cyclin D1 knockout mice. Similarly, in vitro studies have shown that c-MYC is an inhibitor of cyclin D1 expression and c-MYC-dependent pathways do not need Cyclin D1. β-catenin and cyclin D1 are recognized as key components of Wnt/β-catenin signaling. However, in this study, there was no significant expression of β-catenin and cyclin D1 in OSCC.

The primary mechanism involved in initiating cancer proliferation in this study group suggests an alternate hypothesis in which Wnt signaling pathway is underplayed. This hypothesis is also seconded by the observation that there was no significant expression of β-catenin in cancer tissue samples.

When correlating the genetic expression to the risk factors exposed in this study group, there was an increased expression of the Wnt pathway inhibitors (sFRP1, sFRP2, sFRP5, DKK1, WIF1), target genes (c-MYC and cyclin D1), and decreased expression of Wnt-3a and β-catenin among tobacco chewers. This pattern was in exact reversal among the smokers. This expression profile again raises the question that whether Wnt pathway is the primary mechanism of carcinogenesis here, considering the fact that the etiology related to the OSCC in most patients in this study group was tobacco chewing. About 35%–40% of tobacco consumption in India is in smokeless forms. Over 50% of chewing-tobacco-related oral SCCs in India have a 5- to 10-fold DNA amplification of one or more of the c-MYC genes. c-MYC is mainly an immortalizer, and over recent years, their focus is changing from being an oncogene to their cell-transforming ability. The overexpression of c-MYC predisposes cells to apoptosis under nutrient or growth factor deprivation conditions although the critical sets of genes involved remain elusive.

There were six patients who had recurrence and died. Among them, three of them had tumor size <4 cm (T2) but had nodal metastasis and graded in Stage IV. Whether the presence of Wnt-3a, which is already quite known for its stemness, influences the nodal spread and aggressiveness
needs to be further explored. In this way, Wnt-3a can be a prognostic biomarker.

**Conclusion**

Analyze of alterations in the expression of molecular biological factors investigated, herein revealed that Wnt-3a and c-MYC may be an indicator of poor prognosis in OSCC. Moreover, the hypothesis that the canonical Wnt signaling pathway plays an important role in OSCC among South Indian population is questionable and further genetic and epigenetic studies are needed to validate the same.

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**Conflicts of interest**

There are no conflicts of interest.

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