Expression of *WNT10A* Gene in Oral Squamous Cell Carcinoma

LP Kalinke[1], LE Alvares[2], JL Schussel[1, 4], AR Ávila[3], DP Pavoni[3], MA Krieger[3], BV De Oliveira[4], FD Nunes[5], VS Sotomaior[1], GP Garlet[6], PC Trevilatto[1]

ABSTRACT

Objective: *Oral squamous cell carcinoma (OSCC) constitutes the most frequent malignant tumour of the oral cavity. Considering the significant roles the wingless-type mammary tumour virus integration site family (WNT) plays in physiological and pathological events, this study aims to investigate WNT10A gene expression in OSCC.*

Methods: *The cohort was composed of 59 specimens: 49 with OSCC and 10 controls. Total ribonucleic acid (RNA) from the samples was extracted, cDNA was synthesized and real-time polymerase chain reaction (PCR) analyses were performed, with human gene an protein symbol ACTB/ACTB (β-ACTIN) as internal control.*

Results: *The number of OSCC samples for the four tumour, node and metastases (TNM) stages was: 5 (10.2%) stage I, 10 (20.4%) stage II, 9 (18.3%) stage III and 25 (51.1%) stage IV. For real-time PCR analysis, significant difference was found between control samples and OSCC stage IV (*$p < 0.01$*).*

Conclusion: *Higher levels of WNT10A expression was observed in OSCC stage IV, suggesting a potential role in tumour progression.*

Keywords: Oral cancer, signal transduction, squamous cells carcinoma, Wnt proteins

Expresión del Gen *WNT10A* en el Carcinoma de Células Escamosas Orales

LP Kalinke[1], LE Alvares[2], JL Schussel[1, 4], AR Ávila[3], DP Pavoni[3], MA Krieger[3], BV De Oliveira[4], FD Nunes[5], VS Sotomaior[1], GP Garlet[6], PC Trevilatto[1]

RESUMEN

Objetivo: *El carcinoma de células escamosas orales (CCEO) constituye el tumour maligno más frecuente de la cavidad oral. Teniendo en cuenta los importantes papeles que la familia Wnt, miembro de la familia del sitio de integración de virus del tumour mamario de tipo wingless, desempeña en los procesos fisiológicos y patológicos, el presente estudio tiene por objeto investigar la expresión del gen WNT10A en el CCEO.*

Métodos: *La cohorte estaba compuesta por 59 especímenes: 49 con CCEO y 10 controles. El ácido ribonucleico (ARN) total fue extraído de las muestras, el ADN fue sintetizado y se realizaron análisis de reacción en cadena de la polimerasa (RCP) en tiempo real con genes humanos y proteína símbolo ACTB/ACTB (β-actina) como control interno.*

Resultados: *El número de muestras de CCEO para las cuatro etapas del tumour, nodo y metástasis (TNM) fue: 5 (10.2%) en el estadio I; 10 (20.4%) en la etapa II; 9 (18.3%) en la etapa III; y 25 (51.1%) en la etapa IV. En el análisis RCP en tiempo real, se encontraron diferencias significativas entre las muestras del control y etapa IV de CCEO (*$p < 0.01$*).*

Conclusión: *En la etapa IV de CCEO se observaron niveles más altos de expresión de WNT10A, sugiriendo un rol potencial en la progresión del tumour.*

From: [1]School of Health and Biosciences of Pontificia Universidade Católica do Paraná (PUCPR), Curitiba, PR, Brazil, [2]Department of Histology and Embriology, Institute of Biology, State University of Compinas (UNICAMP), Compinas, SP, Brazil, [3]Carlos Chagas Institute (ICC) – FIOCRUZ, Curitiba, PR Brazil, [4]Head and Neck Department of Surgery of Erasto Gaertner Hospital, Curitiba PR, Brazil, [5]Oral Pathology Department of the Dental Faculty of University of Sáo Paulo, SP, Brazil, and [6]Oral Biology Department of the School of Dentistry of Bauru, University of Sáo Paulo (USP), Bauru, SP, Brazil.

Correspondence: Dr C Trevilatto, School of Health and Biosciences Pontificia Universidade Catolica do Parana (PUCPR) Rua Imaculada Conceicao, 1155 80215-901, Curitiba PR Brazil. E-mail: pctrev@yahoo.com.br, juhanaschussel24@gmail.com

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INTRODUCTION

Cancer is a significant and increasing health problem in many parts of the world (1). Currently, in Brazil, it is the second cause of death, and over 14 000 cases of head and neck cancer were estimated for 2012, with 45% of death due, the disease (2). Oral squamous cell carcinoma (OSCC) represents the 6th most common type of cancer in developed countries, and constitutes the most frequent malignant tumour of the oral cavity (2). Furthermore, there has been modest improvement in survival of head and neck cancer patients in the past three decades (3), only 50% of patients are cured with initial therapy (3).

Oral cancer is a multifactorial complex disease. There are several well-documented agents involved in oral cancer aetiology, such as exposure to sunlight, smoking and alcohol consumption (1, 4). The mechanisms underlying oral cancer progression are still poorly understood (4). The poor prognosis of patients with OSCC is mainly due to late diagnosis and the invasiveness of its cells, which may promote early regional lymph node invasion and subsequent distant metastatic spread (5). The lack of pre-malignant markers for early detection and risk assessment is clearly reflected by the fact that more than 50% of all OSCC patients are diagnosed in advanced stages of the disease (1).

There has been a significant progress in identifying genetic and molecular changes that occur during the transformation to malignant cells (6). Among the genes involved in tumour cell proliferation and survival, those coding the Wnt signalling molecules are of particular interest because of their important biological functions. In mammals, the Wnt family is composed of 19 members that share a characteristic cysteine pattern plus other conserved residues defining their functional properties (7). These proteins control several aspects of prenatal development, such as cell growth and differentiation, as well as some processes in homeostasis in adult tissues (6). Wnt pathway signalization is responsible for β-catenin cytoplasm accumulation, and consequent activation of nuclear transcription factors. Originally discovered as a proto-oncogene, Wnt/β-catenin pathway has been identified as the main pathway responsible for the cellular events that ultimately result in cancer (8).

In addition, the Wnt/β-catenin signalling pathway has been found to be associated with head and neck squamous cell carcinomas [HNSCC] (9, 10). The expression of 11 of 19 Wnt family members has been identified in oral carcinomas (6, 9). Wnt/β-catenin signalling promotes HNSCC cell scattering and invasion, which are typical changes for HNSCC cells during tumour progression and invasion (5). Wnt signals also play an important role in the differentiation and uncontrolled proliferation of tumour cells during OSCC genesis (11).

Wnt-10a is a key molecule involved in the early cascade that controls the epithelial-mesenchymal interactions in the oral cavity of vertebrate embryos, being expressed in the oral epithelium from which teeth develop (12). In humans, *WNT10A* mutations have been associated with ectodermal dysplasia, a condition characterized by defects in the morphogenesis of skin, hair, nails, glands and teeth (13, 14). In culture of "chick embryo fibroblasts" (CEF), Wnt-10a was shown to increase the cytoplasmaic concentration of β-catenin to levels equivalent to those induced by Wnt-3a, a classical activator of the Wnt/β-catenin pathway (15).

In spite of the important roles that the Wnt gene family plays in physiological and pathological events, there are only a few studies reporting *WNT* gene expression in oral cancer, and no studies analysing *WNT10A* gene so far. Therefore, this study aims to investigate the expression of *WNT10A* gene in OSCC.

SUBJECTS AND METHODS

Study sample

This prospective study included all patients who underwent surgery for tumour excision during the year of 2007 in the Head and Neck Surgery Department at the Erasto Gaertner Hospital (EGH), Curitiba, Brazil. Exclusion criteria were: subjects should not have had chronic usage of anti-inflammatory drugs, immunosuppressive chemotherapy or radiotherapy, reoccurrence of cancer, history of any diseases known to severely compromise immune function, active infection and current pregnancy or lactation. After the histopathological analysis, a convenience sample comprised of 49 specimens, divided into four stages according to TNM classification. Regarding the control samples, 10 specimens were obtained: five samples from the safety tumour margins (STM) after histopathological analysis (two from the tongue base, two from the soft palate and one from gingival tissues), and five samples from the oral mucosa (gingiva) of healthy individuals, removed at the time of tooth extraction (GT) at the Oral and Maxillofacial Surgery Department at the EGH. All samples were collected in compliance with the EGH (register n° 1216) and Catholic University of Paraná (register n° 723) Research Ethic Committees.

Ribonucleic acid extraction – cDNA synthesis

Immediately after surgery, 3 mm samples were taken from the removed surgical specimens. Total RNA from the samples was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the protocol supplied by the manufacturer. Ribonucleic acid total concentration was determined by the optical density at a wavelength of 260 nm using the Genequant (Pharmacia Amersham Biosciences, Piscataway, NJ, USA). Complementary DNA (cDNA) was synthesized using 1 µg of total RNA and oligodT as primer, with the kit ImProm-ll™ Reverse Transcriptase System (Promega Co., WI, USA).

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) analyses were performed in a MiniOpticon system (BioRad, Hercules, CA, USA) using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) for quantitation of amplicons as previously described (9). Primer pairs were built using Primer3 software (version 0.4.0) for *WNT10A* (sense 5'-GGGAGCGCTTTTCTAAGGAC -3', antisense, 5'- CCG-CATGTTCTCCATCACTG -3'), and housekeeping gene *β-ACTIN* (sense 5'- ATGTTTGAGACCTTCAACA -3', antisense 5'- CACGTCAGACTTCATGATGG -3'). Real-time PCR conditions for each target were optimized in regard to primer concentration, absence of primer dimmer formation, and efficiency of amplification of target genes and house-keeping gene control. SYBR Green PCR Master Mix (Applied Biosystems), 400 nM specific primers, and 2.5 ng cDNA were used in each reaction. The standard PCR conditions were 95 °C (10 min), and then 40 cycles of 94 °C (1 min), 56 °C (1 min) and 72 °C (2 min), followed by the standard denaturation curve. The threshold for positivity of real-time PCR was determined based on negative controls (reactions performed without RNA and without reverse transcriptase). For mRNA analysis, the relative levels of transcripts for *WNT10A* from duplicate measurements were calculated in reference to housekeeping genes β-ACTIN, using the cycle threshold (Ct) method and the $2^{-\Delta\Delta Ct}$ formula (16).

Statistical analysis

Statistical tests were performed with GraphPad InStat 3:05 and GraphPad Prism 3.0 software (GraphPad Software Inc.). Analysis of possible differences between the controls and experimental subgroups were evaluated by analysis of variance (ANOVA), followed by Tukey's test, p-values < 0.01 were considered significant.

RESULTS

Sample

A total of 59 patients were included in the study. Most of them were males (90%), and 95% were Caucasian with an average age at the time of diagnosis of 57.4 years. Cigarette smoking was prevalent in 79.6% of patients and alcohol consumption in 61.2% (Table 1).

The most prevalent OSCC lesion site was the floor of the mouth followed by the tongue and gingiva. The number of OSCC samples with respect to the TNM classification was: 5 (10.2%) were stage I, 10 (20.4%) stage II, 9 (18.3%) stage III, and 25 (51.1%) stage IV (Table 2).

***WNT10A* expression**

Expression analysis showed no statistically significant difference between control samples and early OSCC stages, whilst stage IV OSCC showed statistically significant increase of *WNT10A* levels when compared to the control samples (p < 0.01) [Figure], using b-ACTIN as the internal control gene. Similar results were observed when using other internal control genes such as GAPDH and RNA POL II (results not shown).

DISCUSSION

South and Southeast Brazil have the highest rates in OSCC deaths, and between 1975 and 2002, there was 0.29% increase in the death rate per year in the South of Brazil (17). Erasto Gaertner Hospital is located in South Brazil, and OSCC was the sixth most frequent tumour among almost 44 000 tumours

Table: Baseline clinical parameters of the population sample

	Tumour n = 49	**%**	**Age mean (range)**	**Control** n = 10	**%**	**Age mean (range)**	***p-value***
Ethnicity							
Caucasians	46	93.9	58.2 (18–81)	10	100	49.3 (35–63)	0.989
Afro-Americans	3	6.1	61.6 (71–54)	0	0	0	
Gender							
Male	37	75.5	53.2 (41–77)	6	60	57.6 (50–63)	0.538
Female	12	24.5	59.7 (18–81)	4	40	44.2 (35–62)	
Smoking							
Yes	40	81.7	59.9 (41–81)	7	70	52 (35–63)	0.687
No	9	18.3	58.7 (18–79)	3	30	53 (62–36)	
Alcohol							
Yes	27	61.2	59.7 (41–76)	6	60	54.8 (63–44)	0.780
No	19	38.7	58.1 (18–81)	4	40	48.5 (36–62)	
Not known	02						
Stages							
Stage I	5	10.2	54.8 (42–71)				
Stage II	10	20.4	55.9 (41–73)				
Stage III	9	18.3	64.4 (49–79)				
Stage IV	25	51.1	60.4 (18–81)				

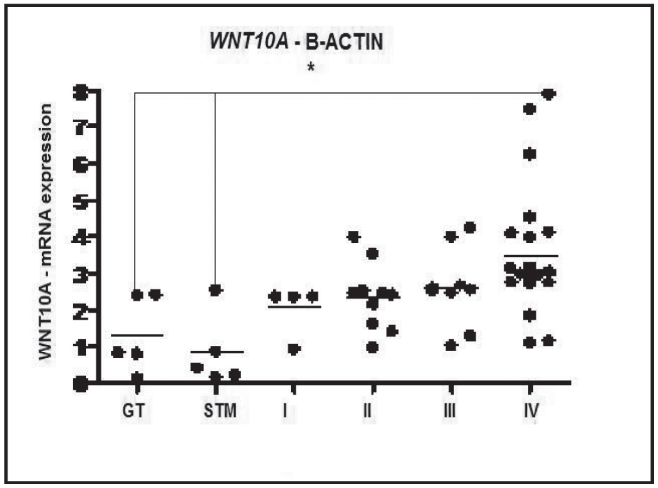

Figure: Quantitative expression of WNT10A in control samples (GT and STM) and in tumour stages (I, II, III and IV) using β-ACTIN as the internal control. Real-time polymerase chain reaction (PCR) analyses were performed using the SYBR-green for quantitation of amplicons. Experimental conditions for each target were optimized in regard to primer concentration, absence of primer dimmer formation, and efficiency of amplification of target genes and housekeeping gene controls. The threshold for positivity of real-time PCR was determined based on negative controls (reactions performed without RNA and without reverse transcriptase). For mRNA analysis, the relative levels of transcripts for WNT10A from duplicate measurements were calculated in reference to housekeeping gene β-ACTIN using the cycle threshold (Ct) method and the 2-ΔΔCt formula.

diagnosed in 2012. Our sample was consisted of high-risk patients, mostly males more than 50 years old with a history of tobacco and alcohol consumption. Between 1975 and 2000, the incidence among males decreased from 21.2% to 15.9%, with an average of 18.8 out of 100 000, while among females, the decrease was from 7.1 to 6.5%, with an average of 7.2 out of 100 000 (18).

Among the environmental aspects involved in OSCC carcinogenesis, smoking is the most significant aetiological risk factor (19). Although in Brazil there has been a decrease in tobacco consumption in the last thirty years, about 21% of males and 13% of females are smokers in the Brazilian population (1). The percentage of smokers among the patients in this study (79.6%) is much higher than that observed in the general Brazilian population, confirming the role of this aetiological factor in OSCC development. Individuals who consume alcohol and tobacco have 100 times more risk of developing head and neck cancer (1).

A high prevalence of advanced disease – stages III and IV (69.3%) – was observed among patients included in this study. Oral squamous cell carcinoma makes-up over 90% of oral tumours, being associated with a substantial cancer morbidity and death rate (17). According to the National Cancer Institute, data regarding OSCC patients' survival time has remained similar for over three decades. Eighty-two per cent of patients present with localized disease with a survival rate of five years, 51% have the disease spread to lymph nodes and other organs, with a survival rate less than five years, while 27.6% have the disease in other organs and lymph nodes with a decreased survival time and increased death rate (18). The present results reinforce that in Brazil, diagnosis occurs in people over the 5th decade of life and frequently at an advanced stage of disease with high invasive potential in regional lymph nodes and subsequent distant metastatic spread (5, 20).

However, in spite of the high incidence of OSCC, its molecular mechanisms are still poorly understood (5, 21). Molecular Biology breakthroughs have implicated genes involved in cancer onset and progression (4). High expression levels of *WNT* genes have been observed in many tumours and may be involved in the differentiation of epithelium (22). One of the first reports demonstrating *WNT* expression evaluated oral tumours of squamous cells compared to non-tumoural epithelial cells. These results suggested that tumour survival and growth may depend on *WNT* signalling (6). Moreover, invasive OSCC presented an active Wnt/β-catenin signalling pathway, reinforcing the role of β-catenin activation in cancer progression and metastasis (25). Wingless-type mammary tumour virus-1 (WNT1) expression in OSCC was associated with tumour differentiation (23).

Wingless-type mammary tumour virus-10A has been associated with the development of ectodermic structures in chicken and mouse embryos (24). In humans, *WNT10A* plays a crucial role in the development and regulation of ectodermic derivatives and mutations in this gene have been associated to ectodermal dysplasia (12). This gene is expressed in embryonic stem cells, moderately expressed in the placenta and adult spleen, and weakly expressed in lung, prostate and ovarian tissue (25). *WNT10A* expression was also noted in epithelial tissue tumours such as gastric and colorectal carcinomas (25). However, to the best of the authors' knowledge, this is the first study reporting *WNT10A* expression in OSCC.

The results showed a gradual increase in *WNT10A* expression according to disease stages, with highest levels of transcripts being observed in stage IV, with statistically significant difference when compare to the control group. An analysis of variance between the different stages showed that this significant difference was not due to the higher number of cases in stage IV (data not shown). Stage IV represents the most advanced phase of disease progression, with invasion of adjacent structures and/or lymph node and/or distant metastasis. Although we cannot exclude the possibility that *WNT10A* is expressed by cells other than those of the tumour, such as endothelial or inflammatory cells of the haematopoietic lineage, our results strongly suggest an association between *WNT10A* expression and tumour progression. Immunohistochemistry or *in situ* hybridization analysis should be performed in order to verify if the *WNT10A* expressing cells are restricted to tumour.

The high expression of WNT molecules results in cytoplasmaic β-catenin accumulation, which in turn enters the nucleus and activates the expression of genes involved in cell proliferation, adhesion and apoptosis, which may lead to tumour progression (7). High-levels of β-catenin are believed

to be fundamental for tumour cell invasion. However, the molecular basis of the differential gene expression induced by β-catenin is not fully understood (7). It has been suggested that WNT signalling may play a crucial role in stimulating invasive cell growth, and an association of WNT signalling with invasive OSCC has been observed in another work (11). Studies on the WNT pathway in oral cancer suggest that its activation may be a cause of oral cancer due to the nuclear transport of β-catenin and activation of specific target genes (26). Thus, it is possible that in the present study's samples, a higher level of *WNT10A* is a major factor in promoting tumour progress, since *WNT10A* has been associated with β-catenin signalling in embryonic cells (15). It is known that the metastatic capacity may also be stimulated by the WNT signalling by the increase of adhesion receptors, promoting cellular adherence to extracellular matrix components (7). Moreover, the Wnt/β-catenin pathway has been shown to have a major impact on epithelial-mesenchymal transition (EMT) during development and cancer progression (27). Indeed, during embryogenesis, *WNT10A* plays a crucial role in the epithelial-mesenchymal interaction required to form the ectodermal derivatives. Likewise, the activation of *WNT10A* has recently been associated with cell migration, invasion, and metastasis, which characterize EMT in cancer (28).

In summary, a significant increase in the *WNT10A* gene expression was observed in OSCC stage IV, suggesting a potential role in tumour EMT and progression. Therefore, it can be suggested that *WNT10A* signalling through β-catenin pathway may have a potential role in OSCC progression. These results might contribute to the understanding of the WNT signalling in oral tumourigeneses. In the future, experimental strategies might be created to antagonize the effects of the WNTs in the tumourigenic process.

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