Original Article - Prospective Study

Expression of CD24 as Cancer Stem Cell Marker in the Diagnosis of Oral Squamous Cell Carcinoma – A Prospective Study

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Abstract

Introduction: Oral squamous cell carcinoma (OSCC) is an aggressive and recurrent malignancy. Identification of unique and overexpressed cell surface antigens is important in the diagnosis and development of cancer vaccines and various therapies for OSCC. We have used real-time polymerase chain reaction (RT-PCR) for the expression of cell surface protein CD24 in both tissue samples and in blood samples to study the clinicopathological features as well as to determine the gene expression profile of CD24 in OSCC and explore its role as a potential target of clinical therapy. **Materials and Methods:** In this prospective study, the expression of CD24 was evaluated in 20 blood (3 ml) and tissue samples of OSCC specimens by quantitative RT-PCR. Student's *t*-test was used for statistical analysis. The significance level was considered <0.05. **Results:** CD24 was found to be upregulated amongst the cases for both the tissue and the blood. CD24 was statistically significant with $P < 0.05$. Fold change was calculated to assess the quantity of the difference in expression amongst cases when compared to controls. Results were supportive of CD24 being a reliable biomarker, hence blood samples can also be used in screening and diagnosis of OSCC. **Discussion:** CD24 expression is significantly upregulated in blood and tissue samples in OSCC. In addition, CD24 overexpression is highly associated with adverse prognostic parameters such as lymph node involvement, advanced clinical stages and worse overall survival. Our findings have important implications in future practice, overexpression of CD24 in OSCC was associated with poor prognosis correlating to the clinical findings, large-scale comprehensive studies are needed further to confirm our findings. In addition to histological features, CD24 can be used as marker for OSCC.

Keywords: Biomarker, CD24, circulating tumour cells, oral squamous cell carcinoma, real-time polymerase chain reaction

Introduction

Oral squamous cell carcinoma (OSCC) accounts for the sixth most common malignancy amongst human malignancies.[1] It is the most common cancer occurring in head-and-neck region involving the oral, nasal cavity, pharynx and larynx.[2,3] Tobacco and alcohol use still remain the greatest risk factor. Treatment is with surgery, radiation or both, though surgery plays a major role in treatment. The overall survival rate of five years is <50%.[4] Despite advances in therapeutics and surgical procedures, there is no significant difference in the overall survival rate in the past 30 years and it is mainly because the early stages of the disease are asymptomatic and lack of awareness of patients to report early.[5] Early diagnosis remains the key in limiting the spread and in treating OSCC.

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Adapting the use of sensitive and specific non-invasive biomarkers along with tissue sampling would be an easy and inexpensive method in the early detection of OSCC.

OSCC arises as the series of different molecular events that starts from individual genetic predisposition, immunodeficiency and external agents such as dietary factors, human papillomavirus and Epstein–Barr virus.

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Genetic and epigenetic factors contribute to tumourigenesis in humans.

Cancer stem cells (CSCs) are small groups of cells present within the tumour which have self-differentiation, self-renewal and tumourigenesis potential. CSC has been identified in embryonic stem cells and adult human stem cells. These cells have the capability to transform to any type of specialised cell type. CSCs can be differentiated inside the tumour from other cells by their symmetry of cell division and alteration in their gene expression.[6] CD24, CD44 and CD133 are few cell surface markers which are used to identify and enrich CSC.

There is recent evidence that CSCs are resistant to conventional chemo and radiotherapy and are likely to cause relapse and cancer metastasis.[7,8]

Lack of universal expression of surface markers limits their usage and no best combination of markers has yet been confirmed to identify CSCs capable of initiating and metastasising tumours.

Many CSCs have been identified in different tumours by specific cell markers CD44 for head-and-neck cancer,[8-10] CD24 for ovarian cancer,[10,11] CD133 for brain tumour and CD44 and CD24 for breast cancer.[12]

CD24 a heat-stable antigen is a glycosylphosphatidylinositol sialoglycoprotein which is expressed in both haematopoietic and non-haematopoietic cells. It is located on chromosome 6q2. CD24 has shown to promote tumour cell proliferation, invasion and metastasis in many types of carcinomas.[13]

In OSCC, a minor subset of tumour cells called CSCs express CD24 and the CD24+ cell population not only drives tumour initiation and expanding tumour growth,[14,15] but also is responsible for recurrence following treatment. Because of the inherent chemo-and radioresistance of these cells[16] and the ability to self-renew and differentiate, it gives rise to heterogeneous, differentiated cancer cells making up the bulk of the tumour mass. Therefore, the identification and removal of CD24+ CSCs may lead to the development of effective treatment.

In the present study, we looked for the expression of CD24 markers in the blood and tissue samples of patients with OSCC. The expression was correlated with the clinical presentations of the disease.

Materials and Methods

Study samples

A total of 20 biopsy-proven cases of OSCC (n = 20) were included in this study. Oral premalignant lesions and carcinoma *in situ* were excluded from the study and tissue showing definite dysplasia was included. Tissue biopsies and peripheral blood samples were collected from the Department of Oral and Maxillofacial Surgery, Saveetha Dental College, Chennai. Written consent was obtained from all participants of the study.

Demographic and clinical information from all cases were obtained through review of all forms from patient record files submitted along with the specimens and included gender, age, time interval before diagnosis (in months), clinical aspect, location and size (in centimetres) of the tumours and nodal status and risk factors (tobacco and alcohol use). Amongst the risk factors, tobacco/betel nut chewing and alcohol consumption history were recorded.

The study was approved by the Institutional Ethics committee of Saveetha University, Chennai, India (001/06/2018/IEC/SMCH), in the meeting held in July 2018. Tissue samples were immediately placed in RNA later solution. Blood was collected in an ethylenediaminetetraacetic acid vacutainer and transported to the laboratory within six hours from collection.

The tissue sample stored in RNA later was carefully removed with tissue forceps for homogenisation. Tissue was placed directly into mortar and pestle in liquid nitrogen and ground to fine powder. The tissue powder and liquid nitrogen were decanted into RNase-free and liquid nitrogen-cooled extract into 2-mL microcentrifuge tube. The liquid nitrogen was allowed to evaporate but without thawing. 600 µL of RNA lysis buffer was added and homogenised by passing the lysate at least five times through a blunt 20 gauge needle fitted to an RNase-free syringe. The lysate was centrifuged at full speed for three minutes. One volume of 70% ethanol was added to the lysate. After repeated decanting, adding buffer and centrifuging RNA in the microcentrifuge tube was used for the further experiment (if the expected RNA yield was <30 µg, elution step was repeated until appropriate quantity of RNA was obtained).

Genotyping

DNA was isolated from all the blood samples using QIAamp DNA Mini Kit (Qiagen, Germany), which was performed using 100 ng of DNA, 1X Taq buffer, 200 µM of dNTP, 1.5 units of Taq DNA polymerase (Genei, Bangalore, India) and 30 pM of primers (forward and reverse).

Gene	Forward primer	Reverse primer
b-actin_1	CCCTGGACTTC GAGCAAGAGAT	GTTTTCTGCG CAAGTTAGG
b-actin _2	GTGAAGGTGAC AGCAGTCGGTT	GAAGTGGGGTG GCTTTTAGGAT

Genotyping of the SNPs was performed by direct dye-terminator sequencing and the raw data obtained were analysed using Seqscape v2.7 software (Applied Biosystems, USA).

Quantitative real-time polymerase chain reaction analysis

Total RNA was extracted from the samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA using verso cDNA synthesis kit (Thermo Fisher Scientific Inc., MD). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using the start universal SYBR Green master mix (Roche, Basel, Switzerland) on the 7500 fast real-time polymerase chain reaction (RT-PCR) system (Applied Biosystems, Foster City, CA, USA). Data were normalised with β-actin.

Statistical analysis

All information was descriptively analysed, and statistical analysis was performed using a standard programme (Statistical Package for Social Sciences, SPSS version 17.0, Chicago, IL, USA), with statistical significance level of 5% ($P < 0.05$). Comparison of means was performed with Student's *t*-test.

Results

Gene expression analysis was performed for target gene CD24 using qRT-PCR amongst the cases. CD24 was found to be upregulated amongst the cases and for both the tissue and the blood. The Student's *t*-test was performed and found CD24 to be statistically significant with $P < 0.05$ [Table 1]. Fold change was calculated to assess the quantity of the difference in expression.

Gene expression

The present study analysed the expression profiles of CD24 pathologically confirmed OSCC patients. CD24 was found to be upregulated amongst the cases for both the tissue and the blood group, and CD24 was revealed to be statistically significant with $P < 0.05$. Fold change was calculated to assess the quantity of the difference in expression amongst cases when compared to controls. The mean age of all patients was 54 years.

Around 65% of the patients were diagnosed in their 60s and only 5% of the patients were younger than 40 years [Table 2]. The mean time of complaint with the lesions reported by the patients before diagnosis was 12 months. Most patients reported to have noticed the lesions up to six months before diagnosis (14 cases, 70%). The mean time of complaint was longer for females three years more than for males.

Histological diagnosis and grade of the tumours rendered after analysis of the HE-stained slides revealed that, from nine cases (45%) of lateral border of tongue OSCC, two cases (22.2%) were classified as well-differentiated (WD) OSCC, 1 case (11.1%) as moderately differentiated tumours and 6 (66.6%) as poorly differentiated (PD) tumours. Two patients with alveolar mucosa/gingiva/retromolar area were classified as PD-OSCC. From eight cases (40%) with tumours located at buccal mucosa/buccal sulcus, two patients were classified as WD-OSCC (25%), while six patients were classified as WD-OSCC (75%). Of the total 20 cases, only one patient showed lip cancer (5%) [Table 3].

Site distribution showed that the most common location of the tumours was the lateral border of the tongue (45%), followed by the buccal mucosa/buccal sulcus (40%) and alveolar mucosa/gingiva/retromolar area (10%) and lower lip (5%) [Table 4].

Clinical aspects of the tumours revealed only ulcers in all the patients. The mean size of the tumours showed that 70% of OSCC patients were diagnosed with 2.0–4.0 cm in their greater diameter, 10% of the patients displayed 4.0–6.0 cm diameter while 20% of the patients were diagnosed with <2.0 cm diameter in tumour size [Table 4]. There was no difference on the mean size of the tumours when comparing affected males with females.

Information about tobacco (present or past) use was seen in 13 patients (65%). Amongst them, 11 patients (73.3%) were males and two patients (40%) were females. Information on alcohol use was seen in 13 patients (86.7%) (present or past alcohol users) and all of them were males [Table 4].

Discussion

The clinical and demographic characteristics (age, gender, time of complaint, site of lesion, clinical aspect and size of tumour) of patients were included and correlated. Number

Table 1: Gene expression in tissue and blood

Gene	Fold change	*P*
CD24	Cancerous tissue group=1.65	0.0071
CD24	Cancerous blood group=1.96	0.0082

t=2.50196, *P*=0.0071, the result is significant at *P*<0.05; *t*=2.44685, *P*=0.0082, the result is significant at *P*<0.05

Table 2: Demographic and clinical features of oral squamous cell carcinomas from the studied sample

Parameters	Number of cases (%)
Gender (*n*=20)	
Males	15 (75)
Females	5 (25)
Age (*n*=20) (years)	
<40	1 (5)
>40–60	12 (60)
>60	7 (35)
Time of complaint (*n*=20)	
0–6 months	14 (70)
12 months	5 (25)
>12 months	1 (5)
Site of lesion	
Border of tongue	9 (45)
Alveolar mucosa/gingiva/ retromolar area	2 (10)
Floor of mouth/ventral tongue	Nil
Soft palate/tonsil area	Nil
Buccal mucosa/buccal sulcus	8 (40)
Lower lip	1 (5)
Others	
Clinical aspect (*n*=20)	
Ulcer	20 (100)
Leukoerythroplakia	Nil
Ulcer + leukoerythroplakia	Nil
Size of the tumours (*n*=20) (cm)	
<2.0	4 (20)
2.0–4.0	14 (70)
4.0–6.0	2 (10)
>6.0	Nil

Table 3: Distribution of the histological grade of the tumours according to the site of the lesions*

Site of the tumours	Histological grade			Total, *n* (%)
	WD, *n* (%)	MD, *n* (%)	PD, *n* (%)	
Border of tongue	2 (22.2)	1 (11.1)	6 (66.6)	9 (45)
Alveolar mucosa/gingiva/retromolar area	0	0	2 (100)	2 (10)
Buccal mucosa/buccal sulcus	2 (25)	0	6 (75)	8 (40)
Lower lip	1 (100)	0	0	1 (5)
Total				20

*Histological grade and distribution according to the site of the lesion. WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated

Table 4: Distribution of the clinical aspect of all oral squamous cell carcinoma, site of the lesions, history of tobacco and alcohol use, and histological grade of the tumours according to the gender of the affected patients

Parameter	Males, *n* (%)	Females, *n* (%)	Total, *n* (%)
Ulcers			
Yes	15 (75)	5 (25)	20 (100)
No			
Site of the lesions			
Border of tongue	7 (46.7)	2 (40)	9 (45)
Alveolar mucosa/ gingiva/ retromolar area	1 (6.7)	1 (20)	2 (10)
Buccal mucosa/ buccal sulcus	7 (46.7)	1 (20)	8 (40)
Lower lip	1 (6.7)		1 (5)
Tobacco use			
Yes (present or past)	11 (73.3)	2 (40)	13 (65)
No	4 (26.7)	3 (60)	7 (35)
Alcohol use			
Yes (present or past)	13 (86.7)		13 (86.7)
No	2 (13.3)		2 (13.3)
Histological grade*			
WD	3 (20)	2 (40)	5 (25)
MD	1 (6.7)		1 (5)
PD	9 (60)	5 (100)	14 (70)

*Histological grade and distribution according to the site of the lesion. WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated

of males in this study was more compared to females/lesion was most commonly seen in the lateral border of tongue 45%, and buccal mucosa/buccal sulcus 40%, followed by alveolar mucosa/retromolar trigone 10% and lower lip 5%.

CD24 is not present in adult human tissues, it is present in wide range of malignancies (B-cell lymphoma, renal cell carcinoma, small cell and non-small cell lung carcinoma, nasopharyngeal carcinoma (NPC),[17] hepatocellular carcinoma,[18] glioma, epithelial ovarian cancer and breast cancer).[19] It is not only expressed in developing or regenerating tissues but also in granulocytes, pre-B cells, keratinocytes and renal tubules and plays an important role in cell selection and maturation during haematopoiesis. CD24 has been identified as an alternative ligand to P-selectin, an adhesion receptor on activated endothelial cells and platelets.

Hung Yang *et al*.[17] showed that CD24 cells isolated from human NPC cell lines through flow cytometry and tumour sphere formation assay express stem line genes TW02 7 TW04 showed activation of the Wnt/β-catenin signalling pathway. They identified increased expression of the Wnt/β-catenin pathway in isolated CD24+ than parenteral CD24- cells. CD24+ cells showed enhanced clone/sphere formation with enhanced resistance to chemotherapeutic drugs and also the expression of higher levels of metalloproteinase responsible for tumour invasion.

Salvador DR *et al*.[20] retrieved 69 files of patients with salivary gland malignant neoplasm (SGMN) and collected information on age, gender, primary anatomic location, history of smoking, alcohol intake, histological classification, nodal status, treatment tumour recurrence, disease-free survival (DFS) and overall survival. Positive controls were taken from tonsil tissue. IHC showed SGMN with CD44+/CD24+ represented the tumours with the most aggressive behaviour and prognosis and was associated with tumour size and lymph node metastasis.

A study by Kwon *et al*. revealed that high CD24 expression is correlated with the presence of lymph node metastasis and advanced pathological stage, suggesting that CD24 is associated with aggressive breast cancer.[21]

The relationship between CD24 expression with DFS or distant metastasis-free survival raises the possibility that targeting CD24 may inhibit local recurrence or distant metastasis in triple-negative breast cancer. A recent study by Abraham BK *et al.* further supports the feasibility of CD24-targeted therapy in the treatment of cancer. This study illustrated that targeting CD24 with a monoclonal antibody inhibits tumour growth in xenograft models of lung and ovarian cancers through changes in tumour cell proliferation and angiogenesis.[22]

Studies have revealed that tumour recurrence can be reduced if the CSCs are specifically targeted. These CSCs are resistant to conventional treatment and developing drugs to target these are highly challenging. Recent studies which caused depletion of CD24 using siRNA in hepatocellular carcinoma have

demonstrated a reduction of tumourigenesis, hence suggesting CD24 can be used as a prognostic biomarker.

In our present study, we evaluated the expression of CD24 in both blood and tissue samples with clearly defined clinical parameters. Our analysis using RT-PCR revealed that CD24 expression was higher in tissue and blood in OSCC.

To summarise, CD24 can be used as a reliable biomarker and blood sample in the early screening and diagnosis of OSCC.

Conclusion

Our results indicate that CD24 expression is significantly upregulated in blood and tissue samples in OSCC. In addition, CD24 overexpression is highly associated with adverse prognostic parameters such as lymph node involvement, advanced clinical stages and worse overall survival. Large scale comprehensive studies are needed to further confirm our findings.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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