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Association of salivary soluble CD44 in patients with oral leukoplakia: A case control study

Running title: Salivary soluble CD44 in patients with oral leukoplakia

Authors

1. Dr. Megha.D.B
Assistant Professor, Oral Medicine and Radiology
Coorg Institute of Dental Sciences, Virajpet, Karnataka, India

2. Dr. Seema Patil
Professor and Head of the Department, Oral Medicine and Radiology
D A P M R V Dental college, Bangalore, Karnataka, India

3. Dr. Asha R Iyengar
Principal, Oral Medicine and Radiology,
D A P M R V Dental college, Bangalore, Karnataka, India

4. Dr. Sanjana M
Assistant Professor, MDS, Oral Medicine and Radiology
Sibar Institute of dental sciences, Guntur, AP, India

5. Dr. Aparna K
Private Practitioner, Oral Medicine and Radiology, Kakinada, AP, India

6. Dr. Ritika Agarwal
Private Practitioner, Oral Medicine and Radiology, Patna, Bihar, India

7. Dr. Subash BV
Reader, Department of Oral Medicine and Radiology, DAPMRV Dental College Bangalore, India

8. Dr. Revan Joshi
Assistant Professor, Department of Oral Medicine and Radiology, DAPMRV Dental College, Bangalore, India

Corresponding author

Dr. Megha.D.B
Assistant Professor, MDS, Oral Medicine and Radiology
Coorg Institute of Dental Sciences, Virajpet, Karnataka, India

Abstract

***Background*:** Leukoplakia is the most common potentially malignant disorder of the oral mucosa. Latest developments in the field of molecular biology have greatly increased our knowledge of the role of various bio markers in carcinogenesis and prognosis of precancerous and cancerous lesions.

Aim: The present study aims to assess the expression of CD44 in saliva of subjects with leukoplakia by quantitative Real Time-polymerase chain reaction.

***Methodology*:** 30 subjects with leukoplakia (clinically and histopathologically confirmed) and 20 healthy controls were included in the study. Quantitative real time PCR was run on the salivary samples of these study subjects.

***Results*:** An individual pattern of down regulation of CD44 was noted in 60% of the subjects with leukoplakia and upregulation was noted in 90% of controls by absolute quantification method. The gene fold expression ratio by relative quantification method between subjects with leukoplakia and controls delta ct method was found to be 5.777/5.827= 0.9914 and by double delta ct method was found to be 28.4151/28.0694=1.0123. No statistical significant difference was noted in the expression of CD44 and grades of epithelial dysplasia.

***Conclusion*:** Although an individual pattern of down regulation of CD44 was noted in subjects with leukoplakia, the gene fold expression ratio between subjects with leukoplakia and controls by both the methods was approximately 1 suggesting no difference in the gene fold expression ratio between subjects with leukoplakia and controls. CD44 was not found to be a reliable marker in determining the malignant transformation of leukoplakia.

Keywords: Leukoplakia, CD44, q RT-PCR, Bio-markers, Saliva.

Introduction

Oral cancer is the 15th most prevalent cancer with the age standardized incidence rate of 3.9 per 100,000 populations worldwide. The Indian subcontinent accounts for one-third of the world burden of this malignancy. Oral cancer is the most common form of cancer and accounts for increasing number of cancer related deaths among men in India.[1] Oral cancer mostly occurs in the buccal mucosa and of the Indian population as compared to the western countries where oral cancer mainly occurs in the lateral border of the tongue. This may be due to the fact that the Indian population is more prone to use of smokeless variety of tobacco.[2]

Leukoplakia is the most common potentially malignant disorder of the oral mucosa. The annual incidence of oral leukoplakia among subjects greater than 15 years of age ranges from 0.2% to 11.7% in different populations of India.[3] It is defined by the World Health Organization in 2005 as a "A white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer". In an explanatory note it has been explicitly stated that the term leukoplakia is unrelated to the absence or presence of epithelial dysplasia.[4]

Studies have shown that dentists and other health care providers are in a tremendous need of systematic educational updates in oral cancer prevention and early detection. [2]

The ability to clinically predict malignant transformation is difficult.[5]A number of diagnostic kits have been developed for the early detection of oral cancer namely OralCDx Brush Biopsy, ViziLite Plus, toluidine blue staining etc. Although these tests can assist in identification of dysplastic features or frank malignancy, they are associated with either false positive or false negative results and are not conclusive. [6] Hence a need for confirmation of their malignant potential by histopathological evaluation arises. However histopathological diagnosis has limited prognostic value and disadvantage of patient discomfort and fear. [5]

A latest development of various bio-markers in carcinogenesis of precancerous and cancerous lesions helps in prediction of rate of malignant transformation and prognosis of the lesion.[6] These biomarkers have generally been detected either in the serum or tissue

specimens. Some of these biomarkers can also be detected in saliva as saliva may be considered as a reflection of serum.[7] Various biomarkers namely Ki-67, argyrophilic nucleolar organizer regions, p53 helps to predict the malignant potential of and as a prognostic tool in oral cancer.[8]

CD44 is a biomarker expressed as a cell surface glycoprotein by a large variety of tissues such as colorectal tissues, urinary bladder,lungs head and neck epithelia etc.. It is also released from mast cells, lymphocytes, and hence are detectable in circulation as well. CD44 acts as a receptor for the extracellular matrix components namely hyaluronate, laminin , collagen etc.[9,10]

Studies have evaluated the role of CD44 in tissue samples of HNSCC with the help of various methods such as innmuno- histochemistry, PCR and flow cytometry. An increased expression of CD44 in saliva of subjects with HNSCC as compared to normal study subjects has been reported in four studies. However one study found no difference in expression of CD44 in both HNSCC and normal study subjects. This was attributed to the fact that HNSCC is a multi-factorial disease which involves a number of risk factors.[11]

There are limited studies on the expression of CD44 in saliva of subjects with leukoplakia. Hence this research was done to assess and compare the levels of CD44 in saliva samples of soluble CD44 in saliva of leukoplakia group with and controls by quantitative Real Time-polymerase chain reaction (RT-PCR) technique and to evaluate the correlation between the salivary soluble CD44 and epithelial dysplasia in subjects with leukoplakia.

Materials and method

Study design

This case control study was done in the department of Oral medicine and Radiology after obtaining the approval from institutional ethics committee.

In this study with Null hypothesis H_o: $m_1 = m_2$ vs. Alternative hypothesis H_a: $m_1 = m_2 +$ d where d is the difference between two means and n1 and n2 are the sample size for Group I and Group II such that N=n1+n2. The ratio r=n1/n2 is considered whenever the researcher needs unequal sample size due to various reasons, such as ethical, cost, availability etc.

Sample size estimation

In this study for an outcome variable CD44s with difference of 1.0(SD=1.40), 90% statistical power and 5% statistical significance, the total sample size of 50 can be derived by systematic random sampling.

Then the total sample size for the study is as follows

$$N = \frac{(r+1)(Z_{\alpha/2} + Z_{1-\beta})^2 \ \sigma^2}{r\, d^2}$$

Where Z_α is the normal deviate at α level of significance(Z_α is 1.96 for 5% level of significance and 2.58 for 1% level of significance) and $Z_{1-\beta}$ is the normal deviate at 1-β% power with β% of type II error(0.84 at 80% power and 1.28 at 90% statistical power). r=n1/n2 is the ratio of sample size required for two groups, generally it is one for keeping equal sample size for two groups, If r=0.5 gives the sample size distribution as 1:2 for two groups. σ and d are the pooled standard deviation and difference of means of two groups.

Methodology

Total 50 samples were divided into, 30 subjects with leukoplakia (clinically and histopathologically confirmed) and 20 healthy controls groups.

Non stimulated salivary sample was collected from both the groups and subjected for investigation. The expression of CD44 in saliva of subjects with leukoplakia was assessed by quantitative Real Time-polymerase chain reaction (PCR) method for both the groups.

Results

In the present study, the difference in CD44 gene fold expression ratio between subjects with leukoplakia and controls was calculated by the following two methods; Delta ct method: Delta ct mean of the subjects/ Delta ct mean of controls and Double delta ct method: RQ mean of subjects/ RQ mean of controls. In both the above mentioned methods, values <1 indicate that the gene fold expression of cases are less than controls where as values >1 indicate that the gene fold expression in cases is more than controls. Values =1 indicate no change in gene fold expression.

In the present study, the gene fold expression ratio by delta ct method was found to be 5.777/5.827= 0.9914 and by double delta ct method was found to be 28.4151/28.0694=1.0123. As the ratio between subjects with leukoplakia and controls was found close to 1 it can be interpreted as gene fold expression between the cases and controls is not significant / or no change is observed between the subjects with leukoplakia and control group in terms of gene fold expression.

Table 1 and 2 indicated distribution of subjects based on gender, age. Males were 27 with lekoplakia and 17 in control group whereas 3 each with leakoplakia and control group (Table 1). Subjects were evaluated with age range of 21-40, 41-60,and >60 (table 2).

Table 3 indicates distribution leukoplakia lesions based on their clinical form and location. Highest leukoplakia form was homogenous one 24 (Buccal mucosa) and 4 as non homogenous one (4 in buccal mucosa, 1 each in soft palate and gingival). There was 18 cases with no dysplasia, 6 mild for homogenous variety. In non homogenous variety, 3 were no dysplasia, 2 were mild and 1 moderate dysplasia (Table 4).

Based on the quantitative expression of CD44 gene, subjects with leukoplakia had 12 cases of upregulation and 18 with down regulation. In control group there was 18 upregulation and 2 down regulation (Table 5). CD44 gene expression based on clinical forms of leukoplakia lesions indicated in table 6 with highest cases with homogenous compared to speckled type.

Table 7 indicates CD44 gene expression based on degree of dysplasia. There was 9 cases diagnosed as no dysplasia, 3 with mild and 1 with moderate dysplasia.

Discussion:

Oral cancer is the most common type of cancer of the head and neck, with an annual worldwide incidence of 300 000 cases. The disease is an important cause of death and morbidity, with a 5-year survival of less than 50%. Most of the oral cancers arise from potentially malignant disorders. Leukoplakia is the most common potentially malignant disorder of the oral mucosa. The prevalence of oral leukoplakia ranges from 0.4% to 0.7% of the population worldwide.[1,3]

The present study evaluates and compares the CD44 gene for diagnosis of leukoplakia. In the present study 16% of the subjects presented with epithelial dysplasia. The frequency of epithelial dysplasia in leukoplakia varies between < 1 and > 30% across literature. The lowest frequency of 0.3% was reported in a population-based study in India in which all clinically diagnosed leukoplakias were biopsied and a very small portion of the subjects presented with epithelial dysplasia.

In the present study, the age of the subjects with leukoplakia ranged between 25-77 years. This is in accordance with two Chinese [12,13] studies in which the age of subjects with leukoplakia ranged from 21-84 years and 24-83 years respectively. In the present study the mean age of subjects with leukoplakia was found to be 48.4 years which is in similarity with studies conducted in India [14], where in the mean ages were found to be 48, 47,years respectively. However studies in countries like Brazil[15], China[13], Taiwan[16], Netherlands [17] reported a slightly higher mean age of the subjects ie. 54 years, 55 years, 52 years, 51 years respectively.

In the present study, most of the subjects with leukoplakia were in the 5th decade of life. An extensive early survey in India and studies conducted in China[13] and Taiwan[16] reported a peak incidence of leukoplakia in the fifth decade of life in concurrence with the present study.

It has been reported that the age at which leukoplakia occurs in an individual is an important risk factor that affects the malignant outcome of leukoplakia. The malignant potential of leukoplakia was assessed in a Chinese study[13] and it was found that the malignant transformation rate of leukoplakia was 72.6% higher in the elderly patients as compared to the younger age groups.

A Hungarian study[18] reported a peak incidence of leukoplakia in the 6th decade of life. This could be due to the prolonged duration of exposure to tobacco amongst the older age groups.

Tobacco usage is the most common etiological factor in the development of leukoplakia. In the developing world, tobacco and areca nut use, either alone or in combination, accounts for the vast majority of leukoplakias. In the present study, 90% of the subjects were smokers and 6% of the subjects used smokeless tobacco habit alone and 3% of subjects used both. The findings of the present study are in agreement with previous reports from Brazil[15], Hungary. [18]

Cˇema et al evaluated the role of pan-CD44 protein expression in leukoplakia tissues. They concluded that CD9 antigen expression in the exosomes of the oral epithelium explained the intercellular flow of SolCD44 and other fluids in the leukoplakia area. [19] Franzmann et al assessed salivary soluble CD44 (solCD44) expression in HNSCC patients and concluded that, salivary solCD44 ELISA seems to effectively detect HNSCC at all stages. [20] Gadge et al stated that among CD44s and its variant isoforms,v5, v6, variant isoform v6 may serve as a marker in detecting high-risk leukoplakias.[21]

Ghazi et al found that diagnostic test's accuracy for identification of OSCC and dysplastic leukoplakia from non-dysplastic leukoplakia and normal tissues and recognition of OSCC from dysplastic leukoplakia showed optimum sensitivity and specificity. [22]

Eighty percent of the study subjects presented with a homogenous variety of the disease whereas 17% of subjects presented with a speckled variety of leukoplakia. One subject i.e.,3% presented with verrucous variety of leukoplakia.

In the present study 16% of the subjects presented with epithelial dysplasia. The frequency of epithelial dysplasia in leukoplakia varies between < 1 and > 30% across literature. The lowest frequency of 0.3% was reported in a population-based study in India in which all clinically diagnosed leukoplakias were biopsied and a very small portion of the subjects presented with epithelial dysplasia.

CD44 expression has been assessed in the head and neck region by various methods such as immuno-histochemistry, western-blot analysis, flow cytometry and polymerase chain reactions in various mediums such as the tissue samples, serum and saliva as a diagnostic and prognostic bio-marker. Discrepancies in the expression of CD44 are probably due to the use of different immunohistochemistry protocols and also due to the differences in the patient population examined.

It has been observed that in epithelia that show loss of integrity such as in HNSCC, low solCD44 levels are initially observed in serum and saliva, which leads to the hypermethylation of the CD44 promoter resulting in increased expression of CD44. Hypermethylation status together with increased CD44 levels may be used as an early marker for HNSCC in cases of oral potentially malignant disorders such as leukoplakia.

Hence, an attempt was made to assess the expression salivary soluble CD44 in subjects with potentially malignant disorders such as leukoplakia. The present study showed no change in CD44 gene fold expression when compared between subjects with leukoplakia and controls although an individual pattern of down regulation and up regulation was noted in the cases and the control population. The results of the present study could not be compared with studies in literature as no studies have assessed the expression of CD44 in serum and saliva samples of subjects with leukoplakia.

Further validation of role of CD44 expression in potentially malignant lesions can be substantiated by determining the role of CD44 in tissue samples of oral leukoplakia. The deterministic establishment of role of CD44 observed in tissue samples, would pave way for further studies in serum in saliva samples. This would help in clarifying the role of CD44 in predicting the malignant potential of premalignant lesions .Studies investigating the effect of smoking cessation and reversal of the lesion on the levels of CD44 are strongly recommended. Success in any of these areas could revolutionize oral cancer screening, by providing a simple and reliable measurement of oral cancer risk assessment that can alert primary care providers, dentists, and other frontline screeners to individuals most in need of skilled oral exam at a stage when the process can be more easily treated or perhaps even reversed with behavioral modification.

Conclusion

It was concluded that, an individual pattern of down regulation in 60% of the cases with leukoplakia and up regulation was noted in 90% of the controls by absolute quantification method, and the gene fold expression ratio by relative quantification method between subjects with leukoplakia and controls by delta ct method was found to be 5.777/5.827= 0.9914 and by double delta ct method was found to be 28.4151/28.0694=1.0123. There was no difference in the gene fold expression ratio of CD44 between subjects with leukoplakia and controls as the gene fold expression ratio value between subjects with leukoplakia and controls was found to be approximately 1. No statistical significant difference was noted in the expression of CD44 and grades of epithelial dysplasia. CD44 were not found to be a reliable marker in determining the malignant transformation of leukoplakia.

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Table 1: Distribution of subjects based on gender.

GENDER	MALES		FEMALES		TOTAL
	n	%	n	%	
SUBJECTS WITH LEUKOPLAKIA	27	90%	3	10%	30
CONTROLS	17	85%	3	15%	20

Table 2: Distribution of subjects based on age.

AGE RANGE	SUBJECTS WITH LEUKOPLAKIA		CONTROLS	
	n	%	n	%
21-40 YEARS	8	26.7%	5	25%
41-60 YEARS	16	53.3%	10	50%
>60 YEARS	6	20.0%	5	25%
TOTAL	30	100%	20	100%

Table 3: Distribution of leukoplakia lesions based on their clinical form and location.

TYPE OF LEUKOPLAKIA	BUCCAL MUCOSA		LABIAL MUCOSA		TONGUE		SOFT PALATE		GINGIVA	
	n	%	n	%	n	%	n	%	n	%
HOMOGENOUS (24)	24	100%	0	0.0%	0	0.0%	0	0.0%	0	0.0%
NON-HOMOGENOUS (6)	4	66.6%	0	0.0%	0	0.0%	1	16.7%	1	16.7%

Table 4: Distribution of leukoplakia lesions based on degree of dysplasia.

TYPE OF LEUKOPLAKIA	NO DYSPLASIA		MILD DYSPLASIA		MODERATE DYSPLASIA	
	n	%	n	%	N	%
HOMOGENOUS (24)	18	75%	6	25%	0	0.0%
NON HOMOGENOUS (6)	3	50%	2	33.33%	1	16.66%

Table 5: Distribution of subjects based on the quantitative expression of CD44 gene.

	UPREGULATION		DOWNREGULATION	
	n	%	n	%
SUBJECTS WITH LEUKOPLAKIA (30)	12	40%	18	60%
CONTROLS (20)	18	90%	2	10%

Table 6: CD44 gene expression based on clinical forms of leukoplakia lesions.

SUBJECTS WITH LEUKOPLAKIA	UPREGULATION		DOWNREGULATION	
	n	%	n	%
HOMOGENOUS LEUKOPLAKIA (24)	10	41.6%	14	58.3%
SPECKLED LEUKOPLAKIA (6)	2	33.33%	4	66.66%

Table 7: CD44 gene expression based on degree of dysplasia.

SUBJECTS WITH LEUKOPLAKIA	UPREGULATION		DOWNREGULATION	
	n	%	n	%
NO DYSPLASIA (21)	9	42.85%	12	57.14%
MILD DYSPLASIA (8)	3	37.5%	5	62.5%
MODERATE DYSPLASIA (1)	1	100%	0	0.0%