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Immunoexpression of C4 Binding Protein in Oral Leukoplakia and Oral Squamous Cell Carcinoma

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ABSTRACT

Background: The immune evasion of dysplastic cells plays an important role in suppressing the immune response and progression of malignancy. The role of the complement inhibitors in the development of oral epithelial dysplastic lesions and squamous cell carcinoma (SCC) is still unclear.

Objective: This study aimed to assess the expression of C4 binding protein (C4BP) as a complement inhibitor in oral squamous cell carcinoma and leukoplakia.

Methods: In this study, 94 samples were classified into four groups: leukoplakia with mild to moderate dysplasia, leukoplakia with severe dysplasia or carcinoma in situ, early invasive SCC, and invasive SCC. The expression of C4BP marker was evaluated by immunohistochemistry (IHC) and real-time PCR. The results were analyzed by the Kruskal-Wallis, Bonferroni adjusted Dunn's multiple comparison, and one-way ANOVA tests.

Results: The results of IHC revealed the expression patterns of C4BP in oral dysplasia and SCC, and indicated that the C4BP expression was not significantly different between different histopathological grades in epithelial cells and vessels (P=0.157 and P=0.123, respectively) but, it was significantly different in fibroblasts and lymphocytes (P=0.017 and P=0.043, respectively). The real-time PCR showed a significant correlation between the dysplasia grade and expression of C4BP (P<0.05).

Conclusion: According to the results, C4BP is expressed in the cancerous tissue by the tumor cells and their surrounding stroma. In addition, upregulation of the C4BP gene as an inhibitor of the complement system is a possible strategy adopted by the tumor cells to evade the immune system.

Keywords: Carcinoma in Situ Complement C4b Binding Protein, Head and Neck Squamous Cell Carcinoma

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INTRODUCTION

Squamous cell carcinoma (SCC) is the most common cancer of the oral cavity. The 5-year survival rate of oral SCC (OSCC) patients is about 50% (1). Oral leukoplakia is the most common type of oral premalignant lesion. The prevalence of its transformation to OSCC ranges from 0.13% to 36.4% (2).

The complement system is part of the innate and adaptive immune systems; however, its role in cancer control is not clear. Traditionally, it is accepted that the antitumoral role of the complement system is mediated by distinguishing the "self" cells from the "non-self" cells, and killing of the "non-self" cells by the membrane attack complex (MAC) or phagocytosis of opsonized tumor cells by the neutrophils and macrophages. However, the cancer cells evade the immune system in several ways such as loss of surface antigens and overexpression of the complement regulatory proteins (3-5). The C4 binding protein (C4BP) is a complement inhibitor that regulates the complement cascade and affects the classical, lectin, and alternative pathways (6, 7). The C4BP can inhibit the complement system and control the formation of MAC by binding to C4b and inhibiting it in three different ways namely by serving as a cofactor in the factor I-mediated inactivation of C4b, preventing the assembly of the C3 convertase, and facilitating the breakdown of the classical C3 and C5 convertases (8-10).

Evidence shows that the binding of C4BP to Caov-3, SW626, and SKOV-3 in cell lines of the ovarian adenocarcinoma inhibits the complement activation (11). It is reported that the X protein of the hepatitis B virus protects the hepatoma cells against the complement attack by activation of C4BP (12). C4BP was recently introduced as a serum marker for early detection of pancreatic ductal adenocarcinoma (13). The serum level of C4BP also increases in epithelial ovarian cancer (14), non-small cell lung cancer (15), and squamous cell lung carcinoma (16).

To improve the quality and prognosis of OSCC treatment, the defense mechanisms of the tumor cells against the immune system should be further elucidated. This study aimed to assess the expression of C4BP as a complement inhibitor in pre-malignant (leukoplakia) and malignant (SCC) lesions to investigate the role of this protein in their progression.

MATERIALS AND METHODS

Selection of Samples

A total of 97 available formalin-fixed paraffin-embedded (FFPE) oral samples diagnosed with dysplastic leukoplakia or SCC between 2012 and 2015 were retrieved from the archives of the Department of Oral and Maxillofacial Pathology of Shahid Beheshti University of Medical Sciences. Two oral pathologists observed the hematoxylin and eosin-stained sections of all samples with adequate underlying connective tissue under an optical microscope (CH2; Olympus, Japan), and graded them as mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ, early invasive SCC, or SCC according to the World Health Organization classification system (17). One case had a suspected diagnosis and two cases had no sufficient connective tissue for assessment. Thus, these three samples were excluded from the study. Finally, 94 samples were selected and categorized into four groups: 25 cases with mild to moderate dysplasia, 26 cases with severe dysplasia or carcinoma in situ, 20 cases with early invasive SCC, and 23 cases with invasive SCC. Details of the clinicopathological data of the study population are provided in Table 1.

The ethics committee of Shahrekord University of Medical Sciences (IR.SKUMS.REC.1394.288) approved this study.

Immunohistochemistry (IHC)

The standard IHC protocols were followed for IHC staining. Firstly, 3-mm-thick sections

Table 1. Clinicopathological data of the study population

Group	Mean age±std. deviation	Gender (number of samples)		Site (number of samples)				
		Male	Female	Buccal Mucousa	Gingiva	Tongue	Palate	Other
Mild	53.4±11.7	52% (13)	48% (12)	32% (8)	20% (5)	24% (6)	8% (2)	16% (4)
Carcinoma	59.9±13.0	26.9% (7)	73.1% (19)	42.3% (11)	19.2 (%5)	30.8% (8)	0 (0)	7.7% (2)
Early	61.3±14.0	45% (9)	55% (11)	25% (5)	30% (6)	10% (2)	20% (4)	15% (3)
Invasive	64.6±15.2	56.5% (13)	43.5% (10)	17.4% (4)	26.1% (6)	26.1% (6)	8.7% (2)	21.7% (5)

of the tissues were mounted on SuperFrost slides (Fisher Scientific, Pittsburgh, PA). Then, the slides were deparaffinized in xylene. Next, they were rehydrated in graded alcohol solutions and immersed in 4% H_2O_2 for 5 minutes. The slides were then rinsed with phosphate-buffered saline. Antigen retrieval was performed in l0 mM citrate solution (pH 6.0) at 750 W in a microwave oven for l0 minutes. Then, the slides were incubated with C4BP antibody (Rabbite polyclonal antibody, PA5-28017, Thermo Scientific, USA) at a 1:500 dilution ratio in a humidified chamber at room temperature for 1 hour, followed by treatment with Dako Envision TM. Next, the samples were incubated with 3, 3 diaminobenzidine (K3468; DAKO) for 3 to 4 minutes at room temperature. Finally, the samples were counterstained with hematoxylin, dehydrated, and mounted (18, 19). The primary antibody (C4BP) was omitted as the negative control, and a hepatitis tissue specimen was used as the positive control. Figure 1 shows representative images of the negative and positive controls for IHC.

Evaluation of Immunoreactivity

The immunoreactivity of the epithelial cells was rated using a semi-quantitative method. The immunoreactivity score was defined as the positive proportion score multiplied by the intensity score. The positive proportion score was assumed 0 if 0-20% of the stained epithelial cells were positive, 1 if 21-40% of the cells were positive, 2 if 41-60% were positive, 3 if 61-80% were positive, or 4 if more than 80% were positive. The intensity was scored as 0, 1, 2, 3, and 4 for negative, weak, moderate, strong, and very strong intensity staining, respectively. The immunoreactivity of fibroblasts, lymphocytes, and connective tissue vessels was assessed using the intensity score (18-20).

Quantitative Analysis of C4BP Transcripts Using Real-time PCR

All tissue specimens were well annotated and immediately stored as FFPE blocks at -80°C for further analyses. Afterward, the samples were cut into 5-10-µm thick sections and placed in RNase-free tubes. The FFPE sections were deparaffinized using 500 µL of n-heptane for 15 minutes and methanol for 10 seconds at room temperature. High-quality total RNA was isolated using an RNeasy FFPE extraction kit (Qiagen, Germany), and 1 µg of RNA was used for cDNA synthesis (QuantiTect Reverse Transcription Kit,

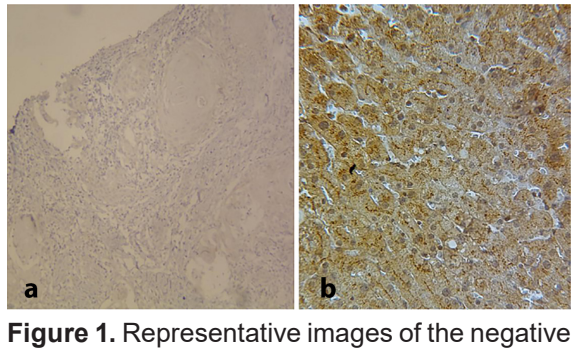

Figure 1. Representative images of the negative control (magnification ×100) (a) and positive control (magnification ×200) (b) of the primary antibody (C4BP).

Qiagen) according to the manufacturer's instructions. Relative quantification of C4BP was conducted on a Rotor-Gene Q 6plex using a 2× QuantiTect SYBR Green PCR kit (Qiagen, Germany) in a total volume of 20 µL. The real-time amplification steps included initial enzyme activation for 10 minutes at 94°C, followed by 40 cycles of amplification (94°C for 15 seconds, annealing temperature for 15 seconds, and 72°C for 20 seconds) and a subsequent normal melting step to assess the purity of the amplified amplicons. C4BP (sense 5'–AAG GAG GTG GAA GGA CAG ATT- 3'; antisense 5'–CCA AGC GGC ACT CAG TAG- 3' annealing 56 °C; NCBI accession: NM_000716.3) and β-actin (sense 5'–TCA TGA AGT GTG ACG TGG ACA TC- 3'; antisense 5'–CAG GAG GAG CAA TGA TCT TGA TCT- 3'; annealing 60 °C; NCBI accession: NM_001101.3) primer pairs were used in the experiments. The primers were designed using GeneRunner software version 3.05 (Hasting software, Inc.); then, they were verified using the Primer-BLAST tool at the National Center of Biotechnology Information database. All quantification reactions were run in triplicate, and the results were normalized to β-actin. Severe dysplastic, carcinoma *in situ*, or early invasive changes were reported compared with the mild dysplasia group as the control group. The real-time efficiency was analyzed according to the standard Pfaffl method (E≥1.9 and R-squared≥0.95), and the relative expression folds were reported (21).

Statistical Analyses

Descriptive results were reported as number (percentage) and mean±standard deviation. The intensity of expression of C4BP by the fibroblasts, lymphocytes, and vessels was compared between different histopathological grades using the Kruskal-Wallis test and Bonferroni adjusted Dunn's multiple comparisons test. One-way ANOVA was applied to compare the immunoreactivity scores and quantitative real-time PCR results between the histopathological groups. SPSS version 22 was used for statistical analyses. P values<0.05 were considered statistically significant.

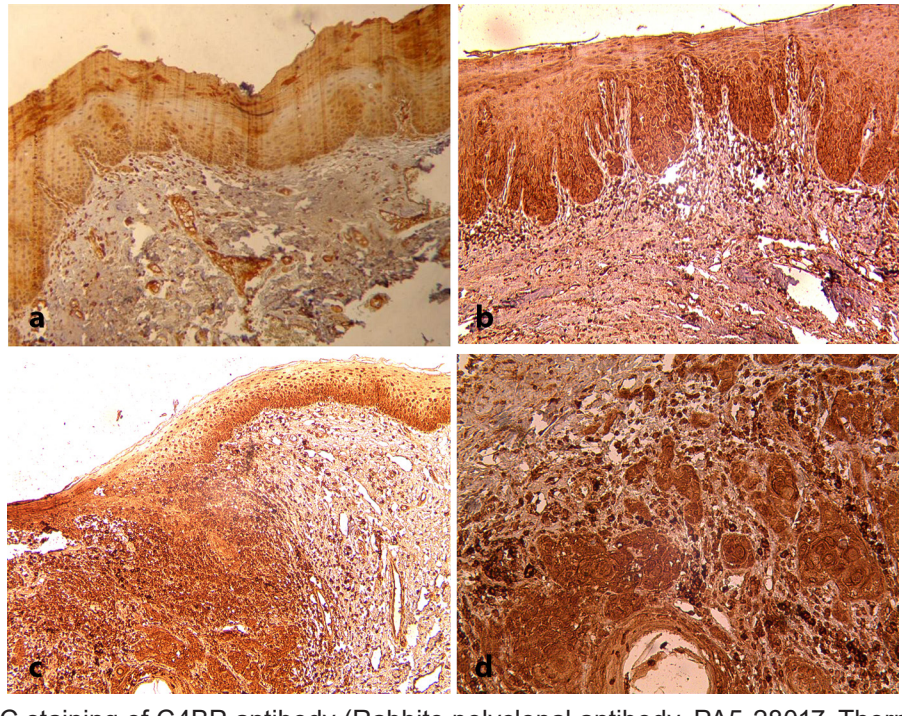

Figure 2. IHC staining of C4BP antibody (Rabbite polyclonal antibody, PA5-28017, Thermo Scientific, USA) in moderate dysplasia, C4BP expressed in the lower tow-thirds of the epithelium (magnification ×100) (a), severe dysplasia, expression of C4BP in the thickness of epithelium (magnification ×100) (b), early invasive SCC (magnification ×100) (c), and invasive SCC, epithelial dysplastic cells and elements of connective tissue reacted with C4BP (magnification ×200) (d).

RESULTS

Immunohistochemical Analysis

All premalignant and malignant specimens showed positive expression of C4BP (Figure 2). The mean immunoreactivity for C4BP in the mild to moderate dysplasia group was 7.24±5.06, and C4BP was expressed by the lower two-thirds of the epithelium. An increase in C4BP expression was showed when comparing mild to moderate dysplasia and severe dysplasia to carcinoma in situ (8.00±3.91). The latter samples noted expression of C4BP in the full thickness of the epithelium. A small decrease in C4BP expression was noted from severe dysplasia to carcinoma in situ to early invasive SCC (7.10±3.41). An increase in expression of C4BP was observed in the invasive SCC group (9.61±3.50). However, the C4BP expression in the epithelial cells was not significantly different between different histopathological grades (P=0.157).

In the underlying connective tissue, the differences in the intensity of expression of C4BP by fibroblasts and lymphocytes were significant (P=0.017 and P=0.043, respectively). There was a significant difference between mild to moderate dysplasia versus early invasive SCC and invasive SCC in the severity of C4BP expression by fibroblasts (P=0.04, P=0.029). A significant difference in the severity of C4BP expression by lymphocytes was also observed between mild to moderate dysplasia versus early invasive SCC (P=0.026). The vessels also showed expression of C4BP but the difference between the groups was not significant (P=0.123).

Table 2 presents the distribution of the intensity of expression of C4BP by fibroblasts, lymphocytes and vessels.

C4BP Transcript Quantification

The relative quantification of the C4BP gene in the samples was assessed using quantitative real-time PCR. The amplification conditions were optimized to obtain the best

Table 2. Distribution of intensity of expression of C4BP by fibroblasts, lymphocytes and vessels

Intensity score	Fibroblasts (P=0.017)					Lymphocytes (P=0.043)					Vessels (P=0.123)				
	0	1	2	3	Mean rank	0	1	2	3	Mean rank	0	1	2	3	Mean rank
Mild to Moderate Dysplasia	10 (40%)	2 (8%)	10 (40%)	3 (12%)	28	2 (8%)	3 (12%)	9 (36%)	11 (44%)	30	4 (16%)	3 (12%)	10 (40%)	8 (32%)	29.78
Severe Dysplasia	12 (46.2%)	0 (0%)	6 (23.1%)	8 (30.8%)	31.77	0 (0%)	2 (7.7%)	12 (46.2%)	12 (46.2%)	32.69	2 (7.7%)	0 (0%)	8 (30.8%)	16 (61.5%)	42.04
Early Invasive	2 (10%)	2 (10%)	4 (20%)	12 (60%)	46.15	0 (0%)	0 (0%)	2 (10%)	18 (90%)	47.8	2 (10%)	0 (0%)	4 (8%)	14 (70%)	44.05
Invasive SCC	5 (21.7%)	0 (0%)	6 (26.1%)	12 (52.2%)	42.67	0 (0%)	1 (4.3%)	7 (30.4%)	15 (65.2%)	39.26	2 (8.7%)	1 (4.3%)	10 (43.5%)	10 (43.5%)	35.85

reaction efficiency for each primer sequence (E=1.89 and R-squared=0.95 for C4BPB and E=1.99 and R-squared=0.98 for β-actin) based on the standard Pfaffl method.

Figure 3 shows a significant correlation between the dysplasia stage and C4BP gene expression. The C4BP expression in the invasive SCC was measured to be approximately 5 times more than that in mild to moderate dysplasia (P<0.01).

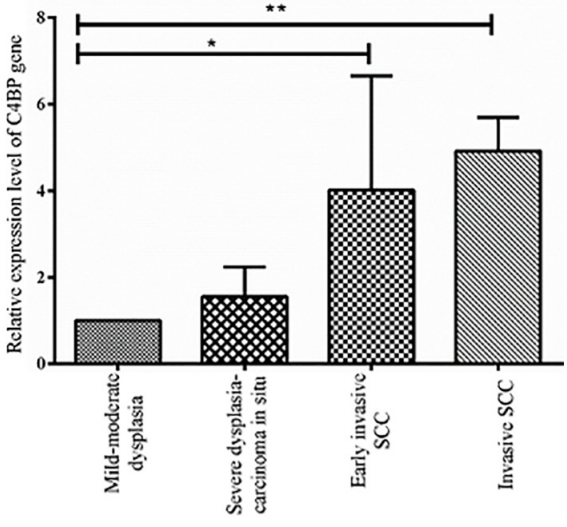

Figure 3. Relative quantification of C4BP transcript variants in different stages of dysplasia, the values indicate the three independent experiments in triplicate (mean±Standard Error). The results were reported as mean±SD of tissue samples in triplicate. Symbols (**) and (*) represent the mean differences between the mild to moderate dysplasia and other groups at P<0.01 and P<0.05, respectively.

DISCUSSION

The complement system is known as the first defense line against the "non-self" cells. As a result, it is part of the immune surveillance against cancer. However, tumor cells can develop mechanisms to escape from complement-mediated elimination. It is believed that cancer cells employ complement inhibitors for immune evasion (22-25). In this regard, Ravindranath et al. showed that the complement inhibitor factors including CD46, CD55, and CD59 that are expressed in the head and neck SCC can evade the complement-dependent cytotoxicity (26).

Considering all the above, we investigated the role of C4BP as a complement inhibitor in oral carcinogenesis. We used real-time PCR and IHC to quantify and analyze the expression patterns of C4BP in oral dysplasia and SCC. The obtained real-time PCR results showed a significant correlation between the dysplasia grade and C4BP gene expression. The IHC study results indicated that with the increased severity of the lesion, the number of epithelial layers expressing C4BP increased. Additionally, following the invasion of the epithelial cells to the underlying connective tissue, the elements of the connective tissue also expressed C4BP. On the other hand, fibroblasts and lymphocytes showed significant differences in expression of the C4BP in different groups. This finding indicates the occurrence of carcinogenesis and progression of tumors as a result of crosstalk between the tumor cells and their surrounding stroma, such as cancer-associated fibroblasts and chronic inflammatory cells (27, 28).

Trouw et al. suggested that C4BP protects the apoptotic cells against excessive complement attack (10). Thus, it may prevent secondary necrosis and increased inflammation in dysplastic lesions, and can potentially lead to the progression of oral dysplastic lesions. This finding is in agreement with our results that showed a correlation between C4BP expression and the severity of the lesion.

It has been suggested that C4BP induces B-cell activation by activating the CD40 ligand (29). The increase in B lymphocyte infiltration is associated with the progression of hyperkeratosis to dysplasia and carcinoma of the oral epithelium (30); hence, it may be involved in the pathogenesis of oral cancer. This statement can justify our observations. Williams et al. (31) reported that C4BP can increase cell survival by modulating CD40/sCD154 interactions, and may have a role in malignancies. This process may induce the progression of oral dysplasia to carcinoma as well.

In summary, expression of C4BP may be one strategy adopted by the dysplastic cells

to evade the immune system and may play a role in the progression of oral dysplasia to carcinoma. This finding can be used to find more effective strategies for the management of OSCC.

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Conflicts of Interest: None declared.

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