Original Article

The Incidence of CD28 Gene Polymorphism (rs3116496) and Gene Expression with Oral Carcinoma in Some Iraqi Samples

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Abstract

Background: Oral carcinoma is one kind of cancer that develops in the cavity of the oral, lip, tongue, gingiva, and oropharynx. The World Health Organization (WHO) illustrated that oral carcinoma is among the most prevalent cancers in the world, considered the sixteenth most malignant, which is commonly prevalent, and the fifteenth reason that causes mortality in the world. **Objective:** This study aimed to investigate the association between oral carcinoma with single-nucleotide polymorphism (rs3116496) and CD28 (cluster differentiation 28) gene expression. **Materials and Methods:** Samples were collected from 61 control and 61 patients with early squamous oral carcinoma from the gum diseases department/Fallujah specialized center. Then we investigated the variation of CD28 polymorphism and gene expression of CD28 using real-time PCR. **Results:** The results showed that there are significant differences between patients and control in the TT genotype of codominant models with $P = 0.003$ and OR = 8.89. There were significant differences in the dominant and recessive models with $P = 0.03$ and 0.008, and OR = 2.4, 7.2, respectively. In addition, the T allele showed significant differences between patients and control with $P = 0.002$ and OR = 2.61. However, in the CD28 gene expression there were no significant differences between patients and control. **Conclusion:** The genotype TT and allele T of SNP of the CD28 gene are considered risk factors in oral carcinoma.

Keywords: (rs3116496), CD 28, gene expression, oral carcinoma

Introduction

Noninfectious diseases represent most of the world's mortality. Nowadays, cancer is predicted to be the major reason for mortality and the most critical hurdle to increasing life expectance in the 21st century.[1] The multireaction of numerous gene loci and various environmental agents has a significant role in the incidence and cancer evolution. The mechanisms in tumorigenesis and the development of cancer represented by anomalous T cell activation lead to disabling immune surveillance function and the deficiency of antitumor responses.[2] The activation of T cells relies on the homeostasis between co-inhibitory and co-stimulatory signals emitted by the co-signaling molecules. It was shown that CTLA-4 (cytotoxic T lymphocytes antigen-4) and (CD28) differentiate cluster 28 was one of the immune factors contributing to malignancy evolution.[3] CD28 is expressed in T cells and is considered the primary T cell co-stimulatory molecule that induces T-cell stimulation, and proliferation.[4]

Oral carcinoma is one kind of cancer that develops in the cavity of oral, lip, tongue, gingiva, and oropharynx.[5] The World Health Organization (WHO) showed that oral carcinoma is among the most prevalent cancers in the world, considered the sixteenth of malignant that is commonly prevalent and the fifteenth reason that causes mortality in the world, with an occurrence range higher in males than females; the development risk of the disease increases after the age of 45.3 years.[6]

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The various habits of individuals, education, and medical services impact oral carcinoma occurrence.[7] Oral squamous cell cancer is the most prevalent type of oral carcinoma (approximately 90%), while 10% of oral tumours include salivary gland cancer, lymphoma, and skin cancer.[8] Several different risk agents are related to oral carcinoma,[9] including use of alcohol, cigarette, and betel.[10]

CD28 is a significant immunomodulation protein encoded via the same chromosome locus of CTLA-4 with homology of amino acid at 31%. Disturbance in the pathway of CD28 causes the inability of oncogenic antigens to tolerance. Substitution of T/C at site +17 for the CD28 gene exists on the third intron (rs3116496). There is no proof appears that CD28 (rs3116496) polymorphisms have an effect on the CD28 gene expression and its function; this single-nucleotide polymorphism was found close to splice acceptor location, which indicates a possible impact on CD28 signaling and stimulation of T cells.[11] Therefore, this study aimed to investigate the association between oral carcinoma with SNP (rs3116496) and CD28 gene expression.

Materials and Methods

Samples collection

The blood samples were collected from 61 patients, who were diagnosed with early squamous oral carcinoma in the gum diseases department in Fallujah specialized center (from February to April 2023), and it was collected 61 samples from healthy individuals as a control group. Questionnaire forma written informed permission and medical histories. Three mL of whole blood was transferred immediately to the EDTA tube for DNA extraction and 2 mL was transferred to tube with Trizol for RNA extraction.

DNA extraction and genotyping

DNA of whole blood was extracted using Quick-DNA Blood MiniPrep kit. Nanodrop was used to determine the purity(1.8-2) and the concentration of DNA. The genotyping of SNP (rs3116496) was determined via real-time thermal cycler Sa Cycler - 96, Sacace Biotechnologies, Italy. It used probes custom Taq Man SNP genotyping from Thermo Fisher Scientific Company/USA (catalog No. 4351379; Assay ID: C_25922478_10). Also, the TaqMan master mix were provided by Kapa Biosystems Company. The real-time PCR components were final volume (20 µL), which included TaqMan master mix 2X (12.5 µL), SNP genotyping assay 20X (1.25 µL), and DNA sample (11.25 µL). Also, the condition reaction was 95°C for 10 min, denaturation at 95°C for 15 s (40 cycles), and annealing/extension at 60°C for 1 min (40 cycles).

Detection of gene expression

Total RNA extracted from whole blood in)TRIzol Reagent/ Thermo Fisher Scientific/ USA). Prime Script RT Reagent Kit(Cat:RR037B/Takara company/Japan) used to synthesize cDNA. The Kapa Syber Fast qPCR master mix (Kapa Biosystems Company/USA (and cDNA as a template were used for the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). CD 28 gene primers were forward GAGAAGAGCAATGGAACCATTATC and reverse TAGCAAGAGAGGACTCCACC AA. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) reference gene primers were forward AGGTCA TCCCTGAGCTGAA and reverse CTGCTTC ACCACC TTCTTGAT.[12]

The reaction mixture was a final volume of 20 µL included: 10 µL Kapa Sybr Fast qPCR master mix (2X), 0.4µl of each primer, 3 µL cDNA, and completed to final volume by nuclease-free water. The mixture was transferred to a real-time thermocycler (Sacace Real-time PCR System, Italy), which was programmed for the following optimized cycles: initial denaturation for 5 minutes at 95°C (one cycle), 40 cycles of denaturation (20 s at 95°C), annealing (20 s at 60°C), and extension (20 s at 72°C), and finally one cycle of melt curve (15 s at 90°C). The expression was given as $2^{-\Delta\Delta Ct}$, which represents the relative fold change. Therefore, the results were expressed as a fold change in the expression level of a target gene that was normalized to endogenous control (housekeeping gene) and relative to a calibrator, which is the target gene in control subjects.

Statistical analysis

The results of gene expression were mentioned as mean ± standard deviation and analyzed via one-way ANOVA, which was performed by Graph Pad Prism version 9.3. Odds ratio (OR) was determined for the detection of risk associated with genotypes and alleles; it is calculated by using the statistical software epidemiological (WINPEPI). *P* Values are significant differences when less than 0.05. Hardy–Weinberg equilibrium (HWE) for SNP was determined by the online calculator of Michael H. Court (2005– 2008). Where if *P*-value is more than 0.05, the population consistent with HWE.

Ethical approval

The consent from participants was obtained, and it was approved by the ethics committee of the Iraqi Ministry of Health, 6999 on April 2023.

Results

DNA was extracted from the fresh blood samples for patients and apparently healthy subjects, by Quick-DNA Blood MiniPrep kit. The extracted DNA result is shown in Figure 1.

The purity (260/280) of DNA 1.87 and the concentration of 67 (ng/mL).

Genetic polymorphism of SNP (rs3116496)

The results demonstrated there are significant differences between patients and control in TT genotype of codominant models with $P = 0.003$ and OR = 8.89. Also, it was observed significant differences in the dominant and recessive models with $P = 0.03$, 0.008, and OR = 2.4, 7.2, respectively. In addition, T allele appeared significant differences between patients and control with $P = 0.002$ and OR = 2.61 as listed in Table 1 and Figure 2A and B.

Gene expression of CD28

Table 2 shows the results of CD28 gene expression there are no significant differences between patients that folding was 1.23 and control that folding was 1 ($P > 0.05$(.

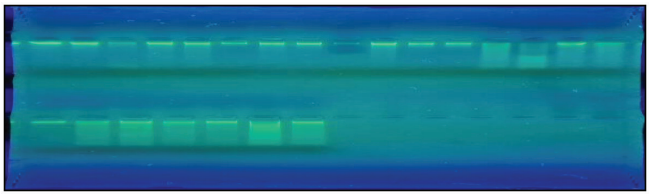

Figure 1: Gel electrophoresis of genomic DNA extraction from blood, 1.5% agarose gel at 5 vol/cm for 1:15 h

Discussion

The immune response is considered significant for the host's defenses against tumors. Particularly, strict monitoring of cellular immunity via cytotoxic T lymphocytes and natural killer cells represent critical factors for antitumor immunity.[13] It increased studies that showed the relation between different cancers and genetic polymorphisms in genes that controlled the function of NK cells and T cells. CD28 is essentially expressed on the surface of the T-cell and provides co-stimulation signals that are demanded for activation of the T-cell.[14] This study has observed that the odds ratio for T allele and TT genotype was more than 1 which indicates the response to the risk increase.[15]

There are no previous studies in Iraq about the genetic polymorphism of SNP (rs3116496) in Iraqi patients with oral carcinoma. However, this study is accordant with one study that studied two other SNPs of CD28 (rs3181100,and rs3181113) in 83 patients with oral carcinoma in Germany. The results showed that there was no association with these SNPs in patients compared with control.[16]

Table 1: Genetic polymorphism of SNP (rs3116496)

Genetic model	Genotype and allele	Control ($N = 61$)	Patients ($N = 61$)	OR (CI: 95%)	*P* Value
Codominant	CC ref	40(65.6%)	27(44.3%)	1	1
	CT	19(31.1%)	22(36.1%)	1.72(0.79- 3.73)	0.23
	TT	2(3.3%)	12(19.7%)	8.89(1.9- 42.11)	0.003
Dominant	CC ref	40(65.6%)	27(44.3%)	1	1
	CT/TT	21(34.4%)	34(55.7%)	2.4 (1.16- 4.95)	0.03
Recessive	CT/CC ref	59(96.7%)	49(80.3%)	1	1
	TT	2(3.3%)	12(19.7%)	7. 2(1.56-33.41)	0.008
Overdominant	CC/TT ref	42(68.9%)	39(63.9%)	1	1
	CT	19(31.1%)	22(36.1%)	1.25(0.59- 2.63)	0.7
Allele	Cref	99(81.1%)	76 (62.3%)	1	1
	T	23(18.9%)	46(37.7%)	2.61(1.46 - 4.66)	0.002
HWE ≥ 0.05		0.9	0.07		

OR = odds ratio, CI = confidence interval; $P < 0.05$ mean significant

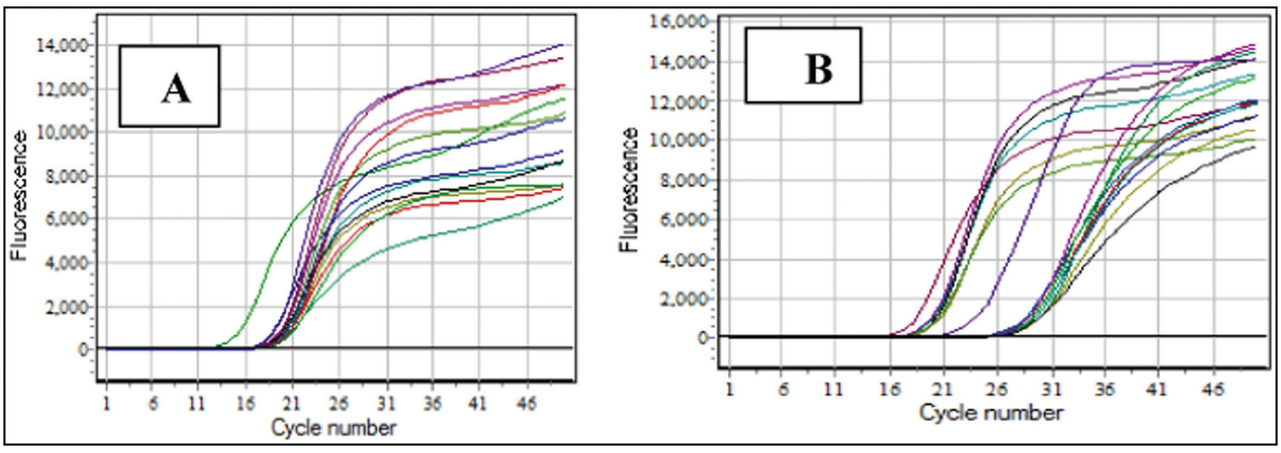

Figure 2: (A) Curve of allele C. (B) Curve of allele T

Table 2: Gene expression of CD28

Groups	Ct Means ± SE	Δ Ct	$2^{-\Delta Ct}$	Folding
Control	34.85 ± 0.15	14.5	4.32	1
Patients	34.34 ± 0.21	14.2	5.31	1.23
P Value		0.453		

Ct = threshold cycle, SD = standard deviation; $P < 0.01$ mean significant

In addition, our study was concordant with other studies that studied different cancers.

Yahya *et al.*[17] showed that CT genotype (rs3116496) was higher in larynx squamous cell cancer patients when compared with the control. In addition, the CT genotype has been associated strongly with clinical pathology and disease risk, indicating this genotype may be related to co-stimulation T-cell in larynx squamous cell cancer.

Although one Iraqi study showed for the genetic polymorphisms of SNP CD28 (rs3116496) in Behçet disease patients, there was no association between controls and patients in allele and genotype frequencies for SNP rs3116496 ($P > 0.05$).[18]

Moreover, this study agreed with another study that studied the effect of SNP (rs3116496) on breast cancer. Zeng and Lai[19] showed in their study CD28 expression was downregulated in patients with breast carcinoma compared with control and rs3116496 for CD28 gene had a positive association with the excess risk of breast carcinoma.

Another study determined that expression of CD28 was lower in tissue free from tumors that surround head and neck cancer compared to tumor tissue, the effect of variants gene was not examined. Zhang *et al.*[20] found in their meta-analysis study that the SNP (rs3116496) had a significant association with the risk of cancer, particularly in the Asian population, and also strongly related to the elevated risk of breast carcinoma, leukemia, and colorectal carcinoma. Another study illustrated that the genetic polymorphism of CD28(rs3116496) did not have a significant role in Turkish patients with stomach carcinoma.[21] However, this study accorded preliminary outcomes on the impact of CD28 in Iraqi patients with oral carcinoma despite the small sample size. In a future study, it is demanded to relate serum levels of CD28 with gene expression in oral carcinoma. Furthermore, this study had been limited due to the small sample size that was collected from patients with oral carcinoma, therefore, it is required to increase the sample size.

Conclusions

This study represents the first study in Iraq about the association of genetic polymorphism of SNP (rs3116496) in Iraqi patients with oral carcinoma. It was shown that the genotype TT and allele T of SNP of CD28 gene represent a risk factor in oral carcinoma. However, the gene expression of CD28 in patients had no significant difference compared with control.

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Conflicts of interest

There are no conflicts of interest.

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