Original Research Article

Polymorphic Investigation of *ADCY2* and *C7* genes in Local Population of Oral Cancer

ABSTRACT

Single nucleotide polymorphisms (SNP) are responsible for genetic mutations. We studied genetic molecular variations and found an association of oral cancer with SNP of Adenylate Cyclase 2 (*ADCY2)* rs252546 and complement C7 (*C7)* rs1450656 genes in people of Southern Punjab, Pakistan. The study involves 100 cases of oral cancer and 100 healthy individuals. *ADCY2* is found as a membrane-associated enzyme and *C7* is involved in innate immunity. The process of genotyping was carried out by Tetra ARMS Primer PCR. The genetic variant of *ADCY2* rs252546 has allelic origin G/A and *C7* rs1450656 with C/T. The statistical analysis showed that the 51-60 years age group is significantly associated with oral cancer. The allelic frequency of *ADCY2* rs252546 and *C7* rs1450656 was calculated through Hardy Weinberg equilibrium. The homozygous mutant allele G of *ADCY2* was more prevalent in cases and C allelic genotype was equally found in cases and controls. Other demographic and polymorphic studies indicated a significant association of variants of *ADCY2* and *C7* with oral cancer in the local population of Punjab. Variations in *ADCY2* and *C7* can be used as potential biomarkers and biological targets for oral cancer management strategies.

Keywords: *ADCY2* and *C7;* Genotyping; Oral cancer; SNP; Tetra Arm PCR

INTRODUCTION

Oral cancer (OC) is a subtype of head and neck cancer that develop at the lips, buccal surface, floor of buccal cavity, salivary gland, tongue, gingival, orophyranx, palate, alveolar mucosa, labial mucosa and other intraoral location resulting in oral malignancies[1, 2]. The evolution and pathogenesis of oral cancer is still not fully known. OC is the sixth most common type of cancer throughout the world [2], which is more common in developing countries including Pakistan, Sri Lanka, and Taiwan [3-5]. In Pakistan and India, oral cancer accounts for 8-10% of all cancers and it is the second most common cancer in Pakistani men and women [6].

Genetic and environmental are the main contributory factors that progress the disease [7]. Other includes consumption of alcohol, tobacco (smoke and smokeless tobacco (betal nuts, naswar),

nutritional deficiency, viral infections, and poor mouth care. The heritable changes which are not coded in the DNA sequence but can change gene expression are known as epigenetic changes. Certain chemical changes in DNA and its associated proteins change the expression of a protein without altering its sequence. The epigenetic changes which occur in humans involve histone modification, DNA methylation responsible for disease progression [8]. Genetic variations play an important role in the development of cancer, tumor suppressor genes are inactivated, and oncogenes are activated as a result of alterations in the copy number of chromosomes. The process of apoptosis, cell cycle, metastasis, and proliferation are positively and negatively regulated by these genes [9]. The single nucleotide variation is a common type of genetic variation and is referred as SNP and exists with a frequency of 1% at least within two variants. On average, the human genome contains 1 SNP per 1000 base pairs and are associated with linkage studies and identifies the relation between genes and disease. SNPs directly affect gene products as they are present within the gene and have a low rate of mutations in complex genomes, thus are preferred for microsatellite markers [9, 10]. Single base changes in the genome can be identified easily by single nucleotide polymorphisms, which act as stable DNA markers for association studies related to personalized medicine involving cancer and other diseases [11-13].

The objective of this study is to find the association of genetic variants of ADCY2 (rs252546) & C7 (1450656) with oral cancer in the population of Southern Punjab. As, single nucleotide polymorphisms of ADCY2 and C7 genes are associated with different types of cancers [14]. Susceptibility to cancer by genes or SNPs is still not well identified [15], so there is a need for the development of new and consistent procedures for SNP analysis for developing therapeutic strategies for oral cancer [13, 16].

MATERIALS AND METHODS

Ethical approval and Blood Sample Collection

The protocol of this study was reviewed and approved by the Ethical review committee of Institute of Molecular Biology and Biotechnology. The study was conducted and a questionnaire for the consent of the sample population was filled. Blood samples were collected from MINAR and Nishtar Medical Hospital, Multan, Southern-Punjab, Pakistan. The study designed involved 200

subjects including 100 affected-individuals as cases and 100 non-affected or normal / healthy

Genotype	Primers	Sequence	Designed Tm

individuals as controls.

DNA Extraction and Quantification

Extraction of blood sample of subjects was carried out by the in-organic method[17]. Quantification of the extracted DNA was done by UV-spectrophotometer (Perkin Elmer Lambda 25) at 260nm using this formula:

DNA microliter/nanogram = Dilution factor × A260 × 50 (250 nanogram/micro liter is the standard concentration of DNA for further use).

Genotyping and Primer Designing

Genotyping was conducted by Tetra ARMS (tetra armed refractory mutational system) PCR for SNP rs252546 of *ADCY2* and rs1450656 of *C7*. The DNA fragment of the samples were amplified by inner forward and reverse primers and outer forward and reverse primers to analyze the two allelic variations. The primers for the respective SNPs were designed by Tetra ARMS software (Primer-1) as showed in Table 1. The amplified fragment size of each allele was visualized on 2% agarose gel. The bands that appear were either heterozygous or homozygous genotypes. The amplicon size compared with 100 base pair DNA Molecular-ladder.

ADCY2 rs252546 G/A	Inner primer (Forward)	372 AAGGGATGATAGATTTTACATTATGTAGTG 401	59^{0}C
	Inner primer (Reverse)	427 GAAGGATTAGTGTTTTACTATGACGCT 401	59^{0}C
	Outer primer (Forward)	157 AAGGGGAAACATATTTGATAAGGTAATA 184	59^{0}C
	Reverse primer (outer)	566 AGAGCTTATTAATTCTAAATTCAGGCAT 539	59^{0}C
C7 rs1450656 C/T	Inner primer (Forward)	374 AAATTAAACATGTCTCTTTTACATTGTT 401	57^{0}C
	Inner primer (Reverse)	425 TCGACTGAATTAAAATCTAGGGTAG 401	57^{0}C
	Outer primer (Forward)	285 TATCATGTCACTCTTGATTAGATGG 309	57^{0}C
	Outer primer (Reverse)	573 GTAAAAAAAAAAGAAGTTAAGCTTGC 548	57^{0}C

Table 1: Primer Sequence for ADCY2 rs252526 and C7 rs1450656 variants

Statistical Analysis

This study design included the investigation of genetic variants of *ADCY2* (rs252546) and *C7* (rs1450656) in our sample population with associated risk factors for OC. The allele frequency for the genetic variants of *ADCY2* and *C7* was calculated from Hardy-Weinberg equilibrium for wild and mutant type allele in case and control subjects. Statistical analysis was done through the chi-square "Goodness of Fit" test with degree of freedom (distribution of sample), as via SPSS v22 software to analyze all associated the risk factors with our genetic variants and OC. The odd ratio (OR), its standard error and 95% confidence intervals (CI) was measured from the online MedCalc Odd Ratio Calculator (www.medcalc.org). The probability value (P-value) was calculated from Social Sciences Statistics- an online calculator (www.socscistatistcs.com) at significance level 0.05, using chi-square and degree of freedom. Association of oral cancer with different risk factors in the sample population was calculated.

RESULTS

The amplification of genetic variants of *ADCY2* (rs252546) and *C7*(rs1450656) by the tetra-primer ARMS PCR revealed, homozygous wild and mutant along with heterozygous genotypes for *ADCY2* (rs252546) and *C7*(rs1450656) are shown in (Fig.1).

Demographic Factors of Oral Cancer

Different demographic factors were found associated with oral cancer. 73% control individuals included in the study were male and 27% were female, as compared to cases with 53% and 47% males and females, respectively. Our analysis showed that male gender is more susceptible towards the disease compared to females (P-value<0.003399). Age is another important associated risk factor for oral cancer. In our study, 15% individuals of the control sample population were found in the age group of 20-30 years. 40% of control individuals were found in the age group of 31-40 years. In the control population, 21% individual falls in the age group of 41-50 years whereas 18% case population was found in this age group. Individuals with an age group of 51-60 years were 21% in the control population and 25% in the case population. It shows that increasing age is another possible associated risk factor for OC. Among the different subtypes of oral cancer, the frequency of oral cavity carcinoma is more in males (18%) compared to females (14%), buccal mucosa was found more common in females (10%) compared to males (6%). Similarly, carcinoma of the tongue was more common in females (10%) and males (9%) (Fig. 2).

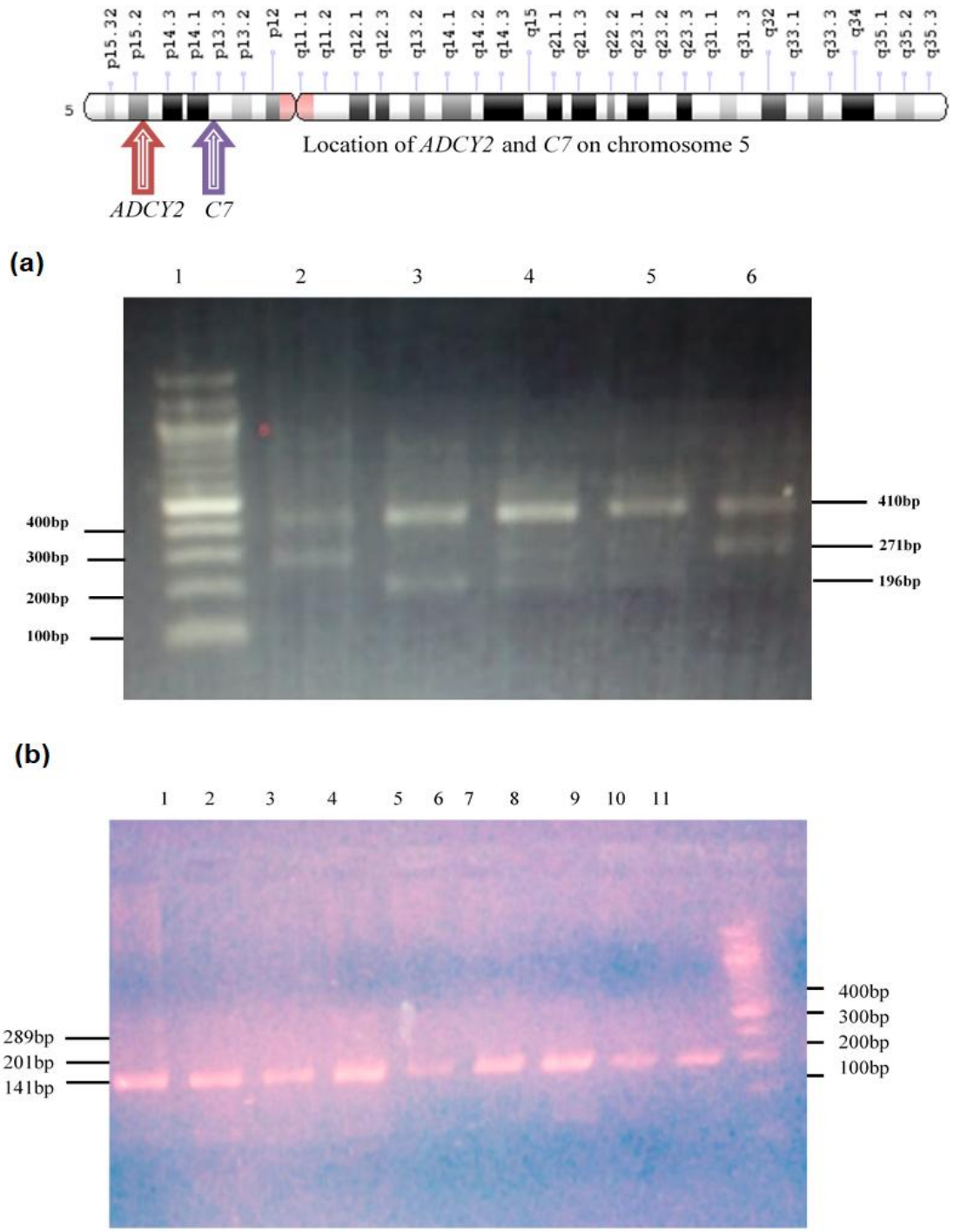

Fig. 1. (a) PCR Products Lane 1: 100bp Molecular Ladder, Lane 2: 271bp (Wild-type), Lane 3: 196bp (Mutant-type), Lane 4: (271bp&196bp) Heterozygous Genotype of ADCY2
(b) PCR Products Lane:1 201 bp (ancestral allele), 141 bp (wild type), 201bp (heterozygous genotype), Lane: 11 100bp ladder. Genotype of C7

Smoking is the most significantly associated risk factor for oral cancer but due to less sample population size, our study revealed it as non-significantly associated with the disease (32% patients as smokers and 28% controls were smokers). Betal leaf were found to have marginally significant association with oral cancer, 20% patients were betal leaf users (p-value<0.00134). Naswar (Snuff) may also have an impact on oral cancer, 18% patients in our study were naswar user, and 6% controls were naswar users (p-value<0.009024).

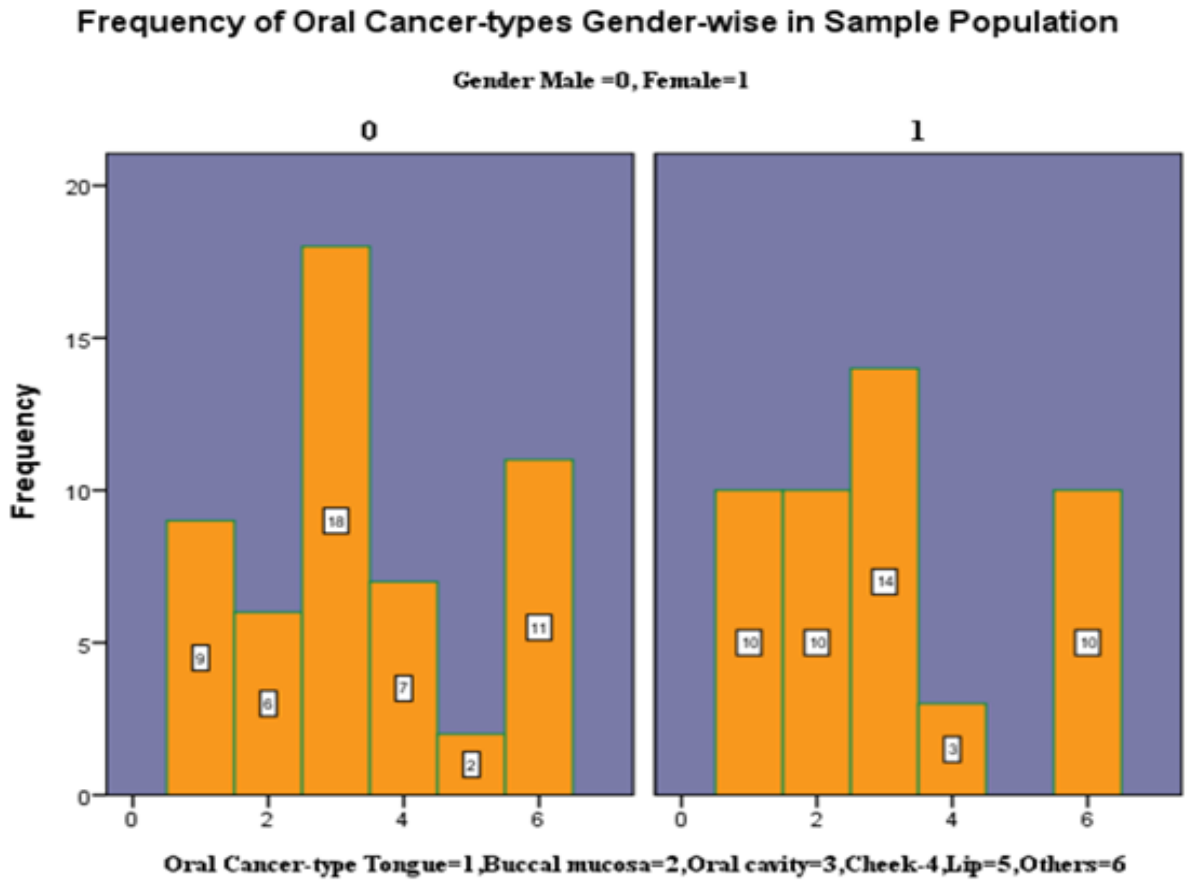

Fig. 2. Frequency distribution of oral cancer types gender wise in our population

Betel quid (Gutka) is also one of the importantly associated risk factors and its usage is not a common practice in

Southern Punjab. Recurrent mouth ulcers, gingivitis, and jagged teeth are found significantly associated with OC (p-value<0.00001). 73% patients were from rural areas compared to 27% from urban areas. Rural area patients are more prone towards developing OC. As the rural area, residents are frequently smokers or smokeless tobacco users (Table 2).

Table 2. Oral Cancer associated Risk factors

Factors	Category	Controls (N=100)	Cases (N=100)	Chi-sq	Df	P-value
Gender	Male Female	73 (73%) 27(27%)	53(53%) 47(47%)	8.580	1	0.003399*
Age in Years:	20-30 31-40 41-50 51-60 61 and above	15 40 21 21 3	3 22 18 25 32	37.833	4	0.00001***
Smoking	Yes No	28 72	32 68	0.381	1	
Betal nuts	Yes No	4 96	0 100	4.082	1	0.043342 ^{NS}
Betal leaves	Yes No	5 95	20 80	10.286	1	0.00134**
Gutka Usage	Yes No	0 100	2 98	2.020	1	0.155239^{NS}
Naswar (Sniff)	Yes No	6 94	18 82	6.818	1	0.009024**
Alcohol	Yes No	5 95	3 97	0.521	1	0.470415^{NS}
Mouth Ulcer	Yes No	2 98	47 53	54.737	1	0.00001***
Jagged Tooth	Yes No	15 85	55 45	35.165	1	0.00001***
Gingivitis	Yes No	4 96	45 55	45.439	1	0.00001***
Gums Bleeding	Yes No	3 97	16 84	9.828	1	0.001719**
Oral hygiene	Yes No	75 25	28 72	44.220	1	0.00001***
Area of Residence	Urban Rural	64 36	27 73	27.604	1	0.00001***
Family history of	Yes No	6 94	13 87	2.850	1	0.091374^{NS}

Any other Cancer						

Highly Significant at p<0.005=***; Moderately Significant at p<0.05= **; Non-significant (NS)

Genotyping and Allelic Frequency

The allelic frequencies of *ADCY2* rs252546 showed that homozygous wild type AA is more common in controls (23%) compared to cases (18%) and homozygous mutant type GG (51%) and heterozygous AG (31%) were more in cases compared to controls (29% and 48%). In our case the population frequency of homozygous wild type A is 0.335 and 0.375 in controls, whereas the frequency of homozygous mutant type G in cases is 0.665 and 0.625 in controls. The allelic frequencies of *C7* showed that homozygous wild type TT is more common in controls (14%) compared to cases (7%), whereas heterozygous genotype TC is 49% in controls and 28% in cases. Homozygous mutant type CC is 65% both in controls and cases. In our case population, the frequency of homozygous wild type T is 0.21 and 0.3008 in controls, whereas the frequency of homozygous mutant type C in cases is 0.79 and 0.6992 in controls (Table 3).

Table 3. Allelic Frequency for *ADCY2* rs252546 *& C7* rs1450656

***ADCY2* rs252546**						
Genotypes	AA	AG	GG	A Freq.	G Freq.	Significance
Cases	18(0.18)	31(0.31)	51(0.51)	0.335	0.665	X^2 =0.7673 DF=2 P=0.681472NS
Controls	23(0.23)	29(0.29)	48(0.48)	0.375	0.625	
***C7* rs1450656**						
Genotypes	TT	TC	CC	T	C	Significance

				Freq.	Freq.	$X^2 = 3.333$ DF=2 P=0.188907NS
Cases	7(0.07)	28(0.28)	65(0.65)	0.21	0.79	
Controls	14(0.14)	49(0.49)	65(0.65)	0.3008	0.6992	

In statistical analysis, the genotypes of our genetic variants were stratified by different genetic and environmental risk factors to find the probability of the disease. Non-significant associations existed between *ADYC2* rs252546, *C7* rs1450656, and smokers. As the mutant genotype GG in smokers for *ADCY2* rs252546 was observed in an equal ratio of 15% in cases and controls. While mutant genotype CC in smokers with *C7* rs1450656 was found more prevalent in cases 21% than 17% in controls (OR = 1.2101, 95% Cl = 0.6602-2.2179). The statistical analysis between the betel leaf usage and genotype were also found non-significant (OR =6.0000, 95% Cl = 1.9699-18.2752). As only 13 cases were found with mutant genotype GG with *ADCY2* rs252546, whereas 9 cases were betel leaf users in *C7* rs1450656. The area of residence were found significantly associated with the disease and genotype in *C7* rs1450656 (X2 = 9.8267, p-value<0.007348) (OR =5.0212, 95% CI= 2.7471-9.1779). The mutant genotype CC for *C7* rs1450656 was observed with high ratio of cases as 48%, all of which were residents of rural area. While the heterozygous genotype TC (*C7* rs1450656) observed with a ratio of 22% in rural area residents. Although, the non –significant association had been found with *ADCY2* rs252546 and rural/ urban area residents. However, its mutant type GG and heterozygous AG were more observed with a ratio of 38% and 21%, respectively, in rural area residents (Table 4).

Associations of different demographic factors including age, area of residence, oral hygiene, jagged teeth, recurrent ulcers, and gingivitis with oral cancer showed significant results.

Table 4. Polymorphisms of *ADCY2* and *C7* variant: rs252546 & rs1450656 associated with OC risk stratification

ADCY2 rs252546	Smokers				Non-Smokers				Significance
Genotypes	Cases	Controls	Chi-sq.	P-value	Cases	Controls	Chi-sq.	P-value	Odd ratio (95% CI)
AA	8	6	0.2702^{NS}	0.873607^{NS}	11	16	0.8268	0.661395	1.2101 / 0.6602-2.2179
AG	9	7			22	22			
GG	15	15			35	34			
C7 rs1450656									
TT	3	3	0.1551	0.925392	6	11	2.7583	0.251795	
TC	8	8			19	13			
CC	21	17			43	48			
ADCY2 rs252546	Betal Leaf Users				Betal Leaf -Non-Users				Significance
Genotypes	Cases	Controls	Chi-sq.	P-value	Cases	Controls	Chi-sq.	P-value	Odd ratio (95% CI)
AA	1	1	NS	NS	17	23	0.1823NS	0.912889NS	6.0000 / 1.9699-18.2752
AG	6	0			25	29			
GG	13	3			38	44			
C7 rs1450656									
TT	2	1	0.9382	0.625571	5	13	2.5596NS	0.278097NS	-
TC	9	1			19	20			
CC	9	2			56	63			
ADCY2 rs252546	Naswar (Sniff) Users				Naswar (Sniff) Non-Users				Significance
Genotypes	Cases	Controls	Chi-sq.	P-value	Cases	Controls	Chi-sq.	P-value	Odd ratio (95% CI)
AA	3	1	2.0532^{NS}	0.35822^{NS}	15	22	1.1466NS	0.563671NS	3.2088 / 1.2086-8.5192
AG	8	1			23	28			
GG	6	4			45	44			
C7 rs1450656									
TT	1	0	NS	NS	6	14	3.3481 NS	0.187484NS	
TC	6	3			22	18			
CC	10	3			55	62			

DISCUSSION

Human genetic studies revealed that mutations in the germ lines result in an increased risk for oral cancer. Changes in carcinogen metabolizing enzymes may increase the risk for oral cancer, even when exposed to a lower dose. Apart from single nucleotide polymorphism, changes in carcinogen activated and metabolizing enzyme expression, exposure to carcinogenic ingredients of tobacco, changes in xenobiotic metabolizing enzymes, repair mechanism of DNA and tumor suppressor genes are also known to cause oral cancer [18]. Previous studies revealed an association of *ADCY2* with lung cancer, colorectal cancer, urinary bladder cancer, and prostate cancer. However, complement protein *C7* is known as part of innate immunity. Some studies revealed its association with ovarian and liver cancer. In our study, we tried to find the association of the single nucleotide polymorphism of *ADCY2* rs252546 which is A to G transition of intronic region. The genetic variant of *C7* rs1450656, which is T to C transition of the intronic region, is associated with oral cancer.

Our study revealed that homozygous mutant genotype GG of *ADCY2* rs252546 was higher in cases compared to controls and homozygous ancestral genotype AA were 23% in controls and 18% in cases and heterozygous genotype AG was 31% in cases. The allelic frequency of homozygous mutant allele G in our population is 66%, which is greater as compared to 27% in Africans, 35% in Chinese, 42% Indians, and 42% in Japanese. Our statistical analysis showed that *C7* rs1450656 homozygous ancestral genotype TT was 14% in controls compared to 7% in cases. The homozygous mutant genotype CC was 65% in both cases and controls. Our study showed some significant association between *ADCY2* and *C7* with some of the risk factors of oral cancer. In our investigation, the interaction of genotype of C7 rs1450656 was found non-significant with betel leaf and betel nut which may be due to a smaller number of users found in our area of sample collection.

Previous studies revealed a strong association between oral cancer and these smokeless tobaccos in South East Asian countries and its prevalence in Karachi, Pakistan [19]. In the United Kingdom, oral cancer is more common in individuals aged 50 years or above [20]. Risk for oral cavity cancer increases two to three folds in individuals who drink alcohol than non-drinkers [21]. Jagged teeth, mouth ulcers and gingivitis were found significantly associated with our disease. Family history of any cancer type was found nonsignificant with oral cancer in our study. Significant association between family history of head and neck cancer and oral cancer was found in Europe [22]. The significant association of genetic variants of *ADCY2* and *C7* with OC progression in the local population of Punjab. Hence, the small sample size, as OC is not very common in our region of sample collection, was a limitation in our study. However, such investigations can be useful to identify the role of genetic variants as potential biomarkers and biological targets for oral cancer pathology.

CONCLUSION

We found that genetic variants of *ADCY2* rs252546 and *C7* rs1450656 are associated with oral cancer. As the minor allele of both genetic variants was more in case. The association of various risk factors with *ADCY2* and *C7* was found significant. Hence, such investigations can be useful to identify the role of genetic variants as potential biomarkers and biological targets for oral cancer pathology.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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