

Association study of NAT2 gene polymorphism and risk of oral cancer in Southern Punjab, Pakistan

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Abstract

Objective: To investigate the single nucleotide polymorphic variations of N-acetyltransferase 2, phase-II metabolising enzyme, and associated risk factors for oral cancer.

Method: The case-control study was conducted from November 2017 to April 2018 after approval from the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan, and comprised oral cancer patients and healthy controls. Single nucleotide polymorphism of the N-acetyltransferase 2 gene associated with oral cancer was analysed. Factors assessed using tetra-primer amplification refractory mutation system polymerase chain reaction included age, smoking, naswar, betel leaves and nuts.

Results: Of the 201 subjects, 94(47%) were patients and 107(53%) were controls, while 108(54%) were aged 10-30 years. Single nucleotide polymorphism rs1208 of N-acetyltransferase 2 gene was primarily A803G and Lys268Arg, with allelic frequency of G/A. Age range 51-70 was significantly ($p=0.00001$) associated with the prevalence of oral cancer in terms of genotypic relationship with A803G. Substantial allelic association was found between the gene and oral cancer ($p=0.006895$). Smoking increased the cancer risk 7-fold (odds ratio: 7.0).

Conclusion: The genetic variant of N-acetyltransferase 2 rs1208 was found to be significantly associated with oral cancer progression and development of associated risk factors.

Keywords: Oral cancer, N-acetyltransferase 2, SNP, Tetra arms PCR. (JPMA 71: 1954; 2021)

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Introduction

Oral cancer (OC), or mouth cancer, is a subtype of head and neck cancer developing a tumour in the oral cavity.¹ Approximately at around 3% of all malignancies, OC occurrence worldwide is around 500,000 cases annually,² making it the second leading cause of death in both men and women.³ In India and Pakistan, 8-10% (1/3rd) of all cancers occur in the oral cavity.⁴⁻⁶ OC is more frequent after age 40, peaking at 60 years. It is more lethal in males than females with a ratio of 2:1.⁷⁻⁹ There is a need to develop therapeutic strategies to cope with this situation.

The N-acetyltransferase 2 (NAT2) metabolising gene is present on chromosome 8p21 region¹⁰ that contains two exons with one long intron of about 8.6 kb. Exon 1 is very short (100 bp), and the entire protein coding region is contained within the 870-bp exon 2. NAT2 alleles having high and low activities are associated with a variety of malignancies.^{11,12} The NAT2 gene is a well-known phase-II xenobiotic metabolising enzyme (XME) and polymorphism of XME genes is strongly associated with OC risk.¹³ OC is a multi-factorial disease having interaction with various

genetic and environmental factors.¹⁴ The single nucleotide polymorphism (SNP) rs1208 of NAT2 gene mutates A803G and Lys268Arg with the allele origin of G/A, with A being the ancestral allele.¹⁰ This SNP was observed in rapid acetylators, and the risk allele for it is G.¹⁵ A803G is not associated with a reduction in O- and N- acetyltransferase catalytic activity and also not in protein expression and stability.

NAT2 is a highly polymorphic enzyme affecting the activity of the enzyme and differentiating the individuals as rapid, intermediate and slow acetylators.^{12,16-19} The frequency of OC is getting higher due to population growth, aging and irrational lifestyle.²⁰ In Asian countries, tobacco is extensively used, and about 90% OC cases are caused by tobacco and its products.^{5,21} Diet lacking in fruits and vegetables, antioxidants, vitamins, minerals, and comprising processed meat is carcinogenic, leading to OC.^{22,23}

The current study was planned to find the genetic polymorphisms of OC associated with phenotypic and genotypic variations in a specific region.

Patients and Methods

The case-control study was conducted from November 2017 to April 2018 after approval from the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya

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University (BZU), Multan, Pakistan, and comprised oral cancer patients and healthy controls. The sample size and statistical analysis was calculated using a MedCalc for Windows, version 19.6.4 (MedCalc Software, Ostend, Belgium).²⁴ SNP was analysed with an odds ratio (OR) 2 at 95% confidence interval (CI), 5% minor allele frequency with case-control ratio 0.909 ratio, and error rate in an allelic test 6.9%. The sample size for a common SNP was significant ($p < 0.05$). The sample was raised using opportunity sampling technique.

The subjects were categorised into control Group 1 having unaffected, normal and healthy individuals without any clinical signs and symptoms of OC, and patient Group 2 having clinically-diagnosed OC patients.

After taking consent, data was

Table-1: Associated phenotypic risk factors of oral malignancy in southern Punjab.

Factors	Category	Controls (n=107)	Cases (n=94)	Significance
Gender	Male	39 (36.44%)	48 (51.06%)	X ² =4.35; D.F.=1; P=0.037009
	Female	68 (63.55%)	46 (48.93%)	
Age in Years	10-30	98 (91.58%)	10 (10.63%)	X ² =134.334; D.F.=3; P=0.00001
	31-50	8 (7.47%)	36 (38.29%)	
	51-70	1 (0.93%)	43 (45.74%)	
	71+	0 (0%)	5 (5.31%)	
Smoking	Yes	5 (4.67%)	26 (27.65%)	X ² =20.27; D.F.=1; P=0.00001
	No	102 (95.32%)	68 (72.34%)	
Betel Leaves/Nuts	Yes	3 (2.80%)	21 (22.34%)	X ² =18.165; D.F.=1; P=0.00002
	No	104 (97.19%)	73 (77.65%)	
Areca Nuts (Gutka)	Yes	1 (0.93%)	16 (17.02%)	X ² =16.72; D.F.=1; P=0.000043
	No	106 (99.06%)	78 (82.97%)	
Naswar	Yes	0 (0%)	14 (14.89%)	X ² =17.12; D.F.=1; P=0.000035
	No	107 (100%)	80 (85.10%)	
Alcohol	Yes	0 (0%)	0 (0%)	0
	No	107 (100%)	94 (100%)	
Jagged Tooth	Yes	3 (2.80%)	13 (13.82%)	X ² =8.303; D.F.=1; P=0.003958
	No	104 (97.19%)	81 (86.17%)	
Gums Bleeding	Yes	7 (6.54%)	20 (21.27%)	X ² =9.343; D.F.=1; P=0.002238
	No	100 (100%)	74 (78.72%)	
Oral Hygiene	Yes	104 (97.19%)	37 (39.36%)	X ² =79.93; D.F.=1; P=0.00001
	No	3 (2.803%)	57 (60.63%)	
Area of Residence	Yes	106 (99.06%)	28 (29.78%)	X ² =108.07; D.F.=1; P=0.00001
	No	1 (0.93%)	66 (70.21%)	
Family History of Oral Cancer	Yes	0	9 (9.57%)	X ² =10.725; D.F.=1; P=0.001057
	No	107 (100%)	85 (90.42%)	

Table-2: Polymorphisms of N-acetyltransferase 2 (NAT2) variant associated with oral cancer risk stratified by various risk factors..

NAT2 Genotyping									
Genotype	Controls		Cases		p-value		Chi Sq.		Odd Ratio 95%CI Smokers and Non-smokers
	Smokers	Non-smokers	Smokers	Non-smokers	Smokers	Non-smokers	Smokers	Non-smokers	
AA	0	0	0	2	0.52197 (Non-significant)	0.010722 (Significant)	0.41	9.071	7.000/2.8549 to 21.3111
AG	1	16	9	21					
GG	4	86	17	45					
Genotype	Betel leaf users	Non-users	Betel leaf users	Non-users	Betel leaf users	Non-users	Betel leaf users	Non-users	Betel leaf users and non-users
AA	0	0	0	2	0.568618 (Non-significant)	0.00132 (Significant)	0.325	13.261	9.9726/2.8681 to 34.6760
AG	1	16	4	26					
GG	2	88	17	45					
Genotype	Snuff users	Non-users	Snuff users	Non-users	Snuff users	Non-users	Snuff users	Non-users	Snuff users and non-users
AA	0	0	0	2	Cannot be Calculated	0.022281 (Significant)	6.015	7.608	38.7267/2.2762 to 658.8767
AG	0	17	7	23					
GG	0	90	7	55					
Genotype	Gutka users	Non-users	Gutka users	Non-users	Gutka users	Non-users	Gutka users	Non-users	Gutka users and non-users
AA	0	0	0	2	Cannot be Calculated	0.007432 (Significant)	0.443	9.804	21.7436/2.8234 to 167.4508
AG	0	17	5	25					
GG	0	89	11	51					

collected related to age, smoking, and consumption of naswar, betel leaves and nuts using the standard interviewer-administered questionnaire.

SNP primers were designed using Soton Primer software. Tetra-primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) amplification procedure was included: forward inner (G-allele) CTGAGGAAGAGGTTGAAGAAGTGCTTAG, forward outer TGCCAAAGAAGAAACACCAAAAAATATA, reverse inner (A-allele) TTCTCCCAAGGAAATCTTAAATATATGTT and reverse outer TTCTTCAAAATAACGTGAGGGTAGAGAG with 60°C for annealing temperature. Deoxyribonucleic acid (DNA) was extracted from blood samples as per standard protocols. The extracted genome was quantified by UV spectrophotometer (Perkin Elmer Lambda 25). Tetra-ARMS PCR refractory mutational-based thermo-cycler (PCR Biometra T-Gradient) was used to study the genetic and polymorphic variations.²⁵ Only a single nucleotide variant of NAT2 (rs1208) was investigated for polymorphic association. In this process, four SNP-specific primers, including 0.5 µl inner-forward, 0.5µl inner-reverse, 1µl outer-forward, and 1µl outer-reverse, were used to amplify the NAT2 genetic variant rs1208 and to observe its allelic frequencies.

Genotypic frequencies of NAT2 rs1208 with OC were calculated. The allelic frequency of the genetic variant of NAT2 rs1208 was calculated using Hardy-Weinberg equilibrium for the wild type allele, and mutant type allele in both the cases and the controls. Statistically, values were analysed using the chi-square goodness of fit test with a degree of freedom to study the associated risk factors with genetic variants and OC. OR, standard error (SE) and 95%CI were measured using the online MedCalc statistical calculator with a significant cut-off value $p=0.05$.^{25,26} Hardy Weinberg equilibrium (HWE) was used to calculate the frequency of NAT2 variations either for mutant or wild type alleles.

Results

Of the 201 subjects, 94(47%) were patients and 107(53%) were controls, while 108(54%) were aged 10-30 years. Male gender, smoking, age and family history were significantly associated with OC-progression (Table 1). NAT2 SNP displayed three bands and indicated heterozygous A/G allele (Figure).

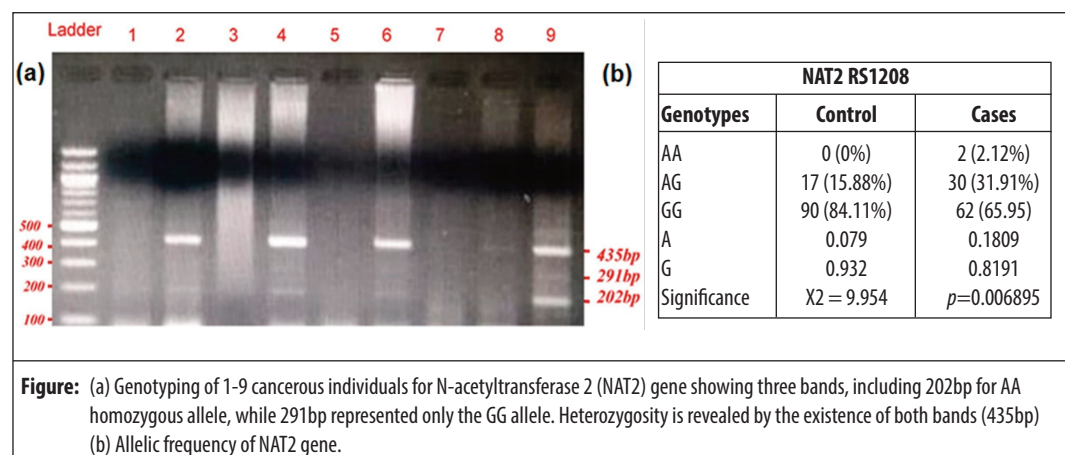
Smoking increased OC risk 7-fold, while betel leaf and naswar usage were not found to be significant (Table 2).

Discussion

Oral cancer is a multi-factorial disease having interaction with various genetic and environmental factors.¹⁴ The current study analysed SNP of NAT2 associated with OC in southern Punjab, and found the association of genetic polymorphisms of NAT2 with various demographic risk factors. The findings of the current study are in line with literature.^{2,7,19,27}

We have observed the distribution of NAT2 alleles in population of the Southern Punjab and accounted for most NATs in other nations.²⁸ In control and cancer patients, risk factors including gender, age, smokers, chewers, and others have not been similar. It has been reported that gender is an important factor and males have been found to be more vulnerable than females to disease development ($p=0.037009$).^{2,7} This study shows the association of phenotypes with the high risk of oral cancer. The interaction between the NAT1 and NAT2 polymorphisms with these phenotypes is probably due to the role of NATs in nitrosamine metabolism as the most significant carcinogen in the use of tobacco and other components of the molecules.²⁹ The frequencies of genotypes and phenotypes are different in our population, however the distribution of normal, rapid and slow acetylators in the control population was found similar in African population, but it was different from Japanese and Turkish populations.^{30,31} NAT2 alleles having high and low activities

are associated with a variety of malignancies. This gene has SNPs affecting the activity of the enzyme and thus differentiating the individuals as rapid, intermediate, and slow acetylators.^{11,12} It has been shown that the homozygous ancestral allele is more prevalent in cases as compared to the control-subjects.⁷



We observed significant outcomes about the homozygous distorted G allele. It is dominant in both controls and cases. A recent study among a Chinese population found a CC genotype of NAT2 rs1565684 T>C polymorphism indicating significantly higher risk of oral cancer.³² We found statistically meaningful association of oral cancer with NAT2 polymorphism.

Conclusions

Genetic variant of NAT2 rs1208 was found to be significantly associated with OC. Genetic and environmental risk factors, like smoking tobacco, gender, aging and family history were major risk factors.

Disclaimer: None.

Conflict of interest: The person who signed the ethical review statement is also a co-author.

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