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The Common polymorphisms in P21 and MDM2 genes as a risk factor for susceptibility and poor prognosis of non-small cell lung cancer in Iraqi population

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ABSTRACT

Background: Cyclin-dependent kinase inhibitor (P21/WAFI) and murine double minute 2 (MDM2) genes regulate cell growth. In malignant tumors, altered expression of these gene products is associated with poor prognosis, this study was designed to determine genotyping of P21 and MDM2.

Methods: This case-control study included 140 lung cancer patients (101 males and 39 females) diagnosed with non-small cell lung carcinoma, matched with 150 healthy individuals(105 males and 45 females). The study was done between 2018 to 2022. The current study aimed to investigate P21-rs1801270 and MDM2-rs2279744 genes polymorphisms with lung cancer risk in the Iraqi population using the PCR-RFLP technique.

Results: Compared with the P21-98 C and MDM2-309T genotypes, we found that P21-98A and MDM2-309G variants were associated with a high risk of NSCLC in Iraqi patients (OR= 5.0, C.I= 3.2-14.2, P<0.0001) for AA and (OR= 6.7, C.I= 4.0-12.4, P<0.0001)for GG. Also, AA and GG genotypes were associated with poor prognosis and significant associations were observed with a stage (p= 0.02) and metastasis status (p =0.003) for the P21 gene, (p= 0.01) and (p= 0.04) for the MDM2 gene respectively of NSCLC in elderly and smokers. Our results obviously showed the multiplicative interaction of P21 AA and MDM2 GG genotypes in the risk of developing lung cancer.

Conclusions: The presence of AA variant alleles for p21 and GG for MDM2 increased the risk of lung cancer in males mainly those smokers older than 45. For this reason, these gene mutations may have a role as markers for susceptibility to lung cancer in those groups of people.

Keywords: *NSCLC, gene, polymorphism, P21, MDM2, Iraq*

BACKGROUND

Lung cancer is the most common malignancy diagnosed worldwide. In Iraq, it represents the principal cause of malignancy related deaths [1,2]. Lung cancer can be classified into two types; non small cell lung cancer (NSCLC) accounts 85% and the rest is small cell lung cancer (SCLC). Nearly two-thirds of NSCLC cases were diagnosed in advanced stages, locally or more frequently metastasized [3, 4]. It is well-established that Tobacco smoking and alcohol consumption are important risk factors for lung cancer [2]. Even though the carcinogenic effects of cigarette smoking and alcohol drinking

are apparent but not all individuals exposed developed lung cancer, suggesting that genetic factors may also contribute to lung cancer pathogenesis. Many single nucleotide polymorphisms have appeared to change the risk of this lethal disease separately or in combination, as well as to environmental exposure [2-4].

The human WAF1/CIP1 gene encodes the p21/WAF1 protein, which is one of the checkpoints in the cell cycle to preserve genetic integrity by cell cycle arrest for allowing the genetic errors to be repaired. The P21 gene is located on chromosome 6 p21.2, comprising 2 introns and 3 exons, and encodes the p21 protein. The function of this protein is a universal inhibitor of cyclin-dependent kinases (CDKs) and its role in controlling the cell cycle [3], p21 is participated in mechanisms of DNA repair such as base excision repair and nucleotide excision repair thus preventing DNA replication and arresting cell cycle in G1[4].

A transversion polymorphism (C/A) at the third base of codon 31 in the p21 gene, leading to the substitution of Ser to Arg amino acid, has been recorded [5]. The alteration of the p21 gene may be interrupting the pathway of the p53 gene which mediates cell cycle arrest and increase susceptibility to cancers. A series of epidemiological studies found that p21 codon 31 polymorphism was related to increased risk of different types of cancers and was designated as a tumor suppressor gene in the lung, colon, and brain cancer, it was revealed that p21 gene stimulates tumor growth suppression throughout p53 normal allele type activity [6, 7]. The protein encodes by the murine double minute 2 (MDM2) gene which is the essential component in the signaling pathway of p53, MDM2 regulates p53 by autoregulatory negative feedback loop and its main role in regulating the progression of the cell cycle and apoptosis. MDM2 is an important oncogene that has been identified to play a key role in a variety of human cancers [8]. The human MDM2 gene is located on chromosome 12q13-14 with 34 kb, it has three promoters, second and third promoters are responsive to the p53 gene [9]. In humans, expression levels of MDM2 protein seem to be important for the regulation of p53 in response to DNA damage. In tumors, MDM2 gene overexpression can substitute by p53 inactivation when lack of p53 mutations, in sporadic tumors the point mutation in the MDM2 gene was recorded, including lung cancer [10, 11]. To our knowledge, there is no information regarding the relationship between lung cancer and the mutation of these genes in our country (Iraq). Therefore, this study aimed to investigate the effect of two more common gene polymorphisms in P21 (98C/A) and MDM2 (309T/G) on the risk and susceptibility of NSCLC in the Iraqi population.

METHODS

Informed consent was obtained from all participants, then blood specimens were collected. This case-control study was conducted during the period from 2018 to 2022 in order to determined genes mutation frequency in non small cell lung cancer of Iraqi population, it was carried out in the molecular biology laboratory in the department of Anatomy and Histology/ Faculty of Medicine/ Kufa University.

A total of 140 patients (101, 72.14 % males and 39, 27.86% females) were confirmed histopathologically with NSCLC, they were collected from the oncology center in AL-Sadder medical City teaching hospital in Al-Najaf Province/ Iraq, they were compared with 150 sex and age-matched control group. The mean ± SD for the ages of the patient and control groups were 59.5± 6.3 years (range 28-84) and 58± 4.6 years (range 30-85) respectively. They were Arabian race, from the middle Euphrates region of Iraq. Individuals with other types of malignancies or chronic diseases were not involved in the current study. Two milliliters of blood were drawn from all individuals who participated in this study, which were collected in an EDTA tube for genotyping analysis by using (ReliaPrepTM promega) kit according to the manufacturer's instructions.

The status of the p21 and MDM2 genes polymorphism was determined by PCR-RFLP. The amplification was done according to the procedure published previously [12]. The primers and parameters for PCR-RFLP were

summarized in Table 1. Amplification conditions were: At 95°C for 3 minutes to initial denaturation, followed by 35 cycles [95°C for 30 seconds to denaturation; 63.7°C for 30 seconds to annealing; 72°C for 30 seconds to extension] and 72°C for 5 minutes to final extension. Both PCR and RFLP products were separated on 3% agarose gel stained with ethidium bromide and imaged by a UV transilluminator.

Statistical analysis

The variations in means of parameters between patients and control groups were determined by continuous variables that were expressed by mean ± SD. Interactive Chi-Square and students t-test were used to examine the demographic characteristics. Genotype frequencies were tested first for Hardy Weinberg equilibrium, the differences in selected demographic variables with P21 and MDM2 genotyping frequencies of patients and healthy control groups were assessed by using the Chi-square tool, the associations of P21 and MDM2 genes variants and risk of lung cancer were measured by odds ratios (ORs) and 95% confidence intervals (95% C.I) according to multinomial logistic regression analyses which done by using SPSS (v. 20.0) software (SPSS Inc., Chicago, IL), P <0.05 was the level of significance in all the analyses.

RESULTS

The demographic characteristics variables between cases and healthy controls are summarized in Table 2. Only a few patients (16.43%) presented with a family history of lung or other cancer. Histopathological studies revealed 80 NSCLC cases with Squamous cell carcinoma (SCC) and 60 NSCLC cases with adenocarcinoma (AC), more than 60% of patients were diagnosed in advanced stages with 27% cases positive for distant metastasis.

Genotype and allele distribution with risk of lung cancer in studied SNPs

An association between the genotypes and the risk of lung cancer was estimated by using unconditional logistic regression analysis, the allele distribution frequencies of P21 C98A and MDM2 T309G genotypes were compared between patients and control groups. The genotype frequencies of P21 were 42 in wild, 60 in heterozygous, and 38 in homozygous genotypes for patients compared with 90 in wild, 48 in heterozygous, and 12 in homozygous genotypes for the control group respectively. furthermore, the heterozygous genotype increased the risk of lung cancer by approximately three times (OR= 2.7, 95%C.I= 1.5-4.5, P=0.0003) and the homozygous genotype by five times (OR= 5.0, 95%C.I= 3.2-14.4, P=0.0001) when compared with wild genotype. While the genotype frequencies of MDM2 were 33 in wild, 61 in the heterozygous, and 46 in the homozygous patients compared with 88 in wild, 52 in the heterozygous, and 10 in the homozygous for the control group respectively. furthermore, the heterozygous genotype increased the risk of lung cancer by approximately four times (OR=3.5, 95%C.I=1.8-5.4, P=0.0001) and the homozygous genotype by seven times (OR=6.7, 95%C.I= 4-12.4, P=0.0001) as shown in Table 3.

The association of P21 (rs:1801270) gene polymorphism with risk of lung cancer in NSCLC patients

The frequencies of P21 C98A in wild, heterozygous, and homozygous genotypes were statistically significant with respect to Age, smoking status, family history, TNM stage, and metastasis status among patients with increased risk of disease in elderly (>45 years) and smoking patients (OR=1.9, 95%C.I= 0.85-3.3, P=0.04 and OR=3.6, 95%C.I= 1.7-6.5, P=0.03), also in those have positive family history by 4 times more than those with negative family history (OR=4.2, 95%C.I= 2.1-8.6, P=0.03), TNM stage with increased risk in patients of advanced stages by 5 times (OR=5.0, 95%C.I= 2.9-7.0, P=0.02) and increased risk by approximately 4 times in poor differentiated squamous cell carcinoma and 2 times in adenocarcinoma (OR=3.5, 95% 95%C.I= 2.8-5.9, P=0.04 and OR=2.1, 95%C.I= 1.5-3.8, P=0.03) respectively. Whereas there were no significant differences in sex as shown in Table 4.

The association of MDM2 (rs: 2279744) gene polymorphism with risk of lung cancer in NSCLC patients

The frequencies of MDM2 T98G in wild, heterozygous, and homozygous genotypes were statistically significant with respect to Age, smoking status, family history, TNM stage, and metastasis status among patients with increased risk of disease in individuals who were smoking by approximately 2 times more than not smoking patients (OR=1.8, 95%C.I= 1.5-4.8, P=0.048), also increased risk of disease in subjects who have positive family history by 3 times more than those with negative family history (OR=3.3, 95%C.I= 2.2-6.8, P=0.04), TNM stage with increased risk in patients of advanced stages by about 3 times (OR=2.8, 95%C.I= 1.5-4.8, P=0.01) and increased risk by two times in poor differentiated squamous cell carcinoma and 3 times in adenocarcinoma (OR=2.15, 95%C.I= 1.7-6.8, P=0.019 and OR=3.0, 95%C.I= 1.7-5.8, P=0.04) respectively. While there were no significant differences with respect to age and sex as shown in Table 5.

Gene-Gene interaction between P21 and MDM2

In the next step, we evaluated whether there were statistical interactions between p21 and MDM2 polymorphisms as appeared in Table 6. We found that the patients who carried the mutant GG genotype of MDM2 were more likely to carry the P21 mutant Arg/ Arg genotype than control individuals (7.2% and 0.7%) respectively.

The presence of the TG genotype in MDM2 and the Arg/Arg genotype in P21 was linked with an increased risk of lung cancer compared to the lack of such genotype (OR=2.2, 95% C.I= 1.2-3.8, and OR=1.3, 95% C.I= 1.0–2.9) respectively. Though, the presence of both MDM2 GG and P21 Arg/Arg genotypes were combined with increased risk of lung cancer by 5 folds (OR= 5.2, 95% C.I= 3.0-7.6, P< 0.05) for homogenous genotypes in comparison to those who lacked both genotypes, these results were indicated a multiplicative interaction between MDM2 GG genotypes and P21 Arg/Arg genotype in risk of lung cancer development.

DISCUSSION

Lung cancer is still to be a serious worldwide health problem and is one of the causes leading to cancer death globally [13]. The number of individuals with lung cancer seems to be growing rapidly each year, behind this phenomenon the SNPs have been studied with a particular interest in this area and many SNPs have been related to increasing risk of cancer [14].

This molecular study was conducted to predict genetic mutations in P21 and MDM2 genes in combination or alone related to the risk of development of lung cancer. Our results were obtained by analyzing 140 lung cancer patients and 150 controls to demonstrate that the functional polymorphisms in the promoter region of MDM2 and P21 codon 98 have a significant contact on the risk of cancer development.

Indeed, our results revealed the Arg/Arg genotype in p21 and GG genotype in the promoter region of MDM2 have a significant impact on the risk of NSCLC development, Arg/Arg with 5.0 folds and GG with 6.7 folds respectively higher risk of NSCLC and were more predominant in individuals with advanced stage ($p<0.02$ for p21 and $p<0.01$ for MDM2) and poor metastasis status at diagnosis ($p<0.04$ for SCC and $p<0.03$ for AC) for p21 Arg/Arg genotype and ($p<0.019$ in SCC and 0.04 for AC) for MDM2 GG genotype of NSCLC.

In fact, the results showed smoking patients have a significant association between p21 gene/ Ser31Arg polymorphism and elevated susceptibility to the development of lung cancer in patients of NSCLC. On the other hand, it appears that the Arg allele may be associated with increased lung cancer risk in people older than 45 years in smokers. These results indicate that polymorphic variants might be low efficient, in consequence, the control management for cell cycle by the p21 variant is limited when compared with the wild type.

Ser allele of p21 or one T allele of MDM2 were at lower risk of NSCLC when compared with Arg/Arg alleles of p21 or GG alleles of MDM2, the approximately 3.6 times increased risk associated with P21 Arg/Arg genotype compared to the P21 Ser/Ser genotype and 5.7 times

increased risk associated with the MDM2 GG genotype compared to the MDM2 TT genotype among elderly smokers (>45 years) are excellent examples of the gene-environment interaction that may play an important cause of lung cancer in the Iraqi people.

Our results agree with many studies, Islami et al. 2015 and Buthainah et al .2018 [15, 16], who found a strong association between cigarette smoking and lung cancer in the Iraqi population mainly in the last decades, and they were attributed to the economic status and cultural environment. Another study from Saudia Arabia, which was reported by Dilshad et. al 2015 [17], found light or intermittent active cigarette smoking or prolonged passive smoking was a bigger threat for many cancers mainly lung. AlReza et.al 2020 [18], found an increased risk of lung cancer related to the GG genotype of MDM2 SNP309, especially in the Asian population and Park et.al 2006 [19], were showed that individuals carrying GG at MDM2 were at higher risk of lung adenocarcinoma compared with individuals carrying TT genotype. Wang et. al 2015 [20], also found MDM2 SNP309 was associated with lung cancer risk in the Northeastern Chinese population. Furthermore, Zhang et. al 2006 [21], reported the association of MDM2 SNP309 with the risk of lung cancer display multiplicative gene-environment interaction with smoking. Xie et.al 2014 [22], who were found cigarette smoking and p21 overexpression were associated with poor prognosis of NSCLC patients. Meta-analysis was done by Liu. et. al 2011 [23] from 49 publications and 66 case-control studies that explored an association between Ser 31 Arg polymorphism of the p21 gene and cancer risk in the recessive model when compared with the wild genotype.

In contrast, our results disagree with Li et.al 2003 [24], who reported no significant association between NSCLC and SNP309 and Li. et.al 2003 [25] found no association between P21 codon 31 gene polymorphism and lung cancer risk. Our results also disagree with those reported by other studies of lung, gastric, and endometrial cancers for which the p21/ Ser allele increased the risk [26, 27], which may be explained by the differences in allele frequency between Asian and European populations [28].

There were a number of limitations in our study, the sample size is small relatively, ethnicity and race of patients should be included; therefore we need further studies with large sample sizes and from different areas in order to reach final conclusion.

CONCLUSION

We concluded from this study, that possessing both variant alleles (p21 98A and MDM2 309G) increased the risk of lung cancer in males, especially those older than 45 years and smokers. So that mutations of these genes may be used as markers for lung cancer susceptibility.

Abbreviation

A: Adinine, AC: Adenocarcinoma, BIpI: Bacillus lentus (C. Polisson), C: Cytosine, Dx: Diagnosis, F: Forward, G: Guanine, MDM2: Murine double minute 2, MspAI: Modified Sparse Approximate Inverses, P21: Tumor suppressor 21, R: Reverse,rs: Reference SNP, RFLP: Restriction fragment length polymorphism, SCLC: small cell lung cancer, SCC: Sequamous cell carcinoma, SD: Standard deviation, T: Thymine

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TABLE 1: primer sequences, length of PCR products, and restriction enzymes for P21 and MDM2 genes.

Primer sequence	Gene	PCR Product (bp)	Restriction Enzyme	Allele size (bp)	Ref.
F:GTC AGA ACC GGC TGG GGA TG R: CTC CTC CCA ACT CAT CCC GG	p21/ C98A	272	BIpI	wild: 183 +89 Hetero:272+183+89 Homo: 272	[5]
F:CGC GGG AGT TCA GGG TAA AG R: CTG AGT CAA CCT GCC CAC TG	MDM2/ T309G	157	MspAI	wild: 157 Hetero:157+110+47 Homo: 110+47	[10]

TABLE 2: General characteristics of NSCLC patients and control groups

Parameter	NSCLC patients=140 (%)	Control group= 150 (%)	P – value
Sex Males Females	 101 (72.14) 39 (27.86)	 105 (70) 45 (30)	 0.78
Age (year) ≤ 45 > 45	 18 (12.86) 122 (87.14)	 20(13.33) 130(86.67)	 0.1
BMI <18,5 18.5-25 >25	 20 (14.29) 42 (30.00) 78 (55.71)	 18 (12.00) 45 (30.00) 87 (58.00)	 0.74
Smoking status Non smoker smoker	 21 (15) 119 (85)	 130 (86.7) 20 (13.3)	 0.001
Clinicopathological parameters in NSCLC patients			
parameters	No. of patients	Percentage %	
Family histry Positive Negative	 23 117	 16.43 83.57	

Cytological type SCC (n=80) Well Moderately Poor AC (n=60) Well Moderately Poor	 20 50 10 10 39 11	 25 62.5 12.5 16.67 53.33 30
TNM stage Early stage (I and II) Advanced stage (III and IV)	 52 88	 37.1 62.9
Metastasis Negative Positive	 38 102	 72.9 27.1

SCC: Sequamous cell carcinoma, AC: Adenocarcinoma

TABLE 3: Genotype frequency of P21 (C→A) and MDM2(T→G) gene polymorphism in NSCLC patients and control.

P21 Genotype C → A	Patients No=140	%	Control No=150	%	OR	95% 95%C.I	P-value
CC	42	30	90	60	1.00		
CA	60	42,86	48	32	2.7	1.5-4.5	0.0003
AA	38	27.14	12	8	5.0	3.2-14.2	0.0001
MDM2 Genotype T →G							
TT	33	23.57	88	34.67	1.00		
TG	61	43.57	52	58.67	3.5	1.8-5.4	0.0001
GG	46	32.86	10	6.66	6.7	4.0-12.4	0.0001

Multinomial logistic regression, No: number; OR: odds ratio, 95%C.I, confidence interval, P<0.05 statistically significant

TABLE 4: Association of P21 gene polymorphism and NSCLC risk in patients

Variables (no.)	CC=42 no. (%)	CA=60 no.(%)	AA=38 no.(%)	C allele	A allele	O.R	95%C.I	Value
Gender Male (101) Female(39)	 29(28.7) 13(33.3)	 44(43.56) 16(41.02)	 28(27.7) 10(25.64)	 O,49 0.49	 0.51 0.51	1.2	0.47- 3.3	0.6
Age atDx (year) ≤ 45 (18) > 45(122)	 6(33.3) 36(29.5)	 7(38.89) 53(42.86)	 5(27.78) 33(27.05)	 0.53 0.51	 0.47 0.49	1.9	0.85- 3.3	0.04
Smoking status Non(21)	 6(28.57)	 9(42.85)	 6(28.57)	 0.50	 0.50	3.6	1.7-6.5	0.02

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Smokers(119)	36(30.25)	51(42.86)	32(22.9)	0.52	0.48			
Family history Positive(23) Negative(117)	 10(43.48) 32(27.35)_	 7(30.43) 53(43.3)	 6(26.08) 32(27.35)	 0.59 0.50	 0.41 0.50	 4.2	 2.1-8.6	 0.03
TNM stage Early I&II (52) Advanced III&IV (88)	 12(23.1) 10 (11.4)	 25(48.1) 50 (56.8)	 15(28.8) 28 (31.8)	 0.54 0.56	 0.46 0.44	 5.0	 2.9-7.0	 0.02
Cytological type SCC = 80 Well 20 Moderate 50 Poor 10 AC = 60 Well 10 Moderate 39 Poor 11	 5(25) 18(36) 3(30) 2(20) 11(28.2) 3(27.27)	 9(45) 20(40) 4(40) 4(40) 19(48.71) 4(36.36)	 (30)6 12(24) 3(30) 4(40) 9(23.08) 4(36.36)	 0.48 0.56 0.50 0.40 0.53 0.45	 0.52 0.44 0.50 0.60 0.47 0.55	 3.5 2.1	 2.8-5.9 1.5-3.8	 0.04 0.03

P<0.05 statistically significant, Dx: diagnosis, SCC: Squamous cell carcinoma, AC: Adenocarcinoma

TABLE 5: Association sof MDM2 gene polymorphism and NSCLC risk in patients

Variables (no.)	TT = 33 no.(%)	TG = 61 no.(%)	GG = 46 no.(%)	T allele Frequency	G allele Frequency	O.R	95% C.I	P Value
Gender Male (101) Female(39)	 22(21.78) 11(28.21)	 43(42.57) 18(46.15)	 36(35.64) 10(25.64)	 0.43 0.51	 0.57 0.49	 0.55	 0.2-1.7	 0.3
Age atDx/year ≤ 45 (18) > 45(122)	 5(27.78) 28(22.95)	 7(38.39) 54(44.26)	 6(33.33) 40(32.79)	 0.47 0.45	 0.53 0.55	 1.74	 0.6-2.4	 0.04
Smoking status Non(21) Smokers(119)	 8(38.1) 25(21)	 8|(38.1) 53(44.54)	 5(23.81) 41(34.45)	 0.57 0.43	 0.43 0.57	 5.7	 3.8-7.2	 0.02
Family history Positive(23) Negative(117)	10(43.48) 23(19.66)	7(30.43) 54(46.15)	6(26.01) 40(34.19)	0.59 o.43	0.41 0.57	3.3	2.2-6.8	0.04
TNM stage Early I&II (52) Advanced III&IV (88)	 20 (38.5) 13 (14.8)	 24 (46.2) 37(42.04)	 8 (15.4) 38 (43.2)	 0.43 0.52	 0.57 0.48	 2.8	 1.5-4.9	 0.01
Cytological type SCC = 80 Well 20 Moderate 50 Poor 10 AC = 60 Well 10 Moderate 39 Poor 11	 7(35) 10(20) 3(30) 4(40) 6 (15.38)	 8(40) 25(50) 4(40) 3(30) 16(41.03)	 5|(25) 15(30) 3(30) 3(30) 17(43.59)	 0.55 0.45 0.50 0.55 0.36	 0.45 0.55 0.50 0.45 0.64	 2.15	 1.7-6.8	 0.019

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	3(27.27)	5(45.45)	3(27.27)	0.50	0.50	3.0	1.7-5.8	0.04

P<0.05 statistically significant, Dx: diagnosis, SCC: Squamous cell carcinoma, AC: Adenocarcinoma

TABLE 6: Risk of NSCLC associated with MDM2 by P21 genotypes.

Genotypes MDM2 P21 T309G C98A(Ser>Arg)		Patients No. (%)	Control No. (%)	OR	95% C.I
TT	Ser/ Ser	7 (5.0)	17 (11.3)	1.00	Ref.
TT	Ser/ Arg	16 (11.4)	22(14.7)	1.2	(0.7-1.5)
TT	Arg/ Arg	8(5.7)	6(4.0)	1.3	(1.0-2.9)
TG	Ser/ Ser	25(17.9)	24(16.0)	1.8	(1.1-2.5)
TG	Ser/ Arg	30(21.4)	35(23.3)	1.7	(1.3-2.4)
TG	Arg/ Arg	17(12.1)	15(10.0)	2.2	(1.2-3.8)
GG	Ser/ Ser	11(7.9)	18(12.0)	1.9	(1.4-3.1)
GG	Ser/ Arg	16(11.4)	12(8.0)	1.8	(1.1-2.7)
GG	Arg/ Arg	10(7.2)	1(0.7)	5.2	(3.0-7.6)

P<0.05 statistically significant