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Association of *MDM2* SNP309 and *TP53* Arg72Pro Polymorphisms with Risk of RA in the Algerian Population

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ABSTRACT

This study examines two common, functional, single nucleotide polymorphisms (SNP) in the genes coding the human homolog of murine-double-minute-2 (MDM2) and P53 in patients with rheumatoid arthritis (RA) based on the hypothesis that P53 may be an important negative regulator of the pro-inflammatory transcription factor nuclear factor kappa b (NFkB).

Genomic DNA was obtained from 101 patients with RA who fulfilled at least 4 ACR criteria and from 91 healthy controls. Mdm2 SNP309 and p53 P72R were genotyped by polymerase chain reaction and restriction enzyme analysis.

In RA patients the frequencies of the p53 P72R, a significant difference in the frequencies of TP53 R72P genotypes or the 72P allele was observed between cases and controls (76.24% of 72 R/P and P/P genotype in cases and 20.88% in controls, p<0.05; 63% of 72P allele in cases and 12.09% in controls, p<0.05). Concerning MDM2 SNP309 G allele, it was associated with an increased risk of RA with OR of 1.80 (95% CI 1.20–2.70), p=0.004. A combined analysis of both polymorphisms revealed a statistically significant association was observed between the increased risk of RA and the combined genotypes of TP53 Arg/Pro +Pro/pro and MDM2 TT ; TP53 Arg/Pro +Pro/pro and MDM2 TG+GG (OR 3.9, 95%CI 1.30- 11.61, p= 0.01)

We can conclude that, there is an association of MDM2 SNP 309 G and/or p53 homozygous genotypes Pro/Pro with the risk of developing RA among Algerian patients.

Key words: *Rheumatoid arthritis, TP53, MDM2, polymorphism, West Algerian population.*

Running title: MDM2 and TP53 polymorphisms and RA

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1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial tissue proliferation with progressive joint destruction and pannus formation, which is the invasion of cartilage and bone by proliferating fibroblast-like synoviocytes (FLS) . These cells exhibit some features of transformation in RA, including loss of contact inhibition, anchorage-independent growth, oncogene activation, autonomous invasion into cartilage and somatic gene mutations (1, 2).

Potentials genes that might contribute to this phenotype is the tumor suppressor gene *P53* and its regulator negative *MDM2* which are both key players involved in multiple pathways including apoptosis, cellular transcriptional control, and cell cycle regulation (3). They are expressed in many inflammatory and autoimmune diseases , and it may serve a protective function by suppressing cytokine production and matrix destruction (4, 5)

Moreover, recent *key* research results from *in-vitro and in vivo* studies indicated that P53 has an inhibitory effect on pro-inflammatory transcription factor nuclear factor kappa b *(NF-κB)* . The transcription factor *NF-κB* is involved in the control of the pro-inflammatory cytokines *TNF and IL*-1activated upon inflammation. Unregulated expression of these cytokines induces the activation of *osteoclasts* and collagenases and subsequently, erosive bone lesions in many rheumatic diseases (6, 7).

The MDM2 is an ubiquitin ligase that binds to P53 and directly blocks P53 function and induces

its destruction by proteosome. On the other side, The *MDM2 expression* is induced by *P53*, there is a *MDM2-P53* feedback auto regulatory loop. Therefore, both P53 and MDM2 expression and their activity in normal circumstances are under control and remain in basal levels (8).

Functional single nucleotide polymorphisms (SNPs) have been described in the two genes. A *TP53* polymorphism at codon 72 of exon 4, encoding an Arg or a Pro (rs1042522, c.215C>G, p.R72P) has been shown to affect some p53 activities in vitro. The Arg variant is more potent in inducing apoptosis (9). The *MDM2* SNP309 (rs2279744) is a T>G transfusion found at position 309 in the first intron of *MDM2* gene, which serves as a transcriptional enhancer region. The G allele increases the affinity of the transcription factor Sp1, which leads to the increased transcription and expression of MDM2 and inhibition of p53 stress response (10).

In this reported study, we examined the relation of SNP309 and *TP53* R72P polymorphisms to rheumatoid arthritis (RA) in the West Algerian population.

2. MATERIAL AND METHODS

2.1 Patients

One hundred and one consecutive RA patients fulfilling the 1997 ACR criteria (11) were collected in Rheumatologic department of University Hospital of Oran. All of these patients were from West Algeria. No ethnic differences were found between the patients and controls. The medical records of the patients were reviewed for the presence of rheumatoid factor. The control group consisted of 91 healthy subjects who were unrelated volunteers blood donors matched for age and sex with the patients. The ethics committee of the University Hospital of Oran approved the study and informed consent was obtained from all patients.

2.2 DNA extraction

Genomic DNA from blood samples was prepared using a simple salting out procedure, as described in (12).

Genotyping

The *TP53* R72P polymorphism was determined by PCR-RFLP, using primers: sens:5'CGTTCTGGTAAGGACAAGGGTT3'and antisens:5'TCCATGAGACTTCAATGCCTGG3'. The product of amplification expected was 441bp in length. 200ng of DNA were used as template in a 25 µl PCR reaction mixture containing: 1.5 µmol MgCl2, 2 µmol of primers, 1 U Taq polymerase (Perkin Elmer Applied Biosystems, Weiterstadt, Germany). Following an initial denaturation step (2 min at 95°C), samples were subjected to 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec with a final extension time of 5 min at 72°C. The PCR products were digested with restriction endonuclease BtgI, (New England Biolabs, Ipswich, MA, USA) and restriction fragments were analyzed on 2% agarose gel. The fragment of homozygote Arg/Arg (G/G) gave only an undigested band of 441bp, the homozygote Pro/Pro (C/C) gave 2 bands of 235 bp and 206 bp

while the heterozygote Arg/Pro (C/G) gave 3 bands of 441bp, 206 bp and 235 bp

Genotyping of the SNP309 polymorphism was determined according to the methods described elsewhere (13), using primers 5′CGG GAG TTC AGG GTA AAG GT3′ (forward) and 5′AGC AAG TCG GTG CTT ACC TG3′ (reverse). The PCR product of 352 bp was digested with MspA1I, resulting in fragments of 187, 88, 46 and 31 bp for the G allele, and 233, 88 and 31 bp for the T allele.

After the completion of genotyping in all the study's subjects, 40 subjects were randomly selected for each polymorphism with 20 subjects for each genotype. Genotypes in the repeat sample were in complete agreement with those in the first assay regarding the two polymorphisms.

2.4. Statistical analysis

The $\chi 2$-test was used to evaluate the homogeneity of genotype frequency distributions among the two groups. Genotype frequencies were cross checked with Hardy-Weinberg expectations by the $\chi 2$-test. Significance was set at $p<0.05$. The statistical software "EpiInfo™ 7 " package was used for these statistical data analyses.

3. DISCUSSION

In RA, joint damage is associated with the transformation of the synovial membrane in a pseudo- tumor tissue called pannus which will gradually invade and destroy the joint. Pannus formation is mainly due to the hyper proliferation of fibroblast-like synoviocytes (FLS) . This transformation is defined by the loss of contact inhibition, by the activation of oncogenes and inhibition of apoptosis (14). Thus, synoviocytes proliferate randomly causing thickening of the synovial membrane. They participate in the maintenance of inflammation and joint destruction To our knowledge, this is the first study that evaluates the association of MDM2 SNP309 and P53 Arg72Pro polymorphisms, alone or in combiantion, with the risk of RA development compared to healthy population, within the Algerian population.

This study demonstrates the association between the MDM2 SNP 309G and P53Pro/Pro and synovial hyperplasia characteristic of RA in our population. Only three diverse populations have evaluated the association between P53 codon 72 polymorphism or MDM2 SNP309 and RA susceptibility (15, 16). In a Korean study no significant differences were found in the distribution of this polymorphism in 114 RA patients and 114 healthy controls. In a study conducted in Germany (16), the frequency of P53 Arg/Pro codon72 polymorphism in a series of 16 patients with juvenile chronic arthritis (JCA) and 15 patients with RA was evaluated and no association was observed. Contrariwise, the German study describes a significant protective effect of the *MDM2* SNP309G allele in most individuals (17). A complementary study on the same population confirmed these results by measuring the apoptotic activity of synoviocytes. They found that the rate of cells apoptosis was decreased in individuals with the T allele compared to those carrying the G allele (18). The

result of another study in an Italian RA population confirmed the absence of an influence of the p53 codon72 polymorphism on the occurrence of RA but it is associated with the structural damage of the disease (19). These conflicting results could be explained by the involvement of additional genetic and environmental factors implicated in the pathogenesis of RA.

Concerning the 309G allele, it was often suggested as a risk factor associated with early onset of various types of cancer and certain autoimmune diseases. This is the result of a higher affinity between the Sp1 transcriptional factor and the G allele, which generates higher concentrations of MDM2 protein and an inhibition of P53 transcriptional activity(10).

Indeed, absence of P53 protein, decrease the apoptotic activity of synoviocytes and activate the proinflammatory transcription factor NF- KB (17). This factor regulates the expression of various adhesion genes ICAM- 1, VCAM -1 and E–selection. Overexpression of adhesion molecules results in the recruitment of inflammatory cells such as neutrophils and eosinophils, and T lymphocytes to the site of inflammation (20).

Otherwise, the NF-KB once activated will induce overexpression of pro-inflammatory cytokines TNF- α and IL1 beta (21), they are key cytokines of articular inflammation. They control the production of many other cytokines such as growth factors (VEGF, TGF, PDGF), and are able to produce auto antibodies such as rheumatoid factor or anti- citrullinated (22). Furthermore, NF-KB is required for the anti-apoptotic expression genes such as cFLIP protein (23). FLIP expression protects FLS of apoptosis induced by TNF - α (24).

Moreover, for P53 Pro allele, different studies in cell lines containing inducible version of alleles encoding the Pro72 and Arg72 variants and in cells with endogenous P53 have shown that the Arg72 variant of P53 is able to induce apoptosis at least five times better than the Pro72 variant. This enhanced apoptotic potential seems to be correlated with the greater ability of the Arg72 variant to localize to the mitochondria and induce the release of cytochrome C into the cytosol (9)

On the other hand, suggestions that the P53 functions in the RA patients could have been indirectly suppressed by the heightened MDM2 levels, making them more vulnerable to RA development. Together, these data support the model whereby SNP309 enhances the affinity of the transcriptional activator Sp1 to the MDM2 promoter gene, resulting in heightened transcription. Heightened levels of MDM2 lead to the direct inhibition of P53 transcriptional activity, which could lead to RA development (25).

According to our data, it should be noted that genetic risk contributing to the development of this disease has been determined with a significant expression of MDM2 T 309G protein (G / G, G) correlated with low anti -proliferative activity of isoform P53 72P (Pro). However, these results are more significant by increasing sampling. Only one study has evaluated the association of rheumatoid arthritis with Mdm2 SNP309 and p53 P72R, and they conclude that the function of MDM2 depends

on the p53 P72R genotype, resulting in either an increased or reduced risk for RA. We suggest that in most cases MDM2 stabilizes the conformation of P53, whereas in P53 PP-positive subjects MDM2 supports the degradation of P53 (17).

In 2011, Knappskog and *al*. report unveils a second SNP in position 285 (SNP285G>C) of *MDM2* promoter, which forms an haplotype with SNP309 (SNP285C//SNP309G). Furthermore, SNP285C reduces the risk of both ovarian and breast cancer (26). While the G allele enhances the transcription, the C allele reduces the binding of specific protein 1 transcription factor on the promoter. Thus, we cannot state the influence of other modifying genes and/or environmental factors which can affect the implication of *MDM2* in the occurrence of RA. It is necessary to study the interaction with the haplotype SNP285C/SNP309G in future investigations.

4 RESULTS

This analysis included 101 RA and 91 cancer-free control subjects and all samples were successfully genotyped for both polymorphisms. The compiled data analysis of the two groups is summarized in table 1. The genotype distributions for both of the SNPs tested were in Hardy-Weinberg equilibrium (TP53 R72P: $\chi 2$= 2,71, p=0.09; MDM2 SNP309: $\chi 2$=0.54, p= 0.462)

4.1. Frequency of TP53 Arg72Pro polymorphism in RA group

The frequency distribution of TP53 R72P genotypes among cases and controls, as well as the estimates of RA, are presented in Table 2. A significant difference in the frequencies of TP53 R72P genotypes or the 72P allele was observed between cases and controls (76.24% of 72 R/P and P/P genotype in cases and 20.88% in controls, $p<0.05$; 63% of 72P allele in cases and 12.09% in controls, $p<0.05$). Since the 72P/P genotype was the infrequent genotype among our population, we combined it with the 72R/P genotype. Furthermore, as compared with the 72R/R wild genotype, a significantly positive association was observed between the combined genotypes and RA risk (OR 12.57, 95%CI 6.14–24.05, $p<0.05$).

4.2. Frequency of MDM2 SNP309 polymorphism in RA group

The genotype frequency of the MDM2 SNP 309 among cases, controls is presented in Table 3. The percentages of the Homozygous TT genotype in cases, healthy controls were 20.8 and 35.17 respectively. The percentage of the heterozygous TG genotype was 44.55 and 45.05 in cases and controls respectively. The homozygous GG percentage within cases and controls was 34.65 and 19.78 respectively. There was statistically significant variation between cases and controls in the frequency of the GG genotype (OR 2.96, 95% CI 1.34–6.53, p = 0.006). Additionally, The MDM2 G allele was associated with an increased risk of RA with OR of 1.80 (95% CI 1.20–2.70), p=0.004.

4.3. Frequency of MDM2 SNP309 polymorphism and Arg72Pro in RA group

The effect of combined genotypes was examined and a statistically significant association was observed between the increased risk of RA and the

combined genotypes of TP53 Arg/Pro +Pro/pro and MDM2 TT ; TP53 Arg/Pro +Pro/pro and MDM2 TG+GG (OR 3.9, 95%CI 1.30- 11.61, p= 0.01; OR 13.09 95%CI 4.96-34.54,p=0, respectively) (Table4).

CONCLUSION AND PERSPECTIVES

We conclude that an association of MDM2 SNP 309 G and/or P53 homozygous genotypes Pro/Pro with the risk of developing RA among Algerian patients. In the future, a larger sample size will be expected to further confirm the effects of these two polymorphisms on RA in our population.

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Table 1. Compiled data analysis of patients and controls

	Healthy controls	RA patients
All	91	101
Gender		
Male	31	11
Female	60	90

Table 2. Frequency distribution of TP53 codon 72 polymorphism between cases and controls and its association with risk of RA.

	Controls (n=91) N. (%)	RA Patients (n=101) N. (%)	OR (95% CI)	P Value
Genotype				
Arg/Arg	72 (79.12)	24 (23.76)	1 a	
Arg/Pro+ Pro/Pro	19 (20.88)	77 (76.24)	12.57 (6.14 to 24.05)	0*
Allele				
Arg	160 (87.91)	74 (36.63)	1 a	
Pro	22 (12.09)	128 (63.37)	12.57 (7.40 to 21.36)	0*

*N: number, %: percentage, OR: odds ratio, CI :confidence interval, p: significance, a: genotype saved as reference category, *: p<0.05 considered as statistically significant.*

Table 3: Frequency distribution of MDM2 SNP309 between cases and controls and its association with risk of RA

	Control (n= 91) N. (%)	AR Patients (n=101) N. (%)	OR (95% CI)	P Value
Genotype				
TT	32 (35.17)	21 (20.80)	1	
TG	41 (45.05)	45 (44.55)	1.67 (0.83 to 3.34)	0.14
GG	18 (19.78)	35 (34.65)	2.96 (1.34 to 6.53)	0.006* 0.02
Allele				
T	105 (57.69)	87(43.07)	1	
G	77 (42.31)	115 (56.93)	1.80 (1.20 to 2.70)	0.004*

*N: number, %: percentage, O: odds ratio, CI: confidence interval, p: significance, a: genotype saved as reference category, *: p<0.05 considered as statistically significant.*

Table 4. Analysis of joint effects for TP53 Arg72Pro and MDM2 SNP309 genotypes on RA risk

Genotypes		Controls (n=91) N.(%)	AR Patients (n=101) N. (%)	OR (95% CI)	P Value
TP53	MDM2				
Arg/Arg	TT	30 (32.96)	10 (9.9)	1 a	
Arg/Arg	TG+GG	40 (43.95)	30 (29.7)	2.25 (0.95 to 5.30)	0.06
Arg/Pro +Pro/pro	TT	10 (11)	13 (12.87)	3.9 (1.30 to 11.61)	0.01*
Arg/Pro +Pro/pro	TG+GG	11 (12.09)	48 (47.52)	13.09(4.96 to 34.54)	0*

*N: number, %: percentage, OR: odds ratio, CI: confidence interval, p: significance, a: genotype saved as reference category, *: p<0.05 considered as statistically significant.*

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