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Original Research Article

Genetic analysis of the M2/ANXA5 haplotype in Syrian healthy population

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ABSTRACT

Background: Annexin A5 (ANXA5) is an abundantly and ubiquitously expressed protein showing the highest levels of concentration in kidney, liver and placenta. ANXA5 plays a central role in the machinery of membrane repair by enabling of a protective 2D bandage at membrane damaged site, has properties anti-inflammatory, pro-fibrinolytic and anti-thrombotic. Four polymorphisms have been identified in the ANXA5 promoter, which were transmitted as a joint haplotype (M2). M2 haplotype decrease the ANXA5 gene promoter activity and mRNA expression which causes several troubles.
Methods: The aim of this cross-sectional study is to determine the frequency of M2/ANXA5 haplotype in healthy Syrian individuals and compare the genotype and allele distribution with other populations. In this study 94 (female, 71 and male, 23) unrelated healthy Syrian nationals were involved. 94 DNA samples have been collected in order to determine the spread the genotype M2 haplotype using allele specific polymerase chain reaction (AS-PCR).
Results: Our results indicate that the distribution of the alleles and genotypes of M2 haplotype vary considerably in different populations. In the Syrian population the distribution of M2 and wide type (WT) were M2/M2 9.6%, M2/WT 44.7%, WT/WT 45.7%. The M2 haplotype was found in 45 of women (allele frequency 31%) and in 15 of men (allele frequency 32%). The distribution of the ANXA5 genotypes in Syrian study group conformed to Hardy-Weinberg equilibrium (p=0.92).
Conclusions: No significant differences were found at frequency distribution of different genotypes and M2 allele between women and men within this Syrian cohort. In comparison with the results of other studies, the results of this study demonstrate that the frequency and distribution of the M2 haplotype in Syrian population are different from most other populations worldwide.

Keywords: Annexin A5, M2 haplotype, SNP, Syrian population

INTRODUCTION

Annexins (ANXs) are a large family of proteins with calcium-dependent affinity for membrane phospholipids.[1] They are widely distributed among eukaryotes but largely absent in prokaryotes and yeasts.[2,3] ANXs participate in numerous membrane-related processes, including exo- and endo-cytosis, vesicle trafficking, regulation of blood coagulation and inflammation, membrane aggregation and fusion, and the regulation of membrane dynamics and

organization.[2,4] Strikingly, although ANXs do not possess a secretory signal peptide, several of them have been found both intracellularly and extracellularly.[5] ANXA5, the most abundant member of this family, was initially localized at the surface of the placental syncytiotrophoblast layer and performs a vital anticoagulant function. ANXA5 is later found in many tissues and in the bloodstream.[6-8] The circulating ANXA5 binds platelets and red cells, and decreases phospholipid availability for the cascade of coagulation factors.[9] Recently, ANXA5 has been detected in several organs of the human male reproductive system, including the testes, epididymis, prostate, and sperm.[10,11] It has been shown that ANXA5 participates in a wide range of pathological and physiological processes, including coagulation, inflammation, signal transduction, cell apoptosis, and cell differentiation.[12] ANXA5 gene located on human chromosome 4q27 and consists of 13 coding exons.[13] The proximal core of the promoter region has two common variations, in the form of haplotypes (M1 and M2). The haplotype M2/ANXA5 comprises 4 consecutive single nucleotide polymorphisms (SNPs): -19G→A (G-467A, rs112782763), 1A→C (A-448C, rs28717001), 27T→C (T-422C, rs28651243) and 76 G→A (G-373A, rs113588187). When only 2 of the 4 variants (rs28717001 and rs28651243) were present, the haplotype was defined as M1.[14] These variants diminish the ANXA5 promoter activity and decrease mRNA expression, with the influence of the haplotype M1/ANXA5 being less pronounced than the one from haplotype M2/ANXA5.[15-17] It has been reported that M2 haplotype is associated with the risk of obstetric pathologies such as preeclampsia, fetal growth restriction, and recurrent pregnancy loss (RPL) a pregnancy complication related to thrombophilia.[18,19] Interestingly, the risk factor status of the haplotype M2/ANXA5 is independent of the parental origin; i.e. there is an equal contribution of the maternal and the paternal allele in the obstetric complications risk.[8,9] Numerous reports have presented the frequency of the M2 haplotype in ANXA5 gene and their association to different diseases in different ethnic groups. This study aims to investigate the distribution of M2/ANXA5 haplotype in unrelated healthy individuals in Syria.

METHODS

Study subjects

This prospective cross-sectional study was conducted at Maternity Hospital, Faculty of Medicine, Damascus University Syria, during six months period from 01 November 2020 to 01 May 2021. This study was approved by the local ethics committee at Damascus University and Health Ministry and complied with the Helsinki declaration of the World Medical Association (2013) and informed consent was obtained from each individual prior to recruitment and collection of blood samples for DNA extraction. Blood samples were collected on EDTA from 94 normal individuals (female, 71; male, 23) from Syrian population. All cases were pregnant females with a normal pregnancy and their birth was normal, in addition to healthy males who did not have any chronic disease, aged between 18 and 36.

Genotyping

DNA was extracted from blood samples using commercial kit (Promega kit, USA). DNA concentration was measured using the Thermo® Nano Drop device. The nested polymerase chain reaction (nested-PCR) was performed on the extracted genomic DNA samples. The primers used to amplify the promoter region of the human ANXA5 gene (GenBank accession number: AC096730) were as follows: Fw, 5`-CCACGCACTATGTTGAGCAC-3`; Rv, 5`-ACCACGCTCTCCTCTCCAG-3`.[20] PCR reaction conditions were optimized using following amplification program: initial denaturation temperature 94 ºC for 5 min, then 35 cycles of the following steps (denaturation step at 94 ºC for 30 sec, annealing at 56 ºC for 30 sec, followed by extension at 72 ºC for 40 sec). Finally, 72 ºC for 5 min to obtain the desired product with a length of 570 bp. The expected length of the amplicon was confirmed using the bioinformatics (Geneious 4.8.4) program. The allele specific polymerase chain reaction (AS-PCR) was performed on the product with a length of 570 bp, in order to detect the haplotype M2 and non-mutated promoter region. This is done through two separate reactions and by applying two PCR reactions according the following conditions: initial denaturation at 94 ºC for 5 min, then 35 cycles of the following steps (denaturation at 94 ºC for 30 sec, annealing at 68ºC for 30 sec, followed by extension at 72 ºC for 30 sec).

Finally, 72 ºC for 5 min to obtain the required product with a length of 139 bp. The expected length of the amplicon was confirmed using the bioinformatics (Geneious 4.8.4) program. The following primers were used to detect the pattern normal primers (F: 5`-TGGCGCGGCCGGCCTGCGGTTGG-3`; R:5′-GAGATGCAGACGCTGAAGGATC-3`) and M2/ANXA5 primers (F: 5`-TGGCGCGGCCGGCCTGCG GTTGA-3` ; R:5`- GAGATGCAGACGCTGAAG GATCT-3`.[21] The PCR products were resolved on a 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide using a gel electrophoresis system (Biorad, USA) at 100 V for 20–30 min. The gel was visualized under UV light using a transilluminator system (Cleaver Scientific Ltd., UK).

Statistical analysis

The statistical analyses were performed using statistical package for the social sciences (SPSS) (version 25.0) "IBM" statistical program. The qualitative variables were given as frequency and percentage. Deviations from Hardy–Weinberg equilibrium (HWE) among the genotypes were first evaluated using the χ^2 test. The distribution of alleles, genotypes and haplotypes was compared between Syrian population and the inhabitants of some countries of the world using two-tailed Fisher's exact test and (Pearson Chi-square). Tests were assumed significant whenever $p<0.05$.

RESULTS

Genotyping

The genomic DNA of 94 samples was extracted and used in nested-PCR reaction to obtain a fragment of 570 bp (Figure 1A). Later, this amplicon was used as a template for AS-PCR reaction, to discriminate the M2 and the normal haplotypes accurately. In result, a fragment of 139 bp (Figure 1B) was obtained, and the individuals having the non-mutated promoter region (WT/WT), presenting the haplotype M2 (M2/M2) and heterozygous carriers of the haplotype M2 (WT/M2) were identified.

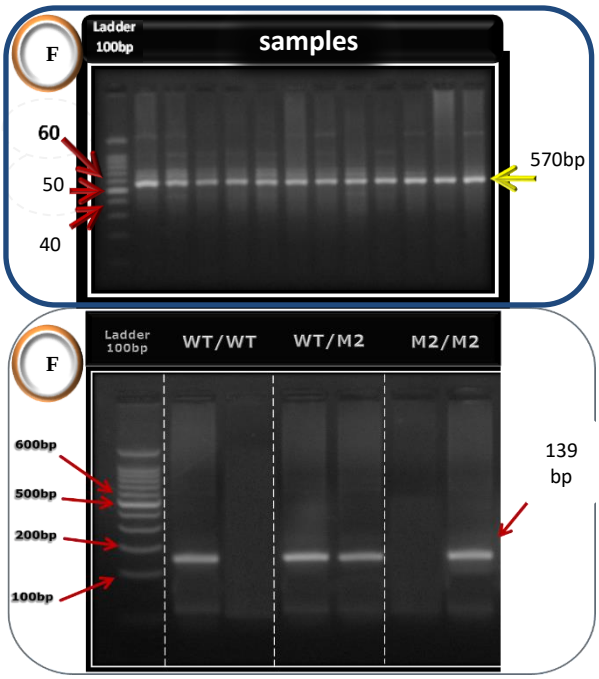

Fig. 1. A: agarose gel electrophoresis of the 570 bp nested-PCR products (lanes 1-12), Lane M indicates the 100 bp DNA size ladder. B: Agarose gel electrophoresis of PCR products for the different genotypes: homozygous WT/WT, heterozygous WT/M2 and homozygous M/M. genotype, lane M: indicates the 100 bp DNA size ladder.

Statistical analysis

The genotype frequencies and allele frequencies in the Syrian population are shown in Table 1. Using two-tailed Fisher's exact test and Pearson Chi-square, no significant differences in the distribution of different genotypes (M2/M2, M2/WT and WT/WT) and M2 allele were found between female and male as shown in Table 1. Out of the 94 subjects (female, 71; male, 23) analyzed for M2 haplotype, the following genotypic frequencies were obtained: M2/M2 9.6%, M2/WT 44.7%, WT/WT 45.7%. The allelic frequency of M2 vs WT was 31.9% vs 68.1%. The distribution of the ANXA5 gene genotypes in Syrian population was conformed to Hardy-Weinberg equilibrium (p=0.92).

Table 1: Genotype and allele frequencies of ANXA5 gene polymorphism (M2 haplotype) in Syrian population.

Index	Study group n (%)	Female n (%)	Male n (%)
Genotype	n=94	n=71	n=23
M2/M2	9 (9.6)	6 (8,4)	3 (13)
M2/WT	42 (44.7)	33 (46.5)	9 (39)
WT/WT	43 (45.7)	32 (45.1)	11 (48)
Allele	n=188	n=142	n=46
AF M2	60 (31.9)	45 (31.7)	15 (32.6)
AF WT	128 (68.1)	97 (68.3)	31 (67.4)

Comparison of the genotype and allele frequencies of the haplotype M2/ANXA5 was done between Syrian population and other populations that mentioned in different studies such as Portuguese, Netherlander, German, Malaysian, Estonian and Danish, populations using χ^2 test.[20-24] In this study, significant differences in the distribution of genotypes and alleles of M2 haplotype were found between Syrian individuals and each of the populations of Portugal, Netherlands, Germany, Estonia and Denmark as shown in Table 2. No significant difference in the genotype frequency of M2 between Syrians and Malaysians was observed, while significant difference was found in the M2/ANXA5 allele frequencies between these two populations as shown in Table 2.

Table 2: Comparison of genotypes and allele frequency of ANXA5 gene polymorphism (M2 haplotype) between Syrian population and other different populations.

Popula-tions	No.	Genotype n (%)			P value		Allele n (%)		P value	
		M2	M2/WT	WT			AF M2	AF WT		
Syria	n=94	9 (9.6)	42 (44.7)	43 (45.7)	Ref		60 (31.9)	128 (68.1)	Ref	
Portugal	n=84	1 (1.19)	12 (14.3)	71 (84.5)	3.9771E-7 =0.0	*3	14 (8.4)	154 (91.6)	2.2466E-8 =0.0	*3
Nether-lands	n=131	1 (0.8)	27 (20.6)	103 (78.5)	4.9966E-7 =0.0	*3	29 (11.15)	233 (88.85)	7.5669E-8 =0.0	*3
North German	n=533	5 (0.9)	77 (14.4)	451 (84.7)	3.3034E-18=0.0	*3	87 (8.2)	979 (91.6)	1.5664E-16=0.0	*3

Continued.

Popula-tions	No.	Genotype n (%)			P value		Allele n (%)		P value	
		M2	M2/WT	WT			AF M2	AF WT		
West German	n=500	10 (2)	31 (6.2)	459 (91.8)	3.3034E-18=0.0	*[3]	51 (5.1)	949 (94.9)	2.9378E-23=0.0	*[3]
Malaysia	n=360	18 (5.0)	134 (37.2)	208 (57.8)	0.059473	NS	170 (23.6)	550 (76.4)	0.023699	*
Estonia	n=97	3 (3)	23 (23.7)	71 (73.3)	0.000456	*	29 (15)	165 (85)	0.000099	*[3]
Denmark	n=115	2 (1.7)	25 (21.7)	88 (76.6)	0.000014	*	29 (12.6)	201 (87.4)	0.000002	*[3]

AF=allele frequency, NS=not significant, *p<0.05, **p<0.01, ***p<0.001

DISCUSSION

Ethnic differences in the distribution of M2/ANXA5 haplotype have been reported in literature. The M2 allele has been shown to be present in a substantially lower frequency in Portuguese and German populations compared to Netherlander and Danish populations and Malaysians.[25] In this study, the genotypes and allele frequency of M2/ANXA5 haplotype in Syrian healthy population were demonstrated and compared with those reported in different studies worldwide as shown previously in Table 2.

Our results showed that the genotype distribution of the M2/ANXA5 haplotype in Syrian population is similar to the Malaysians, since there was no significant difference in the genotypes frequency, while allelic frequency was significantly different between the two populations. However, statistically significant differences in the genotype and allele frequencies of M2/ANXA5 haplotype between Syrians and Europeans from Portugal, Netherlands, Germany, Estonia and Denmark were observed.

Recently the M2 haplotype, a sequence variation in the promoter region of ANXA5 gene was identified as a risk factor for adverse pregnancy outcomes such as premature birth, small for gestational age, pre-eclampsia, placental abruption and recurrent pregnancy loss (RPL).[14] These obstetric pathologies collectively complicate up to 15% of pregnancies and are the leading causes of maternal and fetal morbimortality in developed countries.[26] In fact, M2 haplotype was originally found to be associated with RPL in a German patient cohort.[27] Subsequent studies confirmed the M2/ANXA5 association with RPL among Italian, German, Bulgarian, UK white European, Japanese, Malay and other populations.[25]

Reporter gene assays have demonstrated a reduction in the activity of the ANXA5 promoter region when the M2 haplotype is present.[16,28] Since the syncytiotrophoblast, which carries the ANXA5 protein at its surface, is of fetal origin it has been proved that reduced *ANXA5* expression was independent of M2 allele parental origin, making the M2 haplotype a hereditary factor causing pregnancy pathology by affecting embryonic-induced anticoagulation.[16,17]

Furthermore, ANXA5 expression has been detected in many parts of mal reproductive system.[13,17] In rabbits, ANXA5 protein is the main protein component of seminal plasma and influences sperm concentration, motility, and morphology.

Limitations

Cross-sectional studies are usually simple to do and inexpensive. Furthermore, these usually do not pose much of a challenge from an ethics viewpoint. However, this design does carry a risk of selection bias or measurement bias, i.e. the results of the study may not represent the true situation in the population.

CONCLUSION

In this study, we have determined the frequency of M2/ANXA5 haplotype in Syrian population. Further studies need to be conducted to structure between the M2/ANXA5 haplotype and adverse pregnancy outcomes in Syria.

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