GENETIC ANALYSIS OF 5 α REDUCTASE TYPE II ENZYME IN RELATION TO OXIDATIVE STRESS IN CASES OF *ANDROGENETIC ALOPECIA* IN A SAMPLE OF EGYPTIAN POPULATION

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Abstract

Objective: To study the genetic polymorphism of 5-α reductase type II enzyme in relation to oxidative stress in cases of androgenetic alopecia (AGA) in a sample of Egyptian population.

Materials and Methods: This study was conducted on 45 patients with different grades of AGA,and 45 healthy subjects as control group. Laboratory tests included DNA extraction from blood, amplification of the 5-α reductase type II by PCR and V89L mutation analysis by restriction endonuclease enzyme RsaI, and estimation of the levels of plasma catalase and erythrocyte lysate superoxide dismutase (SOD) enzymes by colorimetric methods.

Results: The studied subjects carrying the homozygote(LL) and the heterozygote (VL) genotypes were of no risk of developing AGA.(OR=0). Regarding the leucine allele, the studied subjects carrying the (L) allele were at about 3.7 higher risk of AGA .(OR=3.692), and the results were statistically significant (p<0.001). There was significant increase in the level of SOD and catalase in patients than in control group(p=0.005),and (p<0.001) respectively,plasma catalase is significantly higher in patients with LLvariant than inVL variant (p=0.020). Asignificant relations was found between the severity of the disease and age and family history (p=0.037), and (0.036) respectively, there was no significant correlation between the level of catalase enzyme and SOD in one hand and the severity of the disease among patients.

Conclusions: There is a possible association between AGA and V89L genetic polymorphism of 5-alpha reductase type II enzyme, patients carrying the mutant leucine (L) allele have a risk for developing AGA. Also there is possible association between AGA with oxidative stress.

Key words: androgenetic alopecia; 5-α reductase type II; oxidative stress

Introduction

Androgenetic alopecia (AGA), the most common type of baldness in both males and females, it is a hereditary thinning of the hair induced by androgen in genetically susceptible individuals that has its onset in late adolescence. AGA is found to be a stressful condition affecting the psychological functioning of an individual [1-3]. About 50% of male population shows some degree of AGA around 50 years of age, and about 12% of women by the age of thirty and up to 41% of women by the age of seventy. The global incidence varies among the different ethnic groups [4-6].

The most important etiological factor for AGA is genetic predisposition; The suspicion of polygenic inheritance is under investigation [7-9]. The 5-α reductase enzyme is responsible for the chemical reaction that converts the hormone testosterone in to the more potent Dihydrotestosterone (DHT) hormone. There are two isoenzymes of the enzyme, steroid 5-α reductase, type 1 and type II [10–13]. Cases with androgenic alopecia have higher levels of 5-α reductase which is present predominantly in the scalp,increased sensitivity of hair follicles to Dihydrotestosterone (DHT), which in turn causes miniaturization of the hair follicles [7,10].

Environmental factors play a role in the development of AGA by causing oxidative stress, which is a disturbance in the normal redox (oxidation–reduction) state of cells that can cause toxic effects that damage all components of the cell [14,15].

Superoxid dismutase and catalase are enzymes which are an important antioxidant defense in nearly all cells exposed to oxygen. The decrease in their levels or activities can cause an oxidative stress status of the cells of the body including hair keratinocyets [14,16].

Aim

The aim of the work is to study the genetic polymorphism of 5-α reductase type II enzyme in relation to oxidative stress in cases of androgenetic alopecia in a sample of Egyptian population.

Materials and Methods

This study enrolled 90 candidates, divided into two groups, (group I) including 45 patients with different grades of androgenetic alopecia. (group II) as control group,including 45 individual who were not suffering from androgenetic alopecia. Age ranged from 20 to 60 years in both groups. Individuals suffering from increased steroid hormones activity were excluded from the study.

The patients were selected from the dermatology outpatient clinic of the main university hospitals, faculty of medicine, university of Alexandria. After obtaining the approval of ethical committee, both patients and control subjects were subjected to full history taking, dermatological examination, assessment grades of AGA using Hamilton-Norwood's classification in males and Ludwig Classification of Female Hair Loss,and laboratory investigations.

1. Molecular analysis:

Genomic DNA was extracted from peripheral blood anticoagulated with EDTA, using a spin column protocol [GeneJET™ Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific Inc. http://www.thermoscientific. com/fermentas)]. The total DNA yield was determined by spectrophotometer through absorbance at 260nm (A260) using Tris-EDTA (TE) buffer as a blank. The quality of DNA was assessed as well by measuring the absorbance at both 260nm and 280nm (A260/A280 ratio) [17].

Polymorphism of 5-α reductase enzyme type II is analysed by PCR-restriction fragment length polymorphism (RFLP). RsaI RFLP was determined by amplification of a 349bp fragment of exon 1 using forward primer 5'-CGCCTGGTTCCTGCAGGAGCT-3'and reverse primer 5'GTGAAGGCGGCGTCTGTG-3' (Thermo Fisher Scientific Inc. http://www.thermoscientific.com/ fermentas). PCR amplification of genomic DNA was carried out using 25µl of PCR master mix, 0.5 µM of forward primer, 0.5 µM of reverse primer,50 nanogram of extracted genomic DNA. The volume is completed to 50µl with deionized water. The thermal conditions required for the reaction were

Initial denaturation at 95ºC for 10 minutes. Followed by 35 cycles of 95ºC for 1 min, 60ºC for 1 min, 72ºC for 30 s, followed by a final extension at 72ºC for 10 min. The products were then digested with 5 unites of RsaI (Thermo Fisher Scientific Inc. http://www.thermoscientific.com/fermentas). Digested products or the restriction fragments were separated by electrophoresis on 2% agarose gels containing ethidium bromide and visualized by UV illumination [Biometra. http://www.biometra.com] [18].

2. Estimation of SOD in erythrocyte lysate by a colorimetric method [19].

This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. The lysate was diluted with distilled water so that the % inhibition falls between 30% and 60%. Measurement of the increase in absorbance was performed at 560 nm for 5 minutes for control (Δ Acontrol) and for sample (Δ Asample) at 25ºC. Where: (Δ Acontrol) = the change in absorbance at 560 nm over 5 minutes following the addition of PMS to the reaction mixture in the absence of sample (Δ A sample) = the change in absorbance at 560nm over 5 minutes following the addition of PMS to the reaction mixture in the presence of sample.

$$\text{Percent inhibition} = \frac{\Delta A \text{ control} - \Delta A \text{ sample}}{\Delta A \text{ control}} \times 100$$

SOD Activity U/ ml = % inhibition × 3.75.

3. Determination of plasma catalase level by a colorimetric method [20].

Catalase reacts with a known quality of H2O2. The reaction is stopped after exactly one minute with catalase inhibitor. In the presence of peroxidase (HRP), the remaining H2O2 react with 3,5-dichloro-2-hydroxybezene sulfonic acid (DHBS) and 4-aminophenazone(AAP) to form a chromophore. It was read at 510nm and the color intensity is inversely proportional to the amount of catalase in the original sample.Samples were read against a sample blank (Asample), and a standard was read against a standard blank (Astandard) at 510nm.

$$\text{Catalase activity in plasma (U/L)} = \frac{A \text{ standard} - A \text{ sample}}{A \text{standard}} \times 1000.$$

Results

Clinical characteristics of patients and controls were illustrated in Table I. The severity of AGA in male patients was determined according to the Hamilton Norwood classification, 7 (25.9%) patients were of grade III, 2 (7.4%) patients were of grade IV, 6 (22.2%) patients were of grade V and 12 (44.4%) patients were of grade VI. For the female patients, the severity was determined according to the Ludwig classification of female hair loss:7 (38.9%) patients were of grade I,10 (55.6%) patients were of grade II, and 1 (5.6%) patient was of grade III.

Genotyping was performed in AGA patients and control subjects for the 5 α reductase type II enzyme gene polymorphism using RFLP-PCR and gel analysis. There are three possible genotype for the type II 5-α reductase; a normal homozygote for valine residue (VV), a mutant homozygote for leucine residue (LL), a polymorphic heterozygote which has both valine and leucine (VL). The homozygote (VV) genotype produces 2 bands at 93 bp and 256 bp, while the homozygote (LL) genotype produces 2 bands at 93bp and 236 bp, and the heterozygote (VL) genotype produces 3 bands at 93bp, 236bp and 256bp (Fig. 1).

The frequency of the three genotypes is illustrated in Table II. Based on chi-square, Fisher Exact and odd ratio tests, the studied subjects carrying the homozygote (LL) and the heterozygote (VL) genotypes were of no risk of developing AGA. (OR=0). Regarding the leucine allele, the studied subjects carrying the (L) allele were at about 3.7 higher risk of AGA. (OR=3.692), and the results were statistically significant (p<0.001). These results are presented in Table III. The relation between genotype with sex and family history of androgenetic alopecia is illustrated in Table IV.
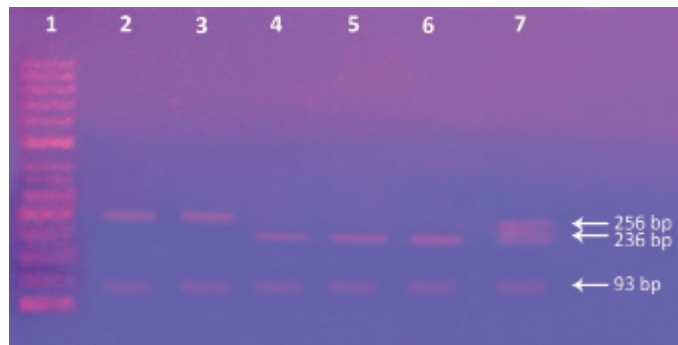
Figure 1. The V89L genetic polymorphism of 5 alpha reductase type II enzyme on agarose gel after digestion by RsaI enzyme. Lanes 2 and 3 showing 2 bands at 93 and 256 bp representing the homozygote (VV) genotype. Lanes 4,5,and 6 showing 2 bands at 93 and 236 bp representing the homozygote (LL) genotype. Lanes 7 showing 3 bands at 93,236 and 256 bp representing the heterozygote (VL) genotype. Lane 1 represents Gene rulerTM Ultra Low Range DNA Ladder, fermentas, Canada.

	Patients		Control		Test of sig.
	No.	%	No.	%	
Sex					
Male	27	60.0	14	31.1	χ^2p = 0.006*
Female	18	40.0	31	68.9	
Age					
<30	10	22.2	23	51.1	
30 – 50	19	42.2	20	44.4	χ^2p <0.001*
>50	16	35.6	2	4.4	
Mean ± SD	43.47 ± 12.86		31.71 ± 10.50		^{t}p <0.001*
Duration of AGA					
Mean ± SD	13.37 ± 9.03				
Family history of AGA	No		%		
-ve	13		28.9		
+ve	32		71.1		

Table I. Clinical characteristics of patients with androgenetc alopecia (AGA) and controls.

p: p value for comparing between the two studied group; * significant at p ≤ 0.05; χ^2: Chi square test;

t: t-test

	Patients (n=45)		Control (n=45)		Test of sig.
	No.	%	No.	%	
Genotype					
VV	0	0.0	9	20.0	^{FE}p = 0.003*
VL	26	57.8	36	80.0	χ^2p = 0.023*
LL	19	42.2	0	0.0	χ^2p <0.001*
P	<0.001*				
Allele frequency					
V	26	28.9	54	60.0	χ^2p <0.001*
L	64	71.1	36	40.0	

Table II. Comparison between the two studied groups according to the genotype and alleles frequency.

p: p value for comparing between the two studied group; * significant at p ≤ 0.05; χ^2: Chi square test;

MC: Monte Carlo test; FE: Fisher Exact test

	Patients (n=45)		Control (n=45)		Test of sig.	OR	95% CI (LL-UL)
	No.	%	No.	%			
Genotype							
VV	0	0.0	9	20.0	^{FE}p = 0.003*		
VL	26	57.8	36	80.0	χ^2p = 0.023*	-	-
LL	19	42.2	0	0.0	χ^2p <0.001*	-	-
MCp	<0.001*						
Allele frequency							
V ®	26	28.9	54	60.0	χ^2p <0.001*		
L	64	71.1	36	40.0		3.692	1.984 – 6.870

Table III. The risk of having V89L polymorphism and the leucine allele in relation to androgenetic alopecia.

p: p value for comparing between the two studied group; * significant at p ≤ 0.05; χ^2: Chi square test;

MC: Monte Carlo test; FE: Fisher Exact test

	Genotype							
	Patients (n=45)				Control (n=45)			
	LL		VL		VL		VV	
	No.	%	No.	%	No.	%	No.	%
Sex								
Male	12	63.2	15	57.7	13	36.1	1	11.1
Female	7	36.8	11	42.3	23	63.9	8	88.9
Test of sig	χ^2(p) = 0.137 (0.712)				^{FE}p= 0.236			
Family history								
-ve	7	36.8	6	23.1				
+ve	12	63.2	20	76.9				
Test of sig	0.341							

Table IV. Relation between genotype with sex and family history of androgenetic alopecia.

p: p value for Chi square test for comparing between the two studied group; * significant at p ≤ 0.05; χ^2: Chi square test;

FE: Fisher Exact test

Regarding the level of lysate SOD,and plasma catalase,there was significant increase in the level of SOD and catalase in patients than in control group (p=0.005), and (p<0.001) respectively Table V. The relation between plasma catalase enzyme and erythrocyte lysate SOD% and sex, family history and smoking in patients group is illustrated in Table VI. The relation between antioxidant markers and genotype is presented in Table VII, regarding catalase, there was a statistically significant difference between the homozygote (LL) genotype and the heterozygote (VL) genotype, where p = 0.020. While in SOD enzyme, non statistically significant difference was found between the homozygote (LL) genotype and the heterozygote (VL) genotype, where p = 0.530.

Relations between the severity of androgenetic alopecia with genotypes, age, smoking, and family history of androgenetic alopecia in patients group is presented in Table VIII.

	Patients	Control	p
Catalase (U/L)			0.005*
Min. – Max.	15.60 – 384.0	173.0 – 391.0	
Mean ± SD	243.19 ± 88.35	288.33 ± 57.93	
SOD (%)			<0.001*
Min. – Max.	38.0 – 90.0	72.0 – 99.0	
Mean ± SD	67.60 ± 12.49	85.60 ± 6.60	

Table V. Comparison between the two studied groups according to plasma catalase enzyme and erythrocyte lysate SOD enzymes.

p: p value for comparing between the two studied group; * significant at p ≤ 0.05

Catalase					
Sex		Family history		Smoking	
Male	Female	-ve	+ve	No	Yes
238.76 ± 97.83	249.83 ± 74.09	265.08 ± 71.23	234.30 ± 93.99	243.24 ± 83.25	243.13 ± 96.56
P = 0.685		P = 0.295		P = 0.997	
SOD%					
Sex		Family history		Smoking	
Male	Female	-ve	+ve	No	Yes
66.59 ± 13.63	69.11 ± 10.75	66.38 ± 12.70	68.09 ± 12.58	67.60 ± 12.63	67.60 ± 12.65
P = 0.514		P = 0.682		P =1.000	

Table VI. Relation between plasma catalase enzyme and erythrocyte lysate SOD with sex, family history and smoking in patients group.

p: p value for comparing between the two studied group; * significant at p ≤ 0.05

	Genotype	
	Patients	
	LL	VL
Catalase (U/L)		
Min. – Max.	161.0 – 358.0	15.60 – 384.0
Mean ± SD	275.63 ± 48.22	219.48 ± 103.35
P	0.020*	
SOD%		
Min. – Max.	49.0 – 85.0	38.0 – 90.0
Mean ± SD	66.21 ± 12.64	68.62 ± 12.53
P	0.530	

Table VII. The relation between plasma catalase and erythrocyte lysate SOD with the genotype of patients with androgenetic alopecia.

p: p value for comparing between the two studied group; * significant at p ≤ 0.05

Regarding the correlation between the severity (grading) of AGA with catalase enzyme, there was no significant correlation between the level of catalase enzyme and the severity of the disease among patients, where p= 0.107 and 0.668, and r= 0.125 and 0.066 in male and female patients respectively. Also there was no significant correlation between the level of SOD enzyme and the severity of the disease among patients, where p= 0.123 and 0.500 and r= 0.072 and -0.239 in male and female patients respectively.

Discussion

A relationship between the V89L genetic polymorphism of 5-α reductase enzyme and androgenetic alopecia (AGA) has been suggested. In our work we assumed that the genetic polymorphism of the 5-α reductase type II enzyme, would be in close proximity to the etiologic genetic mutations that cause AGA.

In this work, there was a statistically significant difference between the two studied groups regarding the frequency of the three genotypes, where the patients group had a higher frequency of the abnormal mutant polymorphic (LL) genotypes than the control subjects, while the control group had a higher frequency of the normal (VV) genotypes and polymorphic heterozygote (VL) type than the patients group.

Regarding the individual valine and leucine alleles, there was also a statistically significant difference between the two groups. The frequency of (V) allele was higher in the control group, while the frequency of allele (L) was higher in the patients group. We also found that the studied subjects carrying the (L) allele, which was higher in patients group, were at about 3.7 higher risk of developing AGA.

This work also revealed no statistically significant difference between the different genotypes of 5-α reductase type II enzyme, with either the sex, or the family history of AGA. Regarding the severity of AGA, we found that there was no statistically significant difference between the different grades of AGA in relation to the genotypes carried by the patients group.

As AGA being an androgen dependant condition, a relation between the increase of androgens levels, and the V89L genetic polymorphism of the 5-α reductase type II enzyme has been suggested. In 1997, Vilchis et al [21] revealed that the V89L polymorphism of type II 5α reductase gene represents a silent polymorphism which does not alter the phenotypical development among a sample of Mexican population.

	Grading (severity) of AGA													
	Male								Female					
	III (n = 7)		IV (n = 2)		V (n = 6)		VI (n = 12)		I (n = 7)		II (n = 10)		III (n = 1)	
	No	%	No	%	No	%	No	%	No	%	No	%	No.	%
Genotype														
VV	5	71.4	1	50.0	3	50.0	3	25.0	4	57.1	3	30.0	0	0.0
LL	2	28.6	1	50.0	3	50.0	9	75.0	3	42.9	7	70.0	1	100.0
VL	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
MCp	0.225								0.463					
Age														
20 – 30	2	28.6	0	0.0	0	0.0	1	8.3	5	71.4	1	10.0	1	100.0
31 – 40	3	42.9	1	50.0	2	33.3	0	0.0	0	0.0	3	30.0	0	0.0
41 – 50	1	14.3	0	0.0	1	16.7	1	8.3	2	28.6	5	50.0	0	0.0
51 – 60	1	14.3	1	50.0	3	50.0	10	83.3	0	0.0	1	10.0	0	0.0
MCp	0.037*								0.075					
Smoking														
No	2	28.6	1	50.0	2	33.3	2	16.7	7	100.0	10	100.0	1	100.0
Yes	5	71.4	1	50.0	4	66.7	10	83.3	0	0.0	0	0.0	0	0.0
MCp	0.624								-					
Family history														
-ve	3	42.9	0	0.0	0	0.0	0	0.0	5	71.4	5	50.0	0	0.0
+ve	4	57.1	2	100.0	6	100.0	12	100.0	2	28.6	5	50.0	1	100.0
MCp	0.036*								0.466					

Table VIII. Relations between the severity of androgenetic alopecia with genotypes, age, smoking, and family history of androgenetic alopecia in patients group.

MC: Monte Carlo test; p: p value for Kruskal Wallis test; * significant at p ≤ 0.05

In 2001 Allen et al [22] stated that the 5-α reductase type II enzyme V89L polymorphism is not a strong determinant of serum androgens concentrations in Caucasian men.However, In 2010, Jiang et al [23] found that there was an association between the 5-α reductase V89L variants and the increase of the concentration of serum androgens in Chinese elderly men.

There was a study by Ellis et al [24] which revealed that polymorphic amino acid substitution of the 5-alpha reductase enzyme was shown to influence the activity and pharmacogenetic variation of the enzyme encoded by the mutants of 5-alpha reductase enzyme gene, however, it did not show a significant differences between cases and controls in allele, genotype, or haplotype frequencies, the findings in this study showed that there was no association between AGA with the 5-α reductase genetic polymorphism.Similar results were found by Seog-Jun et al [18] who were not been able to discover that association as well.

A relationship between oxidative stress and AGA has been suggested. SOD and catalase are enzymes which are an important antioxidant defense in nearly all cells exposed to oxygen, thus, the decrease in their levels or activities can cause an oxidative stress status of all body cells including hair keratincyets,which may respond to oxidative stress from irritants, pollutants, and UV irradiation, by producing nitric oxide, and by releasing intracellularly stored IL-1α. This pro-inflammatory cytokine by itself has been shown to inhibit the growth of isolated hair follicles in culture [25].

In this work, the mean of both erythrocyte lysate SOD and plasma catalase levels were significantly lower in patients group than those in control group.We also found that the mean of SOD enzyme was lower in patients carrying the mutant (LL) genotype, than in those carrying the (VL) genotype, but the difference was not statistically significant between the two genotypes.

On the contrary, the mean of plasma catalase enzyme was higher in patients who are carrying the homozygote (LL) genotype, than those who are carrying the heterozygote (VL) genotypes, and there was a statistically significant difference between both groups.

A study performed by Bahta et al, using cultured dermal hair papilla cells (DPC) from balding and non-balding scalp, demonstrated that balding DPCs grow slower in vitro than non-balding DPCs. Loss of proliferative capacity of balding DPCs was associated with changes in cell morphology, and nuclear expression of markers of oxidative stress including catalase and SOD enzyme [26].

There was another study by Upton et al [27] demonstrated that oxidative stress may exacerbate the onset of androgenic alopecia by affecting TGF-β secretion, which is a known inhibitor of hair follicle growth and an inducer of catagen phase.Another study by Naziroglu et al [28] provided some evidence for a potential role of increased lipid peroxidation and decreased antioxidants in alopecia.

In this work there was a positive correlation between the severity of AGA and the positive family history of male patients, but the correlation was non significant in female.

In 1999, Tosti et al [29] revealed that family history predisposes to the early development and rapid progression of AGA. In 2004, Chumlea et al [30] found that men with fathers who had hair loss, were twice as likely to have hair loss than men whose fathers had no history of hair loss.

In 2009, Harvard medical school released a publication which stated that the risk of AGA rises with age, and it's higher in women with a history of hair loss on either side of the family [31]. In 2010, Fatemi et al [32] mentioned that family history is considered one of the important criteria which are needed for the diagnosis of AGA.

In short, our study provides support for the possibility of an association of androgenetic alopecia with the V89L genetic polymorphism of type II 5-α reductase enzyme, also supported the correlation between AGA and oxidative stress, and there was a significant difference between the two studied groups regarding the levels of the antioxidant enzymes.

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