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Exploring skin aging-associated genotypes; Moving toward delivery of precision medicine-based care more than beyond skin deep care: a genome-wide association study

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Abstract

Background Oxidative damage is the principal cellular disturbance in the skin aging. Missense polymorphisms strengthen or weaken detoxification enzyme activity. Determination of deleterious functional effects of polymorphisms in detoxification genes (*NQO1* and *EPHX1*) in skin aging was the overall purpose of conducting this hospital-based research.

Methods Cases recruitment on dermatological examination-based evidence performed sequentially between November 2022, and April 2023 at the Motahari Hospital Dermatology Outpatient Clinic. Genotype analysis was performed using PCR–RFLP and T-ARMS -PCR. All statistical analyses were performed using SPSS software, and differences were taken as significant at $P < 0.05$.

Results This study results implicate that skin aging obtains on a genetic level and in particular the results suggest that His139Arg, Tyr113His and P187S represent true genetic susceptible loci for cutaneous aging related traits. We found that these new susceptibility loci exhibit sex- and age-specific effect on aging skin risk as well as implicated in interactions with modifiable risk factors including water intake, micronutrient care, sleeping habits, sun exposure and application of sunscreen cream, in the development of an increased risk of aging skin.

Conclusions Molecular defects associated with the His139Arg, Tyr113His and P187S polymorphisms manifest as an observable change in the external appearance of the skin. This study underscores the need to move toward scrutinizing the ageing skin changes at molecular levels.

Keywords Aging Skin, Geriatric Skin, Polymorphism, His139 Arg, Tyr113His, P187S

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Introduction

Skin suffers progressive changes to the morphology with increasing age, however, essentially skin aging cannot be seen only in old age, i.e., aging skin is not the entirely result of getting older [1, 2]. The aging of skin can be the inevitable effect of intrinsic factors. Intrinsic factors that promote skin aging are genetic influences (up to 60%) that can give rise to the marked changes in aged skin phenotype include, uneven pigmentation, skin wrinkles, lax appearance, reduced fat tissue, and benign skin neoplasm [3–6]. Genetic profile contributes to the alteration of specific biochemical properties that, at last, causes skin

Table 1 Skin aging symptoms included in the skin aging score 'SCINEXA'

	Skin aging score: SCINEXA Skin aging symptoms	Evaluation
Intrinsic skin aging items	Uneven pigmentation	0/3
	Fine wrinkles	0/1/2/3
	Lax appearance	0/1/2/3
	Reduced fat tissue	0/1/2/3
	Benign skin tumors	0/1/2/3

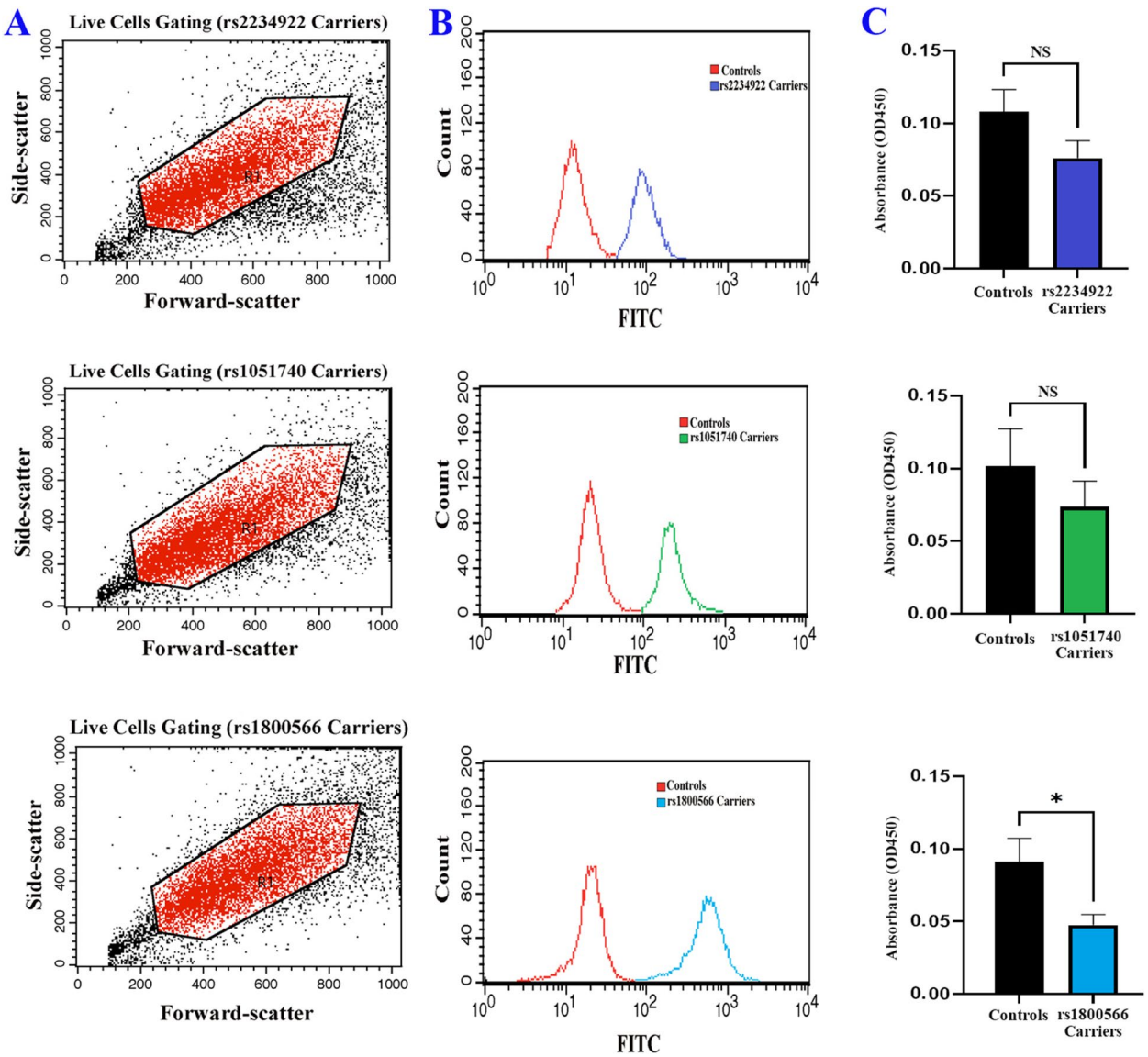

Fig. 1 Flow Cytometry and Enzyme-linked immunosorbent assay analyses using anti-NQO1/EPHX1 antibodies; **A** FSC and SSC, Flow Cytometry gating for single and live cells; **B** Histogram showing cells incubated with anti-NQO1/EPHX1 antibodies for 30 min at 20 °C; **C** Plots show quantitative determination of human Microsomal Epoxide Hydrolase 1 (harboring the Tyr113His and His139 Arg SNPs) and NADPH-quinone oxidoreductase 1 (harboring the P187S SNP) concentration

Table 2 Primers and conditions used in rs2234922 (A > G), rs1051740 (T > C) and rs1800566 (G > A) genotyping

Description	General Characteristics
EPHX1	**rs2234922 (Missense)**
Type of polymorphism	Single-base A > G
Site of polymorphism	His139 Arg
PCR primers	
Forward	5`- AGG GTG GCA GGA CTC AAT ATC TA –3`
Reverse	5`- AGG ATG CCT CTG AGA AGC CAT AG –3`
PCR conditions	
Denaturation	95 °C, 5 min
Annealing	58 °C, 20 s
Extension	72 °C, 15 min
No. of cycles	30
Restriction enzyme	Csp6I (CviQI), (G ↓ TAC sequence)
Restriction Enzymes Product Size (bp)	AA (319 bp)
	AG (153, 166 and 319 bp)
	GG (153 and 166 bp)
EPHX1	**rs1051740 (Missense)**
Type of polymorphism	Single-base T > C
Site of polymorphism	Tyr113His
PCR primers	
Forward outer primer	5`- GTT TTC TGG AAA CAG ACT TTG CTC TTG T –3`
Reverse outer primer	5`- GAT TTG TTG TGA CTG CCA CCA TAT TTT TGCA –3`
Forward inner primer (T allele)	5`- AGA AGC AGG TGG AGA TTC TCA ACA TAT –3`
Reverse inner primer (C allele)	5`- ACC TTC AAT CTT AGT CTT GAA GTG AGG TTG –3`
PCR conditions	
Denaturation	95 °C, 5 min
Annealing	58 °C, 30 s
Extension	72 °C, 30 s
No. of cycles	35
Product Size (bp)	T (234 bp)
	C (261 bp)
	Common (436 bp)
NQO1	**rs1800566 (Missense)**
Type of polymorphism	Single-base G > A
Site of polymorphism	P187S
PCR primers	
Forward outer primer	5`- ATT CTC TAG TGT GCC TGA GGC CTC CT –3`
Reverse outer primer	5`- TGC CTG GAA GTT TAG GTC AAA GAG GCTG –3`
Forward inner primer (G allele)	5`- GCA TTT CTG TGG CTT CCA AGT CTT AGC AC –3`
Reverse inner primer (A allele)	5`- GTG CCC AAT GCT ATA TGT CAG TTG CGA –3`
PCR conditions	
Denaturation	95 °C, 5 min
Annealing	57 °C, 30 s
Extension	72 °C, 30 s
No. of cycles	35
Product Size (bp)	G (176 bp)
	A (196 bp)
	Common (316 bp)

progressive declination. Significant molecular-chemical change driving this process is oxidative stress [6]. Oxidative stress reflects excess production of reactive oxygen species (ROS) and inability to detoxify these reactive products [7]. Most studies suggest that mitochondria is the majority source of intracellular ROS production, which plays the principal role in aging. To ROS scavenging, cutaneous cells utilize endogenous (free radical detoxifying enzyme systems), and exogenous (antioxidant molecules) defense systems [7]. However, genetic variation in the antioxidant enzymes may affect the efficacy of the endogenous antioxidant defense system and susceptibility to oxidative stress, which contributes to excessive oxidative stress. Hence, discovery of genetic single nucleotide polymorphisms (SNPs) in the important participating enzymes in oxidative stress pathways contributes to our understanding of premature aging skin [8]. For instance, genotypes that are associated with "decrease or increase" activity of detoxification (detox) enzymes like Microsomal Epoxide Hydrolase 1 (EPHX1) and NADPH-quinone oxidoreductase 1 (NQO1) which play a crucial role in fighting mitochondrial radicals, may eventually determine individual's susceptibility to genetically induced aging skin [9, 10]. In elaboration, rs1051740 T > C (113 Tyrosine → Histidine in exon 3) and rs2234922 A > G (139Histidine → Argininein exon 4) loci which have been shown to be polymorphic in the reference population (allele frequencies in general population according to the UK Biobank, rs1051740 = 0.35, rs2234922 = 0.22), result in critical amino acid substitutions that are associated with a decrease (39%) or increase (25%) in EPHX1 enzyme activity, respectively [11]. Also the SNP variant of NQO1 enzyme (187Proline → Serine in exon 6) with reduced enzymatic activity is very unstable and thereby rapidly ubiquitinated and degraded by the proteasome (allele frequencies in general population according to the UK Biobank, rs1800566 = 0.32) [12]. To screen out these deleterious missense SNPs of the *NQO1* and *EPHX1* detox genes and determine their functional effects in the aging skin, we herein investigated the relation of the most studied and clinically important missense SNPs

in *EPHX1* (rs2234922 A > G and rs1051740 T > C) and *NQO1* (rs1800566 (G > A)) gene to skin aging.

Methods

Subjects and DNA extraction and enzyme activity assay

Volunteers (40 men and 160 women) aged 35–49 with prevailing intrinsically aged skin based primarily on visual examination, an assessment of the elasticity and extensibility of the skin with physical examination in areas protected from sun (the shadowed areas under the nose and chin, inner side of the upper arm, web spaces between fingers, lower chest region under the thoracic, back and back of upper thigh), and determination of the type of skin aging according to SCINEXA scale (Score for Intrinsic and Extrinsic skin Aging) [13, 14], with determined phototype on the Fitzpatrick's Scale, were sequentially recruited between November 2022, and April 2023, from clients attending Motahari Hospital dermatology clinic (Shiraz, IRAN). Eligible participants with intrinsically aged skin symptoms included those with Fitzpatrick

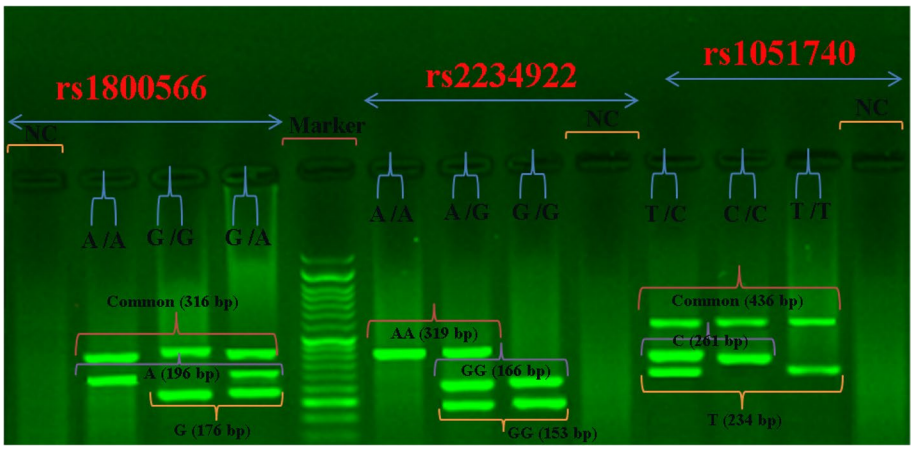

Fig. 2 The pattern of observed bands for rs2234922 (A > G), rs1051740 (T > C) and rs1800566 (G > A) SNPs after agarose-gel electrophoresis (The gel displayed here were cropped slightly and just their ruptured edges have been removed, and were presented without high-contrast (overexposure)

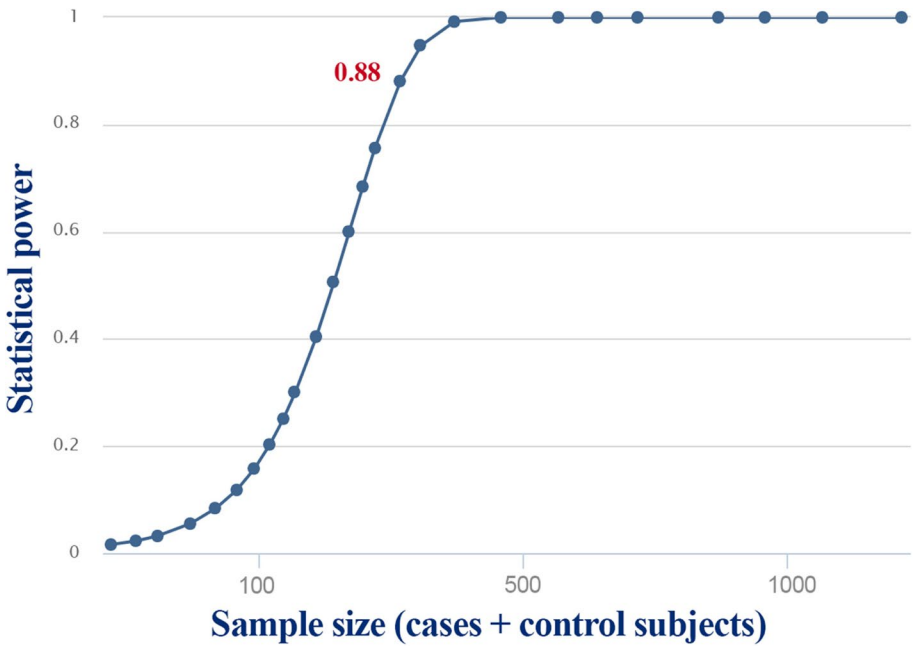

Fig. 3 Power analysis for His139 Arg, Tyr113His, and P187S polymorphisms with α = 0.05

skin types III and IV whom their clinical signs of skin ageing were graded using ordinal scales as follows: 0 (none), 1 (mild), 2 (moderate) and 3 (severe) and binary scale "Yes" (present = 3) or "No" (absent = 0) which was used for uneven pigmentation (for details see Table 1). In general, in the cohort with intrinsically aged skin, subjects who had received any facial esthetic treatments, history of skin cancer, history of immune deficiency or autoimmune disease and history of diabetes, were excluded. Following all participants were fully informed of the study's objectives, 5 ml blood was taken from individuals and stored at room temperature for DNA extraction within the same working day and also at refrigerator for later uses. Subsequently, genomic DNA (g DNA) was extracted by salting out method basis of standard protocol described by Miller and co-workers in 1988 [15]. In brief, a small amount of each of the samples were poured directly into a new 1.5 ml micro tube (Bio plus, Brazil) and heated at 84 °C for 20 min. 100 µl of sucrose 50% (Merck, Darmstadt, Germany) was mixed with the sample and was collected by centrifugation at 14,000 rpm for 15 min at 4 °C. Pellets were resuspended in 100 µl of phosphate buffered saline (Bio idea, Iran), by shaking for 15 s. Finally, the precipitate was obtained by spin down at 8000 rpm for 1 min at 18o and suspended with 50 µl of deionized water for beginning of the PCR processing. NanoDrop ND-2000 UV spectrophotometer (Thermo, Wilmington, NC, USA) was used for quantification of g DNA [16].

To develop a better understanding of the role of EPHX1 and NQO1 polymorphisms in aging skin, we analyzed the enzymes activity in each carrier group of each SNP compared with non-carriers (non-carriers (n = 200) were drawn from the same population as so to be similar to the cases except for not having complication, and providing relatively unbiased estimates). The concentrations of EPHX1 and NQO1 in each group were quantified by Flow Cytometry and Enzyme-linked immunosorbent assay according to a standardized protocol (using Antibodies specific for NQO1 (Abcam Plc., Cambridge, UK) and EPHX1 (Abcam Plc., Cambridge, UK)), in collaboration with Muhammad Rasulullah Research Tower of Shiraz University of Medical Science (Shiraz, IRAN). EPHX1 and NQO1 activity was lower in SNPs carriers than in non-carriers and was significant (p 0.0312) for NQO1 (rs1800566 (G > A)) variant (Fig. 1).

Primer design and PCR analysis

Primers were designed for His139 Arg, Tyr113His and P187S regions with Allele ID 6.0 software and their specificity check was performed running Primer-BLAST on each primer separately.

Here we describe the use of two independent PCR methods that have been utilized for genotyping selected polymorphic sites (The details of the primers and genotyping assay are shown in Table 2);

His139 Arg/rs2234922 (A > G) SNP was amplified by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR–RFLP). The digestion reaction mixture contained a total of 10 µL of PCR product, 1µL *Csp6I* (*CviQI*) restriction enzyme, 2µL buffer, 7µL 10X Buffer Tango, 2µL BSA, and 8µL nuclease-free water. The mixture then incubated for 16 h at 25 °C. Next, 15µL of each digested PCR product was run into a lane of the 3% agarose gel. After the DNA formed a sharp band, the Uvitec gel documentation system (UVITEC, UK) was used to visualize the gel image (Fig. 2, Supplementary file). Finally, the genotypes of rs2234922 SNP were determined [17, 18].

Tyr113His/rs1051740 (T > C) and P187S/rs1800566 (G > A) were genotyped through Tetra-primer amplification refractory mutation system-PCR (T-ARMS-PCR). PCR was performed in a total volume of 25µL containing 1µL template DNA, 0.7µL of each common (outer) primers in combination with 0.7µL of each two inner, allele specific primers, 12.5µL PCR Master Mix and 8.7µL nuclease-free water. The PCR products bands were visualized under UV light (UVITEC, UK) and photographed (Fig. 2).

Statistical analysis

The Kolmogorov Smirnov test was applied to determine normal distribution of data for statistical analysis and then the chi-square test, one way analysis of variance test, and Post Hoc Tukey HSD (high significance difference) were used. All gating and FACS plots were analyzed using FlowJo v10 software (FlowJo LLC, USA). P-values were considered significant at < 0.05. All data analysis was using SPSS version 19.0.

Table 3 Kolmogorov–Smirnov test the distribution of continuous variables

Kolmogorov–Smirnov Test		
	N	**200**
Most Extreme Differences	Mean	16.2000
	Std. Deviation	5.98323
	Absolute	0.073
	Positive	0.073
	Negative	−0.031
	Asymp. Sig. (2-tailed)	0.0047

Table 4 Hardy–Weinberg equilibrium for genetic sample with rs2234922 (A > G), rs1051740 (T > C) and rs1800566 (G > A) polymorphisms

SNP	Gene		Genotypes					Ancestral allele	MAF	χ^2	HWE *p*-value
			AA	AG	GG	A	G				
rs2234922	*EPHX1*	Observed	6	97	99	54	146				
		Expected	106	79	15	Not detected		A	G = 0.2155/1079 (1000 Genomes)	0.001	8.948
			TT	TC	CC	T	C				
rs1051740	*EPHX1*	Observed	27	75	98	65	135	T	C = 0.3133/1569 (1000 Genomes)	0.0926	4.762
		Expected	92	87	21	Not detected					
			GG	GA	AA	G	A				
rs1800566	*NQO1*	Observed	11	63	126	43	157	G	A = 0.2889/1447 (1000 Genomes)	0.6844	0.269
		Expected	124	67	9	Not detected					

MAF Minor allele count, *HWE* Hardy–Weinberg equilibrium

Results

Utilizing QUANTO v.1.2.4 software, we conducted a power analysis to evaluate the adequacy of a sample size of 400 individuals for detecting significant associations with the aging skin phenotype [19]. Our statistical power estimation revealed that this sample size achieves a power of 0.88 for identifying associations between the His139 Arg, Tyr113His, and P187S variants and aging skin in both the case and control groups (Fig. 3). This power level is generally accepted as sufficient, exceeding the 80% threshold, and is appropriate for uncovering genotype–phenotype associations.

Since for continuous data, normality is an important step for deciding the measures of central tendency and statistical methods for data analysis, in this study, the Kolmogorov–Smirnov test was used to test normality of the distribution of continuous variables. Results showed that data were not normally distributed as Kolmogorov–Smirnov test ($p = 0.0047$) was statistically significant, that is, data were considered with non-normal distributions (Table 3).

With an aim of effectively reducing false positive findings of variants underlying aging skin traits, the Hardy–Weinberg equilibrium (HWE) test performed at the candidate polymorphic loci among affected individuals. According to the results, no deviations from Hardy–Weinberg equilibrium could be found at all three polymorphic His139 Arg ($p = 8.948$), Tyr113His ($p = 4.762$) and P187S ($p = 0.269$) sites (Table 4).

Furthermore, the fixation index (F-statistics; Fst) was employed to assess genetic differentiation and to analyze the relationships among subpopulations [20]. The interpopulation genetic structure revealed an average FST of 0.11 for polymorphic loci. These values (HWE & Fst) suggest a low degree of differentiation among the study population.

The frequencies of the genotypes of the His139 Arg, Tyr113His and P187S in the aging skin affected population are summarized in Table 5. The population was polymorphic for the risk genotypes of His139 Arg, Tyr113His and P187S SNPs. Genotypic frequencies were on the whole remarkably similar for His139 Arg GG homozygous ($p < 0.001$) and AG heterozygote ($p < 0.001$) risk genotypes and were significantly frequent. The risk CC homozygous genotype of Tyr113His was more significantly frequent ($p < 0.001$) than the wild TT homozygous

Table 5 Single nucleotide polymorphisms and variant frequencies in the sample population

SNP	Genotypes	Total cases (n)	Total control (n)	Chi (χ2) square	*p*-value
rs2234922 (A > G)					
	AA	6	198	0.089	0.765
	AG	97	2	−0.044	< 0.001
	GG	97	-	−0.044	< 0.001
	A	109	396	0.058	0.049
	G	291	4		
rs1051740 (T > C)					
	TT	27	200	0.124	0.724
	TC	75	-	−0.062	< 0.001
	CC	98	-	−0.033	< 0.001
	T	129	400	0.072	0.026
	C	271	-		
rs1800566 (G > A)					
	GG	11	199	0.44	0.507
	GA	63	1	−0.23	< 0.001
	AA	126	-	−0.01	< 0.001
	G	85	398	0.46	0.039
	A	315	2		

genotype as well as the risk TC heterozygous genotype showed a high significant frequency ($p < 0.001$) in the affected individuals. AA homozygous genotype was the most significant frequently ($p < 0.001$) identified risk genotype for P187S SNP. In Addition, the frequency of GA heterozygous genotype was significantly higher ($p < 0.001$) in participants than the wild GG homozygous genotype.

As shown in Table 6, Study's participants with the polymorphic His139 Arg, Tyr113His and P187S genotypes had an increased risk of developing intrinsic skin aging symptoms including uneven pigmentation, fine wrinkles, lax appearance, reduced fat tissue and benign skin tumors. We found a significant association between skin aging symptoms severity and risk genotypes in all three polymorphic sites.

The identification of risk factors is an important stage in the development of strategies for prevention and treatment of any clinical complications. At the base of aging skin there are non-modifiable (NMRFs) and modifiable (MRFs) risk factors, including gender, age (NMRFs) and water intake, micronutrient care, sleeping habits, sun exposure and sunscreen cream usage (MRFs) which in the present study were considered as potential confounding factors and included as covariates in the statistical analysis. A significant association of the low EPHX1 activity harboring the Tyr113His and His139 Arg SNPs, and low NQO1 activity (p 0.0312) harboring the P187S SNP with MRFs and NMRFs were found in this study (for details see Tables 7, 8 and 9).

Discussion

The overproduction of ROS under oxidative stress conditions can have deleterious effects on cellular constituents stemming from multiple interconnected processes based on genetic programs, biochemical reactions, and external stimulation. Alteration of oxidative balance is the principal cellular perturbation in the skin driving aging [21, 22]. Cutaneous cells utilize the endogenous detox

Table 6 Comparison of intrinsic skin aging items between genotypes

Skin aging score: SCINEXA	Genotype								
Skin aging symptoms	rs2234922			rs1051740			rs1800566		
	AA	AG	GG	TT	TC	CC	GG	GA	AA
Score of uneven pigmentation									
3	1	97	97	3	75	98	0	63	126
0	5	0	0	24	0	0	11	0	0
p-value	0.22	0.03	0.03	0.51	0.02	0.03	0.46	0.01	< 0.001
Score Fine wrinkles									
3	1	49	79	2	43	51	0	39	84
2	1	38	14	4	30	33	1	13	33
1	2	10	4	11	2	14	1	11	9
0	2	0	0	10	0	0	9	0	0
p-value	0.12	0.03	0.07	0.61	0.03	0.01	0.29	0.03	0.05
Score Lax appearance									
3	0	52	58	1	14	61	0	22	48
2	0	32	27	1	59	28	0	35	63
1	1	13	13	22	2	9	9	6	15
0	5	0	0	3	0	0	2	0	0
p-value	0.11	0.06	0.04	0.14	0.01	0.05	0.33	0.04	0.07
Score Reduced fat tissue									
3	0	22	39	0	27	41	0	19	79
2	0	54	51	0	38	29	6	44	33
1	3	21	7	25	10	28	3	0	14
0	3	0	0	2	0	0	2	0	0
p-value	0.21	0.02	0.01	0.23	0.03	0.04	0.59	0.08	0.06
Score Benign skin tumors									
3	2	13	15	0	6	23	0	11	31
2	3	55	43	0	37	5	0	35	59
1	1	29	39	8	30	70	4	17	26
0	0	0	0	19	2	0	7	0	10
p-value	0.24	0.03	0.06	0.19	0.01	0.03	0.12	0.03	0.04

Table 7 Relationship between the risk genotypes of rs2234922 SNP and Non-modifiable and modifiable risk factors associated with intrinsic skin aging

Risk factors		Genotype				
		rs2234922				
		AG or GG carriers				
		Mean difference (I – J)	Std. error	Sig.	95% confidence interval	
					Lower bound	Upper bound
Non-modifiable risk factor						
	Gender					
	Male	-3.48936[a]	.92556	.000	-5.3146	-1.6641
	Female	-4.95299[a]	1.19105	.000	-7.3018	-2.6042
	Age (≥35)	26.1184	.42002	.000	25.2846	26.9521
Modifiable risk factors						
	Water intake					
	≥ 8 glass	1.47028	.98301	.136	-.4683	3.4089
	≤ 5 glass	-3.26756[a]	1.71496	.049	-6.6496	.1145
Micronutrient Care						
	Yes	3.35000	1.89700	.186	-1.1533	7.8533
	No	6.52425[a]	1.13781	.000	3.8232	9.2253
Sleeping habits						
	≥ 7 hours	-.04464	1.92529	.982	-3.8415	3.7522
	5-7 hours	-3.01282	1.95939	.126	-6.8769	.8512
Sun exposure						
	Extensive	-3.40951[a]	1.20134	.005	-5.7786	-1.0404
	Un-extended	.74723	.98270	.448	-1.1907	2.6852
Application of Sunscreen cream						
	Yes	2.47955[a]	.99944	.014	.5086	4.4505
	No	3.55821[a]	1.06068	.001	1.4665	5.6500

[a] The mean difference is significant at the 0.05 level

or biotransformation enzymes to scavenge reactive oxygen species. However, growing evidence has shown that defective detox due to genetic factors such as polymorphisms in genes that code for efficient detox enzymes can result in damaged skin and reflect the aging process [23]. From a genetic perspective, this study proposes an update on the role of additional genetic variants in the clinical manifestation of aging skin, as well as interplay between genetics and external factors holds significant sway over aged skin pathogenesis. EPHX1 is a protective enzyme involved in general oxidative defense against stimulants. Our results suggest that genotypes of both Tyr113His and His139 Arg polymorphisms that are associated with low enzymatic activity will lead to inefficient metabolizing of ROS, and may induce faster skin aging manifestation. No previous studies have analyzed *EPHX1* genetic Tyr113His and His139 Arg polymorphisms and aging skin association, nevertheless, the association of these polymorphisms and other oxidative stress-related complications has been reported, for example, a role have reported for *EPHX* gene polymorphisms in reproductive system and susceptibility to preeclampsia and spontaneous abortion [24]. Cancer researchers have also reported consistent findings. To mention a few of them, these SNPs are reported to be important risk factors for susceptibility to prostate cancer, lymphoblastic leukemia and lung cancer [25–27]. Besides, our findings suggested that the NQO1 protein encoded by the risk homozygous and heterozygous genotypes increased the risk of premature aging skin. This found association is consistent with the results of another similar study, where rs1800566 SNP suggests a strong association with gastric cancer or hepatocellular and renal carcinoma through changes in redox status (minor genotype leading to a reduction in enzymatic function) inside the cells [28]. Similarly, some studies showed an association between oxidative

Table 8 Relationship between the risk genotypes of rs1051740 SNP and Non-modifiable and modifiable risk factors associated with intrinsic skin aging

Risk factors		Genotype				
		rs1051740				
		TC or CC carriers				
		Mean difference (I – J)	Std. error	Sig.	95% confidence interval	
					Lower bound	Upper bound
Non-modifiable risk factor						
	Gender					
	Male	3.48936[a]	.92556	.000	1.6641	5.3146
	Female	-1.46364	1.31430	.267	-4.0555	1.1283
	Age (≥35)	27.2056	.46490	.000	26.2828	28.1284
Modifiable risk factors						
	Water intake					
	≥ 8 glass	-1.47028	.98301	.136	-3.4089	.4683
	≤ 5 glass	-4.73783[a]	1.84418	.011	-8.3747	-1.1010
Micronutrient Care						
	Yes	2.62500	2.07374	.417	-2.2979	7.5479
	No	-2.53725[a]	.24701	.000	-3.1236	-1.9509
Sleeping habits						
	≥ 7 hours	.04464	1.92529	.982	-3.7522	3.8415
	5-7 hours	-2.96818[a]	.86029	.001	-4.6647	-1.2716
Sun exposure						
	Extensive	-4.15674[a]	1.31838	.002	-6.7567	-1.5568
	Un-extended	-.74723	.98270	.448	-2.6852	1.1907
Application of Sunscreen cream						
	Yes	1.07866	1.11381	.334	-1.1179	3.2752
	No	-2.47955[a]	.99944	.014	-4.4505	-.5086

[a] The mean difference is significant at the 0.05 level

stress-related complications risk and NQO1 polymorphism. Sharma et al. results indicate that rs1800566 genotype may increase susceptibility to diabetic nephropathy in north Indian subjects [29]. Three studies identified the association between NQO1 (rs1800566) polymorphism and digestive tract cancer risk [30–32]. Overall, precision medicine can help in the stratification of subjects with aged skin on the basis of molecular pathogenesis responsible for aging skin to benefit the best available [33]. Our study had a shortcoming regarding the sample size (specially over the male subjects) and second, there was no includes I, II, V and VI Skin Phototypes. Regarding the former one, although we asked top dermatologists to refer some more eager male subjects for molecular analysis, they claimed that men tended to be less looking for treat wrinkles and other signs of aging skin than women, and their majority referred subjects are females. In addition, we also asked many primary care physicians who referred their patients to dermatologists for specialized care. In elaboration, a hundred and thirty-four blood samples were obtained from male subjects of non-obvious-intrinsically aged skin presentation. Forty samples from participants with confirmed intrinsically aged skin signs were included and ninety-four samples were excluded from this study. We addressed this research gap by consultation with scientists in the field and two of the Associate Professor of Statistics at The University of Isfahan, IRAN, who suggested the statistical fact "For populations below 1,000, a random sample of 30 percent is thought to correctly represent the larger population. Nonetheless, when we reached 30% of a total 134, i.e. forty male subjects, enrollment stopped before reaching the larger male sample size because of poor recruitment.

Table 9 Relationship between the risk genotypes of rs1800566 SNP and Non-modifiable and modifiable risk factors associated with intrinsic skin aging

Risk factors	Genotype				
	rs1800566				
	GA or AA carriers				
	Mean difference (I – J)	Std. error	Sig.	95% confidence interval	
				Lower bound	Upper bound
Non-modifiable risk factor					
Gender					
Male	1.46364	1.31430	.267	-1.1283	4.0555
Female	4.95299[a]	1.19105	.000	2.6042	7.3018
Age (≥35)	24.2550	1.81010	.000	19.6020	28.9080
Modifiable risk factors					
Water intake					
≥ 8 glass	3.26756	1.71496	.058	-.1145	6.6496
≤ 5 glass	4.73783[a]	1.84418	.011	1.1010	8.3747
Micronutrient Care					
Yes	.42025	.24701	.209	-.1661	1.0066
No	2.95750[a]	.24701	.000	2.3711	3.5439
Sleeping habits					
≥ 7 hours	3.01282	1.95939	.126	-.8512	6.8769
5-7 hours	2.96818[a]	.86029	.001	1.2716	4.6647
Sun exposure					
Extensive	4.15674[a]	1.31838	.002	1.5568	6.7567
Un-extended	3.40951[a]	1.20134	.005	1.0404	5.7786
Application of Sunscreen cream					
Yes	-1.07866	1.11381	.334	-3.2752	1.1179
No	-3.55821[a]	1.06068	.001	-5.6500	-1.4665

[a] The mean difference is significant at the 0.05 level

Though we (statisticians and researchers) believe that a large sample is always helpful in providing more reliable results, accordingly we ensured that the sample size on 30% recruitment of all enrollment would be representative of the entire male population. So, this statement was added to the limitation section to bold this shortcoming and be addressed in future analysis. Concerning the latter, besides inclusion of I, II, V and VI Skin Phototypes, concurrently investigating the relationships between *MC1R* gene polymorphisms and skin color is extremely recommended.

Conclusion

The limitation of traditional therapy of aging skin is possible to overcome by genetic analysis, which requires gathering as much information as possible regarding the genetic determinants of skin aging. Moreover, thanks to the advances in the biotechnology field, limitations concerning enzyme therapy including the activation of immune responses, unwanted adverse effects and toxicity, exposure to endogenous degrading mechanisms, as well as poor tissue specificity, are being overcome. Enzyme encapsulation approaches, such as membrane vesicles, liposomes, erythrocytes and nanoparticles, targeted enzyme modification technology, such as PEG conjugation, and small molecules which by directly binding to a given enzyme correct its dysfunction and increase the transcriptional output of enzyme are some innovative biotechnology strategies are being developed which could solve enzymatic drawbacks of ageing skin.

Abbreviations

ROS	Reactive oxygen species
SNPs	Single nucleotide polymorphisms
Detox	Detoxification
EPHX1	Microsomal Epoxide Hydrolase 1
NQO1	NADPH-quinone oxidoreductase 1
SCINEXA	Score of intrinsic and extrinsic skin aging
gDNA	Genomic DNA
PCR–RFLP	Restriction Fragment Length Polymorphism Analysis of

	PCR-Amplified Fragments
T-ARMS-PCR	Tetra-primer amplification refractory mutation system-PCR
HWE	Hardy–Weinberg equilibrium
Fst	F-statistics
NMRFs	Non-modifiable risk factors
MRFs	Modifiable risk factors

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
N.S. and N.B. designed the study and critically reviewed the manuscript. N.S, K.V.K, K.V.S, and N.L. performed formal analysis. N.S. and N.B. administrated project. K.V.K wrote the manuscript. The final manuscript has been approved by all authors.

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Data availability
The datasets generated and/or analyzed during the current study are available in the [dbSNP] repository (http://www.ncbi.nlm.nih.gov/SNP)" and SNPs can be searched for using the dbSNP ID (rs2234922, rs1051740 and rs1800566). The UK Biobank Allele Frequency Browser (https://afb.ukbiobank.ac.uk/) was used as a resource of variants allele frequencies in general population.

Declarations

Ethics approval and consent to participate
The study protocol was approved by the Islamic Azad University- Kazerun Branch Ethics Committee. All methods were performed in accordance with the guidelines and regulations of the Islamic Azad University- Kazerun Branch. Written informed consent was provided by all the participants before entering the study groups.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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