Int J Clin Exp Med 2017;10(2):4042-4048
www.ijcem.com /ISSN:1940-5901/IJCEM0029342

Original Article

Genetic susceptibility to keloid scarring in Chinese Han population: NEDD4 gene single nucleotide polymorphism

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Received March 28, 2016; Accepted September 7, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Keloid disease (KD) is a benign fibroproliferative dermal tumor of unknown aetiology. The increased familial clustering in KD, its increased prevalence in certain races and increased concordance in identical twins suggest a strong genetic predisposition to keloid formation. Recently, a genome-wide association study (GWAS) identified four single nucleotide polymorphisms (SNPs) in Japanese patients with keloids. To detect the association between KD and SNPs in the Chinese Han population, six SNPs were selected (rs2303579, rs2303580, rs10518830, rs8032158, rs4774833 and rs17819300) for replication in 50 cases and 52 controls by using Sequenom MassArray system. We found SNPs located in NEDD4 showed significant association with the susceptibility of keloid: 15q21.3 (rs2303579, *P*=0.005; rs2303580, *P*=0.006 and rs10518830, *P*=0.013, respectively). We also analyzed the haplotypes of rs2303579 and rs2303580: CT (OR=2.72, *P*=0.0008) and TC (OR=0.37, *P*=0.0008). Our study confirmed previously reported loci 15q21.3 for keloid in the Chinese Han population, which suggested the common genetic factor predisposing to the development of keloid shared by the Chinese Han and Japanese populations. Identification of genetic markers in candidate genes such as NEDD4 may be of significant importance in diagnosis, prognosis and development of new therapies in the management of keloid scarring.

Keywords: Keloid scarring, polymorphisms, NEDD4, susceptibility

Introduction

Keloid disease (KD) is a benign fibro proliferative scar that continually grows beyond the confines of the original wound and invades into surrounding healthy skin [1]. The excessive accumulation of extracellular matrix and overabundant formation of collagen can be observed in keloids. Although the pathogenesis of KD has remained mysterious today along with a non-effective clinical management, it is widely noticed that there is a strong genetic susceptibility to keloid scarring. Keloid scarring is really common among individuals with a darker pigmented skin [2]. The highest incidence of keloids is found in the black population, where it has been estimated to be 16% in black Africans [3]. Meanwhile, familial heritability together with the common occurrence in twins also support the idea of the genetic susceptibility to KD [4].

Several studies that analyze the single nucleotide polymorphisms (SNPs) of genes which involved in the process of KD have been done worldwide to identify the responsible gene in the KD. Genetic polymorphisms of TGF-β1, TGF-β2, TGF-β3 and TGF-β receptor I, TGF-β receptor II, TGF-β receptor III have been testified to find some relationship with the predisposition of KD, but there is no clear evidence to show any potential loci that susceptible to KD [5-8]. Meanwhile, the research on the SMAD genes 3, 6 and 7 cannot detect the possible SNPs related to KD as well [9]. Genome-wide association studies (GWAS) have been proven to be a powerful tool to identify susceptibility genes for common diseases. In a genome-wide association study performed by the Japanese scientists, there are four susceptibility loci that associated with KD in Japanese population were identified. Among the results, a SNP rs8032158 located in the neuronal precursor cell-

Table 1. Characteristics of the patients with KD and normal controls in this study

Characteristics	Case (N=50)	Control (N=52)	Total
Gender, No. (%)			
Male	16 (32)	19 (36.5)	35
Female	34 (68)	33 (63.5)	67
Age, No.			
<30	25	28	53
≥30	25	24	49
Mean age ± SD	30.56 ± 14.09	27.70 ± 19.96	
Diseased region, No. (%)			
Neck	5 (10)	-	5
Chest	23 (46)	-	23
Shoulder	10 (20)	-	10
Face	5 (10)	-	5
Groin	4 (8)	-	4
Belly	2 (4)	-	2
Upper arm	6 (12)	-	6
Foot	1 (2)	-	1
Aimpit	1 (2)	-	1

Table 2. Basic information of the six SNPs in this study

SNP	Chr (Gene)	Position	Alleles A/B	MAF	HWE *P*-value
rs2303579	15 (NEDD4)	56152729	T/C	0.168	0.200
rs2303580	15 (NEDD4)	56152872	C/T	0.168	0.309
rs10518830	15 (NEDD4)	56211218	G/C	0.072	0.138
rs8032158	15 (NEDD4)	56194877	T/C	0.188	0.856
rs4774833	15 (NEDD4)	56210168	A/G	0.091	0.235
rs17819300	15 (NEDD4)	56210929	G/A	0.009	0.620

A/B stands for minor/major alleles. Abbreviations: MAF, minor allele frequency.

expressed developmentally downregulated 4 (NEDD4) gene on chromosome 15, showed significant association with KD. This study first indicated the NEDD4 as a potential gene that participated in the formation of KD [10]. A following study performed by the same Japanese scientists' team revealed the possible involvement of NEDD4 in keloid formation. It confirmed that the expression of fibronectin and type 1 collagen were upregulated by NEDD4, which results in the excessive accumulation of extracellular matrix [11].

NEDD4 is a member of NEDD4 family which belongs to the E3 ubiquitin ligase. It involved in process of protein ubiquitination which is a post-translational modification. E3 ubiquitin ligases are crucial in this process because it can specifically recognize a substrate for modification [12]. NEDD4 participate in the modulation of several important signaling pathways such as EGF, IGF, VEGF et al. It also involved in the regulation of PTEN and p63. These factors are all in close relation to KD.

Because of all these studies done above, we performed a case-control study to comprehensively examine six SNPs in NEDD4 gene and their associations with KD in a Han Chinese population. 3 of them are in the promotor region (rs10518830, rs17819300, rs4774833), 1 in the introns (rs8032158) and the other 2 in the exons (rs2303579, rs2303580).

Methods

Study subjects

A Chinese Han population-based case-control study comprised of diagnosed keloid disease patients were recruited from Xijing Hospital, Fourth Military Medical University. The keloid-free control subjects were individuals who had come to the hospital for cosmetic surgery and had no blood relationship with the KD patients. Additionally, Only Han Chinese patients were included in this analysis to avoid the variance of genotype frequencies among different ethnic groups. The 52 controls consisted of 33 females and 19 males with a mean age of 27.7 years. The 50 KD cases consisted of 34 females and 16 males with a mean age of 30.6 years. Detailed demographic information is shown in **Table 1**. All subjects signed informed consent forms. Blood (3 ml) was collected from each subject according to the study protocol approved by the Clinical Research Ethics Committee of the Fourth Military Medical University. The study was conducted according to the Declaration of Helsinki Principles.

Polymorphisms and genotyping

A total of six SNPs from NEDD4 gene were selected for our study, including rs2303579,

Table 3. Single SNP association with keloid scarring (logistic regression, adjusted by gender, age)

SNP	Model	Genotype	Case	Control	OR (95% CI)	*P*-value
rs2303579	Co-dominant	C/C	30 (60.0%)	15 (28.8%)	1	0.005
		T/C	15 (30.0%)	24 (46.2%)	0.31 (0.13-0.76)	
		T/T	5 (10.0%)	13 (25.0%)	0.19 (0.06-0.64)	
	Dominant	C/C	30 (60.0%)	15 (28.8%)	1	0.002
		T/C-T/T	20 (40.0%)	37 (71.2%)	0.27 (0.12-0.62)	
	Additive	--	--	--	0.41 (0.23-0.73)	0.002
rs2303580	Co-dominant	T/T	30 (60.0%)	15 (28.8%)	1	0.006
		C/T	15 (30.0%)	25 (48.1%)	0.30 (0.12-0.73)	
		C/C	5 (10.0%)	12 (23.1%)	0.21 (0.06-0.70)	
	Dominant	T/T	30 (60.0%)	15 (28.8%)	1	0.002
		C/T-C/C	20 (40.0%)	37 (71.2%)	0.27 (0.12-0.62)	
	Additive	--	--	--	0.41 (0.22-0.74)	0.003
rs10518830	Co-dominant	C/C	34 (68.0%)	21 (40.4%)	1	0.013
		G/C	13 (26.0%)	21 (40.4%)	0.38 (0.16-0.92)	
		G/G	3 (6.0%)	10 (19.2%)	0.19 (0.05-0.75)	
	Dominant	C/C	34 (68.0%)	21 (40.4%)	1	0.005
		G/C-G/G	16 (32.0%	31 (59.6%)	0.32 (0.14-0.72)	
	Additive	--	--	--	0.41 (0.22-0.76)	0.005
rs8032158	Co-dominant	C/C	13 (26.0%)	6 (11.6%)	1	0.048
		T/C	25 (50.0%)	23 (44.2%)	0.50 (0.16-1.55)	
		T/T	12 (24.0%)	23 (44.2%)	0.24 (0.07-0.79)	
	Dominant	C/C	13 (26.0%)	6 (11.6%)	1	0.059
		T/C-T/T	37 (74.0%)	46 (88.4%)	0.37 (0.19-1.07)	
	Additive	--	--	--	0.41 (0.27-0.87)	0.016
rs4774833	Co-dominant	G/G	37 (74.0%)	37 (71.2%)	1	0.406
		A/G	12 (24.0%)	11 (21.2%)	1.09 (0.43-2.78)	
		A/A	1 (2.0%)	4 (7.6%)	0.25 (0.03-2.34)	
	Dominant	G/G	37 (74.0%)	37 (71.2%)	1	0.747
		A/G-A/A	13 (26.0%)	15 (28.8%)	0.87 (0.36-2.07)	
	Additive	--	--	--	0.76 (0.38-1.53)	0.446
rs17819300	Co-dominant	A/A	42 (84.0%)	42 (80.8%)	1	0.669
		G/A	8 (16.0%)	10 (19.2%)	0.80 (0.29-2.23)	
		G/G	0 (0)	0 (0)	--	
	Dominant	A/A	42 (84.0%)	42 (80.8%)	1	0.669
		G/A-G/G	8 (16.0%)	10 (19.2%)	0.80 (0.29-2.23)	
	Additive	--	--	--	0.80 (0.29-2.23)	0.669

P<0.05 indicates statistical significance. Abbreviations: OR, odds ratio; CI confidence interval.

rs2303580, rs10518830, rs8032158, rs4774833 and rs17819300. DNA was extracted from peripheral blood samples using the QiAmp DNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA concentrations were measured using a NanoDropTM 2000 (Thermo Scientific, Waltham, MA, USA). The genotype of all subjects were determined by the MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry) technic. There are multiple PCR steps in the process, and all the reaction conditions followed the standard protocol of MALDI-TOF MS SNP detection technic.

Statistical analysis

In controls, each SNP was tested to determine whether it conform to the Hardy-Weinberg equi-

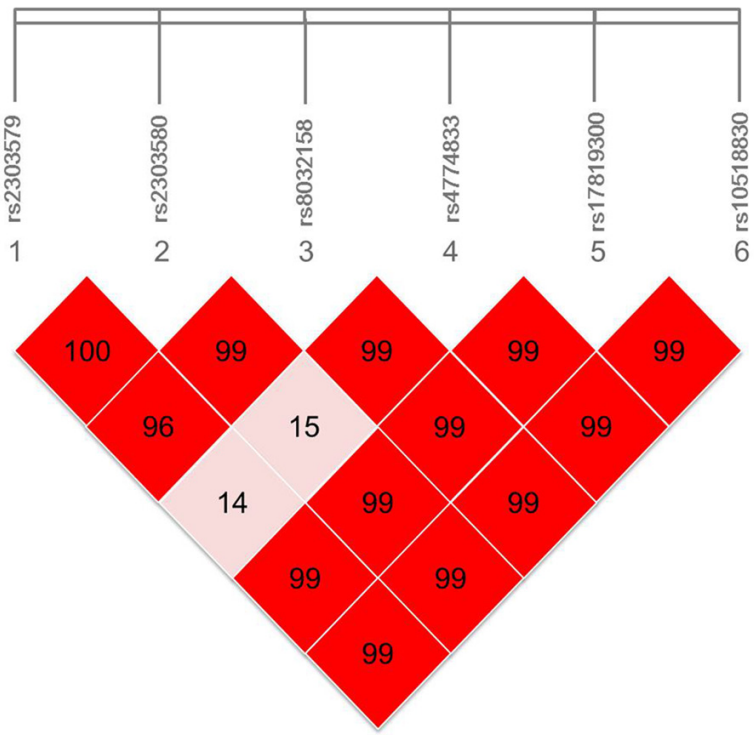

Figure 1. D values demonstrate the extent of disequilibrium between SNPs on NEDD4 in keloid disease cases and controls. LD is indicated using standard color schemes with white (no LD) to bright red (high LD).

librium (HWE). χ^2 tests were used to evaluate the differences in genotype of the NEDD4 polymorphisms. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using unconditional logistic regression models under unrestricted, additive and dominant genetic modes of inheritance. LD of the candidate SNPs was analyzed using Haploview v4.2. Pairwise linkage disequilibrium and haplotype constructions were performed using the SHEsis software (http://analysis.bio-x.cn/myAnalysis.php). Two-tailed tests of statistical significance were performed using the SPSS 17.0. *P* value of less than 0.05 were consider statistically significant.

Results

The distributions of demographic and characteristics among subjects are shown in **Table 1**. KD cases were older (mean age 30.6 ± 14.1) and more likely to be female 34 (68.0%) relative to controls (mean age 27.7 ± 17.0, 33 (63.5%) female).

In initial analyses, we found that all six SNPs conformed to Hardy-Weinberg Equilibrium proportions in the controls ($P > 0.05$). Based on χ^2 tests, rs2303579 (P=0.005), rs2303580 (P=0.006), rs10518830 (P=0.013), rs8032158 (P=0.048) correlated with KD risk (**Table 2**).

In the genetic model analyses, we found the minor allele "T" of rs2303579 was associated with reduced risk of keloid scarring, based on results from the additive model (OR=0.41; 95% CI= 0.23-0.73, P=0.002) and the genotype "CC" may significantly increase KD risk in the dominant model (P=0.002). Similarly, the minor allele "C" of rs11874392 was associated with reduced risk of keloid scarring as revealed by the additive model (OR=0.41, 95% CI=0.22-0.74, P=0.003) and the genotype "TT" may significantly increase KD risk in the recessive model (P=0.002). Additionally, we found the minor allele "G" of rs10518830 was significantly associated with reduced KD risk, with an OR=0.41, 95% CI=0.22-0.76, P=0.005 under the additive model and the genotype "CC" may significantly increase KD risk in the dominant model (P=0.005). Finally, the minor allele "T" of rs8032158 was associated with reduced risk of keloid scarring, based on results from the additive model (OR=0.41, 95% CI=0.27-0.87, P=0.016) and the genotype "CC" may significantly increase KD risk in the co-dominant model (P=0.048). The results with rs8032158 are consistent with those of the previously described GWAS study (**Table 3**).

Under the help of SHEsis software, we performed the linkage disequilibrium analysis on 6 chosen SNPs on NEDD4. D values and r^2 values demonstrate the extent of LD observed in the cases and controls (**Figures 1** and **2**). Because the D' value largely dependents on the sample quantity and the meaning could be exaggerated, so under the consideration of r^2 value, we found the rs2303579 and rs2303580 are in linkage status.

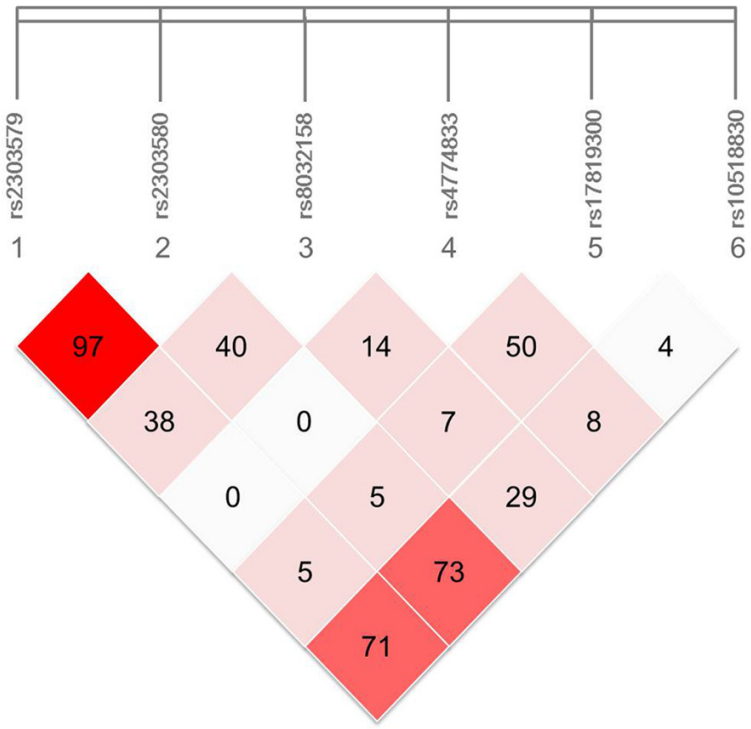

Figure 2. r^2 values show linkage disequilibrium (LD) between SNPs on NEDD4 in both keloid disease cases and controls. LD is indicated using standard color schemes with white (no LD) to bright red (high LD).

Table 4. Association between NEDD4 haplotypes and risk of KD

rs2303579/rs2303580	Case Freq	Control Freq	OR	95% CI	*P*
T/C	0.2500	0.4712	0.37	0.20-0.67	0.0008
C/T	0.7500	0.5192	2.72	1.50-4.94	0.0008

Based on the result of linkage disequilibrium analysis, we further analyzed the haplotypes of rs2303579 and rs2303580 (**Table 4**). Because these two SNPs were in linkage status, there were only two haplotypes: the first kind of haplotype (C allele on rs2303579 and the T allele on rs2303580) was associated with an increased risk of KD (OR=2.72, *P*=0.0008); whereas the second one (T allele on rs2303579 and the C allele on rs2303580) was associated with an decreased risk of KD (OR=0.37, *P*=0.0008).

Discussion

Compared with proliferative scar and other pathological scar, keloids has a stronger genetic predisposition [1]. As the third generation of genetic markers, SNP are widely used in disease research, including studies associated the susceptibility of keloids [13]. Mainly aimed at the promoter region and exon region coding SNPs on NEDD4, we analyzed the relationship between these functional SNPs and the predispositions of the onset of keloids.

In 2010, Japanese researcher Nakashima and his team found a SNP, rs8032158 which located on the intron region of NEDD4, associated with the susceptibility of keloids. Meanwhile, they also reported that the NEDD4 gene might participate in the process of keloids formation [10, 11]. Their works first introduced the NEDD4 to the keloids research. The NEDD4 gene responsible for the encoding process of NEDD4 proteins which belongs to the ubiquitin ligase E3 family. Ubiquitination pathway widely exists in the protein degradation, and the process including Ub and three important enzymes (E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligases). E3 enzymes function as the substrate recognition modules of the system and are capable of interaction with both E2 and substrate, so its function is really crucial in the Ubiquitination pathway. As one of the E3 family member, Nedd4 possess the same function as other E3 enzymes. There are lots of substrates that are specially recognized by Nedd4, and some of them play important role in the onset of KD. It is stated that IGF, VEGF, EGF, PTEN/Akt pathway and Wnt pathway were all detected the participation of Nedd4 [12, 14-18]. It is assumed that Nedd4 may regulate the KD through the impact of these relative molecules or pathways.

Base on the information from SNP database, NCBI (National Center for Biotechnology information), we chose 5 unstudied SNPs which

located on the promotor region or on the exon region and 1 reported SNP which suggested to associate with KD in Japanese on NEDD4 gene. After detection of the genotypes of the 6 SNPs by MALDI-TOF MS technic and the statistically analysis, there were 3 new SNPs (rs2303579, rs2303580, rs10518830) in NEDD4 that associated with KD. Meanwhile, the rs8032158 which associated with Japanese KD was also relevant to the Chinese KD. Other 2 SNPs (rs4774833, rs17819300) located on the promotor region were not associated with KD in our study.

Among SNPs we found related to KD, rs2303579 and rs2303580 located on the exon region of NEDD4. They belong to the missense part of exon, so the variation of the genotype on these 2 loci could change the amino acid type. Based on the data from NCBI we acknowledged that, rs2303579 located on the fourth exon of NEDD4, coding the 279th amino acid on Nedd4 protein. When the genotype is T, the coding amino acid is asparagine (Asn); but when the genotype is C, the coding amino acid is serine (Ser). What's more, we also known that rs2303580 located on the fifth exon of NEDD4, coding the 260th amino acid on Nedd4 protein. When the genotype is C, the coding amino acid is arginine (Arg); but when the genotype is T, the coding amino acid is glutamine (Gln). In the structure of Nedd4 protein, the 260th amino acid and the 279th amino acid located between the first WW domain (from 196th amino acid to 224th amino acid) and the second WW domain (from 351th amino acid to 380th amino acid) in Nedd4 protein. The main function of WW domain in the protein is to specifically recognize the substrate. Meanwhile, rs2303579 and rs2303580 were in a state of strong linkage disequilibrium, the haplotype rs2303579-C and -rs2303580-T associated with KD and possessed a higher risk to get the KD. When the type of the two amino acid in the WW domain joints changed, the space conformation of the corresponding coding NEDD4 protein may change too, which make the specific combination of the downstream proteins unusual. Then, the abnormal process of the target protein hydrolysis can cause the abnormal molecule levels in cells, which finally results in the unusual signal pathway and leads to KD. In all, the two missense coding region SNPs which relevant to the onset of KD is of great importance in the research of KD, and its effect to KD needs further validation by some SNP functional experiments.

The rs10518830 which located on the promotor region of NEDD4 is also relevant to the susceptibility of KD. The change of the genotype in rs10518830 (G>C) may participate in the regulation process of the NEDD4 gene expression. The abnormal of the NEDD4 expression level would results in the change of Nedd4 protein level. The variation of Nedd4 protein level could on one hand impact the biological behavior of fibroblast to cause KD, on the other hand, it could influence the specifically recognized substrate protein level which could participate in the onset of KD [11]. But this hypothesis also needs future testify.

The Japanese researchers found a relevance of rs8032158 and the susceptibility of KD [10]. We studied this SNP again in the Chinese population, and testified that it also associated with the susceptibility of KD in Chinese. Whereas, the function of SNP in the intron region has not been studied before, so we have no idea of its function or role in KD.

From the case-control study, we first found 3 new SNPs in NEDD4 gene that relevant with the susceptibility of KD: 2 of them located on the exon missense area which could impact the coding amino acid type and 1 of them located on the promotor region which may participate in the gene expression process. Our study provide new ideas of KD research as well as new target for KD prevention and cure.

Acknowledgements

National Natural Science Foundation of China (No. 81000840).

Disclosure of conflict of interest

None.

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