An in-Depth Analysis Identifies Two New Genetic Variants in 22q11.2 Associated with Vitiligo in the Chinese Han Population

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Abstract

Background Vitiligo is a complex disease in which patchy depigmentation results from autoimmune loss of melanocytes in affected regions. Previously, we provided significant linkage evidence on 22q12 by genomewide linkage analysis of vitiligo in the Chinese Han population. Our aim is to identify susceptibility variants associated with vitiligo at an expanded region for 22q12.

Methods and Results A deep analysis of the expanded region for 22q12 locus was performed in a large GWAS dataset consisting of 1117 cases and 1701 controls by imputation. Eight nominal SNPs were selected and genotyped in an independent cohort of 2069 cases and 1370 controls of Chinese Han by using the Sequenom MassArray iPLEX1 system. Data were analyzed with PLINK 1.07 software. The C allele of rs730669 located in *ZDHC8/RTN4R* was observed a strong evidence of association for vitiligo ($P = 3.25 \times 10^{-8}$, OR = 0.81). The C allele of rs4820338 located in *VPREB1* and A allele of rs2051582 (a reported SNP in our previous study) located in *IL2RB* were suggestive evidence of association for vitiligo ($P = 1.04 \times 10^{-5}$, OR = 0.86; $P = 1.78 \times 10^{-6}$, OR = 1.27). The three identified SNPs showed independent associations with vitiligo by conditional logistic regression (all $P < 1.0 \times 10^{-5}$; all $D' < 0.05$, $r^2 < 0.0001$).

Conclusions The study identifies that two novel variants rs730669 (*ZDHC8/RTN4R*) and rs4820338 (*VPREB1*) in 22q11.2 might confer susceptibility to vitiligo and affect its disease phenotypes. The presence of multiple independent variants emphasizes an important role in the genetic pathogenesis of disease.

Background

Vitiligo is a complex disease in which patchy depigmentation results from autoimmune loss of melanocytes in affected regions. It is characterized by the loss of epidermal melanocytes leading to localized or generalized depigmentation or loss of plaque in the epidermis and mucous membranes [1]. The epidemiological surveys have shown that the incidence of vitiligo is about 0.5-1% [2]. Its underlying pathobiology may be related to the genetic, immune and environments. Linkage and association studies in multi-ethnic populations provided strong support for vitiligo susceptibility genes on chromosome (such as, 4q13-q21, 1p31, and 17p13) [3, 4]. In addition, the linkage evidence on 22q12 was found for vitiligo by our previous genomewide linkage analysis in the Chinese Han population [5]. So far, sixty genetic loci associated with disease susceptibility were identified by several genome-wide association studies (GWAS) of vitiligo in the Caucasian and Chinese [6, 7, 8, 9, 10, 11, 12], most of which harbor genes involved in regulation of biological functions (melanocyte, apoptosis and immune). These emphasized in a common model of melanocyte autoimmune pathogenesis [13].

To find the susceptibility loci in 22q12 identified locus by linkage analysis with vitiligo [5], a fine imputation analysis was conducted by our previous GWAS dataset [11], and performed further validation analysis in an independent cohort of Chinese Han population, which provided additional information on the locus implicated in vitiligo genetic pathogenesis.

Materials And Methods

Subjects

In total, the data of our previous vitiligo GWAS from 1117 vitiligo patients and 1701 controls were used [11,12], while 2069 cases and 1370 controls were recruited for replication in this study (Table 1). All (the Chinese Han population) were collected from the multiple hospitals in China. After the written informed consent was obtained from all individuals, the blood samples, clinical and demographic information (a previous designed questionnaire) were collected from cases and controls. Based on the Declaration of Helsinki principles, this study was approved by the Institutional Ethical Committee of Anhui Medical University. According to the diagnostic criteria of the Vitiligo European Task Force [14], the clinical diagnosis of all cases were confirmed by at least two dermatologists. All controls were healthy individuals without vitiligo, family history (including first-, second- and third-degree relatives) of vitiligo, and any other autoimmune diseases or systemic disorders. Common factors (ethnicity, age and gender) were matched in case and control groups. According to the manufacturer's instructions, genomic DNA was extracted from peripheral blood lymphocytes by using QIAamp DNA Blood kit (Qiagen, Valencia, CA, USA) and was diluted to working concentrations of 20-25 ng/ml for the replication study.

SNP selection for replication

Based on our previous vitiligo GWAS [11,12] and the reference panel of 1000 Genomes Project (Mar.2012 release) [15], the imputation for a expanded region of 22q12 locus (expansion of 5 Mb of upstream and downstream for this locus, chr 22: 20000000-42000000, hg19) was performed in 1117 vitiligo patients and 1701 controls by using Impute v2.0 [16]. The following conditions will be excluded from further analysis: 1) SNPs with low imputation confidence (INFO score ≤ 0.5), 2) Significant Hardy-Weinberg disequilibrium ($P < 0.05$), 3) A MAF $< 1\%$, 4) A low call rate $< 95\%$. At last, 8 SNPs at the region showed nominal significance of association ($P_{\text{initial}} < 0.01$) in our vitiligo GWAS and were then selected for this replication study.

Genotyping analysis of the replication study

The detection primers for the 8 SNPs were designed using the MassARRAY Assay Design 3.0 software (Sequenom). Approximately 15-20 ng of genomic DNA for each sample was used to genotype. Eight SNPs were genotyped by using the Sequenom MassArray iPLEX1 system (the Key Laboratory of Dermatology, Ministry of Education, China). The DNA samples were amplified by multiplex PCR reactions, and then the PCR products were used for locus-specific single-base extension reactions. The resulting products were desalted and transferred to a 384-element SpectroCHIP array. Allele detection was performed using MALDI-TOF MS. The mass spectrograms were analyzed by the MassARRAY Typer software (Sequenom). SNPs with MAF $< 1\%$, a call rate $< 95\%$, and deviation from Hardy-Weinberg equilibrium ($P < 0.05$) in the control subjects were excluded from further association analysis.

Statistical analysis

Association of each SNP with disease phenotype was tested in the discovery and replication samples using PLINK version 1.07 [17]. Principal component analysis was used to assess population outlier and stratification in the dataset [11]. GWAS and replication association analyses were carried out by using the Cochran-Armitage trend test. Conditional logistic regression was used to determine whether independent effects existed and was carried out by SNPTTEST (V2). The regional plot of association result was generated using LocusZoom based on the information of JPT and CHB populations from hg19/HapMap Phase II. The level of associated significance was assigned at P values of less than 5.0×10^{-8} (criterion for genome-wide significance). The sub-phenotype analyses of the associated SNPs were also performed according to the clinical features which included gender, family history, age of onset (early onset ≤ 20 years and late onset > 20 years), clinical classification (non segmental and segmental) and autoimmune disease involvement (such as, systemic lupus erythematosus, alopecia areata, thyroid disease, rheumatoid arthritis, myasthenia gravis, and scleroderma), P values of less than 5.0×10^{-2} were considered to be statistically significant.

Bioinformatics analysis

Several bioinformatics tools were utilized in this study. The HaploReg4.1 (<https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php>) was adopted to select the strong linked SNPs and evaluated the potential biological significance for targeted SNPs. Single Nucleotide Polymorphism database (dbSNP) was used for gene mapping (<http://www.ncbi.nlm.nih.gov/snp>). In addition, the expression quantitative trait loci (eQTL) studies data based on the Genotype-Tissue Expression (GTEx) database, version 7 (<http://www.gtexportal.org/home/>) were adopted (Consortium, 2013).

Results

Association between SNPs and vitiligo

For the selected 8 SNPs, their call rates were $> 95\%$ and there were no significantly deviated from Hardy-Weinberg equilibrium ($P > 0.05$) in the control subjects for the replication study. Table 2 showed the association analysis for the 8 SNPs on 22q12 for cases and controls. In replication study, the minor alleles of rs730669, rs4820338 and rs2051582 were weakly associated with vitiligo ($P = 1.34 \times 10^{-4}$, OR = 0.82, 95%CI: 0.74-0.91, $P = 7.28 \times 10^{-3}$, OR = 0.88, 95%CI: 0.80-0.97, $P = 4.54 \times 10^{-4}$, OR = 1.27, 95%CI: 1.11-1.45). The combined analyses of GWAS dataset and replication stages, which showed a strong evidence of association for SNP rs730669 ($P = 3.25 \times 10^{-8}$, OR = 0.81, 95%CI: 0.75-0.87, reaching genome-wide significance) and suggestive evidence of association for SNPs rs4820338 ($P = 1.04 \times 10^{-5}$, OR = 0.86, 95%CI: 0.80-0.92) and rs2051582 ($P = 1.78 \times 10^{-6}$, OR = 1.27, 95%CI: 1.15-1.40, a reported association signal in our previous study [12]).

Conditional logistic regression analysis

To have a better understanding of the associations for the three significantly associated SNPs, we performed conditional logistic regression analysis by controlling the genetic effect of associated SNPs (Table 3). Controlling the reported SNP rs2051582, the two newly identified SNPs (rs730669 and rs4820338) also had significant associations with vitiligo ($P = 4.15 \times 10^{-8}$ and $P = 9.13 \times 10^{-6}$). Controlling the SNP rs730669 or rs4820338, the other two SNPs (rs2051582 and rs4820338, rs730669 and rs2051582) also had significant associations with vitiligo ($P = 2.17 \times 10^{-6}$ and $P = 5.12 \times 10^{-6}$, $P = 7.87 \times 10^{-9}$ and $P = 3.07 \times 10^{-6}$). Among the three SNPs, the paired SNPs were no correlated with each other in current study population (all $D' < 0.05$, $r^2 < 0.0001$).

Genotype and subphenotype analysis

The analyses of genotype-phenotype (age of onset, gender, family history, clinical classification, concomitant autoimmune diseases and Koebner phenomenon) of the three SNPs were also performed. For the SNP rs4820338, the frequency of genotype ($P = 2.30 \times 10^{-2}$) and allele ($P = 7.78 \times 10^{-3}$, OR = 0.87, 95% CI, 0.79-0.97) was significantly different between patients with early onset and late onset (Table 4). However, no statistical difference in different subgroups was observed for the SNPs rs730669 and rs2051582 by comparing the genotype and allele frequencies (data not shown).

Functional annotation and bioinformatics analysis

The two associated SNPs rs730669 and rs4820338 are both located at 22q11.2 (Fig 1). Rs730669 is located in 66kb downstream of *Zinc finger DHHC-type containing 8 (ZDHHC8)* and 27kb downstream of *reticulin 4 receptor (RTN4R)*, rs4820338 is located in 78kb upstream of *V-set pre-B cell surrogate light chain 1 (VPREB1)*. The eQTL analysis showed that only SNP rs730669 was associated significantly with the expression level of *ZDHHC8* in cultured fibroblasts cells ($P = 9.10 \times 10^{-6}$) by using the HaploReg4.1 and GTEx database.

Discussion

To explore the contribution of genetic variation at 22q12 previously identified by our linkage analysis with vitiligo, we further performed imputation analysis for the expanded region of this locus in the discovery dataset and then replicated them in an independent cohort from Chinese Han population. This study identified that two novel genetic variants (rs730669 and rs4820338) at 22q11.2 were associated with vitiligo, and confirmed our previous reported variant (rs2051582) at 22q12.3 [12]. Several susceptible variants at 22q13.1 were also reported to be associated with vitiligo in the Caucasian population [6]. This study also showed the three independent associated signals at 22q12 for vitiligo.

The most strongly associated SNP rs730669 at the 22q11.2 locus, is located in a linkage disequilibrium (LD) block that includes multiple genes (Fig. 1), however, most of these genes function have remained largely unknown. SNP rs730669 at 22q11.2 is located in an intergenic region between *ZDHHC8* and *RTN4R*. The *ZDHHC8* contains 11 exons and encodes a palmitoyltransferase that share a conserved cysteine-rich signature catalytic domain (belonging to the 23-member family of enzymes). The genetic variants in *ZDHHC8* have been reported to be associated with schizophrenia [18, 19], epilepsy [20] and total

anomalous pulmonary venous connection [21]. The eQTL data shows that SNP rs730669 is associated with significant effect on the expression level of *ZDHHC8* in cultured fibroblasts cells. Fibroblasts might affect melanocyte or epidermal pigmentation functionality by secreting skin aging-associated proteins from fibroblasts which favoring their loss and progression of vitiligo [22, 23]. It is well known that catecholamines (dopamine) acts as neurotransmitter which plays important role in pathogenesis of vitiligo [24]. The dopamine pathway probably affects directly melanogenesis or by the melanocortin pathway. *ZDHHC8* can interact with the D2 dopamine receptor (D2R), which might influence the status of palmitoylation D2R [25]. The differentiation of mRNA expression for D2R gene is observed in skin for patient with vitiligo [26]. These indicate that *ZDHHC8* might participate in the pathogenetic mechanisms for vitiligo by the above biological pathway.

For this locus, another gene *RTN4R* (also known as the neurite outgrowth inhibitor 66 (NgR)), contains 2 exons and encodes a glycosylphosphatidylinositol (GPI)-linked protein which plays an important role in the inhibition of myelin-mediated axonal growth by interacting with NgR [27]. The genetic variants at *RTN4R* have been reported to be associated with the anterior limb of the internal capsule and the psychosis with 22q11.2 deletion syndrome [28, 29]. Some studies also show that the differentiated expression of NgR on multiple immunological cells (monocytes, dendritic cells, T/B cells) influence the subsequent regulation of above cells adhesion or stimulation, which acts an important role in the pathogenesis for neuroinflammatory diseases such as multiple sclerosis [30, 31]. Together, These further emphasize that neural-inflammatory mediators are implicated in vitiligo pathogenesis [32].

The associated SNP rs4820338 at 22q11.2 is located in an LD block that only contains *VPREB1* (Fig. 1). *VPREB1* contains 2 exons and encodes a protein which belongs to the immunoglobulin superfamily. It is expressed selectively at the early stages of immune B cell development and to regulate the level of Ig gene rearrangements in the immune B cell differentiation. These show that transcriptional and metabolic pre-B cell receptor-mediated ingredients acts as important roles in pathogenesis for autoimmune diseases [33]. The deletion of *VPREB1* has been reported to be associated with chronic autoimmune arthritis [34] and rheumatoid arthritis [35], and acute lymphoblastic leukemia [36, 37]. The lymphoid immune surveillance participates in vitiligo lesions and results in the lesion extension by triggering antigen-specific T and B cell responses, which supports the involvement of immune response in disease development [38]. It is well known that the biological function of melanin-concentrating hormone receptor 1 (MCHR1) has associated with the B-cell autoantigen located on the membrane of melanocytes by blocking the response of the receptor to MCH [39]. Vitiligo has been reported to react against multiple epitopes on MCHR1 [40, 41]. Therefore, it is speculated that the abnormal encoded proteins by *VPREB1* might involve a variety of immunobiological pathways by the interaction between MCHR1 and pre-B cell receptor-mediated ingredients, which further indicates that genetic and immune factors play an important role in the pathogenesis of vitiligo. Notably, early vitiligo onset is specifically associated with SNP rs4820338, which might contribute substantially to the heritability difference between the early-onset and late-onset subgroups.

This study identifies two novel independent variants rs730669 (*ZDHHC8/RTN4R*) and rs4820338 (*VPREB1*) at 22q11.2 that contributes to vitiligo susceptibility, which further emphasizes that the related- neuroimmunology genes play an important roles in the pathogenetic mechanisms for disease. The current findings provide an indirection for functional studies of candidate gene for this locus in future. It is still necessary to identify the causal gene(s) by the targeted sequencing and functional studies.

Declarations

Conflict of interest statement: All authors have no conflicts of interest to declare.

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Author contribution: Fengli Xiao and Xianfa Tang designed research; Hui Cheng, Lu Cheng and Bo Liang were responsible for sample selection and genotyping; Mengyun Chen and Xiaodong Zheng undertook data processing, statistical analysis and bioinformatics investigations; Xianfa Tang wrote the paper.

Ethics approval: This study was approved by the Institutional Ethical Committee of Anhui Medical University.

Consent to Participate (Ethics): Respect for participants and Informed consent to participate in this research.

Consent to Publish (Ethics): All authors agree to publication in this Journal and it is not under consideration for publication elsewhere.

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Tables

Table 1 Summary information of vitiligo patients and controls

Analysis	Cases			Controls		
	Sample Size	Mean Age (s.d.)	Male (%) / Female (%)	Sample Size	Mean Age (s.d.)	Male (%) / Female (%)
GWAS	1117	28.73(±15.00)	656(58.73) / 461(41.27)	1701	35.90(±14.81)	928(54.56) / 773(45.44)
Replication	2069	28.82(±14.41)	1082(52.30) / 987(47.70)	1370	31.43(±14.74)	754(55.04) / 616(44.96)
Total	3186	28.79(±14.60)	1738(54.55) / 1448(45.45)	3071	33.47(±14.63)	1682(54.77) / 1389(45.23)

GWAS, genome-wide association study.

Tables 2 Association evidence for 8 SNPs in the GWAS, the replication study, and the combined analysis.

SNP	Chr_pos	Gene	MA	GWAS					Replication study					Comb	
				1117 cases, 1701 controls					2069 cases, 1370 controls						3186
				MAF		OR (95%CI)	P	Call rate	MAF		OR (95%CI)	P	Call rate	OR (95%CI)	
				Case	Control				Case	Control					
rs730669	20214431	ZDHC8/RTN4R	C	0.32	0.37	0.81 (0.73-0.91)	2.99E-04	1.00	0.32	0.36	0.82 (0.74-0.91)	1.34E-04	0.99		0.81 (0.75-0.87)
rs5750483	22080038	IGLV8-61	A	0.23	0.27	0.82 (0.73-0.93)	1.94E-03	1.00	0.25	0.25	0.96 (0.86-1.08)	5.23E-01	0.95	0.89 (0.82-0.97)	
rs4820338	22166665	VPREB1	C	0.44	0.48	0.83 (0.75-0.93)	9.18E-04	0.99	0.44	0.48	0.88 (0.80-0.97)	7.28E-03	0.99	0.86 (0.80-0.92)	
rs490362	27335509	LOC102724900	C	0.2	0.17	1.27 (1.11-1.46)	6.13E-04	1.00	0.18	0.18	1.03 (0.91-1.17)	6.35E-01	0.99	1.13 (1.03-1.24)	
rs133937	32999866	SYN3	C	0.21	0.25	0.83 (0.73-0.95)	5.03E-03	1.00	0.24	0.25	0.96 (0.86-1.08)	4.91E-01	0.99	0.92 (0.85-1.00)	
rs2051582	37162316	IL2RB	A	0.17	0.14	1.24 (1.07-1.45)	4.26E-03	1.00	0.17	0.14	1.27 (1.11-1.45)	4.54E-04	0.99	1.27 (1.15-1.40)	
rs5756587	37282163	CYTH4	T	0.15	0.18	0.79 (0.68-0.91)	1.46E-03	1.00	0.17	0.16	1.04 (0.91-1.19)	5.50E-01	0.97	0.92 (0.84-1.01)	
rs4820287	37394071	ELFN2	C	0.24	0.28	0.81 (0.71-0.91)	6.65E-04	0.99	0.26	0.26	1.04 (0.93-1.16)	5.40E-01	0.99	0.92 (0.85-1.00)	

GWAS, genome-wide association study, SNP, single-nucleotide polymorphism, Chr_pos, chromosome position, MA, minor allele, MAF, minor allele frequency, OR, odds ratio, CI, confidence interval.

Tables 3 The results of conditional regression analysis.

SNP	r ²	D'	Condition on SNP	P	OR
rs730669	4.51E-04	3.17E-02	rs2051582	4.15E-08	0.81
rs4820338				9.13E-06	0.85
rs2051582	1.13E-05	7.25E-03	rs730669	2.17E-06	1.27
rs4820338				5.12E-06	0.84
rs730669	8.28E-05	2.93E-02	rs4820338	7.87E-09	0.80
rs2051582				3.07E-06	1.26

SNP, single-nucleotide polymorphism, OR, odds ratio.

Tables 4 Distribution of genotypes and alleles for SNP rs4820338 in subgroups.

Subphenotypes	Genotype frequency (%)				Allele frequency (%)			
	CC	CG	GG	<i>p</i> ^a	C	G	<i>p</i> ^b	OR(95%CI)
Age at onset								
Early onset <=20 years(n=1703)	291(17.09)	873(51.26)	539(31.65)	2.30E-02	1455(42.72)	1951(57.28)	7.78E-03	0.87(0.79-0.97)
Late onset >20 years(n=1447)	286(19.77)	761(52.59)	400(27.64)		1333(46.06)	1561(53.94)		
Gender								
Male(n=1714)	320(18.67)	884(51.58)	510(29.75)	0.85	1524(44.46)	1904(55.54)	0.72	1.02(0.92-1.13)
Female(n=1436)	257(17.90)	750(52.23)	429(29.87)		1264(44.01)	1608(55.99)		
Family history								
Positive(n=414)	89(21.50)	213(51.45)	112(27.05)	0.15	391(47.22)	437(52.78)	0.07	1.15(0.99-1.33)
Negative(2736)	488(17.84)	1421(51.94)	827(30.22)		2397(43.80)	3075(56.20)		
Clinical classification								
Non segmental(n=2297)	439(19.11)	1173(51.07)	685(29.82)	0.14	2051(44.65)	2543(55.35)	0.31	1.06(0.95-1.19)
Segmental(n=853)	138(16.18)	461(54.04)	254(29.78)		737(43.20)	969(56.80)		
Autoimmune disease involvement								
Positive(n=92)	20(21.74)	49(53.26)	23(25.00)	0.50	89(48.37)	95(51.63)	0.25	1.19(0.88-1.59)
Negative(n=3058)	557(18.21)	1585(51.83)	916(29.96)		2699(44.13)	3417(55.87)		
Koebner phenomenon								
Positive(n=236)	51(21.61)	121(51.27)	64(27.12)	0.34	223(47.25)	249(52.75)	0.17	1.14(0.94-1.38)
Negative(n=2914)	526(18.05)	1513(51.92)	875(30.03)		2565(44.01)	3263(55.99)		

OR, odds ratio, CI, confidence interval.

^a For genotype using a 2×3 contingency table. ^b For allele using a 2×2 contingency table.

Figures

Figure 1

Regional association plot for the associated SNPs rs730669 and rs4820338 at 22q11.2 region. SNP is plotted by chromosomal position (GRCh38/hg19, x axis) and association with vitiligo from current study ($-\log_{10}P\text{-value}$, y axis). Estimated recombination rates (based on the combined CHB and JPT samples from the HapMap project) were plotted in light blue. Genes are indicated in the lower panel of the plot.