Genetic Variation of Promoter Sequence Modulates XBP1 Expression and Genetic Risk for Vitiligo

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Abstract

Our previous genome-wide linkage analysis identified a susceptibility locus for generalized vitiligo on 22q12. To search for susceptibility genes within the locus, we investigated a biological candidate gene, X-box binding protein 1(XBP1). First, we sequenced all the exons, exon-intron boundaries as well as some 5′ and 3′ flanking sequences of XBP1 in 319 cases and 294 controls of Chinese Hans. Of the 8 common variants identified, the significant association was observed at rs2269577 ($p_{trend} = 0.007$, OR = 1.36, 95% CI = 1.09–1.71), a putative regulatory polymorphism within the promoter region of XBP1. We then sequenced the variant in an additional 365 cases and 404 controls and found supporting evidence for the association ($p_{trend} = 0.008$, OR = 1.31, 95% CI = 1.07–1.59). To further validate the association, we genotyped the variant in another independent sample of 1,402 cases and 1,288 controls, including 94 parent-child trios, and confirmed the association by both case-control analysis ($p_{trend} = 0.003$, OR = 1.18, 95% CI = 1.06–1.32) and the family-based transmission disequilibrium test (TDT, p = 0.005, OR = 1.93, 95% CI = 1.21–3.07). The analysis of the combined 2,086 cases and 1,986 controls provided highly significant evidence for the association ($p_{trend} = 2.94 \times 10^{-6}$, OR = 1.23, 95% CI = 1.13–1.35). Furthermore, we also found suggestive epistatic effect between rs2269577 and HLA-DRB1*07 allele on the development of vitiligo (p = 0.033). Our subsequent functional study showed that the risk-associated C allele of rs2269577 had a stronger promoter activity than the non-risk G allele, and there was an elevated expression of XBP1 in the lesional skins of patients carrying the risk-associated C allele. Therefore, our study has demonstrated that the transcriptional modulation of XBP1 expression by a germ-line regulatory polymorphism has an impact on the development of vitiligo.

Introduction

Vitiligo (OMIM*193200) is a depigmentary disorder characterized by the appearance of white patches as a result of the loss of functional melanocytes and melanin from skin and hair. It is one of the most common pigmentation disorders with a prevalence of 0.1% to 2% in different geographic regions and ethnic groups [1]. Clinical and epidemiological investigations indicated that vitiligo is a multifactorial disorder [2]. The aetiology of vitiligo remains obscure, but several factors have been implicated in the development of the disease including self-destructive, biochemical, neural, autoimmune and genetic susceptibility factors [3].

In the past few years, linkage studies identified several potential susceptibility loci, such as 1p31(AIS1), 7q(AIS2), 8p(AIS3), and 17p13(VAMAS1) in Caucasian population and 4q13-q21(AIS4), 6p21-p22 and 22q12 in Chinese population [4–8]. A number of candidate genes were also suggested to be associated with vitiligo, including HLA, AIRE, VIT1, CAT, FOXD3, ESR1, COMT,

PTPN22 and NALP1 [9–14]. However, most of these candidate genes are not located within the identified linkage regions, complicating the interpretation of the significance of these candidate genes, with the possible exception of the FOXD3 gene within the AIS1 locus and the NALP1 gene within the VAMAS1 locus in Caucasian population.

Our genome-wide and fine-mapping linkage analyses in the Chinese families of generalized vitiligo identified a susceptibility locus on 22q12 [7,8]. Within the 22q12 locus, the linkage evidence was largely confined to a 10 Mb interval (D22S1167–D22S283) that contains 84 genes and 35 uncharacterized transcripts, including X-box binding protein 1 (XBP1). XBP1 encodes a DNA-binding protein whose downstream target is the X boxes of HLA DR-α and DP-β [15]. XBP1 acts as a transcription factor by recognizing the X2 promoter element of both human DR-α and DP-β. Deletion of X1 and X2 sequences eliminates the expression of HLA class II genes in vitro and in transgenic mice. With a view to the autoimmunity feature of vitiligo supported by the significant

Author Summary

Vitiligo is a genetically complex skin disorder that afflicts 0.1%–2% of the population throughout the world. Linkage and association studies in different populations have implicated several genomic regions and candidate genes that are linked to the development of vitiligo. Our previous genome-wide linkage analysis identified a genetic susceptibility locus for vitiligo on 22q12 in a Chinese population. In this study, we conducted a genetic and molecular study of a biologically plausible candidate gene, XBP1, within the region. We first performed a progressive association analysis in three independent samples of vitiligo, which revealed significant association between a putative promoter polymorphism, rs2269577, and vitiligo. Then, our functional analysis also demonstrated that the risk-associated allele of this variant has a stronger promoter activity and was associated with a significantly elevated expression of XBP1 in lesional skins in patients carrying the risk-associated allele. Our findings have provided the first evidence for XBP1 to play an important role in the development of vitiligo.

linkage to the MHC region on 6p21-p22 and evidences for the association of HLA-DR with vitiligo, XBP1 is a strong biological candidate gene for vitiligo due to its plausible role in the development of the disease through its interaction with HLA-DR.

Here, we performed a genetic and molecular study of XBP1 in a Chinese Han population. Through a series of genetic association and functional analyses, we have provided strong evidence for XBP1 to be a susceptibility gene for vitiligo.

Results

Direct Sequence Analysis of Exons, Exon-Intron Boundaries, and 5′ and 3′ Flanking Sequences

To evaluate the role of regulatory and coding variants within XBP1 in the development of vitiligo, we sequenced all the 5 exons, exon-intron boundaries as well as 362 bp 5′ upstream (containing putative regulatory elements) and 946 bp 3′ downstream sequences of XBP1 in 319 patients of vitiligo and 294 normal controls (Figure S1). To increase the likelihood of identifying vitiligo susceptibility-related genetic variants, all the 319 patients were chosen to have a positive family history of disease, meaning with at least one first-degree relative carrying the same disease. We identified 34 variants,

including 32 single nucleotide polymorphisms (SNPs), one single-base deletion and one three-base insertion (Table S1). Of the 34 variants, 9 are known ones (rs2269578, rs2269577, rs2269576, rs2269575, rs5762809, rs2228260, rs35873774, rs34842534, rs2097461,) reported in the dbSNP database (dbSNP BUILD 129), and 25 are novel variants (g.−209C>A, g.−186G>A, g.−175C>T, g.−137C>G, g.−105G>A, g.−26T>C, g.−14delG, g.17G>C, g.24G>A, g.27G>T, g.30C>T, g.63−65insGGC, g.129C>T, g.1374A>G, g.1392C>A, g.1522A>G, g.3352A>G, g.3393A>G, g.3554C>G, g.3626T>C, g.4262G>A, g.4725G>T, g.4746C>T, g.5409A>G, g.5597T>C). 8 SNPs (6 known and 2 novel SNPs) were common with a minor allele frequency of more than 1% in our Chinese Han samples and were selected for further association analysis (Table 1 and Figure S1).

Association Analysis of 8 Common Variants

Association analysis of the 8 SNPs was performed by using genotype-based trend and allele tests. Of the 8 SNPs, the association was only detected at rs2269577 ($p_{trend} = 0.007$, OR = 1.36, 95%CI = 1.09–1.71; Table 1) locating at 197 bp upstream of the first exon, presumably within the promoter sequence of XBP1 (Figure S1). As a follow-up, we sequenced the SNP in additional 365 sporadic patients and 404 healthy controls and confirmed the association ($p_{trend} = 0.008$, OR = 1.31, 95%CI = 1.07–1.59). To further validate this association, we genotyped rs2269577 in a third independent sample of 1402 cases and 1288 controls using a TaqMan assay. The second follow-up analysis provided the further supporting evidence for the association ($p_{trend} = 0.003$, OR = 1.18, 95%CI = 1.06–1.32). The analysis of the combined 2086 cases and 1986 healthy controls revealed a highly significant association between rs2269577 and vitiligo with the minor allele C to be associated with increased risk ($p_{trend} = 2.94 \times 10^{-6}$, OR = 1.23, 95%CI = 1.13–1.35; Table 2). Allelic association analysis of the three samples also provided supporting and consistent evidence for the association (Table 2). Furthermore, of the 2086 cases analyzed in this study, there were 94 patients whose parental DNA samples were collected, allowing us to perform a family-based association analysis (TDT) that believe to be more robust than population-based case-control analysis. The TDT analysis of rs2269577 revealed a significant evidence for the over-transmission of the C allele to the affected individuals (T:U 52:27, p = 0.005, OR = 1.93, 95%CI = 1.21–3.07). Taking together, our progressive association analysis in three independent samples provided strong evidence for the association between rs2269577 and vitiligo susceptibility in the

Table 1. Association analysis of 8 common SNPs within the coding and promoter sequences of XBP1 in the initial 319 cases and 294 controls.

SNP	Alleles	Position	Location	Cases (319) (MAF)	Controls (294) (MAF)	P_trend	OR(95% CI)
rs2269578	C→G	8587412	promoter	0.18	0.22	0.06	0.77 (0.58–1.01)
XBP1snp1	C→A	8587338	promoter	0.03	0.04	0.35	0.74 (0.40–1.40)
rs2269577	G→C	8587326	promoter	0.41	0.34	0.007	1.36 (1.09–1.71)
XBP1snp10	G→T	8587103	5′UTR	0.06	0.06	0.80	0.94 (0.59–1.50)
rs2269575	C→T	8587095	5′UTR	0.26	0.28	0.53	0.93 (0.73–1.18)
rs5762809	G→A	8587063	exon1	0.18	0.21	0.18	0.83 (0.62–1.10)
rs2228260	C→T	8586875	exon1	0.09	0.07	0.40	1.20 (0.79–1.82)
rs2097461	G→A	8582448	intron4	0.42	0.38	0.16	1.18 (0.93–1.50)

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Table 2. Summary of the association results for rs2269577 in three independent samples as well as the combined sample.

		Number	Allele (Frequency)		Genotype (Frequency)			P_allele	P_trend	OR (95% CI)
			C	G	C/C	C/G	G/G			
Initial Sequence Analysis	Cases	319	264 (0.41)	374 (0.59)	57 (0.18)	150 (0.47)	112 (0.35)	0.005	0.007	1.36 (1.09–1.71)
	Controls	294	198 (0.34)	390 (0.66)	41 (0.14)	116 (0.39)	137 (0.47)			
Follow-up By Sequencing	Cases	365	298 (0.41)	432 (0.59)	81 (0.22)	136 (0.37)	148 (0.41)	0.005	0.008	1.31 (1.07–1.59)
	Controls	404	274 (0.34)	534 (0.66)	45 (0.11)	184 (0.46)	175 (0.43)			
Follow-up By TaqMan	Cases	1402	1195 (0.43)	1609 (0.47)	271 (0.19)	653 (0.47)	478 (0.34)	0.002	0.003	1.18 (1.06–1.32)
	Controls	1288	993 (0.39)	1583 (0.62)	187 (0.15)	619 (0.48)	482 (0.37)			
Combined Analysis	Cases	2086	1757 (0.42)	2415 (0.58)	409 (0.20)	939 (0.45)	738 (0.35)	2.01×10^{-6}	2.94×10^{-6}	1.23 (1.13–1.35)
	Controls	1986	1465 (0.37)	2507 (0.63)	273 (0.14)	919 (0.46)	794 (0.40)			

OR: per allele odds ratio from trend test.
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Chinese Han population. The genotypes of the 8 SNPs in the first control samples were found to be in Hardy-Weinberg equilibrium (HWE) (p values>0.05), and the genotypes of rs2269577 were also found to be in HWE in the controls of the two follow-up samples (p values≥0.05).

Stratified Association Analysis of rs2269577 by Clinical Subtypes

To further investigate the association at rs2269577, we performed the stratified association analysis in different patient subgroups by age of onset, family history, severity of disease (body surface involvement) and association with autoimmune disorders. The results were summarized in Table 3. Rs2269577 was found to be associated with the severity of disease. Patients carrying the risk allele are more likely to show extensive body surface involvement (>20%) than limited body surface involvement (<20%) (OR = 1.65, p = 0.02). We did not see the association of rs2269577 with age of onset, family history and the related autoimmune disorders.

Table 3. Stratified association analysis of rs2269577 by clinical subgroups.

		Genotype					
Samples	Total	CC	CG	GG	OR	95%CI	P
Age of onset							
≤20 years	1241	269	536	436	1.12	0.99–1.26	0.07
>20 years	813	136	384	293			
Family history							
Familial	487	91	239	157	1.06	0.92–1.21	0.45
Sporadic	1592	318	695	579			
Body surface involvement							
>20%	40	11	20	9	1.65	1.07–2.54	0.02
≤20%	1167	236	634	528			
Associated autoimmune disorders							
With	71	9	39	23	0.99	0.71–1.38	0.95
Without	1314	238	585	491			

OR and P were from the comparison between the clinical subgroups by using the trend test.
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Analysis of Interaction between rs2269577 and HLA-DRB1*07

Given the possible functional interaction between XBP1 and HLA-DR, we investigated whether the genetic effect at rs2269577 is modified by the HLA-DR risk allele for vitiligo. We successfully genotyped the DRB4*01, DRB1*04, DRB1*07, DRB1*12 alleles by PCR-SSP in 1755 cases and 1742 controls and found the HLA-DRB1*07 allele to be significantly associated with vitiligo (p = 4.53×10^{-21}, OR = 1.98, 95%CI = 1.72–2.29, Table S3). We then tested the epistatic interaction between rs2269577 and HLA-DRB1*07 using logistic regression analysis and found that the full model with both the main and interactive effects was better than the model with only the main effect to fit the data (p = 0.033). Furthermore, we performed the stratified association analysis of rs2269577 by HLA-DRB1*07 allele. The association at rs2269577 was significant in both the patients carrying and not carrying the HLA-DRB1*07 allele (Table 4). However, the association seems to be stronger in the patients carrying the HLA-DRB1*07 allele. For example, the C/G genotype of rs2269577 showed a significant association with increased risk in the patients carrying the HLA-DRB1*07 allele (OR = 1.44, p = 0.007), but did not show the association in the patients not carrying the allele (OR = 0.94, p = 0.496). The CC genotype of rs2269577 also showed a stronger association in the carriers of the HLA-DRB1*07 allele (OR = 2.05) than in the patients not carrying the allele (OR = 1.58), but the difference is not statistical significant.

Molecular Analysis of rs2269577 and XBP1 Expression

Because rs2269577 is located at the promoter region of XBP1, we performed a series of functional analysis to evaluate the SNP's impact on gene expression. First, we performed an *in-vitro* reporter assay analysis to evaluate whether the promoter sequences carrying different SNP alleles have differential promoter activity. We cloned the promoter sequences of XBP1 carrying different SNP alleles into luciferase reporter vectors (pGL3) and then transiently transfected the two reporter vectors into the HaCat and Mel-RM cells. Luciferase activity was measured after 48 hrs incubation. As shown in Figure 1, the cloned promoter sequence carrying the risk-associated C allele can strongly induce the expression of the reporter gene, whereas the cloned promoter sequence carrying the wild-type G allele show a much weaker, if any, induction of the reporter gene expression. In both cell lines, the difference in the induction of the reporter gene expression between the two alleles is statistically significant (P<0.05).

Table 4. Logistic Regression odds ratio estimates of XBP1 genotype stratified by HLA-DRB1*07 in 3497 samples.

| | | HLA-DRB1*07 | | | | | |
| | | + | | | − | | |
	Genotype	OR	95% CI	P	OR	95% CI	P
XBP1	G/G	1.00			1.00		
rs2269577	C/G	1.44	1.10–1.87	0.007	0.94	0.78–1.12	0.496
	C/C	2.05	1.46–2.88	3.19×10^{-5}	1.58	1.23–2.02	2.85×10^{-4}

OR is compared to the reference genotype.
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We subsequently analyzed the expression of XBP1 mRNA in the paired lesional and non-lesional skins from 38 patients by using quantitative real-time PCR analysis. Overall, there was a moderate but statistically significant increased level of XBP1 mRNA in the lesional skins compared with the non-lesional skins of patients (p = 0.0007, Figure 2). More interestingly, the elevated expression of XBP1 mRNA in the lesional skin seemed to be correlated with rs2269577's genotypes (Figure 3). The elevated expression of XBP1 mRNA in the lesional over the non-lesional skins was only observed in the patients carrying either one (average fold difference = 3.61, p = 0.0004) or two copies of the risk-associated C allele (average fold difference = 4.80 p = 0.07). In contrast, in the patients not carrying risk-associated allele (with GG genotype), there was no difference in mRNA level between the lesion and normal skins (average fold difference = 1.39, p = 0.93). The average fold difference between the paired lesional and non-lesional samples in the patients carrying GG genotype (average fold difference = 1.39) was significantly lower than the average fold difference in the patients carrying either GC or CC genotypes (average fold difference = 3.88) (p = 0.003). Therefore, both the *in-vitro* and *in-vivo* transcriptional analyses provided consistent results, indicating that the risk-associated C allele is associated with a higher expression of XBP1 (than the wild-type G allele) which in turn is associated with the lesion development in vitiligo patients.

We also performed immunohistochemistry analysis using formalin-fixed paraffin embedded skin biopsy samples from another 37 patients to assess the XBP1 protein level in the lesional and non-lesional skins. As shown in Figure 4 and Figure 5, there was a moderately enhanced XBP1 antibody staining in the lesional skin compared to the non-lesional skin (p = 0.003). Furthermore, the enhanced staining of XBP1 antibody in the lesional skin is more prominent in the patients carrying either CC (p = 0.04) or GC (p = 0.03) genotype. In the patients carrying the GG genotype, there was no significant enhancement (p = 0.41). An example of the enhanced XBP1 antibody staining in the lesional over the non-lesional skin of a patient is provided in Figure 6.

Discussion

To search for susceptibility gene(s) within a genomic region showing a strong linkage to vitiligo, we interrogated a biologically plausible candidate gene, XBP1, within the region through a series of genetic association and molecular analyses. The progressive genetic association analysis in three clinical samples of vitiligo provided strong evidence for the association between a putative promoter polymorphism, rs2269577 (−197G>C to the first exon), and vitiligo susceptibility in the Chinese Han population. Our subsequent functional analysis has further demonstrated a
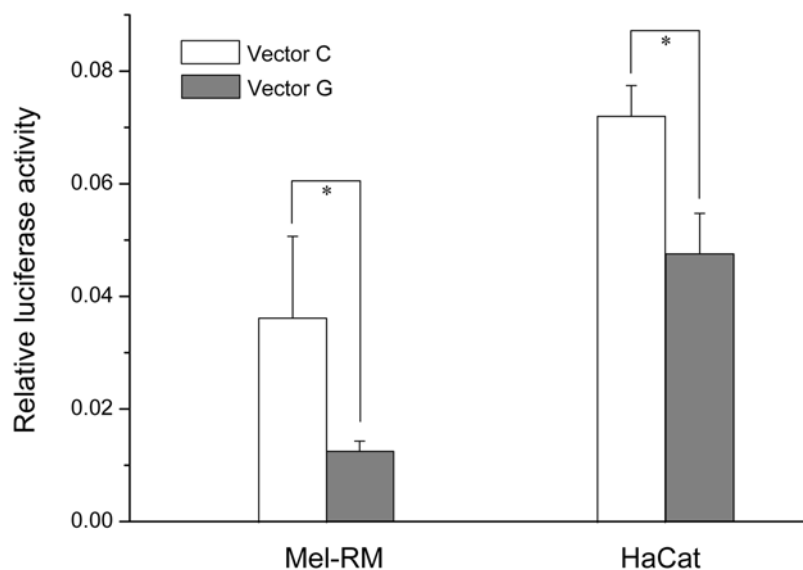

Figure 1. Difference of the promoter activity between the G and C allele of rs2296577 in Mel-RM cells and HaCat cells. Each column represents mean±s.d. of at least three experiments. *Two-tailed P<0.05 by Student's *t*-test.
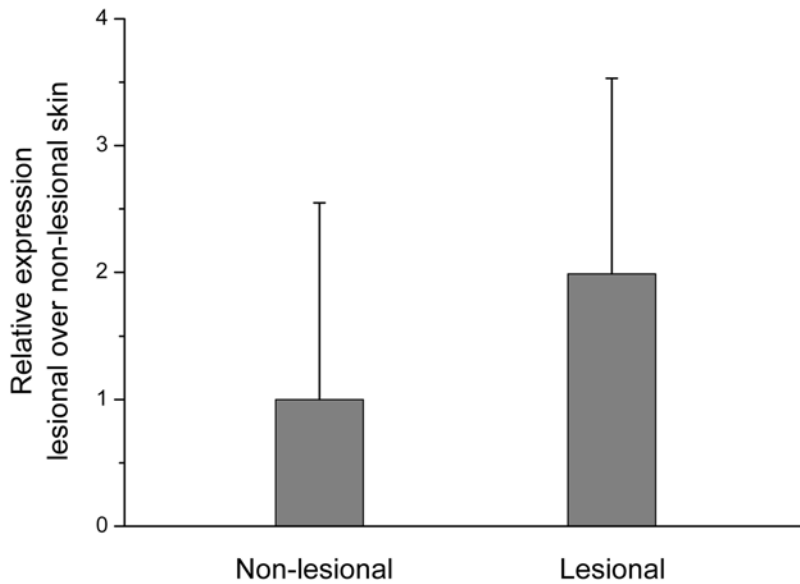doi:10.1371/journal.pgen.1000523.g001

Figure 2. XBP1 mRNA expression in the paired lesional and non-lesional skins from 38 patients. XBP1 mRNA was measured by using quantitative real-time PCR. Data represent the normalized XBP1 mRNA expression level by 18S rRNA. The normalized expression data was standardized so that the average of the normalized expression in the non-lesional skins is equal to 1. The comparison of XBP1 mRNA expression between the 38 paired lesional and non-lesional skins were performed by using nonparametric Wilcoxon Signed Ranks Test.
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plausible molecular mechanism for this association that the risk-associated C allele has a stronger promoter activity (than the wild-type G allele) and may induce a higher expression of XBP1, which in turn promotes the development of lesions in vitiligo patients. Therefore, we have demonstrated that the transcriptional modulation of XBP1 expression by germ-line regulatory polymorphisms can influence the development of vitiligo.

HLA genes have been shown to be associated with vitiligo. Although the results of previous studies are generally moderate and variable, the evidence for the association of HLA-DR alleles with vitiligo was demonstrated in several different populations.

HLA-DRB1*03, HLA-DRB1*04 and HLA-DRB1*07 alleles were found to be associated with vitiligo in Turkish patients, and the association of HLA-DRB4*0101 and HLA-DRB4*0303 alleles was found in Dutch patients [16,17]. Furthermore, Fain et al found that in multiplex vitiligo families, the HLA-DRB1*04-HLA-DQB1*0301 haplotype is associated with both an increased risk of vitiligo and the early onset of the disease [18]. Our previous linkage analysis also implicated the linkage to 6p21-p22 with a NPL value above 4 [8]. Given XBP1's known function as a transcription factor controlling the expression of HLA-DR, we posit that XBP1 may influence the development of vitiligo, at least
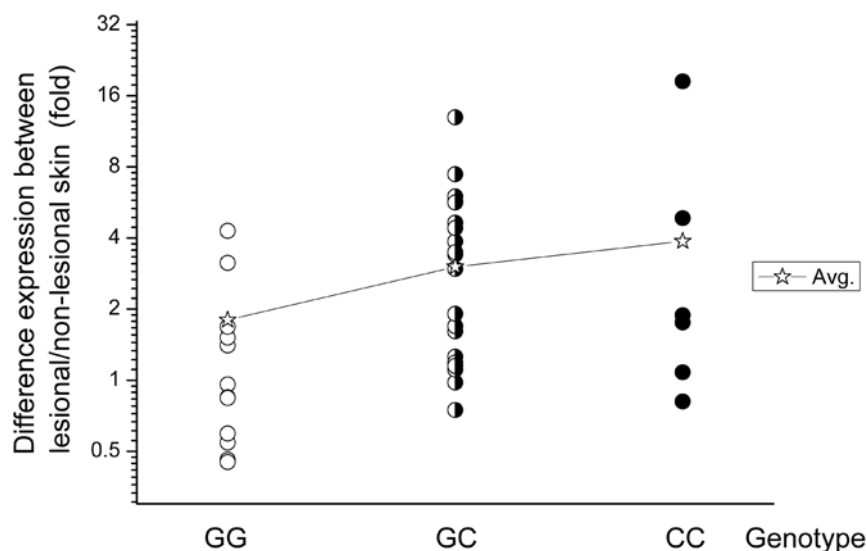

Figure 3. Relative expressions of XBP1 in the lesional and non-lesional skins (fold) of three patient groups with different genotypes of rs2269577: 12 patients with GG genotype, 20 patients with GC genotype, and 6 patients with CC genotype. The Avg. indicates the mean of the difference between the paired lesional and non-lesional skins within each of three genotypes. The y axis is in log2 scale.
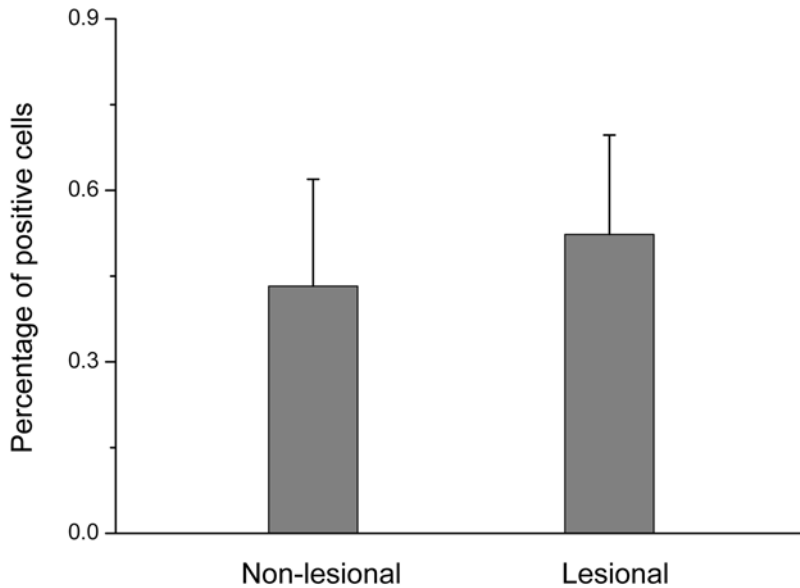doi:10.1371/journal.pgen.1000523.g003

Figure 4. XBP1 protein level determined by immunohistochemical staining in the paired lesional and non-lesional skins from 37 patients with vitiligo. Data represent the percentage of positive cells. The comparison of XBP1 protein level between the 37 paired lesional and non-lesional skins was done by using Paired Samples T Test.
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partially, through its interaction with HLA-DR molecules. Consistent with this hypothesis, we indeed observed the evidence for the modification of the genetic effect at rs2269755 by the risk allele HLA-DRB1*07 and the epistatic interaction between the two risk alleles in the Chinese Han population. However, due to their moderate significance, our results on the interaction between XBP1 and HLA-DRB1 risk alleles need to be validated by additional studies in large samples.

Rs2269577 was first found to be associated with bipolar affective disorder in Japanese patients and over-transmitted to affected offspring in trios of NIMH Bipolar Disorder Genetic Initiative [19]. The follow-up studies of the SNP in diverse populations, however, provided inconsistent results [20–22]. The SNP was also suggested to be associated with schizophrenia [23–26]. Intriguingly, the common G allele was found to be associated with a reduced promoter activity of XBP1 and increased susceptibility to bipolar disorder in the previous study [19], whereas the G allele was found to be associated with a reduced promoter activity but a decreased susceptibility to vitiligo in our study. Upon the confirmation by further studies, these results indicate that the transcriptional modulation of XBP1 function may lead to different physiological outcome. XBP1 has a diverse biological function. It was found to be widely expressed and is essential for fetal survival, hepatogenesis, neurological develop-
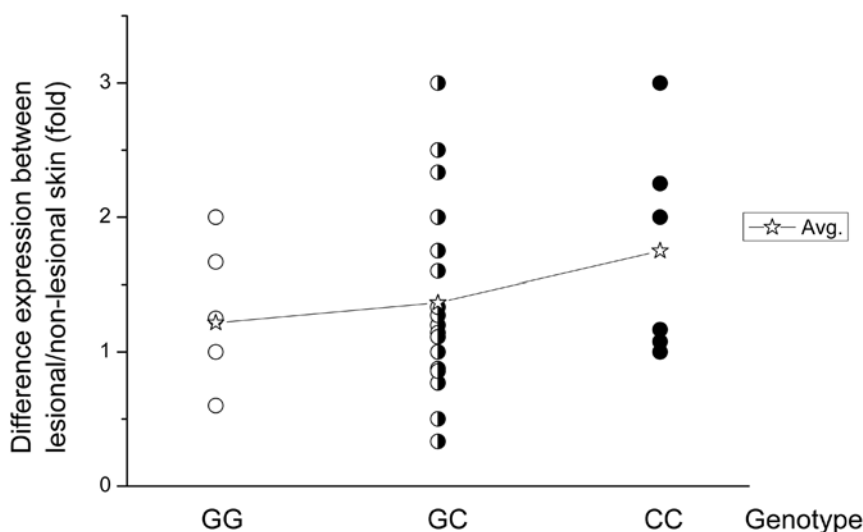

Figure 5. The difference of XBP1 protein level (fold) between the paired lesional and non-lesional skins in three groups of patients with different genotypes of rs2269577: 7 patients with GG genotype, 24 patients with GC genotype, and 6 patients with CC genotype. The Avg. indicates the mean of the difference between the paired lesional and non-lesional skins within each of three genotypes.
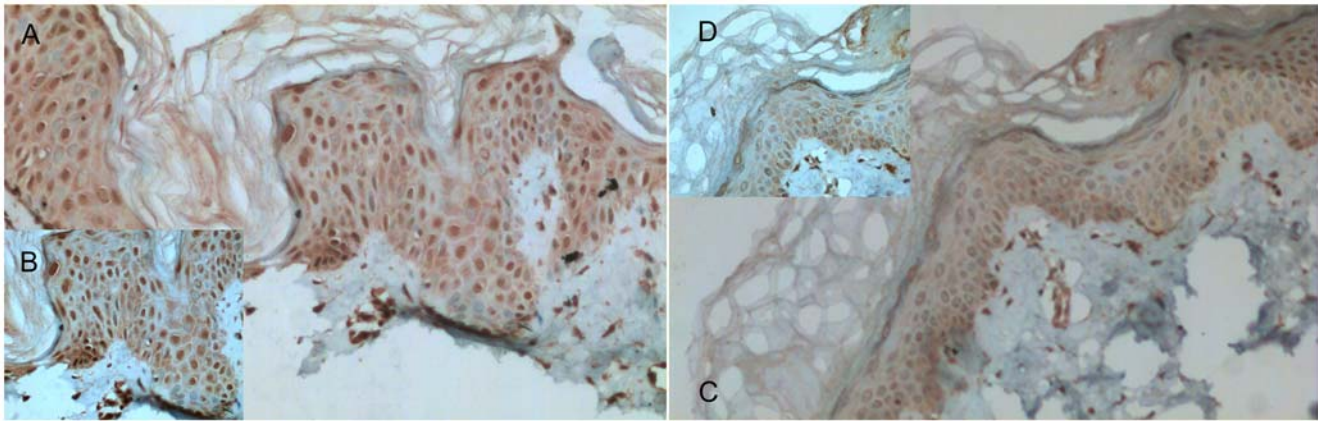doi:10.1371/journal.pgen.1000523.g005

Figure 6. Immunohistochemical staining of XBP1 protein in the paired lesional (A,B) and non-lesional skin (C,D) of a patient with vitiligo (magnifications: (A,C)×200; (B,D)×400).
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ment, bone growth and immune system activation in mouse [27,28]. It was also shown to be required for the development of highly secretory cells such as plasma cells and pancreatic and salivary gland epithelial cells and adaptation of tumor cells to hypoxic conditions [29–31] and the mammalian unfolded protein response (UPR) or endoplasmic reticulum (ER) stress response [32]. As a transcription factor, it regulates functionally distinct targets, including MHC class II genes, through different sequence motifs and often in cell type, cell cycle and tissue specific fashion [33–35]. Consistent to its diverse biological function, XBP1 has also been shown to be involved in many disorders including diabetes, inflammatory bowel disease, bipolar disorder, schizophrenia and multiple myeloma [19,24,36–38]. Therefore, the diverse physiological outcome from the transcriptional modulation of XBP1 by same germ-line mutations is biologically plausible.

There are several limitations that need to be considered for interpreting the results of this study. First, the sample size of our study is moderate, which has limited our study to the common variants. The moderate sample size is also likely responsible for the moderate significance of our results for gene-gene interaction. Furthermore, the contribution of a genetic risk variant to disease development is influenced by both the genetic effect and the population frequency of the risk variant that often vary from population to population. Therefore, the results from the current study need to be validated by additional studies in larger samples of diverse populations. Second, although the coding sequences of XBP1 were well covered in the sequencing and genetic association analyses, the coverage of the promoter sequences is rather limited. Consequently, other regulatory risk variants may have been missed in this study. Furthermore, although our molecular analyses do support rs2269577 to be functional and thus causal, the functional results are generally suggestive and can be further improved. For example, the quantification of the XBP1 antibody staining was done manually, largely due to the limited resource available for the current study, and can be improved by morphometric analysis that digitally analyzes the levels of immunohistorchemical staining. Therefore, further functional studies and fine mapping analyses will be needed to validate the current results.

In conclusion, we found a common regulatory variant, rs2269577, in the promoter region of XBP1 that is associated with increased risk for vitiligo by carrying out a series of association analysis in three independent samples of the Chinese Han population. We further demonstrated that the association is

probably due to the transcriptional modulation of XBP1 expression which was shown to be important for the development of vitiligo lesion. Our findings have therefore not only provided insight into the genetic basis of vitiligo but also advanced our understanding on XBP1's function.

Materials and Methods

Subjects

A total of 4260 individuals were included in this study, consisting of 2086 vitiligo patients, 1986 unrelated healthy controls and 94 parent-child trios. Samples were respectively collected from the Department of Dermatology at First Affiliated Hospital of Anhui Medical University at Hefei, Anhui, China, the Vitiligo Clinic of the Railway Hospital at Xiangfan, Hubei, China, and the Department of Dermatology at Affiliated Xijing Hospital of Fourth Military Medical University at Xian, Shanxi, China. The diagnosis of vitiligo was made on the basis of the presence of typical clinical features (discrete, well-circumscribed depigmented patches), and only patients with clear signs of acquired patches on the trunk, extremities, central face, genitalia, or other area was scored as affected. Consensus diagnosis of vitiligo was performed by two experienced dermatologists who carefully checked phenotypes by history, lesion maps, physical examination and/or photographs. Any individual whose phenotype was questionable was excluded from the study. All controls were healthy individuals without vitiligo, any autoimmune disorders (such as psoriasis, Graves'disease, Systemic lupus erythematosus, Rheumatoid arthritis, etc), systemic disorders, and any family history of vitiligo (including first-, second- and third-degree relatives) and recruited from health check-up centers. All the cases and controls are self-declared Chinese Han and were collected from the same geographic regions of China. Cases and controls were matched on gender. The cases (average age = 25.70±14.36 years) were younger than the controls (average age = 31.95±15.72 years), but the difference is very moderate. Approval to undertake this study was granted by the Ethics Committee of the Anhui Medical University and was conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained from each recruited subject.

Sequence Analysis and SNP Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a standard procedure [39]. We designed primers flanking the

entire coding sequence, exon–intron boundaries, 362 bp sequence of its putative promoter and 946 bp sequence downstream from the 3′ end of XBP1 gene by using the web-based version of the Primer 3.0 program (http://primer3.sourceforge.net/). Primer sequences were listed in Table S2. Products were amplified by polymerase chain reactions (PCR) with touchdown program(95°C for 15 min; 40 cycles of 94°C for 40 s, 57°C for 60 s, 72°C for 70 s, 72°C for 10 min, except that in the first 12 cycles the annealing temperature decreased from 63°C to 57°C by 0.5°C per cycle). PCRs were carried out in an ABI 9700 Thermal Cycler in 10 μl reaction volume containing 20 ng of genomic DNA, 0.3 mM dNTPs, 0.3 μM of each primer, 3.0 mM MgCl$_2$ and 0.1 units of Hotstar Taq DNA polymerase (Qiagen). After the amplification, products were purified using a QIAquick PCR Purification Kit (Qiagen) and directly sequenced on ABI PRISM(R) 3730 automated sequencer (Applied Biosystems). All the variants identified by sequence analysis were checked against the dbSNP data (version 129) for determining the novelty of variants, and the novel variants were named according to their positions to the first exon of the gene.

SNP genotyping was performed using the 5′ nuclease allelic discrimination assay (TaqMan Assay) on an ABI PRISM 7900HT Sequence Detection System. The primers and the discrimination probes were designed and purchased from GeneCore (Shanghai, China). PCR reactions were performed in 384-well plates, each well containing 20 ng DNA, 5 μl of 2×TaqMan Universal PCR Master Mix, 0.9 μM of each primer, 0.25 μM of each probe. Automatic allele calling was performed by ABI PRISM 7900HT data collection and analysis software, version 2.1.

HLA-DR Alleles Typing

Polymerase chain reaction/sequence specific primer (PCR/SSP) method was used for analyzing HLA-DR alleles. We genotyped DRB4*01, DRB1*04, DRB1*07, DRB1*12 alleles, which amplifying primers were described by Olerup et al [40]. Control primers giving rise to a 796-bp fragment from the third intron of HLA-DRB1 were included in all PCR reactions as positive control for PCR amplification. PCRs were performed with a touchdown program in a 10 μl reaction that contained 20 ng of genomic DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.3 mM dNTPs, 3.0 mM MgCl$_2$, 0.3 μM of the allele and group-specific primers, 0.06 μM of the control primers, and 0.1 units of Hotstar Taq DNA polymerase (Qiagen). PCR conditions were: Taq activation at 95°C for 15 minutes, followed by 37 cycles, each having denaturation at 94°C for 20 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 60 seconds, except that in the first 12 cycles, the annealing temperature decreased from 64 to 58°C by 0.5°C per cycle, and the final extension was 10 minutes. PCR products were electrophoresed in 2.0% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were run for 40 min at 5 V/cm in 0.5×TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM EDTA, pH 8.0). Gels were visualized using Gel Documentation and Analysis (Advanced American Biotechnology, Fullerton, CA, USA) after electrophoresis.

Luciferase Assay Analysis

We amplified a 1132-bp fragment (−1017 to +115 bp from the first exon) of XBP1 by PCR and cloned it into the KpnI/HindIII site of pGL3-Basic vector (Promega). We constructed two reporter plasmids, carrying either −116C (pGL3-C) or −116G allele (pGL3-G) of rs2269577. The allele status of the reporter plasmid was confirmed by sequencing. We transfected Human keratinocyte HaCat cells and melanoma Mel-RM cells with the pGL3-basic vector (control without the cloned promoter sequence) or the

reporter plasmid (pGL3-C or pGL3-G). pRL-SV40 plasmid (Promega) was used as an internal control for transfection efficiency. After 48 hours of incubation, cells were harvested and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). All transfections were carried out in triplicate.

Real-Time Quantitative RT–PCR Analysis

Human skin biopsies (3 mm) were collected in the Department of Dermatology at First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China with informed consent. Paired biopsies were obtained from each of 38 patients: one from the lesional skin (at least 2 cm from the lesional boarders), the other from the symmetrical normal skin (also more than 2 cm from the lesional boarders). Total RNA was isolated by using RNeasy Mini kit (Qiagen) with treatment of DNase, according to the manufacturer's instructions. We carried out reverse transcription according to the Superscript protocol (TaKaRa). Real-time PCR was performed using the iCycler (Bio-Rad). Reactions were performed in a 10-μl volume including diluted cDNA samples, primers, and SYBR Green I Mastermix (TaKaRa). For real-time PCR analysis, XBP1 was amplified with forward primer 5′-TGAGCTGGAACAGC AAGTGGT-3′ and reverse primer 5′-CCCAAGCGCTGTCTTAACTCC-3′. 18 s RNA served as an endogenous control and was amplified with forward primer 5′-GTAACCCGTTGAACCCCATT -3′ and reverse prime 5′-CCATCCAATCGGTAGTAGCG -3′. Dissociation curve analyses were performed to confirm specificity of the SYBR Green signals in each experiment. Real-time PCR data were collected using iCycler software (version 3.1, Bio-Rad). Both 18 s RNA and XBP1 were tested in triplicate for each sample. The measurement (Ct value) of XBP1 expression was normalized by using 18 s' measurement (Ct value). For the overall comparison of XBP1 expression between the lesional and non-lesional skins, all the normalized measurements (ΔCt) were standardized so that the average of the ΔCts of non-lensional skin samples was equal to 1. For the comparison of XBP expression between the paired lesional and non-lesional samples from same patients, the normalized measurements (ΔCt) were used to calculate the difference (ΔΔCt) between the lesional and non-lesional skin samples from the same patient. The fold change in the expression level was calculated by using the formula of $2^{ΔΔCt}$.

Immunohistochemistry Analysis

Lesional and non-lesional skin samples were collected (as the same as above) from another 37 patients, and fixed in formalin. Fixed tissues were embedded routinely in paraffin, serially sectioned at 5 μm, and placed on poly-L-lysine coated slides for immunohistochemistry with an ABC Staining kit (Santa Cruz Biotechnology, CA, USA) in accordance with the manufacturer's instructions. The sections were de-paraffinized, rehydrated and reacted overnight at 4°C with the primary Rabbit anti-human XBP1 polyclonal antibody (ab37152, abcam, CA, USA) at a dilution of 1:200. The biotin-labeled secondary antibody (sc-2491, Santa Cruz Biotechnology, CA, USA) was added at a dilution of 1:100. Diaminobenzidine (DAB) was used for staining development and the sections were counterstained with haematoxylin. The staining intensity and the percentage of positive cells were evaluated using regular light microscopy by two observers who were blind to the clinical data (the lesion or the normal) independently.

Statistical Analysis

We performed Armitage Trend test to assess genotype-phenotyoe association as well as allelic association analysis using

the Plink 1.02 software [41]. Association analysis of the combined samples was performed using Cochran-Mantel-Hanezel stratification analysis [42]. Hardy-Weinberg proportion was tested in control samples to ensure genotyping quality. The TDT analysis was performed to test association in case-parent triad data using the Haploview version 3.2 [43]. The comparison between the clinical subgroups was performed by testing the association of rs2269577 with clinical sub-phenotypes using genotype-based trend test. Interaction analysis was performed by comparing the logistic regression model of only main effect against the model of both the main and interactive effects. Chi-square test was used for estimating the significance of the statistics of the log-likelihood ratio test. To examine the pattern of the interaction, we performed the analysis of XBP1 gene stratified by HLA positive and negative.

Student's t test was used to examine the differences in luciferase reporter gene expression. Differences in mRNA level of XBP1 between lesional and normal skins (in all the patients as well as within different genotype groups) were tested for significance with the nonparametric Wilcoxon signed-rank test. Correlation between mRNA levels of XBP1 and genotype (GG vs GC+CC) was analyzed using Mann-Whitney U test. Differences in protein level between lesional and normal skins within different genotype groups were tested using Paired Samples T Test. All the statistical analyses were done in SPSS 10.0.

Supporting Information

Figure S1 Genomic structure of the XBP1 gene with the location of 8 SNPs subjected to genetic association analysis. The gray bars (underneath the genomic structure) indicate the genomic regions that were amplified for sequencing analysis.

Found at: doi:10.1371/journal.pgen.1000523.s001 (1.34 MB TIF)

Table S1 A list of all the variants identified by the initial sequencing analysis of XBP1.
Found at: doi:10.1371/journal.pgen.1000523.s002 (0.07 MB DOC)

Table S2 Primers used for the sequencing analysis of the exons, exon-intron boundaries, and some promoter sequences of XBP1.
Found at: doi:10.1371/journal.pgen.1000523.s003 (0.03 MB DOC)

Table S3 Frequencies of HLA-DR alleles in vitiligo patients compared with control group.
Found at: doi:10.1371/journal.pgen.1000523.s004 (0.03 MB DOC)

Author Contributions

Conceived and designed the experiments: JL XZ. Performed the experiments: YR SX QF CQ YL. Analyzed the data: YR MG ZW JL. Contributed reagents/materials/analysis tools: YR SX MG TG CZ LS YL JH. Wrote the paper: YR JL. Participated with aspects of study design and interpretation of the data: SY WH FZ YZ.

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