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A functional polymorphism in interleukin-1α (*IL1A*) gene is associated with risk of alopecia areata in Chinese populations

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ABSTRACT

Alopecia areata (AA) is an inflammatory hair loss disorder with a major genetic component, which may cause great psychosocial distress for those affected. Studies have shown that interleukin-1 (IL-1) is a very potent inducer of hair loss and a significant human hair growth inhibitor. The 4-bp insertion/deletion (Indel) polymorphism (rs3783553) within the 3′ untranslated regions of *IL1A* gene has been suggested to be associated with risk of various types of cancers, possibly through regulating expression of IL-1α levels. In the current study, we estimated the susceptibility to AA associated with rs3783553 in two independent case–control panels of Eastern and Southern Chinese populations, totally containing 313 AA cases and 626 healthy controls. Logistic regression analysis showed that the heterozygote and the homozygote 4-bp ins/ins confer a significantly lower risk of AA in both panels and total subjects [odds ratio (OR) = 0.55, 95% confidence interval (C.I.) = 0.41–0.75, $P = 6.24 \times 10^{-5}$; OR = 0.47, 95% C.I. = 0.28–0.76, $P = 0.001$, respectively]. Stratification analysis based on age onset showed that the protective roles of ins/del and ins/ins genotype against developing AA was more obvious in AA patients with early age onset (<30 years) under dominant model (OR = 0.48, 95% C.I. = 0.29–0.77, $P = 0.001$). The results of luciferase assay showed that rs3783553 could influence expression of IL-1α in a miR-122 dependant manner. Taken together, our results suggested that the *IL1A* 4-bp indel polymorphism may be a marker for genetic susceptibility to patchy (mild) AA in Chinese populations, likely through miR-122 mediated regulation.

1. Introduction

Alopecia areata (AA) is a common hair loss disorder characterized by patchy hair loss and which affects approximately 1–2% of the general population (Schwartz and Janniger, 1997; Wasserman et al., 2007). Although spontaneous resolution is expected in most patients, a small proportion of cases may evolve into severe and chronic hair loss, which may cause great psychosocial distress for those affected (Garg and Messenger, 2009). Despite extensive research in AA, our current understanding of the pathogenesis of AA is still incomplete. There is a strong association between AA and autoimmune diseases, particularly vitiligo, Hashimoto's thyroiditis, type 1 diabetes, Addison's disease, rheumatoid arthritis and pernicious anemia, suggesting that a shared pathological autoimmune reaction pathway is involved in the development of these disorders (Gilhar and Kalish, 2006; Lu et al., 2006). Results from family and twin studies strongly suggest a complex mode of inheritance (Jackow et al., 1998; Rodriguez et al., 2010). A number of candidate-gene association studies have been performed over the past two decades (McDonagh and Tazi-Ahnini, 2002), and several reports have indicated a significant association between AA and certain human leukocyte antigens (*HLA*) genes such as HLA-DRB1*0401 and DQB1* (Entz et al., 2006; Megiorni et al., 2011). For example, DQB1*0604 allele predisposes individuals to AA in Chinese Han (Xiao et al., 2005). HLA-DQB1*0301 and HLA-DRB1*1104 have been found to be strongly associated with risk of AA (Barahmani et al., 2008). Furthermore, the autoimmune regulator (*AIRE*) as well as the lymphoid-specific protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) have been found to be additional immunoregulatory genes associated with AA (Betz et al., 2008; Tazi-Ahnini et al., 2002). In addition, susceptibility alleles of genes encoding for cytokines and their receptors, such as the interleukin-1 receptor antagonist (*IL1RN*) and chemokines (*MCP-1*) have also been associated with AA (Barahmani et al., 2002; Hong et al., 2006). Genome-wide association studies (GWAS) have greatly contributed to the identification of common

Abbreviations: AA, alopecia areata; AT, alopecia totalis; AU, alopecia universalis; IL1, interleukin-1; Indel, insertion/deletion; 3′UTR, 3′ untranslated region; miR, microRNA; PAGE, polyacrylamide gel electrophoresis; OR, odds ratios; CI, confidence interval.

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genetic variants related to AA. Recent GWAS studies focusing on AA have reported several susceptibility genes such as *CTLA4*, *SPATA5* and *IL13* (Forstbauer et al., 2012; Jagielska et al., 2012; Petukhova et al., 2010).

Interleukin-1 (IL-1) is a primary cytokine involved in mediating inflammatory responses. IL-1 may have a direct effect on hair growth, as demonstrated in hair follicle organ cultures that IL-1 inhibits growth of the hair fiber and induces morphological changes resembling those seen in AA (Philpott et al., 1996). The *IL-1* gene family consists of two major agonistic molecules, namely IL-1α and IL-1β, and one antagonistic cytokine, the IL-1R antagonist (IL-1Ra). Tazi-Ahnini et al. have reported a possible association with *IL1A* + 4845 and AA, suggesting that IL-1α may have a particular role in the pathogenesis of mild cases (Tazi-Ahnini et al., 2001). The rs3783553 is an insertion/deletion (indel) polymorphism (an insertion or deletion of "TTCA" bases) which is located in the 3′ untranslated regions (3′UTR) of *IL1A* and has been suggested to be associated with risk of hepatocellular carcinoma and nasopharyngeal carcinoma, possibly through regulating expression of IL-1α levels (Gao et al., 2009; Yang et al., 2011). To our knowledge, no studies have reported the association between rs3783553 and AA susceptibility. The purpose of this study was to investigate whether rs3783553 in the 3′UTR of *IL1A* plays a role in AA development in a Chinese population.

2. Materials and methods

2.1. Study population

The case–control study was performed on genomic DNA extracted from the peripheral blood of newly diagnosed incident AA cases together with controls matched for sex and age (±1 year) after obtaining their informed consent. All subjects recruited were unrelated ethnic Han Chinese. The case series from Eastern China comprised of 208 AA patients diagnosed in the Second Affiliated Hospital of Soochow University, the Affiliated Suzhou Hospital of Nanjing Medical University and the First People's Hospital of Wujiang from May 2011 to June 2012. The AA cases of Southern Chinese panel were consecutively recruited from October 2011 to August 2012 at the Department of Dermatology, the First Affiliated Hospital of Sun Yat-sen University. The 416 healthy controls from the Eastern Chinese panel and 210 healthy controls were obtained from a community nutritional survey that was conducted in the same regions during the same period as the recruitment of AA patients. The absence of autoimmune diseases in the control and AA populations was guaranteed by specialists with residency in different specific medical fields. Additionally, all the subjects were examined by a panel of antibodies designed for autoimmune disease screening which included anti-nuclear, anti-dsDNA, anti-histones, anti-U1RNP, anti-Ro, anti-Jo1, anti-Ribosomal P protein and anti-Sm antibodies. Only those subjects with negative results of antibody testing were included in the current study. Clinical data were collected from all patients, including age at onset and family history of AA. The inclusion criterion was a diagnosis of AA by standard criteria (Olsen et al., 1999). Those patients suffering from trichotillomania, tinea capitis, syphilis, androgenetic alopecia, congenital alopecia, pseudopelade, cicatricial alopecia, Down syndrome or Turner syndrome were excluded. The clinical subtype of AA was determined according to the AA investigational assessment guidelines (Olsen et al., 2004) and patients were categorized as having mild patchy AA, alopecia totalis (AT) or alopecia universalis (AU). A family history of AA was defined as having at least one first- or second-degree relative with AA. The design of the study was approved by the Ethical Committee of Soochow University.

2.2. DNA extraction and genotyping

Genomic DNA of blood samples were extracted using genomic DNA purification kit (Qiagen). Genotyping was performed without knowledge of case or control status as previously described (20). Briefly, DNA fragments containing the polymorphism were amplified with the forward primer 5′-ATTGGTCCGATCTTTGACTC-3′ and reverse primer 5′-TGATAA CAGTGGTCTCATGG-3′. The PCR products were analyzed by 7% non-denaturing polyacrylamide gel electrophoresis and visualized by silver staining (Allen et al., 1989). The genotypes were determined by the numbers and the length of the band(s) in the gels. Note that the deletion and insertion allele yielded a 132-bp and a 136-bp amplicon, respectively. To validate the genotyping method, we also analyzed 20 randomly selected DNA samples (10 samples with ins/ins genotype and 10 samples with del/del genotype) by direct sequencing; the results for these two methods were 100% concordant. Approximately 10% of the case and control samples were randomly selected and tested in duplicate by independent technicians, with 100% concordance of results.

2.3. IL1A 3′UTR reporter constructs and luciferase assay

The constructed pRL-SV40 vectors containing either the insertion allele or the deletion allele were kindly provided by Professor Yuzhen Gao from the Medical College of Soochow University. The A-375 and CCD-1095Sk cell lines were obtained directly from Shanghai Cell Bank of Chinese Academy of Sciences. Cells were cultured in DMEM with high glucose (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 50 μg/ml streptomycin (Gibco) at a 37 °C incubator supplemented with 5% CO_2. Cells were seeded at a density of 1×10^5 cells per well in 24-well plates (BD Biosciences). 16 h after plating, cells were transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. In each well, 500 ng constructed pRL-SV40 vectors containing either insertion or deletion allele and 50 ng pGL3 control vector were cotransfected with 0, 1, 10, 100 pmol pre-miR-122, respectively. 100 pmol Negative Control #1 from Ambion, Inc. was used as negative controls in every transfection experiment. Six replicates were for each group and the experiment was repeated at least three times. 24 h after transfection, cells were harvested immediately after addition of 100 μl passive lysis buffer. Renilla luciferase activities in cell lysate were measured with the Dual Luciferase assay system (Promega) in TD-20/20 luminometer (Turner Biosystems) and were normalized with the firefly luciferase activities.

2.4. Statistical analysis

The genotype distribution was analyzed for Hardy–Weinberg equilibrium using the Chi-square test. Logistic regression was used to analyze the association between rs3783553 and AA risk, and adjusted for sex and age. In addition, stratified analysis by sex and age at onset was performed using binary logistic regression model. Due to the limited number of ins/ins genotype, dominant model (del/del vs. ins/del + ins/ins) was used in stratified analysis. Student's *t* test was used to examine the differences in luciferase reporter gene expressions. These statistical analyses were implemented in Statistic Analysis System software (version 8.0, SAS Institute). Probability values of 0.05 were used as the criterion of statistical significance.

3. Results

The baseline characteristics of the subjects in the current study are listed in Table 1. No significant differences were found between AA patients and control subjects in terms of sex ($P = 1.00$) and age ($P = 0.99$), suggesting that the frequency matching was adequate. Example output from sequencing and genotyping assays of rs3783553 are shown in Fig. 1. The observed genotypic frequencies for rs3783553 were in agreement with the Hardy–Weinberg equilibrium in both cases and controls ($P = 0.56$ and 0.14 in Eastern Chinese panel; and $P = 0.67$ and 0.46 in Southern Chinese panel, respectively). As shown in Table 2, compared with the del/del genotype, subjects in eastern Chinese panel with the heterozygous ins/del or homozygous ins/ins had a significantly decreased risk of AA under co-dominant model (adjusted OR = 0.58 and 0.55; $P = 0.003$ and 0.031, respectively). Similar trends