Association analysis identifies ZNF750 regulatory variants in psoriasis

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Abstract

Background: Mutations in the ZNF750 promoter and coding regions have been previously associated with Mendelian forms of psoriasis and psoriasiform dermatitis. ZNF750 encodes a putative zinc finger transcription factor that is highly expressed in keratinocytes and represents a candidate psoriasis gene.

Methods: We examined whether ZNF750 variants were associated with psoriasis in a large case-control population. We sequenced the promoter and exon regions of ZNF750 in 716 Caucasian psoriasis cases and 397 Caucasian controls.

Results: We identified a total of 47 variants, including 38 rare variants of which 35 were novel. Association testing identified two ZNF750 haplotypes associated with psoriasis (p < 0.05). We also identified an excess of rare promoter and 5’ untranslated region (UTR) variants in psoriasis cases compared to controls (p = 0.041), whereas there was no significant difference in the number of rare coding and rare 3’ UTR variants. Using a promoter functional assay in stimulated human primary keratinocytes, we showed that four ZNF750 promoter and 5’ UTR variants displayed a 35-55% reduction of ZNF750 promoter activity, consistent with the promoter activity reduction seen in a Mendelian psoriasis family with a ZNF750 promoter variant. However, the rare promoter and 5’ UTR variants identified in this study did not strictly segregate with the psoriasis phenotype within families.

Conclusions: Two haplotypes of ZNF750 and rare 5’ regulatory variants of ZNF750 were found to be associated with psoriasis. These rare 5’ regulatory variants, though not causal, might serve as a genetic modifier of psoriasis.

Background

Psoriasis is a chronic, inflammatory disorder of the skin affecting 2-4% of the Caucasian population [1]. Clinically, psoriasis is characterized by red, scaly plaques typically favoring the elbows, knees, scalp, umbilicus, and gluteal cleft and may be associated with nail dystrophy and arthritis. Histologically, psoriasis is marked by epidermal hyperplasia, dilated vasculature in the dermal papillae, and the presence of T cell infiltrates. Genome-wide association studies and other genetic investigations of psoriasis have identified at least 18 common variants affecting psoriasis susceptibility [2-9]. However, in aggregate, these common variants only explain a fraction of the heritability in psoriasis [10]. Therefore, the missing heritability in psoriasis might be explained by other types of variants not captured by the previous genetic studies, such as rare variants with a low minor allele frequency in the general population.

One candidate susceptibility gene for psoriasis is ZNF750, a gene located at chromosome 17q25 within the PSORS2 locus. ZNF750 has previously been reported to be associated with autosomal dominant forms of psoriasis or psoriasiform dermatitis in two separate, multigenerational families. The first family was a five-generation Jewish Israeli family of Moroccan descent in which affected members displayed clinical features of both psoriasis and seborrheic dermatitis [11]. The causative mutation was identified as a ZNF750 frameshift mutation at residue 19 of this 723-residue protein, resulting in a 44-residue truncated protein which abrogated the zinc finger binding domain. The second family was a five-generation Chinese family with psoriasis [12]. Gene mapping and functional studies identified the
most likely causal mutation as a c.-625A>C promoter variant in ZNF750 that resulted in 42% reduction in promoter activity.

Since Mendelian-pattern psoriasis in these two large families is associated with ZNF750, we sought to examine whether variants in ZNF750 might influence the development of psoriasis in a larger population. In this study, we investigated whether variants within the ZNF750 promoter, 5’ UTR, coding regions, and 3’ UTR were associated with psoriasis in a Caucasian population.

Methods

Patients

DNA samples from 716 unrelated Caucasian psoriasis cases, 397 Caucasian healthy adult controls, and 20 additional family members were collected from the University of California, San Francisco and Washington University, St. Louis. Cases were recruited from outpatient dermatology clinics and the diagnosis of psoriasis was confirmed by a board-certified dermatologist. Healthy controls, recruited from the local community, reported no history of autoimmune disease or cancer according to a written screening questionnaire. All subjects gave written informed consent for study participation in accordance with the institutional review board at their respective institutions.

Sequencing

Each DNA sample was sequenced over 6 amplicons to cover the promoter and 3 exons of ZNF750. PCR was performed using the following primer pairs, sequences listed 5’ to 3’: amplicon 1 (531 bp, forward AATACTG TGCCTCCCAGGTTAT, reverse GTACTTACAGA GTGTTGGCGTGTG); amplicon 2 (710 bp, forward TGT CCTGACCAAAGCTGCAC, reverse CGACTTGAAC AAATGCAGAA); amplicon 3 (698 bp, forward GGCAT CCTGACACCAAGACTGC, reverse CGACTGGAAC GGTGGGCAGTG); amplicon 4 (755 bp, forward CAGGCCA CACCCTGCAAGAG, reverse GGTTTAACCTTGA AGAAATGCAGAA); amplicon 5 (673 bp, forward CCCTCAAC GAGTCTGCATTTT, reverse TGGCTGCCAGGTTTA GACTCG); amplicon 6 (783 bp, forward TGGCTGCCAGGTTTA TCTCT); amplicon 5 (673 bp, forward CCCTCAAC GAGTCTGCATTTT, reverse TGGCTGCCAGGTTTA GACTCG); amplicon 6 (783 bp, forward TGGCTGCCAGGTTTA TCTCT); amplicon 6 (783 bp, forward TGGCTGCCAGGTTTA TCTCT); amplicon 6 (783 bp, forward TGGCTGCCAGGTTTA TCTCT); amplicon 6 (783 bp, forward TGGCTGCCAGGTTTA TCTCT); amplicon 6 (783 bp, forward TGGCTGCCAGGTTTA TCTCT); amplicon 6 (783 bp, forward TGGCTGCCAGGTTTA TCTCT); amplicon 6 (783 bp, forward TGGCTGCCAGGTTTA TCTCT). 10,000 permutations were performed to determine significance for each group tested.

5’ Rapid Amplification of cDNA Ends (5’ RACE)

SMART RACE cDNA amplification kit (BD Biosciences; Franklin Lakes, NJ USA) was used to perform 5’-RACE of ZNF750 gene. RACE-ready cDNA was synthesized according to the manufacturer’s instructions. RACE PCR was performed with gene-specific primers that were used for RT-PCR of ZNF750 (Exon 2, GGACTC- GATCCTGCTCTGA). RACE products were excised from the agarose gel, purified, and cloned into pGEM-T easy vector (Promega Corp; Madison, WI USA). Cloned inserts were sequenced with the T7 and SP6 primers and the DNA sequences obtained were compared to published sequences.

Sequencing was performed in one direction except for amplicon 6 in which the presence of multiple indels required the use of bidirectional sequencing, using the internal reverse primer AGGCCCTTGTAGTTTTG TGTGTT and internal forward primer TGGTTGTAA ACACCTGAATGA. All rare promoter and 5’ UTR variants were sequenced a second time in the forward and reverse directions to confirm their accuracy.

Statistical Methods

Sequencing traces were analyzed with Sequencher (Gene Codes; Ann Arbor, MI USA). Hardy-Weinberg equilibrium p-values were calculated in Haploview to assess sequencing quality and a p-value of 0.05 was used as the significance threshold for exclusion. Individual polymorphisms were tested for association with psoriasis using Fisher’s exact test implemented in PLINK. Haploview was used to identify haplotypes using the confidence intervals method and to calculate haploype associations using a chi-square test. Haploview was also used to perform empirical p-value estimation, using 100,000 permuted experiments. False discovery rate (FDR) q-values were calculated in R. A weighted sum statistic was used to test functionally similar groups of rare variants for association with psoriasis. This approach of combining multiple independent signals has been shown to be significantly more powerful than variant-by-variant or other approaches for the analysis of rare variants [13]. 10,000 permutations were performed to determine significance for each group tested.

Excess dye terminator removal was performed with genCLEAN (Genetix; New Milton, Hampshire, United Kingdom) plates following manufacturer’s instructions before sequencing on an ABI 3730xL DNA Analyzer. Sequencing was performed in one direction except for amplicon 6 in which the presence of multiple indels required the use of bidirectional sequencing, using the internal reverse primer AGGCCCTTGTAGTTTTG TGTGTT and internal forward primer TGGTTGTAAACACCTGAATGA. All rare promoter and 5’ UTR variants were sequenced a second time in the forward and reverse directions to confirm their accuracy.

Reporter Constructs for ZNF750 Promoter Assay

The ZNF750 reference sequence was taken from National Center for Biotechnology Information, with accession number NM_024702. Five candidate mutations in the promoter and 5’ UTR region of ZNF750
were analyzed for changes in luciferase reporter activity. Four novel variants were identified in patients diagnosed with psoriasis and one mutation, c.-625A>C was identified by Yang et al [12]. An 849 bp insert containing the promoter and 5′ UTR (exon 1 portion) of \textit{ZNF750} was amplified from human genomic DNA using the primers 5′-GGCTAGCCAGCAAGCAGTTTTGGT-3′ (\textit{NheI}) and 5′-CACAAGCTTGGATGTGGCCGGTCTTGGT-3′ (\textit{HindIII}), and cloned into pGL3 Basic vector (Promega) using \textit{NheI} and \textit{HindIII} restriction enzymes. QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) was used to create the 5 variations of interest individually. All vectors were sequenced to verify the location of the variation.

**Transfection and Luciferase Assay**

Primary keratinocytes were provided by Dr. Dennis Oh and Dr. Susana Ortiz-Urda (University of California San Francisco, San Francisco, CA). The cells were grown in 500 ml Medium 154CF and Human Keratinocyte Growth Supplement (Invitrogen; Carlsbad, CA USA), and 35 μM of CaCl2. A total of 10^5 cells were seeded in each well of a 6-well plate and were transfected after 24 hours with 500 ng of each reporter construct along with 10 ng of pRL-TK vector (Promega) containing the Renilla luciferase gene as an indicator for normalization of transfection efficiency. Transfections were performed using Lipofectamine™ LTX with PLUS™ Reagent (Invitrogen), according to the manufacturer’s instructions. After 24 hours, the cells were stimulated with 250 ng phorbol-12-myristate-13-acetate (PMA) for the PMA positive set of experiments. Cells were incubated for an additional 24 hours and then analyzed for luciferase activity with the Dual-Luciferase® Reporter Assay System (Promega) and Synergy 2 (BioTek; Winooski, VT USA). Firefly luminescence was normalized to Renilla luminescence and reported as relative luciferase units compared to wild-type sequence. All experiments were performed independently three times using primary keratinocytes from a single donor.

**Results**

**Identification of ZNF750 Variants in Cases and Controls**

To determine whether \textit{ZNF750} is associated with psoriasis, we sequenced \textit{ZNF750} in 716 Caucasian psoriasis cases and 397 Caucasian controls. We sequenced the \textit{ZNF750} promoter region (defined as 400 bp upstream of the transcription start site), 5′ UTR, coding regions which include exons 2 and 3, and 3′ UTR. In total, we identified 9 common variants (minor allele frequency (MAF) \(\geq 2\%\)) in the controls, Table 1 and 38 rare variants (MAF < 2%, Table 2). One polymorphism, a novel 8 bp deletion at position 78381172 (hg18) in the 3′ UTR, was out of Hardy-Weinberg equilibrium and was not included. All identified polymorphisms were successfully genotyped in greater than 94% of samples and the mean genotyping rate per variant was 97.3%. Nearly all variants were single nucleotide variants (SNVs) with the exception of two single base deletions, one located in the 3′ UTR (rs71918228) and the other in the 3′ downstream region (rs35156590). All of the identified common variants were present in dbSNP Build 133 whereas 35 of 38 (92%) of the rare variants were novel.

**Association Testing of Identified Variants**

To analyze the significance of the identified \textit{ZNF750} variations/mutations in psoriasis patients, we first tested each variant alone using a Fisher’s exact test. For the common variants (Table 1), only rs35156590 in the immediate 3′ downstream region showed a significant association with psoriasis (\(p = 0.028\), OR 0.81 [95% CI 0.68-0.98]). However, adjustment for multiple hypothesis testing using empirical permutation testing of this SNP yielded \(p = 0.17\). When tested individually, none of the rare variants (Table 2) achieved a significance association with psoriasis (all \(p > 0.05\)).

**Table 1 Common variants identified in ZNF750**

| Name         | Position | Property (PolyPhen2 impact) | Alleles | F_case | F_control | Fisher P |
|--------------|----------|----------------------------|---------|--------|-----------|----------|
| rs3744165    | 78383731  | Exon 2, 5′ UTR             | C/A     | 0.171  | 0.161     | 0.0541   |
| rs12450046   | 78383677  | Exon 2, 5′ UTR             | C/T     | 0.186  | 0.172     | 0.0478   |
| rs8074277    | 78382917  | Exon 2, M235V (benign)     | A/G     | 0.189  | 0.18       | 0.0640   |
| rs35653278   | 78382757  | Exon 2, P288L (probably)   | C/T     | 0.092  | 0.09       | 0.937    |
| rs34189891   | 78382558  | Exon 2, T354T               | C/T     | 0.021  | 0.021     | 1.000    |
| rs12994879    | 78381781  | Exon 3, P566P              | T/C     | 0.393  | 0.42       | 0.233    |
| rs12938126    | 78381754  | Exon 3, A575A              | A/G     | 0.392  | 0.42       | 0.232    |
| rs71918228    | 7838176-79| Exon 3, 3′ UTR             | CAAA/-  | 0.465  | 0.478     | 0.586    |
| rs35156590    | 78380584  | 3′ Downstream              | -/T     | 0.369  | 0.418     | 0.028    |

Common variants are defined as those with minor allele frequency (MAF) \(\geq 2\%\) in controls. Position is on Chr17 (hg18). UTR, untranslated region. PolyPhen2 impact given as benign, possibly damaging, or probably damaging. Alleles are given as major/minor on the (-) strand. F_case, MAF in cases; F_control, MAF in controls.
Next, we evaluated whether common variant haplotypes of ZNF750 were associated with psoriasis. Two 8-marker haplotypes were significantly associated with psoriasis (Table 3), the risk haplotype CCACCCTG(-) (frequency = 0.019, p = 0.0011, OR 3.70 [1.45-9.46]) which includes the risk allele of rs35156590, and the protective haplotype CCACCCGGT (frequency = 0.374, p = 0.0106, 0.79 [0.66-0.94]) which contains the non-risk allele of rs35156590. Both of these haplotypes remained significant after correction for multiple comparisons using empirical permutation testing (p = 0.0024 and p = 0.0311, respectively) as well as by

### Table 2 Rare variants identified in ZNF750

| Name        | Position   | Property (PolyPhen2 impact) | Alleles   | F_case | F_control | Fisher P |
|-------------|------------|-----------------------------|-----------|--------|-----------|----------|
| Novel_1     | 78391506   | Promoter                    | G/T       | 0 of 1312 | 1 of 745  | 0.365    |
| Novel_2     | 78391367   | Promoter                    | G/A       | 1 of 1309 | 0 of 746  | 1.000    |
| Novel_3     | 78391170   | Exon 1, 5' UTR              | G/A       | 1 of 1309 | 0 of 746  | 1.000    |
| Novel_4     | 78391142   | Exon 1, 5' UTR              | C/T       | 4 of 1306 | 0 of 746  | 0.303    |
| Novel_5     | 78391141   | Exon 1, 5' UTR              | G/A       | 1 of 1311 | 0 of 746  | 1.000    |
| Novel_6     | 78383664   | Exon 2, 5' UTR              | G/C       | 1 of 1349 | 0 of 746  | 1.000    |
| Novel_7     | 78383655   | Exon 2, 5' UTR              | A/G       | 1 of 1349 | 0 of 746  | 1.000    |
| Novel_8     | 78383651   | Exon 2, 5' UTR              | G/A       | 1 of 1349 | 0 of 746  | 1.000    |
| Novel_9     | 78383521   | Exon 2, T33T                 | T/C       | 1 of 1349 | 0 of 746  | 1.000    |
| Novel_10    | 78383438   | Exon 2, R61Q (possibly)     | G/A       | 2 of 1348 | 1 of 745  | 1.000    |
| Novel_11    | 78383402   | Exon 2, P73L (probably)     | C/T       | 0 of 1350 | 1 of 745  | 0.357    |
| Novel_12    | 78383393   | Exon 2, T76I (benign)       | C/T       | 0 of 1350 | 1 of 745  | 0.357    |
| Novel_13    | 78383325   | Exon 2, D99N (probably)     | G/A       | 1 of 1349 | 1 of 745  | 1.000    |
| Novel_14    | 78383272   | Exon 2, E116E                | G/A       | 1 of 1349 | 0 of 746  | 1.000    |
| Novel_15    | 78383216   | Exon 2, A135E (benign)      | C/A       | 0 of 1350 | 1 of 745  | 0.357    |
| Novel_16    | 78383106   | Exon 2, A142T (possibly)    | G/A       | 1 of 1349 | 0 of 746  | 1.000    |
| Novel_17    | 78382899   | Exon 2, E241Q (probably)    | G/C       | 1 of 1357 | 0 of 752  | 1.000    |
| Novel_18    | 78382882   | Exon 2, F246F                | T/C       | 0 of 1358 | 1 of 751  | 0.356    |
| rs35283702   | 78382791   | Exon 2, G277R (probably)    | G/A       | 15 of 1343 | 7 of 745  | 0.825    |
| Novel_19    | 78382759   | Exon 2, H287Q (possibly)    | C/G       | 1 of 1357 | 0 of 752  | 1.000    |
| Novel_20    | 78382489   | Exon 2, F377L (benign)      | C/G       | 0 of 1374 | 1 of 763  | 0.359    |
| rs34687659   | 78382445   | Exon 2, Q392R (possibly)    | A/G       | 0 of 1374 | 1 of 763  | 0.359    |
| Novel_21    | 78382401   | Exon 2, A407T (probably)    | G/A       | 1 of 1373 | 0 of 764  | 1.000    |
| Novel_22    | 78382375   | Exon 2, P415P                | G/A       | 2 of 1372 | 2 of 762  | 0.622    |
| Novel_23    | 78382326   | Exon 2, D432H (possibly)    | G/C       | 2 of 1372 | 0 of 764  | 0.540    |
| Novel_24    | 78382188   | Exon 2, V478I (benign)      | G/A       | 0 of 1374 | 1 of 763  | 0.359    |
| Novel_25    | 78382160   | Intron 2                    | C/A       | 1 of 1373 | 0 of 764  | 1.000    |
| Novel_26    | 78382155   | Intron 2                    | A/C       | 1 of 1373 | 0 of 764  | 1.000    |
| Novel_27    | 78382047   | Intron 2, 5 bp from exon 3  | T/C       | 1 of 1373 | 0 of 764  | 1.000    |
| rs35792712   | 78382015   | Exon 3, P488P               | T/G       | 0 of 1374 | 1 of 763  | 0.359    |
| Novel_28    | 78381714   | Exon 3, G589R (benign)      | G/C       | 1 of 1365 | 0 of 766  | 1.000    |
| Novel_29    | 78381508   | Exon 3, A657A               | G/A       | 1 of 1365 | 0 of 766  | 1.000    |
| Novel_30    | 78381034   | Exon 3, 3' UTR              | G/A       | 1 of 1357 | 0 of 756  | 1.000    |
| Novel_31    | 78380851   | Exon 3, 3' UTR              | A/G       | 1 of 1356 | 2 of 752  | 0.290    |
| Novel_32    | 78380743   | Exon 3, 3' UTR              | A/G       | 2 of 1364 | 0 of 754  | 0.541    |
| Novel_33    | 78380697   | Exon 3, 3' UTR              | T/G       | 1 of 1365 | 0 of 754  | 1.000    |
| Novel_34    | 78380590   | 3' Downstream               | T/C       | 2 of 1318 | 1 of 751  | 1.000    |
| Novel_35    | 78380548   | 3' Downstream               | G/A       | 17 of 1305 | 9 of 743  | 1.000    |

Rare variants are defined as those with minor allele frequency (MAF) < 2% in controls. SNPs Novel_1 through Novel_8 are additionally named by the nucleotide position relative to the translation start site with respect to the cDNA reference. Position is on Chr17 (hg18). UTR, untranslated region. PolyPhen2 impact given as benign, possibly damaging, or probably damaging. Alleles are given as major/minor on the (-) strand. F_case, MAF in cases; F_control, MAF in controls.
calculation of the false discovery rate (q = 0.0077 and q = 0.037, respectively).

Due to the well-recognized difficulty in assessing the significance of rare variants on a variant-by-variant basis due to power limitations [14], we utilized a weighted sum statistic [13] to evaluate functional groups of rare variants for psoriasis association. We tested the following rare variant groups: 5’ regulatory variants (promoter and 5’ UTR), all coding variants, non-synonymous variants, predicted deleterious non-synonymous variants, 3’ UTR variants, and all rare variants. We found that only the 5’ regulatory variants trended towards a significant association with psoriasis (10 variants in cases, 1 variant in controls, unadjusted p = 0.041, Bonferroni threshold p = 0.008, Table 4). The putative association of the rare 5’ regulatory variants with psoriasis was not secondary to their occurrence on a risk haplotype background. Of the 10 patients with rare 5’ regulatory variants, only 1 patient was heterozygous for the risk haplotype CCACCCG(-), while 7 patients were actually heterozygous for the protective haplotype CCACCCGT.

Alternative splicing of ZNF750 mRNA

In order to characterize the ZNF750 promoter and transcripts, the size of ZNF750 mRNA was verified using two methods: reverse transcriptase PCR (RT-PCR) and 5’ Rapid Amplification of cDNA Ends (5’ RACE). Using RT-PCR, the predicted cDNA segments were amplified from mRNA of normal individual skin biopsies. Comparison of the cDNA sequences to NCBI and UCSC databases indicated that there are no alternative splicing variants. 5’ Rapid Amplification of cDNA Ends performed for ZNF750 in primary keratinocytes produced four 5’ RACE PCR products that were cloned and sequenced (data not shown). Alignments of the sequences of the 5’ RACE PCR products with human genomic DNA and known mRNA sequences indicated that only two of the four products were specific to ZNF750. The short product represents the mRNA isoform A as in Refseq gi13375990; NM_024702.1. The long product, previously demonstrated in cDNA from tongue tumor tissue [GenBank:DA436414][15] represents the mRNA isoform B and includes an additional 500 bp of sequence upstream to exon 1 of mRNA isoform A (Figure 1). The expression levels of the 5’-RACE isoforms were studied by RT-PCR with forward primers specific to isoform A and isoform B, respectively, and a reverse primer specific to exon 2. RT-PCR analyses of several cell lines (including primary keratinocytes) showed that the most abundant variant of ZNF750 transcript corresponds to isoform A. Analysis of all 3 reading frames indicated that isoform A and B encode an identical protein: the addition of 500 bp upstream in isoform B compared to isoform A does not result in a different protein. Further investigation of the protein size using Western Blot demonstrated that ZNF750 codes for a single protein of 100 KDa (data not shown). Our data indicate that mRNA isoform A of ZNF750 is the predominant form of ZNF750 expressed.

Table 3 Haplotype association testing between cases and controls

| Haplotype   | Hap Freq | F_case | F_control | P Value | Emp P-Val | Odds Ratio [95% CI] |
|-------------|----------|--------|-----------|---------|-----------|--------------------|
| CCACCCGT    | 0.374    | 0.354  | 0.41      | 0.0106  | 0.0311    | 0.79 [0.66-0.94]   |
| CCACCTAC(-) | 0.246    | 0.253  | 0.234     | 0.3321  | 0.9359    | -                  |
| ACACCTAC(-) | 0.137    | 0.138  | 0.136     | 0.9158  | 1.0000    | -                  |
| CTGCTC(-)   | 0.089    | 0.091  | 0.085     | 0.6426  | 0.9995    | -                  |
| CTGTCTA(-)  | 0.089    | 0.091  | 0.086     | 0.7198  | 0.9997    | -                  |
| ACACTA(-)   | 0.02     | 0.02   | 0.02      | 0.9666  | 1.0000    | -                  |
| CCACCCCG(-) | 0.019    | 0.026  | 0.006     | 0.0011  | 0.0024    | 3.70 [1.45-9.46]   |

The eight marker haplotype, as ordered in the table, consists of: rs3744165, rs12450046, rs8074277, rs35653278, rs34188981, rs12948179, rs12938126, rs35156590. Hap Freq, overall haplotype frequency; F_case, haplotype frequency in cases; F_control, haplotype frequency in controls; Emp P-Val, empiric p-value calculated by permutation testing.

Table 4 Groupwise association testing of ZNF750 functional groups with psoriasis

| Rare Variant Group   | Total Variants, Cases (n = 716) | Total Variants, Controls (n = 397) | P-value, Weighted Sum Statistic |
|----------------------|----------------------------------|-----------------------------------|--------------------------------|
| 5’ Regulatory (Promoter + 5’ UTR) | 10                               | 1                                 | 0.041                          |
| All Coding           | 30                               | 19                                | 0.575                          |
| - Non-synonymous     | 25                               | 15                                | 0.560                          |
| - Predicted deleterious | 24                               | 11                                | 0.314                          |
| 3’ UTR               | 5                                | 2                                 | 0.351                          |
| All Rare             | 67                               | 32                                | 0.192                          |
in keratinocytes and that the 5’ region DNA variants identified in this study lie within the promoter region and 5’ UTR of isoform A.

Functional evaluation of ZNF750 5’ Regulatory Variants
To assess whether the ZNF750 5’ regulatory variants affect ZNF750 expression levels, we conducted a promoter function assay in human primary keratinocytes. Seven rare variants in the 5’ regulatory region of ZNF750 were seen in the psoriasis cases and none of these were present in the controls (Table 2). All of these variants were singletons with the exception of c.-233C>T, which was present in 4 unrelated psoriasis cases. None of these were present in individuals of European descent in the 1000 genomes database. We constructed ZNF750 5’ regulatory region fragments with 4 of the variants cloned into individual luciferase reporter assay constructs. The 4 selected variants are located in either the ZNF750 promoter region (c.-458G>A) or ZNF750 untranslated first exon (c.-261G>A, c.-233C>T, c.-232G>A) (Figure 1). Each construct was transfected into human primary keratinocytes which are known to highly express ZNF750 [11]. As an external control, we also tested the activity of the c.-625A>C variant which was shown to reduce promoter activity in the Taiwanese familial psoriasis study [12]. Interestingly, human primary keratinocytes that have been stimulated with phorbol 12-myristate 13-acetate (PMA) have about 10 fold higher ZNF750 promoter activity than unstimulated keratinocytes. Therefore, we tested the effects of the 4 variants on promoter activity after stimulation of human primary keratinocytes with PMA. We found that all 4 of the variants, as well as the external control, showed a significant reduction in promoter activity of 35-55% compared to the wild type promoter (all p < 0.001, Figure 2B).

Clinical Phenotype of Psoriasis Patients with 5’ Regulatory Variants
To assess whether psoriasis patients with 5’ regulatory variants have a similar clinical phenotype, we clinically characterized the psoriasis patients with the ZNF750 5’ variants (Table 5). Of the 7 affected patients with detailed clinical information available, 5 reported involvement of the scalp or inverse skin folds, and a negative history of psoriatic arthritis, similar to the sebo-psoriasis, arthritis-negative phenotype previously described in the Israeli-Moroccan family with ZNF750 mutation [11].

Discussion
To our knowledge, this is the first large resequencing study of the ZNF750 gene in psoriasis. As ZNF750 resides in the PSORS2 region [16-19] and has been previously reported to cause familial psoriasis forms of psoriasis [11,12], we were motivated to evaluate ZNF750 in a large cohort of cases and controls. We sequenced a total of 1,113 individuals (716 Caucasian psoriasis cases and 397 ethnically matched healthy controls) in the
promoter and exonic regions of ZNF750 and identified 47 polymorphisms, of which 35 were novel. According to estimates of Ionita-Laza et al [20], our study captured greater than 99% of the variants with frequency greater than 0.001 in the CEPH population. However, for perspective, we would have needed to sequence 3,521 individuals to capture 100% of the variants with frequency greater than 0.001.

We conducted an association analysis of our identified variants and found no evidence that individual common polymorphisms (MAF > 2%) were associated with disease. Similarly, imputation of HapMap3/1000 Genomes SNPs using psoriasis GWAS data [7] in the interval 200 kb upstream to 200 kb downstream of ZNF750 did not find any SNPs with association p-values less than $1.0 \times 10^{-3}$ (data not shown), suggesting that no common ZNF750 SNPs are causal for psoriasis. However, our finding that two ZNF750 haplotypes were significantly associated with psoriasis suggests the possibility that other SNPs on 17q in LD with these haplotypes could possibly be associated with psoriasis. We also detected several rare, non-synonymous, potentially deleterious (PolyPhen2) coding variants in ZNF750 that were only present in cases and not in controls, including A142T, E241Q, H287Q, and A407T (Table 2). However, given that each of these variants was only detected in a single

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**Figure 2** Luciferase activity of ZNF750 regulatory region in human primary keratinocytes. Bar chart representing luciferase activity of pGL3 constructs containing ZNF750 promoter and 5' UTR variants relative to wild type promoter (WT). Four variants (c.-458G>A, c.-261G>A, c.-233C>T, c.-232G>A) were detected in psoriasis patients in this study, one variant (c.-625A>C) was detected in a psoriasis family in previous study, and a pGL3 basic (empty vector) was used as a negative control. Constructs were co-transfected with Renilla luciferase into primary human keratinocytes and then treated for 24 h with culture medium (A) or culture medium supplemented with 250 ng/ml PMA (B). After 48 h, cells were assayed for luciferase activity. A reduction in luciferase activity is seen for all 4 promoter and 5' UTR variants as well as the c.-625A>C external control. The results represent the means ± SD of three independent experiments. *** p < 0.001 compared to WT by t-test.
case and absent additional functional data, it is difficult determine whether these variants were truly deleterious. Therefore, in order to more rigorously evaluate the possible association of rare variants in ZNF750 with psoriasis, we used the weighted-sum approach of Madsen and Browning [13] to evaluate functional groups of rare variants for disease association. This method has been found to be robust compared to other rare variant analysis approaches such as CAST, CMC, Variable Threshold, and Li-Leal [13,21]. We found that variants in the promoter or 5’ UTR of ZNF750 were enriched in the cases compared to controls (10 variants vs 1 variant, p = 0.041). Interestingly, the 1 variant found in a control but in none of the cases was c.-597C>T, which was previously reported to be present in 1 of 85 sporadic psoriasis patients screened and 0 of 188 normal controls [12]. In the same study, the promoter variant c.-625A>C was linked to a multigenerational Taiwanese psoriasis family and was reported to reduce activity by 42% [12]. We therefore investigated the functional relevance of only 10% [22]. The existence of rare variants that influence psoriasis risk but which have moderate, non-Mendelian effect sizes is certainly possible but difficult to prove. Alternatively, it is possible that there is no true association of 5’ regulatory variants in ZNF750 with psoriasis and that the nominal association observed is the result of type I error. In this case, the functional effects of these variants on ZNF750 promoter activity demonstrated here may not translate into biological relevance for psoriasis. However, of the 7 patients identified in this study with ZNF750 5’ regulatory variants and for whom detailed clinical descriptions were available, 5 reported scalp psoriasis and 3 reported inverse psoriasis (axillae, genitalia, nails), which is reminiscent of the sebo-psoriasis phenotype in the previously reported Morrocan family with a frame shift ZNF750 mutation [11]. Variants in the promoter and 5’ UTR of ZNF750 which decrease ZNF750 expression might therefore be associated with a seborrhoeic form of psoriasis.

ZNF750 encodes a 723 amino acid protein that contains two nuclear localization sites (NLS), and two histidines and two cysteines that might serve as zinc binding domains. ZNF750 is a putative member of the C2H2

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**Table 5 Clinical characteristics of subjects with rare ZNF750 variants in the 5’ regulatory region**

| ZNF750 Variant | # Pso Cases | Age: Study Entry (Onset) Gender | PsA | Family History of Psoriasis | Description |
|----------------|-------------|-------------------------------|-----|-----------------------------|-------------|
| c.-458G>A      | 1           | NA                            | Female Yes Sister | Confirmed psoriasis, no other information available |
| c.-261G>A      | 1           | 57 (35)                       | Male No Mother, brother, daughter, niece, nephew | Confirmed psoriasis |
| c.-233C>T      | 4           | 41 (25)                       | Female No Mother, sister | Plaque and inverse psoriasis affecting scalp, trunk, extremities, breast folds, axillae, and genitails. |
| c.-232G>A      | 1           | 43 (30)                       | Male No Maternal cousin | Moderate plaque and inverse psoriasis affecting scalp, elbows, legs, axillae, genitalia, and nails |
| c.-45G>C       | 1           | 30 (5)                        | Female No Father | Moderate guttate (originally plaque) and inverse psoriasis affecting scalp, arms, legs, trunk, axillae, and genitalia |
| c.-36A>G       | 1           | 40 (25)                       | Female No Sister | Moderate psoriasis affecting trunk, legs |
| c.-32G>A       | 1           | 65 (NA)                       | Male No NA | Confirmed psoriasis |

All psoriasis cases were of Caucasian ethnicity. PsA, psoriatic arthritis diagnosed by rheumatologist. BSA, affected body surface area. NA, information not available.
subclass of zinc finger transcription factors. ZNF750 is highly expressed in keratinocytes, which are the major skin cell type affected both in seborrheic dermatitis and in psoriasis. In addition, human primary keratinocytes that have been differentiated with Ca²⁺ have increased ZNF750 promoter activity at levels similar to PMA stimulated cells (unpublished data), suggesting ZNF750 may serve an important function in keratinocyte differentiation or immune response in the skin. As keratinocytes in psoriasis or seborrheic dermatitis secrete factors that recruit cells of the immune system and help maintain the inflammatory response [23,24], it is possible that an insufficient level of ZNF750 could lead to a downstream effect that fails to repress a stimulated immune response in psoriasis or seborrheic dermatitis.

**Conclusions**

In summary, we have performed the first re-sequencing study of the candidate psoriasis gene ZNF750 in a large case-control population. Although no individual variants were found to associate with psoriasis, two ZNF750 haplotypes showed a significant association. We also observed a nominal association between rare variants in the 5′ regulatory region of ZNF750 and psoriasis. Collectively, these rare 5′ regulatory variants were seen in 10 of 716 (1.4%) of the psoriasis population surveyed. Functional assays demonstrated that 4 of these variants decreased ZNF750 promoter activity in accordance with previous reports in psoriasis [12] and psoriasiform dermatitis [11]. We found that these variants did not segregate with the psoriasis phenotype within families, suggesting that they are modifier variants rather than causal for psoriasis. Further studies are warranted to determine whether these rare, non-coding variants could play an influencing role in disease expression.

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**Authors’ contributions**

RYB conceived the study, performed and supervised the functional work, and helped write the manuscript. GH performed the luciferase assays and helped write the manuscript. IC performed functional characterization of the promoter region. AP performed the sequencing. HC performed statistical analysis. EL performed statistical analysis. PK provided logistical and financial support. OSB supervised some of the functional work and provided financial support. WL designed the study, contributed cases and controls, supervised the sequencing and 14 analysis, helped write the manuscript, and provided financial support. All authors have read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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