Whole-Genome Sequencing Identifies \textit{STAT4} as a Putative Susceptibility Gene in Classic Kaposi Sarcoma

Mervi Aavikko,1,2 Eevi Kaasinen,1,2 Janne K. Nieminen,4 Minji Byun,4,9ikki Donner,1,2 Roberta Mancuso,12 Pasquale Ferrante,13 Mario Clerici,12,14 Lucia Brambilla,15 Athanasia Tourlaki,16 Ronit Sarid,16 Emma Gutman-Yassky,10,11 Minna Taipale,2,17 Ekaterina Morgunova,17 Pirita Pekkonen,4 Päivi M. Ojala,2,3 Eero Pukkala,6,7 Jean-Laurent Casanova,8,9,18,19 Outi Vaarala,4 Pia Vahteristo,1,2 and Lauri A. Aaltonen1,2

1Department of Medical Genetics, 2Genome-Scale Biology Research Programs Unit, 3Institute of Biotechnology, University of Helsinki, 4Immune Response Unit, Department of Vaccination and Immune Protection, National Institute for Health and Welfare, 5Finnish Cancer Institute, 6Finnish Cancer Registry, Institute for Statistical and Epidemiological Cancer Research, Helsinki, and 7School of Health Sciences, University of Tampere, Finland; 8St. Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller University, 9Howard Hughes Medical Institute, 10Department of Dermatology, and 11Immunology Institute, Mount Sinai Medical Center at Icahn School of Medicine, New York, New York; 12Don C. Gnocchi Foundation, ONLUS, 13Department of Biomedical, Surgery, and Dental Sciences, 14Department of Physiopathology and Transplantation, University of Milan, and 15Dermatology Unit, IRCCS Ca' Granda Foundation–Ospedale Maggiore Policlinico, Milan, Italy; 16Mina and Everard Goodman Faculty of Life Sciences, Bar Ilan University, Ramat Gan, Israel; 17Science for Life Center Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden; 18Laboratory of Human Genetics of Infectious Diseases, Necker Hospital School for Sick Children, and 19Imagine Institute, University Paris Descartes, France

Background. Classic Kaposi sarcoma (cKS) is an inflammatory tumor caused by human herpesvirus 8 (HHV-8) commonly observed in elderly men of Mediterranean origin. We studied a Finnish family of 5 affected individuals in 2 generations. Except for atypical mycobacterial infection of the index case, the affected individuals did not have notable histories of infection.

Methods. We performed genome and exome sequencing and mapped shared chromosomal regions to identify genetic predisposition in the family.

Results. We identified 12 protein-coding candidate variants that segregated in the 3 affected cousins from whom we had samples. The affected mother of the index case was an obligatory carrier. Among the 12 candidates was a rare heterozygous substitution rs141331848 (c.1337C>T, p.Thr446Ile) in the DNA-binding domain of \textit{STAT4}. The variant was not present in 242 Finnish control genomes or 180 additional regional controls. Activated T-helper cells from the HHV-8–negative variant carriers showed reduced interferon γ production, compared with age and sex matched wild-type individuals. We screened \textit{STAT4} in additional 18 familial KS cases and the variant site from 56 sporadic KS cases but detected no pathogenic mutations.

Conclusions. Our data suggest that \textit{STAT4} is a potential cKS-predisposition gene, but further functional and genetic validation is needed.

Keywords. classic Kaposi sarcoma; genetic predisposition; genome sequencing; HHV8; STAT4.
virus (HIV) infection; iatrogenic KS, seen secondary to immuno-nospressive therapy; endemic KS, observed mainly in non-HIV-infected individuals from sub-Saharan Africa; and classic KS, commonly affecting elderly men of Mediterranean, Eastern European Jewish, or Middle Eastern origin.

Classic KS (cKS) is clinically considered the mildest form of KS and is usually a slowly progressing disease involving the skin of the lower limbs and, more rarely, the upper limbs. Familial occurrence of cKS is rare, with about 100 patients reported to date [8, 9]. The reported familial cases have mainly been siblings or cousins of Jewish origin [8].

Although HHV-8 is present in all subtypes of KS, the infection alone is not sufficient for tumor development. Genetic predisposition to cKS has been reported in 4 unrelated children with recessive single-gene inborn errors of immunity. Two of the patients had cKS and other phenotypes due to inherited mutations in IFNGR1 and WAS [10, 11]. Two other patients had isolated cKS due to mutations in STIM1 and TNFRSF4 [12, 13]. Genetic variants in FCGR3A, CXCR2, and IL13 [14, 15], as well as certain HLA-alleles, have also been associated with cKS predisposition at the population level, but these findings remain inconclusive.

We recently reported a frequent occurrence of familial KS in Finland and described a Finnish family of 5 affected individuals with adulthood cKS in 2 generations [9]. The aim of this study was to explore the possible genetic predisposition in the family by genome-wide mutation analysis supported by analysis of shared genomic regions. We report a candidate predisposition variant, c.1337C>T p.Thr446Ile, in STAT4 that segregated with cKS in the family. We further show that the variant carriers display attenuated interferon γ (IFN-γ) production, suggesting that signal transducer and activator of transcription factor 4 (STAT4)–mediated T-helper cells are important in the defense against HHV-8.

**MATERIALS AND METHODS**

**Ethical Considerations**

The study was approved by Ministry of Social Affairs and Health in Finland, as well as by local ethics review committees in Finland, Italy, and Israel. Informed consent was obtained from the patients who donated fresh tissue samples. The use of archival materials was authorized by the Finnish National Supervisory Authority for Welfare and Health.

**Patients and Sample Materials**

The index case (III-5; Figure 1A) received a diagnosis of KS in the right calf and ankle at the age of 58 years. During the following 2 decades, she had multiple KS lesions, some of which were surgically removed and some that were treated with irradiation. At the age of 74 years, she received a diagnosis of atypical mycobacterial infection (due to *Mycobacterium avium intracellulare*) in the lungs. A few months later, her KS metastasized to the submandibular lymph node. After 2 years of unsuccessful treatment of *M. avium intracellulare* infection with antibiotics, treatment was ceased because of leukopenia. Other immunity-related conditions included allergic hypertrophic rhinitis and erysipelas, which was treated during hospitalization. She died during hospitalization at the age of 86 years from normal causes.

One sister of subject III-5 (subject III-1; Figure 1A) was mentally disabled and lived in the same household with subject III-5. She had KS diagnosed in the right knee at the age of 68 years. Similar to subject III-5, she also had consecutive KS lesions, most of which were treated with irradiation. She had osteoarthritis in her left wrist and she was hospital treated twice for erysipelas. The clinical records also described an unspecified pleural scar in the lungs, and tuberculosis was suspected, but she was never examined for that. She died of pneumonia at the age of 89 years.

The other sister of subject III-5 (subject III-2; Figure 1A) had KS diagnosed at the age of 64 years. She died at the age of 67 years from myocardial infarction. Her clinical records were not available. The mother of subject III-5 (subject II-2; Figure 1) had multiple KS lesions diagnosed in the right foot at the age of 70 years. Limited medical records were available from her, and she died from a stroke 3 years after the KS diagnosis.

The cousin of subject III-5 (subject III-6; Figure 1A) received a diagnosis of prostate hyperplasia at the age of 75 years. At the age of 86 years, he had multiple plantar KS lesions diagnosed, which were treated with irradiation. He died of acute myocardial infarction at the age of 88 years.

Samples used in the study are listed in Table 1. From subject III-5, peripheral blood–derived DNA, Epstein-Barr virus–transformed lymphoblastoid cell line, and formalin-fixed paraffin-embedded (FFPE) tumor samples were available, and from subjects III-2 and III-6, only FFPE tumor samples were available (Figure 1A). Samples used in STAT4 screening included FFPE DNA specimens from 6 Finnish KS families [9] (Supplementary Figure 1A), 56 FFPE tumor DNA specimens from Finnish patients with sporadic KS, germ line DNA specimens from a cKS family from Israel [16], as well as germ line DNA specimens from 26 patients with cKS from 13 Italian cKS families.

**Genotyping and Linkage Analysis**

Genotypes of subjects III-5 and III-6 were determined using HumanOmni2.5Quad v1.0 and HumanOmni express FFPE v1.0 platforms, respectively (Illumina, Carlsbad, California). Hybridizations were performed according to manufacturer’s instructions at Finnish Institute for Molecular Medicine. Multipoint parametric linkage analysis with an autosomal dominant model was performed with Merlin v1.1.2 [17]. Regions of consecutive markers with a logarithm of the odds (LOD) score >0
and within a distance of 1 cM from each other were retrieved from the results. Detailed methods are described in the Supplementary Materials.

**Next-Generation Sequencing**

The blood-derived DNA sample from subject III-5 and the FFPE-derived DNA samples from subjects III-2 and III-6

---

**Figure 1.** Family of 5 affected individuals with classic Kaposi sarcoma and flow chart of the genetic analysis. A, Pedigree describing the family. Generations are denoted by Roman numerals. Round and square symbols denote females and males, respectively. The ages at cancer diagnoses are specified in brackets underneath the symbols. The \( STAT4 \) c.1337C>T genotypes and human herpesvirus 8 (HHV-8) statuses are specified underneath the symbols of the studied subjects; plus signs denote HHV-8 positivity, and minus signs denote HHV-8 negativity. The pedigree has been partly modified for confidentiality. B, Description of the genetic analysis and samples used in each step. Abbreviations: FFPE, formalin fixed, paraffin embedded; SNP, single-nucleotide polymorphism.
underwent paired-end sequencing using Illumina sequencing technology. The DNA sample from subject III-5 was fragmented into 2 different sized libraries (approximately 1000 bp and approximately 500 bp), which were extracted from an agarose gel and analyzed using Illumina HighSeq 2000 at the Finnish Institute for Molecular Medicine Technology Center. To increase read depth of the sample from subject III-5, 2 additional genomic libraries and 3 exomic libraries (Agilent SureSelect Human All Exon Kit v1.0 and XT HumanAllExon; Agilent, Santa Clara, California) with fragment sizes of approximately 400 bp were sequenced using Illumina Genome Analyzer II and HighSeq 2000. The exome sequencing of the FFPE tumor specimen from subject III-2 was performed using the Agilent SureSelect XT HumanAllExon kit and Illumina HighSeq 2000 at the Karolinska Institutet (Sweden). Genomic library preparation and sequencing of the FFPE tumor sample from subject III-6 was performed by Illumina (Cambridge, United Kingdom). Data processing and analyses are described in detail in the Supplementary Materials.

**Sanger Sequencing**

Separate polymerase chain reaction primers were designed for analysis of complementary DNA, FFPE tumor specimen DNA, and fresh tissue specimen DNA, using Primer3 [18]. Primer sequences are available upon request. Sequences were analyzed with Mutation Surveyor v4.08 (Softgenetics, State College, Pennsylvania), using the STAT4 (CCDS2310, NM_005427.3) sequence as a reference.

**Flow Cytometry**

Heparinized venous blood samples were collected from 4 carriers of the STAT4 c.1337C>T variant (subjects IV-1, IV-3, IV-4, and IV-6; Figure 1A) and from 8 age- and sex-matched STAT4 wild-type family members (spouses of subjects IV-1, IV-3, and IV-4 and subjects IV-2, IV-5, IV-7, IV-9, and III-16; Figure 1A). See Supplementary Materials for detailed methods.

In brief, for the assessment of STAT4 activation, CD4+ T cells were purified from peripheral blood mononuclear cells (PBMCs), using a CD4+ T-cell isolation kit (Miltenyi Biotec, Gladbach, Germany). Interleukin 12p70 (IL-12p70; R&D Systems, Minneapolis, Minnesota) at a concentration of 20 ng/mL or 100 ng/mL or IFN-α (PBL Interferon Source, Piscataway, New Jersey) at a concentration of 1000 IU/mL was used for the activation of STAT4. Preceding IL-12 treatment, IL-12R expression was induced using anti-CD3/CD28 stimulation (Dynabeads Human T-Expander CD3/CD28; Invitrogen, Carlsbad, California). For detection, PE-conjugated monoclonal antibody against phosphorylated STAT4 (pY693; clone 38/p-Stat4; BD Biosciences, San Jose, California) or an isotype control monoclonal antibody was used. To identify naive and memory T-helper cells, a cocktail of anti-CD4 FITC (SK3) and anti-CD45RO APC (UCHL1) antibodies (BD Biosciences) was used. For the intracellular detection of cytokines, PBMCs were cultured in X-VIVO-15 medium and stimulated with 10 ng/mL of phorbol 12-myristate 13-acetate (PMA) with 1 µg/mL of ionomycin for 1 hour and with brefeldin A for another 4 hours. For detection, anti-CD3 FITC antibody (SK7; BD Biosciences), anti-CD8 PerCP antibody (SK1; BD Biosciences), and, for some samples, also anti-CD45RO APC antibody were used. The samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences). FlowJo software (Tree Star, Ashland, Oregon) was used to analyze the flow cytometry data. The Mann-Whitney U test was used to compare the 2 groups of study subjects. A P value of < .05 was considered statistically significant. Prism 6 software was used for statistical analyses (GraphPad Software, La Jolla, California).

**RESULTS**

**Heterozygous p.Thr446Ile STAT4 Variant Segregates With cKS in the Family**

According to the clinical records, the affected individuals in a Finnish family with 5 affected individuals with cKS (Figure 1A)
did not have notable infectious histories. The index case, subject III-5, however, had a chronic atypical mycobacterial infection concurrent with the cKS. HHV-8 was detected in the FFPE tumor tissue specimens from subjects III-2, III-5, and III-6 by fluorescence immunohistochemical analysis, using antibody against HHV-8-encoded latency-associated nuclear antigen (data not shown).

To exclude chromosomal regions not shared by the affected individuals, we performed linkage analysis with a dominant inheritance model. Because of restricted sample materials, this was done using the samples from subject III-5 and her cousin, subject III-6 (Figure 1A). All chromosomal regions with a negative LOD score and <1 cM in length were excluded, resulting in exclusion of approximately two thirds of the genome from further analyses (Figure 1B and Supplementary Table 1).

Next, we sequenced the genomes of subjects III-5 and III-6 to identify genomic alterations within the shared chromosomal regions. Altogether, 2646 protein-coding variants, excluding synonymous changes, and 326 structural variants (SVs) were identified that were shared by the 2 affected individuals (Figure 1B). After removal of the variants with a minor allele frequency of >0.005 in 242 control genomes, 26 variants remained, of which 12 segregated in the exome of one of the sisters of subject III-5 (Table 2). The affected mother (subject II-2; Figure 1A) of the 3 siblings is an obligatory carrier of the variants. No shared SVs remained after using 49 in-house genomes as controls (Figure 1B).

We analyzed the protein coding regions of the 12 candidate genes from the exomes of 7 patients with KS belonging to 6 Finnish KS families (Table 1 and Supplementary Figure 1A). Additional variants in SLC7A11 (c.604A>G, Ile202Val, rs111500641) and HLA-DRB5 (c.604A>G, Ile202Val, rs111500641) were identified in silico predicted to be benign.

Although these were not present in our initial Finnish genome control set (n = 242), they were listed in the public variant databases with minor allele frequencies (MAFs) of >0.005, and both were in silico predicted to be benign.

Among the 12 candidates, 3 (p.Val36Met in HLA-C, p.Pro212Ala in HLA-DRB5, and p.Thr446Ile STAT4) resided in genes with human immunity–related functions. Of these, c.106G>A (p.Val36Met) in HLA-C and c.1337C>T (p.Thr446Ile) in STAT4 were in silico predicted to be damaging. Both of these variants are also listed in the public databases. The HLA-C c.106G>A (rs2308538) maps to 7 alternative human HLA alleles with an A allele frequency of 0.15 (dbSNP; build 134). STAT4 c.1337C>T (rs141331848), on the other hand, is a very rare variant, with allele frequencies of 0.0001 and 0.0008 in 1000 Genomes and the Exome Variant Server, respectively. The STAT4 c.1337C>T variant was absent from the 484 Finnish alleles that were used in the initial next-generation sequencing data filtering and in 360 population-matched healthy blood donor alleles in Sanger sequencing.

The c.1337C>T substitution encodes a missense change altering the threonine 446 to isoleucine (p.Thr446Ile). The substitution is also located 2 nucleotides from the 3’ exon-intron boundary of exon 16 of STAT4; thus, we tested whether it affects the STAT4 messenger RNA splicing, by complementary DNA sequencing analysis. However, splicing was shown to be normal (Figure 2A).

To study the putative effect of the mutation at protein level, we conducted multiple sequence alignment, which demonstrated that the DNA-binding domains of human STAT1, STAT2, STAT3, and STAT4 are highly homologous and that Thr446 is evolutionarily conserved (Figure 2B). Since no crystal structure exists on STAT4, we made a structural alignment of STAT1

---

### Table 2. 12 Candidate Variants Located Within the Linked Regions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ensembl Transcript No.</th>
<th>Genomic Position</th>
<th>Variation</th>
<th>Genotype</th>
<th>Reference SNP No.</th>
<th>In Finnish Control Genomes (n = 242)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSA10</td>
<td>ENST00000308287</td>
<td>11:60 565 924</td>
<td>c.659T&gt;C</td>
<td>Het</td>
<td>rs139578350</td>
<td>2</td>
</tr>
<tr>
<td>MTMR10</td>
<td>ENST00000435080</td>
<td>15:31 233 884</td>
<td>c.2123A&gt;G</td>
<td>Het</td>
<td>rs200560796</td>
<td>0</td>
</tr>
<tr>
<td>SPTBN5</td>
<td>ENST00000320956</td>
<td>15:42 843 521</td>
<td>c.551G&gt;A</td>
<td>Het/Hom</td>
<td>rs186662969</td>
<td>2</td>
</tr>
<tr>
<td>RANBP2</td>
<td>ENST000003283195</td>
<td>15:42 170 634</td>
<td>c.3376C&gt;T</td>
<td>Het</td>
<td>rs144628608</td>
<td>0</td>
</tr>
<tr>
<td>STAT4</td>
<td>ENST00000392320</td>
<td>15:42 170 634</td>
<td>c.1337C&gt;T</td>
<td>Het</td>
<td>rs141331848</td>
<td>0</td>
</tr>
<tr>
<td>ADAMTS1</td>
<td>ENST00000284984</td>
<td>15:42 843 521</td>
<td>c.1337C&gt;T</td>
<td>Het</td>
<td>rs20015124</td>
<td>0</td>
</tr>
<tr>
<td>C3orf52</td>
<td>ENST00000320956</td>
<td>15:42 843 521</td>
<td>c.1337C&gt;T</td>
<td>Het</td>
<td>rs20015124</td>
<td>0</td>
</tr>
<tr>
<td>NUDT6</td>
<td>ENST00000304430</td>
<td>15:42 843 521</td>
<td>c.1337C&gt;T</td>
<td>Het</td>
<td>rs20015124</td>
<td>0</td>
</tr>
<tr>
<td>SLCTA11</td>
<td>ENST00000320956</td>
<td>15:42 843 521</td>
<td>c.1337C&gt;T</td>
<td>Het</td>
<td>rs20015124</td>
<td>0</td>
</tr>
<tr>
<td>CARD6</td>
<td>ENST00000254961</td>
<td>15:42 843 521</td>
<td>c.1337C&gt;T</td>
<td>Het</td>
<td>rs20015124</td>
<td>0</td>
</tr>
<tr>
<td>HLA-C</td>
<td>ENST00000376228</td>
<td>15:42 843 521</td>
<td>c.1337C&gt;T</td>
<td>Het</td>
<td>rs20015124</td>
<td>0</td>
</tr>
<tr>
<td>HLA-DRB5</td>
<td>ENST00000320956</td>
<td>15:42 843 521</td>
<td>c.1337C&gt;T</td>
<td>Het</td>
<td>rs20015124</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: Het, heterozygous; Hom, homozygous; SNP, single-nucleotide polymorphism.

* The index case (subject III-5) was homozygous for the variant, gene, transcript, and genomic positions from Ensembl 71 (GRCh37).
Figure 2. STAT4 c.1337C>T (p.Thr446Ile) variant segregating in the Finnish classic Kaposi sarcoma family. A, Electropherograms of the wild-type STAT4 and the c.1337C>T STAT4 variant carrier, sequenced from the blood-derived complementary DNA specimens. B, Multiple sequence alignment of STAT4. The Thr at position is highlighted. C, Structural representation of the β-barrel domain of signal transducer and activator of transcription factor 1 (STAT1; blue) and STAT3 (cyan). Conserved Thr446 is represented in spheres and colored by elements (C, yellow; O, red). The loop responsible for the binding of DNA is colored in red, and residues (Ser465 and Asn466) involved in DNA binding are represented in the ball-and-sticks model. The close views represent the network of hydrogen bonds that keep the β-barrel intact and connect it to the coiled-coil domain and the pThr446Ile mutation. Owing to the replacement of the polar Thr with the hydrophobic Ile, the network of interactions with Phe321, His447, and Asn475 cannot be formed, increasing the flexibility of the DNA-binding loop, which may impair and even abolish the DNA-binding properties of STAT4.
and STAT3 (PDB entries 1BF5 [19] and 1BG1 [20], respectively) and modeled the p.Thr446Ile mutation to the structure of STAT3 (Figure 2C). This showed that Thr446 is localized in the core of the DNA-binding domain and forms an H bond with the main chain oxygen of Phe321, keeping the structures connected (Figure 2C). Moreover, Thr446 participates in the extensive network of H bonds, stabilizing the structure of the α-helical segment responsible for the interaction with DNA (Figure 2C). Mutation of the polar Thr446 to hydrophobic Ile possibly breaks down these contacts, creating additional flexibility of the DNA-binding loop (Figure 2C).

**STAT4 Was Not Frequently Mutated in cKS**

To identify STAT4 variants from additional KS cases, we performed Sanger sequencing on the coding region of STAT4 from 1 Israeli cKS patient belonging to a family with four cKS affected siblings and 26 familial Italian patients with cKS (belonging to 13 families) but identified only common intronic polymorphisms. We performed Sanger sequencing on the site of the c.1337C>T variant from 56 FFPE KS tumor tissue specimens. Two tumors harbored a rare c.1338C>A variant encoding the synonymous p.Thr446Thr alteration (rs144421302). The variant is present in the Exome Variant Server and 1000 Genomes with allele frequencies of 0.0025 and 0.0004, respectively.

**Putative KS Predisposing Variants in STAT4-Associated Genes**

Since we did not find additional variants in STAT4, we sought to identify variants in STAT4-related genes from the exomes of the 7 Finnish familial KS cases. We generated a list of genes interacting with, regulating, or regulated by STAT4 (n = 47; Supplementary Table 2) through the use of Qiagen’s Ingenuity Pathway Analysis tool (Qiagen, Redwood City, California; http://www.qiagen.com/ingenuity). Three rare variants (MAF <0.005) were identified, of which 2 were located in TYK2 (c.1642C>T [p.Arg548Cys] and c.329G>A [p.Arg110Gln, rs5609099]) and 1 in CCR5 (c.866A>G [p.His289Arg]). TYK2 c.1642C>T and CCR5 c.866A>G were studied in the samples of the respective affected family members by Sanger sequencing, and the variant in CCR5 was shown to segregate. The variant was predicted damaging by Polyphen2 [21], and it was not present in the 149 Finnish in-house control genomes, in 1000 Genomes, or in the Exome Variant Server. We were not able to study the segregation of the TYK2 c.329G>A variant, since there was no sample available from the respective affected family member.

**STAT4 Phosphorylation in T Cells Is Not Affected by the p.Thr446Ile Variant**

STAT4 is central in the IL-12-mediated signal transduction that drives the differentiation of IFN-γ-producing T-helper cells, central players in the adaptive antiviral and antitumor immune responses. IL-12p70–induced STAT4 phosphorylation (pY693) was therefore evaluated in CD4+ T cells isolated from carriers of the STAT4 p.Thr446Ile variant (subjects IV-1, IV-3, IV-4, and IV-6; Figure 1A) and control subjects (spouses of subjects IV-1, IV-3, and IV-4 and subjects IV-2, IV-5, IV-7, IV-9, and III-17; Figure 1A and Supplementary Figure 1B). We found comparable STAT4 phosphorylation responses in both activated naive and memory T-helper cells between the study groups (Supplementary Figure 1C). This was also the case when IFN-α–induced STAT4 phosphorylation was compared with no prior activation of T-helper cells (Supplementary Figure 1C).

**IFN-γ Responses in T Cells Are Attenuated in Carriers of the p.Thr446Ile Variant**

We hypothesized that T-cell IFN-γ responses would be affected by the STAT4 p.Thr446Ile variant. Therefore, the production of intracellular IFN-γ was assessed by flow cytometry. The intensity of IFN-γ expression following PMA plus ionomycin stimulation was significantly decreased in naive T-helper cells from the p.Thr446Ile variant carriers (Figure 3A), and a trend also existed for lower IFN-γ upregulation in memory T-helper cells (Figure 3A). No differences were found in the percentages of IFN-γ+–positive cells, however (Figure 3A). IL-4 and IL-17 responses in T-helper cells did not differ between the groups (data not shown). In CD8+ cytotoxic T cells, IFN-γ responses were comparable between the variant carriers and controls (data not shown). IL-4 and IL-17 were unexpressed or expressed at very low levels in CD8+ T cells, with no detectable differences between the study groups (data not shown).

**DISCUSSION**

We studied a family with 5 adults with cKS, using whole-genome and exome sequencing analysis. Apart from subject III-5’s atypical mycobacterial infection, the affected family members had seemingly eventless clinical histories, with no striking evidence of immunodeficiency. We identified 12 rare protein-coding candidate variants that were present in 3 of 5 affected individuals from whom we were able to obtain samples. The affected mother of the 3 affected siblings was an obligate carrier of the variants. Two of the variants (c.106G>A in TYK2 c.866A>G [p.His289Arg]) resided in human immunity–related genes and were in silico predicted to be damaging. The variant in TYK2-C, however, seemed an unlikely candidate because of ambiguous genomic mapping and considerably high frequency reported in the dbSNP. The c.1337C>T in STAT4, on the other hand, is a very rare allele with frequencies of 0.0001 and 0.0008 in 1000 Genomes and the Exome Variant Server, respectively. We did not detect any variant carriers among the 242 Finnish genomes used to control the next-generation sequencing data or in the 180 population-matched healthy controls for whom Sanger sequencing was performed.

We sequenced the coding region of STAT4 from 34 familial KS cases and the mutation site from 56 sporadic KS cases. A synonymous c.1338 C>A (p.Thr446Thr; rs144421302) alteration was
identified in 2 sporadic KS cases, but no protein code–altering variants were identified. We also studied mutations of STAT4-associated genes in 7 familial KS cases and found that a novel, potentially damaging heterozygous variant in CCR5 (c.866A>G, p.His289Arg), located at putative transmembrane domain of CCR5 (7tm_1; pfam00001 [22]), segregated in both of the affected family members. CCR5 encodes a CC-chemokine receptor, and STAT4 is known to negatively regulate its expression in mouse T cells [23, 24]. CCR5 is a coreceptor of HIV type 1 (HIV-1) entry [25, 26], and individuals with defective CCR5 alleles have been associated with resistance to HIV-1 infection [27–30].

STAT4 is a one of the 7 members of the STAT family, and it is generally expressed in spermatozoa, myeloid cells, and T lymphocytes [31]. STAT4 is known to be activated by IL-12, interleukin 23, and type 1 IFNs via phosphorylation by Janus kinase 2 or tyrosine kinase 2. Upon activation, STAT4 forms dimers and relocates to the nucleus to regulate its target genes, especially the gene encoding IFN-γ [32, 33]. We studied whether the p. Thr446Ile STAT4 mutation affected IFN-γ production and detected significantly decreased production of IFN-γ upon treatment with PMA plus ionomycin in naive T-helper cells from the mutation carriers. A similar trend was seen in the memory T-helper cells from the carriers. However, there was no difference in the amount of phosphorylated STAT4 in the T-helper cells from the mutant carriers, compared with those from the wild-type individuals. Also, we saw no differences in the IL-4 and IL-17 responses in T cells from the 2 groups.

Germ line mutations in STAT1, IFNGR1, IFNGR2, and IL12RB1 are well-established etiologic factors for Mendelian susceptibility to mycobacterial disease (OMIM: 209950) [34]. Intriguingly, 2 heterozygous alleles affecting STAT1 DNA-binding domain (p.Glu463H and p.ProE320Glu) have been reported in otherwise healthy patients with mycobacterial disease [35]. Furthermore, the one reported child with cKS and IFNGR1 deficiency also had a concurrent severe mycobacterial disease [10]. Thus, it is intriguing to speculate that the p.Thr446Ile variant, located in the DNA-binding domain of STAT4, may also predispose to the atypical mycobacterial infection present in subject III-5, our index case.

Stat4 knockout mice have disturbed IL-12–mediated functions and are susceptible to various intracellular pathogens, including Mycobacterium tuberculosis, Leishmania major, Trypanosoma cruzi, Toxoplasma gondii, Babesia species, and Listeria monocytogenes [36–38]. A recent forward mutagenesis screen in mice

Figure 3. STAT4 c.1337C>T (p.Thr446Ile) variant carriers show attenuated interferon γ (IFN-γ) production in T-helper cells. A, T-helper cells were identified from peripheral blood mononuclear cell cultures as CD3⁺CD8⁻ cells, given that CD4 expression is profoundly downregulated by stimulation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin. For the analysis of intracellular IFN-γ production, cells were further subdivided into populations of CD45RO⁺ memory and CD45RO⁻ naive T-helper cells. A representative example of a wild-type subject is shown. B, Intracellular flow cytometry was used to assess cytokine production in T cells from the p.Thr446Ile variant carriers and wild-type controls at the single-cell level. The amount of intracellular IFN-γ in the responding IFN-γ–positive cells was decreased in the naive T-helper cells from the p.Thr446Ile variant carriers, as evaluated by the fold-increase of geometric mean fluorescence identified in the respective unstimulated samples. In T-helper memory cells, a similar trend was seen, although it was not statistically significant. No differences were observed in the percentages of IFN-γ–positive cells in either memory or naive fractions upon stimulation with PMA plus ionomycin. The percentage of cytokine-positive cells in the respective unstimulated samples, although negligible, was subtracted from the result.
discovered a \textit{Stat4} DNA-binding domain mutation (p.418_E445) that predisposed homozygous mice to acute typhoid-like disease through IFN-\(\gamma\)-mediated immunity [39].

A common haplotype of \textit{STAT4} is associated with susceptibility to several autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and primary Sjögren syndrome [40, 41]. The associated single-nucleotide polymorphism (SNP) (rs7574865) located within the haplotype was also recently associated with the risk of hepatitis B virus–related hepatocellular carcinoma [42]. To our knowledge, variation in \textit{STAT4} gene has not previously been associated with HHV-8 infection. Recently, Svensson et al showed that variation of \textit{STAT4} importantly regulates herpes simplex virus type 2–specific IFN-\(\gamma\) responses in humans [43].

Our data underline the previous findings that acquired immunity—more precisely, T-helper cell responses—are important in the control of HHV-8 infection and the development of cKS. Taken together, we suggest that \textit{STAT4} is a putative cKS-predisposing gene. The predisposition may be caused by an impaired IFN-\(\gamma\)-mediated immune response, but to claim causality the result needs to be validated in other KS cases.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are relevant to the readers. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

\textbf{Acknowledgments.} We thank the patients who participated in the study; Sini Karjalainen, Mairi Kuris, Heikki Metsola, Minna Merikivi, Sini Nieminen, Alison Ollikainen, Sirpa Soisalo, Inga-Lill Svedberg, Sinikka Tsurumi, and Iina Vuoristo, for excellent technical assistance; Maija Lappalainen (HUSLAB), for expertise in virology; the Finnish Institute for Molecular Medicine Genome and Technology Center–IT Center for Science, and the Biomedical Functional Genomics Unit, for their services; and the Nordic Center of Excellence in Disease Genetics, for providing us the Finnish control single-nucleotide polymorphism data.

\textbf{Financial support.} This work was supported by the European Research Council Next Generation of Cancer Predisposition (grant 268648), the Academy of Finland Center of Excellence in Cancer Genetics Research (grant 250345), the Sigrid Jusélius Foundation, the Academy of Finland Center of Excellence in Cancer Genetics Research (grant 266:1865)–IT Center for Science, and the Finnish Institute for Molecular Medicine Genome and Technology Center–IT Center for Science, and the Biomedical Functional Genomics Unit, for their services; and the Nordic Center of Excellence in Disease Genetics, for providing us the Finnish control single-nucleotide polymorphism data.

\textbf{Potential conflicts of interest.} All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**