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CXCR3 Polymorphism and Expression Associate with Spontaneous Preterm Birth

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Spontaneous preterm birth (SPTB) is a major factor associating with deaths and with lowered quality of life in humans. Environmental and genetic factors influence the susceptibility. Previously, by analyzing families with recurrent SPTB in linkage analysis, we identified a linkage peak close to the gene encoding CXCR3. Present objectives were to investigate the association of CXCR3 with SPTB in Finnish mothers (n = 443) and infants (n = 747), to analyze CXCR3 expression levels in human placenta and levels of its ligands in umbilical cord blood, and to verify the influence of CXCR3 on SPTB-associating cytokines in mice. We detected an association between an intronic CXCR3 polymorphism, rs2280964, and SPTB in infants from families with recurrent preterm births (p = 0.009 versus term controls, odds ratio 0.52, 95% confidence interval 0.32–0.86). The minor allele was protective and undertransmitted to SPTB infants (p = 0.007). In the placenta and fetal membranes, the rs2280964 major allele homozygotes had higher expression levels than minor allele homozygotes; decidual trophoblasts showed strong CXCR3 immunoreactivity. Expression was higher in SPTB placentas compared with those from elective deliveries. Concentration of a CXCR3 ligand, CXCL9, was increased in cord blood from SPTB, and the protective rs2280964 allele was associated with low CXCL9. In CXCR3-deficient mice (Mus musculus), SPTB-associating cytokines were not acutely increased in amniotic fluid after preterm birth–inducing dose of maternal LPS. Our results indicate that CXCR3 contributes to SPTB. Activation of CXCR3 signaling may disturb the maternal–fetal tolerance, and this may promote labor. The Journal of Immunology, 2015, 195: 2187–2198.

Preterm birth, defined as birth before 37 completed wk of gestation, affects 5–18% of all deliveries and is the major cause of infant mortality worldwide (1–3). Preterm birth represents a major global health care problem; infants born preterm are at high risk of both immediate- and long-term morbidities, with potentially lifelong consequences (4, 5). Approximately 70% of all preterm deliveries occur after spontaneous onset of labor (6). However, the pathogenesis of spontaneous preterm birth (SPTB) is poorly understood. The process leading to SPTB involves a complex interplay between signals originating both from the maternal and fetal compartments (including the fetal membranes and placenta) (7). Infections are estimated to be involved in 30–40% of all preterm births (8); thus, infection that causes activation of the immune system represents a major factor for SPTB. During pregnancy, the mother tolerates the fetus and extraembryonic fetal tissues as a semiallograft (9). As a consequence, factors affecting maternal–fetal immunotolerance may also influence the onset of preterm delivery. Although a number of acquired factors (e.g., multiple pregnancy and alcohol or narcotic addiction), diseases, and malformations contribute to the risk, the causes are incompletely understood, and there is no effective prevention for SPTB (10).

Aggregation of preterm birth in families suggests that genetic factors play a role in SPTB (11–15). According to large

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The online version of this article contains supplemental material.

Abbreviations used in this article: CI, confidence interval; CYC1, cytochrome c-1; dpc, day postconcept; FU, fluorescence unit; GA, gestational age; HK, housekeeping; MAF, minor allele frequency; OR, odds ratio; PPROM, preterm premature rupture of fetal membranes; qPCR, quantitative PCR; SNP, single-nucleotide polymorphism; SPTB, spontaneous preterm birth; TDT, transmission disequilibrium test; UPL, Universal ProbeLibrary; UTR, untranslated region; WGA, whole-genome amplification; WT, wild-type.

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population-based studies and genetic modeling studies, preterm birth and/or variation in gestational age (GA) are affected by maternal genetic factors (11, 13, 14, 16–19); some of these studies suggest that fetal genes also play a role (16, 17, 19). SPTB is a multifactorial phenotype that is influenced by multiple interacting factors, with the potential involvement of extensive gene–gene and gene–environmental interactions (20). Although several small-scale case-control association studies of selected candidate genes for SPTB have been published, only a few of the detected associations have been replicated in subsequent studies (21). Recently, more comprehensive association analyses [large-scale association studies (22–24) and a family-based analysis (25)] and hypothesis-free approaches, including linkage analyses (26–28), whole-exome sequencing (29), and a genome-wide association study (30), have been used to study SPTB; these studies suggested significant roles for genes involved in multiple pathways, underlining the heterogeneity of the phenotype. Further studies are needed to find out the most important factors associated with predisposition to SPTB.

CXCR3 is a G protein–coupled receptor that plays an essential role in cell-mediated immunity (31, 32). There are three CXCR3 splice variants, as follows: CXCR3-A, -B, and -alt; CXCR3-A and -B are the main variants (32, 33). Five chemokines bind CXCR3, as follows: CXCL9 (also known as monokine induced by IFN-γ), CXCL10 (IFN-γ–induced protein 10), CXCL11 (IFN-inducible T cell α chemoattractant), CXCL4 (platelet factor 4), and CXCL4L1 (platelet factor 4 variant) (34). CXCR3 is preferentially expressed in Th1 cells, but also in other cells, including NK cells, mast cells, and microvascular endothelial cells (31, 32). CXCR3 ligands are upregulated in proinflammatory milieus; they promote migration of circulating leukocytes, mainly Th1 lymphocytes, to sites of inflammation and injury (31, 34). In addition to immune cells, CXCR3-A expression has been detected in the human placenta (35). During labor, immune cells infiltrate the human reproductive and gestational tissues, including the fetal membranes and decidua at the maternal–fetal interface (36–40); several chemokines are expressed by choriodecidual cells (37, 41–43) and upregulated during this process (39, 44). Both spontaneous term and preterm human labor are associated with increased expression of several chemokines in the reproductive and gestational tissues (39, 44–49). These include CXCL10 and its receptor CXCR3, whose levels are increased in choriodecidual cells during spontaneous labor at term (39, 44).

Previously, we used a nonbiased approach to analyze northern Finnish families with SPTB and detected a linkage peak on chromosome X located close to the gene encoding CXCR3 (27). In the current study, we reasoned that variation of CXCR3 may affect susceptibility to SPTB. Therefore, we assessed the role of CXCR3 gene in SPTB in case-control populations. We found an association in the infants between an intronic CXCR3 variant and SPTB. We further analyzed expression of CXCR3 in placentas and fetal membranes from preterm and term births. Next, we analyzed cord blood expression levels of several cytokines, including two CXCR3 ligands, CXCL9 and CXCL11. Finally, we studied cytokine levels in inflammation-induced preterm labor in an experimental model of CXCR3 deficiency. These data support our proposal that activation of CXCR3 predisposes to SPTB.

Materials and Methods

Inclusion criteria for SPTB cases and controls in the genetic analyses

Definition of SPTB. SPTB was defined as preterm birth that occurred at ≤36 completed wk of gestation after spontaneous onset of labor. The duration of the pregnancy was defined on the basis of ultrasound examination performed at most 18 wk after the last menstrual period. Deliveries that occurred after spontaneous onset of labor with intact membranes or after preterm premature rupture of membranes (PPROM, defined as leakage of amniotic fluid before the onset of labor) were included among the SPTB cases. Inclusion criteria for SPTB cases and controls. All medically indicated preterm births without labor (intrauterine growth restriction, pre-eclampsia, and placental abruption) were excluded from the study. To include only SPTB deliveries that involved as few as possible risk factors (other than genetic risk factors) in the study, we excluded deliveries involving the following known risk factors for preterm birth: 1) multiple gestation; 2) polyhydramnios; 3) acute septic infection of the mother (positive blood cultures) and/or evidence of systemic inflammatory response syndrome (WBC count >15,000/mm³ or >10% band cells and fever >38.2°C); 4) diseases of the mother that may affect timing of the onset of delivery (those affecting hepatopulmonary, or endocrine functions and chronic inflammatory disease); 5) alcohol or narcotic use; 6) severe accidents; and 7) fetuses with congenital anomalies. According to our estimates, ≥20% of all preterm deliveries in Finland fulfilled our inclusion criteria (corresponding to ~1% of all deliveries). Term delivery was defined as a delivery occurring at 37–41 wk of gestation (37 wk + 0 d to 41 wk + 6 d); deliveries involving any pregnancy- or labor-associated complications (intrauterine growth restriction, placental abruption, polyhydramnios, pre-eclampsia, fetuses with congenital anomalies, and requirement of special care of the newborn) were further excluded from among the controls. All analyzed subjects were of Finnish origin.

Study populations used for genetic analyses

Original case-control study population from northern Finland (Population I). Cases included mothers with spontaneous preterm deliveries (n = 251) and their SPTB infants (n = 291; GA ≤36 wk). Data regarding mothers and infants were collected retrospectively from birth diaries of Oulu University Hospital (northern Finland) during 1973–2003, and prospectively at Oulu University Hospital during 2003–2005. SPTB case-control association studies of selected candidate genes for genes involved in multiple pathways, underlining the heterogeneity of the phenotype. Further studies are needed to find out the most important factors associated with predisposition to SPTB. Therefore, we assessed the role of CXCR3 gene in SPTB in case-control populations. We found an association in the infants between an intronic CXCR3 variant and SPTB. We further analyzed expression of CXCR3 in placentas and fetal membranes from preterm and term births. Next, we analyzed cord blood expression levels of several cytokines, including two CXCR3 ligands, CXCL9 and CXCL11. Finally, we studied cytokine levels in inflammation-induced preterm labor in an experimental model of CXCR3 deficiency. These data support our proposal that activation of CXCR3 predisposes to SPTB.

DNA sample preparation, single-nucleotide polymorphism selection, genotyping, and sequencing

DNA was extracted from whole-blood and buccal-cell samples using standard methods, as described previously (26). DNA extracted from buccal
cells was whole-genome amplified (WGA) using the Illustra GenomiPhi V2 DNA Amplification kit (GE Healthcare Sciences). WGA products were further purified on Illustra MicroSpin G-50 columns (GE Healthcare Sciences). Purified WGA samples were subjected to extensive quality control, as described previously (26).

**CXCR3** (NG_029076.1) is a small gene (2.6 kb) with only a single common minor allele frequency [MAF] > 0.01) single-nucleotide polymorphism (SNP) (rs2280964) reported in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) and in the 1000 Genomes sequence data (http://www.1000genomes.org) for the Finnish population. We therefore chose this SNP for analysis. Genotyping of SNP rs2280964 was performed with the SNPshot Multiplex kit (Applied Biosystems). For the sake of simplicity, rs2280964 genotypes of hemizygous males (G or A) and homozygous females (GG or AA) are both referred to as GG and AA in the text. To confirm CXCR3 gene in the placental tissues from the population I, the sequence encompassing CXCR3 was further amplified by PCR with eight primer pairs described previously (53). PCR yielded eight partly overlapping PCR fragments that encompassed the whole CXCR3 gene; the first fragment started 2 kb upstream from the first exon, and the last ended ~30 bp downstream from the 3' untranslated region (UTR) of the last exon. The PCR fragments were then Sanger sequenced.

**CXCR3 mRNA and protein analyses**

**Collection of placental samples.** Placental samples were collected at Oulu University Hospital during 2010–2012. There were samples from 21 placentas from preterm deliveries (7 from SPTB and 14 from elective deliveries), GA from 25 wk + 3 d to 35 wk + 4 d. Altogether, these were placentas from twin pregnancies. In addition, there were 27 placentas from term deliveries (14 from spontaneous initiation and 13 from elective induction at 38–42 wk). Samples were collected from the basal plate immediately underneath the placental surface (the maternal side of placenta), from the chorionic plate immediately underneath the placental surface (the fetal side of placenta), and from the fetal membranes. Umbilical cord blood was collected and used for DNA extraction; the SNP rs2280964 genotypes of corresponding infants were determined.

**Quantitative PCR.** RNA was isolated from frozen placental tissue samples according to the manufacturer’s instructions for the High Pure RNA Tissue Kit (Roche Diagnostics). DNA was removed from RNA isolated from fresh placental samples with an RNase-free DNase I kit (Fermentas). RNA was ordered immediately in RNase-free water and stored at −80°C. Reverse transcription (RT) was performed with the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). For RNA isolation, tissue samples were first disrupted with TissueLyser LT (Qiagen) and the stainless steel bead method. The quality and quantity of isolated RNA were assessed by measuring absorbance values at 230, 260, and 280 nm using NanoDrop. Altogether, 250 ng total RNA from each sample was converted into cDNA by the standard RT-PCR procedure with the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). Subsequently, in the corresponding quantitative PCR (qPCR) reactions, the cDNA was used.

Relative quantifications of CXCR3 mRNA levels were done with LightCycler96 (Roche Diagnostics) using CXCR3 splice variant- and cytotome-specific primers and probes; primers were designed to bind at the beginning of the last exon. The PCR fragments were then Sanger sequenced. CYC1 was used as a reference gene based on previous studies (54–56). For example, Drewlo et al. (56) used nine clinical groups (healthy first trimester, healthy second trimester, preterm controls, vaginal and cesarean delivery term controls, severe early onset intrauterine growth restriction, severe pre-eclampsia, and mixed pre-eclampsia–intrauterine growth restriction) and eight commonly used housekeeping (HK) genes to determine the most stable HK genes for placental research. Their data indicated that CYC1 was one of the most stable HK genes for placental research. Additionally, in our study, we followed and confirmed the stability of CYC1 mRNA.

**Western blotting.** Protein homogenates were prepared from placental tissue in lysis buffer (10 mM Tris-HCl [pH 7.5], 250 mM sucrose, and 1 mM EDTA). Cells were lysed by sonication. After centrifugation, supernatants were collected and protein concentrations were measured with Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories) at wavelength 595 nm. For Western blotting analysis, 20 μg total protein from each sample was first separated on a 12% Bis-Tris gel (NuPAGE Novex; Life Technologies) and then electrotransferred onto Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences), according to the manufacturer’s instructions. Polyvinylidene difluoride membranes were blocked overnight at +4 °C. CXCR3 (P49602) was detected with mouse anti-human CXCR3 Ab (R&D Systems; 1:500 dilution) as a primary Ab and goat anti-mouse IgG HRP (Jackson ImmunoResearch Laboratories; 1:1,000 dilution) as a secondary Ab. Detection was done with the ECL Prime Western blotting Detection Reagent kit (GE Healthcare). After CXCR3 detection, membranes were reprobed and β-actin was used as a loading control. β-actin was detected by using mouse anti-β-actin (Sigma Aldrich; 1:3,000 dilution) and goat anti-mouse IgG HRP (GE Healthcare; 1:1,000 dilution) as primary and secondary Abs, respectively, together with the ECL Prime Detection kit. After detection, intensity values of the target proteins were determined with Quantity One (4.6.9.) analysis software (Bio-Rad Laboratories).

**CXCR3 immunohistochemistry and analysis of umbilical cord blood cytokines**

**Collection of placental and umbilical cord blood samples.** Placental and umbilical cord blood samples of newborn infants were collected at Oulu University Hospital during 1998–2002. The population has been described previously (57); it consists of very preterm infants (GA < 32 wk). Infants with major congenital anomalies, metabolic diseases, chromosomal defects, or congenital TORCH (toxoplasmosis, other [syphilis, varicella-zoster, parvovirus B19], rubella, CMV, and herpes) infections were excluded. Full-thickness samples of placental parenchyma were collected immediately after delivery. DNA samples were collected from the basal plate and immediately underneath the placental surface (the maternal side of placenta), from the chorionic plate immediately underneath the placental surface (the fetal side of placenta), and from the fetal membranes. Umbilical cord blood was collected in dry sterile tubes immediately after delivery. Serum was separated by centrifugation and stored at −70°C. Blood samples were used for concentration measurements and DNA extraction; the SNP rs2280964 genotypes of corresponding infants were determined. After exclusion of multiple pregnancies (23 infants) and infants without available concentration data for the cytokines (n = 26), a total of 99 very preterm infants remained for analysis. Of these, 66 were born after spontaneous onset of delivery and 33 after induced delivery or elective cesarean section without signs of an active labor process.

**Immunohistochemistry.** Altogether, 27 and 12 placentas from SPTB and elective preterm deliveries, respectively, were analyzed using immunohistochemistry. Placental samples were embedded in paraffin and cut into 4-μm slices, deparaffinized, and rehydrated. Ag retrieval was done in Tris-EDTA buffer. Endogenous peroxidase activity was blocked in blocking solution (DAKO). Samples were incubated in a 1:2000 dilution of mouse anti-human CXCR3 Ab (R&D Systems; 1:500 dilution) as a primary Ab and goat anti-mouse IgG HRP (GE Healthcare; 1:1,500 dilution) as a secondary Ab. Detection was done with the Envision kit (DAKO). Two monoclonal Abs were used and detected by a microarray scanner. The amount of fluorescence reflects the concentration of bound Abs. Polyvinylidene difluoride membranes were blocked for 1 h in blocking solution (DAKO). Samples were incubated in a 1:2000 dilution of mouse anti-human CXCR3 Ab (R&D Systems; 1:500 dilution) as a primary Ab and goat anti-mouse IgG HRP (GE Healthcare; 1:1,500 dilution) as a secondary Ab. Detection was done with the ECL Prime Western blotting Detection Reagent kit (GE Healthcare). After CXCR3 detection, membranes were reprobed and β-actin was used as a loading control. β-actin was detected by using mouse anti-β-actin (Sigma Aldrich; 1:3,000 dilution) and goat anti-mouse IgG HRP (GE Healthcare; 1:1,000 dilution) as primary and secondary Abs, respectively, with the ECL Prime Detection kit. After detection, intensity values of the target proteins were determined with Quantity One (4.6.9.) analysis software (Bio-Rad Laboratories).

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**Experiments in mice deficient for CXCR3**

**Animals.** Mice deficient for CXCR3 (C57Bl/6J) were obtained from The Jackson Laboratory and back-crossed onto the local C57BL/6J strain for at least four generations prior to experiments. All mice were genotyped, and only CXCR3-deficient mice were used. For the experiments, CXCR3-deficient female mice aged 3–4 mo were mated with males of the same line. Wild-type (WT) C57BL/6 mice served as controls. GA (>12 h) was determined by the presence of a vaginal plug and designated as day 0 of pregnancy.

**Mouse model of preterm birth.** We used our previously established mouse model of LPS-induced preterm birth of live-born offspring (60) to study the role of CXCR3 in inflammation-induced preterm birth. In the current study, the preterm birth–inducing dose of LPS was determined in the ECL model of LPS-induced preterm birth of live-born offspring (60) to study the role of CXCR3 in inflammation-induced preterm birth. In the current study, the preterm birth–inducing dose of LPS was determined in the following preliminary experiment. Timed-pregnant CXCR3-deficient and WT mice were i.p. injected at 17 h postcoitus (dpc) with different doses...
(10–25 μg/mouse) of Escherichia coli LPS (serotype 0111:B4) dissolved in PBS. Control mice (n = 4 for both mouse lines) received an equal volume (100 μl) of PBS. The timed-pregnant mice were randomized to receive either LPS or PBS injections. Dams were followed until the time of delivery, and the viability of the prematurely born pups was observed, as previously described (60). The litters were called live-born when ≥50% of the pups were born alive.

Sample collection. For cytokine analyses, mice were randomized to receive either LPS or PBS injections. Timed-pregnant CXCR3-deficient (n = 5) and WT (n = 4) mice were injected with a preterm birth–inducing dose of LPS (20 μg/mouse) at 17 dpc. Another set of CXCR3-deficient and WT mice received an equal volume (100 μl) of sterile PBS (n = 4 for both mouse lines). Sample size was calculated based on our previous studies (60), and the estimated sample size was 8–10 fetuses. Dams were anesthetized with ketamine-medetomidine and killed with cervical dislocation 3 h after the LPS or PBS injections. Amniotic fluid from the fetuses was collected and stored at −70°C.

Cytometric bead array. Levels of IL-6 and CCL2 in the amniotic fluid of CXCR3-deficient and WT mice were quantitatively measured with the Cytometric Bead Array Mouse Inflammation kit (BD Biosciences), according to the manufacturer’s instructions. In each group, amniotic fluid from 2–5 pups from every litter was analyzed individually. The detection limits for IL-6 and CCL2 were 5 and 53 pg/ml, respectively.

Statistics

Quality control in the genetic study. Before proceeding to association analysis, the absence of Mendelian errors in the genotype data was confirmed using Pedcheck (61). Inconsistent genotypes were recoded as missing.

Statistical analysis in the genetic study. Case-control and family-based association analyses were performed using PLINK, v. 1.07 (62). The significance of allele frequency differences was analyzed with the χ² test, and effect sizes were estimated using odds ratios (OR). To account for the effect of fetal gender, fetal sex was included as a covariate in a logistic regression model. Female individuals were analyzed using genotypic, dominant, and recessive models; analysis with these models was not applicable to male infants because CXCR3 is located on the X chromosome. Allele frequency differences between SPTB infants from deliveries with and without PPROM, and between very (GA <32 wk) and moderate-to-late preterm infants (GA 32–36 wk), were also investigated. Family-based analysis was performed with the transmission disequilibrium test (TDT). Prediction of regulatory effects of the CXCR3 polymorphism was performed with GWAS3D (http://jjwanglab.org/gwas3d) and RegulomeDB (https://regulombd.org), which use public datasets, including data from the Encyclopedia of DNA Elements project (https://www.encodeproject.org), as sources.

Statistical analysis of mRNA, protein, and cytokine studies. RNA, protein, and cytokine analyses were performed with SPSS Statistics 20.0 (IBM Corporation). Expression with the transmission disequilibrium test (TDT). Prediction of regulatory effects of the CXCR3 polymorphism was performed with GWAS3D (http://jjwanglab.org/gwas3d) and RegulomeDB (https://regulombd.org), which use public datasets, including data from the Encyclopedia of DNA Elements project (https://www.encodeproject.org), as sources.

Statistical analysis of mRNA, protein, and cytokine studies. RNA, protein, and cytokine analyses were performed with SPSS Statistics 20.0 (IBM Corporation). Expression level differences between genotype and phenotypic types were assessed by the nonparametric Mann–Whitney U test or Kruskal–Wallis test. Mann–Whitney U test was used to analyze whether cytokine concentrations in umbilical cord blood at time of birth differentiated between SPTB infants (infants born after spontaneous onset of labor) and preterm infants born after elective delivery (without active signs of labor). Receiver operating characteristic curves were used to specify the sensitivity and specificity of the analyzed CXCR3 ligands in predicting SPTB.

Statistical analysis of CXCR3-deficient mice. Quantified protein concentrations were analyzed with SPSS Statistics 20.0 (IBM Corporation). Data were tested for normal distribution, and differences between experimental and control groups were analyzed using either Student t test or Mann–Whitney U test.

Ethical approval

The study had the approval of the Ethical Committees of the participating centers (Oulu University Hospital 79/2003 and 73/2013, Helsinki University Central Hospital 279/E7/2001). Informed consent was obtained from all study subjects. The animal care and experimental procedures were conducted under a protocol approved by the National Animal Experiment Board of Finland (license ESAV/3752/04.10.03/2012).

Results

CXCR3 SNP rs2280964 associated with SPTB

The frequency distribution of CXCR3 SNP rs2280964 differed between SPTB infants from families with recurrent preterm births and term infants from Population I originating from northern Finland (Tables I, II). Minor allele A was underrepresented in SPTB infants (MAF 0.148 versus 0.248, respectively, p = 0.009, OR 0.52, 95% confidence interval [95% CI] 0.32–0.86), indicating a protective effect. Alternatively, the major allele G may have a predisposing effect. A similar trend was detected between mothers with preterm deliveries and those with term deliveries; this did not reach statistical significance (MAF 0.203 versus 0.255, respectively, p = 0.066, OR 0.74, 95% CI 0.54–1.02). Frequencies were similar in mothers who had had recurrent preterm deliveries compared with those who had experienced a single preterm delivery. There was no difference between term infants and infants from families with a single preterm delivery (Table II). Fathers of SPTB infants had a MAF of 0.260, which is close to MAF of the control mothers and infants (0.255 and 0.248, respectively).

To determine whether SNP rs2280964 is also associated with SPTB in the genetically more diverse southern Finnish population, we analyzed SPTB and control infants originating from this region (Population II). Although there was no significant difference in allele distribution between SPTB and term infants, the minor allele was underrepresented in SPTB infants in a manner similar to that in the discovery population (MAF 0.181 versus 0.227, respectively, p = 0.28, OR 0.75, 95% CI 0.45–1.26; Table II). With infants of Populations I and II combined, a similar trend between all SPTB infants and term infants was evident (MAF 0.192 versus 0.238, respectively, p = 0.062, OR 0.76, 95% CI 0.57–1.02). The difference was significant between SPTB infants from families with recurrent preterm deliveries and term infants when the populations were combined (MAF 0.156 versus 0.238, respectively, p = 0.016, OR 0.59, 95% CI 0.39–0.91), further strengthening the evidence for association of this SNP with SPTB.

Next, we determined the MAF of rs2280964 in a large Finnish population control cohort (n = 732; population III) originating from southern and eastern Finland (52) and included these controls in analyses. The MAF in this cohort was 0.234, that is, close to that of our controls. With these controls included, the association was significant in all SPTB infants (MAF 0.192 versus 0.235 in a total of 389 cases and 1090 controls, respectively, OR 0.77, 95% CI 0.61–0.98, p = 0.032) and in infants from families with recurrent SPTB (MAF 0.156, OR 0.60, 95% CI 0.41–0.89, p = 0.011).

Because CXCR3 is located on the X chromosome, we tested separately for association in female and male infants in Populations I and II. The allele frequency difference between SPTB and term infants was detected in both female and male infants. We further analyzed the infants for SNP rs2280964 using logistic regression, with fetal gender as a covariate. Under this model, SNP rs2280964 was a significant factor in predicting susceptibility to SPTB in infants from families with recurrent preterm deliveries, whereas fetal gender did not have predictive value. Analysis of female infants under the dominant model revealed an association with infants of Populations I and II combined (p = 0.017); analysis under the genotypic, dominant, and recessive models did not reveal associations for any other group of female infants. Finally, the frequency distribution of SNP rs2280964 did not differ between preterm infants born PPROM compared with those born after deliveries without PPROM, or between very (GA <32 wk) and moderate-to-late preterm infants (GA 32–36 wk).

A TDT in SPTB families of Population I revealed undertransmission of the minor allele A to SPTB infants in families with recurrent preterm deliveries (p = 0.007, Table III). In accordance with the results of case-control analysis, undertransmission was not detected in families with a single preterm delivery. In the joint analysis of all SPTB families, this test was nearly significant.
UTR was detected in a single SPTB in a premature stop codon in CXCR3-B. In addition, a 1-bp insertion within the mother–infant pairs (Supplemental Table II). This is consistent with 1000 Genomes sequence data (http://www.1000genomes.org), which indicated that no variations within the gene are correlated with this SNP in the Finnish population.

In addition to SNP rs2280964, three rare polymorphic sites were detected within the mother–infant pairs (Supplemental Table II). Two of these have been previously reported (http://www.1000genomes.org): rs56313919 and rs188959001, which results in the 3′ UTR in 15 mothers with preterm deliveries and their SPTB infants and in 10 mothers with term deliveries and their infants from Population I. No variants correlated with the SNP analyzed in the case-control association study (rs2280964) were detected. This is consistent with 1000 Genomes sequence data (http://www.1000genomes.org), which indicated that no variations within the gene are correlated with this SNP in the Finnish population.

Because no further common variants were revealed, we propose that the detected association between CXCR3 SNP rs2280964 and fetal SPTB is likely a direct effect of this SNP. Indeed, based on histone modification marks and ChIP sequence data from the Encyclopedia of DNA Elements project, this SNP maps to a putative enhancer region in several cell lines, and the intrinsic region encompassing the SNP interacts with two transcription factors: NFKB1 and PML. Furthermore, the SNP is predicted (p < 0.05) to affect the binding affinity of transcription factor Sp1.

### Table II. Case-control association analysis of CXCR3 SNP rs2280964 in SPTB

<table>
<thead>
<tr>
<th>Participants</th>
<th>Case/control n</th>
<th>All SPTB Cases vs. Controls</th>
<th>SPTB Cases from Families with a Single Preterm Delivery vs. Controls</th>
<th>SPTB Cases from Families with Recurrent Preterm Deliveries vs. Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern Finnish mothers (I)</td>
<td>251/192</td>
<td>251/192</td>
<td>251/192</td>
<td>251/192</td>
</tr>
<tr>
<td>Case/control minor allele frequency (OR (95% CI))</td>
<td>0.203/0.255</td>
<td>0.208/0.255</td>
<td>0.191/0.255</td>
<td>0.69 (0.42–1.12)</td>
</tr>
<tr>
<td>p</td>
<td>0.066</td>
<td>0.76 (0.54–1.08)</td>
<td>0.69 (0.42–1.12)</td>
<td>0.13</td>
</tr>
<tr>
<td>Northern Finnish infants (I)</td>
<td>291/188</td>
<td>291/188</td>
<td>291/188</td>
<td>291/188</td>
</tr>
<tr>
<td>Case/control n</td>
<td>0.195/0.248</td>
<td>0.232/0.248</td>
<td>0.148/0.248</td>
<td>0.52 (0.32–0.86)</td>
</tr>
<tr>
<td>Case/control minor allele frequency (OR (95% CI))</td>
<td>0.73 (0.51–1.06)</td>
<td>0.92 (0.61–1.38)</td>
<td>0.52 (0.32–0.86)</td>
<td>0.52 (0.32–0.86)</td>
</tr>
<tr>
<td>p</td>
<td>0.098</td>
<td>0.098</td>
<td>0.099</td>
<td>0.099</td>
</tr>
<tr>
<td>Southern Finnish infants (II)</td>
<td>98/170</td>
<td>98/170</td>
<td>98/170</td>
<td>98/170</td>
</tr>
<tr>
<td>Case/control n</td>
<td>0.181/0.227</td>
<td>0.17/0.227</td>
<td>0.17/0.227</td>
<td>0.27/0.227</td>
</tr>
<tr>
<td>Case/control minor allele frequency (OR (95% CI))</td>
<td>0.75 (0.45–1.26)</td>
<td>0.71 (0.41–1.24)</td>
<td>1.00 (0.36–2.84)</td>
<td>1.00 (0.36–2.84)</td>
</tr>
<tr>
<td>p</td>
<td>0.28</td>
<td>0.22</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Infants of Populations I and II combined</td>
<td>389/358</td>
<td>247/358</td>
<td>247/358</td>
<td>247/358</td>
</tr>
<tr>
<td>Case/control n</td>
<td>0.192/0.238</td>
<td>0.212/0.238</td>
<td>0.156/0.238</td>
<td>0.156/0.238</td>
</tr>
<tr>
<td>Case/control minor allele frequency (OR (95% CI))</td>
<td>0.76 (0.57–1.02)</td>
<td>0.86 (0.62–1.19)</td>
<td>0.59 (0.39–0.91)</td>
<td>0.59 (0.39–0.91)</td>
</tr>
<tr>
<td>p</td>
<td>0.062</td>
<td>0.36</td>
<td>0.016</td>
<td>0.016</td>
</tr>
</tbody>
</table>
CXCL9 and CXCL11, in our previously collected cohort of very preterm singleton infants \((n = 99)\) \((57)\). Umbilical cord blood CXCL9 concentrations were higher in SPTB infants than in infants born after elective delivery \(\text{mean FU: 1868 versus 998, median FU: 1101 versus 876, respectively; } p = 0.006\). High CXCL9 concentration \(\text{cutoff value 1231 FU}\) predicted the risk of SPTB with a sensitivity of 47.6% and specificity of 21.2% \(\text{receiver operating characteristic curve shown in Supplemental Fig. 1}\). The SPTB-predisposing \(GG\) genotype of \(\text{CXCR3 SNP rs2280964}\) was associated with higher CXCL9 concentrations \(\text{mean FU for infants with } GG\text{ and } AA\text{ genotypes: 1886 versus 1163, median FU: 1270 versus 761, respectively; } p = 0.021\). Umbilical cord blood CXCL11 concentrations did not differ significantly between SPTB infants and infants born after elective delivery \(\text{mean FU: 886 versus 661, median FU: 554 versus 389, respectively, } p = 0.067\). Moreover, SNP rs2280964 was not associated with CXCL11 concentration. These data indicate that elevated levels of the CXCR3 ligand CXCL9 are associated with SPTB.

We further analyzed a panel of cytokines, including chemokines and cytokines from the hematopoietin, IL-1, and TNF families \(\text{69 cytokines listed in Supplemental Table I}\). Several of the cytokines from the very preterm infants showed increased cord blood levels in SPTB. The cytokines associated most significantly with SPTB are shown in Table IV. Levels of these cytokines were not significantly associated with \(\text{CXCR3 SNP rs2280964}\). IL-6 and CCL2 showed the clearest association with SPTB and were thus analyzed in the subsequent mouse experiment.

**CXCR3 deficiency influenced SPTB-associated cytokines in mice**

We hypothesized that CXCR3 may influence the trafficking of labor-inducing cytokines across the maternal–fetal barrier, and that this may be a conserved event in the highly species-specific labor process. To investigate our hypothesis, we exposed the pregnant mice to LPS and compared the specific responses between WT and CXCR3-deficient strains. CXCR3 deficiency had no detectable influence on the duration of pregnancy or on the survival of prematurely born pups. A preterm birth-inducing dose of maternal LPS was injected at 17 dpc, and the acute influence of LPS on SPTB-associating cytokines in amniotic fluid was studied. IL-6 and CCL2 were included due to their clear association with SPTB in human umbilical cord blood. In WT mice, concentrations of IL-6 and CCL2 in the amniotic fluid were slightly increased 3 h after LPS injection. By contrast, LPS administered to CXCR3-deficient mice decreased the levels of IL-6 and CCL2 \(p = 0.001, \text{Table V}\).

**Discussion**

The causes of SPTB are mostly unknown. Both genetic and environmental factors are involved, but the actual predisposing genes and underlying biochemical pathways are poorly understood \((10)\). In this study, we provide evidence of a role for CXCR3 in preterm labor and premature birth.

We found an association between a fetal CXCR3 polymorphism \(\text{SNP rs2280964}\) and SPTB, with a protective role for the minor allele \(A\), in a Finnish population known to be relatively genetically homogeneous \((51)\). Further sequencing did not reveal any

**Table III. TDT of CXCR3 SNP rs2280964 in families with SPTB**

<table>
<thead>
<tr>
<th>Study Group*</th>
<th>Transmitted/Untransmitted Minor Alleles ((n))</th>
<th>TDT OR ((95% \text{ CI}))</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All families with preterm deliveries</td>
<td>22/36</td>
<td>0.61 ((0.36–1.04))</td>
<td>0.066</td>
</tr>
<tr>
<td>Families with a single preterm delivery</td>
<td>17/18</td>
<td>0.94 ((0.49–1.83))</td>
<td>0.87</td>
</tr>
<tr>
<td>Families with recurrent preterm deliveries</td>
<td>5/18</td>
<td>0.28 ((0.10–0.75))</td>
<td>0.007</td>
</tr>
</tbody>
</table>

\*TDT was performed in families of Population I originating from northern Finland.
polymorphisms that displayed linkage disequilibrium with rs2280964. Because there were no linked variations for rs2280964 either in our sequence data or in that of the 1000 Genomes Finnish population, we conclude that the detected association is most likely a direct effect of this SNP that was previously shown to be a functional polymorphism (expression SNP), associated with expression levels of CXCR3 in human T cells and changes in immune cell responses to chemokine–cytokine signals (63). Therefore, we assessed whether this SNP was associated with expression levels of CXCR3 in the placenta and fetal membranes. Our results demonstrate that on the basal plate of the placenta and in the reflected fetal membranes, minor allele A was associated with decreased CXCR3 mRNA expression. This decreased mRNA expression is consistent with the results of experiments performed in human T cells by Choi et al. (63). We also detected higher expression levels in SPTB placentas compared with placentas from elective preterm or spontaneous term deliveries. It is likely that the associated SNP is located within a regulatory region and the G-to-A change modulates binding of regulatory proteins, which leads to alterations in transcription initiation. Indeed, Choi et al. (63) were able to demonstrate this phenomenon in cultured T cells. Therefore, we suggest that CXCR3 rs2280964 may contribute to SPTB through transcriptional effects in the placenta.

In the placenta, only expression of CXCR3-A mRNA has been previously reported (35). To our knowledge, this is the first study to report expression of all three CXCR3 splice variants in this tissue. Our protein analyses suggest that the ratio of the two main splice variants, CXCR3-A/CXCR3-B, is higher in SPTB compared with spontaneous term delivery. These preliminary data suggest that increased expression of CXCR3-A, in particular, is associated with SPTB, whereas CXCR3-B is expressed at a relatively higher level in the placenta during normal term parturition. This is an interesting finding, because CXCR3-A has been shown to promote chemotaxis and growth, whereas CXCR3-B mediates growth-inhibitory signals (35, 64).

In several abnormal conditions, CXCR3 levels increase. For instance, in renal carcinoma (65) and ovarian cancer (66), the expression level of CXCR3 is correlated with the disease pathogenesis. Additionally, expression of CXCR3 in CD4+ T cells was reported to be increased in the peripheral blood of women with multiple miscarriages (67). Our finding of higher expression of CXCR3 in SPTB placentas suggests a role for this molecule in the

| FIGURE 1. Association of SNP rs2280964 with CXCR3 mRNA levels in placenta and fetal membranes. Relative levels of CXCR3-A, CXCR3-B, and CXCR3-alt mRNA were determined and normalized to expression levels of the HK gene CYC1 using qPCR. Fetal G/GG and A/AA genotypes were compared from the following compartments: basal plate of the placenta (A–C), chorionic plate of the placenta (D–F), and fetal membranes (G–I). G and A refer to genotypes of male fetuses (hemizygotes), and GG and AA to genotypes of female fetuses (homozygotes). Differences between study groups were analyzed by nonparametric Mann–Whitney U test. Median of each group is shown as a horizontal line. Significant change (p < 0.05) indicated by an asterisk. NS, no significant change. |
preterm labor process. This proposed role of CXCR3 in labor is further supported by a study by Gomez-Lopez et al. (39) in which CXCR3 expression in choriodecidual leukocytes was shown to be increased in spontaneous term labor. The placental cells that potentially contribute to the success or failure of a pregnancy may be located in the decidua (maternal side of the placenta), in which fetal and maternal cells are in direct contact (68). We determined that CXCR3 was localized in both decidual and villous cytotrophoblasts, as well as in syncytiotrophoblasts at the fetomaternal interface. Trophoblasts are involved in several key functions of the placenta, including hormone synthesis, molecular transport, metabolic regulation, and immune defense (69). All trophoblasts are of fetal origin, which is intriguing considering that the association of SNP rs2280964 with SPTB was detected in the fetus. Moreover, the chorionic trophoblasts showed significant immunohistochemical staining. This is in concordance with the labor-associated upregulation of several chemokines, including CXCL10, in choriodecidual membranes (39, 44). According to the current proposal, the choriodecidua transmits signals required for the activation of labor-producing uterine contractions. Likewise, fetal membranes overlying the internal orifice of the cervix could potentially transmit signals for cervical ripening (70).

In the decidua, NK cells produce CXCL10, which promotes the migration and invasion of fetal trophoblasts during formation of the placenta (71). The decidua also contains macrophages and T cells. Nancy et al. (72), however, showed that the decidua did not accumulate T cells, and that the T cells could not be recruited from blood in early pregnancy in mice. This was due to low expression of the chemokines CXCL9 and CXCL10, which results from the repressive methylation histone mark that appears upon decidualization.

**FIGURE 2.** Increased CXCR3 mRNA levels in SPTB placentas. The following phenotype groups were compared: SPTB versus elective preterm birth (A–C) and SPTB versus spontaneous term birth (D–F). All samples were from the basal plate of the placenta. Only placentas with the fetal G/GG genotype were included; G and GG refer to genotypes of male (hemizygotes) and female (heterozygotes) fetuses, respectively. As in Fig. 1, relative levels of CXCR3-A, CXCR3-B, and CXCR3-alt mRNA were determined and normalized to expression levels of the HK gene CYC1. Differences were analyzed with nonparametric Mann–Whitney U test. Horizontal line denotes the median of each group. Significant change ($p < 0.05$) indicated by an asterisk. NS, no significant change.
During late human pregnancy, on the contrary, the maternal circulating T cells have been shown to infiltrate into decidua, as reviewed by Gomez-Lopez et al. (73). Another study suggested that CXCR3 ligands play important roles in transplant rejection; Hancock et al. (74) showed that acute cardiac transplant rejection was associated with much higher expression in mice not only of CXCR3, but also of its ligands. This was further supported by the observation that Cxcr3 knockout mice show delayed rejection. Hancock et al. (74) also suggested that this phenomenon was due to infiltration of activated T cells into the site of rejection. Furthermore, in cases of villitis of unknown etiology, the placental inflammatory lesion affecting the villous tissues (rather than decidual tissue in SPTB) has been proposed to be analogous to graft rejection. Indeed, concentrations of CXCR3 ligands in the maternal circulation corresponded with the presence of CXCR3 ligands in the fetal mesenchymal cells of the placenta. This finding was consistent with previous reports that CXCR3 ligands play a role in the regulation of placental development. Additionally, a study by Hancock et al. (74) showed that CXCR3 ligands were expressed in the placenta of women with preeclampsia, a condition associated with high levels of CXCR3 ligands. This suggests a potential role for CXCR3 ligands in the pathogenesis of preeclampsia. Furthermore, a study by Belton et al. (75) showed that CXCR3 ligands were expressed in the placenta of women with preterm labor, indicating a potential role for CXCR3 ligands in the pathogenesis of preterm labor. Overall, the role of CXCR3 ligands in the placenta is an active area of research, with potential implications for the understanding of placental development and the regulation of placental function.

FIGURE 3. Immunoblot analyses of CXCR3 expression levels on the basal plate of the placenta. (A) Two phenotype groups were analyzed: SPTB (samples 1–4) and spontaneous term birth (samples 5–8). All analyzed cases were of the fetal CXCR3 SNP rs2280964 G/GG genotype; G and GG refer to genotypes of male (hemizygotes) and female (heterozygotes) fetuses, respectively. The two forms of CXCR3 receptor, CXCR3-A (41.4 kDa) and CXCR3-B (45.5 kDa), and the loading control (β-actin) are indicated by arrows. For more specific experimental details, see Materials and Methods. (B) Protein expression level ratios of CXCR3-A and CXCR3-B. Intensities of CXCR3-A and CXCR3-B in (A) were determined. Subsequently, the CXCR3-A/CXCR3-B (A/B) ratio was calculated for each of the eight samples in (A). SDs are shown. In term placentas, the A/B ratio was <1, indicating that CXCR3-B was the predominant splice variant. The A/B ratio of preterm placentas was >1. The difference in the A/B ratio between preterm and term placentas was significant (*p = 0.021).

FIGURE 4. Expression of CXCR3 in preterm placentas from infants with SNP rs2280964 A/AA and G/GG genotypes. A simplified illustration of the placenta is shown with sampling sites indicated by black squares. Representative placental tissues were immunostained with CXCR3 Ab. Black arrows show the immunostaining in the cytotrophoblastic cells, and black arrowheads in endothelial cells. Syncytiotrophoblasts are shown in big open arrows, and small open arrows show decidual trophoblasts. Original magnification ×20 for all photomicrographs. (A and B) Samples from SPTB placentas without histological chorioamnionitis. (C and D) Samples from SPTB placentas with histological chorioamnionitis. (E–H) Samples from elective preterm deliveries without histological chorioamnionitis and with cesarean section due to maternal symptoms. G and A refer to genotypes of male fetuses (hemizygotes), and GG and AA to genotypes of female fetuses (homozygotes). Samples from villous (A–F) and decidual (G and H) sections of the placenta. Scale bar, 100 μm.
Table V. Amniotic fluid cytokine levels of WT and CXCR3-deficient fetuses after maternal LPS or PBS injections

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>WT Mice</th>
<th>CXCR3-Deficient Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPS</td>
<td>PBS</td>
</tr>
<tr>
<td>CCL2 (pg/ml)</td>
<td>4611 ± 1531(^a)</td>
<td>3114 ± 3774(^a)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>232 ± 120(^c)</td>
<td>197 ± 371(^c)</td>
</tr>
</tbody>
</table>

\(^{a}\)Levels of CCL2 and IL-6 in amniotic fluid of WT and CXCR3-deficient fetuses after maternal LPS (20 μg/mouse) or PBS injections at 17 dpc are shown.

\(^{b}\)Mean ± SD.
We propose that increased CXCR3 signaling by extraembryonic cells of fetal origin disturbs the normal feto–maternal tolerance, which may promote the onset of preterm labor and delivery.

Acknowledgments

We thank Maarit Haarala for technical assistance; Outi Kajula, Hilkka Puttonen, and Ritta Viikivenmäki for sample collection; Ann Pasanen for help with the reference population data; and Kira O’Day Heller for language editing.

Disclosures

The authors have no financial conflicts of interest.

References


genes in human gestational membranes delivered at term and preterm. Mol. Hum. Reprod. 8: 399–408.
Supplemental Data for the article “CXCR3 Polymorphism and Expression Associate with Spontaneous Preterm Birth”

**Supplemental Table I. Cytokines analyzed from umbilical cord blood of very preterm infants.**

<table>
<thead>
<tr>
<th>Cytokine group</th>
<th>Cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemokines</strong></td>
<td>C-C motif chemokines 1-5, 7, 8, 11, 13-20, 23, 24, 26-28 (CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL23, CCL24, CCL26, CCL27, CCL28) C-X-C motif chemokines 2, 3, 5, 6, 8, 9, 11-13 (CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL9, CXCL11, CXCL12, CXCL13) Fractalcine (CX3CL1) Lymphotactin (XCL1)</td>
</tr>
<tr>
<td><strong>Hematopoietin family</strong></td>
<td>Cytokine receptor common subunit gamma (IL2RG) Fms-related tyrosine kinase 3 ligand (FLT3LG) Granulocyte-macrophage colony-stimulating factor (GM-CSF) Interleukins 2-7, 9, 13, 15 (IL2, IL3, IL4, IL5, IL6, IL7, IL9, IL13, IL15) Interleukin-2 receptor subunit alpha, beta (IL2RA, IL2RB) Interleukin-5 receptor subunit alpha (IL5RA) Interleukin-6 receptor subunit beta (IL6ST; i.e., gp130) Leptin Oncostatin-M (OSM) Stem cell factor (SCF) Stem cell factor receptor (SCFR)</td>
</tr>
<tr>
<td><strong>IL1 family</strong></td>
<td>Interleukin-1 alpha, beta (IL1α, IL1β) Interleukin-1 receptor antagonist (IL1ra) Interleukin 1 receptor-like 1 (IL1RL1) Soluble interleukin-1 receptor 2 (IL1sR2)</td>
</tr>
<tr>
<td><strong>TNF family</strong></td>
<td>Lymphotoxin-alpha (LTA; i.e., TNFB) Tumor necrosis factor (TNF) Tumor necrosis factor ligand superfamily member 6 (FASLG) Tumor necrosis factor receptor superfamily members 1A, 6-8, 10A, 10D, 11A, 14, 21 (TNFRSF1A, TNFRSF6, TNFRSF7, TNFRSF8, TNFRSF10A, TNFRSF10D, TNFRSF11A, TNFRSF14, TNFRSF21)</td>
</tr>
</tbody>
</table>
### Supplemental Table II. Additional CXCR3 genetic variants detected by sequencing.

<table>
<thead>
<tr>
<th>Detected polymorphic site</th>
<th>Location of variant&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Predicted consequence</th>
<th>MAF in 1000 Genomes Finns or ExAC data</th>
<th>Family members in whom variant allele was detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP rs56313919</td>
<td>X:71,619,379; ~860 bp upstream of exon 1</td>
<td>Unknown</td>
<td>0.007&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1 SPTB mother (heterozygote) and her 2 male SPTB infants; 1 male infant born at term and his mother</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 female infant (heterozygote) born at term</td>
</tr>
<tr>
<td>SNP rs188959001</td>
<td>X:71,617,540; exon 2 (amino acid–encoding position in CXCR3-B; position not within protein-coding region for CXCR3-A)</td>
<td>Gln25X (results in premature stop codon in CXCR3-B)</td>
<td>0.006&lt;sup&gt;C&lt;/sup&gt;</td>
<td>1 SPTB mother (who was herself born spontaneously preterm&lt;sup&gt;D&lt;/sup&gt;; heterozygote)</td>
</tr>
<tr>
<td>1-bp insertion (G)</td>
<td>X:71,616,221; 3′ UTR of exon 2</td>
<td>Unknown</td>
<td></td>
<td>1 SPTB mother (who was herself born spontaneously preterm&lt;sup&gt;D&lt;/sup&gt;; heterozygote) and her male SPTB infant</td>
</tr>
</tbody>
</table>

<sup>A</sup>Chromosomal positions refer to human genome build 38.

<sup>B</sup>Minor allele frequency for the Finnish population (n=93) in 1000 Genomes sequence data (www.1000genomes.org).

<sup>C</sup>Minor allele frequency for the Finnish population (n=1,595) in Exome Aggregation Consortium (ExAC) data (http://exac.broadinstitute.org) [02, 2015].

<sup>D</sup>The same mother carried minor allele of SNP rs18895001 and the 1-bp insertion in the 3′ UTR.
**Supplemental Table III. Results of CXCR3 qPCR analyses in tissue samples.**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>No of placentas(^B)</th>
<th>Fold changes in relative mRNA expression levels(^A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal plate of placenta</td>
<td>Chorionic plate of placenta</td>
</tr>
<tr>
<td></td>
<td>CXCR3-(^A)</td>
<td>CXCR3-(^B)</td>
</tr>
<tr>
<td>rs2280964 GG vs. AA genotype (all placentas)</td>
<td>31 / 6</td>
<td></td>
</tr>
<tr>
<td>Preterm infants: rs2280964 GG vs. AA genotype</td>
<td>13 / 3</td>
<td>7.3(^↑) (0.051)</td>
</tr>
<tr>
<td>Term infants: rs2280964 GG vs. AA genotype</td>
<td>18 / 3</td>
<td>1.5(^↑) (NS)</td>
</tr>
<tr>
<td>SPTB vs. elective preterm birth (within GG placentas)</td>
<td>5 / 8</td>
<td>2.6(^↑) (0.040)</td>
</tr>
<tr>
<td>SPTB vs. spontaneous term birth (within GG placentas)</td>
<td>5 / 9</td>
<td>1.4(^↑) (0.062)</td>
</tr>
</tbody>
</table>

\(^A\)Fold changes in the ratio of medians of the corresponding groups are shown. Directions in expression-level differences are indicated by arrows (\(^↑\) for higher and \(^↓\) for lower expression in the first group of each comparison); for example, median of GG placentas/median of AA placentas = 4.8-fold higher mRNA levels in GG placentas. \(^B\)Numbers in each comparison may differ slightly due to unavailable tissue samples from all parts of the placenta from the same delivery. \(^C\)Fold changes appear high due to very low expression levels in AA placentas.
Supplemental Figure 1. Receiver operating characteristic (ROC) curve of CXCL9 concentrations in umbilical cord blood of very preterm infants (GA<32 weeks). CXCL9 concentrations were higher in SPTB infants than in infants born after elective delivery (mean fluorescence units [FU]: 1,868 vs. 998, median FU: 1,101 vs. 876, respectively). High CXCL9 concentration (>1,231 FU) predicted the risk of SPTB with a sensitivity of 47.6% and specificity of 21.2% (area under ROC curve 0.67, p=0.006).