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Research paper

GT-repeat extension in the *IL11* promoter is associated with Hirschsprung's disease (HSCR)

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ABSTRACT

Hirschsprung's disease (HSCR) is a congenital disease characterized by intestinal obstruction due to a defective intestinal neural system. Frequently, the disease is associated with an intestinal inflammation. The most common known underlying genetic alterations are in the *RET* gene but HSCR can also be caused by mutations in other genes that are responsible for the maturation and migration of intestinal neural cells. Recently, a study in an Asian population reported a significant association of several single nucleotide polymorphisms (SNPs) in the *IL11* (interleukin 11) gene with HSCR. We further explored the possible association of genetic alterations in the *IL11* gene with HSCR and HSCR subtypes in an unrelated Caucasian population. We used a targeted sequencing approach to identify a total of 32 SNPs covering the coding region of the *IL11* gene including the proximal part of the promoter and relevant SNPs described in the previous study. Genotype frequencies were compared using additive genetic models in 103 HSCR patients and 128 healthy controls. We failed to observe any significant association of SNPs with HSCR. However, there was a suggestive evidence for an association of the length of a dinucleotide repeat in the *IL11* promoter region with HSCR with an over-representation of > 7 GT repeat subtypes (OR = 4.982 (1.448–17.040), p-value = 0.0111) in HSCR patients. A similar trend was further observed in a subgroup of patients with long-segment HSCR (L-HSCR). These findings need to be replicated in a well-powered study. Changes in *IL11* expression may be a link to the intestinal inflammation frequently observed in patients with HSCR.

1. Introduction

Hirschsprung's disease (HSCR) is a congenital disease that is characterized by intestinal obstruction and inflammation. The obstruction is caused by a lack of ganglion cells of the enteric nervous system (ENS) in the distal part of the intestine. This is caused by a defective migration and maturation of intestinal neuronal precursor cells during embryonal development. Depending on the severity of the migration defect, the length of the aganglionic segment can vary considerably, from S-HSCR (short-segment HSCR), not extending beyond the upper sigmoid and L-HSCR, affecting longer tracts of the colon up to a total colonic aganglionosis (TCA) or even total intestinal aganglionosis (TIA) (Kim et al., 2014). In the aganglionic segment, the coordinated motility of intestine is disturbed. This results in distension of the part of the intestine that is located proximal to the aganglionic segment. Therefore, clinically, the disease also called congenital megacolon. It has an incidence of 1:5000 life births with an over-representation of male patients (Pasini et al., 1996).

Abbreviations: AGT, angiotensinogen; ALDH2, dehydrogenase 2; APC, antigen-presenting cell; CD11c, cluster of differentiation 11c; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CLL, chronic lymphocytic leukemia; DENND3, DENN domain containing protein 3; dbSNP id, SNP database identifier; df, degrees of freedom; DR, dinucleotide repeat; EGF, epidermal growth factor; GDNF, glial derived neurotrophic factor; GOR, genotype odds ratio; gp130, glycoprotein with 130 kDa; GWAS, genome-wide association study; HO1, heme oxygenase 1; HSCR, Hirschsprung's disease; HWR, Hardy-Weinberg equilibrium; IL6ST, interleukin 6 signal transducer; IL11, interleukin 11; IL11alphaR, IL11alpha receptor; JAK, Janus kinase; L-HSCR, long segment HSCR; MAF, minor allele frequency; MAPK, Mitogen-activated protein kinase; m, mosaicism; NA, not available/applicable; NCLN, Nicalin; NRG1, neuregulin 1; NUP98, nucleoporin 98; RET, rearranged during transfection, a membrane-bound tyrosine kinase; REH, relative excess heterozygosity; rs ID, reference SNP ID; SCD, sickle cell disease; SEMA3, semaphorin 3; S-HSCR, short segment HSCR; SNP, single nucleotide polymorphism; SOX-10, Sry-related HMG-box protein 10; STAT, Signal transducer and activator of transcription; TBATA, thymus, brain and testis associated; TIA, total intestinal aganglionosis; Ws4, Waardenburg-Shah syndrome Type 4; Ws, Waardenburg syndrome

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Genetics is known to play a role in predisposition to HSCR. Genetic abnormalities in HSCR are mostly found in genes that are known to be necessary for the development and function of neural crest cells. The most common known genetic causes of HSCR are located in the *RET* gene, accounting for about 50% of familial and 15–20% of sporadic cases of HSCR (Amiel et al., 2008). Several disease-relevant variants have not only been reported from exonic (Fitze et al., 1999) but also from intronic (Fitze et al., 2003a) and the promoter/enhancer region (Emison et al., 2005; Fitze et al., 2003b; Emison et al., 2010) of the *RET* gene. However, in rare cases, other members of the *RET* signaling pathway, such as GDNF (glial derived neurotrophic factor) are also known to be involved (Amiel et al., 2008). SOX10 (Sry-related HMG-box protein 10) is a transcription factor that is necessary for the differentiation of neural crest cells and mediates *RET* transcription (Leon et al., 2009). Mutations in the *Sox10* gene have been found in patients with a combination of HSCR and Waardenburg syndrome (Ws), called Ws4 (Ws type 4) or Waardenburg-Shah syndrome (Pingault et al., 1998; Touraine et al., 2000). These findings were confirmed in mouse models (Herbarth et al., 1998; Southard-Smith et al., 1999).

Apart from the *RET* pathway, also members of the endothelin pathway, like endothelin receptor type B (*EDNRB*) have been identified in HSCR (Puffenberger et al., 1994; Kusafuka et al., 1996; Auricchio et al., 1996; Amiel et al., 1996). In mouse models, it has been shown that the endothelin pathway is necessary for proper development of the ENS (Druckenbrod et al., 2008; Baynash et al., 1994), especially for the migration of ENS precursor cells (Lee et al., 2003). Apart from the *RET* and endothelin pathways, genome-wide association studies (GWAS) identified neuregulin 1 (*NRG1*) as a HSCR susceptibility gene (Garcia-Barcelo et al., 2009; Tang et al., 2011, 2012). NRG1 is a member of the EGF (epidermal growth factor) family and plays a role in neuronal development and neuronal function (Falls, 2003). In a large whole exome sequencing study together with functional Zebrafish studies, several new HSCR predisposing genes were detected. The most prominent signals were from *DENND3* (DENN domain containing protein 3), *NCLN* (Nicalin), *NUP98* (nucleoporin 98) and *TBATA* (thymus, brain and testis associated), with functions in intracellular vesicular traffic, Nodal signaling, nuclear transport and neuronal differentiation respectively (Gui et al., 2017). A large trans-ethnic meta-analysis of GWAS of 507 HSCR cases and 1191 controls confirmed the role of *RET* and *NRG1* in European and Asian ancestries but found that the association of HSCR with *SEMA3* (semaphorin 3) was European-specific (Tang et al., 2016).

Recently, it was described in an Asian population of 187 HSCR patients and 283 unaffected controls that *IL11* SNPs were associated with HSCR (Kim et al., 2015). IL11 is a multifunctional cytokine of the IL6 group that has 5 exons and 4 introns and a maximum transcript length of 2281 bp. It was discovered as a factor that stimulates plasmacytoma proliferation and the development of immunoglobulin-producing B-cells (Paul et al., 1990). It also synergizes with IL3 in supporting megakaryocyte colony formation (Paul et al., 1990) and inhibits adipogenesis (Kawashima et al., 1991). IL11 signals by binding to the IL11alpha receptor (IL11alphaR) and to IL6ST (IL6 signal transducer, also called gp130, glycoprotein with 130 kDa). IL11alphaR is a non-signaling receptor. The IL11 signal is transmitted via IL11ST to the JAK (Janus kinase) (Heinrich et al., 2003). From there, the signal is transmitted to the STAT (Signal transducer and activator of transcription) and/or MAPK (Mitogen-activated protein kinase) pathways (Heinrich et al., 2003). IL11 is necessary for osteoclast differentiation and thereby for normal bone remodeling (Sims et al., 2005).

Since it is involved in inflammatory processes, it may be a link to the intestinal inflammation frequently occurring in patients with HSCR. As a possible further link to HSCR, IL11 is also a neuroprotective cytokine. In a mouse model for autoimmune demyelinating disease it has been shown that it protects from demyelination partly by inducing proliferation of oligodendrocyte progenitors by inhibiting CD11c+ APC (antigen-presenting cell)-mediated lymphocyte activation (Gurfein et al., 2009; Zhang et al., 2006). It has also been shown that IL11 has a direct neurotrophic effect (Thier et al., 1999).

In this study, we examine the role of *IL11* genetic variants in a German population of 103 HSCR patients and 128 controls in a sequencing-based approach and failed to observe an association of *IL11* SNPs with HSCR. However, we observed an over-representation of > 7GT repeat-subtypes in the extension of a GT dinucleotide repeat (DR) in the *IL11* promoter/enhancer of HSCR patients.

2. Patient data/material

We performed a case-control study with patients from two hospitals (Dresden and Erlangen) and referral patients from other hospitals in Germany. Blood specimens from healthy blood donors served as controls. DNA from 103 patients with non-syndromal HSCR and 128 healthy controls was used for the study. For the patients with known length of the segment affected, a separate analysis of S-HSCR and L-HSCR was performed. Patients with an aganglionic segment confined to the rectosigmoid region were classified as S-HSCR (Kessmann, 2006). Aganglionic segments beyond this region were classified as L-HSCR. The study was approved by the local ethics committee of the University Hospital Carl Gustav Carus of the TU Dresden (EK470498). Informed consent was obtained from each patient or its parents.

3. Methods

DNA was extracted from whole blood by hypotonic lysis, proteinase K digestion and phenol-chloroform extraction (Sambrook and Russell, 2001). Primers were selected with the Oligo 6.8 software (MBI, Colorado Springs, Co) and are listed in Supplementary Table 1. The primers were selected to cover all exons as well as those SNPs that have been previously shown to be associated with HSCR (Kim et al., 2015). PCR products were sequenced by Sanger Sequencing on a 3130×/Genetic Analyzer (Applied Biosystems, Foster City, CA) or by Seqlab (Göttingen, Germany). Sequences were analyzed by the Sequencing Analysis (Thermo Fisher, Darmstadt, Germany) and Sequencher (Gene Code Corporation, Ann Arbor, MI) software. Only those SNPs that had overall minor allele frequency (MAF) > 0.05 were carried forward for any further analysis. Derivation from Hardy-Weinberg equilibrium (HWE) was explored in healthy controls using Fisher's exact test (Supplementary Table 2 as implemented in PLINK (https://www.cog-genomics.org/plink2)). In addition, compatibility with HWE was checked using a relative excess heterozygosity (REH) approach (Ziegler et al., 2011) in R, version 3.3.0 (2016-05-03) (Supplementary Table 2). Genotype frequencies were computed in cases and controls and compared using additive genetic models using Cochrane Armitage's Trend test as implemented in StatXat (version 11.0; Cytel Software Corporation, Cambridge, MA). Genotype odds ratios (GOR) based on the additive model with corresponding 95% confidence intervals (CIs) are reported. For the DRs, allelic combinations were compared using χ^2 tests for independence and Fisher's exact tests (as per the relative distribution of individual alleles in each of the comparable groups). All the statistical tests were two-sided, and p values of < 0.05 were considered to be statistically significant unless stated explicitly. No correction for multiple testing was applied.

4. Results

We found 32 SNPs or mutations, five of which had not been yet annotated and had no reference SNP IDs (rsIDs according to the latest dbSNP database, dated 30th April 2016) (Table 1 and Supplementary Table 3). Five mutations were identified (SNPs 2, 12, 14, 26, 28), three of which were exonic (SNPs 12, 14, 26). Two of those were non synonymous resulting in an amino acid exchange (SNP12, c.70C > T, Exon 2 (p.Pro24Ser) and SNP14, c.251C > T, Exon 3 (p.Ala84Val)). Furthermore, a DR in the promoter was further identified. Only 12 SNPs