Original Article

A Study on Genetic Polymorphism of RET Proto-Oncogene in Hirschsprung's Disease in Children

Madhu Kumari, Chhanda Das[1], Madhumita Mukhopadhyay[2], Rishav Dev Patra[3], Pradip Kumar Mitra[4], Biswanath Mukhopadhyay[5]

Pgt 3rd Year, Department of Pathology, IPGME&R, [1]Assistant Professor, Department of Pathology IPGME&R, [2]Professor, Department of Pathology IPGME&R, [3]Associate Professor, Department of Paediatric Surgery, IPGME&R, [4]Professor, Pathology, Health and Family Welfare Department, [5]Professor, Department of Paediatric Surgery Apollo Gleneagles Hospital, Kolkata, West Bengal, India

Abstract

Background: Hirschsprung's disease (HD) is a genetic disorder with a complex pattern of inheritance. Some single-nucleotide polymorphisms (SNPs) are identified to be associated with the risk of Hirschsprung's Disease (HSCR). **Aims and Objectives:** The aim of this study is to know the association between the rearranged during transfection (RET) proto-oncogene polymorphism and HD and to characterize the SNPs of RET proto-oncogene affecting HD. **Materials and Methods:** The study was conducted in the Department of Pathology in association with the Department of Pediatric Surgery. Blood samples were collected from the patients diagnosed with confirmed HD and from age- and sex-matched controls. This case–control study consisted of 53 HSCR cases and 50 controls. Genotypes of rs1800860 and rs1800861 were analysed in by polymerase chain reaction amplification and sanger sequencing. Associations with the risk of HSCR were estimated by odds ratio (OR) and their 95% confidence intervals (95% CI) using. **Results:** We observed that in the case of rs1800860A > G genotype AG was not associated with the increasing risk of disease (OR with 95% CI = 0.568 [0.238–1.356]) while genotype GG was associated with increasing the risk of the disease (OR with 95% CI = 2.278 [0.967–5.366]). In the case of rs1800861G > T genotype GT was associated with lowering the risk of the disease (OR with 95% CI = 0.230 [0.0981–0.539]) while genotype TT was associated with increasing the risk of the disease (OR with 95% CI = 9.647 [3.830–24.302]). The difference in the genotypic distribution of GT and TT at rs1800861G > T between Short segment disease (SSD) cases and Long Segment Disease (LSD) and total colonic aganglionosis was made by Fisher's exact test and it was statistically significant (P = 0.0476 and 0.0054). **Conclusion:** The results of this study support the hypothesis that variations in RET pathway might play an important role in the development of HSCR.

Keywords: Aganglionosis, genetic polymorphism, polymerase chain reaction

Introduction

Hirschsprung's disease (HD) is a genetic disorder with a complex pattern of inheritance and is the major cause of pediatric intestinal obstruction.[1] It occurs due to the failure of migration of ganglion cells from neural crest leads to the development of aganglionic segments, which varies from ultra-short to total intestinal involvement.[2] The incidence of the disease shows significant racial variation occurring in 1 in 5000 live births, with the highest incidence in Asian populations (2.8 in 10,000).[3] HSCR patients can be classified according to the length of the aganglionic segment into the short segment (Short segment Hirschsprung's Disease [SHSCR], 80%), long segment (Long segment Hirschsprung's Disease [LHSCR],15%), and total colonic aganglionosis (TCA, 5%). The male: female ratio (M: F) is ≈ 4:1 among SHSCR patients and ≈ 1:1 among LHSCR patients. The rearranged during transfection (RET) gene, encoding a tyrosine kinase receptor, is the major gene responsible for the development of the enteric ganglia.[4] In this study, we conducted a case-control study to know the association between the RET proto-oncogene polymorphism and HD and to characterize the single nucleotide polymorphisms (SNPs) of RET proto-oncogene affecting HD.

Address for correspondence: Dr. Chhanda Das, 31 Eastern Park, First Road, Santoshpur, Kolkata 75, West Bengal, India.
E-mail: chhhdas@gmail.com

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Materials and Methods

The study was conducted in the Department of Pathology in association with the Department of Pediatric Surgery, I. P. G. M. E. and R, Kolkata. Blood samples were collected from the patients diagnosed with confirmed HD and from age- and sex-matched controls. This case–control study consisted of 53 HSCR cases and 50 controls. Cases were histologically and immunohistochemically diagnosed with either biopsy or surgical resection material for the absence of enteric plexuses. Controls were normal children taking the physical examination in the same hospital. DNA isolation was done by chemagic blood DNA isolation kit. The reference sequence of the RET gene was downloaded from NC0000 10 Chromosome 10 Reference GRch37 primary assembly. From which we came to know that the RET gene is a 53KB gene and its chromosomal location is 43077069–43130351.16 SNPs were found from many Genome-wide association study. From this 16 SNPs, 2 SNPs were chosen rs1800860, rs1800861 because they are synonymous and Utrvariant. They were found by SNPs database. The SNPS were annotated in the GENBANK file. DNA extraction, polymerase chain reaction and Sanger sequencing were done to know the relation between SNPs of RET Proto-oncogene and HD cases.

Results and Analysis

A total of 53 cases were diagnosed with HD. Of which 37 (69.81%) were male and 16 (30.19%) were female. Male-to-female ratio was 2.3:1. Most of the patients 29 (54.72%), were in the neonatal age group, followed by 18 (33.96%) in the age group above 1 year, followed by 6 (11.32%) infants. Twenty-seven (50.94%) patients (50.94%) were Muslim, 25 (47.17%) were Hindu and 1 (1.89%) was Christian. Forty-one (77.36%) cases were of Short segment disease (SSD), 7 (13.21%) cases were of LSD, and 5 (9.43%) cases were of TCA. Forty-three (81.13%) cases were sporadic, 8 (15.09%) cases were familial and 2 (3.78%) cases were syndromic. Eight (15.09%) cases had a family history of HD of which 6 had a similar history in siblings and 2 cases had a similar history in siblings and father also. Two cases were syndromic associated with Down syndrome.

We have analysed 53 cases of HSCR compared to age- and sex-matched controls for the frequency of polymorphic alleles at two loci within the coding region of the RET proto-oncogene. In the case of rs1800860A > G out of 53 cases AG genotype was seen in 12 (22.64%) cases and GG in 41 (77.36%) and out of 50 controls AA genotype was seen in 3 (6.00%), AG in 17 (34.00%) and GG in 30 (60.00%) controls. In the case of rs1800861G > T out of 53 cases GG genotype was seen in 5 (9.43%) cases, GT in 12 (22.64%) and TT in 36 (67.92%) cases, and out of 50 controls GG genotype was seen in 13 (26.00%), GT in 28 (56.00%) and TT in 9 (18.00%) controls. Odds ratio (OR) with 95% confidence interval (CI) was calculated for Genotype frequencies in cases v/s controls, which showed that in the case of rs1800860A > G genotype AG was not associated with the increasing risk of disease while genotype GG was associated with increasing the risk of the disease, but *P* value was not significant ($P = 0.088$) [Table 1]. In case of rs1800861G > T genotype GT was associated with lowering the risk of the disease with a significant *P* value ($P < 0.001$) while genotype TT was associated with increasing the risk of the disease with a significant *P* value ($P < 0.001$) [Table 2].

In the case of rs1800860A > G allelic frequency of wild type allele A was 11% and 23% in cases and controls, respectively. The allelic frequency of A was higher in controls. Allelic frequency of polymorphic allele G was 89% in cases and 77% in controls, which was higher in cases. The difference in allelic distribution at this locus between HSCR cases and controls was statistically significant (Chi-squared with Yates's correction = 4.183, $P < 0.05$).

In the case of rs1800861G > T allelic frequency of wild type allele G was 21% in cases and 54% in controls, which was higher in controls. Allelic frequency of polymorphic allele T was 79% in cases and 46% in controls, which was higher in cases. The difference in allelic distribution at this locus between HSCR cases and normal controls was statistically significant (Chi-squared with Yates's correction = 23.020, $P < 0.0001$).

Out of 41 SSD type of HD in the case of rs18600860A > G genotypic frequency of AG was 26.83% and GG was 73.17%. Out of 12 cases of LSD and TCA genotypic frequency of AG was 8.33% and GG was 91.67%. The difference in genotypic distribution at this locus between SSD cases and LSD and TCA was correlated by Fisher's exact test and it was statistically not significant ($P = 0.2565$). Out of 41 SSD type of HD in the case of rs18600861G > T genotypic frequency of GT was 29.27% and TT was 70.73%. Out of 12 cases of LSD and TCA genotypic frequency of GT was 0% and TT

Table 1: Genotype distribution of the SNPs rs1800860 in case-control

Genotype	Frequency in cases (%)	Frequency in controls (%)	OR with 95% CI
AA	0	3 (6.00)	Reference
AG	12 (22.64)	17 (34.00)	0.568 (0.238- 1.356); *P*=0.273
GG	41 (71.36)	30 (60.00)	2.278 (0.967- 5.366); *P*=0.088

OR: Odds ratio, CI: Confidence interval

Table 2: Genotype distribution of the single nucleotide polymorphisms rs1800861 in case-control

Genotype	Frequency in cases (%)	Frequency in controls (%)	OR with 95% CI
GG	5 (9.43)	13 (26.00)	Reference
GT	12 (22.64)	28 (56.00)	0.230 (0.0981- 0.539); *P*<0.001
TT	36 (67.92)	9 (18.00)	9.647 (3.830- 24.302); *P*<0.001

OR: Odds ratio, CI: Confidence interval

was 100%. The difference in the genotypic distribution of GT and TT at this locus between SSD cases and LSD and TCA was made by Fisher's exact test and it was statistically significant ($P = 0.0476$ and 0.0054).

Discussion

In our study, 37 (69.81%) patients were male and 16 (30.19%) were female. Male-to-female ratio was 2.31. In Hiradfar *et al*.'s[5] study, they found patient's age varied from 2 days to 12 years (mean, 17 ± 4 months) and male-to-female ratio was 2.75. In another study conducted by Kannaiyan *et al*.[6] the male-to-female ratio was 3.2–3.3:1.

In our study, we found 41 (77.36%) cases were of SSD, 7 (13.21%) were of LSD, and 5 (9.43%) were of TCA. In a study done by Zuikova *et al*.[7] they found 75% cases of short segment disease, 16.7% cases of long, 8.3% of TCA which is similar to our study. In another study conducted by Kannaiyan *et al*.[6] 81.7% had short-segment disease, 8.3% had long-segment disease, 10% had TCA. In a study done by Tou *et al*.[8] they found 90% cases of short segment disease and 10% had long-segment disease.

In our study, 43 (81.13%) cases were sporadic, 8 (15.09%) cases were familial and 2 (3.78%) cases were syndromic. In a study conducted by Borrego *et al*.[9] they found all sporadic cases. In a similar study by Seri *et al*.[10] 87.18% were sporadic, 12.82% were familial.

We have analysed 53 cases of HSCR compared to age- and sex-matched controls for the frequency of polymorphic alleles at two loci within the coding region of the RET proto-oncogene. In the case of rs1800860A > G out of 53 cases AG genotype was seen in 12 (22.64%) cases and GG in 41 (77.36%), and out of 50 controls, AA genotype was seen in 3 (6.00%), AG in 17 (34.00%) and GG in 30 (60.00%). In the case of rs1800861G > T out of 53 cases GG genotype was seen in 5 (9.43%) cases, GT in 12 (22.64%) and TT in 36 (67.92%) cases, and out of 50 controls GG genotype was seen in 13 (26.00%), GT in 28 (56.00%) and TT in 9 (18.00%) controls. In a study done by Tou *et al*.[8] they found that in C1296A > G rs1800860, AG genotype was seen in 18.7%, and GG in 81.3% in cases, and in controls AA genotype was seen in 3.6%, AG in 32.1% and GG in 64.3%, which is same as our study. They demonstrated that in rs1800861T > G, rs1800861 GG genotype was seen in 71.6%, GT in 21.1% and TT in 7.3% cases, and in controls, GG genotype was seen in 21.4%, GT in 56.6% and TT in 22.0% which was different from our study due to different risk allele. In our study, OR with 95% CI was calculated for association of genotypic frequencies in cases v/s controls which showed in case of rs1800860A > G, for genotype AG it was 0.568 (0.238–1.356); $P = 0.273$, for genotype GG 2.278 (0.967–5.366); $P = 0.088$. In a study done by Tou *et al*.[8] they found that it was 2.40 (1.38–4.18); 0.002 for genoytype GG, which is the same as in our study but in our study *P* value was not significant. Tou *et al*.[8] found that homozygous RET 1296GG genotype was linked to the risk of HSCR, with the OR of 2.40 (95% CI = 1.38–4.18) compared with the AA or AG genotype. In our study, we also found that RET rs1800860 GG genotype was linked to the risk of HSCR, with the OR of GG 2.278 (0.967–5.366); $P = 0.088$ compared with the AA or AG genotype, but here *P* value was not significant. Liang *et al*.[11] demonstrated that an association was not observed only in the GG/GA versus AA genotype.

In our study, OR with 95% CI was calculated for association of genotypic frequencies in cases v/s controls which showed in case of rs1800861G > T, for genotype GT it was 0.230 (0.0981–0.539); $P < 0.001$, for genotype TT 9.647 (3.830–24.302); $P < 0.001$. In a study done by Tou *et al*.[8] they found that it was 1.08 (0.46–2.53); 0.853 for genotype TG and 9.79 (4.28–22.43); <0.001 for genotype GG. This study showed the elevated risk for the development of HSCR, compared with the counterpart wild genotype, as in our study. In a study by Phusantisampan *et al*.[12] OR (95% CI) for SNP rs1800861 was 3.33 (1.79–6.20), which also showed that it was associated with an increased risk of disease. Liang *et al*.[11] also demonstrated the role of rs1800861 polymorphism in the occurrence of HSCR.

In our study, in the case of rs1800860A > G allelic frequency of wild type allele A in cases was 11% and 23% in controls. Allelic frequency of A was higher in controls. Allelic frequency of polymorphic allele G was 89% in cases, and 77% in controls which was higher in cases. The difference in allelic distribution at this locus between z cases v/s controls was statistically significant (Chi-squared with Yates's correction = 4.183, $P < 0.05$). In a study by Tou *et al*.[8] The frequency of G allele in rs1800860 A > G was 90.7%, which was higher than that of 80.4% in controls. They have shown that this marker to be strongly associated with the HSCR risk. In a study by Wu *et al*.[13] they also found that the frequencies of allele G of rs1800860, was significantly higher in HD patients than in the controls as in our study.

In our study, in the case of rs1800861G > T allelic frequency of wild type allele G was 21% in cases, and 54% in controls which was higher in controls. Allelic frequency of polymorphic allele T was 79% in cases, and 46% in controls which was higher in cases. The difference in allelic distribution at this locus between HSCR cases v/s controls was statistically significant (Chi squared with Yates's correction = 23.020, $P < 0.0001$). In a study by Tou *et al*.[8] the frequency of risk allele in rs1800861was 82.1%, which was higher than that of 49.7% in controls same as in our study. Wu *et al*.[13] also found that the frequency of risk allele was higher in HD patients than controls.

We found that the difference in the genotypic distribution of AG and GG at locus rs1800860 between SSD cases and LSD and TCA was made by Fisher's exact test and it was statistically not significant ($P = 0.2565$), but the difference in the genotypic distribution of GT and TT at locus rs1800861 between SSD cases and LSD and TCA was made by Fisher's exact test, and it was statistically significant ($P = 0.0476$ and 0.0054). In a study by Wu *et al*.[13] they demonstrated that none of the SNPs

were significantly different between LSD and SSD patients, which was different from our study.

Wu *et al*.,[13] Phusantisampan *et al*.,[12] Liang *et al*.,[11] Borrego *et al*.[9] support the hypothesis that common variations in the RET pathway play an important role in the pathogenesis of HSCR and might provide clues to develop screening and surveillance strategies as in our study.

Conclusion

We found that the homozygous polymorphisms of SNPs rs1800861 are significantly associated with an increased risk of HSCR, and the SNPs of rs1800860 polymorphisms may be associated with increased risk of disease. We also found that polymorphisms of SNPs rs1800861 is more in LSD and TCA than SSD.

Limitations

The sample size was relatively small, comprising total of 53 cases for gene sequencing.

This observational study was performed over a limited period of one and a half years and was conducted in a single institute. The genotypic study was expensive, resources were limited so that we have selected only two SNPs.

Future scope

If the disease-causing mutations have been identified in the family, prenatal diagnosis for pregnancies at increased risk is possible by the analysis of DNA extracted from fetal cells obtained by amniocentesis (usually performed at ~ 15–18 weeks gestation) or chorionic villus sampling (usually performed at ~ 10–12 weeks gestation). Preimplantation genetic diagnosis may be an option for some families in which the disease-causing mutation has been identified in an affected family member.

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Conflicts of interest

There are no conflicts of interest.

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