ELECTRONIC LETTER

Association of germline mutations and polymorphisms of the *RET* proto-oncogene with idiopathic congenital central hypoventilation syndrome in 33 patients

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The idiopathic congenital central hypoventilation syndrome (CCHS) was first described by Mellins *et al*[1] and is characterised by an alteration of the ventilatory response to hypercapnia and hypoxia.[1] Whereas normal ventilation is adequate in many of the patients during wakefulness, the alveolar hypoventilation observed during sleep seems attributable to a failure of the central autonomic control of ventilation in the brainstem.[2] CCHS is a rare entity in which affected children show symptoms typically in the newborn period. These symptoms include a period of cyanosis upon sleep induction, a decrease of oxyhaemoglobin saturation with simultaneous life threatening increase of the partial pressure of CO_2 in arterial blood (PaCO_2), which yields no respiratory response, and no arousal reflex by the infants. Primary neuromuscular, lung, or cardiac disorders or an identifiable brainstem lesion are absent.[3] The first extensive series of CCHS patients was described by Weese-Mayer *et al*[4] in 1992.

The apparent functional defect possibly stems from an abnormal migration or differentiation of neural crest derived cells into the autonomic ventilatory control system. Therefore, idiopathic congenital central hypoventilation syndrome may be regarded as a feature of complex neurocristopathies, as confirmed by the observation of a combination of CCHS and other neurocristopathies.[5] Specifically, CCHS has been reported in association with neoplastic as well as dysgenetic neurocristopathies, such as neuroblastomas[6] and ganglioneuromas,[7] but the frequency of such cases is lower than 5%. Furthermore, in 16-20% of the patients, CCHS is combined with Hirschsprung disease (HSCR), a developmental disorder characterised by congenital absence of ganglion cells in the myenteric and submucosal plexuses of the bowel.[6 8–16]

Although most CCHS cases are sporadic, a putative genetic origin for CCHS has also been considered because of the familial occurrence in several reported cases, such as monozygotic female twins,[17] female sibs,[9 18] male-female sibs,[4] and male-female half sibs.[19] Moreover, a case-control family study showed that CCHS patients and their parents are more likely to be affected with symptoms of autonomic nervous system dysfunction (ANSD) than controls and parents of controls. Thus, CCHS is also considered as the most severe manifestation of ANSD. The segregation analysis of ANSD symptoms in families with CCHS affected subjects was consistent with the familial association hypothesis and fits almost equally two genetic models, the major locus model and the multifactorial model.[14 20 21]

In addition to the frequently reported association of CCHS with Hirschsprung disease in humans, a similar phenotype has also been observed in the *ret* knockout mouse model. Whereas heterozygous animals did not show any histopathological signs of aganglionosis, animals with a homozygous functional *ret* deletion showed severe aganglionosis extending to the stomach and also variable defects of the kidneys.[22] Interestingly, *ret* -/- mice died within the first 16-24 hours of

Key points

- The idiopathic congenital central hypoventilation syndrome (CCHS) is characterised by an alteration of the ventilatory response to hypercapnia and hypoxia resulting in a life threatening increase of PaCO_2 during sleep. The reported association of CCHS with Hirschsprung disease in humans, and a similar phenotype observed in the *ret* knockout mouse model, support the *RET* proto-oncogene as a candidate gene for CCHS.

- We analysed the complete *RET* coding sequence in 33 unrelated patients with CCHS by direct DNA sequencing.

- In eight (24.2%), CCHS coexisted with Hirschsprung disease. We detected three *RET* germline mutations, which in two cases were inherited from asymptomatic parents. Additionally, we found an association of *RET* polymorphic variants with the CCHS phenotype. In particular, the association of the c.135A variant with CCHS was statistically significant.

- The identification of *RET* germline mutations in CCHS patients, as well as the association of specific *RET* variants with this phenotype, suggest that this gene contributes to the aetiology of CCHS. Nevertheless, the low mutation rate, incomplete penetrance, variable phenotype, and the involvement of other genes like *GDNF*, *EDN3*, or *BDNF* imply that there are further unidentified genes which may contribute to the aetiology of CCHS in a complex pattern of inheritance.

life, probably not as a result of the above mentioned malformations but rather from altered respiratory CO_2 sensitivity and responsiveness.[23]

In addition, reports of patients with HSCR who harbour germline mutations of the *RET* proto-oncogene have shown that they were primarily point mutations scattered throughout the extracellular domain and within the intracellular tyrosine kinase domain of *RET* in up to 20% of sporadic cases and in up to 50% of familial cases. However, a population based study of Swedish HSCR patients showed a low mutation rate of only 3.2%. The *RET* mutations found in HSCR result in either RET protein truncation, decreased presentation on cell surface, or functional inactivation of the molecule.[24–29]

Based on these observations, the *RET* proto-oncogene may also be considered as a candidate gene for idiopathic congenital central hypoventilation syndrome, although previous analyses of the coding region of *RET* showed no causative mutation (apart from common gene variants at positions c.1296G/A, c.2071G/A, c.2307T/G, and c.2712C/G) in 17 CCHS patients, of whom seven showed a combination with

Table 1 *RET* germline mutations in 33 CCHS patients

Patient	Exon/ intron	Mutation	Nucleotide	Alteration in sequence	Evolutionary conservation	HSCR (phenotype)
1	12	IVS12 + 54C>A	C to A			No HSCR
2	14	P841L	CCG to CTG	Missense	M*	Short
3	13	Y791F	TAT to TTT	Missense	M, C†	Long

*M = mouse. †C = chicken

HSCR.[16 30] In further studies on seven patients, and in an additional case report, two missense mutations of *RET* were detected in patients with the CCHS/HSCR association.[15 31] Moreover, similar to the findings in HSCR patients,[32–35] a missense mutation of the RET ligand *GDNF*[31] and an *EDN3* frameshift mutation[36] were found in patients with isolated CCHS. In a recent sequencing analysis of the *BDNF* gene in 19 CCHS patients, a missense mutation was detected in a patient without HSCR but additional ANSD symptoms.[37]

In this study we report the analysis of the coding *RET* sequence in CCHS patients, detecting three germline mutations in unrelated cases, of whom one had an isolated CCHS. Furthermore, we describe the allele frequencies of seven *RET* polymorphic variants and the association of the c.135A variant with the CCHS phenotype.

MATERIALS AND METHODS

Our population comprised 33 unrelated patients (31 from Germany, one from German Switzerland, and one from Austria) who were followed exclusively in specialised pulmonary care centres and met the criteria for CCHS, evaluated and diagnosed by a polygraphic respiratory recording which detected hypoventilation, high $PaCO_2$, and low PaO_2 during quiet sleep,[3] and for whom the review of medical records yielded no further cause for the hypoventilation. None of them showed additional primary neuromuscular, pulmonary, or cardiac anomalies. Eight of the 33 patients (24.2%) had a concomitant HSCR phenotype. Hirschsprung disease was diagnosed by histological and histochemical evaluation of intestinal biopsies, confirming the absence of enteric ganglia and the increase in acetylcholinesterase in hyperplastic nerve fibres in the aganglionic tract.

One patient in our series had a monozygotic twin with the same clinical presentation of CCHS and another patient had an affected brother. All other patients were seemingly sporadic cases. Samples from previously described healthy German blood donors served as our normal, race and gender matched controls.[38] According to a questionnaire, they did not have any signs of congenital or tumoral disorders. Symptoms of ANS dysfunction were not evaluated. Our control population showed similar allele frequencies of *RET* polymorphisms compared to other unaffected European and North American populations. All studies were conducted with written informed consent from the patients and their families, and all protocols were approved by the Clinical Ethics Commission of the University of Dresden.

Genomic DNA was obtained from leucocytes derived from peripheral venous blood samples and isolated by standard protocols. The 21 *RET* exons were amplified from genomic DNA using primers and reaction conditions described by Ceccherini *et al*[39] for exons 1-4, 11, and 16, and by Mulligan *et al*[40] for exons 5, 7, 9, 10, 12, 13, 17, 18, and 20. To amplify the remaining exons we generated new primer pairs (exon 6: sense 5′-CAGAGCAGCTTGGTGGTCA-3′; antisense 5′-AGTC TACTCTGTGCTGGTTGG-3′; exon 8: sense 5′-GCACTAG CTGGACGCTGG-3′; antisense 5′-GAGACCATCCCAGGCTG GC-3′; exon 14: sense 5′-TGTGTCCACCCCCTTACTCATTGG-3′; antisense 5′-CGTGGTGGGTCAGGGTGTGG-3′; exon 15: sense 5′-CCCCCGGCCCAGGTCTCAC-3′; antisense 5′-GCTCCACTA

ATCTTCGGTATCTTT-3′; exon 19: sense 5′-GCGGACATC AGCAAAGAC-3′; antisense 5′-GCTGGGCAATG TGGTAAT-3′; exon 21: sense 5′-CCGGGCCCACCACATCATC-3′; antisense 5′-AGCCCAAATTAGAGCCAGGTTACG-3′). Amplified DNA fragments were analysed by a direct DNA sequencing approach applying the Thermo Sequenase® Fluorescent Cycle Sequencing kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's protocol. The sequencing primers were the same as the PCR primers with an additional Cy5 labelling, allowing sequence analysis on ALF Express® devices (Amersham Pharmacia Biotech, Freiburg, Germany).

We also investigated the genotype distribution of polymorphisms of positions c.135G/A, c.375C/A, c.1296G/A, c.2071G/A, c.2307T/G, c.2508C/T, and c.2712C/G of the coding region of the *RET* proto-oncogene. The seven investigated DNA fragments were amplified from genomic DNA using primers and methods described previously.[38] All analysed polymorphisms created or abolished a restriction site of an endonuclease, namely, *Eag*I, *Mbo*II, *Bsm*I, *Ban*I, *Taq*I, *Alu*I, and *Rsa*I. Genotypes were determined by digestion of the PCR product and electrophoresis on a polyacrylamide gel. Statistical analysis was performed with the Pearson χ^2 test with a confidence interval of 95%.

RESULTS

Sequence analysis showed a *RET* germline mutation in a female patient with CCHS and central epilepsy, but without any symptoms of HSCR. The patient herself and the relatives investigated did not suffer from constipation, so we had no reason to evaluate the intestinal nervous system histologically. We found a nucleotide change (C>A) at position ds+54 of intron 12. This mutation was transmitted from the healthy father and we also detected this transversion in the asymptomatic brother.

Furthermore, we found a missense mutation of exon 13 codon 791 (TAT→TTT) in a male patient with CCHS in association with total colonic aganglionosis, which resulted in the substitution of a highly conserved tyrosine for phenylalanine. This Y791F mutation has been previously reported in a patient with sporadic HSCR,[28] as well as in a kindred with familial medullary thyroid carcinoma.[41] In our patient, the mutation was inherited from the unaffected father and the family history for HSCR or MEN2 was negative. A third mutation was detected in exon 14 codon 841 (CCG→CTG) in a female CCHS/HSCR patient with short segment aganglionosis. This hitherto unknown C→T transition resulted in the substitution of a proline by a leucine. This mutation was not detected in the healthy mother, while genetic testing was not possible in the father. None of these three mutations was detected in 120 normal controls (table 1).

In addition to the mutations, we found a rare *RET* variant of exon 18 codon 982 (CGC→TGC)[42 43] in a male patient with combined CCHS and HSCR, which substituted a cysteine residue for an arginine. This variant was inherited from the healthy mother and was absent in 156 normal controls.

We have determined allele frequencies for seven *RET* polymorphisms in the CCHS population and compared the results to those in a normal control population. Only one of these

Table 2 Allele frequencies of polymorphic variants of *RET* in 33 CCHS patients and 156 controls

Exon/intron	Nucleotide change/codon position	Restriction site changed	Allele frequency*		Statistics	
			Control population	CCHS population	Chi-square	p value
Exon 2	GCG to GCA c.135G/A	*Eag*I	76.3%	63.6%	4.536	0.044
Exon 3	GTC to GTA c.375C/A	*Mbo*II	98.1%	100%	1.107	0.592
Exon 7	GCG to GCA c.1296G/A	*Bsm*I	72.4%	60.6%	3.656	0.074
Exon 11	GGT to AGT c.2071G/A	*Ban*I	79.8%	80.3%	0.008	1.0
Exon 13	CTT to CTG c.2307T/G	*Taq*I	76.3%	71.2%	0.755	0.432
Exon 14	AGC to AGT c.2508C/T	*Alu*I	96.4%†	93.9%	0.853	0.487
Exon 15	TCC to TCG c.2712C/G	*Rsa*I	80.1%	81.8%	0.099	0.865

*Allele frequencies of the wild type allele, which is underlined in the column "Nucleotide change/codon position".
†For the c.2508 polymorphism only 153 controls were tested.
‡For the intron 2 ds+9 polymorphism only 114 controls were tested.

seven polymorphisms showed a statistically significant association with the CCHS phenotype. The genotype distribution for each of the seven polymorphic loci did not deviate significantly from Hardy-Weinberg equilibrium.

A significant over-representation of the c.135A variant was found within the CCHS population in comparison to the controls (table 2, $\chi^2=4.536$, p=0.044). Furthermore, a similar tendency towards over-representation of the c.1296A variant of *RET* was found in the CCHS population, although the difference was not statistically significant (table 2, $\chi^2=3.656$, p=0.074). The allele frequencies of all other investigated polymorphisms, namely those of codons c.375C/A, c.2071G/A, c.2307T/G, c.2508C/T, and c.2712C/G, did not derive from that detected in our normal control population, which was matched for race and gender (table 2).

DISCUSSION

In this study, we investigated 25 patients with isolated CCHS and eight patients with CCHS in combination with HSCR for *RET* germline mutations in the 21 exons including the adjacent intronic sequences. We detected three germline mutations, one in a patient with a CCHS/epilepsy phenotype but no HSCR and two in patients with a CCHS/HSCR phenotype. Several studies have reported 25 CCHS patients in total comprising 10 patients with a combined CCHS/HSCR phenotype,[15 16 30 31] but *RET* germline mutations were reported solely in CCHS patients in association with HSCR. Amiel *et al*[31] found a heterozygous P1039L mutation in a CCHS/HSCR patient who had inherited the mutation from the unaffected father, and Sakai *et al*[15] also described a heterozygous T706A mutation in a CCHS/HSCR patient.[15]

We report a *RET* mutation in a patient with isolated CCHS/epilepsy phenotype. Although it was located in position ds+54 of intron 12, we did not find this variant in the normal control population. Intronic mutations close to this variant have been reported in association with HSCR.[28 44] Since the mutation derives from a C→A transversion, we hypothesise that it could generate a new splice acceptor site.

Furthermore, we describe a new P841L mutation in a CCHS/HSCR patient. Interestingly, the same amino acid change was found in codon 1039 of a CCHS/HSCR patient by Amiel *et al*.[31] In addition, the substitution of a leucine for a proline amino acid residue was reported in association with isolated HSCR for codons 20, 64, 399, and 973.[24 26 44] In addition to the frequent occurrence of this substitution, all of these proline residues are conserved between humans and mice.

Besides the mutations, a rare *RET* variant of exon 18 codon 982 was found in a patient with combined CCHS and HSCR. This variant was not detected in our 312 control chromosomes, but Mulligan *et al*[40] found this polymorphism in three out of 142 normal chromosomes. Sancandi *et al*[45] detected the heterozygous codon 982 variant in four out of 57 sporadic HSCR patients[45] and Svensson *et al*[29] detected it in a sporadic HSCR patient who harbours an additional *EDNRB* mutation.[29] Although this variant is detected in controls and the phosphorylation activity of the RET molecule is not altered, it is considered not as a mutation but as a polymorphism.[42 43] However, the possibility remains that this polymorphism increases the risk of developing the HSCR or CCHS/HSCR phenotype by interfering with *RET* expression rather than with *RET* function.

We also found an association of a polymorphic *RET* variant with idiopathic congenital central hypoventilation syndrome. In particular, the c.135A variant is significantly over-represented in our CCHS population compared to normal controls. Although the statistical significance of this association is weak, this finding agrees with the strong association of this variant with the HSCR phenotype, as previously reported.[38 46] Interestingly, none of the c.2071G/A, c.2307T/G, or c.2712C/G *RET* polymorphic variants associated with the HSCR (particularly the c.2307G variant) were over- or under-represented in the CCHS population. In contrast, we found a tendency towards an over-representation of the c.1296A variant in our CCHS population. Analysing the allele frequencies of the c.1296G/A and c.135G/A polymorphisms in the isolated CCHS subpopulation and the CCHS/HSCR subpopulation, we found almost the same frequencies in both subpopulations for the c.1296G/A alleles (30 c.1296G and 20 c.1296A alleles in the isolated CCHS subpopulation and 10 c.1296G and six c.1296A alleles in the CCHS/HSCR subpopulation ($\chi^2=0.032$, p=1.000)). Further, the c.135A variant is slightly over-represented in the CCHS/HSCR subpopulation compared to the isolated CCHS subpopulation, albeit not significantly (33 c.135G and 17 c.135A alleles in the isolated CCHS subpopulation and nine c.135G and seven c.135A alleles in the CCHS/HSCR subpopulation ($\chi^2=0.498$, p=0.556)). Additionally, if compared to the normal controls, c.135A showed no statistically significant association with the isolated CCHS population (33 c.135G and 17 c.135A alleles in the isolated CCHS subpopulation and 238 c.135G and 74 c.135A alleles in the control population ($\chi^2=2.421$, p=0.159)). In spite of the limited sample size and absence of ANS dysfunction in

controls and their families, these findings support the hypothesis of an involvement of specific *RET* haplotypes in the aetiology of CCHS and HSCR.

Finally, the identification of additional *RET* germline mutations in CCHS patients and the association of a specific *RET* variant with this phenotype suggest a contribution of *RET* to the aetiology of CCHS as the most severe form of ANS dysfunction. However, the low mutation rate, the incomplete penetrance, the variable phenotype, and the involvement of other genes like *GDNF*, *EDN3*, or *BDNF* further suggest that additional unidentified genes may be present, involved in the aetiology of idiopathic congenital central hypoventilation syndrome in a complex multigenic pattern of inheritance, in which a cumulative effect of mutations in multiple genes contributes to the phenotype.

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