Noradrenergic neuronal development is impaired by mutation of the proneural HASH-1 gene in congenital central hypoventilation syndrome (Ondine’s curse)

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Congenital central hypoventilation syndrome (CCHS, Ondine’s curse) is a rare disorder of the chemical control of breathing. It is frequently associated with a broad spectrum of dysautonomic symptoms, suggesting the involvement of genes widely expressed in the autonomic nervous system. In particular, the HASH–PHOX–RET developmental cascade was proposed as a candidate pathway because it controls the development of neurons with a definitive or transient noradrenergic phenotype, upstream from the RET receptor tyrosine kinase and tyrosine hydroxylase. We recently showed that PHOX2B is the major CCHS locus, whose mutation accounts for 60% of cases. We also studied the proneural HASH-1 gene and identified a heterozygous nucleotide substitution in three CCHS patients. To analyze the functional consequences of HASH-1 mutations, we developed an in vitro model of noradrenergic differentiation in neuronal progenitors derived from the mouse vagal neural crest, reproducing in vitro the HASH–PHOX–RET pathway. All HASH-1 mutant alleles impaired noradrenergic neuronal development, when overexpressed from adenoviral constructs. Thus, HASH-1 mutations may contribute to the CCHS phenotype in rare cases, consistent with the view that the abnormal chemical control of breathing observed in CCHS patients is due to the impairment of noradrenergic neurons during early steps of brainstem development.

INTRODUCTION

Congenital central hypoventilation syndrome (CCHS, Ondine’s curse, MIM 209880) is a life-threatening disorder characterized by persistent hypoventilation during sleep, beginning in the neonatal period and requiring life-long ventilatory assistance (1). While the pathophysiologic mechanisms underlying CCHS remain elusive, several lines of evidence suggest that genetic factors are involved (occurrence in siblings, concordant monozygotic twins and rare vertical transmission) (2). Changes in the integration of afferent inputs from central and peripheral chemoreceptors in the brainstem are the most likely disease mechanisms. In addition, most CCHS patients present a broader defect of the autonomic nervous system (3), including Hirschsprung disease (25–30% of cases, Haddad syndrome, MIM 209880) (2), a malformation of the enteric nervous system that has been ascribed to a mutation at the RET receptor tyrosine kinase locus. In mice, the Ret signaling pathway, which involves the sequential expression of the Mash1, Phox, Ret and TH genes, is responsible for the development of all

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transient or permanent noradrenergic derivatives (4,5). We recently showed that PHOX2B is the major disease-causing gene in both CCHS and Haddad syndromes, with an autosomal dominant mode of inheritance and de novo mutations at the first generation (2). Cross-regulation of the Phox2 and Mash-1 genes has been shown in mice (6,7); moreover, newborn Mash-

.expression was detected in the presumptive enteric nervous system, from the esophagus to the rectum (Fig. 2E–H) and in the adrenal medulla (Fig. 2I and J).

**Functional analysis of HASH-1 mutant alleles**

We investigated the effects of the HASH-1 mutated alleles on their transcriptional activity by means of a luciferase assay. P19 cells were co-transfected with HASH-1 alleles (+/− E47) and the luciferase gene under the control of the Deltal promoter. The transcriptional activities of the P18T and A36–A43del alleles were similar to that of the control, regardless of whether the E47 cofactor was present (data not shown, available on request).

We then overexpressed the wild-type and mutant HASH-1 alleles, and assessed the functional consequences for the differentiation of the noradrenergic lineage, using the PHOX–RET signaling pathway and the tyrosine hydroxylase (TH) phenotype as markers (11,12). We demonstrated that undifferentiated vagal neural crest progenitor cells, isolated from E9 mouse embryos, may be engaged in noradrenergic differentiation, and then sequentially produce Phox2a, Ret, and TH, thereby reproducing the mouse Mash-1 molecular cascade (Fig. 3A). Interestingly, no alternative lineage was obtained, as almost all TH+ cells were derived from PHOX2A-expressing neurons. We then showed that it was possible to reproduce the catecholaminergic differentiation pathway, 72 h after the forced expression of HASH-1 by recombinant adenoviruses (Fig. 3B and C). All the infected cells (100%) were transduced with the HASH-1 adenoviral constructs (Fig. 4), as shown by GFP production (Fig. 4B). The overexpression of wild-type HASH-1 resulted in the differentiation of 52.3 ± 3% (n = 12) of neural crest cells into postmitotic noradrenergic derivatives, as shown by Phox2a production (data not shown). These values are similar to those previously reported for retroviral forced expression (11). We investigated the functional consequences of HASH-1 gene mutations for the noradrenergic lineage (Fig. 4B). All mutated forms of HASH-1 tested led to a significant decrease (20–30%) in the number of neuronal derivatives of the noradrenergic lineage, as shown by Phox2a production (Fig. 4B). In particular, only 34.4 ± 0.7% (p < 0.01) of neural crest progenitors differentiated to give a noradrenergic phenotype using the A37–A41del HASH-1 mutant construct, whereas 39.3 ± 0.6% and 38.7 ± 0.6% of cells produced Phox2a when the P18T and A36–A43del HASH-1 mutant alleles, respectively, were used (p < 0.01; Fig. 4B).

**DISCUSSION**

Taking advantage of the well-characterized specification of catecholaminergic neurons derived from the vagal neural crest...
(5), we recapitulated *in vitro* the molecular cascade involved in the early steps of noradrenergic determination. In that system, we used a forced expression of wild-type and mutant HASH-1 to test the functional relevance of mutated HASH-1 alleles identified in CCHS patients. We showed a significant decrease in the number of noradrenergic neurons generated after expression of mutant HASH-1 alleles. However, this is a limited effect considering that patients are heterozygotes for the HASH-1 mutated allele as opposed to our experimental model. Nevertheless, subtle consequences on the noradrenergic neuronal development could be expected with HASH-1 gene mutations lying outside of the bHLH domain of the protein. Moreover, the HASH-1 mutations were located in highly conserved regions of the protein, although not involved in the basic helix–loop–helix consensus motives (13). Together with the expression pattern of HASH-1 in the developing human embryo, these results cope well with the phenotype of heterozygous *Mash-1*+/−/− mice (10). These data also suggest that variant HASH-1 alleles may contribute to the CCHS phenotype. However, HASH-1 gene mutations are neither necessary (most patients do not have a HASH-1 gene variant), nor sufficient for CCHS to occur (carriers have no phenotypic expression). This observation is reminiscent of the findings in isolated Hirschsprung disease in the Mennonite population, where endothelin B receptor (EDNRB) mutations are neither necessary nor sufficient for the disease to occur and act in conjunction with a yet unknown hypomorphic RET allele (14). The question as to whether HASH-1 acts as a modifier of PHOX2B, and/or a disease-causing autosomal dominant gene with incomplete penetrance remains unresolved.
Polyalanine repeats are common in transcription factors but their normal function is largely unknown. Expansions of polyalanine tracts in transcription factors, including de novo PHOX2B polyalanine expansions in CCHS, have been shown to be responsible for disease in several malformation syndromes in humans (15,16), whereas contractions have not been reported thus far. Polyalanine contractions may therefore act as hypomorphic alleles.

As the CCHS phenotypes of patients with PHOX2B and HASH-1 mutations are undistinguishable, our data suggest that any mutation affecting the HASH-1–PHOX developmental pathway is likely to impair the development of neurons of the

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**Figure 2.** HASH-1 gene expression in developing human embryos. Slides stained with hematoxylin-eosin (A, C, E, G, I) and dark-field illumination of the hybridized adjacent sections (B, D, F, H, J) on day 32 (Carnegie 14, A–D), and day 44 (Carnegie 18, E–J). Parasagittal sections showing weak HASH-1 expression at Carnegie stage 14 and strong expression of this gene at Carnegie stage 18 in mesencephalon (mes), rhombencephalon (rh), and the dorsal area of the spinal cord (sc) (arrowheads, A, B and E–H, respectively). From Carnegie stage 14, expression was detected in cells to the side of the dorsal aorta (arrow, D). HASH-1 expression was also detected in the intestine, from the foregut (arrow, F) to the hindgut (arrow, H), and in the adrenal medulla (arrow, J) at Carnegie stage 18. ad, Adrenal gland; ao, aorta; go, gonad; meta, metanephros; mes, mesencephalon; meso, mesonephros; e, esophagus; re, rectum; rh, rhombencephalon; sc, spinal cord.
Figure 3. In vitro culture model of mouse vagal neural crest cells to analyze the HASH-1–PHOX2B/PHOX2A cascade controlling. (A) Experimental design; NCSCs were isolated from E9.5 mouse embryo neural tubes (NT). Ec, ectoderm; Som, somite; End, endoderm; NC, neural crest. (B) Transcription-factor cascade controlling the RET and tyrosine hydroxylase (TH) lineage of neural crest cells (NCSCs). Cells cultured in the presence of diffusible embryonic mesodermic factors retain the capacity to generate catecholaminergic derivatives, as shown by neurofilament (NF160) and TH production, recapitulating the MASH1–PHOX2B–PHOX2A–RET pathway as in vivo (lower arrow). Conversely, NCSCs cultured in medium containing serum (FCS medium) remain multipotent and retain their proliferative abilities (upper arrow). (C) Sequential expression of the Mash1–Phox2a–Ret–TH gene pathway, studied in cultured NCSCs by immunohistochemistry (first row), peripherin staining (Per, second row), merged immunohistochemistry and peripherin staining (merged, third row) and in situ hybridization (phase, fourth row), from day 1 to day 5 (d1–d5). The Mash1 (d1), Phox2a (d2), Ret (d3) and TH (d5) are shown in red fluorescence; the peripherin (d1–d3) and neurofilament (NF160, NF, d5) are shown in green fluorescence. (D) Temporal expression of the Mash1–Phox2a–Ret–TH gene pathway during NCSCs culture, from day 0 (d0) to day 6 (d6). The expression timing of Mash1, Phox2a, Ret and TH are indicated by solid bars. Peripherin and NF (NF160) are consistently expressed from d1 to d6.
noradrenergic lineage and the resulting defect may modify the respiratory network in the brainstem and periphery.

MATERIALS AND METHODS

Patients

A series of 30 patients fulfilled the inclusion criteria for diagnosis of CCHS namely: (i) hypventilation, hypoxemia and hypercarbia during quiet sleep on polygraphic respiratory recording; with (ii) no cardiac, pulmonary, neuromuscular, EEG or cerebral MRI abnormality (17). Haddad syndrome was diagnosed in 11 cases (36%, five long segment HSCR, two short segment HSCR, four unknown). The histological criteria for HSCR were: (i) absence of enteric plexuses with histological evaluation of the aganglionic tract; and (ii) strong histochemical staining of acetylcholinesterase in nerve fibers. Blood samples were obtained with informed consent and DNA was extracted according to standard protocols.

We screened the coding sequence of the HASH-1 gene by single-strand conformation polymorphism (SSCP) analysis and/or direct DNA sequencing. The PCR products were heated for 10 min at 95°C, loaded onto a Hydrolink MDE gel.

Figure 4. Functional analysis, indicating a decrease in the number of noradrenergic neuronal derivatives obtained from NCSCs after forced expression of the three mutated forms of HASH-1, with respect to the wild-type. (A) HASH-1 adenovirus constructs for the production of both green fluorescent protein (GFP) and wild-type or mutated forms of HASH1 under cytomegalovirus (CMV) promoter control. (B) Left panel: representative samples of infected NCSCs 24 h after infection with adenoviruses producing the wild-type (WT) and mutant (P18T, A36–A43del, A37–A41del) forms of HASH-1. GFP was detected by its green fluorescence and Phox2a was detected by staining with Fast Red. A typical Phox2a-positive cell is indicated by a white arrow; a typical Phox2a-negative cell for is indicated by a white arrowhead. Right panel: percentage of Phox2a positive cells, illustrating the decrease in the number of noradrenergic derivatives obtained from NCSCs after infection with the mutant HASH-1 retroviral constructs (P18T, A36–A43del and A37–A41del).
In situ hybridization in human embryos

Human embryos and fetal tissues were collected from legally terminated pregnancies in agreement with French law and ethics committee recommendations. Tissues were prepared as previously described (18). A 222 bp PCR fragment corresponding to HASH-1 exon 1 was amplified from human genomic DNA with a T7 extension (TAATACGACTCACTAGGGAGA) added to both primers to generate the sense and antisense probes. Probes were labeled with [35S]UTP and purified as previously described (2). Tissues on slides were dehydrated, placed against BIOMAX MR X-ray film (Amersham) for 3 days, and immersed in Kodak NTB2 emulsion for 3 weeks at +4°C. Developed and toluidine blue-counterstained slides were analyzed with dark- and bright-field illumination. No hybridization signal was detected with the sense probe (data not shown).

Construction of adenoviral plasmids

Adenoviral plasmids were constructed using the Adeasy system (19). Polyalanine tract contractions and the control HASH-1 coding sequence were amplified by polymerase chain reaction with the 5′ primer, TCTAGACGCATGGAAA-GCTCTGCCAA, and the 3′ primer, GTCGACTCAGAACC-AGTTGTTGAAGTCTGA, using genomic DNA from controls or patients. PCR-derived fragments were checked by sequencing and subcloned into the pAd-track CMV vector, using the XbaI–SacI restriction sites. pAd-track CMV vectors were used to produce viruses that could be tracked by following GFP fluorescence. Adenoviral plasmids were generated by homologous recombination in electrocompetent E. coli BJ5183, according to the manufacturer’s instructions (Stratagene). Adenoviral plasmids were then cut with PacI and viral stocks were produced in QBI-293A cells.

Mouse neural crest culture

Neural crest cells were cultured as previously described (20). Neural tubes were microdissected from Swiss mouse (Iffa Credo) embryos on day 9 of gestation. Somites and surrounding tissues were removed to obtain neural tube segments corresponding to the first seven somites of the vagal region. These segments were then plated on fibronectin-coated glass coverslips (10 µg/ml, Boehringer Roche) and cultured for 2 days in DMEM (Life Technologies) supplemented with 10% fetal calf serum (AbCys) and 100 IU/ml penicillin/streptomycin (Life Technologies), at 37°C, in a humidified atmosphere containing 5% CO2.

Infection of primary cultures of neural crest cells

We tested various levels of multiplicity of infection (MOI) and decided to use 2000 plaque-forming unit (pfu), which gave 100% infected neural crest cells. Neural crest cells (NCCs) were cultured for 2 days, infected on day 2 and fixed 24 h later.

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