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The genomic structure, chromosome location, and analysis of the human DKK1 head inducer gene as a candidate for holoprosencephaly

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Abstract. Holoprosencephaly (HPE) is the most common developmental defect of the brain and face in humans. Here we report the analysis of the human ortholog of *dkk-1* as a candidate gene for HPE. We determined the genomic structure of the human gene DKK1 and mapped it to chromosome 10q11.2. Functional analysis of four missense mutations identified in HPE patients revealed preserved activity in head induction assays in frogs suggesting a limited role for this gene in HPE pathogenesis.

Holoprosencephaly (HPE) occurs as an early embryonic defect in the division of the eye field and ventral forebrain into distinct left and right halves (Li et al., 1997, reviewed in Muenke and Beachy, 2000). Embryological analysis in animal models has revealed the pathogenesis to be defective patterning during the gastrulation and early neurulation stages of the vertebrate embryo which can be caused by a variety of genetic and environmental perturbations. Although our understanding of these patterning mechanisms is incomplete, mutations in several genes have been identified which result in HPE in humans (Roessler and Muenke, 2000), including Sonic Hedgehog (SHH), SIX3, ZIC2, and probably others such as TGIF and PTC. All of these HPE genes are active during these early embryological stages of anterior brain development.

We considered the human DKK1 gene as a candidate for HPE based on the observation that antibodies directed against the frog head inducer protein, dkk-1 caused cyclopia (Glinka et al., 1998). The *dkk* family of genes have been recently described as coding for secreted proteins of a novel class that act during development to bind and sequester members of the Bmp and Wnt families, effectively establishing developmental zones free of the effects of these powerful morphogens (Glinka et al., 1997). A similarly expressed protein, cerberus, also binds these morphogens in addition to a third factor, nodal (Bouwmeester et al., 1996). Both of these proteins are expressed in the Spemann organizer and are involved in the specification of the anterior brain region during patterning of the anterior-posterior (A-P) axis of the embryo. Therefore, we set out to determine the structure of the human DKK1 gene, its chromosome location, and to analyze the gene for mutations in our panel of HPE patients.

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Materials and methods

Determination of the DKK1 gene structure and its chromosomal location

Based on the sequence of a partial cDNA clone for the human DKK1 gene, an amplicon was designed from the 3′ untranslated region that empirically amplified from commercial genomic DNA the correct sequence representing DKK1. These primers were then used to screen a BAC library from Genome Systems. A single clone address, 233k8, was chosen for further analysis since it cross-hybridized to the 5′ and 3′ subfragments of the original cDNA isolate. Primers were empirically designed and used to directly sequence the BAC clone. This analysis unambiguously identified exons 2, 3 and 4 based on comparison with human ESTs and the known partial cDNA sequence. The sequence of exon 1 was inferred by sequencing of the BAC clone 5′ of exon 2 and comparing its sequence with the mouse cDNA. (DKK1 exons 1 and 2, and DKK1 exons 3 and 4 sequence data can be obtained from GenBank accession nos. AF261157 and AF261158.) An excellent homology match was found 444 bp upstream of exon 2 which was in frame, contained the correct splicing sequences, and contained a consistent context for protein translation.

Fluorescent in situ hybridization (FISH) analysis

Slides with chromosome metaphase spreads were incubated for 1 h at 37 °C in 2 × SSC (0.3 M NaCl and 0.3 M sodium citrate) and then dehydrated sequentially in 70 %, 80 %, and 90 % ethanol. Chromosome DNA was denatured in 70 % formamide, 2 × SSC, for 2 min at 72 °C followed by dehydration in ethanol washes of 70 %, 80 %, 90 %, and 100 %. FISH was performed with probes labeled with Spectrum Orange-dUTP (Vysis, Downers Grove, IL), essentially as described by Pinkel et al. (1986) and Lichter et al. (1988). On each slide 100 ng of labeled DNA was applied. Non-unique and nonspecific DNA hybridization was blocked by preannealing the probes with a ten-fold excess of human Cot1 DNA. Labeled and blocking DNAs were denatured at 75 °C for 10 min and then preannealed at 37 °C for 15 min. The hybridization mixture contained labeled DNA in 10 µl of 50 % formamide, 2 × SSC, and 10 % dextran sulfate at pH 7. Slides were hybridized overnight at 37 °C. Post-hybridization washes were performed at 45 °C as follows: 1) 50 % formamide, 2 × SSC, 20 min, 2) 1 × SSC, 10 min, and 3) 0.1 × SSC, 10 min. Slides were counterstained with propidium iodide-Antifade (Oncor) or DAPI, 250 ng/µl (Boehringer Mannheim, Indianapolis, IN) with Antifade.

Patients and DNA preparation

DNA samples of patients with HPE were obtained by informed consent according to the guidelines of the Children's Hospital of Philadelphia Institutional Review Board. These samples were prepared from blood or lymphoblastoid cells lines by routine methods.

A total of 61 probands with familial HPE and 251 probands with sporadic HPE were chosen for Single Strand Conformational Polymorphisms (SSCP) analysis. These families included individuals with a wide range of HPE phenotypes. When SSCP bandshifts were detected, these samples were sequenced to determine the potential mutations. Where possible, the sequence variations were examined in the parents and siblings to determine the pattern of transmission and also examined in a panel of over 100 normal control individuals (over 200 normal chromosomes) to determine any correlation with the disease state or uniqueness of the sequence variations. All families but one were too small for effective linkage analysis.

Mutational analysis

Amplification of genomic DNA was performed in a 15 µl reaction volume, using 60–100 ng DNA template; 200 µM each of dATP, dGTP and dTTP; 125 µM dCTP; 3.5 µCi [α^{32}P]-dCTP (800 Ci/mmol [10 mCi/ml]); 30 pmol each primer; 1.5 µl 10 × PCR buffer (Gibco); 1.25 µl 10 × PCR Enhancer™ (Gibco); 1.5 mM $MgSO_4$ (Gibco); 1 U Amplitaq polymerase (Perkin Elmer). All of the PCR reactions were performed in a PTC-100 thermal cycler (MJ Research). Four sets of primers were used to screen the entire coding region of the human DKK1 gene. For exon 1, we used the forward primer 164 (5′ TCTTTCTTTCTCCCTCTTGAGTCC 3′) and reverse 165 (5′ GAGCATCCTCTGAGTGCCCGAC 3′); for exon 2 we used 166 (5′ CACCCTCTCCCCGAACCCTTCC 3′) and 167 (5′ TCAAAGGCTGGACAGTTTTAGTGTG 3′); for exon 3 168 (5′ ACCCTGAAGTTAATCACTATTTCC 3′) and 169 (5′ ATTCAAAAGACACTTCTGATGTGC 3′); and finally, for exon 4 we used 170 (5′ TTTTTCCTACTGTCTTCTCCTTCG 3′) and 171 (5′ AGTTCACTGCATTTGGATAGCTGG 3′).

For SSCP analysis, following amplification, 5 µl of each sample was diluted with 10 µl stop solution (9.5 ml deionized formamide [Fluka], 20 mM EDTA, 0.5 % [*wt/vol*] bromphenol blue, and 0.5 % [*wt/vol*] xylene cyanol), denatured for 10 min at 94 °C, and then immediately chilled on ice. The samples were run either with or without 5 % glycerol at room temperature on 0.5 × MDE (FMC) sequencing grade polyacrylamide gel. Sequencing of the amplicons which demonstrated SSCP band shifts was performed on an ABI Prism™ 377 analyzer.

Functional analysis of mutations in the developing frog

Conventional PCR and recombinant techniques were used to prepare synthetic pCR2+ constructs to express the wild type and mutant proteins. In order to enhance the translation of the synthetic transcripts, a synthetic Kozak sequence (5′ GCCGCCACC 3′) was artificially positioned just upstream of the initiator methionine. The structure of all five constructs used in the frog head induction assay was verified by direct sequencing.

Results

Direct sequencing of the BAC clone isolate 233k8 identified the DKK1 gene as consisting of four exons and encoding a protein of 266 amino acids (Fig. 1A). This result is in perfect agreement with the sequence of the human cDNA transcript subsequently published (Fedi et al., 1999). Recently, two additional mouse Dkk homologs (*Dkk2* and *Dkk3*) have been reported (Monagham et al., 1999). An alignment of the predicted protein sequences of these genes confirm that the sequence of DKK1 reported here is most similar to murine *Dkk1* (Fig. 1B). Among the Dkk family members, the most consistent regions of homology reside in the two cysteine-rich domains in the carboxy-terminal region of the protein (Monagham et al., 1999). The chromosome location of the DKK1 gene was determined to be near the chromosome 10 centromere in band 10q11.2 by FISH analysis (Fig. 2).

We identified four different heterozygous sequence alterations in the human DKK1 gene which were not detected in 100 unrelated normal controls. The first sequence variation, 46A→C, predicts an M16L substitution within the signal peptide. An *Nru*I restriction site is lost by this sequence change and could be demonstrated in two different HPE families.

The second sequence variation, 292G→C, predicts a substitution of A98P encoded by exon 2 and confirmed by restriction digestion with *Hha*I. This sequence variation was only identified once in a proband with a medial cleft lip and facial features of HPE. As an incidental finding we observed a common single nucleotide polymorphism affecting A106A which does not alter the primary structure of the protein.

The third mutation, R120L, results from the sequence variation 359G→T which was detected in four unrelated families and not in normal controls. This predicted amino acid substitution occurs adjacent to an invariant cysteine residue. However, a leucine residue is normally present in one of the murine homologs, *Dkk3.*

The final sequence variation which we identified is 469G→T which predicts an S157I substitution. This variation was observed in three unrelated families with HPE and not in normal controls. One of these families was a large five generation family which segregated autosomal dominant HPE and is the only large family that did not show linkage to the SHH gene on human chromosome 7q36 (family 1 in Muenke et al., 1994).

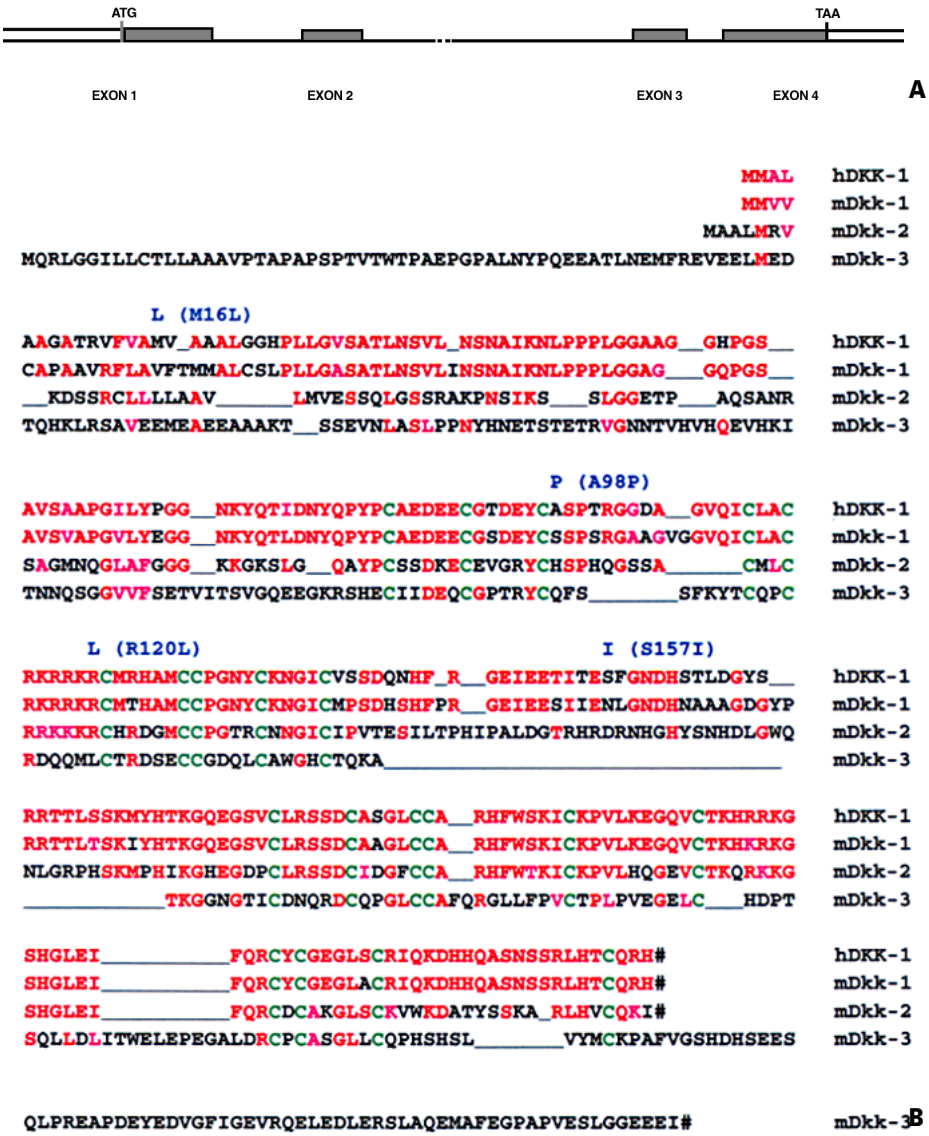

Fig. 1. (**A**) A schematic representation of the human DKK1 gene. The coding region which was screened for mutations is shown in gray. Primers flanking each of the four coding exons were used to amplify genomic segments for mutational analysis. (**B**) An alignment of the protein sequences of the human DKK1 and three murine proteins (Dkk1–3) are shown with identical residues in red, similar residues in pink, and the critical cysteine residues in green. The positions of each of the four mutations are shown in blue above the affected residues.

Interestingly, the presence or absence of the *Hin*dIII RFLP defined by this sequence alteration was correctly correlated with the disease status in 7 out of 8 available family members. Nevertheless, the one failure to correlate with the disease occurred in an obligate carrier male who had an affected child with HPE. Repeat blood samples from this father confirmed the absence of this RFLP. Since it was formally possible that his child was a new sporadic case of HPE, in the context of a familial form, we chose to examine the effects of all four mutations in functional studies.

Dkk1 genes encode potent inducers of head structures when ectopically expressed in the ventral margin of the developing frog embryo. Therefore, constructs were prepared to test the function of the wild type and four mutant proteins identified among patients with HPE. Two assays were employed to test dkk1 variants. The first assay (Glinka et al., 1998) measures the ability of dkk1 to induce ectopic heads when coexpressed with the BMP inhibitor noggin (Smith and Harland, 1992). Four-cell embryos were coinjected with plasmid DNA where the genes are under control of the CMV promoter. Frog and mouse dkk1, as well as wild type human DKK1 was able to induce ectopic heads in Xenopus embryos when coinjected with noggin (Fig. 3A, B). Likewise, all mutant human DKK1 constructs (M16L; A98P; R120L; S157I) were also able to induce secondary heads (data not shown). The second assay measures the ability of dkk1 to inhibit the Wnt signaling pathway (Glinka et al., 1998). mRNA injection of mWnt1 (Fung et al., 1985) in 4-cell stage embryos activates the Wnt pathway. This activation was measured by induction of a coinjected luciferase reporter gene pS01234, carrying the Wnt-responsive elements of the siamois promoter (Brannon and Kimelman, 1996). A 4-fold induction of the Wnt reporter was found following injection of

Fig. 2. The FISH signal with the BAC clone 233k8 containing the DKK1 gene is present near the centromere on human chromosome 10q in this field from a normal individual (see arrows).

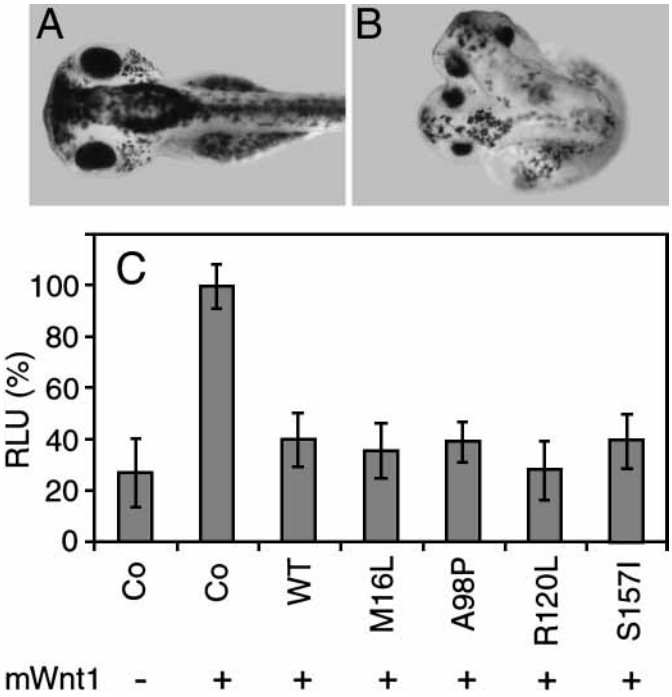

Fig. 3. Wild type and mutant hdkk1 induce ectopic heads and inhibit Wnt signalling. (**A**) Control embryo at 3 days post fertilization. (**B**) Plasmid DNA encoding noggin (2.5 pg/blastomere) and wild type human DKK1 (12.5 pg/bl) were coinjected at the 4-cell stage into two ventral blastomeres. Both wt DKK1 as well as the tested mutant forms induce secondary heads. Likewise, all mutants (M16L, A98P, R120L, S157I) were able to induce secondary heads (not shown). (**C**) Wnt reporter assays. 4-cell embryos were coinjected with the samples described below and luciferase reporter DNA (pS01234; 20 pg/blastomere) in all four blastomeres. At early gastrula (stage 10.5) embryos were extracted with passive lysis buffer (Promega) and 10 µl samples were assayed for luciferase activity (relative light units/embryo, RLU) in quadruplicates as described (Glinka et al., 1996). Reporter DNA was coinjected with (+) or without (–) mWnt1 mRNA (24 pg/bl) and either preprolactin (ppl) negative control mRNA (Co; 140 pg/bl); or with wild-type (WT) or mutant (M16L; A98P; R120L; S157I) human DKK1 mRNA (4 pg/bl) + ppl (112 pg/bl) as indicated.

Wnt1 mRNA (Fig. 3C). Coinjection of all human DKK1 mutants was able to inhibit this activation by mWnt1. In summary, the mutant forms of the human DKK1 protein retained the ability to induce head structures and to inhibit Wnt signaling when tested in the frog system.

Discussion

Animal models of brain development have been extremely useful in suggesting pathogenetic mechanisms of human malformations, such as HPE. In this study, we examined the potential role of DKK1 in HPE and found no direct evidence for its role as a common genetic determinant of this disorder. Such an analysis cannot formally exclude DKK1 as a participant in disease susceptibility, particularly since many cases of HPE do not survive to term. Nevertheless, recent studies on cyclopia phenotypes in animal models suggest that the factors determining cyclopia have roles in dorsal-ventral patterning, such as SHH, and do not directly participate in A-P axis determination (Bouwmeester and Leyns, 1997; Thomas and Beddington, 1996). The recent analysis of the murine *Dkk1*$^{-/-}$ model, which manifests deletion of anterior brain structures (Shrom et al., 1999), suggests that determination of the anterior brain is a separate process from the division of these structures patterned by the axial midline prechordal plate. Our analysis of the human DKK1 gene should facilitate the examination of other brain phenotypes more compatible with our understanding of the function of these developmental genes.

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