

CHARACTERISATION OF CANDIDATE GENE FOR DYT3

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Characterisation of candidate gene for DYT3

Background to the project

X-linked dystonia parkinsonism (XDP) is a severe inherited neurodegenerative disorder. The main clinical feature is the presence of generalised dystonia and in some cases there is also additional Parkinsonism. The disorder was first described in the Philippines, where the mutation was introduced by a common ancestor (founder effect) [Lee et al. 1991].

Although XDP is an uncommon disorder, its study is important for a number of reasons. There are several conditions where Parkinsonism and dystonia co-exist including some genetic conditions such as XDP and idiopathic Parkinson's disease. This suggests that there is an anatomical or physiological link between dystonia and parkinsonism and therefore a common mechanism in their pathogenesis. Studies of these links, by investigating the molecular pathways damaged in XDP, will provide important clues in understanding the pathogenesis of both Parkinsonism and dystonia, the normal function of the basal ganglia and the mechanisms of neurodegeneration within the central nervous system.

In order to identify the gene mutation causing XDP, DNA samples from 47 patients from the Philippines were collected and since no significant chromosomal rearrangements were found a positional cloning approach

was undertaken to isolate the gene causing XDP. This approach identifies disease genes without knowledge of the underlying gene defect, by determining the subchromosomal location of the gene (achieved by genetic linkage studies). Once the location has been determined, construction of high resolution physical and transcript maps of the region is possible. The identified transcripts can then be analysed for mutations in affected individuals.

XDP is inherited as an X-linked recessive condition and the results from linkage analysis mapped the gene, designated DYT3, to the proximal long arm of the X chromosome [Graeber et al., 1992]. This was followed by refined localisation of the candidate region and construction of a 1.8 Mb YAC (Yeast Artificial Chromosome) contig of the disease locus [Haberhausen et al., 1995]

Over the past 3 years, the group I am working with has constructed a sequence-ready HPAC, HBAC and cosmid contig of the region. The critical candidate region is estimated to be 700kb [Németh et al., 1999a]. A novel brain-specific gene was identified using direct cDNA selection on a PAC from the contig of the DYT3 candidate region. The gene is the human homologue of the *Rattus norvegicus* neuroligin 3 gene (NL3). NL3 is one of a family of brain-specific cell-adhesion proteins which are involved in aligning

Special points of interest:

- This project was undertaken as part of the IFCC Fellowship/ Professional Scientific Exchange Programme Scheme
- X-linked dystonia parkinsonism (XDP) is a severe inherited neurodegenerative disorder.
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neurons at the synapse via their interaction with the neurexins and the drosophila discs large family of proteins. The chromosomal location of NL3 and its function at the synapse makes this gene a very good candidate for DYT3.

The aim of my project was to evaluate NL3 as a candidate gene for DYT3. To do this the expression pattern of NL3 was investigated, full length human coding sequence and the intron-exon structure of the gene needed to be determined. Once this had been achieved than mutation analysis was performed.

Results

Expression of the gene:

Results from northern blot analysis using the Neuroligin 3 specific probes (cDNA clones 1934o16 and AA490666, representing the 3'-UTR of the gene) revealed two different transcripts, one of ~3kb and one of ~6kb. The 6 kb transcript was detectable only in lung tissues and the 3 kb transcript - only in brain. As the pathology observed in XDP is brain-specific, the 3 kb transcript was further characterised.

Coding sequence and genomic organisation of human NL3

The sequence of the Rattus neuroligin NL3 was used for nucleotide BLAST searches and additional human ESTs representing the 5'-end of NL3 were identified and sequenced. Some of the coding sequence was obtained using vectorette-PCR [Riley et al 1990] and the remainder was determined by subcloning the HPAC originally used to identify the gene. These techniques were used instead of RT-PCR because the gene was found to be expressed at only low levels in lymphoblastoid cell-lines thus making it difficult to perform direct sequencing on RT-PCR products. Since mutation analysis was not possible using the cell lines we

lected to perform mutation analysis by direct sequencing of the genomic DNA of XDP patients and controls. This had the added advantage of allowing us to examine the splice borders for mutations as well as the coding sequence. The exon intron boundaries were found using a combination of vectorette PCR and Expand Long Template PCR System (Boehringer Mannheim) containing mix of thermostable Tag and Pwo DNA polymerases. Exon/intron junctions conform to the 5'-donor and 3'-acceptor consensus (GT...AG) rule.

The human NL3 gene consists of at least 8 exons and 7 introns. Exon 1 and part of exon 2 code for the 5' - untranslated region (5'-UTR). The start codon (ATG) is located at position 302 in exon 2 and the stop codon (TAG-Amber) is at position 2848 in exon 8. The size of the exons varies from 58 nucleotides for exon 3, to more than 1240 nucleotides for exon 8 containing the 3'-UTR. So far no polyadenylation signal in the 3' -end of the gene is found, suggesting the presence of a longer 3'-UTR.

Analysis of the sequence immediately upstream of the transcription initiation site reveals that NL3 promoter contains CCAAT box (-120 in sense orientation) but lacks a typical TATA consensus motif. In addition, this promoter is highly rich in G+C content and multiple CpG and GpC dinucleotides are located in a region of 700 bp (positions - 500 to + 200). Putative binding sites for transcriptional factors AP-2 and GATA are also present.

The NL3 sequence was found to be highly conserved between rat and human, both on nucleotide and amino-acid level.

Alternative splicing of NL3

RT-PCR of part of NL3 resulted in several PCR products. These were

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subsequently subcloned in pGEM -T vector, amplified and sequenced. They correspond to alternatively spliced products of exons 3 and 4 of NL3. Three alternatively spliced form were present - first, lacking exon 3, second, missing exons 3 and 4, and third - retaining both exons. The alternative splicing is also observed in rat at the same positions in two forms - first, lacking both exons and second - only exon 4.

Mutation analysis of NL3

Since mutation analysis proved to be difficult using lymphoblastoid cell lines and RT-PCR we performed direct analysis of the genomic sequence on patient and male Filipino control DNA's. This involved PCR amplification across all exons and adjacent intronic conserved regions. The PCR products were purified and sequenced, using an ABI 377 automated sequencer.

No difference between patient and control samples was found apart from two nucleotide changes in non-coding intronic sequences. Both polymorphisms were found in healthy controls which suggests that these changes are non-pathogenic.

Human neuroligins 1 and 2

During the characterisation of the human NL3 gene, the highly homologous human neuroigin 1 and 2 genes were identified using a combination of BLAST searches of EST and genomic databases and screening HBAC libraries using the EST clones which were identified. The coding sequence, genomic organisation and chromosomal localisation of these two genes were identified and the results of this investigation are being prepared for publication [Nemeth at al.,1999b].

Further investigations

To completely exclude NL3 as a candidate gene for DYT3 additional experiments are required. These include Southern analysis of patient and control DNA digested with different restriction endonucleases and probed with the NL3 gene. This method has proven to be very useful for identifying mutation larger than about 1kb [Kobayashi at al., 1998]. The promoter region also needs to be examined for mutations.

Since mutations in the NL3 have not been found in XDP patients, other transcripts must now be analysed . Several genes and novel ESTs have been mapped into the XDP region. and are also good candidates for DYT3. The next step will be further characterisation of the genomic structure of these genes, followed by screening for a mutation in XDP patients. To sea rch for mutations in these genes, the same strategies as described above for NL3 will be used.

We also plan mutation screening with DHPLC (denaturing high performing liquid chromatography). The mutation detection rate with a combination of the methods mentioned is expected to be close to 100%. The DHPLC will be used in parallel with automated sequencing of the 700 kb XDP region in search for SNPs (Single Nucleotide Polymorphisms), which will be useful used as markers for further refinement of the critical XDP region.

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