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Detailed mapping, mutation analysis, and intragenic polymorphism identification in candidate Noonan syndrome genes *MYL2*, *DCN*, *EPS8*, and *RPL6*

EDITOR—Noonan syndrome (NS) is an autosomal dominant developmental disorder in which the cardinal features include short stature, typical facies with hypertelorism, ptosis, downward slanting palpebral fissures, and low set, posteriorly rotated ears. In addition, there is a notable cardiac involvement seen in these patients, principally pulmonary valve stenosis and hypertrophic obstructive cardiomyopathy.[1 2] The frequency of NS has been estimated to be between 1:1000-1:2500 live births.[2 3]

Using linkage analysis in a large three generation pedigree, we have previously mapped a gene for NS to an interval of more than 6 cM on 12q24 flanked by the markers D12S1637 and *NOS1*.[4 5] A similar analysis in smaller two generation families showed genetic heterogeneity for this disorder.[4] Despite the relatively high incidence of NS, there appears to be a distinct lack of large families suitable for linkage analysis, possibly resulting from an increase of infertility in males.[6] However, the location of the NS gene has recently been further refined to a 5 cM interval through the identification of additional recombinants in one additional large NS family.[7] No chromosome rearrangements associated with the disease have so far been discovered. In view of this, one approach currently being used to identify the underlying gene responsible for this disorder is examination of candidate genes from within this large region of chromosome 12. We present below the examination of four candidate genes, the precise localisation of three of which, epidermal growth factor receptor pathway substrate-8 (*EPS8*), decorin (*DCN*), and myosin light chain 2 (*MYL2*), had not previously been accurately determined. The fourth, ribosomal protein L6 (*RPL6*) was known to lie within the NS interval on 12q24.[8]

PCR was used to produce gene specific products for FISH (see below) and to produce exonic fragments for SSCP (see below). Sequence information from the cDNA clones of epidermal growth factor receptor pathway substrate-8 (*EPS8*) and decorin (*DCN*) were used to design primers for FISH. Primers used were GACAACTAACAGCATCCAGC (*DCN*-F), GGATTCCTACTTGCCTTGGA (*DCN*-R), CTTCCTTATTCTTGGTGT (*EPS8*-F), and CTCGAACTTGGGTCATTG (*EPS8*-R). The primers used for SSCP analysis of the *MYL2* and *RPL6* genes, and for the FISH of *MYL2* (exon 4 product) are shown in table 1. Thermocycling parameters were 96°C for five minutes, 35 cycles of 96°C for 30 seconds, 55°C (*DCN*) or 50°C (*EPS8*) for 30 seconds, and 72°C for 30 seconds, using 1.5 mmol/l $MgCl_2$. The primers for *DCN*, *EPS8*, and *MYL2* were produced from database sequences. Those for *RPL6* were derived from sequences determined by one of the authors.

The subchromosomal localisation of each gene was determined by hybridisation of fluorescently labelled PCR products to metaphase chromosome spreads.[9] PCR products for *DCN*, *EPS8*, and *MYL2* (exon 4 product) were labelled using the PCR Digoxigenin Probe Synthesis nick translation kit (Boehringer Mannheim). Conditions for hybridisation and immunofluorescent detection were performed according to the manufacturer's instructions.

Primers for SSCP analysis of genomic DNA were designed from intronic sequences such that the entire exon and flanking splice sites could be analysed (table 1). PCR conditions were optimised for each primer set and are available upon request. Amplified fragments were analysed for SSCP on a 30 × 40 cm gel containing 5% acrylamide, 0.25% bisacrylamide, with and without 10% glycerol in TBE (100 mmol/l Tris, 100 mmol/l boric acid, 2 mmol/l Na_2 EDTA, pH 8.3). Electrophoresis was performed at 30 W and 4°C.

EPS8 is highly conserved between species,[10] is widely expressed during mouse development,[11] and had previously been assigned to 12q24.[10] However, our FISH analysis localised the gene to chromosome 12p13.2 (fig 1). To confirm this localisation, the *EPS8* cDNA was used to screen a chromosome 12 specific cosmid library (Lawrence Livermore National Laboratory, kindly provided by Dr Sue Chamberlain). The positive clones obtained also hybridised to chromosome 12p13, confirming the localisation and exclusion of this gene (fig 1).

Through its ability to bind extracellular matrix constituents and growth factors, *DCN* is thought to play an impor-

Table 1 Oligonucleotide sequences flanking each of the exons of the MYL2 and RPL6 genes

	Forward 5'-3'	*Reverse 5'-3'*
MYL2		
Exon 1	CTCACCTATGACTGCCAAAAG	CCCTCGCTTGTAGTGGCTTC
Exon 2	CCCAGAGTAGGGGCCTGACCTAG	CCATCCAGGCGGATGATTCAATAG
Exon 3	CCAGGCTGAGCTGCCAATCAC	CATGCAGGGCTAGAGAGGGGT
Exon 4	CCCTGAGTGTGTGTTTCCTACCC	TTCTGCCAGCCCCCCCGAAGAA
Exon 5	CCCAGCCACCCCCAGTACATGT	CCCGAACGCTGCAGAGAAAGGA
Exon 6	GACACCAACACCTGCTTTCCTTTC	GGAGAACCAGGAGCTGGGTTAGAGG
Exon 7	CTTAGCACGTGTTGCTGGCTCA	CACTCTGCAAAGACGAGCCCA
RPL6		
Exon 1	CCGGCCTAGGATTTACTA	CTCAGTTAGCCTTGGACATG
Exon 2	TTGTTAGAGAGATGACTGGTG	CAATTAAGGTTAAGACATAATGG
Exon 3	CTTAATTGGCATTCTCTTACTG	TTCAAGCATAAACAGGAAATCC
Exon 4	GCTTCTAGTAATCTGAATGGC	GCAGCTGCAGTGAAGCGC
Exon 5	GATGCCTGTGATTTTATGAATTC	AAGTTTCACAGAACATCAC
Exon 6	CACCTAAATTGCAGGATGATG	CAGTGCTAACACAGGAGATG
Exon 7	AAGTAATTTGGTATGTGCCTG	AGTCAGCTATTTAATTAGGTTC

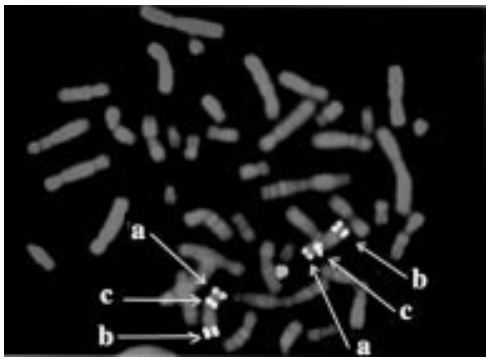

Figure 1 FISH using cosmid c62A3 that contains a fragment of the EPS8 gene. The cosmid (a) hybridised to the distal part of 12p. YACs 887b9 and 955d8 (b) flank the NS critical region at 12q24. A biotinylated 12 α satellite probe (ONCOR) was used as a marker for chromosome 12 (c).

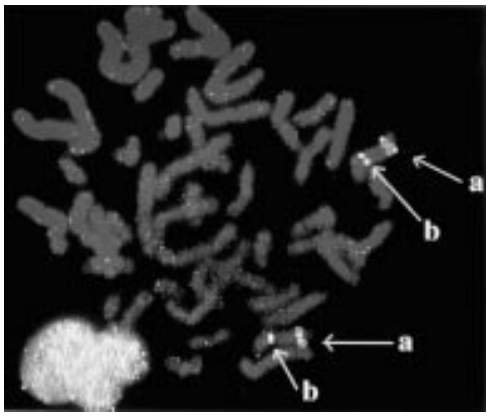

Figure 2 FISH using cosmid 91F7 that contains part of the MYL2 gene. The cosmid hybridised in the NS critical region between the YACs 887b9 and 955d8 (shown together as a). A biotinylated 12 α satellite probe (ONCOR) was used as a marker for chromosome 12 (b).

tant role in the remodelling and maintenance of extracellular matrices.[12 13] Two previous studies, both using radiolabelled in situ hybridisation, suggested different localisations for the *DCN* gene on chromosome 12 at bands 12q21-q22 and 12q23.[14 15] In view of its proposed function, *DCN* would be an excellent candidate for NS if it mapped within the interval. FISH clearly showed that the *DCN* gene maps at 12q13.2q proximal to both of the previous locations, and once again can be excluded as a candidate for NS.

While the genes described above were shown to be located outside the NS locus, this was not the case for the *MYL2* gene. *MYL2* has previously been assigned to chromosome 12q23-q24.3 by in situ hybridisation.[16] Although the precise function of the protein is not understood, MYL2 is known to be critical for the correct regulation of myosin ATPase activity in smooth muscle.[17] The non-muscle myosin II-B is known to be required for normal development of the mouse heart[18] and an increase in ventricular MYL2 has been observed during myocardial hypertrophy in patients with valvular stenosis. In addition, missense mutations within the *MYL2* gene have been identified in patients with a rare variant of cardiac hypertrophy,[17] an intriguing observation in view of the cardiac anomalies associated with NS. As a result of its position and putative function, *MYL2* was regarded as a strong candidate gene for NS.

Using a labelled *MYL2* gene fragment in conjunction with genomic clones that flank the NS critical interval, we were able to show that the *MYL2* gene overlaps the assignment of the NS gene at 12q24 (fig 2). Sequence information from the *MYL2* gene was used to design primers, which were used in SSCP analysis in 22 familial[4] and 44 sporadic NS patients. Primers were designed which flanked each of the seven *MYL2* exons including splice sites (table 1). Three band shifts were detected in these regions (data not shown). However, the same shifts were also seen with a high frequency in normal controls, or the corresponding change in the nucleotide sequence did not lead to an amino acid substitution, indicating that these changes represent normal polymorphisms. Sequencing showed one substitution at codon 44 (ATT to ATC) which does not result in an amino acid change, while the others were the result of variations in a GT repeat immediately 3' to exon 4. The absence of any pathogenic mutations in the coding regions of *MYL2* in any NS patients makes it unlikely that this is the causative gene.

In *Drosophila*, mutations in genes for the ribosomal proteins have been shown to cause the *minute* phenotype, which includes small body size, diminished fertility, and specific somatic abnormalities.[19–21] Furthermore, the ribosomal protein genes *RPS4X* and *RPS4Y* are discussed as "candidate" genes for Turner syndrome.[22–24] Turner syndrome and NS have short stature and webbing of the neck as common symptoms. Heart malformations, although of a different type, are also associated with both disorders. The human *RPL6* gene is located in the NS critical interval,[8 25] suggesting this gene as a candidate.

To check for possible mutations, the six coding exons of the *RPL6* gene as well as the preceding exon containing the 5'UTR were screened by SSCP analysis in the same subset of NS patients as used for the *MYL2* gene. The primers are shown in table 1. In exon 4, a point mutation was found in two unrelated affected subjects from small families with only two parents and two sibs. This mutation is predicted to cause the substitution of lysine, residue 139, for an asparagine (Lys139Asn). While the substitution cosegregates with NS in one family, it does not in the second, in which NS does not cosegregate with the critical region on chromosome 12. No mutations were found in the large family with NS linked to 12q24, and the Lys139Asn substitution was also seen in one out of 150 unaffected controls, showing this A to C transversion to be a rare polymorphism. As for *MYL2*, the analysis suggests that a role for *RPL6* in NS is unlikely. Mutations influencing the expression of these genes cannot yet be excluded as being causative for NS.

In summary, as part of a positional candidate cloning strategy to identify a gene for NS, we have examined a number of potentially interesting candidate genes on chromosome 12. Two were excluded by FISH, while two others located within the NS critical interval showed no causative mutations. In the absence of additional recombination events in NS families which can be unequivocally linked to chromosome 12, a screening strategy geared towards the identification of any chromosomal rearrangements within the NS critical interval is currently being used. In conjunction with this approach, the construction of a genomic contig encompassing the entire NS critical interval, and its sequencing, is also in progress.

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Hall-Riggs syndrome: a possible second affected family?

EDITOR—Definition of the clinical and genetic features of multiple congenital anomalies/mental retardation syndromes is a difficult task that requires identification of a specific phenotype in multiple patients in the general population and within families.

Figure 1 Facial dysmorphism of the two probands, case 1 on the right, case 2 on the left.

We report two sibs possibly affected by a rare MCA/MR syndrome, first observed by Hall and Riggs[1] in 1975. No other cases have been published since then.[2]

Case 1 is an 11½ year old female, the first born to healthy, non-consanguineous parents. Her younger male sib is case 2 of this report. She was the term product of a pregnancy complicated by threatened abortion during the first months and by intrauterine growth retardation. Birth weight was 2400 g, length 46 cm, and OFC 31 cm (all below the 3rd centile). Apgar scores were 7 and 9.

She has had feeding problems, failure to thrive, and severe developmental retardation. She walked unassisted at 6 years and she never achieved any language. Metabolic analysis, including amino acidaemia, amino aciduria, MPS screening, and lysosomal and peroxisomal enzymes has been negative.

Sialotransferrin, cholesterol, and 7-dehydrocholesterol were within normal limits. The EEG showed moderate multifocal irritative anomalies, without evidence of clinical seizures. MRI of the brain showed the presence of a large cyst in the septum pellucidum and a cavum vergae. The high resolution karyotype was normal, 46,XX.

Physical examination at 11½ years showed height 120 cm, weight 23 kg, and head circumference 47 cm (all <<3rd centile). She has severe microcephaly, hypertelorism, a flat nasal bridge, a large nose with a large nasal tip, and anteverted nostrils. The mouth is wide and carp shaped. Both the upper and lower lips are thick and everted, giving a coarse appearance to the lower part of the face (fig 1). The permanent teeth have not yet erupted and