Human Molecular Genetics, 2000, Vol. 9, No. 8 **1251–1255**

Identification of mutations in the *MSX2* homeobox gene in families affected with foramina parietalia permagna

Wim Wuyts+, William Reardon[1], Sabine Preis[2], Tessa Homfray[3], Alberto Rasore-Quartino[4], Hans Christians[5], Patrick J. Willems[6] and Wim Van Hul

Department of Medical Genetics, Universiteitsplein 1, 2610 University of Antwerp, Belgium, [1]Our Lady's Hospital for Sick Children, Dublin, Ireland, [2]Department of Pediatrics, Heinrich Heine University, Dusseldorf, Germany, [3]St George's Hospital, London, UK, [4]Department of Pediatrics and Neonatology, Galliera Hospital, Genova, Italy, [5]Neurology and Psychiatry Practice, Zweibrucken, Germany and [6]Department of Clinical Genetics, Erasmus University, Rotterdam, The Netherlands

Received 28 February 2000; Revised and Accepted 8 March 2000

Foramina parietalia permagna (FPP) is an autosomal dominant condition characterized by cranial defects of the parietal bones. It can be present as an isolated feature, but it is also one of the characteristics of a contiguous gene syndrome associated with deletions on chromosome 11p11–p12. One of the proteins known to be involved in skull development is the MSX2 homeobox protein. Previously, *MSX2* has been shown to be mutated in patients suffering from Boston type craniosynostosis. We have now analyzed the *MSX2* gene in five families affected with FPP. An intragenic microsatellite marker did not reveal any recombination and a cumulated LOD score of +3.2 at $\theta = 0$ was obtained. Sequence analysis further showed that in four out of five families an *MSX2* mutation was responsible for the skull defect. Moreover, it appears that FPP is caused by haploinsufficiency of the *MSX2* gene. This implies that Boston type craniosynostosis and FPP are allelic variants of the same gene, with FPP caused by loss of *MSX2* function and craniosynostosis Boston type due to gain of *MSX2* function.

INTRODUCTION

The *MSX2* gene is a member of the *MSX* homeobox gene family, a small family of homeobox genes related to the *Drosophila* gene muscle segment homeobox (*msh*) (1). At present, two human *MSX* genes (*MSX1* and *MSX2*) have been isolated (2–4), whereas three *Msx* homologues (*Msx*1-3) have already been identified in mouse (5–7). The MSX2 protein appears to play an important role in craniofacial development as is clearly illustrated by expression studies, showing expression at several key sites of the developing skull (1). Moreover, a specific P148H missense mutation in the MSX2 homeodomain has previously been found to cause Boston type craniosynostosis (4). Functional studies have revealed that this mutation affects the DNA binding properties of MSX2 through a stabilizing effect on the DNA–MSX2 binding, leading to a disease-causing gain-of-function (8). This is corroborated by the generation of transgenic mice that overexpress mutated or wild-type *MSX2* and show premature suture closure and multiple craniofacial malformations (9,10).

Another example of abnormal skull development is found in patients affected with foramina parietalia permagna (FPP), also referred to as the 'Catlin mark'. This malformation is characterized by the presence of ossification defects in the parietal bones of the skull (11). These openings, which show great variability among different patients, are mainly located symmetrically to the sagittal suture and their size normally decreases with time (12). Generally, these lesions are of no clinical significance, but on rare occasions the defects can be very large, requiring neurosurgical intervention. FPP can be inherited as an isolated autosomal dominant condition (11,13–16), or it can be present as one of the symptoms in a contiguous gene syndrome associated with microdeletions on the short arm of chromosome 11 (17,18).

We have now analyzed five families with FPP for the presence of mutations in the *MSX2* gene to investigate a potential role for this homeobox gene in this disorder.

RESULTS AND DISCUSSION

In this study, we examined five families affected with isolated autosomal dominant FPP (Figs 1 and 2), in order to identify the causal mutation responsible for this malformation. Potential linkage to the *MSX2* region on chromosome 5q34–q35 was investigated by the analysis of a previously identified dinucleotide repeat (MSX2GT) in intron 1 of the *MSX2* gene (4). No recombinants were observed in any of the five families, and despite the lack of information in some families, a total cumulative LOD score of +3.2 at $\theta = 0$ was obtained.

The *MSX2* coding sequence is contained within two exons, and we sequenced the entire coding region in two patients from every family. In two families a nonsense mutation was found in exon 1 (Table 1). In family 5 mutation of cDNA nucleotides 265 and 266 (with the adenine of the start codon designated +1) generates a premature stop codon (Fig. 3). The truncated

+To whom correspondence should be addressed. Tel: +32 3 820 2630; Fax: +32 3 820 2566; Email: wwuyts@uia.ua.ac.be

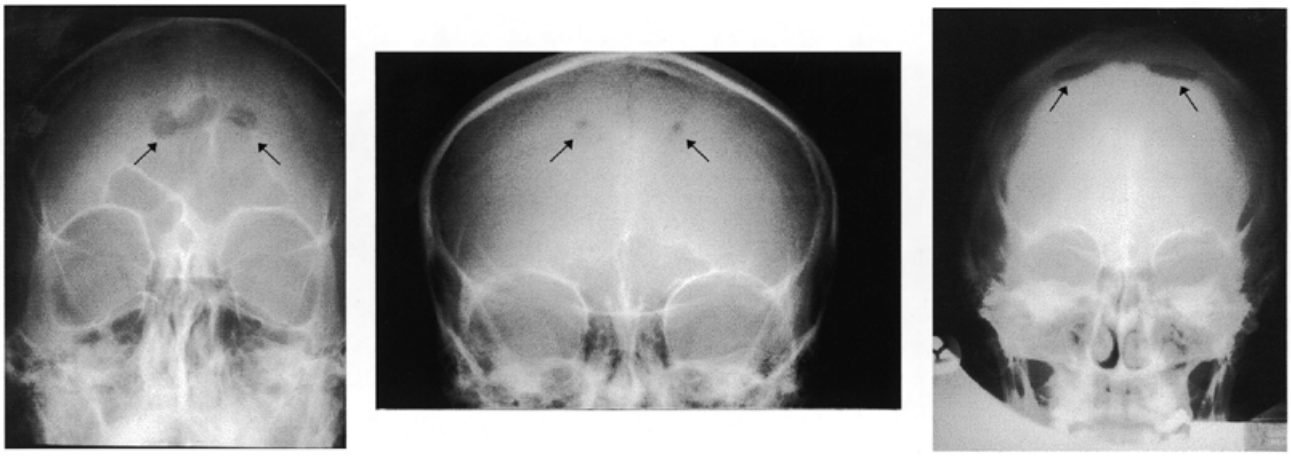

Figure 1. Skull X-rays illustrating the presence of FPP (arrows) in family 1 (left), family 2 (middle) and family 4 (right). Clinical details of families 3 and 5 have been published previously (14,22).

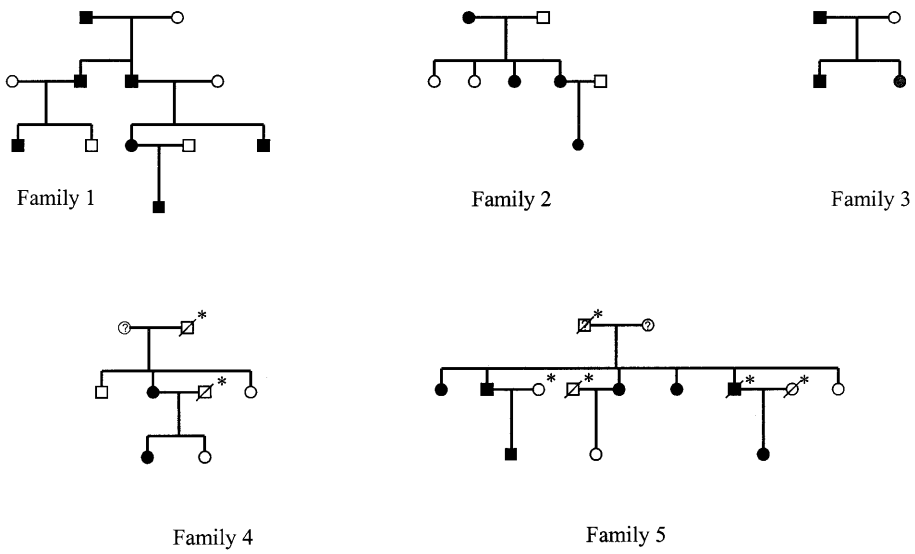

Figure 2. Pedigrees of the five FPP families included in this study. Filled symbols represent affected individuals, open symbols are unaffected individuals. DNA from individuals marked with an asterisk was unavailable for analysis.

MSX2 protein lacks the entire C-terminus containing the homeodomain and has therefore lost the function(s) associated with this conserved domain. Similarly, in family 3, a deletion of one cytosine at position 344 or 345 was identified, resulting in a premature termination of translation after 114 amino acids (Fig. 3). The identification of these inactivating mutations supports the hypothesis that FPP is probably caused by loss of function mutations in the *MSX2* gene, a theory that is corroborated by a recent finding of Wilkie *et al.*, reporting the identification of a large deletion comprising the entire *MSX2* gene in a family affected with FPP (19).

In family 1, a thymine to cytosine missense mutation in exon 2 (Table 1), resulting in a substitution of leucine by proline (L154P), was identified (Fig. 3). A second missense mutation, resulting in the substitution of arginine to histidine (R172H), was detected in family 2 (Fig. 3). The latter mutation is identical to one previously described by Wilkie *et al.* (19), but whether both families are related is not known. For both mutations, allele-specific restriction analysis showed cosegregation

Table 1. Mutations in the *MSX2* gene in FPP

Family	Origin	Location	Base change[a]	Effect
1	UK	exon 2	T461C	L154P
2	UK	exon 2	G515A	R172H
3	Germany	exon 1	344–345delG	W115X
4	UK	?	?	?
5	Germany	exon 1	[G265T];[C266A]	A89X

[a]The adenine of the start codon designated +1.
'?', not known.

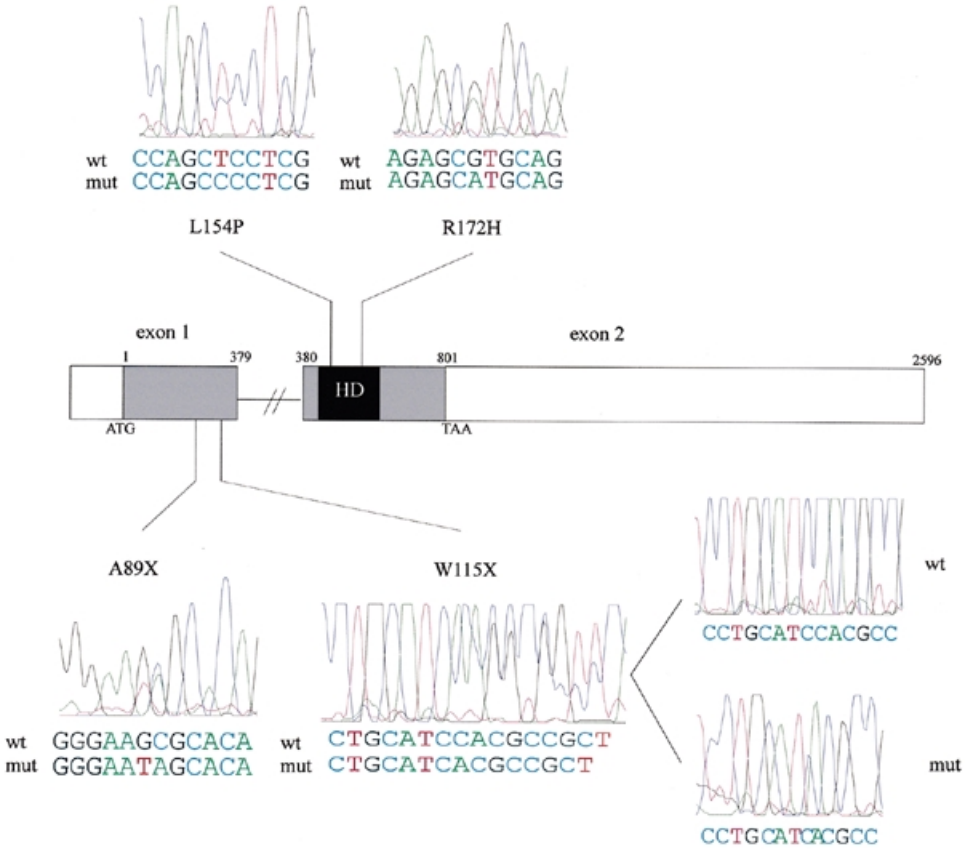

Figure 3. *MSX2* mutations in our FPP patients. Electropherograms of missense mutations in the homeodomain in families 1 and 2 are shown above, mutations in exon 1 in families 3 and 5 below the schematic representation of the *MSX2* gene. Both wild-type and mutant sequences are listed below each electropherogram. All sequences are shown in the forward direction with the exception of the W115X mutation in family 3, for which the reverse strand is shown. For this family, electropherograms of the subcloned wild-type and mutant allele are represented. Exons are drawn to scale with the coding region marked in gray and the homeodomain (HD) in black. The start and stop codon are indicated.

with the disorder in both families and an absence of these substitutions in 100 control chromosomes. The two substituted amino acids, leucine 154 and arginine 172, are located in the homeodomain and are highly conserved between MSX2 of several species from men to *Xenopus*, and between the MSX1 and MSX2 proteins (4), suggesting that they are crucial for proper functioning of the MSX2 protein. Previously, a P148H mutation in the MSX2 homeodomain has been shown to cause an autosomal dominant form of Boston type craniosynostosis (4). This is most intriguing, as craniosynostosis is due to the premature fusion of one or more sutures, whereas FPP is in fact almost the opposite, with a delayed or incomplete closure of the opening between the frontal and parietal bones. The homeodomains from different genes and organisms show highly conserved amino acid sequences and they form a similar three-dimensional structure with three α-helices and an extended N-terminal arm (20). The P148H missense mutation, which causes Boston type craniosynostosis, is located at position 7 in the N-terminal arm of the MSX2 homeodomain, a region specifically involved in DNA binding (20). As shown by the study of *MSX2* transgenic mice (9,10) and analysis of the DNA binding properties of mutant and wild-type *MSX2* (8), the P148H mutation causes craniosynostosis through a gain of function by increasing the stability of the mutant MSX2–DNA complex. The missense mutations identified in FPP families 1 and 2, however, are located in helix I and helix II, respectively. These helices are believed to stabilize the folded structure and to help maintain the relative orientation of the N-terminal arm and helices III, which makes direct contact with the DNA. The stabilizing properties of helices I and II depend on the helix structure and the ionic interactions between opposite charged residues of both helices, and therefore substitution in one of these helices can result in conformational and functional loss (21). It is therefore likely that the mutations in our FPP families have such a destabilizing effect on the MSX2 protein that it can result in loss of MSX2 function, although this has to be confirmed by functional analysis of the FPP-associated missense mutations.

No mutation was detected in family 4. Due to the small size of the family with only two affected patients, we can neither exclude nor confirm linkage to the *MSX2* region on chromosome 5q. Although *MSX2* flanking markers are heterozygous in both patients, we can not exclude a small deletion encompassing the *MSX2* gene with the intragenic marker MSX2GT being homozygous or hemizygous in both patients. We could not distinguish this family clinically or radiologically from those with a proven *MSX2* mutation, and therefore two possibilities remain open to explain the genetic cause for FPP in this family. The phenotype can be caused by a deletion involving the *MSX2* gene or by mutations located outside the *MSX2*

coding region, which we did not analyze. Secondly, the FPP can be caused by mutations in another gene located elsewhere in the genome. Evidence for genetic heterogeneity of FPP has already been provided since we have previously shown by deletion analysis of patients suffering from DEFECT 11 syndrome, that a gene for FPP must be located on 11p, in close proximity to the *EXT2* gene (17,18).

In conclusion, we provide strong evidence that the majority of FPP cases are due to haploinsufficiency of the *MSX2* gene. This corroborates the essential role of the MSX2 protein in the skull ossification process, as illustrated previously in transgenic experiments and functional analysis of the P148H mutation causing craniosynostosis (Boston type).

MATERIALS AND METHODS

Patients

Five families affected with FPP were included in this study (Fig. 2). Family 3 has been described previously by Preis *et al.*, and part of family 5 has been described by Schmidt-Wittkamp and Christians (14,22). The remaining three families originate from the UK. The diagnosis of all patients was based upon skull X-rays (Fig. 1).

Genetic linkage analysis

Linkage to the *MSX2* gene was investigated by PCR amplification of the intragenic MSX2GT repeat (4) using primers MSX2GT1 (5′-TccccTcTcAAcTgAAAgcAc-3′) and M13 reverse-tailed primer MSX2GT2 (5′-ggATAAcAATT-TcAcAcAggAcAcATAgTTTTgAcAAAgg-3′). Additional markers (D5S498, D5S2008 and D5S2030) (23) were analyzed in family 4. The PCR–amplification mixture (10 µl) contained dNTPs (10 mM each), 0.5 pmol of two specific primers (0.5 pmol) and an IRD800 labeled M13-reverse primer (5′-GGATAACAATTTCACACAGG-3′), 1× PCR buffer and *Taq* DNA polymerase. PCR conditions were 5 min at 96°C, 35 cycles of 1 min at 96°C, 45 s at 57°C and 45 s at 72°C, and finally 10 min at 72°C. Amplification products were analyzed on a LI-COR IRD800 detection system. Linkage analysis was performed using the LINKAGE 5.1 program. An autosomal dominant mode with 90% penetrance and a disease frequency of 1/25 000 was assumed.

Mutation analysis

Exons 1 and 2 of the *MSX2* gene were amplified with a GC-rich amplification kit (Clontech, Palo Alto, CA) according to the manufacturer's recommendations, using intronic primers MSX2ex1a-MSX2ex1b (5′-gcTgccgggTTgccAgcgg-3′ and 5′-ccgcTcccTccAgTAcccc-3′) to amplify exon 1 and MSX2ex2a-MSX2ex2b (5′-gTAAcTTTcTTTTgcTAATccg-3′ and 5′-TcgTggAgAgggAgAggAAAccc-3′) to amplify exon 2. For both amplifications 35 cycles were performed at a T_m of 52°C and GC melt concentration 1×. Amplification products were gel purified with Gel Extraction kit (Qiagen, Valencia, CA), and sequenced using BigDye terminator chemistry (Perkin-Elmer, Foster City, CA) on an ABI 377 automated sequencer.

Allele-specific PCR–restriction digestion

Confirmation of mutations in families 1, 2 and 5 was obtained by restriction analysis of exon 2 with restriction enzymes *Alu*I (family 1), or *Nla*III (family 2), and *Hha*I digestion of exon 1 (family 5). To confirm the W115X mutation in family 3 a modified primer MSX2mod1 (5′-ATcggccgggTTccTggATc-3′) was used, creating a new *Bam*HI restriction site only in the wild-type MSX2ex1a-MSX2mod1 amplification product. The amplification products were digested with *Bam*HI restriction enzyme. All restriction digests were analyzed on 12% acrylamide gels.

ACKNOWLEDGEMENTS

We thank patients and families for contributing to this project. W.W. is a postdoctoral researcher for the Fund for Scientific Research, Flanders (FWO). This study was supported by a concerted-action grant from the University of Antwerp to W.V.H.

REFERENCES

1. Davidson, D. (1995) The function and evolution of Msx genes: pointers and paradoxes. *Trends Genet.*, **11**, 405–411.
2. Ivens, A., Flavin, N., Williamson, R., Dixon, M., Bates, G., Buckingham, M. and Robert, B. (1990) The human homeobox gene HOX7 maps to chromosome 4p16.1 and may be implicated in Wolf-Hirschhorn syndrome. *Hum. Genet.*, **84**, 473–476.
3. Hewitt, J.E., Clark, L.N., Ivens, A. and Williamson, R. (1991) Structure and sequence of the human homeobox gene HOX7. *Genomics*, **11**, 670–678.
4. Jabs, E.W., Muller, U., Li, X., Ma, L., Luo, W., Haworth, I.S., Klisak, I., Sparkes, R., Warman, M.L. and Mulliken, J.B. (1993) A mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis. *Cell*, **75**, 443–450.
5. Robert, B., Sassoon, D., Jacq, B., Gehring, W. and Buckingham, M. (1989) Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis. *EMBO J.*, **8**, 91–100.
6. Hill, R.E., Jones, P.F., Rees, A.R., Sime, C.M., Justice, M.J., Copeland, N.G., Jenkins, N.A., Graham, E. and Davidson, D.R. (1989) A new family of mouse homeo box-containing genes: molecular structure, chromosomal location, and developmental expression of Hox-7.1. *Genes Dev.*, **3**, 26–37.
7. Wang, W., Chen, X., Xu, H. and Lufkin, T. (1996) Msx3: a novel murine homologue of the *Drosophila* msh homeobox gene restricted to the dorsal embryonic central nervous system. *Mech. Dev.*, **58**, 203–215.
8. Ma, L., Golden, S., Wu, L. and Maxson, R. (1996) The molecular basis of Boston-type craniosynostosis: the Pro148→His mutation in the N-terminal arm of the MSX2 homeodomain stabilizes DNA binding without altering nucleotide sequence preferences. *Hum. Mol. Genet.*, **5**, 1915–1920.
9. Winograd, J., Reilly, M.P., Roe, R., Lutz, J., Laughner, E., Xu, X., Hu, L., Asakura, T., vander, K., Strandberg, J.D. and Semenza, G.L. (1997) Perinatal lethality and multiple craniofacial malformations in MSX2 transgenic mice. *Hum. Mol. Genet.*, **6**, 369–379.
10. Liu, Y.H., Kundu, R., Wu, L., Luo, W., Ignelzi, M.A.J., Snead, M.L. and Maxson, R.E.J. (1995) Premature suture closure and ectopic cranial bone in mice expressing Msx2 transgenes in the developing skull. *Proc. Natl Acad. Sci. USA*, **92**, 6137–6141.
11. Goldsmith, W.M. (1922) The Catlin mark: the inheritance of an unusual opening in the parietal bones. *J. Heredity*, **13**, 69–71.
12. Murphy, J. and Gooding,C.A. (1970) Evolution of persistently enlarged parietal foramina. *Radiology*, **97**, 391–392.
13. Lother, K. (1959) Familiares vorkommen von Foramina parietalia permagna. *Arch. Kinderheilk.*, **160**, 156–168.
14. Schmidt-Wittkamp, E. and Christians H. (1970) Die lückenbildungen der scheitelbeine. *Fortsch. Gebiete Rontgenstr. Nuklearmed.*, **113**, 29–38.
15. Rasore-Quartino, A., Vignola, G. and Camera, G. (1985) Hereditary enlarged parietal foramina (foramina parietalia permagna). Prenatal diagnosis, evolution and family study. *Pathologica*, **77**, 449–455.
16. Zabek, M. (1987) Familial incidence of foramina parietalia permagna. *Neurochirurgia*, **30**, 25–27.

17. Bartsch, O., Wuyts, W., Van Hul, W., Hecht, J.T., Meinecke, P., Hogue, D., Werner, W., Zabel, B., Hinkel, G.K., Powell, C.M. *et al.* (1996) Delineation of a contiguous gene syndrome with multiple exostoses, enlarged parietal foramina, craniofacial dysostosis and mental retardation, caused by deletions on the short arm of chromosome 11. *Am. J. Hum. Genet.*, **58**, 734–742.
18. Wuyts, W., Di Gennaro, G., Bianco, F., Wauters, J., Morocutti, C., Pierelli, F., Bossuyt, P., Van Hul, W. and Casali, C. (1999) Molecular and clinical examination of an Italian DEFECT 11 family. *Eur. J. Hum. Genet*., **7**, 579–584.
19. Wilkie, A.O., Tang, A., Elanko, N., Walsh, S., Twigg, S., Hurst, J.A., Chrzanowska, K., Wall, S.A., Maas, R.L. and Maxson, R.E. (1999) Mutations of the homeobox gene MSX2 cause symmetric parietal foramina: contrasting effects of loss and gain of function mutations for skull development. *Am. J. Hum. Genet.*, **65**, A47.
20. Kissinger, C.R., Liu, B.S., Martin-Blanco, E., Kornberg, T.B. and Pabo, C.O. (1990) Crystal structure of an engrailed homeodomain-DNA complex at 2.8 Å resolution: a framework for understanding homeodomain-DNA interactions. *Cell*, **63**, 579–590.
21. Isaac, V.E., Sciavolino, P. and Abate, C. (1995) Multiple amino acids determine the DNA binding specificity of the Msx-1 homeodomain. *Biochemistry*, **34**, 7127–7134.
22. Preis, S., Engelbrecht, V. and Lenard, H.G. (1995) Aplasia cutis congenita and enlarged parietal foramina (Catlin marks) in a family. *Acta Paediatr.*, **84**, 701–702.
23. Dib, C., Fauré, S., Fizames, C., Samson, D., Drouot, N., Vignal, A., Millasseau, P., Marc, S., Hazan, J., Seboun, E. *et al.* (1996) A comprehensive genetic map of the human genome based on 5264 microsatellites. *Nature*, **380**, 152–154.