Letters to the Editor

Presymptomatic testing in myotonic dystrophy: genetic counselling approaches

Siv Fokstuen, Jenny Myring, Christine Evans, Peter S Harper

EDITOR—We report the genetic counselling approaches used in a series of 72 presymptomatic genetic tests for myotonic dystrophy undertaken in our centre over an eight year period. The study has identified factors which influenced the counsellor’s approach, and which can provide a basis for further, more systematic research.

Genetic counselling in myotonic dystrophy has always been difficult and complex, owing to the extreme variability of the disorder, in both severity and age at onset, with anticipation between generations and influence of the sex of the affected parent.

The identification of a CTG repeat expansion within the 3’ untranslated region of the myotonic dystrophy protein kinase gene on chromosome 19 as the primary molecular defect has transformed our understanding of the genetic aspects of this disorder and provides the basis for an accurate and specific diagnostic and presymptomatic test. The broad correlation of the size of the CTG expansion with age at onset and severity of the phenotype allows a limited degree of prognosis to be given to those found to have the mutation, particularly for very large or minimal gene expansions. Subjects carrying a minimal expansion (less than 100 repeats) usually show few or no muscle symptoms, but may develop cataract in later life; they contribute to a pool of mutation carriers who may transmit clinically significant disease to their offspring as a result of anticipation.

Presymptomatic genetic testing for late onset dominantly inherited disorders first became possible for Huntington’s disease (HD), for which extensive experience has resulted in widely accepted guidelines for genetic counselling protocols; these comprise two pre-test sessions for information and preparation combined with post-test support. With appropriate adaptation, this has become a model for other late onset genetic disorders of the nervous system and to some extent also for the familial cancers. Although direct presymptomatic testing for myotonic dystrophy has now been available for eight years, we are not aware of studies so far on the counselling approach for this disorder in relation to presymptomatic testing. This lack of knowledge has prompted the present study, in conjunction with experience of direct molecular testing as a service in our centre, reported separately.

Patients and methods
Since the identification of the specific gene mutation in 1992, up to June 2000, out of a total of 287 molecular analyses for the disorder in subjects living in Wales (population 2.9 million), there were 78 presymptomatic tests for myotonic dystrophy; by comparison 205 diagnostic tests on symptomatic subjects were performed and four prenatal tests. The laboratory methods used and the overall composition and outcomes of the series are described in the accompanying paper. For the purpose of the present study, we only included the 72 subjects seen for presymptomatic testing by 11 different clinical geneticists providing the clinical genetics service for Wales over this eight year period, based in the Institute of Medical Genetics, Cardiff, the remaining six samples having been received from neurologists and paediatricians. Data about the counselling approach were ascertained through the clinical genetics service notes and correspondence, supplemented by further discussion with the relevant clinical geneticist. We did not recontact any patients.

No single or specific counselling approach to presymptomatic testing in myotonic dystrophy has been advocated in Wales over this period, though one of us (PSH) has had a long standing clinical and research interest in the disorder. In general, subjects requesting presymptomatic testing were seen in their local medical genetics clinic by the clinical geneticist and genetics nurse specialist with designated responsibility for the particular district, not in a specialist clinic for the disorder.

Results
When grouped according to the perspective of the subject tested, three different presymptomatic test situations could be distinguished (table 1). The largest group of subjects (A, n=58) represented the classical presymptomatic test situation with persons at 50% risk.

Table 1 Presymptomatic testing for myotonic dystrophy: principal indications

<table>
<thead>
<tr>
<th>Presymptomatic test situation</th>
<th>Test results</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Direct request for presymptomatic testing</td>
<td>58 48 10</td>
</tr>
<tr>
<td>(B) Presymptomatic testing considered in relation to possible testing of ongoing pregnancy</td>
<td>5 5 0</td>
</tr>
<tr>
<td>(C) Testing of asymptomatic parent in relation to diagnostic investigation of child</td>
<td>9 3 6</td>
</tr>
<tr>
<td>Total</td>
<td>72 56 16</td>
</tr>
</tbody>
</table>
Table 2  Different approaches for presymptomatic testing used in Wales

<table>
<thead>
<tr>
<th>Approach</th>
<th>Total</th>
<th>Normal/abnormal molecular result</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Two stage approach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Blood taken at second genetic counselling session</td>
<td>7</td>
<td>7/–</td>
</tr>
<tr>
<td>B Offer of a two stage approach, rejected by the person at risk</td>
<td>2</td>
<td>1/1</td>
</tr>
<tr>
<td>(2) Intermediate approach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Blood taken by family doctor or genetic nurse specialist at interval after first genetic counselling session</td>
<td>3</td>
<td>3/–</td>
</tr>
<tr>
<td>B Blood taken at first genetic counselling session, stored until confirmation to proceed from patient.</td>
<td>5</td>
<td>5/–</td>
</tr>
<tr>
<td>(3) One stage approach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Blood taken at first genetic counselling session</td>
<td>46</td>
<td>35/11</td>
</tr>
<tr>
<td>B Blood taken by the genetic nurse specialist at home, genetic counselling clinic session only in case of an abnormal result</td>
<td>9</td>
<td>5/4</td>
</tr>
<tr>
<td>Total</td>
<td>72</td>
<td>56/16</td>
</tr>
</tbody>
</table>

Table 3 Relevant factors for counselling approach in presymptomatic testing for myotonic dystrophy

<table>
<thead>
<tr>
<th>Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Clinical assessment</td>
</tr>
<tr>
<td>Familiarity with disorder</td>
</tr>
<tr>
<td>Family dynamic</td>
</tr>
<tr>
<td>Subject’s perception of possible symptoms</td>
</tr>
<tr>
<td>Time pressure (pregnancy)</td>
</tr>
</tbody>
</table>

Ten proved to have an abnormal result. Two of them showed minimal clinical abnormalities at the time of testing, but did not regard their symptoms as abnormal. Patients with an ongoing pregnancy formed a separate subgroup (B, n=5). All five (four females, one male) were healthy subjects at 50% risk asking for a presymptomatic test during an ongoing pregnancy. Three of them wished for a prenatal diagnosis in case of an abnormal presymptomatic test result. Results proved normal in all of them. The other three (one couple and a mother) had lost a fetus with unexplained arthrogryposis and/or neuromuscular abnormalities and wanted to know the risk for a further pregnancy. All three were completely healthy at clinical examination and the test result was normal in all of them.

As summarised in table 2 and discussed below, three different counselling approaches to presymptomatic testing for myotonic dystrophy could be identified. The most common practice (n=46) was to take the blood sample at the end of the initial genetic counselling session. The factors which decided the choice of the approach are listed in table 3.

Discussion

There is a general consensus that presymptomatic testing for late onset, mendelian, genetic disorders should not be considered as a purely laboratory procedure, but that it should be linked to appropriate genetic counselling in order to achieve a proper foundation of information, preparation, and support. How this can be ensured will vary according to the disorder and a universal model cannot be applied. There is, however, a range of issues common to most genetic disorders of late onset. These issues include the implications of an abnormal result for reproductive decisions, the genetic implications to offspring and other relatives, consequences for future employment and insurance, strategies for coping with an abnormal result, and the availability of support from family, friends, and professionals. Most reported experience so far has come from Huntington’s disease, which represents an extreme situation regarding its severity and current lack of therapy. A two step approach has generally been recommended, both to allow the complex issues to be discussed fully and to give space to think about the testing process.

For myotonic dystrophy, however, no guidelines have been produced so far, and the approaches used have been based on individual patterns of clinical practice. One clear difference between the two disorders relates to the penetrance of the gene, since whereas in Huntington’s disease a considerable risk of serious disease exists for healthy subjects at risk who have passed 50 years of age, for myotonic dystrophy the great majority of offspring of an affected person will show definite clinical features of the disorder by early adult life, while most late onset cases are clinically mild. Brunner et al showed in a total of 139 clinically normal offspring of myotonic dystrophy patients that the residual risk of carrying the myotonic dystrophy gene mutation is approximately 8% between the ages 20 and 39 and a comparable risk (8.6%) was shown in a linkage based series. In the present series, the proportion of abnormal presymptomatic test results in clinically normal subjects (seven out of 69, 10.1%) was comparable. However, in only two such cases (males aged 19 and 37 years) was the abnormal expansion in the range likely to be associated with significant neuromuscular symptoms (>100 repeats). Thus, likelihood of a clinically normal myotonic dystrophy relative carrying a mutation significant for their own health is small, especially after the age of 40 years.

We can now consider in turn the different approaches to genetic counselling identified in this series (table 2). The factors listed in table 3 are among those particularly relevant to the approach that may be most appropriate.

The two stage approach, corresponding to that used for Huntington’s disease and comparable late onset neurodegenerative disorders, was used in only seven of the 72 cases, being offered but rejected in two others. Factors favouring this approach include unfamiliarity with the disorder and its consequences, complexities of family relationships and dynamics, and young age of the person to be tested, giving a greater likelihood that an abnormal result might indicate relatively severe disease. In general, we would advocate it in any situation where the person is uncertain about a decision to be tested and where the amount or complexity of new
information to be considered is too great to be given satisfactorily in a single session or where a period of reflection is needed.

In contrast to Huntington’s disease, where this complex situation is almost invariably present, this is not always the case for myotonic dystrophy, so we see no need for a two step approach to be recommended in all presymptomatic testing situations for myotonic dystrophy; equally, where it is suggested but declined, as occurred in two cases in our series, we see no reason why it should be insisted on.

In the great majority of presymptomatic tests in the present series (46 out of 72), a blood sample for analysis was taken at the end of the initial genetic counselling session. This approach seems particularly appropriate where the subject is familiar with the disorder, where there are no family complexities, or where their age (over 40 years) makes the likelihood of an abnormal result small and where even an abnormal result is unlikely to have major clinical significance. This last factor contrasts strongly with presymptomatic testing for Huntington’s disease, where the chance of an abnormal result remains high at a relatively advanced age and where the clinical consequences of the disorder are serious regardless of age at onset. In myotonic dystrophy older subjects detected as having clinically insignificant expansions should perhaps be considered gene carriers rather than affected patients.

In eight instances in this series, an intermediate approach was used (2A and B in table 2), where opportunity was given for reflection following the interview by either storing the blood sample until the person had confirmed that they wished testing to proceed (five cases) or by arranging for the blood sample to be taken after an interval by the family doctor or genetics nurse specialist (three cases). This would allow the family doctor or genetics nurse specialist to provide a useful perspective when the case does not clearly fall into either the two stage or single stage situations.

In five cases, the request for presymptomatic testing was made in the context of an ongoing pregnancy, with prenatal diagnosis being wished for in three of these. There was thus a situation of time pressure, making a full two step approach difficult even if considered desirable, a problem not infrequently met also in presymptomatic testing for Huntington’s disease. In each of these three cases, pregnancy was taken and a normal result so that no further action was required. In the two cases where prenatal diagnosis was not wished for, an intermediate approach as outlined above was used.

In nine cases in this series, blood was taken at a home visit by the genetics nurse specialist before the genetic counselling session attended, this only being arranged if the result was abnormal. This was considered appropriate at the time in cases where no special complexities were expected and it is likely that some unanticipated difficulties would have been detected had the home visit been omitted, allowing the sampling to be postponed. However, we would not now recommend this approach for a number of reasons. First, it gives no opportunity for clinical assessment before testing is undertaken, a factor of considerable importance in view of the high frequency of asymptomatic subjects at risk who show clinical abnormalities. Second, while some of the relevant issues would have been able to be discussed at the home visit, it is unlikely that a full picture of the potential consequences of testing would have been obtained in this way. Thirdly, by seeing only those subjects with an abnormal result in clinic, no opportunity was given to those with a normal outcome to discuss the disorder and its consequences for affected family members, something that might be relevant to them as a relative, even though not affecting themselves.

Two areas which require particular discussion are the role of clinical examination in the presymptomatic testing process and the importance of the subject’s perception as to whether testing was presymptomatic or diagnostic. Previous family studies have clearly shown that a considerable proportion of asymptomatic family members show definite clinical abnormalities on examination (17.6% in one early study). In the present study, some clinical abnormalities were present in nine of the 16 cases with an abnormal presymptomatic test result, in the total series of 78 cases (see accompanying paper for details). By contrast, the likelihood of an abnormal genetic test result in a carefully examined and clinically normal adult is low (around 8% in the studies discussed above). The figure of seven out of 78 cases in the present series is comparable. It can thus be argued that clinical assessment is likely to make a greater contribution to outcome than laboratory analysis and should always be undertaken before genetic testing. On the other hand, since some people request testing primarily for reproductive reasons and may not recognise the possibility of being affected, this possibility and the reasons for clinical assessment will need careful explanation before testing is performed, with the timing of the assessment depending on the individual situation.

A related important issue is the possible difference in perception of the person being tested and the clinician involved as to whether the testing is “presymptomatic” or “diagnostic”. We have considered as presymptomatic in our series all those cases who had no complaints, even though abnormal or suspicious clinical features might have been present at the time of testing. When the referring clinician has recognised these features it is possible that the test situation may be handled as a diagnostic confirmation. A particular issue in myotonic dystrophy exists for the mothers of children suspected of having congenital myotonic dystrophy, who may be tested as part of their child’s diagnosis, whereas for the mother, the situation is likely to be presymptomatic. Example 1 illustrates this.

Example 1 illustrates the difficulty in disclosing the diagnosis in minimally affected subjects with no subjective complaints and who are not familiar with the disorder. The reaction of the mother clearly shows that she was caught in a dilemma and did not have enough space to understand what was going on or to think about
Letters

Example 1
A 3 year old boy, accompanied by his mother, was referred by a paediatrician for evaluation of a possible genetic cause for his unexplained motor difficulties. The mother wanted to know the diagnosis in her son and the risk for further children. The family history was unremarkable. Clinical examination of the son showed reduced muscle tone and slight developmental delay. The mother had grip myotonia on examination, but had not previously regarded it as abnormal. She stated that she and her son slept without fully closing their eyes. For the clinical geneticist involved, the diagnosis of myotonic dystrophy was clear. A possible clinical connection between the symptoms of both were discussed and blood was taken for molecular testing for myotonic dystrophy from the mother only. At the second appointment, the test result, which confirmed the diagnosis in the mother, was discussed. The mother was very upset about the result and a pause for reflection was necessary before testing the son. At the third appointment, all medical and reproductive implications were discussed in detail and blood was taken from the son for molecular analysis. The result showed an abnormal expansion.

her own health. The diagnosis became shifted towards herself although the clinical request had concerned her son. For the clinician, the mother’s situation appeared diagnostic. The view of the authors, however, is that as such people consider themselves healthy, a presymptomatic rather than diagnostic approach seems more appropriate. This means that there is a need for information and counselling before molecular testing. It seems appropriate to answer first the question of the diagnosis in the child and postpone the blood sampling in the mother until she is informed, prepared, and supported.

No instance of presymptomatic testing of a healthy young child was recorded in our series, the youngest being aged 16 years. In general, the policy recommended for late onset disorders was followed with full discussion, clinical assessment where required, but with postponing of genetic testing until an age was reached where the implications of testing could be understood and consent given.

In general, this policy was understood and supported by parents, but in a small number of cases a strong and persistent wish for testing of a healthy child was expressed, as outlined in example 2.

Example 2 shows how perceived barriers can interfere with full appreciation of the issues involved in a difficult situation. One possible explanation of the outcome might be that removal of the perceived barrier allowed a fuller understanding of the potential problems, even though these had been repeatedly explained on previous occasions.

A final counselling issue requiring mention relates to the potential need for disclosure of genetic test results in relation to insurance, a topic of considerable debate in the UK and elsewhere. Although most of the period covered by our retrospective study predated the emergence of this issue, it is now a topic that requires discussion with people before testing. However, the small number of subjects in the series (two out of 78) who were clinically normal, yet had an abnormal test result of clinical significance to themselves, suggests that there is little need for insurers to have access to such results, and that a normal clinical assessment is a more relevant factor.

In conclusion, the counselling approach in relation to presymptomatic testing for myotonic dystrophy needs to be flexible, so as to respond to the variable and complex issues that may arise. Adherence to a two stage process does not seem required for the majority of situations, but each instance requires careful consideration and at least one full interview giving opportunity for detailed discussion of the issues would seem essential. While the retrospective nature of the present study makes it unwise to draw definitive conclusions, the issues raised should provide the starting point for further, more systematic study, as well as giving a general framework that may be useful for those involved with presymptomatic testing for this important and exceptionally variable condition.
We thank all the clinical staff of the Institute of Medical Genetics for permission to report information on their patients and, in particular, Professor Angus Clarke and Dr Helen Hughes for helpful discussions. Sin Fokstuen was supported by a grant from the Swiss Academy of Medical Sciences.


Trinucleotide repeat contraction: a pitfall in prenatal diagnosis of myotonic dystrophy

Jeanne Amiel, Valérie Raclin, Jean-Marie Jouanno, Nicole Morichon, Hélène Hoffmann-Radvanyi, Marc Dommergues, Josué Feingold, Arnold Munnich, Jean-Paul Bonnefont

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Editor—Myotonic dystrophy (DM) is a common autosomal dominant disorder characterised by myotonia, muscle weakness, ECG abnormalities, cataracts, hypogonadism, and frontal balding in the typical adult form (MIM 160900). The genetic defect consists of the amplification of an unstable CTG trinucleotide repeat in the 3′ untranslated region of the dystrophia myotonica protein kinase gene (DMPK), which maps to 19q13.3.2 Normal subjects have five to 37 repeat copies while affected subjects have over 50 repeats.3 There is some correlation between repeat length and clinical symptoms, especially with respect to the age at onset.4–6 In the vast majority of cases, the number of repeats increases during parent-offspring transmission of the mutant allele, thus providing some molecular basis to the observation of anticipation (increased severity of the disease in successive generations).7 8 However, a decrease in repeat size is occasionally observed in the offspring, mostly in the case of paternal transmission of an expansion of over 600 trinucleotide repeats,7 but contraction of a parental expanded repeat back to the normal range when transmitted to offspring seems to be an extremely rare phenomenon.9 10 Here we report such a case and emphasise the direct impact of this situation on prenatal diagnosis (PND) of DM.

Material and methods

PatiEnts

DM was diagnosed in II.2 (fig 1), who presented with mild atrophy of the head and neck muscles, myotonia of the hands, and frontal balding at the age of 37 years, while his first wife (II.1) was pregnant. The sister of patient II.2 (II.4) was more severely affected, with marked impairment of walking from the age of 43 years. Their father, I.2, had no symptoms of DM at the age of 61 years. III.1, the first offspring of II.2, was severely affected with muscular weakness and mental retardation. II.2 was first referred to our unit during the pregnancy of his second wife (III.3) for first trimester PND.

Methods

DNA was extracted from leucocytes of II.2 and II.3 and from CVS performed at 11 menstrual weeks. Study of the CTG repeat size was carried out by Southern blotting (EcoRI/PM10M6 and PstI/PM10M6)13 and PCR amplification with primers flanking the CTG repeat in parental and fetal DNA.14 Poly (CA) microsatellite markers were used both for linkage analysis at the DM locus and to rule out false paternity (D19S223, D19S412, D19S606, D19S596, D19S879, D20S194, D12S878, with heterozygosity of 81, 80, 81, 53, 76, 91, and 91% respectively, data available through Genebank).

Results

The healthy mother (III.3) had two alleles of 10 CTG repeats, while the father (II.2) displayed a wild type allele of 13 CTG repeats and a mutated allele of approximately 200 CTG repeats (figs 2 and 3). The DM allele in I.2, II.4, and III.1 had previously been estimated as approximately 60, 400, and 600 CTG repeats in size, respectively (data not shown for II.4 and III.1). PCR amplification of the CTG repeat region from the CVS DNA showed two normal alleles, a 10 trinucleotide repeat allele inherited from the mother and a 30 trinucleotide repeat allele not found in the father. Fetal DNA testing by Southern blotting failed to detect any expanded allele (fig 3). Results of the CVS DNA analysis were confirmed both by
PCR amplification of the CTG repeat region and Southern blotting of DNA extracted from cultured amniocytes. Linkage analysis in I.2, II.2, II.3, and III.3, using poly (CA) microsatellite markers flanking the DMPK gene, showed that the fetus had inherited the paternal DM allele (fig 1). The probability of false paternity was assessed to be less than 10^-5. The parents were informed about this unusual situation implying some uncertainty regarding the fetal status and subsequently decided to continue the pregnancy.

Discussion

Here we report on the contraction of a large expanded DM allele to the normal range during a father-offspring transmission. To our knowledge, this is the first case where contraction of a parental expanded allele back to the normal range has been detected during the prenatal period. No somatic mosaicism could be identified in either choriocytes or amniocytes. A recombination event at the DM locus appears unlikely, based on both haplotype analysis and PCR amplification of the CTG repeat, indicating that the fetus did not inherit the paternal wild type DM allele (fig 1). Such a contraction of a DM allele back to the normal range has seldom been reported. In one case, a discontinuous gene conversion between the wild type and the DM allele was shown. In the other three cases, as in the case reported here, this mechanism appears unlikely, because of the different numbers of CTG repeats in the father/offspring wild type alleles. A contraction of the DM allele or a double recombination disrupting the CTG repeats is a possibility. These events could be either meiotic or early mitotic. It is worth mentioning that in all cases reported to date, the contraction event was paternal in origin, making the hypothesis of a meiotic event more likely. In one case, long term follow up established that the offspring remained asymptomatic at or beyond the age of disease onset in the transmitting parent, also favouring the hypothesis of a meiotic event. Understanding when and how the contraction event occurs would be of importance to appreciate both the risk for the children of developing the disease and their own risk of transmission of DM to their offspring.

A partial reduction in size of a trinucleotide repeat above the normal range during parent-offspring transmission seems to be far more frequent than a reduction back to the normal range. In a large series of 1489 DM parent-offspring pairs reported by Ashizawa et al, a partial reduction was noted in 6.4% of cases. Such a contraction was more frequently observed in paternal than in maternal transmission (10% versus 3%, respectively). In these cases, the size of the parental DM allele varied from approximately 500 to 1500 CTG repeats. Interestingly, the observed number of sibships whose members had inherited a contracted CTG repeat was greater than expected. This could argue either for a predisposition to reduction during transmission of an expanded allele in some subjects or for negative selection against sperm carrying the largest CTG expansions. This last hypothesis has been raised in FRAXA, where males carrying a full mutation in their somatic cells transmit only premutated alleles to their daughters (MIM 309550). While expansion of a mutated DM allele during parent-offspring transmission is almost invariably associated with clinical anticipation, the rare events of contraction raise difficult issues with respect to PND and genetic counselling in DM. In the large series of Ashizawa et al,
Figure 3  Southern blotting (EcoRI/ PM10M6) in patients III.3, II.3, I.2, and II.2. The expanded allele in II.2 is not found in his offspring II.3.

approximately half of the offspring who inherited a contracted allele showed clinical anticipation despite the reduced CTG repeat size. In all these cases, however, the size of the contracted allele remained above the normal range. Moreover, Southern blot analysis showed some overlap between the boundaries of the “smear” in some parent-offspring pairs. Conversely, in the four cases where the transmitted DM allele reverted to the normal range, the clinical phenotype seemed to be normal, taking into account the absence of data regarding long term follow up in these cases.9–12

Taken together, these data strengthen the well known fact that direct analysis of fetal DNA should be used as the primary approach in PND, since a reliable prediction of the seriousness of the phenotype cannot be based upon haplotyping using polymorphic markers linked to the DMPK locus. Moreover, the detection of a contraction event in a fetus by Southern blotting warrants further molecular investigations in order to assess the size of the CTG repeat accurately. Indeed, while detection of a DM allele remaining above the normal range does not preclude clinical anticipation, the observation of a contracted allele back to normality should allow reassurance of couples at risk for transmitting DM.


Psychological studies in Huntington’s disease: making up the balance

Magdalena Duisterhof, Rutger W Trijsburg, Martinus F Niermeijer, Raymund A C Roos, Aad Tibben

EDITOR—Huntington’s disease (HD) is an incurable neurodegenerative disease, characterised by involuntary movements, changes in behaviour and personality, and cognitive impairment, leading to death 15 to 20 years after its onset.1 HD is an autosomal dominantly inherited disorder, the gene for which is localised on the short arm of chromosome 4.2 Subjects carrying the gene will develop the disease in the absence of other causes of death. The mean age of onset is 40 years, by which time gene carriers may have passed on the gene to their offspring. The age of onset ranges from 2 to 75 years3 so that those at risk (that is, risk carriers at 50% or 25% genetic risk) can never be sure of having escaped HD. Since 1986, presymptomatic DNA testing using genetic linkage analysis has made it possible for risk carriers to have their risk modified to approximately 98% or 2%. After
identification of the HD gene mutation in 1993, CAG repeat size analysis of the huntingtin gene allowed complete certainty of either having or not having HD. Risk carriers, being raised in a family in which HD played a major role, could be expected to have specific adjustment problems. Yet, only one study addressed the psychological functioning of people at risk for HD before presymptomatic testing was introduced. Most psychological studies were started when clinicians and researchers became concerned about the effects of a presymptomatic test on people at risk.

The aim of this article is to review studies addressing psychological and psychiatric adjustment of people at risk for HD. The methods used by the studies (that is, objectives, inclusion and exclusion criteria, recruitment, assessment, design, and statistical analyses) and their results are presented. General trends and limitations of the present work are described and a direction for future research is presented.

The term “carriers” is used to designate all subjects who underwent linkage or mutation analysis and were found to have an increased risk or were identified with a pathological repeat length of the IT15 huntingtin gene. The term “non-carriers” denotes those with a decreased risk result or those having a normal repeat size of the IT15 gene.

Methods
A search of published reports was conducted in the MEDLINE and PsycLIT databases using the keywords “Huntington’s disease”, “psychological”, “psychiatric”, “predictive testing”, “adjustment”, and “family”. Cross references in identified papers were also used. Quantitative studies on the psychological wellbeing of those at risk were included; this could be conducted by questionnaire or by interview. Studies addressing attitudes are included when they indirectly refer to wellbeing in the pre- and/or post-test period. Case descriptions or clinical impressions were excluded from this analysis as well as neurological and pharmacological studies.

Results
A total of 18 articles provided a quantitative analysis on the wellbeing of subjects at risk for HD. Characteristics of the studies are summarised in table 1.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample size</th>
<th>Carrier/ non-carrier/ uninformative*</th>
<th>Mean age</th>
<th>Measurement time</th>
<th>Objective</th>
<th>Statistical methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boston</td>
<td>15</td>
<td>4/7/5</td>
<td>—</td>
<td>Baseline, 3 mth, 9 mth</td>
<td>Description</td>
<td>Percentages</td>
</tr>
<tr>
<td>Vancouver</td>
<td>51</td>
<td>NA</td>
<td>39.3</td>
<td>Before test</td>
<td>Course</td>
<td>Percentages</td>
</tr>
<tr>
<td>Wiggins et al†‡</td>
<td>135</td>
<td>37/58/40</td>
<td>37.5</td>
<td>Baseline, 1 wk, 6 mth, 12 mth</td>
<td>Prediction, description</td>
<td>ANOVA, Kruskal-Wallis test, chi-square test</td>
</tr>
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<td>Baltimore</td>
<td>112</td>
<td>NA</td>
<td>26.7</td>
<td>Baseline, 1 mth, 6 mth, 9 mth, 12 mth</td>
<td>Description, baseline, course</td>
<td>Percentages, chi-square test</td>
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<tr>
<td>Brands et al</td>
<td>55</td>
<td>12/30/13</td>
<td>35.4</td>
<td>Baseline, 1 mth, 6 mth, 9 mth, 12 mth</td>
<td>Description</td>
<td>Percentages</td>
</tr>
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<td>Folstein et al 11</td>
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<td>—</td>
<td>Baseline, 3 mth, 6 mth, 9 mth, 12 mth</td>
<td>Clinical impressions</td>
<td>Description</td>
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<td>Codori and Brandt</td>
<td>68</td>
<td>17/51</td>
<td>37.7</td>
<td>8 visits in 3 y after test</td>
<td>Prediction</td>
<td>Percentages</td>
</tr>
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<td>Codori et al</td>
<td>160</td>
<td>52/108/—</td>
<td>34.3</td>
<td>Baseline, 3 mth, 6 mth, 9 mth, 12 mth</td>
<td>F tests</td>
<td></td>
</tr>
<tr>
<td>Rotterdam/Leiden</td>
<td>18</td>
<td>9/9</td>
<td>35.9</td>
<td>12 mth</td>
<td>Description, comparison</td>
<td>Percentages, clinical impressions</td>
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<td>Tibben et al</td>
<td>63</td>
<td>29/44</td>
<td>31.6</td>
<td>Baseline, 6 mth</td>
<td>Prediction</td>
<td>Backward regression analysis</td>
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<td>63</td>
<td>24/39</td>
<td>31.6</td>
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<td>Percentages</td>
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<td>73</td>
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<td>Course</td>
<td>MANOVA</td>
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<td>Tibben et al</td>
<td>49</td>
<td>20/29</td>
<td>32.2</td>
<td>Baseline, 1 wk, 6 mth, 3 y</td>
<td>Course</td>
<td>MANOVA</td>
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<td>DudokdeWit et al†‡</td>
<td>25</td>
<td>9/16</td>
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<td>Baseline, 6 mth</td>
<td>Prediction</td>
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<td>Leuven</td>
<td>53</td>
<td>22/31</td>
<td>34</td>
<td>Baseline, 1 mth, 12 mth</td>
<td>Prediction, course</td>
<td>Multiple regression analysis, t tests</td>
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<td>19</td>
<td>5/14</td>
<td>36.9</td>
<td>Baseline, 3 mth, 6 mth, 9 mth, 12 mth</td>
<td>Comparison of groups</td>
<td>Mann-Whitney U test</td>
</tr>
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</table>

*Genetic status not assessable in linkage test.
†‡§Same population.
NA: not applicable.
other major psychiatric illness, or, by history, being at risk for suicide (Baltimore Group, USA,23 Boston, USA,24 Indianapolis, USA,24 Leuven Group, Belgium,25 Rotterdam/Leiden Group, The Netherlands,26 Vancouver Group, Canada27). In the study by Meissen et al,13 secondary exclusion criteria were: a recently experienced stressful event, moderate depression, a suicide attempt more than 10 years before testing, or a family history of suicide.11 The Leuven group included risk carriers with a psychiatric history, provided that social support was available and that the risk carriers were receiving psychiatric treatment (M Decruyenaere, 1999, personal communication).

Results of reviewed studies
STUDIES UNRELATED TO PRESYMPTOMATIC TESTING
Children of HD patients had a high rate of psychiatric disorder (25% conduct disorder or antisocial personality disorder, 18% major depression).11 Most conditions (anxiety and depression) were mild or occurred only in adolescence (conduct disorder).17 Introversive  

Abbreviations are shown in the Appendix.
Students of psychology, extraversion and neuroticism were similar to those in the general population.15

DESCRIPTIVE STUDIES RELATED TO PRESYMPTOMATIC TESTING

Psychological wellbeing of test applicants before disclosure of test result

The mean scores of psychological wellbeing and Huntington specific distress before disclosure of the test result (baseline level) fell within the normal range.4 5 7 9 19–21 In the Dutch studies,10 21 the mean scores of risk carriers indicated mild signs of hopelessness; this could not be confirmed in other studies.7 15 Approximately 20% of the risk carriers scored at mild levels of depression and hopelessness, whereas very few scored at the level of moderate or severe depression.7 For about 20% of the risk carriers, their scores on the GHQ-60 indicated the possible presence of psychiatric morbidity.16 20

Most test applicants had a normal psychological profile.11 In comparison to the general population, they were more socially extraverted, had higher ego strength, and reacted more with active coping, palliative coping, social support seeking, and comforting ideas. Later identified carriers and non-carriers did not differ in general wellbeing and Huntington specific distress.5 8 15 19–21

Course of psychological wellbeing after the test result

General measures of psychological wellbeing and Huntington specific distress

Analysis of distress in identified gene carriers at seven days post-test showed more depression, hopelessness, and a decrease in general wellbeing.8 9 19–21 However, their mean scores remained in the mild range. A return to baseline levels of anxiety and depression occurred in the first month.8 Hopelessness, depression, and general wellbeing returned to baseline level within six months.19 21 and remained there one and three years post-test.8 20 21 Although not differing from baseline, Wiggins et al.16 found linear declines for distress and depression over a 12 month period. Only Brandt et al.17 reported a slight increase in general distress after one year. However, because the dropout rate in their sample was extremely high (75%), this finding should be interpreted with caution.

The non-carriers were more optimistic regarding their future at seven days post-test; however, this more positive view of the future disappeared after six months and three years.18 20 On the other hand, anxiety and depression decreased from baseline one month and one year after the test result.7 Also, general distress, assessed by means of the GSI index, decreased in the first year after the test result.7

In comparison to carriers, non-carriers reported less general distress, less depression, less hopelessness, and a greater sense of wellbeing one week after the test result.16 21 This difference disappeared in the first year. At six months follow up, only general wellbeing was significantly greater for the non-carriers, but this difference disappeared at 12 months up to three years after the test result, returning to baseline level.21 31 Only Quaid and Wesson15 found a higher general wellbeing for carriers than non-carriers after 12 months.

In comparison to a “no change” group, consisting of 23 subjects who did not want to take the test and 17 subjects for whom the test was uninformative, both carriers and non-carriers scored lower for depression and higher for wellbeing.15 However, it cannot be inferred from these findings that testing has benefits, since particularly an uninformative result can lead to an increase in distress, the wish for certainty about carrier status being frustrated. A subgroup of both carriers and non-carriers had difficulties adjusting to their new carrier status. About 10-20% of both carriers and non-carriers showed psychological problems in the post-test period.15 19 20 21 32 35 Interviews with carriers, three months after the result, indicated that half of the carriers had periods of severe depression, whereas the other half had suffered moderate depression.14 Therapists identified a minority of carriers and non-carriers as having psychiatric symptoms in the first year after the test.7 However, very few people committed or attempted suicide or needed psychiatric hospitalisation after predictive testing.34

With regard to Huntington specific distress, carriers showed a slight increase of avoidance behaviour in the first six months, which returned to baseline level after three years.20 Non-carriers showed a decrease in avoidance during the first six months post-test,19 21 which returned to baseline level at the three year follow up.7 10 For both groups, intrusive thoughts decreased in the first six months,15 whereas these increased to baseline level at the three year follow up.20

The observations by Lawson et al.31 underline the general impression that both carriers and non-carriers have problems in adapting to the test result, but at different moments in time. The number of adverse events was similar for carriers and non-carriers. For the carriers, adverse events took place within 10 days after the test result, whereas for non-carriers adverse events occurred six months after the result or later. Seventy percent of these events were identified by clinical criteria, that is, suicidal ideation, depression lasting longer than two months, substance abuse, or a breakdown of an important relationship, either alone or in conjunction with a raised score on one or more questionnaires.15

Attitudinal studies regarding the test result

Before a test result was given, risk carriers expressed concern about the future and guilt about the possibility of passing on the gene.4 After six months, half of the carriers stated that the results had not influenced their lives and half of them also rarely thought of the result, indicating that denial plays a role.18 The non-carriers expressed relief in the first weeks after the test result was given, but after six months half of the non-carriers appeared to
deny the impact of the test result, as was reflected by absence of relief and emotional numbness. Some of them have expressed survivor guilt. Compared to presymptomatic testing protocol. In general, wellbeing of the group of test applicants was normal before test disclosure. Both carriers and non-carriers had difficulties in adapting to the test result, but at different moments in time. Distress in carriers increased in the first weeks post-test, which returned to baseline level within one year. The relief non-carriers expressed in the first weeks disappeared afterwards; they experienced most distress at six months. Within one year, non-carriers seemed to be somewhat less distressed than they were before test disclosure, but they had not developed more optimistic future expectancies.

A subgroup of both carriers and non-carriers had long lasting adaptation problems. Those reporting to be distressed before test disclosure most often had problems in adapting to the test result. Although wellbeing seemed to be independent of test outcome, wellbeing was

Comparison of at risk population and partners
At baseline, spouses reported more depression than their at risk partners, whereas hopelessness was comparable for carriers and their partners. During the three year follow up, carriers and their partners showed similar patterns of avoidance, intrusion, and hopelessness, whereas non-carrier partners reported less avoidance, intrusion, and hopelessness than the non-carriers. After three years, partners of carriers were still showing more avoidance than partners of non-carriers. In contrast, Quaid and Wesson found comparable distress for carrier partners and non-carrier partners in the first year after disclosure of the test result. Whereas distress was similar for carriers and their partners, their attitudes towards the test result differed. Carriers did not report an increase in problems after they received an unfavourable test result. Their partners did mention having problems, but expressed reluctance to seek help or to talk about it with their spouse, owing to feelings of guilt and not wanting to hurt them. This was especially the case for those who became aware of the risk for HD at a later stage, for example, after marriage. For the non-carriers, most of them did not experience relief, whereas their partners did.

Having children proved to be an additional stress factor for partners during and after the test procedure. At baseline, partners with children were significantly more hopeless than partners without children. One week after the test, carrier partners with children reported significantly more hopelessness, avoidance thoughts, and intrusive feelings than carrier partners without children. At six months and three years after the test, this difference in avoidance thoughts and intrusive feelings was sustained.

Prediction of wellbeing
Five studies aimed to identify pre-test variables that predict the way subjects adapt to their test result (for the variables see table 3).

Test result
In general, test outcome did not predict psychological adjustment. Only Codori et al found carriers to be more likely to be pessimistic about their future than non-carriers.

General and Huntington specific distress
The level of psychological adaptation after the test (anxiety, depression, hopelessness, intrusion, and avoidance) was predicted by the same measures at baseline. The more depressive symptoms reported at baseline, the more distress subjects reported at the one year follow up, and the greater the chances that they were rated as having experienced an adverse event, as defined by Lawson et al. Pessimism, a low avoidance, and dissatisfaction with available support at the moment of testing predicted pessimism at six months. Those severely anxious before the test were more likely to show low intrusion six months after disclosure.

Biographical variables
Having children predicted post-test intrusion and hopelessness. Women showed more intrusion and avoidance than men six months after disclosure of the test result. For carriers, being married or having children predicted hopelessness, as did the estimated years to onset of HD.

Social support
Subjects who were satisfied with the perceived quality of support of others felt less hopeless after either test result. The more pre-test avoidance and the less satisfaction with available support, the more avoidance behaviour was reported six months post-test. However, the larger the number of persons perceived as being supportive before the test, the more avoidance was reported post-test.

Risk perception
Risk perception refers to the expectations one has about the test result. No support was found for the hypothesis that for identified carriers, those with a low perceived risk of being a carrier would have a less favourable adjustment than persons with a high perceived risk.

Personality measures and coping strategies
Ego strength was associated with a lower general anxiety and depression level one year after the test. Moreover, ego strength in combination with the coping strategy “comforting ideas” predicted a lower general anxiety.
related to having children, certain personality traits (ego strength, coping), and the subjective estimation of the number of years before onset of HD.

These findings have been shown to be helpful in guidance and counselling of risk carriers in testing programmes. However, the research still has some serious limitations that need to be overcome for progress to be made in this research field. Limitations and a promising new direction will be discussed below.

STUDY POPULATION
In the study of Folstein et al., 60% were not willing to participate in the study, leading to a possible underestimation of the problems of risk carriers. The study population in other studies consisted of risk carriers who visited a genetic centre and/or applied for a predictive test. The percentage of those at risk who requested testing when approached by registries or testing centres varied from 9% in Wales, 10% in Indiana, 16% in the Manchester area, to 20% in the Vancouver area. In The Netherlands, 752 out of 1032 subjects at risk, applying for presymptomatic testing in the period 1987 to 1997, decided to be tested, which is 24% of the at risk persons registered in the Leiden Roster for HD. It was suggested and confirmed that persons who participate in the studies on testing form a resourceful self-selected group. Those who decided not to be tested had more frequent expectations of unfavourable emotional reactions and showed more hopelessness than tested subjects.

On the other hand, the level of anxiety, ego strength, and coping strategies were not different between the tested and untested groups. Also, the untested participants form a self-selected resourceful group; both tested and untested participants had a higher ego strength than others.4 5 6 Little is known about the wellbeing of those who do not seek testing and who do not participate in psychological studies. Therefore, bias seems to be involved in the estimation of adaptation in HD risk carriers.

Although we need to be careful to generalise the findings to the whole HD population, we should take into account that differences were observed within the group of test applicants. Some subjects acknowledged the burden of HD, but saw themselves as being able to face the truth. Others denied a burden of HD in their lives and disagreed that the results had a profound impact on their lives.4 6 7 8

Moreover, the dropout rates in most follow up studies are high. Information from relatives about the wellbeing of these dropouts suggest that those who declined participation in follow up research, both carriers and non-carriers, often have serious problems they do not want to disclose, indicating that risk carriers applying for the test may have more problems than the studies suggest.

GLOBAL MEASUREMENT
Another problem is that most measures used are global ones. The IES is the only Huntington specific questionnaire that provides insight into the process of a person’s working through the situation. Research with more specific and sensitive measures is needed for assessment of the process of adjustment between and within test applicants in the post-test period.

THEORETICAL FRAMEWORK
Tibben et al9 and DudokdeWit et al10 used the stress response theory of Horowitz et al11 to formulate their hypotheses. In other studies, it is unclear which underlying theoretical assumptions are used, the design and statistics not being guided by clear hypotheses. A theoretical framework is needed to provide more insight into the observations.
psychological model or theory will contribute to our understanding about the psychological dynamics that characterise this study population.

LACK OF FAMILY PERSPECTIVE ON WELLBEING OF RISK CARRIERS
HD is a family disease.12 The initial onset of symptoms is usually between 30 and 50 years, a period when people are raising a family. People at risk were generally familiar with the disease from early childhood, knowing the symptoms in the parent and/or other family members. Clinicians have shown how the presence of HD in a family can affect the family dynamics.55–56 In some of the reviewed studies, the influence of HD on family dynamics can be inferred. Post-test studies indicated the difficult and different processes test participants and their partners go through. Marriage and career need to be reconsidered43 and the necessary social support may no longer be available. Having children is an additional stress factor for both carriers and their partners.50

However, wellbeing in HD risk carriers has rarely been related to their childhood experiences. Folstein et al.11 investigated how childhood experiences contribute to a more or less favourable adaptation in later life. They found conduct disorder in adolescents and antisocial personality disorder in adults to be related to experiences of having lived in a disorganised household. No relation was found between anxiety or depression and family factors. Recently, Decruyenaere et al.2 found a low but significant correlation between the participants’ age at which the parent showed the first symptoms and psychological functioning before test disclosure. Psychological adjustment to the test result was not correlated with the age of the participant at onset of HD in the parent.

To identify adjustment problems in adult risk carriers, childhood experiences and family dynamics need to be taken into account. In our opinion, the attachment theory53 provides a meaningful theoretical framework for describing childhood experiences in HD families and generating hypotheses concerning the influence of childhood experiences on later adaptation.

ATTACHMENT THEORY
Attachment theory, developed by John Bowlby, postulates a universal human need to form close affectionate bonds. It is a normative theory of how the “attachment system” functions in all humans.56 The attachment theory concerns the nature of early experiences of children, and the impact of these experiences on aspects of later functioning. The central assumption of attachment theory is that individual social behaviour may be understood in terms of generic mental models of social relationships constructed by the person.57 These models, although constantly evolving and subject to modification, are strongly influenced by the child’s experiences with the primary caregivers. The attachment system serves as a primary mechanism for the regulation of infant safety and survival and is highly activated in times of danger.58 An infant is considered securely attached if he or she regards the parents as people to rely on when facing a frightening situation. A responsive and sensitive way of parenting generally gives rise to a secure attachment pattern. Secure infants are able to explore new situations and to experience proximity and comfort in times of distress, illness, or tiredness. Insecure attachment is often found in those who in childhood have experienced rejection or neglect by one or both parents, or who were asked to take care of the parent instead of being taken care of.59 Insecure attachment in either infancy or adulthood is related to the occurrence of psychopathology in adulthood.60

Three types of insecure attachment can be discerned. An avoidant (dismissing) attached infant shifts attention away from rejecting caregivers and minimises displays of distress. An ambivalent (preoccupied) attached infant is highly focused on the caregiver and maximises distress through insistent demands for care and attention. A third group of infants appear to exhibit a range of seemingly undirected behavioural responses giving the impression of disorganisation and disorientation.61 These infants may display (momentarily) bizarre and contradictory behaviour. Frightening experiences with caregivers who behaved in threatening, frightened, or dissociated ways and experiences of loss and trauma may lead to a disorganised attachment pattern.62 It is generally held that for such infants the caregiver has served both as a source of fear and as a source of reassurance, thus the arousal of the attachment behavioural system produces strong conflicting motivations. Not surprisingly, a history of severe neglect or physical or sexual abuse is often associated with the manifestation of this pattern.63–65 It is generally held that the patterning of attachment related behaviour is underpinned by different strategies adopted by children to regulate their emotional reactions.66 As affect regulation is acquired with the help of the child’s primary caregiver, the child’s strategy will be inevitably a reflection of the caregiver’s behaviour towards him/her. Established attachment patterns or working models guide a person’s response to frightening situations and interpretation of the caregiver’s response.

The stability of early childhood attachment patterns is well demonstrated. During development from infancy to childhood, attachment working models become difficult to change. However, current experiences with attachment figures continue to influence the attachment working model.67–69

How can the attachment theory be relevant for people dealing with Huntington’s disease? We speculate that the presence of HD in a family involves specific stressors, which might influence the attachment relationship between parents and their children for different reasons. First, the affected parent in the onset phase of HD may become preoccupied with the diagnosis, their own future, and the frightening recollections of his/her parent or other relatives going through the HD disease progression. As
the disease progresses, the patient is less receptive to the questions of the children and may become depressive or aggressive. These mood and personality changes, together with the choreic movements, may frighten or alienate their offspring. Second, the disease may lead to changes in the family system. The unaffected parent will experience a change in responsibilities and dependency of the spouse in the relationship; the affected spouse becomes a person who insidiously needs care. Some healthy partners may feel unable to take up this task and will leave the household. Changes in the household may lead to neglect of the children. Some children may take up the care of the ill parent. The unaffected parent may seek one of the children as a substitute partner. Third, the fact that the children are at risk for developing HD also puts stress on parent-child bonding. The parents may be concerned about the carrier status of the child and may have feelings of guilt by having passed on the gene. Knowing that their children may get the disease can also create an emotional distance. Some parents also have predictions or even fantasies about their children, thinking that they may or may not develop HD. The healthy parent often has the difficult task of rearing these children and informing them about their risk without the help of the partner. With regard to testing, having children is an additional stress factor for the healthy parent. To summarise, a family burdened by a genetic disorder may have to deal with several types of loss: loss of the physical capacity of the affected person, loss of his or her own personality, loss of the old family system, and loss through death. This may be accompanied by shame, secretiveness, and social isolation.

Mental representations of attachment in adults are assessed by means of the Adult Attachment Interview (AAI). The AAI asks subjects about childhood attachment relationships and the meaning which a person currently gives to attachment experiences. The instrument is rated according to the scoring system developed by Main and Goldwyn which classifies people into Secure/Autonomous, Insecure/Dismissing and superimposed on these Insecure/Preoccupied, Unresolved/Disorganised with respect to loss or trauma, categories according to the structural qualities of their reports of early experiences. The assessment of attachment by means of coherence may help to overcome problems with self-report measures in previous studies. Next to the attachment representation, the Adult Attachment Interview generates information about the psychodynamics and defences in a person.

Although Huntington’s disease is a highly dramatic disorder with an increased chance for children to become traumatised, we speculate that the findings are also important for other genetic disorders that have been passed on to consecutive generations. This does not need to be restricted to autosomal dominant, late onset diseases with full or partial penetrance, but also for X linked disorders and diseases with recessive inheritance patterns. There is further important evidence that attachment relationships may play a key role in the transgenerational transmission of hardship and deprivation. People categorised as secure are three or four times more likely to have children who are securely attached to them. This turns out to be true even in prospective studies where parental attachment is assessed before the birth of the child. These findings also emphasise the importance of quality of parenting in determining the child’s attachment classification. Investigation of the attachment relationship in HD families and its influence on adult functioning may contribute to a greater understanding of earlier research findings and serve to improve genetic counselling and intervention.

### Appendix

#### Measures

- **AQ, Attitude Questionnaire**
- **BDI, Beck Depression Inventory (BDI)**
- **BHS, Beck Hopelessness Scale (BHS)**
- **EPI, Eysenck Personality Inventory**
- **FACES, Family Adaptability and Cohesion Evaluation Scales**
- **GHQ, General Health Questionnaire**
- **GSI, Global Severity Index**
- **GWS, General Wellbeing Scale**
- **HADS, Hospital Anxiety Depression Scale**
- **IERS, Impact of Event Scale**
- **MCMI-2, Millon Clinical Multiaxial Inventory II**
- **MHQ, Mental Health Questionnaire**
- **MMAD, Minneapolis Multiphasic Personality Inventory**
- **PDDI, Positive Symptom Distress Index (SCL-90)**
- **PST, Positive Symptom Total (SCL-90)**
- **SCL-90, Symptom Checklist 90**
- **SSQ, Social Support Questionnaire**
- **STAI, State Trait Anxiety Questionnaire**
- **UCL, Utrecht Coping List**

### References


A novel 3' mutation in the APC gene in a family presenting with a desmoid tumour

Diana Eccles, John Harvey, Adrian Bateman, Fiona Ross

EDITOR—Desmoid tumours, also known as infiltrative fibromatoses, are rare benign tumours which often recur after local resection and can cause death through local infiltration of vital structures. The estimated incidence in the general population of such tumours is 1-2 per million but in familial adenomatous polyposis (FAP) they occur in up to 15% of cases. Likely precipitating factors include trauma and female sex hormones, since females are more often affected than males. The majority of desmoid tumours in FAP (over 90%) arise in the mesentery of the bowel or in the abdominal wall musculature. In recent years, several families have been described where the predominant phenotype is of desmoid disease and where the colonic phenotype is minimal. We describe another such family with a novel protein truncating mutation in the 3' end of the APC gene.

Methods and results

CLINICAL DETAILS

The index case presented at 29 years of age with a firm, slightly tender swelling within the right rectus abdomen muscle. A 6 x 5 cm tumour was locally excised and conventional histological examination showed infiltrative fibromatosis. The tumour recurred after six years and was resected again along with 30 cm of adherent small bowel. A year later, a further abdominal wall recurrence was resected and on this occasion fresh tissue was submitted for cytogenetic analysis. Full colonoscopy before referral to the genetics service showed no evidence of colonic adenomas throughout the colon. Repeat colonoscopy after the gene mutation was identified still failed to show any colonic pathology, although contrast dye spray was not undertaken on either occasion. The only relevant family history was that her father had a previous history of a sigmoid colectomy and where the predominant phenotype is of desmoid disease and where the colonic phenotype is minimal. We describe another such family with a novel protein truncating mutation in the 3' end of the APC gene.

Molecular analysis

DNA from the index case was examined for mutations in the APC gene using denaturing high performance liquid chromatography (DHPLC). A single base substitution G>A was identified at nucleotide position 7511, codon 2504, which changes tryptophan (TGG) to a stop codon (TAG). The mutation was present in both the index case and her father.
The colonic tumour and the desmoid tumour were examined for β-catenin expression using immunohistochemistry. This showed intense nuclear and cytoplasmic β-catenin expression in the colonic tumour. A similar, although less intense, pattern of nuclear expression with some cytoplasmic expression was apparent in the desmoid tumour from the index case.

**Discussion**

Classical familial adenomatous polyposis coli (FAP) is recognised by the presence of many hundreds of colonic adenomas throughout the colon. Since the identification of the gene responsible for FAP in 1991, it has been possible to recognise certain genotype/phenotype correlations which may not only allow the clinician to direct the molecular geneticist towards the most likely region where a mutation may be located, but conversely may predict the likely natural course of the disease associated with a given mutation and direct management strategies accordingly. Where mutations fall into the 5' and 3' extremes of the gene, an attenuated colonic phenotype is usually seen with fewer and later onset polyps. Whereas mutations at the 5' end of the gene (exons 1-4) are not associated with desmoid tumour development, mutations arising in the 3' half of the gene are more often associated with desmoid tumours, and in rare families this is the dominant phenotype with little else to suggest the diagnosis of polyposis on examination of the colon. Although cytogenetic analysis of desmoid tumours is technically difficult and requires fresh tumour tissue, it may be a useful additional investigation where the phenotype for FAP is absent. Cytogenetic deletion of 5q in desmoid tumours is more frequently associated with FAP than apparently sporadic tumours, so, even in the absence of typical FAP features, a cytogenetic deletion of 5q in the tumour may direct a careful search for possible underlying APC gene mutations.

APC is involved in many critical cellular functions including cell adhesion (via E-cadherin) and in negatively regulating β-catenin levels. β-catenin is involved in the transcriptional activation of several genes involved in cell cycle entry and progression. APC mutation leads to reduced degradation of β-catenin (reflected in overexpression of β-catenin shown using immunohistochemistry in the tumours described in this case).

The natural history of desmoid tumours is variable but they can follow a highly aggressive course. However, even in families where the desmoid phenotype is 100% penetrant, the clinical course is unpredictable. The explanation for the particular prediction of 3' mutations to be associated with desmoid tumour formation is still a matter of speculation. Low levels of truncated protein product have been reported in similarly distal mutations, possibly owing to an unstable mRNA; the stability of the mRNA may vary according to tissue type. There are likely to be both environmental and genetic modifiers exerting an effect on APC gene mutations in view of the variation in phenotype between subjects with the same mutation. It is possible that the effect of these putative genetic modifiers will differ according to tissue type. Detection of such modifiers and determination of their mode of action could be extremely helpful in selecting potential medical treatment for patients with desmoid tumours, since surgery is best avoided if the tumours are not life threatening. A recent paper looking at second hits in adenomas and desmoid tumours in FAP included data only for patients with germline mutations 5' of codon 1462. In desmoid tumours from these patients, there was either loss of the wild type allele or a somatic APC mutation. The somatic mutations were more often 3' of codon 1462. In our patient, there was loss of heterozygosity indicated by a cytogenetic deletion but a 3' germline mutation. This suggests that the siting of first and second hits may be interchangeable with respect to the end result (a desmoid tumour), but if somatic mutations in colonic epithelium are in some way reliant on the germline mutation this may explain the very different clinical phenotype presented by many patients with unusually 3' mutations.

For families where the desmoid phenotype is the dominant clinical feature and the risk of colorectal cancer is lower, the frequency of adenomatous polyps is frequent, colorectal resection should be reserved for situations where there is significant colonic pathology. There may be an argument for favouring subtotal colectomy and ileorectal anastomosis if colectomy becomes necessary. Giving clear information to at risk members of the family reported here is difficult, as the position of the mutation leads us to suspect a high risk for desmoid tumours although the clinical picture is not of 100% desmoid tumour penetrance. Although the colon cancer risk must also be increased, the association of desmoid tumours with trauma (and surgery in particular), the late onset, and the presence of only a few adenomas in the only identified gene carrier with colon cancer and the normal appearance of the colonic mucosa in the index case in her late thirties all suggest that a conservative approach to surgery is appropriate.

We have offered genetic testing to at risk family members and suggested that a screening colonoscopy every two years starting at 20 years, with dye spray or random mucosal biopsies to determine whether any microadenomas are present, would be a reasonable course of action for a gene carrier or for a family member at 50% risk who chose not to have a predictive genetic test.

We know this disease is caused by a mutation in the APC gene and could therefore be called familial adenomatous polyposis coli (FAP) or perhaps better attenuated adenomatous polyposis coli (AAPC). This acknowledges that there is an increased colon cancer risk; however, this potentially underplays the significance of the desmoid tumour phenotype which may be a very important factor to take into account in making decisions about surgery.
letters

A silent mutation in exon 14 of the APC gene is associated with exon skipping in a FAP family

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EDITOR—Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited disorder characterised by the development of hundreds to thousands of adenomatous polyps in the colon and rectum. If left untreated, there is a very high risk of colorectal cancer. Adenomatous polyps may also develop proximally in the stomach and the distal part of the duodenum. FAP is also associated with a variety of extracolonic benign and malignant manifestations, including congenital hypertrophy of the retinal pigment epithelium (CHRPE), dental abnormalities, desmoid tumours, osteomas, epidermoid cysts, hepatoblastoma, and thyroid neoplasia.1 Germline mutations of the APC gene localised on chromosome 5q21.22 are responsible for FAP.2 3 APC is a tumour suppressor gene encoding a 2843 amino acid protein, which contains multiple functional domains and which mediates growth regulatory signals by its association with a variety of cytoplasmic proteins. More than 300 different APC mutations have so far been identified distributed throughout the whole gene, with a higher concentration in the 5' part of exon 15 (codons 713-1597).4 The majority of mutations are predicted to introduce premature termination signals resulting from single nucleotide alterations, small insertions or deletions, or splice site mutations that lead to truncation of the normal protein product.4 Missense mutations have rarely been reported and their functional implications are often unclear.5 7 Larger deletions and insertions have been described, as well as genomic rearrangements resulting from recombinations mediated by Alu elements which cause inappropriate exon splicing.6 10 Isoforms of APC transcripts lacking exon 9, exon 10A, and exon 14 encoded sequences have been reported.6 11–13 Isoforms lacking exon 9 or exon 14 owing to splice site mutations have also been associated with a FAP phenotype.14 17

In this study, we describe a G→T transversion at nucleotide position 1869 in exon 14 which gives rise to a silent mutation, since both normal and mutated alleles encode an arginine residue at codon 623. This exonic mutation induces complete skipping of exon 14, leading to truncated APC protein and resulting in a FAP phenotype.

Materials and methods

The index case of family GE08 (IV.1, fig 1, table 1) was referred to our institution for genetic counselling. The patient underwent a total colectomy for diffuse polyposis. A diagnosis of FAP was made on the basis of family history and histopathological results.


After informed consent had been obtained from numerous family members, medical records were reviewed to confirm the diagnosis of polyposis, polyposis and cancer, and the age of occurrence. The clinical information obtained is shown in table 1. The pedigree was constructed as shown in fig 1. Peripheral blood samples were obtained from the proband and family members III.5, IV.1, IV.2, IV.3, IV.4, IV.5, IV.6, IV.15, IV.16, IV.18, IV.19, IV.20, IV.21, IV.22, IV.26, V.1, V.2, V.3, V.4, V.11, and V.12.

Mutation analysis was carried out on genomic DNA prepared from isolated leucocytes using SNAP Whole Blood DNA Isolation Kit (Invitrogen, Carlsbad, CA). DNA was also extracted from paraffin embedded normal tissue from subject III.5. The protein truncation test (PTT) was performed to detect truncating mutations in exon 15. Mutation screening of the entire APC gene were amplified by PCR using the primer pairs reported elsewhere. SSCP was carried out as described previously. The variant conformer was confirmed in at least three different samples from the same person and by direct DNA sequence analysis using an ABI PRISM TM 377 DNA Sequencer (Perkin Elmer, Foster City, CA).

mRNA was isolated from IL-2 transformed lymphoblastoid cell lines by using the MicroFast Track mRNA Isolation Kit (Invitrogen, Carlsbad, CA). First strand cDNA was synthesised with 2 μg of polyA+ RNA in a reaction mixture containing 1.5 mmol/l MgCl2, 10 mmol/l Tris HCl, 50 mmol/l KCl (pH 8.3), 200 μmol/l each dNTP, 200 U of M-MLV Reverse Transcriptase (Ambion, Austin, TX), 25 U RNase OUT (Life Technologies, Gaithersburg, MD), 50 μmol/l of primer RV7-A10 and primer 15A-RP3, and 0.25 U Taq polymerase in a volume of 50 μl. Primers RV7-A and 15A-RP enabled the APC region corresponding to codon 493 to 759 to be amplified. The PCR products were visualised by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The band intensity was measured.

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**Table 1** Characteristics and clinical data from FAP family GE08

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Clinical presentation*‡</th>
<th>Age at diagnosis polyp/cancer</th>
<th>Number of adenomas</th>
<th>Exon 14 mutation</th>
</tr>
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<tbody>
<tr>
<td>II.1</td>
<td>†Symptomatic</td>
<td>?</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>II.3</td>
<td>†Symptomatic</td>
<td>?</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>III.1</td>
<td>†Symptomatic</td>
<td>?</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>III.4</td>
<td>†Symptomatic</td>
<td>?</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>III.5</td>
<td>Symptomatic</td>
<td>61/61 &gt;100</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>IV.1</td>
<td>Symptomatic</td>
<td>46 &gt;100</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>IV.2</td>
<td>Call up</td>
<td>44</td>
<td>Absent</td>
<td></td>
</tr>
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<td>IV.3</td>
<td>Call up</td>
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<td>IV.4</td>
<td>Symptomatic</td>
<td>40</td>
<td>Present</td>
<td></td>
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<td>Call up</td>
<td>36</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td>IV.6</td>
<td>Call up</td>
<td>34</td>
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<td></td>
</tr>
<tr>
<td>IV.9</td>
<td>NK</td>
<td>34</td>
<td>NK</td>
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<tr>
<td>IV.10</td>
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<td>IV.12</td>
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<td>IV.13</td>
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<td>38</td>
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<tr>
<td>IV.14</td>
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<td>34/34 &gt;100</td>
<td>Present</td>
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<td>IV.15</td>
<td>Call up</td>
<td>39</td>
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<td>IV.16</td>
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<td>36/36 &gt;100</td>
<td>Present</td>
<td></td>
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<tr>
<td>IV.17</td>
<td>NK</td>
<td>32</td>
<td>NK</td>
<td></td>
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<tr>
<td>IV.18</td>
<td>NK</td>
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<td>NK</td>
<td></td>
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<tr>
<td>IV.19</td>
<td>NK</td>
<td>29</td>
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<td></td>
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<td>IV.20</td>
<td>Call up</td>
<td>25</td>
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<td></td>
</tr>
<tr>
<td>IV.21</td>
<td>Call up</td>
<td>15</td>
<td>Present</td>
<td></td>
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<tr>
<td>IV.22</td>
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<td>10</td>
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<tr>
<td>IV.25</td>
<td>NK</td>
<td>2</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

*Symptomatic, patients showing symptoms of FAP and/or CRC.
†Symptomatic, no data available.
‡No extracolonic manifestations were present in affected subjects.
§Unaffected subjects and asymptomatic gene carriers, actual age, and age at DNA test are given.
Call up, called for endoscopic examination as at risk subjects; NK, not known; NT, not tested.

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*Una*
Letters

865

are present in the family. No upper gastrointestinal tract lesions, from six to more than 100 in a
cousins, both possible carriers of the disease. IV.16 (table 1). These patients belonged to a
rence and the multiplicity of polyps. The age at
some peculiarities regarding the age of occur-
The pedigree of family GE08 (fig 1) shows
FAP and underwent colectomy at the age of 46.
The index patient had a clinical diagnosis of
and a control carrying a nonsense mutation at
codon 563 were analysed.
using 10% SDS PAGE gel. EBV transformed
products were separated by 6% silver stained
denaturant and the alternative spliced transcript lacking exon 14. GAPDH gene coamplification was carried out
as an internal control. Moreover, the PCR
products were separated by 6% silver stained
doublet of 793 and 578 nucleotides were cut o
polyacrylamide gel electrophoresis. The bands
of 793 and 578 nucleotides were cut off and eluted in water overnight at 37°C. One µl of
each mixture was then used to amplify the two bands separately with primers RV7-A and
15A-RP. The identity of the PCR products was
ascertained by direct sequencing. A stretch of
intronic 40 nucleotides upstream of exon 14 was also sequenced for analysing the acceptor
site branch point.
Protein extraction and western blot analysis
were performed according to Gismondi et al
using 10% SDS PAGE gel. EBV transformed
cells of subjects IV.1, IV.16, a normal control,
and a control carrying a nonsense mutation at
codon 563 were analysed.
Results
The index patient had a clinical diagnosis of FAP and underwent colectomy at the age of 46.
The pedigree of family GE08 (fig 1) shows
some peculiarities regarding the age of occurrence and the multiplicity of polyps. The age at
which polyps and cancer appeared is later than in
classical polyposis, except for IV.14 and
IV.16 (table 1). These patients belonged to a
branch of the family in which a consanguineous marriage had taken place between first
cousins, both possible carriers of the disease.
The number of adenomatous polyps ranged
from six to more than 100 in affected members.
No upper gastrointestinal tract lesions,
CHRPES, or other extracolonic manifestations
are present in the family.
During mutation screening of the APC coding
region, a G→T transversion at nucleotide position 1869 was detected by SSCP and direct
sequencing of exon 14 in the index patient
IV.1. This mutation changes codon 623 from
CGG to CGT, both encoding arginine (R623R). Since the entire open reading frame of the APC gene was analysed by SSCP and
PTT several times without detecting any additional sequence variation, this is the only
detectable DNA alteration unique to this
patient.
The sequence at the branch point of the exon
14 acceptor site also did not show any alteration. Subsequent screening for the mutation in
21 additional relatives showed the presence
of the mutation in three successive generations, as shown in fig 1, and its segregation
with the disease. In patient III.5, the mutation was detected on paraffin embedded tissue.
Direct sequencing of DNA from patients
IV.14 and IV.16 did not show homozygosity for the R623R mutation. This allelic variant was not observed in any of 100 DNA
samples from normal controls.
 Messenger RNA purified from transformed lymphoblastoid cell lines from three affected
subjects (IV.1, IV.4, IV.16) and from three nor-
mals controls was examined. The analysis of
cDNA with primer sets RV7-A and 15A-RP showed the two isoforms representing the
mRNA transcript containing exon 14 and the
alternatively spliced transcript lacking exon 14 leading to a stop codon in exon 15A.
The densitometric ratio between the two
isoforms of 793 and 578 bp amplicons was
comparable in all normal controls, being 4.2.
By contrast, the band intensity of 578 bp was
greater in the three patients examined, with a
densitometric ratio of 0.6 (p<0.03) showing a
dramatic increase in the alternatively spliced iso-
form of exon 14 (fig 2A).
 Direct DNA sequencing of the two bands showed only the wild type allele in the 793 bp
mRNA isoform containing the normal spliced RNA while the G→T transversion was not
present. In the retrotranscribed, short 578 bp
isoform, the mRNA showed a complete lack of
exon 14.
Western blot analysis was performed on protein extracts from a normal control and from
subjects IV.1 and IV.16. In addition, a FAP
patient with a nonsense mutation at codon 563
was analysed as an APC protein truncated control (fig 2B). Using 10% SDS-PAGE
analysis, the APC wild type protein was not

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116 kDa
84 kDa
58 kDa
52 kDa
36 kDa

Figure 2 (A) Agarose gel electrophoresis showing the expression of the two exon 14 isoforms of 793 and 578 bp; φX174,
DNA molecular marker; C, normal controls; IV.4, IV.1, IV.16, affected subjects. (B) Western blot analysis showing the 64
kDa APC truncated protein in patients IV.1 and IV.16 and the 62 kDa APC protein in the truncated control in M; C is
the normal not truncated protein.
Letters

The exonic silent mutation R623R reported here creates a complete skipping of exon 14 of the APC gene and gives rise to a stable truncated APC protein. This mutation has not been described previously and does not represent a polymorphic gene variant as it has been tested on more than 100 subjects. This mutation occurs as the only DNA change in all affected members of a large three generation FAP family. Clinically, the family is characterised by an unaggressive form of the disease in terms of age of onset and number of polyps (table 1, fig 1), except for the branch derived from a consanguineous marriage, both parents being possible carriers of FAP. This difference might occur because of the interfamilial variability of FAP or because of some unknown recessive modifier gene inherited from both parents. Although alternative skipping of exon 14 is a physiological event observed in normal subjects, a splice acceptor site mutation leading to increased expression of an APC mRNA isoform without exon 14 has been previously described in a FAP family.10 In our family, splice sites do not show any modification and, furthermore, to rule out the possibility of an intron alteration in the tract near the acceptor splice site, which might eliminate lariats formation at the branch point, a stretch of 40 nucleotides upstream of the exon 14 acceptor site was also sequenced.

Our data indicate that the mutation harbouring allele is expressed exclusively in the alternative isoform and that the DNA exonic silent mutation might be able to produce a complete skipping of exon 14, suggesting the existence of a mechanism of splicing regulation distinct from splice junctions. Silent mutations not leading to amino acid change are generally considered to be normal variants and are thought to have no role in disease. However, a few papers have described exonic silent mutations able to induce exon skipping in the fibrillin-1 gene, the MLH1 gene, and the human phenylalanine hydroxylase gene, all associated with the corresponding diseases.27-29 Recent studies have indicated that sequence elements that are distinct from the splice sites are also needed for normal splicing. These elements are required for efficient splicing and may affect splice site recognition during constitutive and alternative splicing.26-27 They are found within coding exons and are called exonic splicing enhancers (ESE). Since no consensus sequence that describes ESE is recognised, these elements are difficult to identify, not least because they are not as purine rich as was originally thought.28 Silent point mutations that affect ESE and lead to inappropriate exon skipping have been described in human genetic diseases. Frontotemporal dementia linked to chromosome 17 missense mutation affects splicing of tau gene exon 10 by acting on an ESE.29 Carbohydrate deficient glycoprotein syndrome is associated with a missense mutation that disrupts a splicing enhancer sequence, resulting in exon 5 skipping.30 Splicing difference between the two forms of spinal muscular atrophy, SMN1 and SMN2, is attributed to a silent mutation in an ESE located in the centre of SMN exon 7.31 Very recently, Liu et al2 reported aberrant exon skipping in the BRCA1 gene resulting from nonsense, missense, or translationally silent mutations, which have disrupted a critical ESE. The G→T silent genomic DNA mutation is the only change found in family GE08 and it is located in a purine rich DNA sequence (ACCG-GAGCCGAG). This region in the middle of exon 14 might represent an ESE sequence necessary for the control of the alternative splicing and the one base substitution might disrupt this, thus provoking total exon 14 skipping. Experiments are being carried out to test enhancer activity of the exon 14 APC sequences by inserting them into another transcript that could be assayed by in vitro splicing and/or by transfection,32 followed by mutagenesis of the sequence, to identify the elements that compose the putative enhancer.

- We describe a silent third base codon mutation in exon 14 of the APC gene, a G→T transversion at nucleotide 1869 (R623R), associated with complete skipping of exon 14 and production of a stable truncated APC protein. The R623R mutation segregates with the disease in a large FAP family. This mutation is the sole variation found in the entire APC coding sequence, is not a polymorphism, and has not been described before.
- To evaluate whether the silent genomic DNA variation could affect RNA transcription, RT-PCR analysis was carried out in affected subjects. Although exon 14 is subject to alternative splicing, an unbalanced expression of the two exon 14 isoforms was found. Sequence analysis of the two isoforms showed that the mutant APC allele expressed exclusively mRNA lacking exon 14. Western blot analysis showed that the mutant mRNA was translated into a stable truncated protein.
- Our findings show a possible new model of APC mutation causing disease and suggest that exonic elements might modulate the splicing of APC gene exon 14. They therefore underline the importance of investigating the significance of silent mutations.

This work was supported by MURST, Ministero dell’Università e della Ricerca Scientifica e Tecnologica, Convenzionamento 1998, Ricerca Finalizzata-Ministero Sanità 1999, IC30301RF99.34. We also acknowledge Galliera Genetic Bank, Progetto Telethon C42.

Evidence of somatic mosaicism for a MECP2 mutation in females with Rett syndrome: diagnostic implications

Violaine Bourland, Christophe Philippe, Thierry Bienvenu, Bernadette Koenig, Marc Tardieu, Jamel Chelly, Philippe Jonveaux

Editor—Rett syndrome (RTT) (MIM 312750) is an X linked dominant neurodevelopmental disorder that occurs almost exclusively in females. Affected girls are considered to have a normal perinatal period followed by a period of regression, loss of acquired purposeful manual and speech skills, hand wringing, gait disturbance, and growth retardation.1 2 A gene for RTT has been identified in the Xq28 region which encodes the methyl-CpG

References

binding protein 2 (MeCP2) involved in transcriptional silencing. This disorder most frequently occurs sporadically and results from a de novo mutation, although a few familial cases have been reported. Many studies have shown that the MeCP2 gene is mutated in approximately 80% of patients with classical RTT and the MeCP2 mutation spectrum includes missense, nonsense, and frameshift mutations, as well as larger rearrangements like deletions encompassing a few hundred bp.

The failure to detect MeCP2 mutations in the remaining 20% may indicate the presence of mutations in unexplored regions of the MeCP2 gene, such as regulatory elements or non-coding regions, notably in the new first exon or in an additional RTT locus.

Here, we report for the first time mosaicism for a somatic MeCP2 mutation found in two unrelated females affected with RTT. These two girls were diagnosed according to the international criteria of the Rett Syndrome Diagnostic Criteria Work Group.

Case reports

The first patient (case 1) is 13 years old. She suffers from classical Rett syndrome with 7/9 of the necessary criteria, 4/8 of the supportive criteria, and none of the exclusion criteria. More specifically, she had a normal neonatal period and head circumference at birth and a phase of social withdrawal at the age of 12 months when she lost purposeful hand skills and developed stereotypic hand movements, ataxia, and apraxia. She suffered from breathing dysfunction and peripheral vasomotor disturbances. She had severely impaired development but acquired independent walking at the age of 24 months. However, she did not acquire microcephaly or develop epilepsy.

The second patient (case 2) was reported as an atypical case of RTT without any period of regression. Both mental and motor development were very slow. At the age of 4 years, she had acquired microcephaly (−2 SD) and had very limited ambulation, but her hand use was correct without hand wringing movements. She developed epilepsy and progressive scoliosis. She is a placid girl without useful speech but she communicates well by eye movements.

Methods and results

For case 1, an initial study on DNA extracted from a lymphoblastoid cell line by denaturing gradient gel electrophoresis (DGGE) and sequencing showed that she carried a 26 bp deletion starting at position 1165. To confirm this mutation, DNA was extracted from a fresh blood sample and the deletion was assessed by direct sequencing. Surprisingly and despite a careful examination of the sequence, we did not find the 26 bp deletion with DNA extracted from leucocytes. This sample was reanalysed by DGGE and heteroduplexes were detected while the homoduplex corresponding to the deleted band was absent (fig 1A). We confirmed this result by conformation sensitive gel electrophoresis (CSGE) analysis, which showed the heteroduplexes but not the mutant homoduplex (fig 1B). The results obtained from peripheral blood lymphocytes suggested mosaicism for a somatic mutation.

In order to determine the level of mosaicism, we used a semi-quantitative approach based on fluorescent PCR. The MeCP2 gene exon 3 portion containing the deletion was PCR amplified, the reverse primer being conjugated...
to 6-FAM (6-carboxy-fluorescein). PCR products were analysed on an ABI 310 sequencer and peak areas were generated by ABI GeneScan and Genotyper software. The ratio between the deleted and normal peak areas showed that only 36% of lymphocytes harboured the deletion, that is, 18% of X chromosomes bore the 26 bp deletion (fig 2A). This semiquantitative approach confirms that case 1 does have somatic mosaicism for the MECP2 deletion. The relatively low level of somatic mosaicism could explain the normal sequencing result. Thus, mosaicism was quantified in different tissues. DNA was extracted from buccal mucosa cells\(^1\) and hair bulb cells.\(^{20}\) The level of mosaicism was about the same in buccal mucosa cells (30%) as in lymphocytes, but lower in hair bulbs cells (17.5%) (fig 2A).

**Discussion**

On the basis of these results, we hypothesised that some patients with RTT may in fact carry a somatic mutation. Small deletions (from 7 to 170 bp) within the region between bp 1096 and 1165 of the MECP2 gene have been recurrently identified.\(^5\)\(^7\)\(^9\)\(^10\)\(^12\)\(^15\)\(^16\) They do not affect the two functional domains but result in the loss of one fifth of the protein. Interestingly, it has been shown that the deletion of the carboxy-terminal 63 amino acids of the MeCP2 protein impairs binding with the nucleosomal DNA during the transcription regulation process.\(^21\) These recurrent deletions may be the result of palindromic and quasipalindromic sequences within this region, which are believed to form secondary structures that render the region vulnerable to deletions. Therefore, using our fluorescent PCR approach, we reanalysed the 3' region of the MECP2 gene, between bp 1096 and 1165, in a cohort of 29 patients diagnosed as typical or atypical RTT; for these patients, we failed to detect any mutation using a bidirectional sequencing strategy of the entire MECP2 coding region. A second somatic mosaicism for a 27 bp deletion was identified in peripheral blood lymphocytes from case 2 with atypical RTT; the mosaicism rate was quantified with our fluorescent approach to be about 37% (fig 2B). We confirmed this result by CSGE analysis (fig 1B).
In both cases, numerical aberrations of the X chromosomes as a cause for the uncommon fluorescent PCR patterns were excluded by the presence of a normal 46,XX karyotype. These two patients show a similar deletion with an equivalent mosaicism rate in blood, but a distinct clinical presentation. X inactivation study on proband 1 with typical Rett syndrome showed a random pattern of inactivation in the peripheral blood. Although the results have to be extrapolated from the peripheral blood cells, it would suggest that in the brain the majority of mutated X chromosomes may remain active in the girl with classical Rett syndrome. Our results illustrate clearly once again the difficulty in establishing a correlation between genotype and phenotype in RTT.

Recently, a boy with a mosaic mutation has been described.22 To our knowledge, we show for the first time that somatic mosaicism for MECP2 mutation in girls is not infrequent (two somatic mutations on 102 putative RTT cases studied) and may cause different phenotypes. These clinical and molecular findings suggest that multiple forms of mosaicism (X inactivation, somatic and somatic mosaicism) may be present in a single patient with RTT. Mosaicism has been documented for chromosomal abnormalities, mitochondrial mutations, triplet repeats,23 and in a growing number of dominant and recessive X-linked genes, such as Duchenne muscular dystrophy,24 haemophilia B,25 Conradi-Hünermann-Haplo syndrome,26 and double cortex/lassoencephaly syndrome.27 Because a proportion of cells carry the mutation not only in blood but also in tissues deriving from other cell lineages, it must be assumed that the mutation occurred very early during embryogenesis.

Finally, the detection of mosaic mutation depends mainly on the method used for the identification of mutations within the MECP2 gene. Nowadays, the method of choice for identifying deleterious mutations relies on direct DNA sequencing. The ability of this method to detect mosaic mutations is poor, which is particularly true when the mosaicism rate is low. Our findings underline the need for at least two complementary approaches, such as methods based on heteroduplex analysis and sequencing, for an efficient screening of the MECP2 gene.

We thank Dr Deblay for critical advice, Dr Florence Rousselet for her technical contribution, and l'Association Française du Mal des 46,XY for their financial support.


Temperature sensitive acyl-CoA oxidase import in group A peroxisome biogenesis disorders

Atsushi Imamura, Nobuyuki Shimozawa, Yasuyuki Suzuki, Zhongyi Zhang, Toshiro Tsukamoto, Tadao Orii, Takashi Osumi, Naomi Kondo

Editor—Peroxisome biogenesis disorders (PBDs) are lethal genetic diseases characterised by a number of peroxisomal metabolic abnormalities, including the oxidation of very long chain fatty acids (VLCFAs), biosynthesis of bile acids and plasmalogen, and detoxification of H₂O₂. Peroxisomal matrix proteins are synthesised on free polyribosomes and directed to the organelle by cis acting peroxisome targeting signals (PTSs). PTS1 is a C-terminal tripeptide Ser-Lys-Leu (SKL) sequence and the consensus sequence was broadened to (S/A/C/K/N)-(K/R/H/Q/N/S)-L, based on subsequent studies. Acyl-CoA oxidase (AOX) has SKL and D bifunctional protein has AKL. PTS2 is an N-terminal cleavable peptide (-R/KLX5Q/HL) that resides in peroxisomal 3-ketoacyl CoA thiolase (PT), alkyldihydroxyacetonephosphate synthase, and phytanoyl-CoA hydroxylase. PBDs are genetically classified into at least 12 complementation groups (CGs) and each CG contains various clinical phenotypes, for example, Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD). ZS patients have severe neurological defects, liver dysfunction, and renal cysts and die before 1 year of age. NALD patients have symptoms similar to ZS patients, but they survive a little longer, and IRD patients show milder abnormalities in the central nervous system and survive even longer.

We identified the restoration of peroxisome biogenesis in a temperature sensitive manner in fibroblasts from milder forms of ZS patients. Temperature sensitive peroxisome import was detected by immunofluorescence microscopy in cells cultured at 30°C but not at 37°C. Peroxisomal biochemical functions were also restored. However, virtually no peroxisomes were formed in ZS patients cultured at 37°C. We also observed that peroxisomal activities were reduced or absent at 37°C, whereas they were severely reduced or absent at 37°C (fig 1C, D, table 1).

Materials and methods

CELL LINES

Skin fibroblasts from the patients belonging to CG-A (CG8) including four with ZS (A-02, 06, 10, and 14), two with NALD (A-05 and 08), and one with IRD (A-04) were cultured at 37°C or 30°C in an atmosphere of 5% CO₂ in MEM supplemented with 10% fetal calf serum. A-02, A-06, and A-14 were Japanese babies diagnosed as ZS with typical dysmorphic features, who died at a few months of age. The clinical data of A-04 and A-08 have been previously reported, whereas those of A-05 and A-10 have not.

In addition, ZS fibroblasts belonging to CG-C (C-08), CG-E (E-14), and CG-F (F-01) were cultured under the same condition (the numbers and clinical data of these patients have been previously described). All cell lines were classified by complementation analysis as previously described.

IMMUNOFLUORESCENCE STUDY

For the detection of peroxisomes and the import of PTSs, cells were fixed after 72 hours’ incubation at either 37°C or 30°C, permeabilised with 0.1% Triton X-100, and processed for indirect immunofluorescence staining.

The first antibodies we used were rabbit antibodies to human catalase, AOX, D bifunctional, protein, and PT, and in double immunofluorescence rabbit anti-rat PMP70 antibody was used.

BIOCHEMICAL ASSAYS

Peroxisomal VLCFA oxidation in fibroblasts was assessed by the ratio of lignoceric acid (C24:0)/palmitic acid (C16:0) oxidation activity. The activity of DHAP-AT, the first enzyme in the pathway leading to plasmalogen biosynthesis, was measured as described previously using 14C labelled DHAP as substrate. Continuous cell labelling with 35S-methionine and immunoprecipitation of AOX with rabbit anti-human AOX antibody was performed as described previously.

Results

IMMUNOFLUORESCENCE STUDY IN FIBROBLASTS FROM CG-A PATIENTS

The fibroblasts from PBD patients belonging to the CG-A were examined by immunofluorescence microscopy to determine the import of PTS1 and PTS2 containing peroxisomal matrix proteins. The immunoreactivity of these proteins in control cells showed the same punctate pattern as previous reports (data not shown). The most striking result was that in the fibroblasts from ZS patients, the import of AOX was rescued apparently after incubation at 30°C, whereas it was severely reduced or absent at 37°C (fig 1C, D, table 1).
The AOX and 70 kDa peroxisomal membrane protein (PMP70) were co-localised in these cells (data not shown). In other CGs, none of the ZS cell lines showed such TS characteristics. There was a little cross reactivity of AOX antibody with mitochondria (fig 1C) co-localised with anti-mitochondria antibody (data not shown). The import of catalase (fig 1A, B), D bifunctional protein (fig 1E, F), and PT (fig 1G, H) was defective after incubation at 30°C (table 1). In cell lines from NALD and IRD patients, the import of catalase, AOX, D bifunctional protein, and PT greatly improved after incubation at 30°C, whereas import of these enzymes was deficient at 37°C (table 1).

**BIOCHEMICAL ANALYSIS OF FIBROBLASTS FROM CG-A PATIENTS**

The relative VLCFA oxidation capacity was expressed as a ratio of C24:0/C16:0 fatty acid oxidation activity of the cell line. Using this assay, a lower ratio of C24:0/C16:0 fatty acid oxidation indicates lower peroxisomal VLCFA oxidation activity. The import of DHAP-AT indicates lower plasmalogen biosynthesis capacity of peroxisomes. The VLCFA oxidation ratio and the activity of DHAP-AT in ZS patients belonging to CG-A showed a very low level after incubation at 30°C, although a slight increase in activities was observed. Cell lines from NALD patients, showing TS import of PTSs, showed marked improvement in activities after incubation at 30°C, whereas import of these enzymes was deficient at 37°C (table 1).

The AOX and 70 kDa peroxisomal membrane protein (PMP70) were co-localised in these cells (data not shown). In other CGs, none of the ZS cell lines showed such TS characteristics. There was a little cross reactivity of AOX antibody with mitochondria (fig 1C) co-localised with anti-mitochondria antibody (data not shown). The import of catalase (fig 1A, B), D bifunctional protein (fig 1E, F), and PT (fig 1G, H) was defective after incubation at 30°C (table 1). In cell lines from NALD and IRD patients, the import of catalase, AOX, D bifunctional protein, and PT greatly improved after incubation at 30°C, whereas import of these enzymes was deficient at 37°C (table 1).

**Table 1** The import of peroxisome matrix proteins of fibroblasts from complementation group A (CG-A) patients

<table>
<thead>
<tr>
<th>No</th>
<th>Type</th>
<th>Catalase</th>
<th>AOX</th>
<th>D bifunctional protein</th>
<th>PT</th>
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<tr>
<td></td>
<td></td>
<td>37°C</td>
<td>30°C</td>
<td>37°C</td>
<td>30°C</td>
</tr>
<tr>
<td>A-06</td>
<td>ZS</td>
<td>0</td>
<td>10</td>
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<td>ZS</td>
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<tr>
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<tr>
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<td>90</td>
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</tr>
<tr>
<td>A-04</td>
<td>IRD</td>
<td>0</td>
<td>60</td>
<td>0</td>
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</tr>
</tbody>
</table>

Cells were cultured for 72 hours at either 37°C or 30°C and immunostained by antibodies of peroxisome matrix proteins. Immunopositive cells were defined as over 100 immunoreactive particles stained by each antibody in a cell. Immunopositive cells among 20 cells were counted in five view fields at a magnification ×1000 and data are averages of these in percentages.

**Discussion**

The diagnosis in PBD patients is based on clinical features combined with a series of tests...
to assess peroxisomal function and structure, and the absence or reduction of both PTS1 and PTS2 protein imports are observed in the vast majority of cells from patients with PBDs. In fibroblasts from milder forms of PBDs, we elucidated the TS restoration of peroxisome biogenesis including the import of PTSs after incubation at 30°C. The temperature-dependent manner of peroxisome biogenesis has been identified in cells from mildly affected patients belonging to various different CGs including CG-A in PBDs, whereas it has never been seen in those from ZS patients with these CGs. In this study, we clarified the restoration of AOX import and processing after incubation at 30°C in fibroblasts from patients with ZS, NALD, and IRD belonging to CG-A. AOX is one of the PTS1 proteins, and is transported to the peroxisome by binding with Pex5p, a PTS1 receptor. Pex5p is severely reduced in the fibroblasts of patients with CG-A, C, and E. However, cells with those CGs share the ability to import small amounts of both PTS1 and PTS2 proteins. The genes responsible for CG-C and E, PEX6 and PEX1, encode Pex6p and Pex1p, respectively. These proteins belong to the AAA (ATPases associated with diverse cellular activities) family of proteins and are required for stability of the PTS1 receptor in yeast as well as in human cells. The product of the CG-A gene, which is still unknown, may also physically function with Pex5p. The biochemical functions of peroxisome as measured by VLCFA oxidation and DHAP-AT activity are restored apparently in the fibroblasts from milder NALD patients, whereas they showed lower levels in the ZS cells even at 30°C as was the case with other CGs. This might be caused by the defective import of peroxisomal beta oxidation enzymes other than AOX. The impairment in fibroblast VLCFA beta oxidation and the clinical manifestations were more severe in bifunctional protein deficient than AOX deficient patients. Therefore, ZS patients with CG-A showed as severe a clinical course and prognosis as those with other CGs, notwithstanding the AOX import was rescued at 30°C in the cells from patients with CG-A. TS import of AOX in ZS fibroblasts is unique to CG-A. A peroxin encoded by the candidate gene for CG-A may interact with Pex5p independently or with the Pex5p-AOX complex, and may lead to the characteristic import of AOX at 30°C. Isolation of the PEX gene for CG-A will help to clarify the function of peroxin for CG-A and mechanism of the selective TS import of AOX in CG-A patients.

We thank T Hashimoto for anti-human catalase, acyl-CoA oxidase, and D-bifunctional protein antibodies. This work was supported in part by a Grant in Aid for Scientific Research (16070118, 16070721) from the Ministry of Education, Science, Sports and Culture of Japan, by Health Science Research Grants from the Ministry of Health and Welfare of Japan, by the Mother and Child Health Foundation, and by the Ichiro Kane hashara Foundation.


Novel mutations of FOXP3 in two Japanese patients with immune dysregulation, polyendocrinopathy, enteropathy, X linked syndrome (IPEX)

Ichiro Kobayashi, Reza Shiari, Masafumi Yamada, Nobuaki Kawamura, Motohiko Okano, Asao Yara, Akihiro Iguchi, Nobuyoshi Ishikawa, Tadashi Ariga, Yukio Sakiyama, Hans D Ochs, Kunihiko Kobayashi

Editor—Immune dysregulation, polyendocrinopathy, enteropathy, X linked syndrome (IPEX), also known as X linked autoimmunity-allergic dysregulation syndrome (XLAAD), is characterised by enteropathy and involvement of the endocrine system, such as insulin-dependent diabetes mellitus (IDDM) and thyroiditis, which develop in association with autoantibodies in early infancy (MIM 304930, 304790). 1,2 IPEX has been mapped to chromosome Xp11.23-Xq13.3.3,4 Recent studies have indicated that FOXP3, a member of forkhead/winged-helix proteins, is a causative gene for both IPEX and an equivalent mouse, scurfy.5,6 Human FOXP3 consists of 11 exons and encodes 431 amino acids containing a zinc finger (Zn) domain, a leucine zipper (Zip) motif, and a forkhead domain.7,8 We have previously reported two unrelated Japanese patients with X linked autoimmune enteropathy associated with tubulonephropathy and endocrinopathy.9,10 We report here novel mutations in the FOXP3 gene of these patients.

Patients and methods
Clinical and laboratory findings of our patients have been previously reported.5,6 Briefly, patient 1, a boy, now 11 years old, was diagnosed as having autoimmune thyroiditis, autoimmune haemolytic anaemia, and autoimmune enteropathy at the age of 2 weeks, 2 months, and 5 months, respectively. Renal tubular dysfunction was also noted. His maternal uncle and his older brother had died of a similar diarrhoeal disease complicated by IDDM, suggesting X linked inheritance of the disease.1 7 He has been treated with a combination of tacrolimus (0.3 mg/day) and beta-methasone (0.3-0.5 mg/day).8 Hypocalcaemia and hypokalaemia often develop in spite of supplementation with calcium, potassium, and vitamin D, which suggests renal damage resulting from either the underlying disease or a side
Patient 1 showed a deletion of a single nucleotide T227 in exon 2, which results in a frameshift and generates a premature stop at codon 128 (fig 1A). Accordingly, the truncated protein in patient 1 lacks all of the domains and is apparently non-functional. This mutation was not observed in his healthy brother, sister, or father. His mother was found to be heterozygous for this mutation. Seven mutations of \( \text{FOXP3} \) have been reported in IPEX. Three cases carry single amino acid substitutions in the forkhead domain, whereas another has a single amino acid deletion in the Zip domain. \(^6\) Deletion of the forkhead domain was reported in one case, which resulted from exon 9 skipping and a frameshift accompanied by premature termination. \(^8\) The remaining two cases involve deletion of a stop codon which results in the addition of new residues. \(^6,7\) Patient 2 showed an A1087G substitution in exon 10, which results in an Ile363Val substitution (fig 1B). Wildin \textit{et al}. \(^6\) reported that no sequence variations are found in exons 10-11.

**Table 1. Primers used in the analyses of the FOXP3 gene**

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Exon 1</td>
<td>5'-AGTATCTCATGCGTCTCTCTCTGTCTTTCCAGCTCCAAGGCCAAGGAGGTGTG-3'</td>
</tr>
<tr>
<td>Exons 2–3</td>
<td>5'-AGATGCACAGAAAATTAGAAATTGACACAAGACAGTTG-3'</td>
</tr>
<tr>
<td>Exons 4–5</td>
<td>5'-AGGCTTTCTGCCACTACACCTCTCAGCTTTAGGACCAAGACAGTTG-3'</td>
</tr>
<tr>
<td>Exon 6</td>
<td>5'-GGGATAGTGCGAAACC-3'</td>
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<tr>
<td>Exon 7</td>
<td>5'-GGGATAGTGCGAAACC-3'</td>
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<tr>
<td>Exon 8</td>
<td>5'-GGGACAGAAGCAAGCTGCACTGTA-3'</td>
</tr>
<tr>
<td>Exon 9</td>
<td>5'-GGGACAGAAGCAAGCTGCACTGTA-3'</td>
</tr>
<tr>
<td>Exons 10–11</td>
<td>5'-AGATGCATGGCCGCTCTAAACCCCGAGTTTGTG-3'</td>
</tr>
</tbody>
</table>

*Letters*


table 1

Sequence analyses of exon 2 of patient 1 (A) and exon 10 of patient 2 (B). Arrowhead indicates a deletion of T at nt 227 in patient 1. Underline indicates an A to G transition at nt 1087 in patient 2.

**Results and discussion**

Patient 1 showed a deletion of a single nucleotide T227 in exon 2, which results in a frameshift and generates a premature stop at codon 128 (fig 1A). Accordingly, the truncated protein in patient 1 lacks all of the domains and is apparently non-functional. This mutation was not observed in his healthy brother, sister, or father. His mother was found to be heterozygous for this mutation. Seven mutations of \( \text{FOXP3} \) have been reported in IPEX. Three cases carry single amino acid substitutions in the forkhead domain, whereas another has a single amino acid deletion in the Zip domain. \(^6\) Deletion of the forkhead domain was reported in one case, which resulted from exon 9 skipping and a frameshift accompanied by premature termination. \(^7\) The remaining two cases involve deletion of a stop codon which results in the addition of new residues. \(^6\) Patient 2 showed an A1087G substitution in exon 10, which results in an Ile363Val substitution (fig 1B). Wildin \textit{et al}. \(^6\) reported that no sequence variations are found in exons 10-11.

**Figure 1** Sequence analyses of exon 2 of patient 1 (A) and exon 10 of patient 2 (B). Arrowhead indicates a deletion of T at nt 227 in patient 1. Underline indicates an A to G transition at nt 1087 in patient 2.
Maternal uniparental isodisomy 11q13–qter in a dysmorphic and mentally retarded female with partial trisomy mosaicism 11q13–qter

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Editor—Partial trisomy mosaicism describes the presence of a normal cell line together with an unbalanced translocation in a second cell line. Its incidence is not known. Only a few cases have been published, almost all with developmental delay and a pattern of dysmorphism. The presence of a normal cell line points towards postzygotic formation, but the origin and mechanism of formation have so far only been investigated in one case of partial trisomy 16p mosaicism and in another case of partial trisomy 21q mosaicism. In the former, a complex formation by trisomy first, translocation second, and uniparental disomy and partial trisomy third was inferred. In the latter, paternal meiotic origin of der(21)t(11;21)(q10;q10) mosaicism (46,XX,der(21);21)(q10;q10,+21) in a girl with mild Down syndrome was described.

Here, we report on a 25 year old woman with mental retardation, dysmorphic features, partial trisomy 11q13–qter mosaicism (46,XX, der(19)t(11;19)(q13;p13.3)/46,XX), maternal uniparental isodisomy 11q13–qter in the
normal cell line, and two maternal and one paternal segment(s) 11q13→qter in the abnormal cell line.

Case report
The female patient is the second child of a healthy, unrelated, white couple. An older brother is healthy. At the proband’s birth, her mother was 38 years old and her father was 39 years old. Following information, the parents opted against prenatal cytogenetic diagnosis.

Delivery by caesarean section took place at 42 weeks of a normal gestation. Weight (2500 g) and length (48 cm) were below the 10th centile. A right inguinal hernia, ipsilateral pes equinovarus, and left hip dysplasia were surgically corrected. At the age of 6 years, height (1.7 m) was on the 10th centile, weight (22 kg) on the 75th centile, and occipitofrontal head circumference (OFC) (51 cm) on the 50th centile. At the last re-examination at the age of 25 years, height (1.50 m) was below the 3rd centile and OFC (55 cm) was between the 50th and 75th centile. Dysmorphic features included deep set eyes, downward slanting palpebral fissures, broad nasal root, large nares, flat and broad philtrum, diastema of the lower incisors, small and low set ears, contractures at both elbows with inability to supinate or pronate, ulnar deviation of both hands at the wrists, slender fingers, clinodactyly of fingers IV and V, and small feet with hypoplastic nails and partial 2/3 syndactyly (fig 1). Severe developmental delay was obvious.

Methods
Chromosome analysis was performed on GTG banded fibroblast and lymphocyte cell cultures at a level of about 550 bands according to standard procedures. Fluorescence in situ hybridisation (FISH) was done according to the manufacturer’s instructions (Vysis Inc, Downers Grove, IL, USA).

Table 1 Results of molecular investigations of genomic DNA from lymphocytes and fibroblasts using microsatellites mapping to chromosome 11

<table>
<thead>
<tr>
<th>Marker</th>
<th>Location</th>
<th>(M/P blood/P fibroblasts/F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D11S2071</td>
<td>p15.5</td>
<td>ab/a/ac</td>
</tr>
<tr>
<td>D11S922</td>
<td>p15.5</td>
<td>bd/ad/ad/ac</td>
</tr>
<tr>
<td>D11S1318</td>
<td>p15.5</td>
<td>ac/ab/bc</td>
</tr>
<tr>
<td>D11S1334</td>
<td>p15.4</td>
<td>a'/ab/b</td>
</tr>
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<td>D11S860</td>
<td>p15</td>
<td>ac/ab/bc</td>
</tr>
<tr>
<td>D11S1324</td>
<td>p14</td>
<td>bc/ab/bc</td>
</tr>
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<td>D11S905</td>
<td>p12-p13</td>
<td>bc/ab/ab/ab</td>
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<tr>
<td>D11S436</td>
<td>p12-p11.22</td>
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</tr>
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<td>q13-q23</td>
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Informative markers are underlined. F = father, P = patient, M = mother.

Figure 1 Face of the proband aged 6 months (A) and 6 years (B, C).

Table 1 Results of molecular investigations of genomic DNA from lymphocytes and fibroblasts using microsatellites mapping to chromosome 11

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<td>D11S860</td>
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<tr>
<td>D11S527</td>
<td>q13.57</td>
<td>ac/ab/ab/ab/ab</td>
</tr>
<tr>
<td>D11S2002</td>
<td>q21-q23</td>
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</tr>
<tr>
<td>D11S901</td>
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<td>D11S876</td>
<td>q14.1-q23.3</td>
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<td>D11S940</td>
<td>q22</td>
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Informative markers are underlined. F = father, P = patient, M = mother.

Figure 2 Molecular results of marker D11S940 with two distinct maternal alleles (A) and two copies of the paternal allele (B) in genomic DNA from the parents, only one maternal allele in DNA from nucleated blood cells of the patient (B), but an additional paternal allele in genomic DNA from the patient’s fibroblasts (C), and only the paternal allele in the derivative chromosome 19 microdissected from the patient’s fibroblasts (D). The additional weak signal in DNA from nucleated blood cells of the patient (B) is either an artifact or represents low-level mosaicism for a paternal allele.
was microdissected from 10 metaphases according to standard procedures, the dissected chromosomes were collected in a PCR tube containing 2 µl collection drop solution (10 mmol/l Tris/ HCl, 10 mmol/l NaCl, 3 mg/ml proteinase K PCR grade), and incubated for two hours at 60°C. Subsequently, proteinase K was inactivated at 90°C for 10 minutes. Whole genome amplification was performed according to a slightly modified (no gelatine, 500 µmol/l dNTP, 1 mmol/l MgCl₂, 100 µmol/l totally degenerated, 15 nucleotide long primer) primer-extension-preamplification polymerase chain reaction (PEP-PCR) protocol. Finally, multiple highly polymorphic microsatellites were analysed by time release PCR (0.1 mmol/l dNTP, 0.24 µmol/l primers, 1.25 U AmpliTag Gold®, 1.5–2.5 mmol/l MgCl₂, using 5 µl aliquots of the preamplified DNA in a final volume of 50 µl in a Technne Progene® thermocycler for 56 cycles: 60–64°C for four minutes, 94°C for one minute), run on a 6% polyacrylamide gel, and bands visualised by silver staining.

**Results**

Chromosome analysis from lymphocytes at the age of 6 years showed partial trisomy 11q mosaicism (46,XX,der(19)(t(11;19)(q13;p13.3)) [17]/46,XX). Twenty years later, blood chromosome analysis showed a 46,XX karyotype in 40 metaphases. In fibroblasts, mosaicism was also present (46,XX,der(19)(t(11;19) (q13;p13.3)de novo)[30]/46,XX[20]). Involvement of chromosomes 11 and 19 in the rearrangement was confirmed by FISH with libraries of chromosomes 11 and 19 (Vysis® Inc, Downers Grove, IL, USA). FISH with a 19p subtelomeric probe (Vysis® Inc) showed signals on both chromosomes 19 in the normal cell line and on the normal and the derivative chromosome 19 in the abnormal cell line, and FISH with an “all telomeres” probe (Vysis® Inc) failed to show a telomere at the translocation breakpoint. Therefore, the breakpoint on 19p must be distal to the subtelomeric locus of this probe, and the rearrangement led to duplication of 11q13–qter without significant concomitant 19p deletion in the abnormal cell line. The karyotypes of both parents were normal.

Molecular investigations performed at the age of 25 years showed maternal uniparental isodisomy 11q13→qter in DNA from nucleated blood cells (table 1). In DNA from the patient's fibroblasts, paternal bands of weaker intensity of markers mapping to 11q13 were found (fig 2). The breakpoint was determined between markers D11S916 and D11S527. In blood and fibroblasts, biparental inheritance of chromosome 19 markers was shown. The results of investigations with several microsatellites from various other chromosomes were in agreement with correct paternity (data not shown). Following microdissection of the derivative chromosome 19 from 10 metaphases, primer-extension-preamplification, and subsequent microsatellite analysis, only a paternal allele and no maternal allele was found at markers D11S912 and D11S940 (fig 2).

For molecular investigations, DNA was extracted from blood and cultured fibroblasts of the patient and from blood of both parents. PCR amplification of highly polymorphic microsatellites (Research Genetics®, Huntsville, AL, USA) was carried out under standard condition. Bands were visualised by silver staining.

For a more detailed investigation of the origin of the normal chromosomes 11 and of the segment 11q13→qter translocated onto one chromosome 19, a new technical approach recently developed in our laboratory was applied. Briefly, the derivative chromosome 19
weak signal in DNA from the patient’s blood at the level of the paternal allele was interpreted as either an artefact or as representing low level mosaicism for the der(19) chromosome.

Discussion
The unexpected molecular results obtained in the present case could theoretically be explained by three different mitotic and two meiotic mechanisms of formation.

(1) Each homologue of chromosome 11 was regularly duplicated in the late interphase of a normal mitosis (fig 3, left, line 2). Both paternal sister chromatids broke and simultaneously one chromatid segment 11q13→qter was translocated to one chromosome 19, while her sister chromatid segment was lost (line 3a). In the anaphase, one normal maternal chromatid and one deleted paternal chromatid segregated to each of the two daughter cells, as well as the derivative chromosome 19 to one of the daughter cells (line 3c) and the normal chromosome 19 to the other (line 3b). Finally, mitotic reduplication of the maternal segment 11q13→qter in both daughter cells (line 4a and b) resulted in maternal uniparental isodisomy 11q13→qter in the normal (line 5a) as well as in the abnormal cell line (line 5b).

(2) Again, in a normal 46,XX zygote, inheritance of chromosome 11 was biparental and each homologue of chromosome 11 was duplicated in the late interphase of a regular mitosis (fig 3, right, line 2). Subsequently, after separation of the sister chromatids, a break occurred in only one paternal chromatid at 11q13, and the segment 11q13→qter was translocated to chromosome 19. The complete paternal chromatid segregated together with the derivative chromosome 19 regularly to one daughter cell (line 3c), while the isolated part of the 11q chromatid segregated with the other daughter cell together with two normal chromosomes 19 (line 3d). Finally, mitotic reduplication of the maternal segment 11q13→qter in the daughter cell containing the deleted 11 (line 4) resulted in maternal uniparental disomy (UPD) (11q13→qter) in the now (through correction) normal cell line (line 5c) and, in contrast to the first mechanism, in biparental inheritance of the whole chromosome 11 in the abnormal cell line (line 5d).

(3) Considering the third mechanism, two independent mitotic events must be postulated (fig 4). The first is characterised by an exchange between two non-sister chromatids (line 3a) resulting in two daughter cells with opposite segmental UPD (11q13→qter) (line 6a and b). The cell line with maternal UPD (11q13→qter) would persist as shown in blood (line 6a), whereas the cell line with paternal segmental UPD (11q13→qter) would not be viable (line 6b). In another cell with biparental inheritance of chromosome 11 (line 2b), a break in either one (line 3c) or in both (line 3b)
chromatids would have occurred. In the latter case, one segment would have been translocated to one chromosome 19 (line 4), whereas the other would have been lost (line 6e). The cell line with del(11)(q13qter) would not have been viable (line 6d). In the other cell line, maternal segmental UPD (11q13→qter) (line 6c) would have been formed by reduplication during interphase (line 5). In the alternative case of breaking of only one chromatid (line 3c), one cell line with del(11)(q13qter) (lethal) (line 6g) and a second cell line with biparentally inherited chromosomes 11 and an additional translocation of the paternal segment 11q13→qter would have arisen (line 6f). This partial mechanism is less likely, because the molecular investigations showed a weaker intensity of the paternal versus the maternal alleles.

(4) A fourth mitotic mechanism (fig 5) would be characterised by a normal zygote and segmental mitotic reduplication on the maternal chromosome. Then, a crossing over between one paternal chromatid and the reduplicated segment as well as translocation of the paternal segment to 19p occurred. Segregation resulted in the karyotype described.

(5) A fifth, in part meiotic mechanism would require a balanced 11;19 translocation already present in the paternal gamete (fig 6, left, line 1a). Thereafter, again a mitotic reduplication of the maternal segment 11q13→qter must have happened (line 2) and, in addition, in the normal cell line the loss of the translocated paternal segment 11q13→qter (line 5a) or even the loss of the entire derivative chromosome 19 combined with mitotic reduplication of its normal homologue must be postulated. The latter was excluded by the biparental inheritance of chromosome 19 in blood and fibroblasts (data not shown).

(6) A sixth, again in part meiotic mechanism would require a trisomic zygote resulting from maternal non-disjunction (fig 6, right, line 1b). Mitotic crossing over between the paternal chromatid and one maternal chromatid (line 4b) would result in two different daughter cells (line 5e and f). One with biparental chromosomes 11 and translocation of the paternal segment 11q13→qter to one chromosome 19 (line 5e), and a second with exclusively maternal 11q13→qter material (line 5f). In addition, in both cell lines, one chromosome must have been lost, the chromosome with del(11) (q13→qter) in one (line 5d), and a second complete maternal chromosome 11 in the other (line 5g). This mechanism is supported by the advanced maternal age of 38 years at delivery; on the other hand, complete isodisomy would better fit a postmeiotic formation.

In total, each mechanism requires a minimum of three subsequent or in part simultaneous events. With the presently available molecular investigations, it was not possible to define one single possible mechanism of formation. Isodisomy in the normal cell line could be considered to indicate mitotic formation. Apart from paternal UPD (11p15) in up to 20% of cases with Beckwith-Wiedemann syndrome,6 segmental UPD has rarely been reported.7–11 Similarly, paternal UPD of the whole chromosome 11 has been described only twice, in a fetus with severe intrauterine growth retardation, intestinal malrotation, and confined placental mosaicism,12 and in a mosaic state in a girl with Beckwith-Wiedemann syndrome.13 To the best of our knowledge, maternal segmental UPD of chromosome 11 has not been reported so far. Full trisomy 11 is not viable and non-mosaic duplication of 11q13→qter has been reported only rarely. The phenotype of the latter is characterised by cardiac anomalies, restricted elbow movements including supination and pronation, facial dysmorphism, small and low set ears, and mental retardation.1

The clinical consequences of the complex rearrangement found in our patient are difficult to evaluate. The phenotype strongly resembles several cases with duplication of 11q13→qter, and thus could be caused only by this. On the other hand, an additional effect of mosaicism for the loss of the normal homologue of an imprinted gene and/or of homozygosity for a mutated allele of a recessive gene cannot yet be fully excluded.

We are grateful to the family for their cooperation. The study was supported by the Swiss National Foundation, grant Nos 32-45604.95 and 32-56053.98.
Mitosis


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High frequencies of ICF syndrome-like pericentromeric heterochromatin decondensation and breakage in chromosome 1 in a chorionic villus sample

Melanie Ehrlich, Fern Tsien, Delma Herrera, Viola Blackman, Jennifer Roggenbuck, Cathy M Tuck-Muller

EDITOR—The immunodeficiency, centromeric region instability, and facial anomalies syndrome (ICF) usually involves mutations affecting the catalytic domain in DNMT3B, one of the three human genes known to encode DNA methyltransferases.1–3 ICF always results in defective immunity, a high frequency of chromosomal abnormalities in the vicinity of the centromere (pericentromeric region) of chromosome 1 and/or chromosome 16 in mitogen stimulated lymphocytes, and hypomethylation of a small portion of the genome.4–6 ICF symptoms are manifested often from infancy and this syndrome can cause early childhood death from infections. The DNA sequences targeted for undermethylation in ICF include the heterochromatin adjacent to the centromeres of chromosomes 1 and 16 (1qh and 16qh), where a high incidence of chromatin decondensation, chromosome and chromatid breaks, and rearrangements to form multiradial chromosomes are characteristically seen in mitogen stimulated ICF blood cultures and in ICF lymphoblastoid cell lines.6–9 These aberrations are more common in chromosome 1 than in chromosome 16 and only infrequently observed in chromosome 9.5–8 We describe an unusual primary culture from a chorionic villus (CV) biopsy in which a high frequency of ICF-like chromosomal abnormalities was observed. However, follow up indicated that the infant did not have ICF.

A 30 year old, gravida 4, para 0, ab 3 white female was referred for genetic counselling and CV sampling because of a previous pregnancy with trisomy 13. Both the patient and her husband were phenotypically normal and healthy apart from their reproductive history. The patient’s first two pregnancies ended in spontaneous abortion at 15 and 10 weeks. No fetal studies had been performed; parental chromosomes were analysed and reported to be normal. The third pregnancy was found to be affected with trisomy 13 (47,XY,+13) and was then terminated. During the fourth pregnancy, the patient was offered and accepted CV sampling for prenatal diagnosis of trisomy conditions.

In this CV sample, routine cytogenetic analysis of 20 metaphases from an eight day culture in Chang B medium with Chang C supplement (Irvine Scientific) showed four cells with pericentromeric breaks in chromosome 1, one with a deletion of the long arm of chromosome 1, and seven with decondensation of 1qh (fig 1B, C). The high frequency of chromosome 1 abnormalities led to the examination of an additional 100 metaphases from the eight day culture. Forty-one percent of the 120 cells examined had abnormalities in the pericentromeric region of chromosome 1. Decondensation of the pericentromeric heterochromatin of chromosomes 1, 16, and 9 was seen in 38, 5, and 0.8% (one cell) of the metaphases, respectively (fig 1). Eleven cells (9%) had chromosome breaks in the pericentromeric heterochromatin of chromosome 1 such that both arms were present but widely separated, one cell (0.8%) had a similar break at 16qh, and one (0.8%) had a pericentromeric break of only one chromosome 1 chromatid. Three cells (2.5%) had a deletion of 1q. In addition, a triradial(1)p,q,q was observed (fig 1D).

We passaged these CV cells three times at 1:5 splits after the clinical analysis and examined 100 additional metaphases. The frequency of 16qh decondensation increased from 5% to 26% and decondensation of 1qh increased from 38% to 49% at passage 3 compared to the initial eight day primary culture. At passage 3, there were 11 metaphases with a deletion of 1q, six with a chromosome break at 1qh, two with a multiradial chromosome (a triradial(1)p,p,q in one and a quadradial(1)p,p,q in the other), one with an isochromosome composed of two pericentromerically fused 1q arms, and one with a deletion of 16q.

Because the ICF syndrome, in which these specific chromosomal anomalies are found, is always accompanied by hypomethylation of limited portions of the genome, including the major DNA sequence in 1qh and 16qh, satellite 2 (Sat2), we examined methylation of chromosome 1 Sat2 DNA sequences in the...
Letters

883

The present patient was counselled that the cytogenetic findings on the CV sample could reflect a culture artefact, a fetus affected with ICF, or other unknown aetiology. Given the high frequency of ICF-like chromosomal anomalies in the patient’s CV cells, a follow up study of amniotic fluid at 16 weeks was done and showed no cytogenetic anomalies. At 39 weeks of gestation, the patient delivered a healthy male infant, weighing 3856 g, with no anomalies in the patient’s CV cells, a follow up study of amniotic fluid at 16 weeks was done and showed no cytogenetic anomalies. However, the majority of the CV samples (58%) displayed low levels of these anomalies with 1-8% (median 2%) of their metaphases showing these chromosome 1 or chromosome 16 aberrations. Ninety-four percent of these anomalies were decondensation of 1qh or 16qh, with 3.4-fold more 1qh than 16qh decondensation. The only pericentromeric rearrangements seen were two whole arm deletions of 1q and one break at 1qh resulting in separated 1p and 1q arms. No clonal abnormalities except for the chromosome 1 and chromosome 16 aberrations were observed in these metaphases from randomly chosen CV samples. These CV samples were obtained over several years and included samples tested at the same time as the patient’s sample and all were analysed by the same method. Also, no change in the lot of medium or fetal calf serum can explain the different results obtained from the patient’s sample and the random samples.

Anecdotal observations of these types of pericentromeric chromosome 1 and 16 anomalies in normal CV metaphases are common although, to our knowledge, they have been described by only one group in detail and mentioned by another. In the latter case, Bjorck et al stated without elaboration that “heterochromatic decondensation can occasionally be seen in both amniocytes and cultured chorionic villi without any pathological significance.” The former group, Miguez et al, found in a study of 244 24 hour CV cultures that about 9% of the metaphases displayed chromosomal lesions, usually breaks or gaps at various fragile sites. The most common site was at 1qh (1q12) or 1q21.1 although the quality of the chromosomes allowed only 36% of the preparations to be “successfully banded” and the frequency of cases with this anomaly was not given. In another report, this group described decondensation in 1qh, 9qh, 16qh, or 1qh in 2.4, 3.6, and 0.3, and 0.2%, respectively, of the 5820 examined metaphases, with 47% of 339 24 hour CV cultures displaying such condensation in at least one metaphase. In contrast to those investigators who observed decondensation in 9qh to be most frequent, we found DNA from the CV cultures. Amniocytes, like postnatal somatic tissues, are derived from epiblast cells of the embryoblast. A high level of methylation of this sequence was seen in DNA from the patient’s amniocyte culture as well as in random amniocyte samples (data not shown). These results are incompatible with the patient harbouring ICF type DNMT3B mutations because all tested ICF cell populations from diverse tissues or cultured cell types display Sat2 hypomethylation. We compared the frequency of ICF-like pericentromeric chromosome 1 anomalies in this patient’s CV sample to others. Retrospective examination of 2250 metaphases from 26 clinical CV samples (50-100 metaphases each) from eight day random CV cultures, which had been interpreted as normal, showed that only 47 of the metaphases (2%) had a pericentromeric abnormality of chromosome 1 or 16. However, the majority of the CV samples (58%) displayed low levels of these anomalies with 1-8% (median 2%) of their metaphases showing these chromosome 1 or chromosome 16 aberrations. Ninety-four percent of these anomalies were decondensation of 1qh or 16qh, with 3.4-fold more 1qh than 16qh decondensation. The only pericentromeric rearrangements seen were two whole arm deletions of 1q and one break at 1qh resulting in separated 1p and 1q arms. No clonal abnormalities except for the chromosome 1 and chromosome 16 aberrations were observed in these metaphases from randomly chosen CV samples. These CV samples were obtained over several years and included samples tested at the same time as the patient’s sample and all were analysed by the same method. Also, no change in the lot of medium or fetal calf serum can explain the different results obtained from the patient’s sample and the random samples.

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Figure 2 Southern blot analysis showing hypomethylation of Sat2 DNA from chromosome 1 in term placenta, CV samples, and ICF cells but not in postnatal somatic tissues. BstBI digested DNA from the following samples was hybridised to a chromosome 1 Sat2 specific probe and autoradiographed: term placenta; the patient’s CV(CV1) culture from passage 3; four random CV samples from passage 3 (CV A, CV G, CV G, and CV H); lung and thymus from normal trauma victims; and a lymphoblastoid cell line (LCL) from an ICF patient.
only a single metaphase exhibiting decondensation of 9qh in this study and that was in the CV sample with the high frequency of 1qh decondensation. This difference between their results and ours could be because of the use of one day rather than eight day cultures. The longer culture time affords better quality metaphase chromosomes but selects for extraembryonic mesodern cells (which are derived from hypoblast cells of the embryo in the CV samples as opposed to mostly cytrophoblast metaphases (which are trophoblast derivatives) in the one day CV cultures.16 Cell cultures from hypoblast cells of the embryoblast in the embryonic mesoderm cells (which are derived from them in this study and that was in the patient who gave such a high percentage of chromosome 1 anomalies in the corresponding CV culture. The chromosome 1 (and chromosome 16) pericentromeric aberrations observed in this clinical CV sample may have formed during culture in vitro. However, there was nothing remarkable about the CV tissue used for cell culture or in this culture's hypomethylation in the ICF syndrome. Whatever the cause of the anoma-
lies in this patient's sample, our analysis of CV samples indicates that ICF-like chromosomal abnormalities are part of the normal spectrum for CV chromosomes and need not indicate any clinical condition. Furthermore, we conclude that CV sampling should not be attempted to prenatally diagnose ICF by the chromosome abnormalities used to diagnose this syndrome in mitogen treated blood samples from immunodeficient patients. In families at risk for ICF, prenatal analysis for DNMT3B mutations1 would be preferable.

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Supernumerary marker chromosome (1) of paternal origin and maternal uniparental disomy 1 in a developmentally delayed child

Benno Röthlisberger, Tatjana E. Zerova, Dieter Kotzot, Tamara I. Buzhievskaya, Damina Balmer, Albert Schinzel

Editor—At least 168 cases with a supernumerary marker chromosome (SMC) from all chromosomes not including chromosome 15 have been documented. Birth prevalence is estimated at 0.14 to 0.72 per 1000. Subjects with a SMC have a partial trisomy (duplication) and in some cases a partial tetrasomy (triplication) of the genetic material contained in the SMC. The risk of an abnormal phenotype associated with a randomly ascertained de novo SMC derived from acrocentric autosomes (excluding chromosome 15) is estimated to be approximately 7% compared with approximately 28% for SMCs derived from non-acrocentric autosomes. The great variability of clinical findings in patients with SMCs originating from the same chromosome is probably the result of variation in size and genetic content, the degree of mosaicism, and uniparental disomy of the normal homologues of the chromosome from which the SMC derived.

Evidence that subjects with SMCs might have an increased risk for UPD of the structurally normal homologues of the SMCs has been reported by several authors. To the best of our knowledge the coexistence of SMCs with UPD has been described for chromosomes 6, 7, 15, 20, and X. Here, we describe a further patient with multiple congenital anomalies, developmental delay, and the unique finding of coexistence of SMC 1 mosaicism and maternal uniparental disomy 1.

Case report

The female patient was born at term after an uneventful pregnancy. At her birth, her mother was 33 years old and her father was 47 years old. Birth weight was 2500 g and length 49 cm. At the age of 6 years, she was investigated because of mental retardation. Height (1.14 m) and weight (17 kg) were within the normal range, but head circumference (44 cm) was far below the 3rd centile. Additional findings were temporal narrowing, downward slanting palpebral fissures, long eyelashes, high palate, pointed chin, low set and dysplastic ears, hip dysplasia, and tapering fingers with clinodactyly of fingers 2, 4, and 5.

Methods

Table 1 Results of molecular genetic investigations

<table>
<thead>
<tr>
<th>Primer</th>
<th>Locus</th>
<th>cM</th>
<th>Alleles</th>
<th>SMC</th>
<th>Origin of alleles</th>
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</table>

The alleles are given in the order patient, mother, father. Allele designations (a-d) are arbitrary. Genetic mapping according to the sex averaged linkage map by the Genetic Location Database (LDB). Mat=mataural, iso=isodisomy, NP=not performed, NI=not informative.
additional small marker chromosome (SMC) in 14 of 40 metaphases (35%) investigated (fig 1A). Chromosome analysis of the mother and the father showed normal karyotypes. In order to determine the origin of the SMC, a microFISH experiment with reverse painting to normal metaphases was performed. It could be shown that the SMC was formed by a region of chromosome 1, 1p21.1→1q12 (fig 1B).

Application of the “all telomeres” probe (Vysis® Inc, Downers Grove, IL, USA) showed no telomeric signal on the extra chromosome indicating that it is a ring chromosome (data not shown).

Microsatellite analysis on genomic DNA of the patient and the parents showed that the patient had inherited two maternal alleles (heterodisomy and isodisomy respectively at different loci) but no paternal allele at different loci of chromosome 1 (table 1, fig 2A, B). Investigations of several microsatellites from various other chromosomes were in agreement with correct paternity. Based on these results, the karyotype of the patient may be described as 47,XX,UPD(1)mat,+der(1)(p21.1q12)/46,XX,UPD(1)mat.

Probably owing to the patient’s relatively low level mosaicism of leucocytes with the SMC, the microsatellite analysis with pericentromeric markers did not result in an unambiguous determination of the parental origin of the SMC. Although some markers showed a weak paternal band, this was not obvious enough for unequivocal parental determination. Therefore, a new approach combining microdissection of the SMC with subsequent PEP-PCR and conventional microsatellite PCR was performed. All investigated markers mapping
to the duplicated region clearly showed one paternal band and no maternal band and were thus in agreement with paternal origin of the SMC (fig 2B). In addition, by investigating the genomic DNA with these pericentromeric markers, three markers mapping within the duplicated region on the short arm of chromosome 1 within a 10 cM distance from the centromere and also two markers at the distal end of the short arm of chromosome 1 showed maternal isodisomy (table 1).

Discussion
The great variability of clinical symptoms in patients with a SMC of the same chromosomal origin is probably the result of variation of the genetic content, the degree of mosaicism (especially, as in our case, in ring chromosomes), and uniparental disomy of the normal homologues from which the SMC is derived.1,2

To allow any karyotype-phenotype correlation, comparison of a sufficient number of accurately investigated cases is necessary. The best way to characterise a SMC is a multistep approach: (1) classical cytogenetic procedures, which provide information about the degree of mosaicism by investigating many cells, if possible, from different tissues; (2) microFISH, which gives detailed information about the chromosomal content of the SMC; (3) microsatellite analysis of the normal homologues, which gives information about uniparental disomy and a clue towards the mechanism of formation; and (4) the absence of telomeres indicated that the SMC is a ring.

In most cases with an SMC originating from chromosome 1, neither FISH analysis with locus specific probes nor molecular investigations with microsatellites was performed.3,4 The patients described show great variability of their phenotypes and so far no distinct syndrome has been associated with the group of cases with a supernumerary marker chromosome originating from chromosome 1.

The phenotype of four patients with maternal UPD(1) reported so far is normal and therefore suggests that maternally imprinted loci on chromosome 1, if existing at all, probably do not influence the phenotype. Thus, the congenital anomalies in our patient are probably not the result of the maternal UPD of chromosome 1, but are more likely related to the mosaic duplication of chromosome 1 (p21.1→q12).

Several authors assumed that subjects with SMCs might have an increased risk for UPD of the structurally normal homologues from which the SMCs derived. However, the incidence of UPD associated with a SMC is not known, and so far only one case each of chromosomes 6, 7, 20, and X,3,5,6 as well as nine cases of chromosome 15, seven with Prader-Willi syndrome, and two with Angelman syndrome, have been reported.6,7,8,9,10,11,12

Coexistence of UPD with a SMC is mainly explained by the following two mechanisms:13

1. First, duplication of the normal homologue in a zygote which has inherited a SMC in place of the normal corresponding chromosome “rescues” an aneuploidy. In this case, UPD arises by mitotic non-disjunction and therefore complete isodisomy should always be observed.
2. Second, the zygote may have originated as a trisomy with the single parental chromosome being lost through a breakage event or, alternatively, a disomic gamete was fertilised by a gamete with a SMC formed during meiosis.

Heterodisomy, as reported in our case, can only be explained by the second mechanism. The distribution of isodisomic and heterodisomic segments in our case, pericentromeric and more distal, respectively, indicates an error in maternal meiosis II resulting in the maternal UPD(1). Whether the SMC was formed during paternal meiosis or in an early somatic cell division cannot be differentiated. If it was formed during paternal meiosis, the coexistence of a SMC with UPD may be considered as a coincidence. Even if it was formed postzygotically, our case might at best not contradict the hypothesis that subjects being uniparentally disomic might have an increased risk of bearing a SMC of the same homologue (rather than the reverse). In other words, the presence of two maternal (or paternal) chromosomes in the zygote might constitute a risk for the formation of a paternal (or maternal) SMC through a breakage event.

Parental origin of the normal chromosomes and of the SMC is sometimes difficult to determine. In many instances, only quantitative results can be obtained (mitotically formed mosaicism, supernumerary marker chromosomes). The new technical approach applied in the case reported here allows the unequivocal tracing back of informative alleles from a dissected abnormal chromosome to one of the parental homologues.

In conclusion, we present a case with coexistence of a SMC 1 with maternal UPD(1) and hence mosaicism for complete maternal uniparental disomy and partial trisomy for a small pericentromeric segment of chromosome 1.

- To the best of our knowledge, 10 cases of supernumerary marker chromosome 1 (SMC 1) mosaicism have been reported so far. Most of these ring shaped markers were identified by fluorescence in situ hybridisation (FISH) with centromere specific probes.
- We report another case with SMC 1 mosaicism identified by combining chromosome microdissection with reverse painting to normal metaphases (microFISH).
- Microsatellite analysis showed heterodisomic maternal uniparental disomy (UPD) of the normal chromosomes 1. In addition, a new approach combining chromosome microdissection, primer-extension-preamplification polymerase chain reaction (PEP-PCR) and standard PCR of highly polymorphic microsatellites showed paternal alleles only within the region of the SMC. This indicates that the SMC is of paternal origin.
1 combined with uniparental disomy for the rest of the chromosome. By a new technical approach, which allows the parental origin of the marker chromosome to be determined, even in cases with low level mosaicism, we were able to unequivocally show the paternal origin of the marker.

The authors are grateful to the family for their cooperation. The study was supported by the Swiss National Foundation, grants 32-45604.95, 32-56053.98, and 71PS1778.

Sponastrime dysplasia: presentation in infancy

A C Offiah, M Lees, R M Winter, C M Hall

EDITOR—The case of a white female with sponastrime dysplasia is presented.

Case report
The patient is the first and only child of healthy, non-consanguineous parents. During routine ultrasound scanning, the fetus was noted to have extremely short limbs and a tentative diagnosis of achondroplasia was made. The pregnancy was otherwise uncomplicated. The baby was delivered at 40 weeks’ gestation by emergency caesarian section because of breech presentation. Birth weight was 2460 g and length 44 cm (both below the 3rd centile). During infancy and early childhood, her height remained well below the 0.4th centile.

Examination showed a small baby with midface hypoplasia and a small, slightly upturned nose. With increasing age, the prominent forehead, saddle shaped nose, and midface hypoplasia became more obvious. There was rhizomelic and mesomelic shortening of her upper and lower limbs (fig 1). She was noted to have short, broad hands and feet with deep palmar creases, short toes, and dimples in the elbows and knees. In addition, she had marked generalised joint laxity except at the elbows where extension was limited. A skeletal survey at 5 months was not diagnostic, but achondroplasia was excluded.

Chromosomal analysis was normal (46,XX). At 4 months of age, she was noted to have an eczematous skin rash, and there followed several hospital admissions for recurrent chest infections; investigations showed hypogammaglobulinaemia. Both the skin rash and the hypogammaglobulinaemia gradually normalised, effectively excluding a diagnosis of a short limbed dwarfism syndrome in association with immunodeficiency. Despite a dysplastic (but not dislocated) left hip, crawling and walking were not delayed; however at 2 years of age she developed a waddling gait, and at 2 years 5 months a dislocated left hip was diagnosed. At 3 years, an open varus reduction and derotation osteotomy was performed. She made good recovery from her operation, and at 4 years 3 months the internal fixator was removed.

Developmental milestones were reached at appropriate ages, and mental development was normal. Radiographic findings
The radiographic findings are illustrated in figs 2-7.

Spine (figs 2 and 3)
These x rays show the typical changes in the shape of the vertebral bodies as described by Langer et al in 1996 and again in 1997:

platyspondylly improving with the patient’s age; a distinct junction (apparent in early/mid-childhood) between the anterior and posterior parts of the vertebral bodies (this is as a result of the anterior portions having convex end plates compared to the straight end plates of the posterior portions); a central anterior beaking of the vertebral bodies; and increasing concavity of the posterior surfaces of the vertebral bodies (posterior scalloping). There is a progressive kyphoscoliosis and mild osteopenia. Additionally, there is loss of the normal increase in interpedicular distance from L1 to L5 which can be appreciated in the radiograph taken at birth (fig 2A).

Long bones (figs 4-7)
Characteristic changes in the proximal femora consist of a pronounced bony projection of the lesser trochanter, short femoral necks, and loss of the normal metaphyseal flare. These give a “spanner-like” appearance to the proximal
femora (fig 4A), which becomes less marked with time (fig 4B). There was progressive development of coxa vara deformity, which although complicated by dislocation on the left, could be appreciated bilaterally.

Vertical metaphyseal striations are best seen around the knee and in the distal radius. These striations developed with age, and were not demonstrable radiologically until 4 years 8 months (figs 5 and 6).

There is retardation of bone age (fig 5) when compared to the standards of Greulich and Pyle (2 years 6 months at a chronological age of 4 years 8 months, SD 11.65 months). A pseudoeiphipysis of the first metacarpal is seen, a finding which has also been seen in several other patients. All epiphyses are small for age and slightly irregular.

Other features include a predominantly rhizomelic shortening of the limbs and flaring of the distal humeral metaphyses, giving them a rather bulbous appearance which becomes more pronounced with time. In addition, there is a curious appearance of the proximal humeral diaphyses (fig 7), consisting of a linear radiolucency affecting the medial cortex and running obliquely. Beneath this, there is cortical thickening (buttressing) and mild angulation of the humeral shaft. This appearance, reminiscent of focal fibrocartilagenous dysplasia, was bilaterally symmetrical.

Discussion

Sponastrime dysplasia is a rare but distinct entity which can be categorised as a spondyloepimetaphyseal dysplasia. The acronym was derived by Fanconi et al from the spondylar and nasal alterations which occur in addition to the striations of the metaphyses. It has been documented in several sets of sibs, but in the 13 cases reported to date (including ours) the parents have been non-consanguinous. It would appear that sponastrime dysplasia is inherited as an autosomal recessive disorder although germline mosaicism is a possibility.

Other than ours, 12 cases of true sponastrime dysplasia have been reported, of which only one, described by Langer et al, was an infant. Comparing the findings of Langer et al to those in our patient, there would appear to be specific findings which may allow the diagnosis of sponastrime dysplasia to be made at birth and in infancy. Clinically these are non-specific and include midfacial hypoplasia, a saddle shaped nose, short limbs, and short stature. Radiological features, however, are more specific; the proximal femora have a characteristic radiological “spanner-like” appearance with a bony projection of the lesser trochanter, short curved femoral necks, and loss of the normal metaphyseal flare. This appearance of the proximal femora becomes less apparent with age. In the spine there is a significant platyspondyly with loss of the normal progressive widening of the interpedicular distances from L1 to L5. While present in all eight of the previous cases in which the result of spinal radiography was available, this is the first time that the gradual reduction in interpedicular distance has been
confirmed in the neonate and infant. As in other reported cases, 1–6 patients may develop a scoliosis, which, as in our patient, may be significant.

Lachman et al 5 obtained biopsies from the iliac crests of two patients with sponastrime dysplasia. Light and electron microscopic examination findings suggested a specific morphological appearance for sponastrime dysplasia. Unfortunately, despite open surgery, a histological sample was not obtained in our patient to confirm this appearance.

Short stature is a universal finding. The severity of short stature seen in our patient (fig 1) was made worse by the development of a progressive (and significant) kyphoscoliosis (fig 2B).

Our patient developed bilateral coxa vara deformity, which has been documented in two other patients. 7 There have been two published cases of patients who both developed thoraco-lumbar scoliosis warranting surgery; 8 our patient has also developed a significant scoliosis.

Including our patient, mild osteopenia has been described in all eight of the patients in whom this information is available.

This is the first case of sponastrime dysplasia in which transient hypogammaglobulinaemia has been reported.

In our patient, there was a predominantly rhizomelic limb shortening and prominence of the distal humeral metaphyses which had a rather bulbous appearance. These changes have not previously been described in sponastrime dysplasia.

The appearance of the proximal humeri was reminiscent of focal fibrocartilaginous dysplasia, a known cause of tibia vara. On plain film it appears as a tongue-like cortical defect. It affects the medial cortex and leads to varus deformity centred at the lesion. On MRI, there is no associated soft tissue mass. 13 Although it has previously been reported in the upper limb, 13 to our knowledge it has never been seen bilaterally. Histologically, there is abnormal growth and remodelling of fibrocartilaginous tissue at the growth plate interface between the tendon and its bony attachment. 15 16 Comparing this with the histological findings of Lachman et al 4 in sponastrime dysplasia, we wonder if a similar (abnormal) process is occurring in the two conditions. Interestingly, neither the humeral shaft changes nor the metaphyseal striations around the wrist and knees were radiologically obvious at birth or in infancy, but became apparent in mid-childhood (4 years 8 months). The natural history of focal fibrocartilaginous dysplasia is that of spontaneous resolution 17; as we have neither histology nor radiographs beyond 4 years 8 months in our patient, we feel that radiographic follow up may be useful.

Fig 8 shows the patient’s metacarpophalangeal pattern profile (MCPPP) performed at 4 years 8 months according to the method of Garn et al. 18 All 19 bones had negative Z values, confirming shortening. Z scores ranged from −1.2 (distal phalanx 3) to −3.6 (metacarpal 3). The normal Z score range for the metacarpals is +2 to −2. All metacarpals in this patient had a value of less than −2, again confirming...
brachymetacarpy as a feature of sponastrime dysplasia. Fig 8 illustrates an up and down variation in the hand pattern, particularly affecting the phalanges. In our patient, this is not as pronounced as in the cases of Cooper et al and Fanconi et al. The MCPP obtained by Camera et al shows a much flatter pattern, further supporting the likelihood of a different condition in their patient. The pattern variability index (OZ) in this patient, based on the method described by Garn et al, was calculated to be 0.51. A score greater than 0.7 is said to indicate hand dysmorphogenesis; Cooper et al calculated a value of 0.73 for their patient.

Their patient was 6 years 7 months old and ours 4 years 8 months; age is therefore an unlikely explanation for the differences and MCPP analyses are required in more patients with sponastrime dysplasia, in order to evaluate its use in aiding the diagnosis.

Intelligence in patients with true sponastrime dysplasia is normal. Several patients reported as cases of sponastrime dysplasia are likely to be cases of spondyloepimetaphyseal dysplasia (SEMD) with large joint dislocations (first characterised by Hall et al). The two sisters described by Camera et al and the patient described by Verloes et al do not seem to have either sponastrime dysplasia or SEMD with large joint dislocations. Table 1 summarises and compares the findings in these conditions.

As previously reported, the metaphyseal striations are not a prominent feature in the early stages, and in our patient were not radiologically apparent until the child was almost 5 years old.

Table 1 Differential diagnosis of sponastrime dysplasia

<table>
<thead>
<tr>
<th>Feature</th>
<th>Sponastrime</th>
<th>SEMD with large joint dislocations</th>
<th>Camera et al</th>
<th>Verloes et al</th>
</tr>
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<tbody>
<tr>
<td>Intelligence</td>
<td>Normal</td>
<td>Normal</td>
<td>Severe retardation</td>
<td>Severe retardation</td>
</tr>
<tr>
<td>Short stature</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Midface hypoplasia</td>
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<td>+</td>
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<tr>
<td>Saddle nose</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Rocker bottom feet</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Joint laxity</td>
<td>+</td>
<td>++</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Hypotonia</td>
<td>+</td>
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<tr>
<td>Large joint dislocations</td>
<td>–</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Osteopenia</td>
<td>+</td>
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<td>Microcephaly</td>
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<td>–</td>
<td>+</td>
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<td>Wormian bones</td>
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<tr>
<td>Striated metaphyses*</td>
<td>+/++</td>
<td>+</td>
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<tr>
<td>Irregular metaphyseal margins*</td>
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<tr>
<td>Epiphysial involvement</td>
<td>+</td>
<td>++</td>
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<td>–</td>
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<tr>
<td>Delayed bone age</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Scoliosis</td>
<td>+/+++</td>
<td>-</td>
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</tr>
<tr>
<td>Characteristic age related vertebral changes*</td>
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<tr>
<td>Lumbar lordosis</td>
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<td>Reduction in interpedicular distances from L1 to L5</td>
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</tr>
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<td>Brachymetacarpia</td>
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<td>–</td>
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<tr>
<td>Pseudoepiphyses of metacarpals</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>Long slender phalanges</td>
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<td>–</td>
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<tr>
<td>MCPP</td>
<td>Up - down variation</td>
<td>Relatively flat</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Major radiological diagnostic features of sponastrime dysplasia: + mild; ++ moderate; +++ severe; – absent; ? not mentioned in paper.
years of age, so sponastrime dysplasia was not considered as a possible diagnosis; indeed this patient has previously been presented by Slaney et al as a new syndrome of spondyloepimetaphyseal dysplasia, eczema, and hypogammaglobulinaemia. We therefore tend to agree with Langer et al3 who feel that less emphasis should be placed on these striations and more on the findings in the spine. They suggest that the condition be called “spondylometaphyseal dysplasia with midface hypoplasia and depressed nasal bridge”. However, because of the mild epiphyseal abnormalities, and because it is now present in textbooks and databases as sponastrime dysplasia, we feel that “spondyloepimetaphyseal dysplasia (SEMD), sponastrime type” is a more appropriate term.