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Prenatal Diagnosis in Rett Syndrome

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Key Words
Rett syndrome · *MECP2* gene · Prenatal diagnosis, Rett syndrome · De novo mutation · Germline mosaicism · Genetic counseling

Abstract
Background/Aim: Rett syndrome (RTT) is an X-linked neurodevelopmental dominant disorder that affects almost exclusively girls. The disease is mainly sporadic, caused by de novo mutations at *MECP2* gene (Xq28), but a low percentage of familial cases have been reported. We present the results of RTT prenatal diagnosis in three families and discuss the usefulness of such analyses in diseases caused mainly by de novo mutations. ***Methods:*** For adult individuals, DNA was extracted from peripheral lymphocytes; for fetus analysis it was obtained from cultured amniotic fluid or from chorionic biopsy specimens. Mutation detection at *MECP2* gene was first carried out in the patients by SSCP/HD analysis and subsequent sequencing. Family studies and prenatal diagnoses were done by direct analysis of previously characterized patients' mutations using SSCP/HD or restriction analysis. ***Results:*** Heterozygous mutations identified in the 3 patients were: 1061del96bp, 473C→T, and 763C→T, respectively. Mutations were not present in the mothers' DNAs obtained from peripheral lymphocytes. None of the 3 fetuses analyzed carried the mutation of the affected sister. ***Conclusions:*** Recurrence within RTT families can be due to asymptomatic nonpenetrant carrier mothers or to parental germinal mosaicism for the *MECP2* mutation. Since germline mosaicism can neither be predicted nor detected, families with 1 affected patient whose RTT-causing mutation has been previously identified can benefit from prenatal diagnosis which contributes to a decrease in the recurrence risk in a new pregnancy comparable to that of the normal population.

Introduction

Rett syndrome (RTT; MIM * 312750) is an early-onset neurodevelopmental disorder that accounts for 10% of severe mental retardations in females. In classic RTT, neurological regression becomes apparent around the 1st year of life, after a neonatal period of normal development. It is characterized by loss of acquired speech and motor skills, stereotypical hand movements, seizures, autistic behaviour, and brain growth impairment [1–3]. Mainly sporadic, the disease has an estimated prevalence of 1/10,000–1/15,000 girls [4].

De novo dominant mutations at the X-linked *MECP2* gene have been identified in approximately 70% of the sporadic RTT patients and in some familial cases [5–10]. Genetic analysis of 2 familial cases [6, 8] has shown that

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boys carrying the same *MECP2* mutation as their RTT-affected sisters were affected by severe lethal congenital encephalopathy. These results have demonstrated the lethality of hemizygous *MECP2* mutations causing RTT in females. Some of the rare cases of boys described to date with an RTT phenotype [11–16] had an associated Klinefelter syndrome [15] or else a somatic mosaicism for a *MECP2* mutation [16], thus mimicking the phenomenon of X-chromosome inactivation in females.

The *MECP2* gene encodes methyl-CpG-binding protein 2 (MeCP2) which is widely expressed but differentially regulated among tissues during development [17, 18]. The protein consists of a methyl-CpG-binding domain [19], a transcriptional repression domain [20], and a corepressor-interacting region [21]. MeCP2 selectively binds to methylated CpG dinucleotides and mediates gene transcription repression through interaction with histone deacetylase and the corepressor SIN3A.

Recurrence within families is rare in RTT (~1%). Genetic analysis of several reported familial cases [5, 6, 8] has shown that recurrence can be due to vertical dominant transmission from asymptomatic carrier females or to germline mosaicism in one of the parents for a de novo *MECP2* mutation.

The results obtained from the genetic analysis of the *MECP2* gene in RTT patients demonstrate that the same mutation can yield a broad range of clinical manifestations, from healthy carrier females to severe congenital encephalopathy in males and from women with mild neurological symptoms to congenital forms of RTT. Therefore, the constitutive carrier status of the mother should be individually analyzed in order to evaluate the risk of recurrence in each family. If we bear in mind the fact that germline mosaicism cannot be detected, and that some mothers may be also unaffected by somatic mosaicism [10], genetic counseling must be carefully given, and prenatal diagnosis should be offered to all couples with a previous affected sib.

We present the first report of RTT prenatal diagnosis in three families with a previous affected girl and discuss the usefulness of prenatal testing in diseases caused mainly by de novo mutations.

Subjects and Methods

Subjects

The 3 RTT girls (cases RS17.3, RS30.3, and RS58.3) described in this paper had been diagnosed as having a classic form of RTT, according to the 'diagnostic criteria for Rett syndrome' [22]. Blood samples from the patients and their healthy relatives were obtained from different regions of Spain. The patients are included in a large database series of Spanish RTT patients collected from throughout the country and held at the Genetics Section of Hospital Sant Joan de Déu, where the genetic bases of the disease are being analyzed. Informed consent for genetic research was obtained from all families following the Hospital Ethics Committee's approval of the study.

Fetal samples were obtained from chorionic villi in case RS17.4 and from amniotic fluid in cases RS30.7 and RS58.5.

Methods

Genomic DNA was obtained from peripheral leucocytes by standard phenol extraction and ethanol precipitation protocols. The chorionic villus sample was washed twice in 1 × SSC and 10 m*M* EDTA and incubated overnight at 56 °C in sterile lysis buffer containing 2% SDS and proteinase K (4 mg/ml) prior to phenol extraction. Fetal DNA from amniotic fluid was prepared with the same method after culturing the cells for 3 weeks.

Markers DXS7107, DXS1223, DXS1043, and DXS7593, linked to the X chromosome; GABRA5 and GABRB3 from 15q11–q13; D7S653 and ELN from 7q11.23, and D17S1873 and D17S955 from 17q11.2 were analyzed by PCR. DNA (200 ng) was amplified in a mixture containing 200 μ*M* of each dNTP, 1 p*M* of each primer, 0.8 U of Expand High Fidelity PCR System (Roche), 2 m*M* Tris-HCl, pH 7.5 (25 °C), 10 m*M* KCl, 0.1 m*M* DTT, 0.01 m*M* EDTA, 0.05% Tween 20, 0.05% Nonidet P-40, 5% glycerol, and 2.5 m*M* $MgCl_2$ in a final volume of 25 μl. The PCR amplification protocol was as follows: 5 min denaturation, 30 cycles of 94 °C, 30 s; 52 °C, 45 s, and 72 °C, 15 s; final extension at 72 °C for 5 min.

For each patient, a search for mutations at the coding region of the *MECP2* gene was carried out by means of SSCP (single-stranded conformational polymorphisms) and heteroduplex analysis, followed by sequencing. The three coding exons of the gene were amplified according to the method of Amir et al. [5]. PCR fragments showing anomalous migrating gel bands were purified with the Qiaquik PCR purification kit (Qiagen), and single-strand sequencing was performed using the BigDye™ terminator cycle sequencing ready reaction (Applied Biosystems). Runs were done on an ABI PRISM 377 genetic analyzer (PE Biosystems) at an external center (Serveis Cientificotècnics, University of Barcelona). Sequence analyses were done with the Chromas program and the BCM Search Launcher utilities (http://www.technelysium.com.au/chromas.html and http://www.searchlauncher.bcm.tmc.edu, respectively).

Mutation analysis of familial and prenatal samples was carried out by direct detection of the previously identified patients' mutations. The methods used were SSCP/HD analysis in families fRS17 and fRS58.3 and NlaIII digestion of the PCR fragment 3a of *MECP2* [5] in family fRS30.

Results

During the course of our genetic analysis of RTT in Spain, three pregnancies occurred in families with 1 previously affected girl. The three families (fRS17, fRS30, and fRS58) asked for prenatal testing as soon as they received the molecular diagnosis of the affected daughter. Previous identification of the responsible *MECP2* mutation in the patients allowed us to directly analyze its pres-

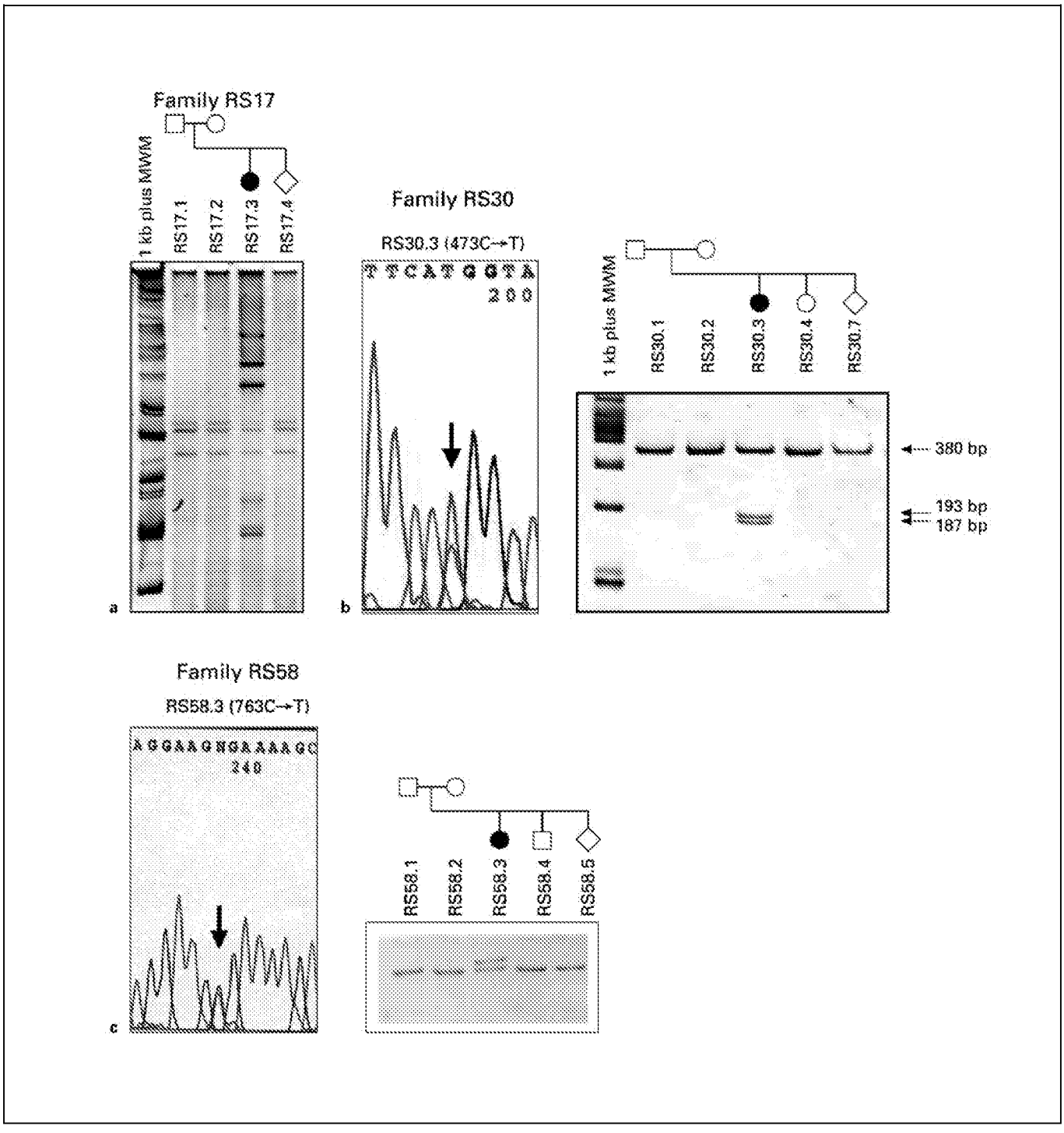

Fig. 1. Genetic detection of patients' *MECP2* mutations, familial analyses, and prenatal diagnoses. **a** SSCP analysis of family RS17. Changes in the SSCP pattern for patient RS17.3 reflect a gross in-frame heterozygous deletion (1061del96bp) which causes the loss of 32 residues at MeCP2 protein, from R354 to P385. **b** Analysis of family RS30. Sequence tracings show the heterozygous state of mutation 473C→T identified in patient SR30.3; familial and prenatal mutation analyses are shown by NlaIII digestion, since the 473C→T mutation creates a new restriction site and generates the presence of two additional bands of 193 and 187 bp, respectively. **c** Analysis of family RS58. Sequencing detection of the heterozygous 763C→T transition in patient RS58.3 and familial and prenatal analysis by SSCP.

ence in the fetuses (fig. 1). Patient RS17.3 had a gross heterozygous deletion (1061del96bp) at the 3′ end of the gene, not previously described (fig. 1a). This in-frame deletion causes the loss of 32 residues at MeCP2, from R354 to P385, including the polyhistidine domain beyond the transcription repressor domain of the protein. Patient RS30.3 carried the 473C→T mutation (fig. 1b) which produces a T158M amino acid change in the methyl-binding domain of MeCP2 [5]. It can be directly detected after NlaIII restriction digestion (fig. 1b). Mutation 763C→T was found in patient RS58.3 (fig. 1c); it causes a predicted truncated protein R255X previously described by Amir et al. [5].

The de novo origin of all three *MECP2* mutations was demonstrated by parental DNA analysis from peripheral blood lymphocytes. The results are shown in figure 1. None of the 3 mothers was a non-penetrant constitutive carrier of the daughter's mutation. Genetic counseling was given to each family, and prenatal diagnosis was offered to the parents in the genetic report because of the potential risk of germinal mosaicism. The three families had requested such.

The fetal samples were obtained as described in the Subjects and Methods section. Prenatal cytogenetic studies showed normal karyotypes: 46,XY for sample RS17.4 and 46,XX for samples RS30.7 and RS58.5.

To ensure freedom from maternal contamination, familial segregation analysis was done by amplifying several microsatellite markers in the parents and the fetal samples. Normal biparental inheritance was observed in all 3 cases (data not shown).

Fetal DNA samples were analyzed by SSCP/HD after amplification of the PCR fragments, where the sisters' mutations had been previously identified. Mutation 473C→T from patient SR30.3 was also analyzed by NlaIII digestion [5]. None of the 3 fetuses carried the sister's mutation (fig. 1).

Discussion

RTT is almost always sporadic. Despite more than 30 cases with more than one affected member have been described [reviewed in 23], estimating the risk of recurrence based on present data would be misleading. After *MECP2* genetic analysis, 4 of the cases where shown to be caused by different independent genetic events, since 1 of the patients carried a mutation, but the affected relative did not. In a high percentage of families, no mutation could be found in the coding region of *MECP2*, a surprising finding if we take into account that mutation detection in sporadic cases ranges between 70 and 90%, depending on the series and the clinical RTT form. To resume, only 6 familial cases have been confirmed, because the same mutation was detected in the different patients, if monozygotic twin pairs are excluded [5, 6, 8]. Parental analysis in these six families showed that 3 cases were due to vertical transmission from clinically unaffected carrier mothers, whereas in the other 3 cases germline mosaicism in 1 of the parents had to be suspected.

Description of asymptomatic females carrying the same constitutive *MECP2* mutation identified in their affected children has important implications and demonstrates that incomplete penetrance can occur in RTT. The X chromosome inactivation status was studied in some of these healthy carrier women and their affected daughters. An extremely skewed pattern was found in mothers' lymphocyte DNA, whereas the patients showed random inactivation, as do most classic RTT patients [9]. Nevertheless, phenotype predictions should not be made on the basis of X chromosome inactivation, since it is known that the X inactivation pattern in blood or other available tissues does not necessarily reflect the brain activity of the mutated *MECP2* gene. Other factors may be involved in the clinical manifestations of the disease, and the existence of other mechanisms not related to *MECP2* underlying RTT has not been ruled out to date.

To give proper genetic counseling to the family, identification of the *MECP2* mutation in an RTT patient should be followed by DNA analysis of the mother and other healthy sisters. Mother's analysis will test the presence of a non-penetrant constitutive *MECP2* mutation which would be associated with a recurrence risk of 50%. A negative result in maternal DNA derived from lymphocytes, however, does not preclude the possibility of recurrence within the family. Recurrence would still exist due to mosaicism for the mutation. The risk of gonadal mosaicism is low in RTT when compared to other inherited disorders such as Duchenne muscular dystrophy, but it cannot be predicted nor can it be assessed experimentally. We have found 1 case of mosaicism during the analysis of 40 healthy sisters of 29 RTT patients with a previously identified *MECP2* mutation or polymorphism. In 1 case, corresponding to 2.5% of the studied meioses, a de novo polymorphism not present in the parental lymphocyte DNA was found in both the patient and her sister. In addition to germline mosaicism, the possibility of somatic mosaicism in the unaffected mother should also be considered [10]. This situation has not been described in females yet, but it has been found in 2 boys: a 14-year-old

boy with classical RTT [16] and a boy with a clinical phenotype of Angelman syndrome [24, 25].

Severity and clinical manifestations of RTT have devastating implications for the patient and her family. Parental suffering is high, and many couples with a first affected daughter decide not to have more children to avoid recurrence. Offering the parents prenatal detection of the daughter's mutation has a great calming effect on their anxiety and encourages them to have more children. A negative result in the prenatal diagnosis reduces the risk of recurrence to approximately 1/12,000 which is the estimated prevalence of RTT.

In conclusion, prenatal diagnosis in RTT has important benefits for the family and should be offered to all couples with a previously identified affected patient, even when the mutation has a de novo origin. Genetic counseling has to properly inform parents about the estimated recurrence risk of the disease and to advise them about the potential risks associated with invasive methods. Prenatal diagnosis should be offered independently of the gender of the fetus, since some boys with mutations at the *MECP2* gene suffering from severe congenital encephalopathy have also been described. The experimental procedure should consist of direct detection of the patient's mutation on the fetal DNA sample. A recurrence risk equal to the prevalence of RTT can be conveyed to the family when the fetus is not a carrier of the patient mutation.

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