Hum Genet (1996) 97:247–250

ORIGINAL INVESTIGATION

Philippe Camus · Naïma Abbadi
Marie-Christine Perrier · Michèle Chéry
Simone Gilgenkrantz

X chromosome inactivation in 30 girls with Rett syndrome: analysis using the probe

Received: 16 January 1995 / Revised: 23 June 1995

Abstract Rett syndrome (RS) is a neurologic disorder with an exclusive incidence in females. A nonrandom X-inactivation could provide insight into the understanding of this disease. We performed molecular analysis based on the differential methylation of the active and inactive X with probe M27β, taking into account the parental origin of the two Xs, in 30 control girls, 8 sisters, and 30 RS girls. In 27 control an 31 RS mothers, the inactivation status of the X transmitted to their daughters was also analyzed. The results showed a significantly increased frequency of partial paternal X inactivation (> 65%) in lymphocytes from 16/30 RS compared with 4/30 controls ($P = 0.001$). These results do not support the hypothesis of a monogenic X-linked mutation but should be taken into account when researching the etiology of this desease.

Introduction

Rett syndrome (RS) is a severe progressive neurologic disorder occurring exclusively in females (Rett 1966; Hagberg et al. 1983). Its incidence is about 1 in 10000–15000 live-born females (Brud et al. 1991; Hagberg 1985). Most of the approximately 2000 cases reported world-wide (Percy 1992) are sporadic, fever than 1% being identified as familial cases (Bühler et al. 1990). Twin studies reveal concordance in monozygotic twins (NZT), except for one pair, and discordance in dizygotic twins (Migeon et al. 1995). A woman wit RS has given birth to a girl who clearly has RS-like manifestions (Hagberg et al. 1992).

Several genetic hypotheses have been proposed: a new mutation on the X chromosome with male lethality and generally no reproduction in the affected female, uniparental disomy, involvement of mitochondrial DNA (Eeg-Olofsson et al. 1989), or metabolic interference of X and autosomal gene products on X-inactivation (Johnson 1980). Two translocations in RS girls have been reported involving the X chromosome: 46,X, t(X;3)(p21.3; q13.31) (Zoghbi et al. 1990a), and 46,X, t(X,22)(p11.22;p11) (Journel et al. 1990). The first is a de novo translocation and the second has also been found in the unaffected mother and the RS "forme fruste" sister.

In the present ignorance of the pathogenetic mechanism of RS, the hypothesis of a monogenic X-linked disorder occurring exclusively in females as a new mutation has led us to study X-chromosome inactivation. The object of the current investigation has been study the differences in the X-inactivation pattern, using the highly informative probe M27β, between RS and control girls taking into account the parental origin of the inherited X, and between RS an control mothers taking into account the daughter's transmitted and non-transmitted Xs.

Materials and methods

Patients and families

30 control families with at least one girl and 34 families with one RS girl each were studied. The patients fulfilled the diagnostic criteria described by Hagberg et al. (1983) and were evaluated by the French Rett Scientific Committee. All patients are sporadic cases.

Laboratory methods

DNA was extracted from peripheral blood collected in EDTA for all controls and 20 RS families in our laboratory, using Jeanpierre's method (1987), or a phenol-chloroform method for 14 RS families in Généthon (Evry-France). First, 20 µg DNA samples were digested with *Bcl*I, separated into two tubes, and digested with *Msp*I or *Hpa*II. The samples were Southern blotted under standard conditions (Southern 1975). The M27β probe (Boyd and Fraser 1990) was labeled with (^{32}P)-dCTP using a random primed DNA labeling kit (Boehringer Mannheim). Autoradiographic films were subjected to densitometry (Sebia densitometer) to determine whether the inactivation process was skewed. The relative band intensity shown by *Hpa*II-cut bands was also measured.

P. Camus · N. Abbadi · M.-C. Perrier · M. Chéry (✉)
S. Gilgenkrantz
Laboratoire de Génétique, C.H.U. de Brabois,
Avenue de Bourgogne, F-54511 Vandoeuvre-Lès-Nancy, France

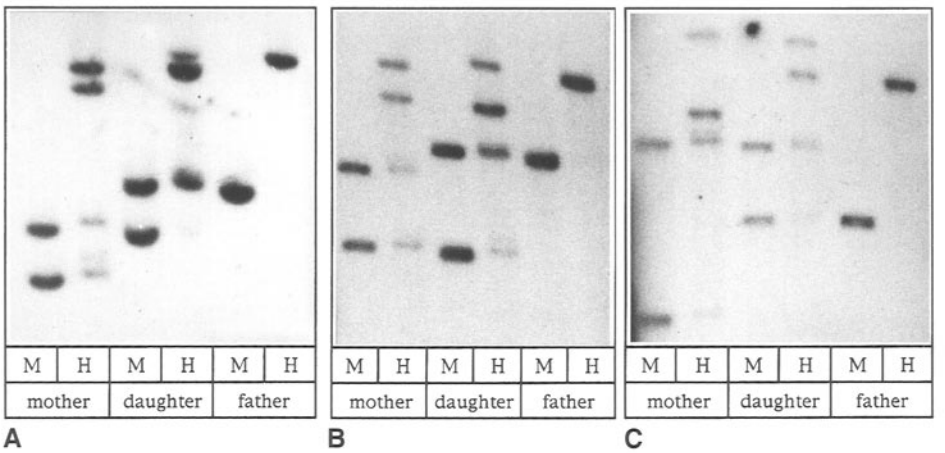

Fig. 1A–C Southern blot of DNA from three families with an RS-affected daughter. *M* Genomic DNA digested by *BclI/MspI*; *H* genomic DNA digested by *BclI/HpaII*. Inactivation profiles are represented by *HpaII* bands at the *right* of *M* bands. **A, B** Two RS cases with a partial preferential paternal X inactivation (cases 4, 18). **C** A RS case with a partial preferential maternal X inactivation (case 12)

Table 1 Percentages of *HpaII*-cut bands at the DXS255 locus, corresponding to the maternal inactive X transmitted to the daughter and the paternal inactive inherited by the daughter, in each group

Inactivation status	Severely skewed	Moderately skewed	Symmetrical	Moderately skewed	Severely skewed
% of maternal inactive X transmitted to the daughter	< 20	20–35	35–65	65–80	> 80
RS mother	2	5	22	2	0
Control mothers	3	2	15	7	0
% of inactive paternal X	< 20	20–35	35–65	65–80	> 80
RS	0	2	12	10	6
RS sisters	0	0	3	2	1
Control girls	0	5	21	3	1

Results

Because of the high level of heterozygosity at the DXS255 locus revealed by M27β after DNA digestion with *BclI/MspI*, maternal and paternal alleles could be distinguished in about 88% of the females studied: 30/34 RS girls, 31/34 RS mothers, 6/8 RS sisters who had all inherited the same allele as their affected sister, 27/30 control mothers, and 30/34 control girls. Therefore, at this locus and for all our informative cases, the hypothesis of uniparental disomy was excluded, as previously described (Benedetti et al. 1992; Webb et al. 1993; Camus et al. 1994; Migeon et al. 1995).

The X-inactivation pattern was studied by analysis of methylation at DXS255. There are several methylation sites at 5′ end of a LINE-1 element at this locus, where the active X is always fully methylated, whereas the inactive X is subject to partial methylation. However, in the majority of cells, CCGG site 2 of the inactive X is unmethylated, and can be digested with *HpaII* (Hendriks et al. 1992). In studying *HpaII*-cut bands, the low proportion of inactive Xs methylated at this site was not taken into account. The proportion of each allele undergoing this methylation was considered to be equivalent.

The labeling density of the *HpaII*-cut bands (Fig. 1) was considered to show the inactivation percentages for the inherited paternal and maternal Xs in RS girls, control girls, and RS sisters, and for the daughters' transmitted and non-transmitted Xs from mothers; 100% selective X inactivation was not observed. The observed X-inactivation patterns were classified into five groups according to previously defined band intensity percentages (Harris et al. 1992): symmetrical from 35% to 65%, and differently skewed (Table 1).

Determination of the inactivation status for the daughters' transmitted X from the mothers showed that the RS mothers transmitted their X independently of its inactivation status, as did the control mothers. No significant difference between distributions of moderately and severely skewed patterns were found in control mothers (respectively 9 and 3/27) and in RS mothers (respectively 7 and 2/31), reflecting no bias of inactivation in their X transmitted to the daughter.

If only severe skewing is considered, then 1/30 control daughters (3.3%), 4/57 control females (7%), or 7/94 non RS females (7.4%) attained 80:20 or more, whereas 6/30 (20%) RS girls reached extreme skewing. Moreover, these extremely nonbalanced RS patterns all favored the maternal X remaining active, whereas only 2/36 (5.5%) non-RS daughters showed a similar pattern.

Some control girls had a skewed inactivation of the maternal (5/30) or paternal (4/30) X, with a main pattern of inactivation that was symmetrical (21/30). In contrast, the majority of RS girls (16/30) showed a preferential inactivation of their paternal X, 12/30 cases had a symmetrical X inactivation, and 2/30 had a moderately skewed maternal preferential X inactivation.

Chi-square tests were used to compare distributions between groups and showed a significant difference be-

tween the number of cases with a partial preferential paternal X inactivation of > 65% in RS (16/30) and control girls (4/30) ($\chi^2 = 10.8$; 1 *df*; $P = 0.001$). The number of RS sisters was not sufficient to allow the chi-square test. But when RS sisters and control girls, were analgamated in a group (7/36) to be compared with RS girls, the difference remained significant ($\chi^2 = 8.3$; 1 *df*; $P > 0.01$). These differences allowed us to suggest a correlation between Rett's syndrome and X-inactivation favoring the paternal allele remaining inactive.

Discussion

No specific bias of inactivation was found in the mothers of our sporadic RS cases, compared with the control mother series. This was in agreement with the observation of random inactivation patterns in leukocytes from a normal mother with an RS mildly affected sister and an RS daughter (Anvret and Wahlström 1992). Neither of these findings supported the hypothesis of nonrandom X-inactivation proposed to explain the familial RS cases (Zoghbi et al. 1990b), based on the totally selective X-inactivation observed in a normal mother with two RS daughters who were half-sisters.

When data were analyzed with respect to the degree of skewing, a marked difference of high bias was observed between RS and control females (20% versus 7%). In a previous study (Zoghbi et al. 1990b), non-random inactivation was also detected in leukocytes of 4/11 (36%) RS subjects, whereas it was found in only 1/13 (8%) control subjects. From the analysis of leukocytes from normal females who were randomly selected an who were not known to carry any X-linked disorder, 4/42 (9.5%) (Harris et al. 1992) to 21/100 (Gale et al. 1994) displayed skewing of 80:20 or greater. Thus, because as many as 7%–21% of normal females show highly skewed inactivation, skewing of inactivation cannot be a characteristic of RS.

When data were analysed with respect to the parental origin of each allele, all the RS girls presented a highly skewed inactivation 6/30 (20%) favoring the paternal allele remaining inactive. In contrast, 2/36 (5.5%) non-RS girls presented the same pattern. No highly skewed inactivation favoring the maternal allele remaining inactive was found. The parental origin of each allele in control females was mentioned in only two previous studies. Severely skewed patterns were attributable to paternal X inactivation in three cases, and to maternal X inactivation in two cases, among the five control females studied (Gale et al. 1994). In the three control mother and daughter pairs where X inactivation was analyzed, no severely skewed patterns were found (Harris et al. 1992).

Furthermore, a significant difference between the distribution of RS cases (16/30) and control girls (4/30) was found, with repect to the moderately and severely skewed X inactivation profiles with a partial preferential paternal X inactivation, thereby supporting the correlation between Rett's syndrome and X-inactivation favoring the paternal allele remaining inactive, as previously described (Camus et al. 1994). In a similar study of nine Rett patients, a moderate preferential paternal X-inactivation was found in two cases, and a symmetrical X-inactivation in the other seven cases (Webb et al. 1993). Alone, the number of RS cases was not enough to allow a chi-squared test. Together with our results, the difference between 18/39 (46%) RS daughters and 7/36 (19.5%) non-RS daughters remained significant ($\chi^2 = 6.0$; 1 *df*; $P < 0.02$). Therefore, the distribution favoring the paternal allele remaining inactive was seen with raised frequency in RS, compared with controls.

Nevertheless, the paternally skewed X-inactivation cannot be the causative factor of RS for several reasons. First, only a group of RS girls presented this result and 100% selective inactivation was not found in our series. The hypothesis of leukocyte selection resulting in paternally biased inactivation pattern is unlikely because the analysis of the proportion of paternal inactive X as a function of age in our RS patients, as in controls, reveals that these two criteria are independent (data not shown). Nonrandom inactivation as a consequence of selective pressure has been associated with X-linked diseases, such as in X-linked severe combined immunodeficiency (Puck et al. 1987), Lesh-Nyhan syndrome (Nyhan et al. 1970), Wiskott-Aldrich syndrome (Fearon et al. 1988), agammaglobulinemia (Conley et al. 1986), adrenoleukodystrophy (Migeon et al. 1981); skewing is marked (< 10% or >90%) and is found in all patients. However, in chronic granulomatous disorder (Windhorst et al. 1967), selection does not occur. Secondly, depending on RS symptomatology, efforts have been made to study the affected brain tissue. Among these studies, X-inactivation has been analyzed and random inactivation profiles have been observed in a total of five RS patients (Zoghbi et al. 1990b; Anvret and Wahlström 1994). Lastly, in one pair of MZT concordant for RS, we found the same moderately skewed paternal X-inactivation (data not included in Table 1). In a recent study on MZT with RS (Migeon et al. 1995), the frequent nonrandom X-inactivation previously observed in MZT was confirmed, but it was associated with twinning rather than RS. Furthermore, as only 1/8 MZT pairs was discordant for RS, whereas generally the discordance is higher in MZT with X-linked disorders (Jorgensen et al. 1992), it was concluded that RS was not transmitted as an X-linked mutation.

Our results do not suport the hypothesis of a monogenic X-linked mutation but should be taken into account in research on the etiology of this disease. The significantly increased incidence of partial preferential inactivation of the paternal X observed in RS girls may reflect a complex secondary role played by X-inactivation in this disease.

Acknowlegements This work was supported by research grants from the French Rett Syndrome Association (A.F.S.R). The authors thank the Preventive Medicine Center of Nancy, and Mireille Malot, Marie-José Grégoire, Agapi Kataki, Christophe Philippe, Dominique Hillaire, Jean-François Prudhomme, and their coworkers from Généthon for their assistance in the collection of blood or DNA.

References

Avret M, Wahlström J (1992) Genetics of the Rett syndrome. Brain Dev [Suppl] 14: S101–S103

Avret M, Wahlström J (1994) Letter to the Editor: Rett syndrome: random X chromosome inactivation. Clin Genet 45: 274–275

Benedetti L, Munnich A, Melki J (1992) Parental origin of the X chromosomes in Rett syndrome. Am J Med Genet 44: 121–122

Boyd Y, Fraser NJ (1990) Methylation patterns at the hypervariable X-chromosome locus DXS255 (M27β): correlation with X-inactivation status. Genomics 7: 182–187

Bühler EM, Malik N, Alkan M (1990) Another model for the inheritance of Rett syndrome. Am J Med Genet 36: 126–131

Burd L, Vesley B, Martsolf JT, Kerbeshian J (1991) Prevalence study of Rett syndrome in North Dakota children. Am J Med Genet 38: 565–568

Camus P, Abbadi N, Gilgenkrantz S (1994) X-inactivation in Rett syndrome: a preliminary study showing a partial preferential inactivation of paternal X with the M27β probe. Am J Med Genet 50: 307–308

Conley ME, Brown P, Pickard AR, Buckley Rh, Miller DS, Raskind WH, Singer JW, Fialkow PJ (1986) Expression of the gene defect in X-linked agammaglobulinemia. N Engl J Med 315: 564–567

Eeg-Olofsson O, Al-Zuhair A, Teebi AS, Al-Essa MMN (1989) Rett syndrome: genetic clues based on mitochondrial changes in muscles. Am J Med Genet 32: 142–144

Fearon ER, Kohn DB, Winkelstein JA, Vogelstein B, Blaese RM (1988) Carrier detection in the Wiskott-Aldrick syndrome. Blood 72: 1735

Gale RE, Wheadon H, Boulos P, Linch DC (1994) Tissue specificity of X-chromosome inactivation patterns. Blood 83: 2899–2905

Hagberg B (1985) Rett's syndrome: Prevalence amd impact on progressive severe mental retardation. Acta Pediatr Scand 74: 405–408

Hagberg B, Aicardi J, Dias K, Ramos O (1983) A progressive syndrome of autism, dementia, ataxia, and loss of pruposeful hand use in girls: Rett's syndrome. Report of 35 cases. Ann Neurol 14: 471–479

Hagberg B, Naidu S, Percy AK (1992) Tokyo symposium on the Rett syndrome. neurological approach – concluding remarks and epilogue. Brain Dev [Suppl] 14: S151–S153

Harris A, Collins J, Vetrie D, Cole C, Bobrow M (1992) X-inactivation as a mechanism of selection against lethal alleles: further investigation of incontinentia pigmenti and X-linked lymphoproliferative disease. J Med Genet 29: 608–614

Hendriks RW, Hinds H, Chen ZY, Craig IW (1992) The hypervariable DXS255 locus contains a LINE-1 repetitive element with a CpG island that is extensively methylated only on the active X chromosome. Genomics 14: 598–603

Jeanpierre M (1987) A rapid method for the purification of DNA from blood. Nucleic Acids Res 15: 22

Johnson WG (1980) Metabolic interference and the +/– heterozygote. A hypothetical form of simple inheritance which is neither dominant nor recessive. Am J Hum Genet 32: 374–386

Jorgensen AL, Philip J, Raskind WH, Matsushita M, Christensen B, Dreyer V, Motulsky AG (1992) Different patterns of inactivation in MZ twins discordant for red-green color-vision deficiency. Am J Hum Genet 51: 291–298

Journel H, Melki J, Turleau C, Munnich A, Grouchy J de (1990) Rett phenotype with X/autosome translocation: possible mapping to the short arm of chromosome X. Am J Med Genet 35: 142–147

Migeon BR, Moser HW, Moser AB, Sprenkle JA, Sillence D, Norum RA (1981) Adrenoleukodystrophy: evidence for X-linkage, inactivation, an selection favoring the mutant allele in heterozygous cells. Proc Natl Acad Sci USA 78: 5066–5070

Migeon BR, Dunn MA, Thomas G, Schmeckpeper BJ, Naidu S (1995) Studies of inactivation and isodisomy in twins provide further evidence that the X chromosome is not involved in Rett syndrome. Am J Hum Genet 56: 647–653

Nyhan WL, Bakay B, Connor JD, Marks JF, Keele DK (1970) Hemizygous expression of G6PD in erythrocytes of heterozygote for Lesch-Nyhan syndrome. Proc Natl Acad Sci USA 65: 214–218

Percy AK (1992) The Rett syndrome: the recent advances in genetic studies in the USA. Brain Dev [Suppl] 14: S104–S105

Puck JM, Nussbaum RL, Smead DM, Conley ME (1987) Carrier detection in X-linked severe combined immunodeficiency based on patterns of X chromosome inactivation. J Clin Invest 79: 1395–1400

Rett A (1966) Ueber ein eigenartiges hirnatrophisches Syndrom bei Hyperammonämie im Kindesalter. Wien Med Wochenschr 116: 724–738

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 88: 503–517

Webb T, Watkiss E, Woods CG (1993) Neither uniparental disomy nor skewed X-inactivation explains Rett syndrome. Clin Genet 44: 236–240

Windhorst DB, Holmes B, Good RA (1967) A newly defined X-linked trait in man with demonstration of the Lyon effect in carrier females. Lancet 1: 737–742

Zoghbi HY, Ledbetter DH, Schultz R, Percy AK, Glaue DG (1990a) A de novo X;3 translocation in Rett syndrome. Am J Med Genet 35: 148–151

Zoghbi HY, Percy AK, Schultz RJ, Fill C (1990b) Patterns of X chromosome inactivation in Rett syndrome. Brain Dev 12: 131–135