Arq Neuropsiquiatr 2009;67(3-A):577-584

GENOTYPE-PHENOTYPE CORRELATION IN BRAZILLIAN RETT SYNDROME PATIENTS

Fernanda T. de Lima[1], Decio Brunoni[2], José Salomão Schwartzman[3], Maria Cristina Pozzi[4], Fernando Kok[5], Yara Juliano[6], Lygia da Veiga Pereira[7]

Abstract – **Background:** Rett syndrome (RS) is a severe neurodevelopmental X-linked dominant disorder caused by mutations in the *MECP2* gene. **Purpose:** To search for point mutations on the *MECP2* gene and to establish a correlation between the main point mutations found and the phenotype. **Method:** Clinical evaluation of 105 patients, following a standard protocol. Detection of point mutations on the *MECP2* gene was performed on peripheral blood DNA by sequencing the coding region of the gene. **Results:** Classical RS was seen in 68% of the patients. Pathogenic point mutations were found in 64.1% of all patients and in 70.42% of those with the classical phenotype. Four new sequence variations were found, and their nature suggests patogenicity. Genotype-phenotype correlations were performed. **Conclusion:** Detailed clinical descriptions and identification of the underlying genetic alterations of this Brazilian RS population add to our knowledge of genotype/phenotype correlations, guiding the implementation of mutation searching programs.

KEY WORDS: Rett syndrome, genotype-phenotype correlation.

Correlação genótipo-fenótipo em pacientes brasileiras com síndrome de Rett

Resumo – **Introdução:** A síndrome de Rett é uma grave doença do neurodesenvolvimento ligada ao X dominante, causada por mutações no gene *MECP2*. **Objetivos:** Identificar mutações de ponto no gene *MECP2* e estabelecer uma correlação entre as principais mutações encontradas e o fenótipo. **Método:** Avaliação clínica de 105 pacientes, seguindo um protocolo estabelecido. A identificação de mutações de ponto foi realizada em DNA de sangue periférico por sequenciamento da região codificante do gene amplificada por PCR. **Resultados:** Em 68% dos pacientes observou-se o quadro clássico da síndrome. Mutações de ponto patogênicas foram encontradas em 64,1% dos pacientes e em 70,42% das pacientes com o quadro clássico. Quatro novas variações de seqüência foram identificadas e sua natureza sugere patogenicidade. Correlações genótipo-fenótipo foram estabelecidas. **Conclusão:** Descrições clínicas detalhadas desta população brasileira de pacientes acrescenta conhecimento às correlações genótipo-fenótipo nesta grave condição, que podem auxiliar na implantação de programas de triagem de mutações.

PALAVRAS-CHAVE: síndrome de Rett, correlações genótipo-fenótipo.

Rett syndrome (RS) is an X-linked dominant neurodevelpmental disorder, with an estimated prevalence of one in 15000 girls[1]. There is a typical evolution of the disease, although some of the girls show a variant evolution, and are described as the atypical forms of RS[1,2].

In 1999, mutations in the *MECP2* gene, that encodes the methyl-CpG binding protein 2 (MeCP2) were described as the cause of RS[3]. MeCP2 binds to methylated CpG dinucleotides, through the methyl binding domain (MBD) and is involved in transcriptional silencing of genes through the transcriptional repression domain (TRD). The nature of the identified mutations indicates that they may

[1]Médica Geneticista Clínico, Doutorado em Ciências, Centro de Genética Médica, Universidade Federal de São Paulo (UNIFESP), São Paulo SP, Brazil; [2]Médico Geneticista Clínico, Livre Docente, Centro de Genética Médica, UNIFESP; Programa de Pós-Graduação em Distúrbios do Desenvolvimento da Universidade Presbiteriana Mackenzie, São Paulo SP, Brazil; [3]Médico Neuropediatra, Doutorado em Medicina, Programa de Pós-Graduação em Distúrbios do Desenvolvimento da Universidade Presbiteriana Mackenzie, São Paulo SP, Brazil, Associação Brasileira de Síndrome de Rett, São Paulo SP, Brazil; [4]Médica Neuropediatra, Mestrado em Medicina, Associação Brasileira de Síndrome de Rett, São Paulo SP, Brazil; [5]Médico Neuropediatra, Pós-doutorado em Medicina, Faculdade de Medicina, Universidade de São Paulo (USP), São Paulo SP, Brazil; [6]Administradora Hospitalar, Doutorado em Ciências, Professora Titular da Disciplina de Saúde Coletiva da Universidade de Santo Amaro, São Paulo SP, Brasil; [7]Física, Doutorado em Ciências Biomédicas, Livre Docente, Laboratório de Genética Molecular, Instituto de Biociências, USP. Financing: IRSA – International Rett Syndrome Association; FADA – Fundo de Auxílio aos Docentes e Alunos da UNIFESP-EPM; CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; FAPESP – Fundação de Amparo a Pesquisa do Estado de São Paulo.

Received 16 February 2009, received in final form 6 May 2009. Accepted 16 June 2009.

Dra. Fernanda Teresa de Lima – Rua Botucatu 740 / Disciplina de Genética / UNIFESP-EPM - 04023-062 São Paulo SP - Brasil. E-mail: ftlima@terra.com.br

cause total or partial loss of protein function[4]. However, results of genotype-phenotype correlations have been inconsistent across groups[5-7].

Since RS is an heterogeneous disorder, the detailed study of different patients helps understanding the clinical and evolutional variability of the disease. Mutation analysis, on the other hand, allows a better comprehension of the mutations' phenotypic effects, broadening the known clinical spectrum. This study was performed to characterize *MECP2* mutations present in a large cohort of Brazilian RS patients, analyzing genotype-phenotype correlations.

METHOD

This study was approved by the Ethics Comittee of Federal University of São Paulo – Paulista School of Medicine (UNIFESP-EPM), protocol number 464/02. It was also approved by the Ethics Comittee of Biomedical Sciences Institute of São Paulo University (USP), protocol number 367. An informed consent was requested to the legal responsables of the patients, and only those with this document signed were included in the study.

All the 140 patients were examined by neuropediatricians and geneticist, following the same clinical protocol. The diagnostic criteria used to classify the patients as classical RS were those recommended by "The Rett Syndrome Diagnostic Criteria Work Group"[8]. The atypical patients fulfilled the Hagberg criteria[1], excet the minimum age of 10 years. The majority of the patients were seen at the Brasilian Association of Rett Syndrome in São Paulo, Brazil.

The data collected was used to estimate the clinical severity and to perform genotype-phenotype correlation. The severity was estimated using the scale modified by Pineda[9].

The molecular investigation was performed at Molecular Genetics Laboratory of Genetics and Evolutive Biology Department of Bioscience Institute of USP. Genomic DNA was obtained from peripheral lymphocytes[10]. Point mutations in the *MEP2* gene were searched by DNA amplification through polimerase chain reaction (PCR) and direct sequencing of the products. PCR and sequencing primers were designed to amplify the coding region of the gene, in 6 overlapping fragments:

Exon 2 F-5'-AAAAGGTCGTGCAGCTCAAT-3';
R-5'-GATGGCCAAACCAGGACATA-3';
Exon 3 F-5'-CTGTTTGGGGGAGGCAGAAG-3';
R-5'-CTCCATGAGGGATCCTTGTC-3';
Exon 4a F-5'- TGCCCTATCTCTGACATTGC-3';
R-5'-CACCACACTCCCCGGCTTTC-3';
Exon 4b F-5'-GAAGCGAAAAGCTGAGGCCG-3';
R-5'-GGAGCTCTCGGGCTCAGGTG-3';
Exon 4e F-5'-CCCCCAAGAAGGAGCACCA-3';
R-5'-ACAATGTCTTTGCGCTCTCC-3';
Exon 4f F-5'-CCCAAGGAGCCAGCTAAGAC-3';
R-5'-CAGAGCCCTACCCATAAGGAG-3'.

Each fragment had specific cycling parameters and all had an inicial denaturing of 94°C for 5 minutes and final extension of 72°C for 10 minutes. Exon 2 and 4a used 30 cycles of 94°C for 45 seconds, 58°C for 45 seconds and 72 °C for minute. Exon 4b used 30 cycles of 94°C for 45 seconds, 60 °C for 45 seconds and 72 °C for minute. Exons 3, 4e and 4f used 14 cycles of 94°C for 30 seconds, 69 °C for 40 seconds lowering 0.5°C per cycle and 72°C for a minute and half, followed by 24 cycles of 94°C for 30 seconds, 62 °C for 40 seconds and 72 °C for a minute and half. Amplification quality was verified in agarosis gel. PCR products were purified and quantified before been submitted to a sequencing reaction, on both strands. Reactions were analyzed in automatic sequencer ABI Prism model 310, version 3.0, using big dye (Amersham-Pharmacia®), according to the manufacturer's instructions. Sequences were compared to normal *MECP2* sequence (Genbank L37298/AF030876). Thirty-five patients were excluded because of structural alterations found in the *MECP2* gene (detected or suspected).

Based on clinical evaluation and point mutations found, a genotype-phenotype correlation was performed, with respect to presence or absence of mutation, type and location of mutations and between the three most frequent mutations.

Statistical analysis of the results was performed using: (1) Chi-square test[11], to study associations between groups and studied variables; (2) Mann-Whitney test[11] to confront diferent mutation types and the presence or not of a mutation in relation to scores on the Pineda scale[9]; (3) Kruskal-Wallis variance analysis[11] to study scores on the Pineda scale[9] and the location of the mutation and between the three most frequent mutations. The level of rejection of null hypothesis was fixed in 0.05 or 5%.

RESULTS

Patients

One hundred and forty female patients were evaluated with clinical hypothesis of RS. Thirty-five patients were late excluded because of large structural alterations found in the *MECP2* gene (detected or suspected). Beetween the remaining patients, there were 2 pairs of concordant twins. Among the 105 patients, 68% had classical RS and 27% were atypical. Five patients were not classified due to their age, or because they showed an initial very suggestive clinical picture, but the diagnostic criteria were impossible to apply; they were categorized as "non-classified". The median age of the patients on the first evaluation was 8 years (SD 5.44 years), ranging from 1 year and 7 months and 27 years and 11 months of age.

Mutations

In total, 84 point variations were found in 74 patients, including the 2 pairs of twins, resulting in a frequency of detection fo 70% (72/103, considering twins as one) (Fig 1). Of them, 68 were considered pathogenic (81% of all variations), and 4 were not previously described. Twelve were silent variations (14.3% of all variations); 1 not previously described. Three missense non-pathogenic polymorfisms

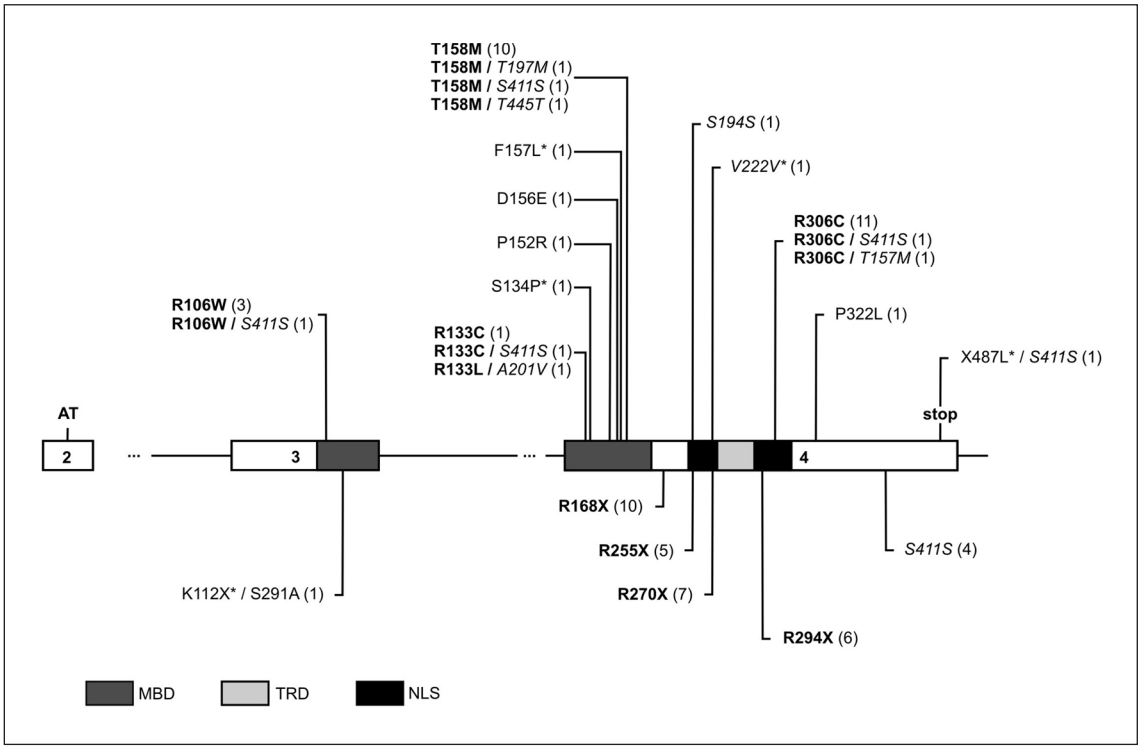

*Fig 1. Mutations and polymorphisms found in the MECP2 gene. Scheme of the MECP2 gene where rectangles represent exons 2–4, and lines introns. The regions encoding the different functional domains of the MECP2 protein are indicated. Missense mutations and silent polymorphisms are shown above the gene. Polymorphisms found in association with a mutation are placed together with the mutation found. Truncating mutations are shown below the gene. Number between parentheses indicate the number of patients with that alteration. In bold, mutations in hot spots, and in italic, polymorphisms. MBD: methyl binding domain; TRD: transcriptional repression domain; NLS: nuclear location signal. *not previously described mutations.*

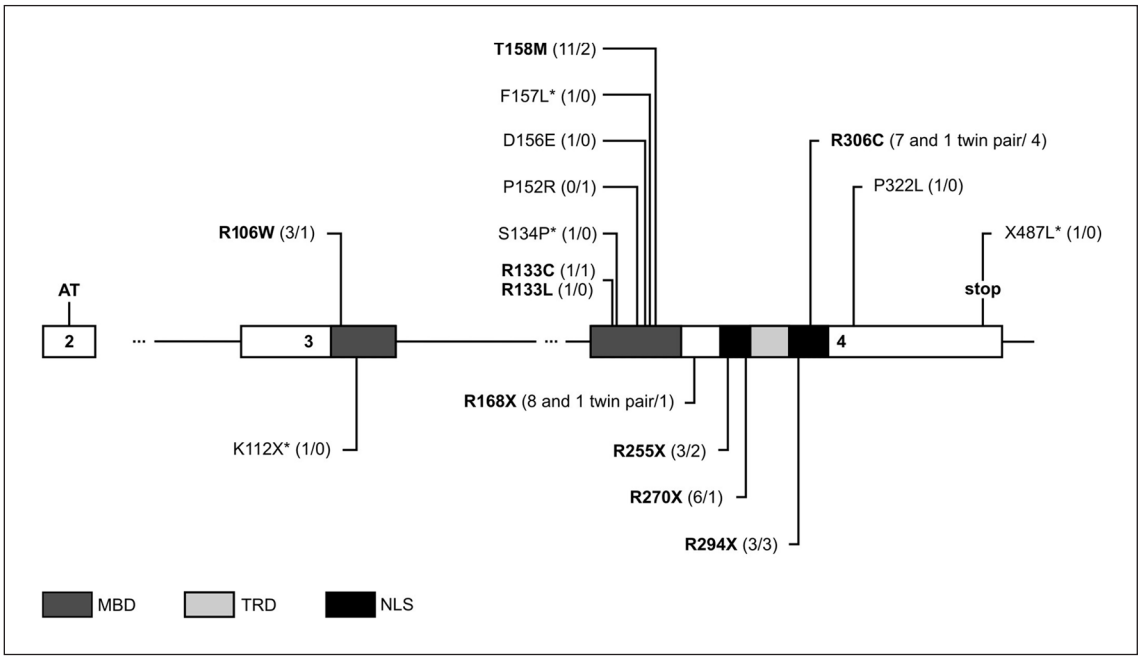

*Fig 2. Distribuition of pathogenic point mutations according to clinical classification of the patients. Scheme of the MECP2 gene where rectangles represent exons 2–4, and lines introns. The regions encoding the different functional domains of the MECP2 protein are indicated. Missense mutations are shown above the gene. Truncating mutations are shown below the gene. The first number between parentheses indicate the number of classical patients with that alteration and the second, the number of atypical or non-classified patients with that mutation. In bold, mutations in hot spots. MBD: methyl binding domain; TRD: transcriptional repression domain; NLS: nuclear location signal. *Not previously described mutations.*

(3.65% of all variations), previously described, and an alteration whose pathogenicity was not been previously determined were also found (1.1% of all variations). Six patients had only non-pathogenic variations (5.8%). C to T transitions at CpG islands totalizes 88.1% (74/84). Most of the pathogenic point mutations were concentrated on the methyl binding domain (MBD) (25/66 – 36%). The frequency of pathogenic point mutations was 64.1% (66/103). The distribution of pathogenic point mutations is shown in Figure 2.

Three patients deserve special consideration. Two were atypical, one had hyperchromic linear spots and normal karyotype and the other had a chromosomal abnormality, t(4)(q11);14(q11). None of them had any detected point mutation. The third patient had a classical phenotype but high levels of lactic and pyruvic acids. This patient had the T158M mutation, previously described. All were included because it was considered interesting to check if they had *MECP2* mutations.

Among the classical patients, the frequency of detection of pathogenic point mutations was 70.4% (50/71, twins considered as 1). Among the atypical patients, the frequency was 50% (14/28), and among the non-classified patients, 40% (02/05). The four most frequent mutations amounted to 68% of the pathogenic point mutations detected in the classical patients and 50% in the atypical ones.

The distribution of pathogenic point mutations detected among the atypical patients was 58.8% (10/17) in those with forme fruste; 33.3% (2/6) in those with congenital variation; 66.7% (2/3) in those with late regression and none in those with early seizure.

Genotype-phenotype correlation

Presence or absence of a pathogenic mutation – Initially, the patients were grouped according to the presence or absence of a detected pathogenic point mutations (Table 1). Patients with pathogenic point mutations are more likely to exhibit the classical form (p=0.056) and breathing irregularities (p=0.006). Patients without detected pathogenic point mutations presented earlier onset of disease (p=0.013).

Type of mutation – The mutations were divided into two different groups: (1) missense mutations and (2) truncating or nonsense mutations (Table 2). Patients with truncating mutations had earlier symptoms, before one year of age (p=0.005), as they had higher scores with Pineda scale[9] (p=0.032) (Table 5).

Location of the mutation – The location of the pathogenic point mutations in the MECP2 protein was divided into four groups: (1) mutations in the MBD; (2) mutations between the MBD and the TRD; (3) mutations in the nuclear location signal (NLS) and (4) mutations in the transcriptional repression domain (TRD) after the NLS (Table 3). Mutations at the terminal carboxi region were not correlated. Patients with mutations at NLS had symptoms before one year and this almost reached statistical significance (p=0.066). Patients with mutations in the TRD after NLS were more likely to maintain ability to walk (p=0.010). Patients with mutations in NLS and between domains had higher scores with Pineda scale[9] in comparison to patients with mutations in TRD after NLS, that had lower scores (p=0.027) (Table 5).

Three most frequent mutations – The three most frequent pathogenic point mutations, T158M, R306C, R168X, were used to perform a more specific genotype-phenotype correlation (Table 4). All of them were more frequent among patients with classical form. Patients with the R306C mutation had late symptoms (p=0.007) and were more likely to maintain the ability to walk (p=0.029). They also reached lower scores with Pineda scale[9] (p=0.021) (Table 5).

Table 1. Rett syndrome patients, with and without detected point mutations, according to studied variables, showing χ^2 test results.

Variables	Patient with detected mutations	Patients without detected mutations	p value
Forms[I]			
Classical	50 (78.13%)	21 (29.58%)	
Atypical	14 (50%)	14 (50%)	0.056*
Onset[II]			
≤ 12 months	32 (48.48%)	26 (74.29%)	
> 12 months	34 (51.51%)	09 (25.71%)	0.013**
Positive variables			
Walk	36 (72%)	14 (28%)	0.139
Loss of hand use	58 (67.44%)	28 (32.56%)	0.221
Word acquisition	45 (69.23%)	20 (30.77%)	0.222
Breathing disorder	48 (75%)	16 (25%)	0.006**
Seizures	42 (67.75%)	20 (32.25%)	0.443
Scoliosis	37 (61.67%)	23 (38.33%)	0.443
Stereotypic hand movements	67 (66.34%)	34 (33.67%)	0.090
Sleep disturbances	23 (63.89%)	13 (36.11%)	0.892

[I]Pair of twins counted as 1, 2 non-classified patients; [II]2 patients without this information; *significance level almost reached; **statistical significance.

Table 2. Studied variables in Rett syndrome patients divided according to the type of detected point mutation, showing χ^2 test results.

Variables	Missense mutations	Truncating mutations	p value
Forms[I]			
Classical	29 (56%)	22 (44%)	
Atypical	8 (57.1%)	6 (42.9%)	0.985
Onset[II]			
≤ 12 months	12 (32.43%)	19 (67.86%)	
> 12 months	25 (67.57%)	9 (32.14%)	0.005**
Positive variables			
Walk	22 (61.11%)	14 (38.89%)	0.434
Loss of hand use	33 (57.89%)	24 (42.11%)	0.642
Word acquisition	28 (63.64%)	16 (36.36%)	0.114
Breathing disorder	24 (51.06%)	23 (48.97%)	0.152
Seizures	24 (57.14%)	18 (42.86%)	0.927
Scoliosis	23 (63.89%)	13 (36.11%)	0.202
Stereotypic hand movements	38 (57.57%)	28 (42.42%)	0.249
Sleep disturbances	12 (54.54%)	10 (45.45%)	0.802

[I]Pair of twins counted as 1, 2 non-classified patients; [II]2 patients without this information; **statistical significance.

Table 3. Studied variables in Rett syndrome patients divided according to the location of detected point mutation, showing χ^2 test results.

Variables	NLS	MBD	TRD after NLS	Between domains	p value
Forms[I]					
Classical	9 (18%)	20 (40%)	12 (24%)	9 (18%)	
Atypical	2 (14.29%)	5 (35.71%)	6 (42.86%)	1 (7.14%)	0.504
Onset[II]					
≤ 12 months	7 (63.63%)	9 (37.5%)	7 (36.84%)	8 (80%)	
> 12 months	4 (36.36%)	15 (62.5)	12 (63.16%)	2 (20%)	0.066*
Positive variables					
Walk	6 (17.14%)	10 (28.57%)	16 (45.71%)	3 (8.57%)	0.010**
Loss of hand use	8 (14.29%)	22 (39.29%)	17 (30.35%)	9 (16.07%)	0.284
Word acquisition	4 (9.30%)	18 (41.86%)	14 (32.56%)	7 (16.28%)	0.087
Breathing disorder	10 (21.74%)	15 (32.61%)	13 (28.26%)	8 (17.40%)	0.442
Seizures	7 (17.07%)	16 (39.02%)	12 (29.27%)	6 (14.63%)	0.987
Scoliosis	6 (17.14%)	16 (45.72%)	10 (28.57%)	3 (8.57%)	0.336
Stereotypic hand movements	12 (18.46%)	25 (38.46%)	19 (29.23%)	9 (13.85%)	0.128
Sleep disturbances	4 (18.18%)	8 (36.36%)	7 (31.81%)	3 (13.63%)	0.981

NLS: nuclear location signal (R255X and R270X mutation); MDB: methyl binding domain (R106W, K112X, R133C, R133L, S134P, P152R, D156E, F157L, T58M mutations); TRD after NLS: transcriptional repression domain after the NLS (R294X, R306C mutations), between domains (R168X mutation); [I]Pair of twins counted as 1, 2 non-classified patients; [II]2 patients without this information; *significance level almost reached; **statistical significance.

DISCUSSION

Patients

It is known that RS is more frequent and shows a higher phenotypic variation than was first realized[1]. Published Brazilian studies grouped few patients[12-14] and none performed a molecular caracterization. This is the first study in a large group of Brazilian RS patients with molecular characterization and genotype-phenotype correlation.

The majority (68%) of the studied patients had the classical form of the disease. Since the evaluation of a patient at an initial stage is more difficult, five of the patients were grouped as non-classified. In three of them, there were no pathogenic point mutations detected, although they have a very suggestive clinical picture. All of these three patients have less than 2 years, and the clinical follow-up will be mandatory for the establishment of a diagnosis.

Mutations

MECP2 mutations have been identified in 70-90% of sporadic patients and approximately 50% of familial cases[15]. In this study, pathogenic mutations were found in 64.1% of all patients, 70.4% of classical RS patients, 50%

Table 4. Rett syndrome patients with the three most frequent detected point mutations, according to studied variables, showing χ^2 test results.

Variables	R306C	T158M	R168X	p value
Forms[I]				
Classical	9 (31%)	11 (37.9%)	9 (31%)	
Atypical	3 (50%)	2 (33.3%)	1 (16.7%)	0.653
Onset[II]				
≤ 12 months	4 (30.77%)	2 (16.67%)	8 (80%)	
> 12 months	9 (69.23%)	10 (8.33%)	2 (20%)	0.007**
Positive variables				
Walk	11 (52.38%)	7 (33.33%)	3 (14.29%)	0.029**
Loss of hand use	11 (35.48%)	11 (35.48%)	9 (29.03%)	0.916
Word acquisition	10 (40%)	8 (32%)	7 (28%)	0.695
Breathing disorder	8 (36.36%)	6 (27.27%)	8 (36.36%)	0.256
Seizures	8 (36.36%)	8 (36.36%)	6 (27.27%)	0.996
Scoliosis	7 (38.89%)	8 (44.44%)	3 (16.67%)	0.306
Stereotypic hand movements	13 (37.14%)	13 (37.14%)	9 (25.71%)	0.263
Sleep disturbances	4 (36.36%)	4 (36.36%)	3 (27.27%)	0.999

[I]Pair of twins counted as 1, 1 non-classified patient; [II]1 patient without this information; **statistical significance.

Table 5. Pineda score means in Rett syndrome patients, divided according the various groups, showing Mann-Whitney and Kruskal-Wallis tests results.

Patients divided according to:	Mean	p value
Presence of point mutation[I]		
With point mutation detected	16.969	
Without point mutation detected	17.543	0.511
Mutation type[I]		
Missense	16.319	
Truncating	18.074	0.032**
Location of mutation[II]		
NLS	17.909	
MBD	16.957	
TRD after NLS	15.316	
Interdomain	18.909	0.027**
Most frequent mutations[II]		
R306C	15.077	
T158M	17.091	
R168X	19.444	0.021**

NLS: nuclear location signal (R255X and R270X mutation); MDB: methyl binding domain (R106W, K112X, R133C, R133L, S134P, P152R, D156E, F157L, T58M mutations); TRD after NLS: transcriptional repression domain after the NLS (R294X, R306C mutations), between domains (R168X mutation); [I]Mann-Whitney test; [II]Kruskal-Wallis test. **statistical significance.

of atypical patients and 40% of non-classified patients (Fig 2). Four pathogenic point mutations were more frequent – T158M, R306C, R168X and R270X, amount to 68% of pathogenic point mutations in classical patients and 50% of atypical patients, indicating that these mutations should be initially searched in screening programs with Brazilian RS patients.

Four not previously described point mutations were found. The first one, K112X, was not described on RettBASE[16], a database that collects *MECP2* mutations. It was found in association with another described alteration, S291A, whose pathogenicity was questioned. The aminoacid K in that position of the *MECP2* gene is conserved in all species and the mutation leads to an early stop codon, and therefore a truncated protein. This suggests that K112X is pathogenic and S291A is not.

The other three new mutations were S134P, F157L and X487L. At codon 134, there have been 2 different mutations described: S134C, pathogenic, and S134F, whose pathogenicity is unknown[16]. This is a conserved residue in MBD in all species, polar, whith a hydrophilic behaviour, and it was substitute by a non-polar hydrophobic aminoacid, which suggest that S134P should be pathogenic. At codon 157, there have also been 2 different mutations described: F157fs, pathogenic and F157I, whose pathogenicity is unknown. This is also a conserved aminoacid in all species, which suggest pathogenicity. The mutation X487L abolishes the final codon, which prevents the stop of the transcription, leading to a large protein, suggesting pathogenicity.

The classical patient with higher lactic and pyruvic acids had the T158M mutation. The elevation of blood levels of lactic acid and/or pyruvic acid is found in a substantial proportion of patients with RS and can be associated with ventilation disturbances[17]. The identification of a pathogenic mutation in this patient reinforces the findings of Huppke et al.[18] , who emphasized that the diagnosis of RS should not be dismissed because some characteristic features are absent or because uncharacterisic symptoms are present.

In this study, only the coding region of MECP2 has been analyzed, and mutations in regulatory elements could account for those cases in which no mutation has been identified[15]. In addition, PCR-based techniques could miss the altered gene if there is large rearrangements. Without am-

plification of the mutated allele, only the normal allele is amplified, resulting in false-negative results, and accounting for detection rates lower than a 100%[19].

A recently published recommended screening strategy is to firstly screen exons 3 and 4 for mutations using PCR-based techniques[20]. If no mutation is identified, further screening of exons 1 and 2 is reccomended. For individuals in whom no pathogenic mutations have been found, additional screening for large deletions is recommended, which may be achieved by Southern analysis, quantitative PCR or multiplex ligation prove amplification (MLPA). Patients who are negative for *MECP2* mutations and have a strong clinical diagnosis of RS, particularly if there are early onset seizures, should be considered for further screening of the *CDKL5* gene, recently associated with this variant[20].

Genotype-phenotype correlations

The discovery of a molecular alteration related to RS raised questions about a possible genotype-phenotype correlation; however various studies in different populations have yielded inconsistent results[6,7,21-24].

There are different genetic factors that are likely to influence the phenotype in RS, including patterns of X chromosome inactivation and the type and location of the mutation. Several studies have demonstrated normal X inactivation patterns in the majority of RS cases, so this should be an important factor in a minority of patients[5,24].

Presence or absence of a pathogenic mutation – Before correlating the different types or locations of the mutations and the phenotype, a correlation between the presence or absence of a pathogenic mutation and the clinical picture was done (Table 1). It was observed that most patients with an unidentified pathogenic point mutation (74.3%) had an earlier onset of symptoms, before one year of age. A similar observation was made in 20 Korean RS patients, where there was a tendency for patients with no detected *MECP2* mutation (30%) to show more severe symptoms and more rapid clinical progression[23]. We suggest that these patients have large structural abnormalities in the *MECP2* gene, not detectable by our screening strategy, causing total loss of protein function and therefore the earlier disease presentation. Additional molecular methodologies are necessary to confirm this hypothesis.

In this study, breathing abnormalities were significantly more frequent in patients with identified point mutations. Breathing abnormalities are a striking feature of RS that occurs during wakefulness[5], and its association with a point mutation reinforces the specificity of the mutations in respect of the classical phenotype. Additional associations observed in patients with detected mutations were pointed by Bienvenu et al.[25], that showed that patients with detected mutation lost more frequently acquired purposefull hand skills, had more frequent peripehral vasomotor disturbances and epilepsy and were more frequently able to walk, although statistically not significant.

Type of mutation – Several groups tried to determine whether different types of mutations in the *MECP2* gene can account for the variability of clinical features. We divided the mutations found in this study in two different groups: missense and truncating mutations (Table 2). In agreement with other studies[6,24,26,27], we found that patients with truncating mutations had earlier symptoms and higher scores with Pineda scale[9] (Table 5), indicating that these mutations are more deleterious to the protein.

Location of the mutation – The mutations were also grouped according to their location in the different regions of the *MECP2* gene (Table 3), and it is expected that mutations in more important functional protein regions lead to more severe phenotypes. Indeed, we found that mutations in the NLS lead to earlier disease onset and higher scores with Pineda scale[9]. In contrast, patients with mutations in the TRD domain after the NLS had lower scores, presenting the atypical form of the disease.

These results are similar to those of Colvin et al.[28], and they indicate that mutations involving the NLS may result in failure of the protein to be directed to the nucleus, resulting in a more significant reduction of MECP2 protein function, which in turn would lead to a more severe phenotype. Proteins carrying mutations distal to the NLS might still be targeted to the nucleus and exert a partial effect on transcription repression[28].

Three most frequent mutations – Finally, the three most common mutations were considered individually (Table 4), searching to a more precise genotype-phenotype correlation. It might happen that a specific mutation could be responsible for a specific symptom or cause a more severe phenotype.

All of the three mutations were more frequent in classic RS. In accordance with other studies[27,29], we observed that the R306C mutation was associated with a milder form of RS that included later disease onset, lower scores with Pineda scale[9], and more frequently retention of the ability to walk. A functional assay showed that R306C mutation retained the ability to repress transcription, accounting for the milder phenotypes[30].

We found that presence, type and location of mutations do not influence all features of RS in the same way, as observed by other studies[5-7,18,20-29]. It is necessary more studies on protein function and genotype-phenotype correlations with higher number of patients to better interpret these findings.

In conclusion, this is the first study with molecular characterization of a large sample of Brazilian RS patients. A genotype-phenotype correlation was shown, providing valuable information necessary for genetic and clin-

ical counseling. Although still tentative, genotype-phenotype correlations helps answering questions raised by parents and caretakers about the meaning of a particular molecular alteration found. When a negative test result is obtained, it should be emphasized that additional molecular alterations could be present, not detected by the methodology used, or different altered genes could be involved and, because of that, the clinical interpretation of this result should be made with caution. The results also show that mutation screening in Brazilian RS patients should begin by searching for the four most frequent mutations identified here. It is also suggested that methods that detect large deletions should be used, mostly when analyzing early onset and atypical patients.

REFERENCES

1. Hagberg B. Clinical criteria, stages and natural history. In: Hagberg B, Anvert M, Wahlström J (Eds). Rett syndrome: clinical & biological aspects. Clinics in Developmental Medicine N 127, London: Cambridge University Press, 1993b:4-20.
2. Schwartzman JS. Síndrome de Rett. Rev Bras Psiquiatr 2003;25: 110-113.
3. Amir RE, Van Den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked *MECP2*, encoding methyl-CpG-binding protein 2. Nat Genet 1999;23:185-158.
4. Dragich J, Houwink-Manville I, Schanen C. Rett syndrome: a surprising result of mutation in *MECP2*. Hum Mol Genet 2000; 9:2365-2375.
5. Amir RE, Van Den Veyver IB, Schultz R, et al. Influence of mutation type and X chromosome inactivation on Rett syndrome phenotypes. Ann Neurol 2000;47:670-679.
6. Cheadle JP, Gill H, Fleming N, et al. Long-read sequence analysis of the *MECP2* gene in Rett syndrome patients:correlation of disease severity with mutation type and location. Hum Mol Genet 2000;9:1119-1129. Erratum in: Hum Mol Genet 2000; 9:1717.
7. Huppke P, Laccone F, Kramer N, Engel W, Hanefeld F. Rett syndrome: analysis of *MECP2* and clinical characterization of 31 patients. Hum Mol Genet 2000;9:1369-1375.
8. The Rett Syndrome Diagnostic Criteria Work Group. Diagnostic criteria for Rett Syndrome. Ann Neurol 1988;23:425-428.
9. Colvin L, Fyfe S, Leonard S et al. Describing the phenotype in Rett syndrome using a population database. Arch Dis Child 2003;88:38-43.
10. Miller SA, Dykes, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Ac Res 1988;16:1215.
11. Siegel S, Castellan Jr NJ. Estatística não paramétrica para ciências do comportamento. 2ª Ed. Porto Alegre: Artmed, 2006.
12. Rosemberg S. Clinical analysis in nine Brazilian cases of the Rett syndrome. Brain Dev 1990;12:44-46.
13. Bruck I, Antoniuk SA, Halick SMS, et al. Estudo retrospectivo e prospectivo de 28 pacientes. Arq Neuropsiquiatr 2001;59:407-410.
14. Pozzi CM, Rosemberg S. Rett syndrome: clinical and epidemiological aspects in a Brazilian institution. Arq Neuropsiquiatr 2003;61:909-915.
15. Shahbazian MD, Zoghbi HY. Rett syndrome and MeCP2: linking epigenetics and neuronal function. Am J Hum Genet 1997; 71:1259-1272.
16. RettBASE: IRSA *MECP2* Variation Database. Western Sydney Genetics Program, Children's Hospital, Westmead, NSW Australia, 2002. URL: http://mecp2.chw.edu.au/mecp2/
17. Clarke A, Schanen, Anvret, M. Towards the genetic basis of Rett syndrome. In: Kerr A, Witt-Engerström I (Eds). Rett disorder and the developing brain. New York: Oxford University Press, 2001:27-55.
18. Huppke P, Held M, Laccone F, Hanefeld F. The spectrum of phenotypes in females with Rett syndrome. Brain Dev 2003; 25:346-351.
19. Ariani F, Mari F, Pescucci C, et al. Real-time quantitative PCR as a routine method for screening large rearrangements in Rett syndrome: report of one case of *MECP2* deletion and one case of *MECP2* duplication. Hum Mutat 2004; 24:172-177.
20. Williamson S, Christodoulou J. Rett syndrome: new clinical and molecular insights. Eur J Hum Genet 2006;14:896-903.
21. Giunti L, Pelagatti S, Lazzerini V, et al. Spectrum and distribution of *MECP2* mutations in 64 Italian Rett syndrome girls: tentative genotype/phenotype correlation. Brain Dev 2001; 23(Suppl 1):S242-S245.
22. Yamada Y, Miura K, Kumagai T, et al. Molecular analysis of Japanese patients with Rett syndrome: identification of five novel mutations and genotype-phenotype correlation. Hum Mutat 2001;18:253.
23. Chae JH, Hwang YS, Kim KJ. Mutation analysis of *MECP2* and clinical characterization in Korean patients with Rett syndrome. J Child Neurol 2002;17:33-36.
24. Hupke P, Held M, Hanefeld F et al. Influence of mutation type and location on phenotype in 123 patients with Rett syndrome. Neuropediatrics 2002;33:63-68.
25. Bienvenu T, Carié A, Roux N, et al. *MECP2* mutations account for most cases of typical forms of Rett syndrome. Hum Mol Genet 2000;9:1377-1384.
26. Monros E, Armstrong J, Aibar E, Poo P, Canos I, Pineda M. Rett syndrome in Spain: mutation analysis and clinical correlations. Brain Dev 2001;23(Suppl 1):S251-S253.
27. Smeets E, Schollen E, Moog U, et al. Rett syndrome in adolescent and adult females: clinical and molecular genetic findings. Am J Med Genet 2003;122:227-233.
28. Colvin L, Leonard H, de Klerk N, et al. Refining the phenotype of common mutations in Rett syndrome. J Med Genet 2004; 41:25-30.
29. Schanen C, Houwink EJ, Dorrani N, et al. Phenotypic manifestations of *MECP2* mutations in classical and atypical Rett syndrome. Am J Med Genet 2004;126A:129-140.
30. Yusufai TM , Wolfe A. Functional consequences of Rett syndrome mutations on human MeCP2. Nucleic Acids Res 2000; 28:4172-4179.