Mutations in the gene encoding methyl-CpG-binding protein 2 cause Rett syndrome

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Abstract

Rett syndrome is an X-linked dominant neurodevelopmental disorder primarily affecting girls. About 80% of classic Rett syndrome is caused by mutations in the gene for methyl-CpG-binding protein (MeCP2) in Xq28. MeCP2 links DNA methylation to transcriptional repression, and MECP2 mutations likely cause partial or complete loss of function of the protein, leading to inappropriate transcription of downstream genes at critical times in brain development. More severe and milder variant forms can all be caused by similar mutations. Most classic Rett syndrome patients have random X-chromosome inactivation (XCI), but skewed patterns are present in a few. All asymptomatic or mildly mentally delayed female carriers studied to date have non-random XCI patterns, suggesting that this attenuates the deleterious effects of the MECP2 mutations in these women. The finding of non-random XCI patterns in some patients with very early truncations is consistent with this observation and supports that many mutations could cause partial and not complete loss of function. Our observation that the mutant mRNA is stable in three patients with truncating mutations supports this possibility. Further studies will have to be performed to better understand the functional consequences of MECP2 mutations in RTT.

Keywords: -Rett syndrome; MECP2; Methyl-CpG-binding domain; Transcription repression domain; Xq28; X-linked dominant; Mutations

1. Introduction

Rett syndrome (RTT) is an X-linked dominant neurodevelopmental disorder that affects about 1/10,000 to 1/15,000 females. RTT is characterized by apparently normal development for 6–18 months [1], followed by a period of regression with loss of purposeful hand use, deceleration of head growth, and onset of repetitive, stereotyped hand movements. Affected girls develop gait ataxia and apraxia, autistic features, seizures, respiratory dysfunction (episodic apnea and/or hyperpnea), autonomic dysfunction and have decreased somatic growth [2]. This period of rapid deterioration is followed in puberty by a stabilization phase that lasts through adulthood. Some girls have variant forms of Rett syndrome: some may have a milder phenotype and retain the ability to walk or speak and others a more severe disease with an earlier onset (congenital form) [3,4].

2. The X-linked inheritance of Rett syndrome and discovery of MECP2 mutations in Rett syndrome

The exact origin of RTT has long been debated [2,5,6], but several observations suggested that it is an X-linked dominant disorder. Although 99% of RTT occurrences are sporadic, a few familial cases were reported in which the disease was inherited through maternal lines [7,8]. X-linked dominant inheritance was also supported by the observation, in three families, of a non-random pattern of X-chromosome inactivation in obligate carrier females without a RTT phenotype [9–11] and the presence in two of these of a male sibling who presented with a severe neonatal encephalopathy and died as an infant [11,12]. Based on these observations, a series of exclusion mapping studies aimed at defining the smallest regions shared amongst affected individuals in familial RTT was used and mapped the Rett syndrome gene to a 10 Mb region telomeric to DXS998 [10–15]. Mutational analysis of candidate genes in the included region was then focused on those with known or potential functions in the development of the CNS [16,17]. The unexpected observation of biallelic methylation of a putative imprinted gene (PEG3) in three patients with Rett
syndrome (Van den Veyver and Zoghbi, unpublished results) drew our attention to the gene encoding methyl-CpG-binding protein 2 (MeCP2) in Xq28. Using heteroduplex analysis and direct sequencing of the coding sequence of the MECP2 gene, we identified mutations in patients with classic Rett syndrome.

3. Function of MeCP2

MeCP2 maps between L1CAM and the RCP/GCP loci in Xq28 and undergoes X-inactivation [18]. The gene is very conserved between species, not only in its coding region, but also in the 3′ and 5′ untranslated regions (UTR) [19]; this has led to the recent identification in human and mouse of a fourth MECP2 exon that encodes 5′UTR sequence [20].

MeCP2 was found to be the molecular link between DNA methylation and transcriptional repression [21,22]. It binds to symmetrically positioned CpG dinucleotides, methylated at position 5 of cytosine (m5CpG). MeCP2 can bind throughout the chromosome, but it preferentially associates with dense heterochromatin, rich in m5CpG [23]. Methylation of CpG dinucleotides in the mammalian genome is important to silence transcription of imprinted genes and genes on the inactive X chromosome, in the immobilization of endogenous transposon sequences and possibly in the control of tissue-specific gene expression [24]. When MeCP2 binds to the methylated CpG islands it recruits a complex containing histone deacetylases (HDAC1 and HDAC2) and the co-repressor Sin3A [21,22]. The tails of core histones in the nucleosomes are deacetylated by HDACs, which leads to compaction of heterochromatin, making it inaccessible to the transcriptional activators and components of the transcriptional machinery, resulting in stable repression of downstream genes [24] (Fig. 1). MeCP2 belongs to a family of methyl-CpG-binding domain-containing proteins [25], of which at least three (MBD1, MBD2a, MBD3) are members of different transcriptional repressor complexes. These may have distinct or overlapping functions with MeCP2 during methylation-dependent epigenetic regulation of transcription [24]. In addition there are at least four different DNA methyltransferases, one of which (DNMT1) plays a role in the stable transmission of DNA methylation during cell division [26,27], and two are de novo methylases (DNMT3a and DNMT3b) [28]. The embryonic lethality seen after gastrulation in mice with null mutations for the DNA methyltransferases, supports the importance of DNA methylation in development [27,28].

4. Mutations in MECP2

To date, we have identified mutations in the coding region of MECP2 in 74 of 92 sporadic patients with classic RTT (81%) and in four of nine RTT families [29,30]. We found 30 missense (MS) mutations, 22 of which are in the methyl-CpG-binding domain (MBD) of MeCP2, 35 nonsense (NS), 12 frameshift (FS) and 1 splice-site mutation. Forty-six of 48 truncating mutations are beyond the MBD, 41 of which affect the transcription repression domain (TRD). Consistent with the sporadic occurrence of RTT, most of the mutations occurred de novo and the majority of nucleotide substitutions are C to T transitions at CpG mutation hotspots (Fig. 1).

We found two mutations that are predicted to truncate the MBD. One is the splice-site mutation at the acceptor splice site of exon 4; we could only detect the wild-type transcript in lymphoblast mRNA from this patient, suggesting that she either has a non-random XCI pattern, or that the mutant RNA is unstable. Unfortunately, the patient was uninformative for the androgen receptor assay used to determine XCI [30]. The other mutation (Y141X), as well as one reported by Wan et al. (411delG) [31], is distal to the DNA-binding surface of the MBD. Both of these Rett patients show a tendency to non-random XCI in genomic DNA from peripheral blood leukocytes or from lymphoblastoid cell lines [30,31]. Interestingly, there were also three larger deletions of 41–170 base pairs distal to the TRD, in the C-terminal region of the coding sequence. This area contains a number of (quasi)-palindromic repeats that may make the region vulnerable to such deletions [32]. Even though systematic screening of the 5′UTR and 3′UTR and a search for larger deletions has not been completed, we found mutations in 81% of sporadic classic RTT patients. Therefore we think that this is most likely the major locus for Rett syndrome. However, one can speculate that rare cases of RTT and other similar disorders may be caused by mutations in other MBD-containing proteins or in additional factors involved in methylation-dependent transcriptional repression. It is interesting that some authors have reported lower incidences of MECP2 mutations in sporadic RTT patients.
[31,33]; these studies may have included patients with milder forms of RTT (such as preserved speech variant) and have used different mutation screening methods, such as denaturing gradient gel electrophoresis (DGGE) or denaturing high performance liquid chromatography (DHPLC). It also remains to be investigated whether those could have mutations in non-coding regions, in other genes with related functions or in MeCP2 target genes (see below).

5. Genotype–phenotype correlation

In order to better understand the pathogenesis of RTT and the role of the different mutations in the phenotype of RTT, we undertook a detailed genotype–phenotype correlation study on 48 RTT patients, 47 of whom fit all the diagnostic criteria for classic Rett syndrome, while one was considered to have a variant type of RTT, because of some preserved speech and gait [30]. We analyzed 13 clinical variables (age of onset, seizures, ambulatory function, respiratory function, head growth, motor function, hand movements, somatic growth, communication, autonomic dysfunction, screaming, self-abuse, scoliosis) as well as typical EEG characteristics, the length of the QTc interval on EKG, and CSF levels of biogenic amines and β-endorphins. Each variable and an overall composite clinical severity score (CCSS) were compared between patients with missense mutations and those with nonsense mutations. The most striking result of this study was that the CCSS as well as most individual variables were similar in the two groups of patients. We only found statistically significant differences in respiratory dysfunction, levels of HVA (both occurred more frequently in nonsense mutations), and scoliosis (more in missense mutations) [30]. Since our report, additional genotype-phenotype correlation studies have been performed and investigators have reported both similar and different results compared to ours. Cheadle et al. [34] describe mutations in 44/55 (80%) of classic Rett syndrome girls and scored for hand use, speech and capacity to walk. These authors found a more severe phenotype associated with truncating mutations compared to missense mutations, as well as with early truncations compared to late truncations. Huppke and colleagues found mutations in 24 of the 31 patients they studied (77.4%). In their comparison of truncating and non-truncating mutations, they did not find statistically significant differences using a similar clinical severity score, but they suggest that there is a trend towards a more severe phenotype with truncating mutations [35]. Bienvenu and colleagues [33] found mutations in 30/46 typical RTT patients (65%) using DGGE and direct sequencing. They did not report any correlation between the mutation type and the phenotypic features within the group of patients with mutations. They did report that girls with known MECP2 mutations more frequently lost purposeful hand skills, had more peripheral vasomotor disturbances and seizures but had less problems with walk-

ing. However, the P values for these features were above 0.05, and significance of this finding is therefore questionable [33]. Additional studies reported by Hoffbuhr et al., Gill et al., Yamakawa et al., Amano et al., Yamashita et al., and Raffaelle et al. at the 2000 World Congress for Rett syndrome (Karuizawa, Nagano, Japan) yielded different results: some groups report more severe phenotypes with truncating mutations compared to missense mutations, while others found the exact opposite, but overall, most studies did not detect an unequivocal genotype–phenotype correlation.

6. Role of X-chromosome inactivation

We studied XCI patterns in leucocyte-derived DNA samples from 34 Rett syndrome patients, informative for the XCI assay at the polymorphic human androgen receptor locus and found that 91% of patients had random patterns of XCI in these samples [30]. We found a skewed pattern of XCI in a patient with a putative early truncation and in an asymptomatic carrier mother [30]. Several other authors have also found that in general, non-random patterns of XCI are associated with milder (variant) phenotypes, asymptomatic carriers and with early more severe mutations [31,36] (Hoffbuhr, et al, 2000; presented at the 2000 World Congers of Rett syndrome; Karuizawa, Nagano, Japan). However some discrepancies exist: while we and others have observed only very skewed XCI patterns in asymptomatic mutation carriers [30,31,33], the data on classic Rett and variant forms with milder or more severe phenotypes are not as clear. Both skewed and random XCI have been observed in each of these groups, even associated with the same mutations [36]. In addition, Hoffbuhr and colleagues reported patients with early truncating mutations and random XCI that have classic RTT, suggesting that this XCI pattern can be associated with near total loss of function of MeCP2.

7. Role of MeCP2 in the pathogenesis of Rett syndrome

The currently favored hypothesis is that the mutated MeCP2 cannot perform its function as a transcriptional repressor, which may lead to inappropriate activation of a number of genes during development. This idea is consistent with the in vitro and cell culture assays that demonstrate that MeCP2 can bind double stranded DNA methylated at cytosine and thereby suppress transcription of specific reporter genes [21,22]. The available evidence to date points towards such a loss of function of MeCP2 as the underlying mechanism leading to the phenotype of Rett syndrome. Most missense mutations seem to interfere with normal function of the MBD or the TRD, while the majority, but not all, of truncating mutations delete at least part of the TRD. The methyl-CpG-binding capacity of four missense mutations (R106W, R133C, F155S, T158M) was studied in
vitro and revealed that the first three reduced binding by over 100-fold, while the T158M mutation only resulted in a 2-fold reduction [37]. Moreover, because of XCI, each cell has either the wild-type or the mutant MECP2 active. This excludes a possible dominant-negative mechanism in which the protein produced from the mutant allele would interfere with the function of its wild-type counterpart. However, it does not exclude the less likely alternative of dominant-negative interference of the mutant MeCP2 with the function of other methyl-CpG-binding complexes. Genes regulated by allele-specific methylation of CpG islands or other regulatory regions usually belong to the class of imprinted genes or of genes that undergo X-inactivation [24]. Within these groups, those that could affect CNS development are good candidates. It is incompletely understood whether CpG island methylation plays a role in the developmental regulation of expression of other genes. Even though this has not been proven in mammalian systems [38], the abnormalities observed in hypomethylated developing Xenopus embryos suggest that methylation plays an important role in very early differentiation and development [39].

The affected target genes are most likely important in normal development of the central nervous system, since neuroimaging, neuropathological and neurochemical data all suggest an arrest in CNS development [40]. Genes that play a role in development and function of specific neuronal systems, such as the serotonergic connections in the brainstem are likely to be important players and could be primarily or secondarily disrupted. An alternative possibility is that the target genes are not neuronal specific, but that the developing brain is more dependent on MeCP2 function than any other tissue. The elevated expression levels of longer forms of the MECP2 mRNA in developing brain supports this possibility [19].

The low percentage of early truncating mutations that affect most or all of the MBD, is interesting and together with our observation that there is a tendency for skewing of XCI in patients with such a mutation suggests that the common mutations seen in classic RTT may cause partial and not complete loss of function. It remains to be determined whether all truncated proteins will enter the nucleus, as the nuclear localization signal is located within the TRD [41]. To support the ‘partial’ loss of function hypothesis, it will be important to determine that the mutant protein is expressed. Towards this goal, we have recently performed RT-PCR on RNA from lymphoblast cell lines from three RTT patients with missense mutations (R168X) and demonstrated by allele-specific restriction enzyme digestion and direct sequencing of the RT-PCR product that the mutant allele is expressed in these cells (Van den Veyver and Zoghbi, unpublished results) (Fig. 2). This mutation is in the last exon of the gene, and the absence of a downstream intron is consistent with the absence of nonsense mediated mRNA decay in the presence of this truncating mutation [42]. We are further evaluating whether mutant truncated protein is being produced.

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**Fig. 2.** Expression of mutant mRNA in three patients with the R168X mutation. Reverse-transcription polymerase chain reaction (RT-PCR) amplification of lymphoblast derived total RNA was performed from three patients with a R168X mutation and one control sample using primers 2bF and 3bR [29]. The C to T change at position 502 creates a HphI restriction enzyme (RE) site. The undigested RT-PCR product is 761 bp (lane ND). RE digestion of the control sample (WT lane) with HphI generates a 505 bp, a 142 bp and a 114 bp fragment. As can be seen in lanes 1.2 and 3 (patient samples), digestion with this enzyme generates additional 371 bp and 134 bp fragments, resulting from the mutant allele. Even though this is a non-quantitative assay, samples 1 and 3 appear to have generated more mutant PCR than WT product.

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**8. Conclusions**

It has been well established that mutations in the X-linked MECP2 gene are the cause for the majority (as much as 80%) of classic RTT. Most mutations are de novo and occur at CpG hotspots. For patients who fit the major and minor diagnostic criteria for classic RTT, the combined published data do not support a clear correlation between the severity of the phenotype and the type and position of the different mutations. The phenotypic spectrum of MECP2 mutations will likely be expanded in the future since affected males as well as women with mild mental delay and asymptomatic carriers who have skewed XCI have been described [31]. The MECP2 mutations most likely lead to partial or complete loss of function of the protein. This is thought to result in inappropriate expression of a number of genes that are crucial for normal CNS development. Further studies in RTT patients and in mouse models will be needed to better understand the functional consequences of the MECP2 mutations. This will hopefully lead to the design of targeted preventive and therapeutic strategies.

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