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# Point mutation frequency in the *FMR1* gene as revealed by fragile X syndrome screening

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ABSTRACT

Fragile X syndrome (FXS) is a common cause of intellectual disability, developmental delay and autism spectrum disorders. This syndrome is due to a functional loss of the *FMR1* gene product FMRP, and, in most cases, it is caused by CGG repeat expansion in the *FMR1* promoter. Yet, also other *FMR1* mutations may cause a FXS-like phenotype. Since standard molecular testing does not include the analysis of the *FMR1* coding region, the prevalence of point mutations causing FXS is not well known. Here, high resolution melting (HRM) was used to screen for *FMR1* gene mutations in 508 males with clinical signs of mental retardation and developmental delay, but without CGG and GCC repeat expansions in the *FMR1* gene and *AFF2* genes, respectively. Sequence variations were identified by HRM analysis and verified by direct DNA sequencing. Two novel missense mutations (p.Gly482Ser in one patient and p.Arg534His in two unrelated patients), one intronic and two 3′-untranslated region (UTR) variations were identified in the *FMR1* gene. Missense mutations in the *FMR1* gene might account for a considerable proportion of cases in male patients with FXS-related symptoms, such as those linked to mental retardation and developmental delay.



## 1. Introduction

Fragile X Syndrome (FXS) is the most frequent X-linked cause of mental retardation [1,2]. Affected patients show heterogeneous and variable degrees of these symptoms, such as autism spectrum disorder as well as behavioural and learning disabilities. Physical characteristics can include macrocephaly, long face and large ears and/or macroorchidism [3,4]. FXS patients lack the FMRP (fragile X mental retardation protein), a synaptic RNA-binding protein. In most cases, FXS is caused by a CGG repeat expansion in the 5′-UTR of the *FMR1* gene, coding for FMRP [5–7], and relates to an expansion of >200 trinucleotide units, referred to as a full mutation, which results in the epigenetic transcriptional silencing of the *FMR1* gene [8,9]. Another type of X-linked mental retardation, called FRAXE, is caused by an expansion of the GCC repeat in the *AFF2* gene. Affected patients present with weaker clinical signs as compared to FXS, such as mild mental retardation with psychotic behaviour, and mostly lack typical overt physical traits [10].

Due to the X-linked nature of this disease, FXS males are often more severely affected than females [3]. Although CGG repeat full mutations in the *FMR1* gene promoter frequently cause FXS in many of the diagnosed patients, further *FMR1* sequence modifications may result in a loss of FMRP function. Large deletions of the *FMR1* gene, including the promoter region, have been described as a cause of FXS [11–13]. Smaller deletions and point mutations in the coding sequence of the *FMR1* gene can also result in a FXS-like phenotype [14–18]. It has been suggested that patients with a clinical FXS-like phenotype, who present with developmental delay but not the *FMR1* gene full mutation, should be routinely tested for further mutations of the *FMR1* coding region [16,18]. However, since the standard FXS diagnostics only comprises the sizing of the triplet repeat block in the 5′-UTR of the *FMR1* gene [3], the prevalence of other mutations in the *FMR1* coding region is still not well known [16,18]. In a large study of 963 developmentally delayed males without CGG expansions, 130 novel sequence variations were identified including one missense mutation (p.Arg138Gln) [14]. Thus, undetected mutations in the *FMR1* gene may account for FXS-like phenotypes. The prevalence of *FMR1* mutations in male patients, tested negative for *FMR1* and *AFF2* repeat expansions was investigated here.

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## 2. Material and methods

### 2.1. Patient samples

Included were 508 genomic DNA samples from male patients, which met the criteria of FXS-like phenotype, mental retardation or developmental delay. All samples had been recruited by neuropediatric specialists and sent to the Department of Human Genetics, Ruhr-University Bochum, Germany, for sizing the *FMR1* CCG blocks over a period of ten years. *FMR1* or *AFF2* full mutations (>200 CGG and >100 GCC, respectively; [19]) had been excluded from all patient samples based on the use of Southern blot and PCR analyses, respectively. The length of *FMR1* trinucleotide blocks in this patient group displayed a median of 30, with a minimum of 16 and a maximum of 51 repeats. Control DNAs were obtained from a group of healthy blood donors of the same geographical region as the patient population. Patients or legal guardians had consented to extend the molecular testing of the patients FXS, and the Ethics Committee of the Ruhr-University Bochum, Germany, approved this study.

### 2.2. PCR amplification

Primers for the 17 exons of the *FMR1* gene were selected based on the GenBank reference sequence NM_002024 (isoform 1). Amplicon sizes were set at <400 base pairs (bp) to increase the sensitivity of HRM. For PCR amplification, we used 10 μl reaction mix that consisted of 50 ng DNA template together (or no template for no-template control) with 1 μl $MgCl_2$-free 10 × PCR buffer, 0.2 mM of each dNTP, 0.1 units of *Taq* polymerase (Genecraft), 0.25 pmol/μl of each primer, 2–3 mM $MgCl_2$and 1 μl of DNA intercalating fluorescent dye 10 x LCGreen Plus (Idaho Technology). Optimized PCR amplifications were performed in a thermocycler (Biometra). Cycling conditions included 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at annealing temperature and 30 s at 72 °C, and a final elongation step for 5 min at 72 °C.

### 2.3. High resolution melting and DNA sequencing analyses

Initially for mutation scanning, HRM analyses were carried out directly after the PCR. Additionally, DNA was mixed 1:1 with an amplified control DNA. Amplification of the GC-rich exon 1 (67%) was achieved without LCGreen. For HRM analysis, 1 μl dimethyl sulfoxide (DMSO) and 1.5 μl LCGreen were added post-PCR, and amplicons were denatured at 98 °C for 30 s. HRM was performed on a LightScanner® 96 System (Idaho Technology) within a temperature range of 20 °C using a melting temperature of up to 98 °C. Data were acquired and evaluated using the Call-It 2.0 (Idaho Technology). For each plate Δ-fluorescence difference curves were created. Exon melting profiles that deviated by >0.02 Δ*F* from the group of profiles containing the majority and/or selected baseline wild type control sample were recorded as sequence variants. These samples where independently amplified again and bidirectionally sequenced (BigDye Terminator v.3.1, Applied Biosystems) in an automated capillary sequencer 3500XL Genetic Analyser (Applied Biosystems).

## 3. Results

### 3.1. High resolution melting

Sequence variations were detected in the *FMR1* gene by HRM according to the melting behaviour of DNA heteroduplexes [20]. The largest PCR fragment was 368 bp, and we detected three melting points. All base changes identified showed curve drifts or obvious differences from wild type samples after plotting. Sequence variations were detected in 13 of the 17 amplified exonic regions and the neighbouring intronic areas. In exons 5, 15 and 17, several sequence variants were detected. At least one polymorphism was identified in each of the ten regions, whereas no sequence variation was detected in exons 3, 4, 7 or 12. Each distinct melting profile was investigated by direct DNA sequencing. If a mutation was discovered in the process, it was used as a positive control in the following HRM analyses. Overall, 17 different point mutations were detected, and five of them had not been described previously in the public SNP database (http://www.ncbi.nlm.nih.gov/SNP/). A summary of all sequence alterations found in the present study and described previously is given in Table 1.

### 3.2. Novel mutations

Five newly discovered base substitutions are listed in Table 2. After extensive database and predictive analyses, no pathogenic effect was obvious for the intronic variant c.1189-39A > *G*, the two variations located in the 3′-UTR, c.*60T > *G* or c.*68T > *G*. The c.*60T > *G* variation had been described before in a similar patient group [14], where it was not considered to be pathogenic. Two novel missense variants were identified in the *FMR1* gene. The first mutation c.1444G > *A* was discovered in a single sample (Fig. 1); it resulted in a glycine to serine substitution at the amino acid position of 482. This amino acid exchange was predicted to be pathogenic using rests from the programs Polyphen-2 (HumDiv: 0.969/

**Table 1**
Summary of known polymorphisms detected by high resolution melting (HRM) curve analysis in the present study, their positions in the *FMR1* gene and their minor allele frequencies in the present patient group; population frequency data are according to dbSNP build 138 (http://www.ncbi.nlm.nih.gov/SNP/). There were no remarkable differences of the frequencies compared with previous studies.

| SNP# | Position (NM_002024) | Position (NP_002015) | Frequency among 508 FXS-like patients (%) | Population frequency in % |
|---|---|---|---|---|
| rs111485627 | c.18G > *T* | p.Val6= | *T* = 0.787 | *T* = 1.271 (54/4247) |
| rs25726 | c.52-112A > *G* | – | *G* = 15.945 | *G* = 9.585 (201/2097) |
| rs29279 | c.271-19A > *G* | – | *G* = 0.197 | *G* = 2.237 (37/1654) |
| rs148872586 | c.309C > *T* | p.Tyr103= | *T* = 0.197 | *T* = 0.045 (2/4494) |
| rs25707 | c.414G > A | p.Arg138= | *A* = 8.268 | *A* = 10.120 (790/7806) |
| rs29281 | c.433G > *T* | p.Ala145Ser | *T* = 0.197 | *T* = 3.396 (284/8362) |
| rs150724379 | c.801 + 31C > *T* | – | *T* = 3.543 | *T* = 1.693 (28/1654) |
| rs25714 | c.990 + 14C > *T* | – | *T* = 8.858 | *T* = 25.784 (1044/4049) |
| rs41311668 | c.991-54A > *G* | – | *G* = 0.197 | *G* = 0.061 (1/1653) |
| rs143889976 | c.1572C > *T* | p.Ser524= | *T* = 0.393 | *T* = 0.147 (9/6110) |
| rs73606790 | c.1737 + 8C > *A* | – | *A* = 0.197 | *A* = 2.053 (34/1656) |
| rs201939954 | C.*32C > *G* | – | *G* = 0.197 | – |