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Beyond Trinucleotide Repeat Expansion in Fragile X Syndrome: Rare Coding and Noncoding Variants in FMR1 and Associated Phenotypes

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Abstract: FMR1 (FMRP translational regulator 1) variants other than repeat expansion are known to cause disease phenotypes but can be overlooked if they are not accounted for in genetic testing strategies. We collected and reanalyzed the evidence for pathogenicity of FMR1 coding, noncoding, and copy number variants published to date. There is a spectrum of disease-causing FMR1 variation, with clinical and functional evidence supporting pathogenicity of five splicing, five missense, one in-frame deletion, one nonsense, and four frameshift variants. In addition, FMR1 deletions occur in both mosaic full mutation patients and as constitutional pathogenic alleles. De novo deletions arise not only from full mutation alleles but also alleles with normal-sized CGG repeats in several patients, suggesting that the CGG repeat region may be prone to genomic instability even in the absence of repeat expansion. We conclude that clinical tests for potentially FMR1-related indications such as intellectual disability should include methods capable of detecting small coding, noncoding, and copy number variants.

Keywords: FMR1; FMRP; fragile X; variant classification; copy number variants

1. Introduction

Fragile X syndrome (FXS) is an X-linked disorder due to a loss of FMR1 function. The phenotype involves both neurological and physical features, including developmental delays (DD), intellectual disability (ID), autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), joint hypermobility, and characteristic facial features such as a long face, large or prominent ears, prominent forehead, and prominent jaw [1,2]. Because not all features are present in all affected individuals, FMR1 pathogenic variants can be found (or may not be recognized) in patients with apparently nonsyndromic ID or ASD. FXS phenotypes are not restricted to hemizygous males; females heterozygous for loss-of-function variants can also be clinically affected [2,3].

Expansions of the CGG repeat located in the FMR1 5’ untranslated region (UTR) account for most cases of FMR1-related disease through both loss- and gain-of-function mechanisms. The “normal” repeat size is 5–44 repeats [4]. “Full mutation” expansions to >200 CGG repeats typically result in gene silencing with methylation of the FMR1 promoter; this loss-of-function mechanism is responsible for most cases of FXS [4]. “Premutations” containing 55–200 repeats confer risk for expansion to full mutations in the next generation but can also themselves cause non-FXS phenotypes due to toxicity of RNA expressed from the premutation allele, which is not silenced and can even display increased levels of transcription [5]. These include fragile X-associated tremor/ataxia syndrome, fragile X-associated primary ovarian insufficiency, and neurodevelopmental and psychiatric conditions. The same gain-of-function mechanism and phenotypes can also occur in individuals...
with unmethylated full mutation alleles that are transcribed [5]. “Intermediate” alleles ranging from 45 to 54 repeats display a higher risk for repeat length change in the next generation but not an expansion to full mutation within one generation.

Most clinical tests and guidelines to date have thus focused on detecting CGG repeat expansions [4]. Some but not all clinical tests for repeat expansion can also detect interspersion of AGG repeats, which stabilize the repeat region and reduce the probability of its expansion [6,7]. However, the common strategy of ordering CGG repeat expansion testing “for FMR1” means that a negative result does not eliminate FMR1-related disorders from the differential diagnosis. Besides random mutations, the FMR1 region is prone to genomic instability since long tracts of CGG repeats are mutagenic on nearby sequences; they fold into non-B-DNA conformations and produce double-stranded DNA breaks from replication fork stalling [8,9]. It is thus important to consider the possibility of loss-of-function FMR1 variants other than repeat expansions when ordering, designing, or interpreting clinical tests for potentially FMR1-related phenotypes such as ID and ASD.

To better understand the range of pathogenic variants in FMR1 other than repeat expansions, we collected previously reported variants other than CGG repeat length changes and analyzed their evidence for pathogenicity. We included the entire range of variation with coding, noncoding, and copy number variants and provided a curated list of evidence relevant to the impact of each variant to assist with independent reassessment of pathogenicity. We further analyzed the inheritance and allelic origin of deletions to understand whether rearrangements originate from repeat expansion alleles.

2. Materials and Methods

All previously reported FMR1 variants other than CGG repeat length changes were collected from PubMed search results for the terms (FMR1) OR (“fragile X”) through August 2021 (8572 results) by manually identifying publications with FMR1 variants identified in humans, followed by filtering for relevant variants as detailed below. Variants were mapped to the GRCh38.p13 chromosome X (NC_000023.11) and FMR1 transcript NM_002024.6 reference sequences. Coordinates from previous assemblies were converted using the UCSC Genome Browser and LiftOver (http://genome.ucsc.edu (accessed on 1 August 2021)) [10]. On these reference sequences, the transcription start site is c.-261 (g.147911919), and the CGG repeat is c.-129 to c.-70 (g.147912051–147912110).

The pathogenicity of collected FMR1 variants was reclassified by board-certified medical geneticists following the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) joint guidelines for sequence variant interpretation (SVI) [11] and ACMG/ClinGen technical standards for constitutional copy number variant (CNV) interpretation [12]. For the SVI criteria, since FMRP has multiple functions and a single standardized functional test does not exist, the PS3 criterion was applied for functional studies showing a deleterious effect on any physiologically relevant function. The BS2 criterion was applied for noncoding/splice variants with putative effects on protein expression given that loss of FMRP expression is fully penetrant in males, but not variants expected to change protein sequence since the penetrance of missense variants in FMR1 has not been established.

For CNVs, all variants that met the inclusion criteria necessarily fell under criteria 1A (contains protein-coding gene) and 3A (<35 protein-coding genes involved) on both the copy number loss and gain scoring metrics; therefore, these criteria are not shown. Section 4 was also inapplicable to the deletion variants, given that FMR1 loss is known to cause disease and is not shown. Criterion 5A, which is subject to interpreter discretion, was assigned 0.15 points for all reported de novo occurrences due to the moderate genetic heterogeneity of fragile X-like phenotypes and since confirmation of maternity was not always explicitly specified.

Interpretation of partial deletions can also vary considerably between different raters based on how the guidelines are interpreted. For consistency, all such deletions involving coding sequence were scored as intragenic variants following the SVI guidelines with
ClinGen recommendations for modifying PVS1, as recommended in the CNV interpretation technical standard [12,13]. The edge case of deletion extending from upstream sequences into part of the FMRP N-terminus within exon 1 was interpreted as PVS1_Moderate regardless of the location of the 5′ breakpoint. Criterion PS2 was applied to all de novo variants, though an argument could be made for assigning a decreased weight when the originating allele is a full mutation expansion. Criterion PM2 was also applied due to the absence of similar coding region deletions in the Database of Genomic Variants; though this approach has limitations, it is often used [14].

On the other hand, all such deletions that ended upstream to c.1, to which most of the SVI criteria are not applicable, were interpreted as 2C-2 under the CNV interpretation scoring metric. The recommendation to upgrade points for deletions involving well-characterized promoter regions was interpreted as a score of 0.30 for large deletions including the promoter and 0 for 5′UTR deletions within exon 1. This left two CNVs with additional benign evidence (normal FMR1 expression in proband) that are noted in Table 1.

Both small variants and copy number variants were included as follows: both coding and noncoding small variants were included if reported in association with a phenotype, while variants found among large sequencing cohorts were only included if absent in the Genome Aggregation Database (gnomAD v3.1.10; https://gnomad.broadinstitute.org (accessed on 1 August 2021)) or specifically discussed in the literature. For CNVs, deletions that extended into the CGG repeat region were included, while deletions entirely within the CGG repeat (i.e., repeat contractions) were out of the scope of this paper. To focus on FMR1-related phenotypes, we excluded larger variants such as cytogenetically visible microdeletions, other CNVs affecting neighboring genes with known phenotypes (e.g., AFF2 (FRAXE) and IDS (mucopolysaccharidosis II) distally) and X-autosome translocations.
| Ref. | Location b | Number Affected with Variant, Sex (M/F) | Inheritance/Originating Allele | Ascertainment | ACMG Criteria [12,13] | Conclusion |
|------|------------|----------------------------------------|--------------------------------|---------------|------------------------|------------|
| [15] | >140198205 (DXS1232-DXS105), <7 Mb upstream | 1M | Maternal (unaffected), de novo in mother (both grandparents lack deletion with normal CGG repeat counts) | ID, obesity | CNV 2A 5G_0.1 | PATH (1.1) |
| [16] | >140784366 (CDR1-sWXD2905), <7 Mb upstream | 1M + mother | Maternal (mildly affected) | DD, clinical suspicion | CNV 2A 5G_0.1 | PATH (1.1) |
| [17] #3 | 142254456, 5.7 Mb upstream | Del het 1F | De novo | Epilepsy research database | CNV 2A 5A_0.15 | PATH (1.15) |
| [18] | 147158490, 753 kb upstream | Mosaic del (90%) | De novo | ID, facies, testicle size | CNV 2A 5A_0.15 | PATH (1.15) |
| [19] | 147653688 c, 260 kb upstream | Del 1M | Mosaic in proband on 23-repeat allele (mother 23, 30 repeats) with breakpoints in LINE1 elements | ID | SVI PVS1_Strong PS2 PM2 | PATH |
| [20] | 147955394, 4 kb downstream | Del | Clinical FXS | CNV 2A 5H_0.1 | PATH (1.1) |
| [21] | >147722126 (DXS548); >DXS477, < cosm id 494; <190 kb upstream | Del 1M | ? (mother “borderline intelligence”) | ID | SVI PVS1_Strong PM2 | LPATH |
| [22] #2 | >147722126 (DXS548); >G9L, <FRAXAC1; <190 kb upstream | Del 1M | De novo on 19-repeat allele (mother 19, 51 repeats) | ID, clinical FXS | SVI PVS1_Strong PS2 PS3 PM2 | PATH |
| [23] | <147722126 (includes DXS548), not mapped further; >190 kb upstream | Del 1M + infant brother | Mat germline mosaicism (2 brothers, absent in mother) (mother has congenital digit amputations) | DD | CNV 2A 5G_0.1 | PATH (1.1) |
| [24] #18072 | 147787231, 125 kb upstream | Del 2M | Maternal (unaffected het) | Autism cohort | CNV 2A5G_0.1 | PATH (1.1) |
| [25] #3 | 147830806, 74 kb upstream | Del 1M | De novo | Clinical lab sample | CNV 2A 5A_0.15 | PATH (1.15) |

Table 1. *FMR1* CNVs reported in the absence of CGG repeat expansions a.
| Ref. | Location b | Number Affected with Variant, Sex (M/F) | Inheritance/Originating Allele | Ascertainment | ACMG Criteria [12,13] | Conclusion |
|------|------------|----------------------------------------|-------------------------------|---------------|------------------------|------------|
| [26] | 15–80 kb upstream (G9L YAC) | 1M | De novo (normal repeat alleles in mother) | DD | SVI PVS1_Strong PS2 PS3 PM2 | PATH |
| [27,28] | 4.4 kb upstream | 1M | Maternal (unaffected mosaic) d; breakpoints in L1MC2 and MIR3 elements | Preschool cohort with ID and >=1 FXS feature | CNV 2A 5G_0.1 | PATH (1.1) |
| [29] #24 | 147910365, 1.5 kb upstream | 4M, 2F | Maternal (unaffected) | Speech delay, hyperactivity | CNV 2C2_0.3 5D_0.3 VUS (0.6) |
| [22] #1 | 147911457, 462 bp upstream of transcription start | Mosaic del (40%) | De novo | Epilepsy, other clinical | CNV 2C2_0.3 5A_0.15 VUS (0.45) |
| [30] | ~147911751 (~300 bp upstream of CGG) Also has 13p+ polymorphism | ? (~400 bp size deletion) | De novo (mother is het for 700–900 repeat full mutation) | ID, aggressive behavior | CNV 2C2_0.5A_0.15 VUS (0.15) |
| [31] | 147911831, 88 bp upstream of transcription start | Del | ID male with >=2 FXS features | Unaffected; prenatal testing | SVI PVS1_Moderate PS3 PM2 LPATH |
| [32] | 147911966, c.-214 | Del | Maternal mosaic (unaffected het for 430–530 repeat full mutation, deletion occurred on full mutation allele by haplotype) | Unaffected, prenatal testing | CNV 2C2_0.5F_0 VUS (0) |
| [33] | 147911981, c.-199 | Del | Transmitted from unaffected female proband to male fetus | Unaffected, individual, population screening study | CNV 2C2_0.5F_0 VUS (0) |
| [34] | 147912140, c.-40 (distal to CGG repeat) In trans with large deletion involving FMR1-46,X,del(X)(q24) | Del | De novo, from maternal full mutation allele | Asymptomatic; due to brother with FXS | CNV 2C2_0.5A_0.3; lymphoblasts are FMRP+ VUS (~0.3) with additional benign evidence |
| [35] | 147912024, c.-156 | Del | De novo, from maternal full mutation allele | Asymptomatic; due to maternal full mutation | CNV 2C2_0.5A_0.3 (unaffected); normal blood FMR1 mRNA level VUS (~0.3) with additional benign evidence |
Table 1. Cont.

| Ref. | Location b | Number Affected with Variant, Sex (M/F) | Inheritance/Originating Allele | Ascertainment | ACMG Criteria [12,13] | Conclusion |
|------|------------|----------------------------------------|-------------------------------|---------------|------------------------|------------|
| [36] | APN26, #1  | <147948682 (Deletion of exon 17)        | Del 3M                       | Maternal (unaffected) | ID sequencing cohort  | SVI PVS1_Moderate P3_S3_Supporting PM2 PP1 LPATH |
| [33] | Undetermined 5' UTR | Loss of exon 1                     | Del 1M fetus                 | Mother is full mutation (280 repeat) het | Population screening study | SVI PVS1_Strong P3 PATH |
| [38] #3666 | Intragenic (~197 bp flanking CGG repeat in exon 1) | Intragenic Del 1M ? (mother has mild ID) | ID cohort CNV 2C2_0 5G_0.1 VUS (0.1) | Potentially including AFF2 (additional reports of confirmed FMR1-AFF2 deletions not shown) |

| Ref. | Location b | Type | Number Affected with Variant, Sex (M/F) | Inheritance/Originating Allele | Ascertainment | ACMG Criteria [12,13] | Conclusion |
|------|------------|------|----------------------------------------|-------------------------------|---------------|------------------------|------------|
| [25] #4 | 142773651, 5.1 Mb upstream | Del het | 1F                                      | De novo | Clinical lab sample | CNV 2A 5A_0.15 PATH (1.15) |
| [17] #2 | 143096757, 4.8 Mb upstream | Del het | 1F                                      | De novo | Epilepsy research database | CNV 2A 5A_0.15 PATH (1.15) |

Duplications

| Ref. | Location b | Type | Number Affected with Variant, Sex (M/F) | Inheritance/Originating Allele | Ascertainment | ACMG Criteria [12,13] | Conclusion |
|------|------------|------|----------------------------------------|-------------------------------|---------------|------------------------|------------|
| [25] #1 | 147707513 (204 kb upstream) | Dup het | 1                                      | De novo | Clinical testing (lab) | CNV 2H_0 4C_0.15x3 5A_0.15 VUS (0.6) |
| [25] | 147709189 (203 kb upstream) | Dup | 1                                      | De novo | Clinical testing for seizures (lab) | CNV 2H_0 4C_0.15x3 5A_0.15 VUS (0.6) |
| [25] #2 | 147796927 (115 kb upstream) | Dup het | 1                                      | De novo | Clinical testing (lab) | CNV 2H_0 4C_0.15x3 5A_0.15 VUS (0.6) |
| [39] | 147894723 (17 kb upstream) | Dup | 1                                      | De novo (Proband also has small 1q44 paternal dup and 4p15 del) | Myoclonic seizures | CNV 2H_0 4C_0.15x3 5A_0.15; normal mRNA in leukocytes VUS (0.6) |
| [40] | 147912003 (c.-177) | Dup | N/A (0.2-0.3% X chromosomes in study) | Finnish population samples with multiple unaffected males | CNV 2I_0 4C_0.15x3 5A_0.15; normal mRNA in leukocytes | VUS (0.6) |

In addition to the listed CNVs, we note that (1) two deletion alleles extending into the CGG repeat region from g.147911980 and g.147912003 were identified in a sequencing cohort without definite information regarding whether they were mosaic, constitutional, or associated with a CGG repeat expansion [11]; and (2) we were unable to access the full text of one report of a deletion in an affected patient in the Ukrainian-language literature (PubMed 9381553). Breakpoints listed as approximate (~) were specified in relation to other sequence landmarks by the authors. Corresponding to the hg19 coordinates 146,735,206-147,036,914 given for the aCGH result; the GRCh38 coordinates and ClinVar entry given in the same publication appear to be for a different region on chr6. Mosaic for deletion in 4% alleles in blood, 8% skin, 11% urine sediment, 12% menses, 33% eyebrow by quantitative polymerase chain reaction (PCR); 4/1000 leukocytes, 213/1600 fibroblasts by FISH. #: Case number of proband in report, if given; ~: approximately; PATH: pathogenic; LPATH: likely pathogenic; ID: intellectual disability; DD: developmental delay; Del: deletion; Dup: duplication; het: heterozygous; N/A: not applicable; M: male; F: female; FXS: fragile X syndrome; ?: unclear.
We determined or estimated breakpoints of CNVs that were published without specific genomic coordinates as follows. If junction sequences were published, they were matched to the reference genome by NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 1 August 2021)); breakpoints were shifted downstream of their published locations if the 3' rule applied. If breakpoint locations were specified in reference to landmarks (e.g., c.1, the CGG repeat, or the other breakpoint) or to sequences such as pE5.1 (Genbank X61378), an approximate value was calculated using the appropriate sequence. If sequence-tagged site markers were specified and primer sequences for those markers were available in the NCBI Probe legacy database (UNISTS_human.sts file at https://ftp.ncbi.nih.gov/pub/ProbeDB/ (accessed on 1 August 2021)), the location of the primer sequences were determined by BLAST and checked against the STS track of the UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgGateway (accessed on 1 August 2021)). For site DXS296 (probe VK21C), while we were not able to find published primer sequences, it is distal to FRAXE and within the **AFF2** transcript [42]. If breakpoints were determined by restriction fragment length polymorphism (RFLP), no re-analysis was performed to avoid possible misidentification due to ambiguity in some of the restriction fragment sizes.

Conservation of protein residues involved in missense variants was visualized using ClustalOmega [43] to align FMR1P, FXR1P, and FXR2P protein sequences (Uniprot Q06787, P51114, P51116) and ConSurf [44].

### 3. Results

#### 3.1. FMR1 CNVs in the Absence of Repeat Expansion

The majority of reported FMR1 CNVs were deletions identified in patients who underwent clinical testing for neurological features such as DD, ID, ASD, and/or epilepsy, with four small duplications only containing **FMR1** (Table 1). Details of the clinical evidence used to assign pathogenicity criteria, and the phenotypes are listed in Supplementary Tables S1 and S2. In summary, pathogenic deletions involving the whole gene or eliminating c.1 were found in both male and heterozygous female probands, with presentations ranging from typical FXS to nonsyndromic epilepsy. On the other hand, most deletions within the promoter/5'UTR remained variants of uncertain significance (VUS) if the CNV interpretation criteria were strictly applied. Four deletion alleles were also found in individuals who were tested for reasons other than the presence of clinical manifestations. Two were in heterozygous females ascertained through a population screening study of pregnant women [33]. Two were in individuals hemizygous for de novo 5'UTR deletions tested due to family history of FXS, a 19-month-old male with c.-156_-69del and no neurological or physical findings, and a 10-year-old female with c.-196_-40del in trans with a large deletion including **FMR1** but no typical phenotype for FXS (only features were low birthweight, early thelarche, hearing loss, perinatal asphyxia with 6-week ICU stay) [34,35].

Proximal breakpoints were generally more frequent near the **FMR1** transcription start site/exon 1, rather than being evenly distributed along the length of the intergenic region upstream of **FMR1**. Breakpoints clustered near a previously described chi-like sequence as well as within long and short interspersed nuclear elements (LINEs, SINEs) and at regions of a few base pairs of microhomology (Table 1) [18].

Deletions were observed to occur on both full mutation and normal-sized CGG repeat alleles. Among eight de novo constitutional deletions for which maternal CGG repeat status was known, three originated from normal-sized alleles (one confirmed by haplotype to be from a 19-repeat allele), three originated from full mutation alleles by haplotype analysis, and two occurred in probands whose mothers were full mutation heterozygotes. Inheritance of deletion alleles from heterozygous and mosaic mothers was also observed, including cases of maternal germline mosaicism with recurrence in subsequent pregnancies despite negative maternal testing [20,23].
3.2. Mosaicism for CNVs with CGG Repeat Expansions

Repeat length instability is well-established in patients with expanded alleles, with somatic mosaicism for premutation to full mutation-sized repeats as well as repeat contractions. However, repeat expansions were also associated with mosaicism for CNVs around the repeat region. We did not apply clinical classification criteria to variants co-occurring with repeat expansions since the majority of cells in these individuals carried known pathogenic full mutation expansions.

All such CNVs reported were deletions, mostly starting proximal to the CGG repeat region (Table 2). While several breakpoints were observed multiple times in unrelated individuals, there was no single recurrent location. Furthermore, while some breakpoints had a few bases of microhomology \([45-47]\), others were reported as lacking sequence features that might explain the breakpoint.
Table 2. Mosaic deletions extending outside the CGG repeat region in patients with FMR1 repeat expansions.

| Ref/Case # | Breakpoints a | c. | Sex | Expansion | % Deletion in Blood | Functional/Phenotypes b | Maternal CGG Repeats |
|------------|---------------|----|-----|-----------|---------------------|------------------------|----------------------|
| [48]       | 147911612     | 147912529 | upstream | c.51+299 | M | 300–350 | No FMRP (blood, hair roots) | ~100, normal |
| [45] #1513 | ~147911695    | ~147912736 | upstream | ~c.51+506 | M | FM | 3–4% mRNA | 99/105, 30 |
| [49]       | 147911883     | 147912368 | upstream | c.51+138 | M | FM | 20–30% | |
| [50] #12   | 147911908(ins(9)) | 147912135 | upstream | c.-45 | M | 250, 510, 710; 170–320 with deletion | 9% (with 22.5% unmethylated, 68.5% methylated) | 11% FMRP, 72% mRNA; Mullen early learning composite 90 |
| [51] #1    | ~147911938    | CGG repeat | ~c.-242 | 30 repeats | M | 300–500 | 17% | |
| [50] #11   | 147911941     | 147912291 | c.-239 | c.51+61 | M | 420, 470, 1040; 84 with deletion | 20% (with 13% unmethylated, 67% methylated) | 6% FMRP, 17% mRNA; IQ 66 |
| [52] #6    | ~147911945    | 147912267 | ~c.-235 | c.51+37 | M | FM | No FMRP (lymphocytes) | FM, normal |
| [50] #9    | 147911960(insTT) | 147912133 | c.-220 | c.-47 | M | 350, 510, 630; 1050; 150–500 with deletion | 13% (with 28% unmethylated, 59% methylated) | 32% FMRP, 64% mRNA; IQ 47, autism spectrum disorder |
| [53]       | ~147911964    | ~147912164 | ~c.-216 | ~c.-16 | M | 70, 463–846 | FXS + FXTAS 97% FMRP (blood) | PM/FM mosaic, normal |
| [52] #5    | ~147911966    | 147912125 ins(7) | ~c.-214 | ~c.-55 | M | FM | No FMRP (lymphocytes) | FM, normal |
| [46] #2    | 147911966     | 147912135 | c.-214 | c.-45 | M | FM | 5–10% | PM, normal |
| [47]       | 147911966     | 147920712 | c.-214 | c.51+8482 | M | ~300 | 90%; 85% fibroblasts (12/14) | FM, normal (affected) |
| [54]       | 147911976     | 147912202 | c.-204 | c.23 | M | 58–60, FM | 2% (23% PM, 75% FM) | |
| [46] #4    | 147911978     | 147912134 | c.-202 | c.-46 | M | FM | 5–10% | |
| [46] #3    | 147911978     | ~147912545 | c.-202 | ~c.51+315 | M | FM | 15% | |
| [55]       | 147911988     | 147912198 | c.-192 | c.19 | F | ~400/normal het | 21% (29% FM, 27% methylated normal, 23% unmethylated normal) | PM, normal |
Table 2. Cont.

| Ref./Case # | Breakpoints a | c. Breakpoints | Sex | Expansion | % Deletion in Blood | Functional/Phenotypes b | Maternal CGG Repeats |
|-------------|---------------|----------------|-----|-----------|---------------------|---------------------------|----------------------|
| [50] #10    | 147911990 (insCG) | 147912138 c.-190 c.-42 | M   | 450, 540, 620, 930, 1080; 118 with deletion | 4% (with 14% unmethylated, 82% methylated) | 5% FMRP, 14% mRNA; IQ 69 |
| [56]        | ~147911990 ~147912137 ~c.-190 ~c.-97 | M   | 170, 230, 450 | 15% (60% methylated FM, 25% unmethylated PM) | 18% FMRP (lymphocytes), 6% FMRP (hair roots), mild (IQ 81) | PM, normal |
| [46] #1, [57] #2, [58] | 147911998 | 147912288 c.-182 c.51+58 | M   | FM | 5–10% | Prader-Willi syndrome phenotype | PM, normal |
| [59]        | 147912010 147912111 c.-170 c.-69 | M   | 165, >200 | 53% (Southern; with 37% PM, 10% FM) | 22% WBCs are FMRP+ Milder FXS than non-mosaic brother | 109, normal |
| [60]        | 147912020 147912171 c.-160 c.-9 | M   | FM | | | |
| [45] #1629  | 147912023 147912115 c.-157 c.-65 | M   | FM | | PM, normal |
| [45] #1033  | 147912023 147912150 c.-157 c.-30 | M   | FM | | 3% mRNA | 99/106, 31 |
| [45] #1337  | 147912039 147912110 insCTGGG c.-141 c.-70 | M   | FM | | 16–19% mRNA | 79, 30 |
| [45] #1234  | 147912044 147912136 c.-136 c.-44 | M   | FM | | 4–6% mRNA | 174/>200, 36 |
| [61]        | ~147912060 147912140 ~c.-120 (4th CGG) c.-40 | M   | FM | | 20–30% | 28% lymphocytes are FMRP+ Typical FXS |
| [52] #7     | >147909424 (RFLP) <147912612 (RFLP) upstream <c.51+382 | M   | FM | | No FMRP (lymphoblastoid cell line) | PM, normal |
| [62]        | >147911959 (primer) <147912181 (primer) >c.-221 <c.2 | M   | FM | | |
| [52] #8     | ? (RFLP) ? (RFLP) entire repeat | M   | FM | | FM, normal |

* Breakpoints listed as approximate (~) were specified in relation to other sequence landmarks by the authors. b As % of wildtype levels, by quantitative reverse transcriptase PCR (qRT-PCR) for mRNA. #: case number of proband in report, if given; c.: coding DNA position; FM: full mutation; PM: premutation; specific repeat lengths shown if given in original publication.
3.3. Coding Region Variants

Missense, in-frame deletion, frameshift, and nonsense variants have all been reported in patients with neurological and/or physical features of FMR1-associated disease (Table 3, Supplementary Table S3). A total of 11 published variants from all of these categories had sufficient evidence to conclude pathogenicity. We reclassified one variant that was reported as pathogenic, as well as three others reported as possibly causing disease, as VUSs under strict application of variant interpretation criteria (Table 3). In particular, c.1550C>T (p.P517L) was reported as a pathogenic stop-gain variant (p.Q406*) in a man with seizures and unilateral cerebral white matter hyperintensity [63]. This variant produces a stop codon in an alternate C-terminus in a different reading frame from that of the canonical isoform, such that we would have interpreted it as a missense VUS rather than loss-of-function based on the updated recommendations for applying the PVS1 criterion [13].

Table 3. Missense, nonsense, and frameshift FMR1 variants reported as pathogenic or possibly disease-causing.

| Ref. | Location | Number Affected with Variant, Sex (M/F) | Other Variants in Patient | Inheritance in Probard | ACMG Criteria [11] | Conclusion |
|------|----------|----------------------------------------|---------------------------|------------------------|---------------------|------------|
| [64] | c.80C>A  | p.S27*                                  | Mat (affected)            | PS1 PS3 PM2 PP1        | PATH                |
| [65] | c.188_193del | p.(D63_E64del)                        | De novo confirmed         | PS2 PM2 PM4 PP3        | LPATH               |
| [66] | c.229delT | p.(C77Af*5)                             | Maternal confirmed        | PS1 PS2 PM2 BP5        | PATH                |
| [67] | #1       | p.(T125Lfs*35)                          | De novo                  | PS1 PS3 PM6           | PATH                |
| [37,68] | c.377T>C | p.(F126S)                               | De novo                  | PS2 PM6 PP3           | LPATH               |
| [69,70] | c.413G>A | p.R138Q                                | Maternal (affected, learning disability/anxiety) | PS3 PP1 PP4; note 7 hemizygotes in gnomAD v3 exomes (rs200163413) | LPATH               |
| [71] | c.413G>A | p.R138Q                                | (1st cousin consanguinity) | Maternal               |                     |
| [72] | c.413G>A | p.R138Q                                | N/A                      |                        |                     |
| [73] | c.413G>A | p.R138Q                                | 1F                       |                        |                     |
| [74] | c.797G>A | p.(G266E)                               | Maternal (unaffected)     | PS3 PM2 PP3           | LPATH               |
| [75,76] | c.911T>A | p.(I304N)                               | Known PHKA2 deficiency (glycogen storage disease IXa1) | PS2 PS3 PM2 PP3       | PATH                |
| [77] | #1       | c.1021–1028delinsTATTGG_p.N341Yfs*7    | 2M                       | Mother not tested; had epilepsy | PS1 PS3 PM1        | PATH                |
| [78] | c.1325G>A | p.(R442Q)                              | 11 kb paternal deletion of part of MYH7 | Maternal (affected)     | PS3 PM2 PP1 PP3    | LPATH               |
| [79] | c.1444G>A | p.(G482S)                              | 1M                       | PM2 BP4                | VUS                 |
Table 3. Cont.

| Ref. | Location | Number Affected with Variant, Sex (M/F) | Other Variants in Patient | Inheritance in Proband | ACMG Criteria [11] | Conclusion |
|------|----------|----------------------------------------|---------------------------|------------------------|-------------------|------------|
| [63] | c.1550C>T | 1M                                     | ?                         | PM2                    | VUS               |            |
| [79] | c.1601G>A | 2M (unrelated), 1F                     | Maternal (unaffected) in both families | PM2 | VUS | |
| [80] | c.1610dup | 1M                                     | ?                         | PVS1 PS3 PM2           | PATH             |            |
| [81] TNX351 | c.1637G>A | 1M                                     | FMR1 c.990+14C>T          | ?                       | PM2              | VUS        |

* New BglII restriction site. b All references in literature are to a variant identified in the DDD study that was not listed in that publication but corresponds to DECIPHER patient #259197. c Reported as NM_001185081.1:c.1216C>T (p.(Q406*)) on an alternate reading frame from the reference transcript. #: case number of proband in report, if given; M: male; F: female; N/A: not applicable; ?: unclear; LPATH: likely pathogenic; PATH: pathogenic.

An expanded analysis of all published variants with the clinical evidence used to assign pathogenicity criteria, including variants from sequencing studies where pathogenicity was not assessed in the original publication, is in Supplementary Table S3, with reported phenotypes in Supplementary Table S4. We also note that one unpublished likely pathogenic variant in ClinVar, #1098346, has informative criteria provided (c.866C>T, p.(P289L), de novo occurrence without reported confirmation of maternity/paternity).

Four pathogenic/likely pathogenic missense variants had functional data supporting pathogenicity, but the effects on FMRP function differed between variants. Nonsense and frameshift variants were observed in patients with neurological and physical features of FXS and absent FMR1 mRNA, presumably due to nonsense-mediated decay. Furthermore, one frameshift variant (c.1610dup) that did not completely eliminate FMR1 mRNA produced a truncated protein that was still nonfunctional; it inappropriately localized to the nucleus due to a nuclear localization signal in the frameshifted C-terminus and produced novel axonal abnormalities in Drosophila [80].

3.4. Noncoding Small Variants

Noncoding variants from the promoter to 3’UTR were reported with varying levels of evidence for disease association (Table 4). Many of the variants initially reported in association with disease are relatively common in genomes from gnomAD, suggesting that they are benign. For brevity, Table 4 shows only noncoding variants initially reported as possibly disease-causing. Supplementary Table S5 contains all published variants and clinical evidence for impact with phenotypes in Supplementary Table S6.
Table 4. Noncoding *FMR1* variants reported as pathogenic or possibly disease-causing.

| Ref. | Location | Number Affected with Variant, Sex (M/F) | Inheritance in Proband | Patient and/or Functional Data | ACMG Criteria | Conclusion |
|------|----------|----------------------------------------|------------------------|--------------------------------|---------------|------------|
| [69] | “c.-332G>C” (promoter) | 1M (male DD cohort) | Maternal (unaffected) | Reduced reporter expression to 5.9% of WT (10 hemizygotes in gnomAD, rs922007219) | BS1 BS2 PS3 | VUS |
| [69] | “c.-293T>C” (promoter) | 1M (male DD cohort) | Maternal (unaffected) | Reduced reporter expression to 29.2% of WT (7 hemizygotes in gnomAD, rs1222840333) | BS1 BS2 PS3 | VUS |
| [69] | c.-254A>G | 1M (male DD cohort) | Maternal (unaffected) | Reduced reporter expression to 36.2% of WT (8 hemizygotes in gnomAD, rs1217601043) | BS1 BS2 PS3 | VUS |
| [69] | CGG repeat 26 of 31 CGG>CCG | N/A (new EagI site) | Maternal (unaffected) | Normal % of FMRP+ lymphocytes, but statistically significant decrease to 76% normal FMRP level in lymphoblastoid cell line | None | VUS |
| [82] | c.18G>T | 2M (unrelated) | Maternal (unaffected) | Normal intron 1 splicing in lymphoblastoid cell line (1 patient), normal FMRP Western blot in lymphoblastoid cell line (1 patient) | BS1 BS2 BS3 | BEN |
| [83] | c.18G>T | 4/508 male ID/DD cohort | Maternal (unaffected) | No normal transcripts; 2 abnormal transcripts with skipped exon 2 and skipped exons 2–3; FMRP absent in lymphoblastoid cell line | PVS1 PS3 PP1 | PATH |
| [67] | c.52-1_52delinsTA | 2 abnormal transcripts | Maternal (affected) | No family history of DD (parental first cousin consanguinity) | PS2 PS3 PM2 | PATH |
| [37] | #3 | r.419_420ins420-7_420-1 (p.(M140Ifs*3)) | Maternal (unaffected, no XCI skewing) | De novo with maternity/paternity confirmed | PS3 PM2 | LPATH |
| [84,85] | c.801G>A (IVS8-1) | iso.810del (p.(S211_G267del)); exon 8 skipping | Maternal (unaffected) | No family history of DD (parental first cousin consanguinity) | PS2 PS3 PM2 | PATH |
| [86] | c.879A>C (IVS9-2) | p.(V293=); reported abnormal splicing intron 9 | Maternal (unaffected) | Inclusion of intron 9 sequence in 23/36 subclones from peripheral blood cDNA | BS2 | VUS |
| [27] | c.879A>C (IVS9-2) | p.(V293=); no splicing abnormality found | Maternal (unaffected) | No abnormal splice amplicons found; normal exon 9–10 junction in RT-PCR product sequence in blood; FMRP present in blood homogenate (1 hemizygote in gnomAD, rs782013865) | BS2 | VUS |
| Ref. | DNA Location | r/p. | Number Affected with Variant, Sex (M/F) | Inheritance in Proband | Patient and/or Functional Data | ACMG Criteria | Conclusion |
|------|--------------|------|---------------------------------------|------------------------|-----------------------------|---------------|------------|
| [77] | c.881-1G>T   | ? (exon 10 splice acceptor) | 1M (clinical suspicion) | Maternal (1.99 skewed XCI, unaffected) | | PVS1 PM2 | LPATH |
| [37] | c.990+1G>A   | r.881_990del (p.K295Nfs*11)) (exon 10 skipping) | 1M (ID cohort) | De novo | Exon 10 skipping in blood | PVS1 PS3 PM2 PM6 | PATH |
| [69] | c.990+4T>C   | ? (intron 10) | 1M (DD cohort) | | | | |
| [81] | c.990+14C>T  | r.881_990del (p.K295Nfs*11)) (exon 10 skipping) | 3 unrelated males (ID, FXS feature cohort) | | Exon 10 skipping on peripheral blood RT-PCR product sequencing in 2 probands (TN-183, TN-351) | |
| [87] | c.990+14C>T  | 81 control individuals | | Observed in many controls from general population | | |
| [79] | c.990+14C>T  | 45/508 in ID/DD cohort | | | | |
| [88] | c.990+14C>T  | 7M/4F among 88 patients with ASD | | Statistically significant (p = 0.0123) higher frequency in ASD patients vs. controls | BA1 BS2 | BEN |
| [89] | c.990+14C>T  | | | Stably inherited in unaffected family members | | |
| [27] | c.*312_313dupT | 1 (ID, 1 FXS feature cohort) | | | | |
| [69,90] | c.*746T>C     | 2M (proband and half-brother from DD cohort) | | Faster mRNA decay with FMRP level 80% of normal in lymphoblastoid cell line; variant sufficient and necessary to decrease reporter gene expression in vitro; loss of metabotropic glutamate receptor-stimulated upregulation of reporter expression in transfected mouse neurons (72 hemizygotes in gnomAD, rs183130956) | PS3 PP1 BS1 BS2 | VUS |

| c.: coding DNA position; r.: RNA position; p.: protein position; ASD: autism spectrum disorder; ID: intellectual disability; DD: developmental delay; N/A: not applicable; M: male; F: female; AF: allele frequency; WT: wildtype; BEN: benign. |
There were no promoter or UTR variants with definite pathogenicity. Five pathogenic splicing variants were reported, including three at canonical splice sites, one at the end of exon 8, and one activating a cryptic splice site in intron 5. However, two variants initially thought to have pathogenic splicing effects, c.879A>C and c.990+14C>T, now have conflicting evidence (Table 4).

No variants have been reported to cause disease through effects on alternatively spliced transcript isoforms. One alternatively spliced transcript with the inclusion of a novel exon 9a, leading to a frameshift that escapes nonsense-mediated decay, was found in the course of evaluation of a patient with an FXS-like phenotype but is also present in multiple control individuals [91].

4. Discussion

4.1. Clinical Presentations of Pathogenic Non-Repeat Expansion Variants

Deletion, missense, nonsense, frameshift, and splice variants were all identified as pathogenic in affected individuals. Figure 1 shows their locations in relation to functional domains of FMRP [92–95]. Consistent with the mechanism of pathogenesis, variants expected to completely eliminate FMRP production (whole-gene deletions, nonsense, frameshift, and frameshifting splice variants) resulted in similar phenotypes to full mutation alleles. They were associated with neurological and at least some physical features of FXS in males, while affected heterozygous females had primarily neurological involvement, which was generally milder than in their sons, and some were ascertained due to these sons rather than their own phenotypes. Patients with pathogenic missense variants, both those impairing FMRP’s canonical role in translational regulation and those affecting other protein functions and promoter-region deletions, were also reported to have some physical features of FXS (Supplementary Tables S1 and S2). However, this may be biased by several studies’ using the presence of FXS characteristics as a recruitment criterion, as well as the greater likelihood of testing FMR1 in a patient with clinical features suggestive of FXS.

The inclusion of FMR1 sequence analysis in gene panels and exome slices (exome-based tests that only analyze a selected group of genes known to be associated with a particular phenotype) for less specific indications such as ID and ASD might broaden the range of missense and deletion phenotypes as more patients are reported.

While the loss-of-function variants were fully penetrant in males, the allele frequency of the R138Q missense variant suggests incomplete penetrance. Seven hemizygous males with this variant are present in gnomAD v.3.1.1, with an allele frequency among ethnic groups of up to ~1/1300 (Latino). On the other hand, the pathogenicity of the R138Q variant is well supported by functional studies, including a knock-in mouse model [70,72]. Three other pathogenic missense variants (G266E, I304N, R442Q) are absent in the general population. This raises the possibility that the R138Q phenotype is more amenable to modification by other genetic or environmental factors due to its different mechanism of pathogenicity.

The R138Q variant interferes with presynaptic functions of FMRP rather than its role in translational regulation at polyribosomes [70]. In contrast, the other three characterized variants interfere with FMRP’s role in translational regulation at polyribosomes: the G266E and I304N variants impair binding to polyribosomes and negative regulation of local protein synthesis (measurable as excessive AMPA receptor internalization in response to metabotropic glutamate receptor 5 signaling), while the R442Q variant protein inappropriately localizes to the nucleus [74,76,78,96]. These effects on function are consistent with the location of G266 and I304 within K-homology RNA-binding domains and of R442 in the nuclear export signal. In summary, all five of the pathogenic missense variants involve conserved residues within established functional domains. The identical amino acid is present in both human autosomal paralogs of FMRP (FXR1P and FXR2P) and is highly conserved among different species (Figure 1B).

Our analysis of deletion-related phenotypes (Table 1) was limited to deletions with breakpoints in or near FMR1. In clinical practice, microdeletions often extend into adjacent
genes that also cause intellectual disability, especially AFF2 located 550 kb downstream and IDS (mucopolysaccharidosis II) 1.5 Mb downstream. These larger deletions can present with additional clinical features related to the neighboring genes, as in proximal deletions including SOX3 [97] and distal deletions including IDS [98–107].

Small duplications of the entire FMR1 gene were also reported in possible association with disease in four patients where the duplicated regions only included FMR1 and a gene of no known function, FMR1NB. Of these, two were males ascertained due to seizures, but clinical information was only available for one with myoclonic and absence epilepsy, who also had speech and motor delay, behavior problems, and fifth finger clinodactyly. Two heterozygous females had developmental delay and other syndromic features. Larger duplications have been reported in patients with different syndromic features that were hypothesized to be from other genes in the duplicated region [108,109].

Figure 1. (A) Pathogenic and likely pathogenic small variants in FMR1 in relation to exons and protein functional domains. The FMR1 coding sequence (c.1-1899) is shown to scale with functional domain locations from the work of [95]. At the top, genetic variation in the general population is graphed with the x-axis showing c. locations and the y-axis showing the total minor allele frequency of missense and canonical splice variants in gnomAD (with higher frequencies extending farther downward, with several common variants clipped to a maximum frequency of $5 \times 10^{-5}$.) Below, protein domains and exons 1–17 (dark and light boxes) are shown with the 11 coding variants above and 5 splice variants below the exon schematic. (B) Protein sequence conservation of the 5 residues involved in pathogenic missense variation. For each location, 11 aa of FMRP sequence around the residue is shown aligned to the paralogous FXR1P and FXR2P sequences. The FMRP sequence is color-colored based on evolutionary conservation from 1 (variable) to 9 using Consurf.
4.2. Noncoding Variants Can Cause Disease, but New Variants Require Functional Confirmation

Pathogenic noncoding variants are a mechanism of FMR1-related disease, with five pathogenic splice variants described (Table 4). However, conflicting evidence for two variants reported as pathogenic indicates a need for caution and the use of multiple functional studies in interpreting new putative splice variants. The c.879A>C (IVS9-2, p.(V293=)) and c.990+14C>T variants were initially reported to be pathogenic due to detection of abnormally spliced transcripts, respectively by cDNA subcloning and by RT-PCR product sequencing [81,86]. Subsequently, another patient with the c.879A>C had no splicing or protein abnormalities, both variants were found in hemizygotes in gnomAD, and the c.990+14C>T variant turned out to be a common polymorphism in the general population, excluding its pathogenicity [27,89]. Protein analysis may thus be needed to determine whether any given splice variant truly alters FMRP in the patient being evaluated.

Interestingly, three promoter variants identified in a male DD sequencing cohort impaired transcription in a reporter gene expression assay yet are present in multiple hemizygotes in gnomAD [69]. Similarly, one 3' UTR variant (c.*746T>C) found in affected half-brothers had clear negative effects on expression in multiple functional studies but is common in gnomAD with 72 hemizygotes [90]. This suggests that decreased transcription and translation of FMR1 may not be fully penetrant or that the range of phenotypic expression includes mild effects on learning and behavior, perhaps analogous to the males with unmethylated full mutation alleles who have very mild phenotypes.

Several additional variants in the promoter region were identified due to alteration of restriction sites on Southern/RFLP testing or disruption of primer binding sites interfering with PCR assays but could be classified as benign based on functional data and/or population frequencies. Benign deletions and duplications in the 5'UTR/promoter region also occurred [34,35,40].

4.3. Rearrangement Breakpoints around the CGG Repeat

Expanded CGG repeats are known to be mutagenic, and deletion breakpoints in humans have previously been noted to cluster around the CGG repeat [8,9,18]. Consistent with this, many deletion events extending outside the repeat region were described, both de novo constitutional deletions and somatic mosaicism in patients with repeat expansion alleles. Besides their potential to affect FMR1 expression and cause or modify FXS-related phenotypes, these deletions can impact routine laboratory testing for CGG repeat expansion in FMR1.

Like the small variants discussed above, deletions overlapping PCR amplicons or restriction sites are rare potential causes of allele misclassifications. Primer locations for PCR may vary between testing laboratories and are generally selected to avoid deletion hotspots as per the most recent ACMG technical standard [4]. For instance, the primer sets cited as examples in the above standard bind to the reference sequence at c.-165_-145/c.-15_+8 (with two mismatches in the forward primer used in one study) [110,111], c.-165_-145/c.-64_-44 [111], c.-250_-221/c.+2_29 [112], and c.-252_-235/c.-20_+6 [113,114]. Any of the whole-gene deletions or other large deletions overlapping at least one primer binding site (Table 1; Supplementary Tables S1 and S5) would produce a failure of amplification. On the other hand, smaller deletions overlapping these primer binding sites are also known to occur and are not necessarily pathogenic, for example, c.-4_+1delGAAGA (Supplementary Table S5), which was detected due to amplification failure with one set of primers and found in a male proband with normal FMRP levels [115]. Deletions wholly within the PCR amplicon could theoretically lead to inaccurate calculated CGG repeat sizes. For example, since full mutations can fail to amplify due to the length of the repeat, some individuals with mosaicism for full mutation and deletion show only the small PCR product originating from the deleted allele [45].

Deletions including part of an expanded CGG repeat can reduce the size of the repeat, restoring expression of the gene, and normal FMR1 expression can be observed even
if some of the adjacent 5′ UTR sequences outside the repeat is deleted [35]. However,
the fact that several of the de novo constitutional deletions occurred on alleles without
repeat expansions suggests that the CGG repeat may be unstable even at normal sizes.
Such deletions, if limited to the 5′ UTR or wholly within the CGG repeat, can reduce the
number of AGG interspersions in the repeat without disrupting FMR1 function, producing
alleles that are not associated with disease but have a higher probability of expansion [7].
Conversely, since AGG interspersion reduces the chance of repeat expansion, it would be
interesting to test whether it also inhibits de novo deletion events, i.e., whether deletions
are associated with fewer AGGs in maternal alleles. This information was not available
in many of the reported cases but could be collected in the future as more laboratories
perform CGG repeat tests that include the determination of AGGs.

Mosaicism is increasingly appreciated as a factor in the occurrence and variability of ge-
netic disease, with mosaic pathogenic variants found in up to 8% of affected probands with
other X-linked neurodevelopmental disorders such as CDKL5 and PCDH19 epilepsy [116].
Besides mosaic FMR1 variants in individuals without repeat expansions, mosaic deletions
occur in patients with full mutations but are of uncertain clinical importance due to the
small number of characterized patients and presence of additional mosaicism for premu-
tations. A few patients had discordant phenotypes from non-mosaic siblings. One had
the Prader-Willi syndrome-like presentation, while his non-mosaic brother had classic
FXS [29,46,57]. Two other patients with mosaicism for deletion, premutation, and full
mutation alleles had milder FXS and higher cognitive functioning than expected [56,59].
These individuals had 18–22% of wild-type FMRP expression in blood. Another patient
with mosaicism for a deletion and full mutation allele, without a reported premutation
allele, had typical FXS despite FMRP staining in 28% of lymphocytes [61]. Therefore,
although constitutional deletions of the CGG repeat can be functional alleles as discussed
above, it is currently difficult to separate any contribution of deletion alleles from that of
unmethylated premutation alleles in mosaic full mutation patients. Furthermore, many such
mosaic deletions may be undetected due to technical factors such as occurrence outside
PCR amplicons used for testing. Using a relatively large 557 bp amplicon, Gonçalves et al.
reported relatively frequent detection of mosaic deletions in the presence of full mutations
(2.02% of a cohort referred for FXS testing, vs. 8.09% found to have full mutation only) [45].

Two large studies indicate the approximate frequency of deletion alleles. A multicenter
study on 1105 families (5062 unique individuals tested) with members diagnosed with FXS
by laboratories in Spain identified three deletion alleles [117]. In the general population,
two deletion alleles were identified in a carrier screening study of 20,188 asymptomatic
pregnant women in Taiwan [33]. One woman was heterozygous for a deletion of the 5′UTR;
the other was heterozygous for a full mutation allele, but testing of her male fetus showed
an exon 1 deletion. The screening test used was CGG repeat-primed PCR, which would
only be expected to observe deletions close enough to exon 1 to be within the PCR product.
Therefore, the frequency of deletion alleles in the general population might be higher.

4.4. Detection of FMR1 Variants Other than Repeat Expansions

While most patients with FXS are expected to have full mutation size CGG repeat
expansion in the 5′ UTR of FMR1, a negative CGG repeat expansion on targeted testing
does not completely exclude the diagnosis of FXS [65,71]. In individuals with features
suggestive of FXS and no pathogenic CGG repeat expansion identified, testing methods
investigating FMR1 sequence variants are recommended. The potential for genomic se-
quencing to simultaneously interrogate FMR1 together with several other genes in the
same assay makes this strategy an ideal follow-up testing approach in these patients [77].
Clinical genomic sequencing can either be targeted with the use of multi-gene panel test-
ing or more comprehensive with exome sequencing (ES) or genome sequencing (GS).
Custom multi-gene panels, including FMR1 and other genes of interest with integrated
deletion/duplication analysis, can allow for effective identification of coding and targeted
noncoding single nucleotide variants (SNVs) and intragenic CNVs. ES or GS, on the other
hand, can help identify coding and potentially deep intronic (applicable to GS) \textit{FMR1} sequence variants \cite{77}. While applications of long-read next-generation sequencing (NGS) technologies have shown great promise in reliably detecting structural variations, non-diagnostic short read-based ES or GS may require follow-up testing methods such as exome array to screen for large exon-level or whole-gene CNVs not reliably detected by current clinical genomic sequencing methods \cite{118,119}.

Finally, the architecture of \textit{FMR1} commends special considerations when analyzing and interpreting the results of genomic sequencing. First, both normal-sized and expanded CGG repeat alleles tend to be unstable, with many deletion breakpoints clustering around the repeat region. Second, short read NGS technologies and single-end sequencing methods face the issues of poor sequencing or sequencing bias across GC-rich genomic regions and inadequate mapping of tandem repeat sequences \cite{118}, all the things that currently make it challenging not only to detect and size CGG repeat expansion but also to identify potential sequence variants within or around the repeat region. These factors can be mitigated for NGS tests through higher coverage and the use of bioinformatic tools capable of analyzing in-repeat reads, allowing information to be obtained about expansions longer than the read length \cite{120,121}.

\textbf{Supplementary Materials:} The following are available online at https://www.mdpi.com/article/10.3390/genes12111669/s1, Table S1: Information supporting pathogenicity criteria for CNVs in Table 1; Table S2: Phenotypes of individuals reported with \textit{FMR1} CNVs; Table S3: All published coding region small variants; Table S4: Phenotypes of individuals reported with \textit{FMR1} coding variants; Table S5: All published noncoding small variants; Table S6: Phenotypes of individuals reported with \textit{FMR1} splice/noncoding variants.

\textbf{Author Contributions:} Conceptualization, S.T.Y. and C.T.-N.; methodology, S.T.Y.; investigation, S.T.Y., C.T.-N. and A.G.; resources, S.T.Y., C.T.-N. and A.G.; writing—original draft preparation, S.T.Y. and C.T.-N.; writing—review and editing, S.T.Y., C.T.-N., B.D.S. and A.G.; visualization, S.T.Y. and C.T.-N.; supervision, B.D.S. All authors have read and agreed to the published version of the manuscript.

\textbf{Funding:} C.T.-N., B.D.S., and S.T.Y. are supported by the NIH Intramural Research Program. This research received no external funding.

\textbf{Institutional Review Board Statement:} Not applicable.

\textbf{Informed Consent Statement:} Not applicable.

\textbf{Data Availability Statement:} The data presented in this study are available in Supplementary Tables S1–S6 of this article.

\textbf{Conflicts of Interest:} The authors declare no conflict of interest.

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