Further identification of a 140bp sequence from amid intron 9 of human *FMR1* gene as a new exon

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Abstract

The disease gene of fragile X syndrome, *FMR1* gene, encodes fragile X mental retardation protein (FMRP). The alternative splicing of *FMR1* can affect the structure and function of FMRP. However, the biological functions of alternatively spliced isoforms are remain elusive. In previous study, we identified a new cryptic exon of 140bp from the intron 9 of human *FMR1* gene. In this study, we further examined the biological functions of this new exon and its underlying signaling pathways. qRT-PCR results showed that this novel exon was commonly expressed in the peripheral blood of normal individuals. Comparative genomics showed that sequences paralogous to the 140bp sequence are only found in the genomes of primates. To explore the biological functions of the new transcript, recombinant eukaryotic expression vectors and lentiviral overexpression vectors were constructed. Results showed that the spliced transcript encoded a truncated protein which was expressed mainly in the cell nucleus. Additionally, a few genes including *BEX1* gene involved in mGluR-LTP or mGluR-LTD signaling pathways was significantly influenced when the truncated FMRP was overexpressed. Our work emphasized the notion that the alternative splicing of *FMR1* gene is complex which may in a large part account for the multiple functions of FMRP protein.

Introduction

Fragile X syndrome (FXS) is a common form of inherited intellectual disability, affecting 1/7000 females and 1/4000 males. Apart from the mild to severe mental retardation, individuals with FXS often have autism-like behaviors, including social anxiety, attention deficit, mood disturbance and sleep disorders. FXS patients also present other prominent physical symptoms, encompassing a long, narrow face with large protruding ears, flat feet, eye-gaze avoidance and macroorchilism. Pathogenically, when the trinucleotide CGG repeat in the 5' untranslated region of the fragile X mental retardation 1 gene (FMR1) expands to more than 200, DNA methylation and transcriptional silencing of FMR1 will be resulted, leading to the absence of FMR1 gene product, fragile X mental retardation protein (FMRP) [1].

In mammals, full-length FMR1 gene sequence encodes a 71kD FMRP protein. It has three motifs that mediate RNA or protein interaction, including two K homology domains (KH1 and KH2) encoded by exon 7–9 and exon 9–13, respectively, and the arginine-glycine-glycine (RGG) box encoded by exon 15–16. FMRP also contains a nuclear localization signal (NLS) and a nuclear export signal (NES). Additionally, lots of researches showed that FMRP has Agent domains at N terminus to combine with methylated H3K9 chromatin[2]. FMRP is also a selective mRNA-binding protein that regulates the process of RNA transcription, splicing and cell apoptosis[3]. It is estimated that FMRP can bind more than 5% mRNA in cells. Yeast three-hybrid assay and microarray have detected as many as 400 potential mRNAs that related to FMRP[4]. FMRP is widely expressed and especially abundant in brain, playing a critical role in synaptic plasticity and neurological signaling pathways as a translational repressor. Among them, mGluR-LTD is one of the most common pathways that can regulate the α-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid receptor (AMPAR) internalization and local protein synthesis in order to prevent deficits in synaptic plasticity[5]. Besides, mGluR-LTP, phosphorylation of FMRP, GABA and Dopamine receptors (DA) signaling pathways are all required for the formation of normal neurological function[6].

Mature transcripts from FMR1 gene have multiple alternatively spliced isoforms in different organs. The most common ways of alternative splicing are the inclusion or exclusion of exons 12 and 14, and the selection of splice acceptor sites at exons 15 and 17 [7]. It has been reported that FMR1 gene can produce more than 20 FMRP isoforms with various structures and functions. Different FMRP isoforms may be involved in different signal transduction pathways, implying significant biological functions. With the incidence of 50–75% of alternatively spliced genes in central nervous system, they have important influence on synaptic plasticity[8], ion channel activity[9], the genesis of dendritic spines[10] and the release of neurotransmitters[11]. FMR1 is also an example of key genes that produces various spliced transcripts in human brain. However, the exact number of alternatively spliced isoforms in various tissues and cells and the specific biological functions of them are still poorly understood.

Previously, we and others reported a novel exon of 140 bp when detected alternatively spliced transcripts from FMR1 gene[12, 13]. This 140 bp sequence comes from intron 9 of human FMR1 gene and introduces a premature stop codon in the target mRNAs. Bioinformatics analysis shows that the novel sequence has the canonical splicing signals, implying that it has the potentiality to be an alternative exon. Furthermore, qRT-PCR shows the cryptic exon can be detected in mature mRNA molecules of from the peripheral blood of non-FXS individuals. Using eukaryotic expression vector and lentiviral vector for truncated FMRP analysis, we find that the inclusion of the novel 140 bp sequence leads to a truncated protein with altered subcellular distribution. RNA microarray analysis on cells in which the truncated FMRP was overexpressed revealed a group of differentially expressed genes that might contribute to FMRP signaling pathways. Our work emphasizes the notion that the alternative splicing of FMR1 gene is much more complex than what we have realized and such complexity may in a large part account for the multiple functions of FMRP protein.

Materials And Methods

2.1. RNA isolation and cDNA synthesis Materials

Peripheral blood samples were obtained from the Department of Laboratory Medicine of 900th Hospital of the Joint Logistics Force. RNA was extracted from the peripheral blood of fifty two non-FXS individuals using RNA isolation kit (Qiagen, Hilden, Germany), following the manufacturer's protocol and stored at -80°C for subsequent use. The quality of RNA was evaluated by Biophotometer (Eppendorf, Hamburg, Germany). Synthesis of the cDNA was carried out following the instructions of the kit manufacturer (Toyobo, Osaka, Japan).

2.2. Real-time reverse transcriptase PCR

qRT-PCR reactions were performed using iTaq SYBR Green Kits, and we followed the 3-step cycles from the manufacturer's protocol (Toyobo, Osaka, Japan). All reactions were carried out in triplicates and the cycles were run on Bio-Rad CFX96 real-time system (Bio-Rad, Hercules, CA, USA). The primers used were listed in Supplementary Tab. S1.

2.3. Bioinformatics

Four softwares were used to evaluate the splicing signals in our study, including ASD (http://www.ebi.ac.uk/asd), HSF (http://www.umd.be/HSF/), BDGP (http://www.fruitfly.org/seq_tools/splice.html), and ASPicDB (http://t.caspur.it/ASPicDB/index.php).

2.4. Western Blot

We used the 10% polyacrylamide gel to separate target proteins, which were extracted from cell lysates using lysis buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor mixture). The protein concentration was determined by bicinchoninic acid assay protein assay (Bio-Rad, Hercules, CA, USA). The proteins were transblotted onto polyvinylidene fluoride membrane (BioRad, Hercules, CA, USA), and the membrane was blocked with Tris-buffered saline (TBS) containing 5% non-fat milk for one hour, and incubated with a mouse monoclonal antibody anti-FMRP (Abcam, Cambridge, MA, USA) at 1:750 dilution for overnight incubation at 4℃. Next day, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Dallas, Texas, USA) at 1:5000 dilution for one hour. After washing for three times, electrogenerated chemiluminescence solution (ECL) (Beyotime, Shanghai, China) was added while exposing the film according to conventional procedures. For the detection of BEX1 protein, a rabbit monoclonal anti-BEX1 (Abcam, Cambridge, MA, USA) primary antibody at 1:1000 and a HRP-conjugated anti-rabbit secondary antibody were used.

2.5. Plasmids construction

HEK293T cells were obtained from Shanghai Cell Bank, Chinese Academy of Science. To identification the subcellular distribution of the end product encoded by the novel transcript containing the 140 bp sequence, full-length coding sequence of FMR1 or fragment containing exons 1–9 and the 140 bp sequence was inserted into the eukaryotic expression vector pEGFP-N2 (BD Biosciences, San Jose, CA, USA). Full-length coding sequence of FMR1 gene was amplified with primers wFMR1-F and wFMR1-R (wFMR1-F: 5'-AAAGAGCTCGATGGAGGAGCTGGTGGTGGA AG-3'; wFMR1-R:5'-ACGCGCGACCGGGTACTCCATTCACGAGTG-3';), while the fragment containing exons 1–9 and the 140 bp sequence was amplified with primers tFMR1-F and tFMR1-R (tFMR1-F: 5'-AAAGAGCTCGATGGAGGAGCT GGTGGTGGAAG-3'; tFMR1-R:5'-GCGTCGACCGACTTCAACCCTACTAAGT TCCTTGGA-3'). All primers contained the restrictive enzyme sites Sac□and Sal□, for convenience of subcloning. For construction of the lentiviral overexpression vector, the target coding fragment was amplified using specific primers tFMR1-PF and tFMR1-PR with Not□and Mlu□sites, respectively (tFMR1-PF: 5'-AAAT ATGCGGCCGCATGGAGGAGCTGGTGGTGGAA-3'; tFMR1-PR: 5'-GCGACGC GTTCAGATCTTCAACCCTACTA A-3'). After the amplified product was linked to the lentiviral vector pLEX-MCS, the packaging plasmids

pMD2.G and psPAX2 (Open Biosystems, Huntsville, USA) were used to co-transfect HEK293T cells using polyethylenimine (PEI) reagent (Sigma, St. Louis, MO, USA).

2.6. Cell culture and Transfection

HEK293T cells were cultured in DMEM medium with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). HEK293T cells were transfected with the recombinant eukaryotic vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Three days later, cells were observed and RNA/protein extracted for subsequent use. As for the recombinant lentiviral vector, transfection of HEK293T cells followed the recommended protocol (Sigma, St. Louis, MO, USA) by use of polyethylenimine (PEI) reagent. The virus supernatant of cells was collected per 24 h for 3 days and then was used to infect a new batch of HEK293T cells. The newly infected HEK293T cells were cultured in DMEM/F12 (1:1) medium with puromycin for about 15 days. Finally, stably transfected HEK293T cells were harvested.

2.7. Immunofluoresence

The subcellular distribution of proteins was examined by immunofluresence staining. Specifically, Cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 for 20 min, and incubated with 3% BSA for an hour. Then cells were stained with the primary antibodies of interest, such as anti-FMRP and anti-BEX1 (Abcam, Cambridge, MA, USA), for overnight at 4℃. Next day, after washing for three times with Phosphate-buffered saline (PBS), cells were incubated with secondary antibodies Alexa Fluor® 594-conjugated goat anti-mouse IgG or goat anti-rabbit IgG for two hours at room temperature (Santa Cruz, Dallas, Texas, USA). Also, the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) dye for 5 min (Beyotime, Shanghai, China). Finally, we observed cells under FV1000 laser-scanning confocal microscope (Olympus, Tokyo, Japan).

2.8. RNA microarray analysis

Total RNA was extracted from two batches of stably transfected HEK293T cells, including HEK293T cells transfected by void lentiviral vector (pLEX-MCS) and those by pLEX-MCS-tFMR1 vector. After the evaluation of RNA quality according to manufacturer's protocol, RNA microarray hybridization was performed at Capital Bio Company (Beijing, China) and Affymetrix Human Genome U133 Plus 2.0 was used for analysis (Affymetrix, Santa Clara, CA, USA). For the functional analysis of the differentially expressed genes, DAVID (http://david.abcc.ncifcrf.gv/) database was also used.

Results

3.1. FMR1 mRNA containing the 140 bp sequence exists universally in the peripheral blood of normal non-FXS individuals

To determine if this alternative exon is commonly expressed in healthy individuals, cDNA was synthesized from the total mRNAs of the peripheral blood of 52 normal non-FXS individuals and real-time reverse transcriptase PCR (qRT-PCR) was carried out. We designed specific primers aiming at the 140 bp sequence to test the RNA expression of this novel alternative exon (Supplementary Tab. S1). The results showed that the 140 bp sequence could be detected in the mRNA molecules of peripheral blood cells of all non-FXS controls. The lowest copy number of the 140 bp sequence was about less 19 fold than that of the internal reference (GAPDH), the median almost less 15 fold and the highest about less 7 fold (Fig. 1). This results indicated that FMR1 mRNA containing the 140 bp sequence exists ubiquitously in the peripheral blood of normal healthy individuals.

3.2. Comparative genomics shows that sequences paralogous to the 140 bp sequence are only found in the genomes of primates

Given that the common expression of the 140 bp sequence was found in normal individuals, we further search the genomes of other species for sequence similar to this new spliced variant. Genomes of FMR1 gene from diverse species were obtained and submitted to DANMAN software. The results showed that Primates, such as Homo sapiens (GenBank: NC_000023), Macaca mulatta (GenBank: NC_012614), Pan troglodytes (GenBank: NC_006491) and Pongo abelii (GenBank: NC_007878), all had paralogues of the 140 bp sequence, although they distributed in different introns of FMR1 gene, for Homo sapiens and Macaca mulatta in intron 9 and for Pan troglodytes and Pongo abelii in intron 10 (Supplementary Fig. S1). However, the 140 bp sequence can not be found in non-Primates, implying that the 140 bp sequence might be associated with the development and evolution of intelligence.

3.3. FMR1 mRNA containing the 140 bp sequence can be translated into a truncated FMRP protein with altered cellular localization

The splicing of the novel exon with exon 9 at the 5’ end and exon 10 at the 3’ end results in FMR1 mRNAs can be translated into a truncated FMRP of 34 kDa, containing 297 amino acid residuals, while lacking a large part of the carboxy-terminal domains, including NES, the second KH domain and RGG box (Fig. 2A). To identify the potential truncated protein and detect the expression of this newly alternative FMR1 transcript, total proteins were extracted from peripheral blood cells of 6 normal non-FXS individuals and Western blot performed using a monoclonal antibody against the N-terminus of FMRP. The result showed that a protein band could be seen in all 6 normal individuals with a molecular weight of approximate 35 kDa, almost half length of that of the full-length FMRP protein (Fig. 2B).

To explore the subcellular localization of the end product from the new alternatively spliced FMR1 transcript, we constructed the recombinant eukaryotic expression vectors with full-length coding sequence (pEGFP-N2-fFMR1, full-length isoform) or coding fragment containing exons 1–9 together with sequence of the 140 bp (pEGFP-N2-tFMR1, truncated isoform), respectively. Western blot showed that HEK293T cells transfected by void plasmid, pEGFP-N2-fFMR1 and pEGFP-N2-tFMR1 all expressed the endogenous FMRP protein of 71 kDa. Besides, HEK293T cells transfected by pEGFP-N2-fFMR1 and pEGFP-N2-tFMR1 expressed FMRP or truncated FMRP fused with EGFP protein, with molecular weights of 90KD and 55 kDa, respectively (Fig. 3A). Immunofluorescence revealed that the full-length FMRP was expressed mainly in the cytoplasm, while the truncated protein mainly in the cell nucleus, demonstrating an altered subcellular localization (Fig. 3B).

3.4. FXS-related signal pathways are significantly influenced when the truncated FMRP is overexpressed

RNA microarray analysis revealed a total of 545 genes with altered expression (the top 20 up-regulated and down-regulated differentially expression genes are showed in Supplementary Tab. S2 and Tab. S3), among which, BEX1 (brain expressed X-linked 1) is the most significantly up-regulated gene and GABRB3 (gamma-aminobutyric acid type A receptor beta3 subunit) the most significantly down-regulated gene (Supplementary Fig. S3A&S3B). To verify the microarray results, 11 genes were selected to perform qPCR, which demonstrated that there were 9 genes (BEX1, MAGE, MAGEB2, PNPLA4, PPP1R1A, GABRB3, NAP1L3, NAP1L2, RGS-7) whose expression levels were altered to an extent similar to that of mRNA microarray analysis (Fig. 4A). Western blot showed that BEX1 gene was overexpressed in HEK293T cells stably transfected by pLEX-MCS-tFMR1 (Fig. 4B). Immunofluresence revealed that BEX1 gene was expressed both in the cytoplasm and cell nucleus, but it was mainly located in the cytoplasm of HEK293T cells, co-localized with the endogenous FMRP (Fig. 4C).

We performed an integrated bioinformatics analysis on 545 differentially expressed genes (DEGs). Ontological classification revealed that the DEGs mainly enriched in 3 categories including biological process (BP), cellular component (CC) and molecular function (MF). In the BP group, 28% proteins were concerned with metabolic process, and 19% with cellular process (19%), while the differentially expressed genes in the CC group were comprised of cell part (37%), organelle (25%) and membrane (16%). For the MF group, the differentially expressed genes were associated with binding (45%) and catalytic activity (31%) (Supplementary Fig. S2C). KEGG analysis demonstrated that the differentially expressed genes were mainly involved in 4 significant signaling pathways, i.e. cancer-related pathways, PI3K-Akt, influenza A and neuroactive ligand-receptor interaction signaling pathways.

Disscussion

About alternative splicing of FMR1 gene

It is estimated that almost 95% human genes would experience certain level of alternative splicing (AS) and contribute to proteome complexity[14]. AS can produce mRNAs that different in their untranslated regions or coding sequence. The mechanisms of AS mainly include exon skipping, intron intention, the use of alternative splice sites and the choice of mutually exclusive exons. The different spliced isoforms might influence mRNA localization, stability and translation. Moreover, some splicing mRNA variants could alter the reading frame and generate various protein isoforms with diverse localizations and functions. The most common place for AS is in the neural tissue, where various spliced transcripts may function as modulators to synaptic functions. Since the cloning of FMR1 gene as the disease gene of FXS in 1991, a large number of FMR1 mRNAs and FMRP isoforms derived from AS were detected in mouse and human[15]. The distribution of different FMRP isoforms in specific cellular roles and different tissues was also relatively well understood. It is reported that a high expression level of different FMR1 mRNAs was found in brain, testis, placenta and lymphocytes, while a lower level of expression in other organs[15]. Besides, there are at least 4 predicted FMRP isoforms identified in mouse brain, which demonstrates that the dissimilar isoforms of FMRP occur together in the same cell type or separately in distinct cell types[16]. At present, the 24 and more predicted mature transcripts were reported, mainly involving the inclusion or exclusion of exons 12 and 14, and the selection of splice acceptor sites at exons 15 and 17[17]. To our knowledge, our work represents the further study that a new cryptic exon from intron 9 of human FMR1 gene was identified.

About subcellular localization of FMRP

It is believed that the longest isoform of FMRP (Isoform 1) is predominantly cytoplasmic and mainly function as a mRNA-binding protein that can directly or indirectly interact with other proteins, regulating the stability of mRNA and maintaining the balance of shuttling between the cytoplasm and the nucleus. The nuclear localization signal (NLS) and nuclear export signal (NES) of FMRP were also associated with the cytoplasmic localization of FMRP. A patient with a novel R138Q mutation in the NLS had developmental delay[18]. The mechanism of this mutation is not very clear, but it may lead to the different distribution of proteins in cytoplasm and nucleus and may indicate the importance of the domain. Besides, the exon 14 of FMR1 gene were shown to encode a cytoplasmic retention domain. Exclusion of exon 14 altered the downstream reading frame, generated two different C-terminal regions, and finally showed a nuclear localization[7]. Furthermore, FMRP C-terminal is one of the determinant factors of nuclear localization and is the key domains that mediate the kinesin and dendrites transmission[19]. FMRP homologous proteins, FXR1 and FXR2, also shuttle between the nucleus and cytoplasm by producing multiple isoforms with different C-terminal[20]. Correspondingly, FMRP N-terminal is highly conserved in species[21]. Banerjee and his colleagues studied the functional difference of long isoforms and short isoforms FMRP of D. melanogaster, showing that the short isoforms, without the C-terminal region, can easily cause short-term or long-term learning and memories disorders[22].

FMRP can bind key protein cytoplasmic FMRP-interacting protein (CYFIP1), a downstream effector of Rac1 in the cytoplasm, remodeling the cytoskeleton and involving in the formation of the translational initiation complex[23]. However, Previous studies presented that FMRP binds its mRNA targets in the nucleus and facilitates the cargo of nuclear proteins, and the export of FMRP from the nucleus depended on mRNA synthesis. Kim and his colleagues knocked down the mRNA exporter Tap/NXF1, resulting in the increase of FMRP protein in nucleus[24]. It also has been proved that FMRP can combine proteins in nucleus, such as NUFIP1 and 82-FIP (FMR1 interacting protein 1), RISC (RNA-induced silencing complex), AGO2 and Dicer (argonaute 2), and eIF5 (eukaryotic translation initiation factor 5), which are the pivotal molecules that mediate translational repression by inhibiting the initiation of translation and causing polyribosomes stalling[25, 26]. Moreover, the combination process of FMRP and ribosomes was presented in the nucleus, being an important mechanism of translational regulation. FMRP has been regarded as chromatin-associated protein. It can coimmunoprecipitate with chromatin confirmed by Chip sequencing, as well as interacting with nucleolin, affecting the transcription of rRNA and the biosynthesis of ribosomes[27].

Due to the missing of nuclear export signal, our newly identified FMRP isoform with a short C-terminal retains in the nucleus. We speculate that the increasing of FMRP alternatively spliced isoforms in the nucleus could break the balance of shuttling between the nucleus and cytoplasm and then affect the translational repression of FMRP.

About new interactors of FMRP in the FXS-related signaling pathways

With the development of several high-throughput approaches, such as microarray analysis, HITS-CLIP and PAR-CLIP, researches revealed that FMRP can interact with about 5% mRNA targets in brain [4]. FMRP is a translational repressor involving in the regulation of synaptic functions via the activation of NMDA receptors, AMPA receptors and GABA receptors, which contribute to the formation of long-term depression (LTD) and long-term potentiation (LTP), according to mGluR theory[28]. It is widely believed that the regulation of neurological function mainly depends on the mGluR-LTD pathway, which mediates the synaptic plasticity and hinges on the local protein synthesis of dendrities. The mGluR theory of FXS emphasizes that FMRP is downstream of mGluRs and upstream of local protein synthesis. It has been suggested that FMRP represses the translation of dendritically localized mRNAs. With the activation of mGluR, FMRP repression would allow the synthesis of local protein in response to synaptic stimulation, resulting in the AMPAR internalization and LTD [28]. For patients with FXS, the absence of FMRP could constructively increase protein synthesis, leading to the over activation of AMPAR internalization and LTD exaggeration. Both of the extracellular signal–related kinase (ERK) and mammalian target of rapamycin (mTOR) signaling pathways are required for the regulation of mGluR-LTD [29, 30]. Studies have showed that antagonizing the mGluR pathway can alleviate the phenotypes of FXS [31]. Therefore, mGluR theory

provides new avenues for the understanding of pathological mechanisms and therapeutic intervention of FXS.

Interestingly, our findings of RNA microarray analysis revealed GABRB3 is the most significantly down-regulated gene and BEX1 the most significantly up-regulated gene. Altered expression of mRNA and protein for GABA receptors has been reported in FMR1 knockout mice, implying the loss of FMRP can affect GABA receptor subunits expression. Recent publications have also identified the absence of FMRP can cause to the upregulation of mGluR signaling, resulting in the lowered expression of GABRB3 protein, which was consistent with our RNA microarray results[32–34]. Therefore, we indicated that overexpression of truncated FMRP protein has profound effects on FMRP-mGluR-GABRB3 signaling pathway. Besides, it is tempting to speculate that BEX1 gene may participate in mGluR-LTD and mGluR-LTP signaling pathways. BEX1 gene was linked to neurotrophin signaling as a interactor of the Trk tyrosine kinases (TrkA, TrkB and TrkC) or p75 neurotrophin receptor (p75NTR), regulating differentiation, growth, and survival of neuronal and glial cells[35]. Trk receptors can be activated by several canonical pathways, including the phosphatidylinositol-3 kinase (PI3K)/AKT/mTOR and Ras/MAP kinase signaling pathways[36]. Additionally, TrkB can be involved in the LTP signaling pathways and mediate the synaptic plasticity[37]. However, the signaling mechanisms of p75NTR are still poorly understood. It has been reported p75NTR can change the functions of the amygdale[38] and contribute to multiple process of cell responses, such as apoptosis, survival, axonal growth and cell death. When BEX1 protein is overexpressed, it can inhibit the NF-κB activity by Trk receptors and p75NTR, without impacting activation of AKT and Erk1/2 signaling[35], which are critical molecules involved in mGluR-LTD signaling pathways. The high BEX1 level in the connection with the mGluR-LTP or mGluR-LTD signaling gives us a new insight into interactions of FMRP in the FXS-related signaling pathways.

In conclusion, our study identified a new cryptic exon from amid intron 9 of human FMR1 gene with widely expressed in normal healthy individuals. In particular, sequences similar to the new exon can be only found in the genomes of primates and the insertion of it can produce a truncated FMRP protein with altered cellular localization. Our preliminary data from RNA microarray analysis points to the possibility that BEX1 gene may be a new player in the FXS-related mGluR-LTP or mGluR-LTD signaling pathways, although the complicated molecular mechanisms of this new alternative exon-influenced roles of FMRP await for further clarification.

Accession Numbers

Insertion sequence of FMR1 gene is available from GenBank MF593118. Sequenced reads have been deposited in the NCBI Gene Expression Omnibus (GEO) database (accession number GSE101830).

Declarations

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Dedications

FL and WY conceived and designed the investigation. WY and JL carried out most of the experiments. XG and XF carried out bioinformatics analyses. YX, DL, AY and DZ performed some of the experiments. WY wrote the manuscript and FL revised it.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

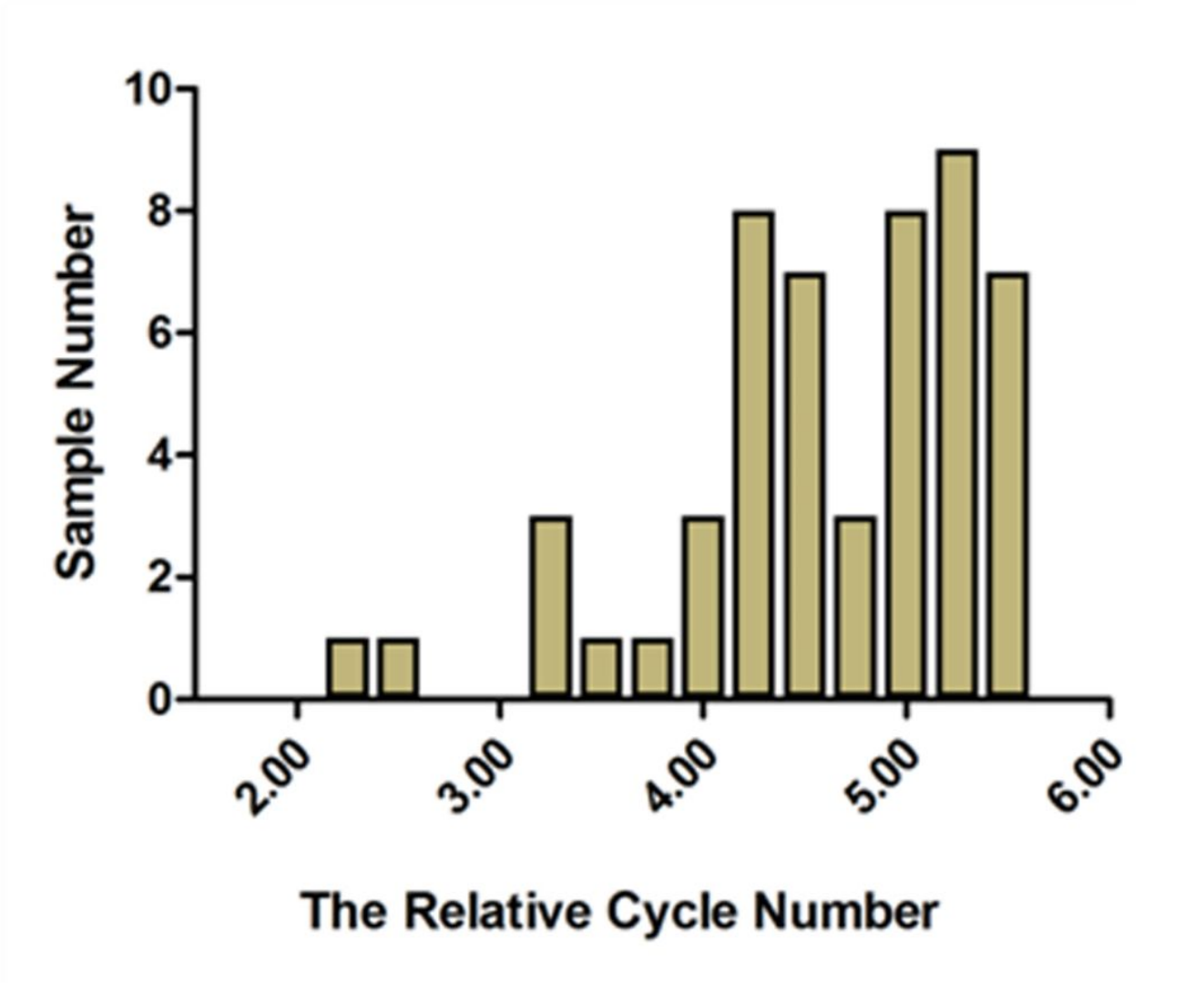

Figure 1

The new exon of 140bp is commonly expressed and distributed in the peripheral blood of normal non-FXS individuals. qRT-PCR results of mRNA expression level of the 140bp sequence in peripheral blood cells of 52 non-FXS controls.

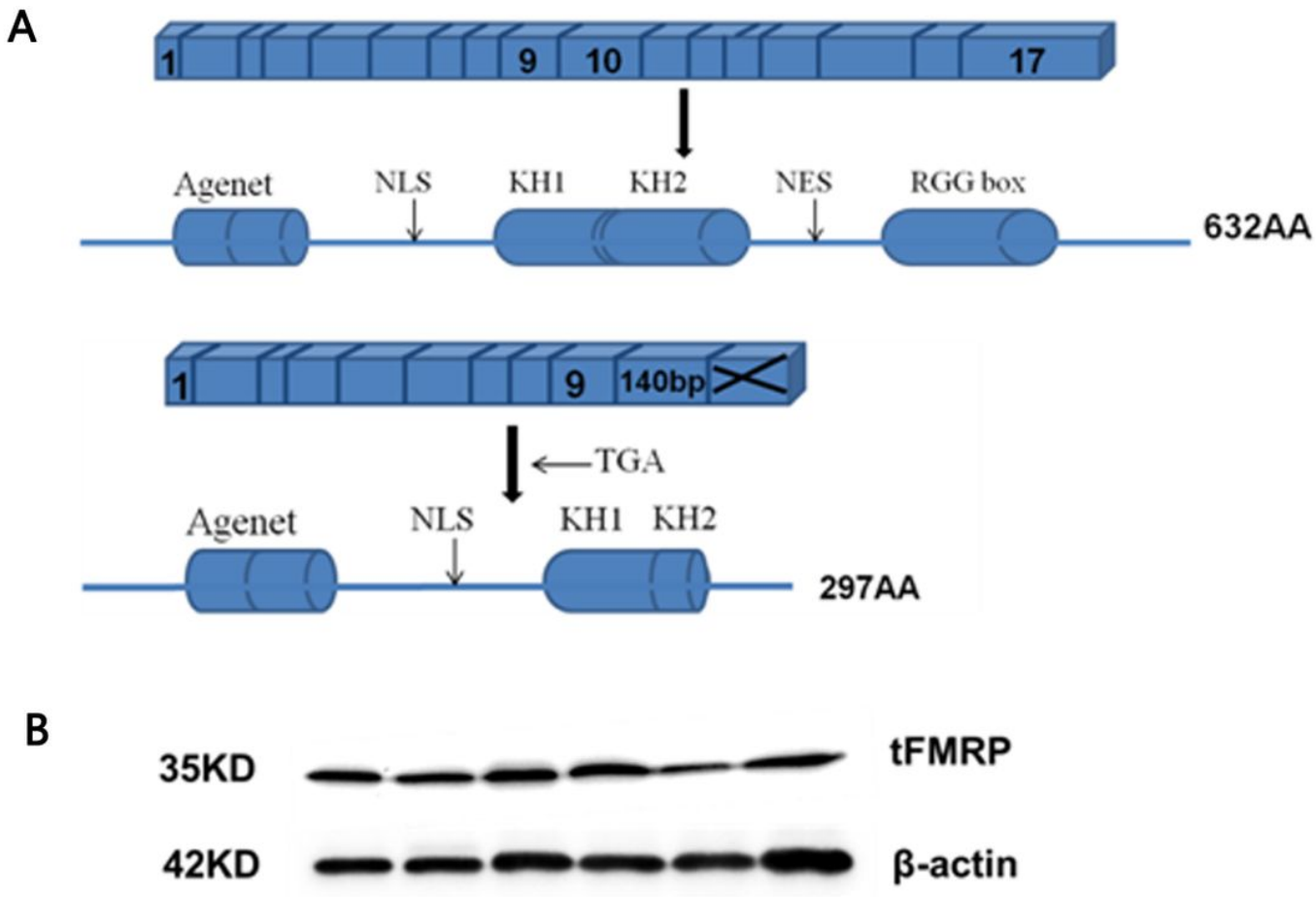

Figure 2

The structure and expression of truncated FMRP protein in healthy individuals. (A) FMRP structure with coding exons (squares) and functional domains (columns). a: FMR1 gene containing full-length sequence encodes 632 amino acids; b: coding fragment containing exons 1-9 and the 140bp sequence. (B) Western blots showing the expression of the new alternatively spliced FMR1 transcript in peripheral blood cells of 6 healthy individuals. tFMRP: truncated FMRP.

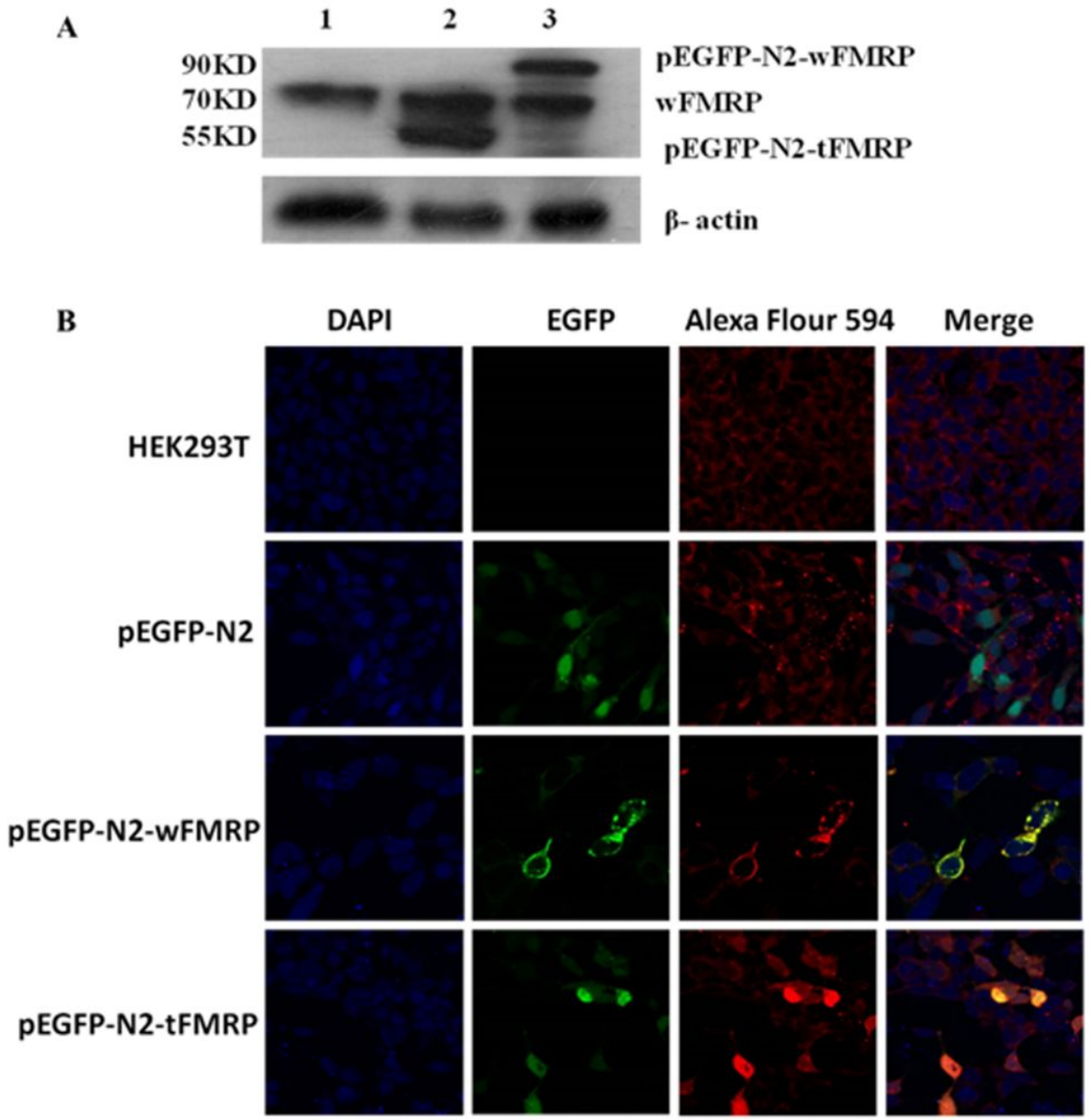

Figure 3

The new spliced transcript containing the 140bp sequence is translated into a truncated FMRP protein with altered subcellular localization. (A) Western blots showing the expression of the wild type FMRP or the truncated FMRP in HEK293T cells. 1: HEK293T cells without transfection; 2: HEK293T cells transfected by pEGFP-N2-tFMR1; 3: HEK293T cells transfected by pEGFP-N2-fFMR1. (B) Immunofluoresence showing the localization of the wild type FMRP or the truncated FMRP in HEK293T cells. HEK293T: HEK293T cells without transfection; pEGFP-N2: HEK293T cells transfected by the void plasmid; pEGFP-N2-tFMR1: HEK293T cells transfected by pEGFP-N2-tFMR1; pEGFP-N2-fFMR1: HEK293T cells transfected by pEGFP-N2-fFMR1; DAPI: the solution suitable for nuclear staining; EGFP: vectors encoding the GFP-tagged protein; Alexa Flour 594: a FMRP antibody fluorescently labeled red.

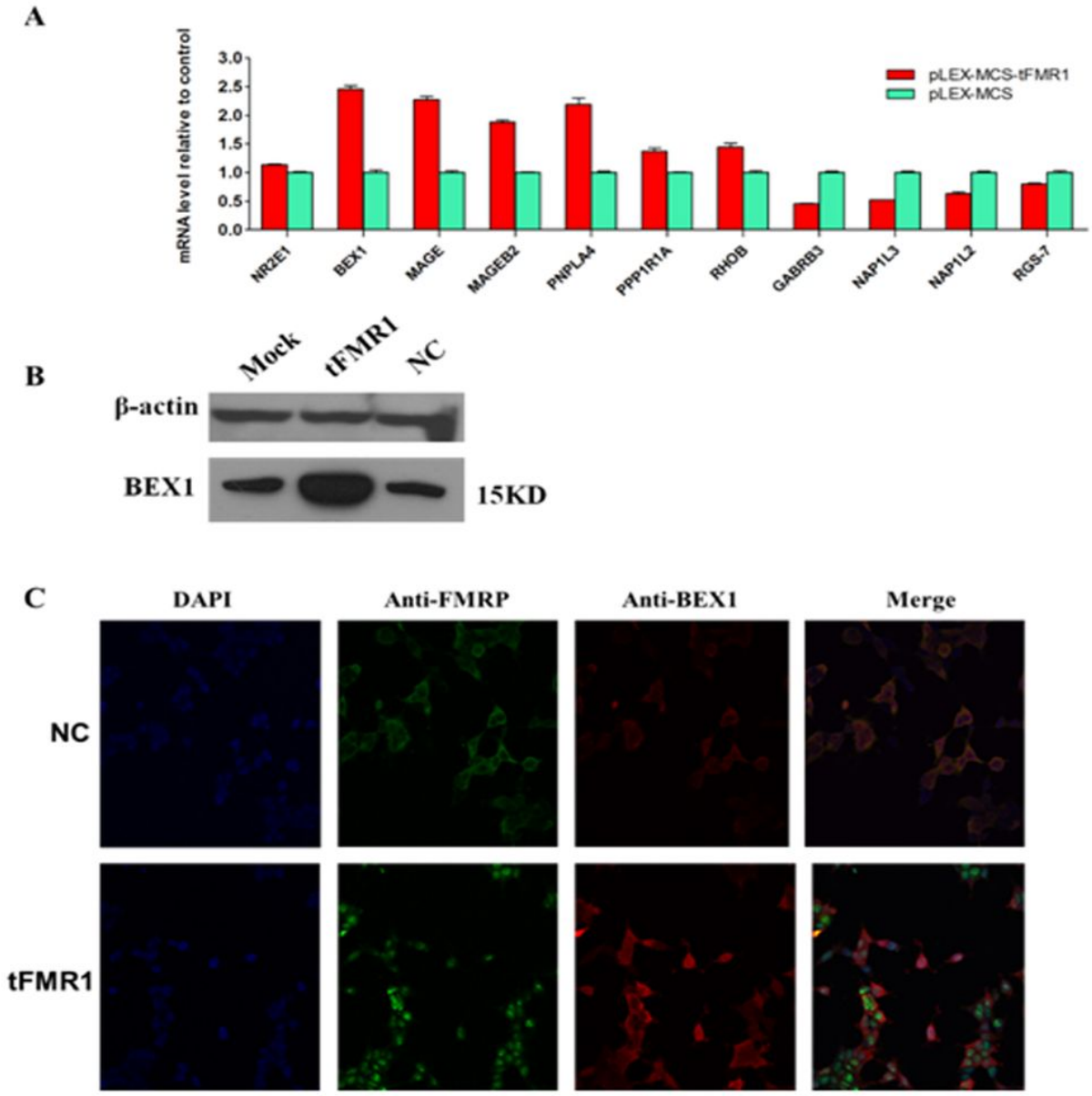

Figure 4

Overexpression of the truncated FMRP protein altered FXS-related signaling pathways. (A) Partial qRT-PCR results of differentially expressed genes. Red: HEK293T cells transfected by pLEX-MCS-tFMR1; Green: HEK293T cells transfected by pLEX-MCS. (B) Western blots showing the overexpression of BEX1 protein in HEK293T cells transfected by pLEX-MCS-tFMR1. 1: HEK293T cells without transfection; 2: HEK293T cells transfected by pLEX-MCS- tFMR1; 3: HEK293T cells transfected by pLEX-MCS. (C) Immunofluoresence showing the localization of BEX1 protein in HEK293T cells. HEK293T: HEK293T without transfection; pLEX-MCS-tFMR1: HEK293T cells transfected by pLEX-MCS- tFMR1. DAPI: the solution suitable for nuclear staining; Anti-FMRP: the primary antibodies of FMRP; Anti-BEX1: the primary antibodies of BEX1.

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