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FMR1 Linked Haplotype Analysis in a Mentally Retarded Male Population

Research Article

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Abstract: Fragile X syndrome is caused by dynamic mutation of *FMR1* gene CpG island CGG repeats. The underlying mutational mechanism is not fully understood. Different microsatellite markers and SNP have previously been reported as markers associated with *FMR1* CGG repeat instability. The aim of the present study was to identify specific haplotypes among Latvian FXS patients and the control group with respect to allelic stability. Eleven male FXS patients and 122 control male patients participated in the study. In total, 27 different DXS548-FRAXAC1-ATL1-FRAXAC2 haplotypes were found. The prevalent haplotype in the control group was 7-4-A-5+ (rel. frequency 0.327). The prevalent haplotype associated with the FXS group was 2-2-G-4 (rel. frequency 0.818; $p < 0.0001$). Grey zone alleles with a long uninterrupted CGG tract at the 3' end were significantly associated with the 2-2-G-4 haplotype ($p = 0.0022$). Our findings suggest that, for the Latvian population, the haplotype 2-2-G-4 is a marker of CGG tract instability. We conclude that a founder effect could not be an explanation for our findings on the basis of heterogeneity exhibited by the Latvian population and lack of studies throughout this geographical region. This data may provide evidence of different mutational pathways of expansion in the Baltic States region.

Keywords: *Fragile X Syndrome* • FMR1 *gene* • *DXS548* • *FRAXAC1* • *FRAXAC2* • *ATL1* • *Haplotype*

1. Introduction

Fragile X syndrome (FXS; OMIM #300624; FRAXA, Xq27.3) is a well known common cause of X-linked mental retardation. The prevalence of FXS full mutation is 1/4000 - 6000 males and 1/8000 - 10000 females. The syndrome is caused by a dynamic mutation of *FMR1* gene CpG island CGG repeats [1-4]. Among individuals from the general population, the polymorphic CGG repeat ranges from 6 to 50 repeats and is usually interspersed every 9–10 repeats with an AGG [5,6]. Premutation alleles have a moderate expansion of the repeat (from 50 to ~200 units), they are unmethylated on an active X chromosome and do not affect *FMR1* expression. CGG repeat expansion over 200 is the basis for CpG island methylation, leading to silencing of the *FMR1* gene [7]. Intermediate or grey zone alleles are poorly defined. Boundaries for the grey zone range vary among studies, from 34 or 35 CGG repeats for the lower boundary to 58/60 repeats for the upper boundary [7-10]. These alleles are often transmitted stably, but are more likely to exhibit unstable transmission with increasing size within this range.

The underlying mutational mechanism is not fully understood and remains a topic of debate. The gender of the parent carrying an expanded repeat (maternal imprinting), the number of repeats (dynamic mutation) and the absence of AGG interruptions in long tracts of CGG repeats have been described as the main factors related to this instability [5,9,11]. The microsatellite markers DXS548-FRAXAC1-FRAXAC2 and the ATL1 SNP have previously been reported as markers associated with *FMR1* CGG repeat instability [5,12-17].

Haplotypes linked to FXS are widely described

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across Western European and Scandinavian populations; however, less is known regarding populations from Eastern Europe, including the Baltic States. The aim of the present study was to identify specific haplotypes among Latvian FXS patients and control mental retardation patients with a normal number of CGG repeats with respect to allelic stability.

2. Material and Methods

2.1. Patients

Eleven unrelated male FXS patients, confirmed by DNA analysis, participated in this study. The control group comprised of 122 unrelated male patients exhibiting mental retardation, referred by clinical geneticists for routine FRAXA screening, with a normal number of *FMR1* CGG repeats. The Latvian Central Committee of Medical Ethics and the Riga Stradins University Committee of Medical Ethics approved the study.

2.2 Analysis of the CGG repeat and AGG interspersion pattern Haplotype analysis

The CGG repeat number was determined by fluorescent PCR on an ABI Prism® 310 genetic analyser following the manufacturer's protocol (Applied Biosystems, USA). Twenty-six patients with grey zone alleles (35–50 repeats) were detected and subsequently selected for AGG interspersion pattern analysis by direct sequencing.

For CGG repeat pattern analysis, the flanking DNA sequences were amplified from genomic DNA using the PCR protocol described by Chong *et al.* [17] PCR products were concentrated and purified for the sequencing reaction by a Millipore Montage PCR filter device.

The sequencing reaction was performed using a BigDye® Terminator v3.1 kit (Applied Biosystems) according to the manufacturer's protocol. Due to the high G/C content of the template, 1 µl of DMSO and 0.5 µl of glycerol were added to the reaction. The primer used was 5'-GAC GGA GGC GCC GCT GCC AGG-3' [19]. The reaction was carried out on a PCR Eppendorf Mastercycler. Subsequent purification of the sequencing products was performed as recommended by the manufacturer. All sequencing reaction products were run on an ABI Prism® 310 genetic analyser and analysed by ABI DNA sequencing software.

2.3. Haplotype analysis

The ATL1 polymorphism (alleles A/G located 5613 bp upstream of the CGG repeat) was analysed by following the allele-specific oligonucleotide PCR protocol described by Dombrowski *et al.* [11].

Case-control haplotype analysis between the FXS patients and the control group was performed using the microsatellite markers DXS548, FRAXAC1 and FRAXAC2. The DXS548 microsatellite is located 189895 bp downstream of the CGG repeat, the FRAXAC1 microsatellite is located 7221 bp downstream of the CGG repeat and the FRAXAC2 microsatellite is located 12418 bp upstream of the CGG repeat. Multiplex PCR for DXS548 and FRAXAC2 was performed in a total reaction volume of 15 µl, containing 1× PCR reaction buffer (75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 7% glycerol, 2 pmol of each primer DXS548A (5'-HEX-AGA GCT TCA CTA TGC AAT GGA ATC-3') and DXS548B (5'-GTA CAT TAG AGT CAC CTG TGG TGC-3') [20], 1 pmol of each primer FRAXAC2A (5'-6-FAM-GAC TGC TCC GGA AGT TGA ATC CTC A-3') and FRAXAC2B (5'-CTA GGT GAC AGA GTG AGA TCC TGT C-3') [20], and 0.15 U True startTM Hot Start *Taq* DNA polymerase. PCR was carried out by an initial step of 2 min at 95°C, then 10 cycles of 30 sec at 95°C, 1 min at 60°C and 1 min at 72°C. This was followed by a second round of amplification comprising 25 cycles of 30 sec at 95°C, 1 min at 55°C and 1 min at 72°C, and a final extension at 72°C for 7 min.

The FRAXAC1 microsatellite marker was amplified separately with 4.5 pmol of primer FRAXAC1A (5'-NED-GAT CTA ATC AAC ATC TAT AGA CTT TAT T-3') and FRAXAC1B (5'-AGA TTG CCC ACT GCA CTC CAA GCC T-3') [20] under the same PCR conditions.

Multiplex reaction product (0.5 µl) was mixed with an equal volume of GeneScan™ ROX 500™ size standard and 24 µl of deionised formamide. The lengths of product fragments were determined on an ABI Prism® 310 genetic analyser.

Genotyping results were analysed by GeneScan® Analysis software. The corresponding peak's length was calculated according to the calibration curve of GeneScan™ ROX 500™ size standard. Nomenclature for alleles was adjusted to the nomenclature recommended by Macpherson *et al.* [14].

Genotyping results were validated by direct sequencing of random alleles for each microsatellite marker. For each marker allele, the same PCR conditions as described above for genotyping were used, except the reverse primers A were not labelled with fluorescent dye. PCR products were concentrated and purified for the sequencing reaction by a Millipore Montage PCR filter device.

The sequencing reaction was performed using a BigDye® Terminator v3.1 kit according to the manufacturer's protocol. The reaction was carried out on a PCR

Eppendorf Mastercycler. Subsequent purification of the sequencing products was performed as recommended by the manufacturer. All sequencing reaction products were run on an ABI Prism® 310 genetic analyser and analysed by ABI DNA sequencing software.

2.3. Statistics

The statistical calculations of AMOVA for the haplotype analysis and level of heterozygosity for all polymorphisms were done using Arlequin 3.5 software [21]. Comparison of obtained data was performed by Fisher's exact test of 2×2 contingency tables and chi-square (http://www.graphpad.com/quickcalcs/index.cfm). The differences were considered statistically significant at $p < 0.05$. In case-control analysis Bonferroni correction was applied for multiple testing.

3. Results

3.1. ATL1 SNP

The control group CGG repeat alleles were analysed with respect to ATL1 alleles. The control group was divided into two subgroups according to CGG allele repeat number – normal size alleles and grey zone alleles (Table 1). The relative frequency (RF) of allele A in the normal subgroup was 0.563, for allele G it was 0.438. In the grey zone subgroup, allele A had a RF of 0.308, for allele G the RF was 0.692. Allele G differed significantly between normal size alleles and grey zone alleles ($p = 0.0271$).

Table 1. Frequencies of the ATL1 SNP in CGG Alleles.

	Normal CGG alleles (16–34 repeats)		Grey zone CGG alleles (35–50 repeats)	
ATL1	n	RF	n	RF
A	54	0.563	8	0.308
G	42	0.438	18	0.692*
Total	96	1.000	26	1.000

*n = number of chromosomes; RF = relative frequency; * p < 0.05*

A statistically significant association between individual CGG alleles and the ATL1 SNP was found for allele 29 and G ($p = 0.001$); 30 CGG repeats and A ($p < 0.0001$) and allele 31 and A ($p = 0.0013$).

All FXS group chromosomes were found to be exclusively associated with ATL1 allele G, and this association was statistically significant ($p = 0.0008$).

3.2. Repeat structures of grey zone alleles

Twenty-six grey zone alleles were sequenced in order to characterise the AGG interruption within the CGG repeat. Twelve chromosomes exhibited a CGG interspersion pattern with three AGG, 12 chromosomes with two AGG, one chromosome with one AGG and one pure CGG tract (Table 2). For all chromosomes, the loss of AGG was detected at the 3' end of the sequence.

Table 2. AGG Interspersion Pattern and Linked DXS548-FRAXAC1-ATL1-FRACXAC2 Haplotype Frequencies of Grey Zone CGG Alleles.

DXS548	FRAXAC1	ATL1	FRAXAC2	CGG	AGG	n	RF
2	2	G	4	38	9+9+18	8	0.308**
				39	9+29		
				40	9+9+20		
				40	9+9+20		
				41	9+9+21		
				45	9+9+25		
				47	9+9+27		
				50	9+9+30		
7	4	A	5+	39	10+9+9+8	6	0.231
				41	10+9+9+10		
				41	10+9+10+9		
				41	10+9+10+9		
				42	10+9+21		
				43	10+9+22		
6	5	G	7+	37	9+10+6+9	2	0.077
				38	Pure		
7	4	G	6+	39	9+9+9+9	2	0.077
				39	9+9+9+9		
7	4	G	5	39	9+9+9+9	2	0.077
				39	9+9+9+9		
6	4	G	5	41	9+9+21	2	0.077
				42	9+9+22		
6	5	A	7	37	9+10+6+9	1	0.038
6	4	A	5+	35	10+6+8+8	1	0.038
3	2	G	4	38	9+9+18	1	0.038
7	4	G	5+	36	10+9+5+9	1	0.038
Total						**26**	**1.000**

*RF = relative frequency; AGG = pattern of CGG tract, the digit corresponds to the CGG repeat number and '+' denotes the AGG interspersion position; ** p < 0.01.*

3.3. DXS548-FRAXAC1-ATL1-FRAXAC2 haplotypes

Several microsatellite markers and the ATL1 SNP were analysed in the FXS patient group and the control group. Seven different microsatellite alleles were detected with respect to the DXS548 locus, four alleles were identified regarding the FRAXAC1 locus and nine alleles were found for the FRAXAC2 locus. The frequencies of the described alleles in the control and FXS group are shown in Figure 1.

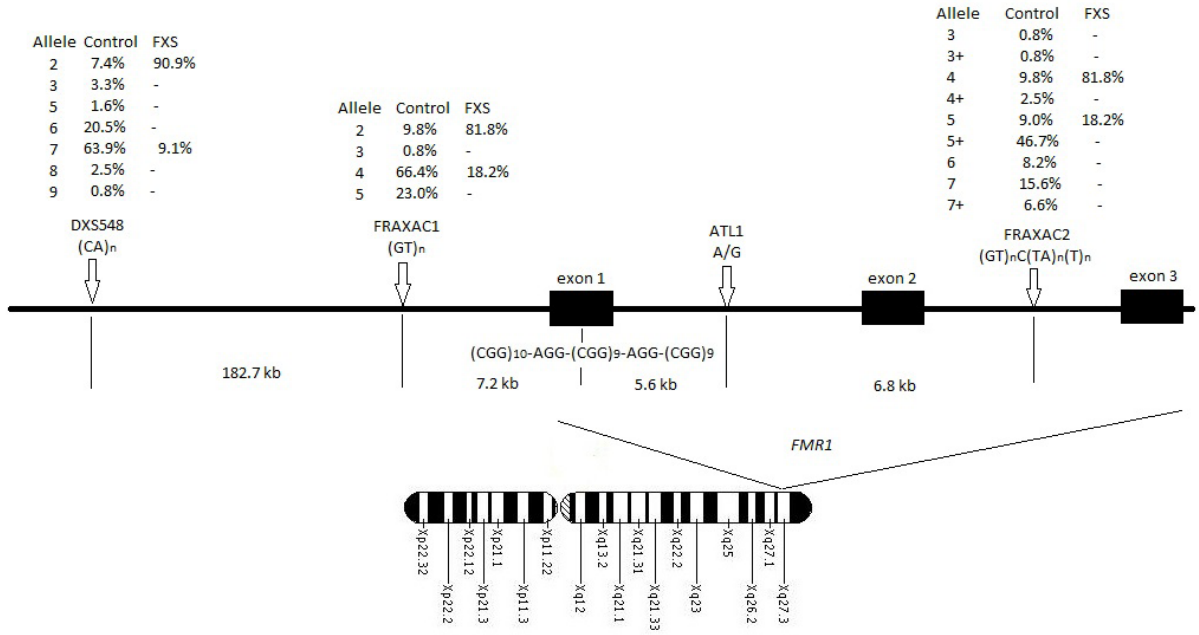

Figure 1. The location, nomenclature and frequencies of *FMR1* linked STR- and SNP- polymorphisms, tested in this study.

The level of heterozygosity for all polymorphisms was examined under finite island model and compared (Table 3).

Table 3. Expected Heterozygosity and Observed Heterozygosity among the Control and FXS Groups.

Locus	Control H_E	FXS H_E	Mean	S.D.	H_T	H_0	X^2	p-value
ATL1 SNP	0.504	0.000	0.252	0.356	0.501	0.696	20.332	<0.0001
FRAXAC1	0.501	0.327	0.414	0.123	0.545	0.863	53.654	<0.0001
FRAXAC2	0.734	0.327	0.530	0.288	0.757	1.055	62.591	<0.0001
DXS548	0.546	0.182	0.364	0.258	0.594	1.009	94.310	<0.0001

HE = expected heterozygosity; HT = total heterozygosity; HO = observed heterozygosity; S.D. = standard deviation; X2 = chi-square.

The identified markers were combined to form haplotypes. In total, 27 different haplotypes were detected – 26 in the control group, three in the FXS group. Only one haplotype from the FXS group was unique (Table 4). The most common haplotype among the control group chromosomes was 7-4-A-5+ (RF = 0.327; $p = 0.0336$). Among FXS patients, the prevalent haplotype was 2-2-G-4 (RF = 0.818; $p < 0.0001$).

Statistical analysis of the data revealed that the molecular variation between the groups was 27.04% and within the groups was 72.96%. The fixation index *Fst*, calculated based on haplotype frequencies between the control and FXS group, was 0.27042.

A haplotype analysis based on the CGG tract pattern in grey zone alleles was performed (Table 2). The most common haplotypes in this subgroup were 2-2-G-4 (RF = 0.308) and 7-4-A-5+ (RF = 0.231). All the alleles with 2-2-G-4 haplotype had a long (≥ 18 CGG repeats) uninterrupted sequence at the 3' end ($p = 0.0022$). Six alleles out of 18 with other detected haplotypes had the same feature.

4. Discussion

Our study is the first study in the Baltic States region regarding *FMR1* linked haplotypes. In the present study, we characterised the microsatellite markers DXS548, FRAXAC1 and FRAXAC2, the ATL1 SNP and the corresponding haplotypes in a mentally retarded male population from Latvia with normal and expanded *FMR1* gene CGG repeats.

Previous studies have suggested linkage of CGG tract instability with the G allele of ATL1, specific microsatellite marker haplotypes and a CGG tract AGG interspersion pattern exhibiting a long uninterrupted CGG repeat at the 3' end [5,11,22-25]. Our results revealed a statistically significant prevalence of the G allele of ATL1 among grey zone alleles and full mutation alleles as an indicator of instability.

The AMOVA data suggested that the differences between detected haplotypes within the control and FXS group were significant. In our Latvian population, 7-4-A-5+ was determined as the prevalent haplotype for nor-

Table 4. Frequencies of DXS548-FRAXAC1-ATL1-FRAXAC2 Haplotypes in the Control and FXS Group.

Haplotype				Control group			FXS group		
DXS548	FRAXAC1	ATL1	FRAXAC2	n	RF	SD	n	RF	SD
7	4	A	5+	40	0.327	0.043	0	-	-
7	5	G	7	11	0.090	0.026	0	-	-
2	2	G	4	9	0.074	0.024	9	0.818***	0.122
7	4	G	5	8	0.066	0.023	1	0.091	0.091
7	4	G	6+	8	0.066	0.023	0	-	-
6	4	A	5+	7	0.057	0.021	0	-	-
6	5	G	7	6	0.049	0.020	0	-	-
6	5	G	7+	6	0.049	0.020	0	-	-
8	4	A	5+	3	0.025	0.014	0	-	-
3	2	G	4	3	0.025	0.014	0	-	-
5	4	A	5+	2	0.016	0.012	0	-	-
7	5	G	7+	2	0.016	0.012	0	-	-
7	4	G	5+	2	0.016	0.012	0	-	-
7	4	A	4+	2	0.016	0.012	0	-	-
6	4	G	5	2	0.016	0.012	0	-	-
6	5	A	5+	1	0.008	0.008	0	-	-
3	4	G	6+	1	0.008	0.008	0	-	-
7	4	A	5	1	0.008	0.008	0	-	-
7	4	G	7	1	0.008	0.008	0	-	-
7	3	A	5+	1	0.008	0.008	0	-	-
6	4	A	4+	1	0.008	0.008	0	-	-
7	4	A	3	1	0.008	0.008	0	-	-
7	4	A	3+	1	0.008	0.008	0	-	-
9	4	A	5+	1	0.008	0.008	0	-	-
6	5	G	6+	1	0.008	0.008	0	-	-
6	5	A	7	1	0.008	0.008	0	-	-
2	4	G	5	0	-	-	1	0.091	0.091
Total				**122**	**1.000**	**0.374**	**11**	**1.000**	**0.304**

*n = number of chromosomes; RF = relative frequency; SD = standard deviation; *** p < 0.0001*

mal CGG alleles. However, after Bonferroni correction, this finding was not considered to be statistically significant. Furthermore, haplotype 2-2-G-4 was found to be in positive association with full mutation CGG alleles and grey zone alleles featuring a long (≥18 repeats) uninterrupted CGG tract at the 3' end. These findings imply that, in our population, haplotype 2-2-G-4 is a marker of CGG tract instability. Grey zone alleles with a long uninterrupted CGG tract at the 3' end associated with this haplotype have a higher likelihood of increasing the number of CGG repeats, leading to premutation or mutation over generations.

Several studies have identified specific haplotypes associated with FXS patients and normal CGG repeat alleles across European populations [22,25-29]. However, only a limited number of these studies focused on populations from Eastern and Northeastern Europe. Different loci were used for these haplotype analyses in European populations. Thus, comparison of our results with these analyses would prove difficult. Therefore, we compared the results presented here with single locus data in the literature.

Allele 7 at the DXS548 locus was the most common allele (RF = 0.639) in our control group. Similar findings, albeit with slightly different frequencies, have been reported from Sweden [27], Czech Republic [28], Finland [30], France [16], Croatia [25], Russia [31], Norway [32] and Poland [29]. In contrast, our finding with respect to the most common allele in FXS patients (allele 2, RF = 0.909) was in agreement with only one of the analyses (Polish population, allele 2; Swedish population, alleles 7 and 6; Czech population, alleles 2, 6 and 7; French population, alleles 2, 6 and 7; Finnish population, almost exclusively allele 6; Croatian population, alleles 7, 6 and 3; Norwegian population, alleles 6 and 2).

For the FRAXAC1 locus, allele 4 was the most common allele (RF = 0.664) in our control group. Analysis of this locus in control chromosomes of a Czech population revealed allele 3 to be the most common allele [28]. This was also the case in populations from Russia [31],

Norway [32] and Croatia [25], while alleles 3 and 4 were prevalent in a Polish population [29]. Regarding Latvian FXS chromosomes, we found allele 2 to be the most common allele (RF = 0.818). However, this was not in line with the findings from other populations (Norwegian, allele 4; Polish, allele 4; Croatian, allele 3; Czech, alleles 3 and 4).

Compared to the microsatellite marker FRAXAC1, the FRAXAC2 locus has been more widely used in studies. Allele 5+ was the most common allele at this locus in our control group, detected at a RF of 0.467. This finding was not replicated in other populations: Finnish, allele 3 [30]; Swedish, Czech and French, allele 4+ [16,26,28]; Polish, alleles 7 and 7+ [29]. The prevalent allele in our FXS patients was allele 4. Allele 3 was identified in half of the investigated chromosomes in a Finnish FXS patient group, while alleles 4+ and 5 were prevalent in a Swedish population, alleles 4+ and 4 in a Czech population, alleles 3 and 4 in a French population, and allele 7 in a Polish population.

In summary, our finding regarding allele 7 being the most common allele at the DXS548 locus in Latvian control patients is in line with several other European population control groups. Furthermore, our FXS patient group finding (i.e. allele 2 being the prevalent allele at this locus) is consistent with data from a Polish FXS population. The FRAXAC1 and FRAXAC2 loci results for our control and FXS group differ to varying degrees from the data reported for other European populations.

As the analysed microsatellite loci and nomenclature assigned to alleles in the literature are different, confusion arises, which may lead to bias in the interpretation of literature data comparing haplotypic results from different populations.

To the best of our knowledge, specific *FMR1*-linked haplotypes in the Baltic State region and Northeastern Europe have not been previously described. The present study is the first to report Latvian population *FMR1* haplotype data. Comparison of the data with those obtained from geographically close European populations highlights differences, particularly with the FXS patient group. Indeed, haplotype 2-2-G-4 appears to be exclusively found in Latvian FXS chromosomes. A small sample size making up the case group may have a negative effect on this result.

We conclude that a founder effect could not be an explanation of our findings on the basis of heterogeneity exhibited by the Latvian population and lack of studies across this geographical region. A larger study of *FMR1*-linked haplotypes in Eastern and Northeastern European regions may provide evidence of a specific mutational pathway for unstable CGG alleles in this geographical region.

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