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A study of GluK1 kainate receptor polymorphisms in Down syndrome reveals allelic non-disjunction at 1173(C/T)

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Abstract. Mechanisms underlying Down syndrome (DS)-related mental retardation (MR) remain poorly understood. In trisomic offspring, non-disjunction may result in the reduction to homozygosity of a susceptibility allele inherited from a heterozygous parent. Accordingly, we sought evidence for allelic non-disjunction in the GluK1 gene that encodes the critical kainite-binding glutamate receptor subunit-5, maps to chromosome 21q22.1 in the DS critical region and is expressed in brain regions responsible for learning and memory. Three polymorphisms of GluK1 [522(A/C) rs363538; 1173(C/T) rs363430 and 2705(T/C) rs363504] were genotyped in 86 DS patient families by means of PCR-coupled RFLP assays and evaluated with respect to allele frequency, heterozygosity, linkage disequilibrium, stage and parental origin of allelic non-disjunction. We report that the distribution of allele frequencies is in Hardy-Weinberg equilibrium. Moderate heterozygosity (0.339) and a major allele frequency of 0.78 render the 1173(C/T) marker informative. Pair-wise comparisons reveal that 522(A/C)-1173(C/T) [$\chi^2 = 31.2$, $df = 1$, $p = 0.0001$; $D' = 0.42$] and 1173(C/T)-2705(T/C) [$\chi^2 = 18.3$, $df = 1$, $p = 0.0001$; $D' = 0.34$] are in significant linkage disequilibrium of weak magnitude. The estimated ratio of meiosis-I to meiosis-II errors arising from allelic non-disjunction of 1173(C/T) is 4:1 in maternal cases and 2:1 in paternal cases. Studies including additional markers and patient samples are warranted to further substantiate present findings.

Keywords: Down syndrome, mental retardation, glutamate, GluK1/GluR5/GRIK1, allelic non-disjunction, parent and stage of origin, risk

1. Introduction

Down syndrome (DS) occurs due to non-disjunction of chromosome 21 and is the leading genetic cause of mental retardation (MR) [12,27,33]. Other than the presence of an additional chromosome, factors such as allelic variation in candidate genes for MR may also contribute to the complex phenotype. Accordingly, dosage imbalance of alleles that have been triplicated or the variable penetrance of alleles in a critical region of chromosome 21q have been postulated to account for the range of MR and observed developmental delay in DS [30]. In the case of trisomic offspring, non-disjunction may also result in the reduction to homozygosity of a susceptibility allele inherited from a heterozygous parent [20]. Given a central role for the neurotransmitter glutamate in mediating physiological processes such as learning, memory and neuronal plasticity [13] we reasoned that polymorphic markers implicated in excitatory glutamatergic neurotransmission are

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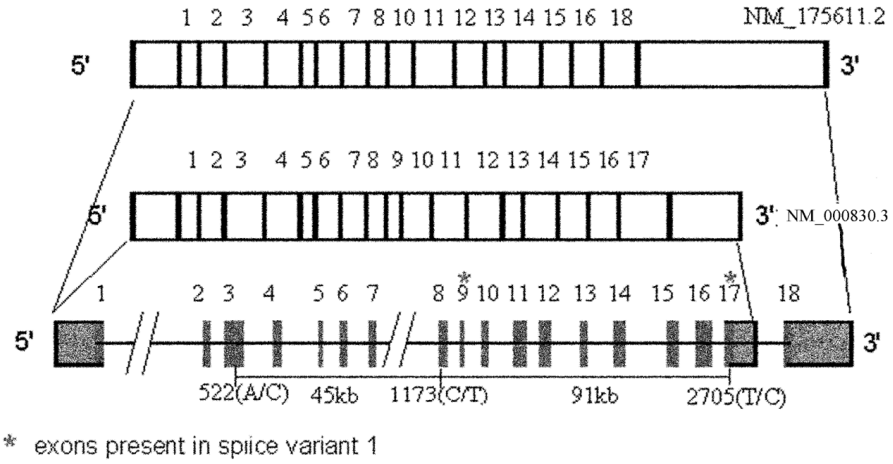

Fig. 1. A schematic representation of the GluK1 gene showing position and relative physical distance between 522(A/C), 1173(C/T) and 2705(T/C) polymorphisms in the alternatively spliced variants NM_175611.2 and NM_000830.3.

likely to influence genetic susceptibility to DS-related MR.

The human GluK1/GRIK1/GluR5 gene codes for the critical subunit-5 of glutamate receptors that bind kainate [1,7], maps to chromosome 21q22.1 [17] and spans 400 kb with 18 exons [1] (Fig. 1). GluK1-containing glutamate receptors form Ca^{2+} channels [2] that are expressed in the hippocampus, cerebellum and cortex [13]. Robust expression of GluK1 mRNA is observed in cortical layers II, III and IV, with a transient peak during the period of greatest developmental plasticity in the somatosensory cortex of rat brain [6,28]. A search of human (T1Dbase) [3,23] and murine (Gene Novartis Foundation) [3] databases reveals region-specific expression of GluK1 mRNA in the cerebellum, striatum, caudate nucleus, hippocampus, amygdala and the cortex.

Since genetic variation in GluK1 may impact behavioral phenotypes [26], we elected to study the role of three commonly reported single nucleotide polymorphisms (SNP) of GluK1 in mediating susceptibility to DS-related MR. The 522(A/C) [rs363538] transversion in exon 3 and the 1173(C/T) [rs363430] transition in exon 8 result in conservative changes in the codons for the amino-acid residues T174 and D391, respectively [25]. These residues are located in the extra-cellular ligand-binding loop [32]. In contrast, the 2705(T/C) [rs363504] transition in exon 17 results in a non-synonymous change (L902S) that affects the intracellular C-terminal domain [25]. Alternative splicing of the primary GluK1 transcript has been reported to generate two distinct mRNA species: NM_000830.3 and NM_175611.2 [1]. Interestingly, sequence alignments and Clustal-X analysis with the genomic contig of GluK1 (NT_011512.10) indicates that the NM_000830.3 transcript includes coding sequences from exons 9 and 17 that are absent, however, in the alternatively spliced NM_175611.2 transcript (sequence alignment data not shown; Fig. 1). The 2705(T/C) polymorphism in exon 17 of GluK1 is thus differentially expressed in the splice variants (Fig. 1).

We genotyped the polymorphisms by means of PCR-coupled RFLP assays and evaluated them with respect to heterozygosity, allele frequency, linkage disequilibrium, as well as the stage and parental origin of non-disjunction in DS patient families. We report that distribution of allele frequencies are in Hardy-Weinberg equilibrium and pair-wise combinations of alleles are in significant linkage disequilibrium of weak magnitude. The 1173(C/T) marker exhibits moderate heterozygosity (0.339) and the non-disjoining allele originates both during meiosis-I and meiosis-II in a bi-parental mode that likely confers risk of DS-related MR. The estimated ratio of meiosis-I to meiosis-II errors is 4:1 in maternal cases and 2:1 in paternal cases suggesting varying risk for non-disjunction.

2. Materials and methods

2.1. Bio-informatics procedures

GluK1 genomic DNA sequence (NT_011512.10 gi: 51475294), alternatively spliced mRNA transcript sequences (NM_175611.2, gi: 59710095; NM_000830.3, gi: 59710094) and corresponding cDNA se-

quences [15] were obtained from the NCBI database (release date: February 29, 2008). Data pertaining to single nucleotide polymorphisms in GluK1 were retrieved from dbSNP (http://www.ncbi.nlm.nih.gov/sites/entrez? db = snp). The expression profile for GluK1 mRNA in human tissues was obtained from T1Dbase [23] database that uses data from Gene Novartis Foundation (GNF) database [3]. Data pertaining to murine brain region-specific expression of GluK1 was also obtained from the GNF database [3]. An Average Difference Value, calculated by the Affymetrix software, is proportional to the mRNA content in the sample and is the estimate of cross hybridization that exceeds specific signal intensity [3]. The GluK1 genomic DNA sequence (NT_011512.10) was aligned with cDNAs corresponding to two alternatively spliced transcripts (NM_175611.2 and NM_000830.3) using Clustal X-v.1.83 [16] under alignment parameters with pair gap penalties of 10.00 for gap opening and 0.10 for gap extension and multiple alignment penalties of 10.00 for gap opening and 0.20 for gap extension. Similar alignment of NT_011512.10 with NM_175611.2 and NM_000830.3 sequences was performed with the help of the Spidey software program [31] that defines windows on the genomic sequence and performs mRNA: DNA alignment separately within each window; the alignment parameter is based on minimum % identity and mRNA length coverage which are set at default value.

2.2. Subject ascertainment and diagnostic procedures

We recruited 86 DS patient families (58 trios and 28 duos) and 13 singleton cases from the Out-Patient Department of Manovikas. The DS patient group comprised of 62 males and 37 females with a mean age of (7.34 $\pm$ 5.38) years. All patients fulfilled criteria for Down syndrome as per SMITH'S Recognizable patterns of human malformation [19] and the criteria for mental retardation as per Diagnostic and Statistical Manual-IV text revised version [4]. Detailed demographic and clinical history was obtained from parents and other participants by means of a structured questionnaire. Venous blood samples ($\sim$ 5 ml) were collected for genomic DNA analysis after securing written informed consent from all participants. For DS cases below 5 years of age ($n = 46$), the developmental delay was evaluated by means of functional assessment; in older children with DS ($n = 53$), the degree of mental retardation was assessed by means of the Wechsler's IQ testing procedure [11]. The study protocol had prior approval of the Institutional Ethical Committee of Manovikas. Approximately 76.5% of all recruited individuals were native speakers of Bengali; the sample studied represents a mixture of various caste and religious affiliations: Hindu [brahmin (26.50%), kayastha (33.44%), baisya (9.46%), vaidya (0.63%), mahisya (2.84%), scheduled caste (16.40%)], Muslim (10.09%) and Christian (0.63%).

2.3. Genotyping procedures

The salting out procedure of Miller et al. 1988 [29] was used to isolate genomic DNA from whole blood lymphocytes. Polymerase Chain reaction (PCR)-based amplification of genomic DNA targets was carried out in the DNA Engine Thermal Cycler (MJ Research PTC-200). For the 522(A/C) polymorphism 10 pmol of each forward (5′-CATACAGACCCGCTGGAAAC-3′) and reverse (5′-TGAGTCCCATACCTGTGCTG-3′) primers were used in reactions containing 100 ng of genomic DNA, 1-X Thermopol-II buffer composed of 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Triton X-100 (New England Biolabs), 1.5 mM $MgSO_4$, 200 μM dNTPs, 0.2 unit Taq DNA polymerase and 0.01% gelatin in a total volume of 20 μl. The cycling conditions were as follows: heat denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec, elongation at 72°C for 30 sec, and a final elongation step at 72°C for 10 min. After PCR amplification, restriction enzyme digestion was carried out with 200 ng PCR product, 0.2 unit of *Btg I* (New England Biolabs) enzyme, 1X NEB3 assay buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM Dithiothreitol and 1X nuclease free BSA in 20 μl reaction volume at 37°C for 16 h. For amplification of the 2705(T/C) polymorphism, forward (5′-CCATCCAACCAACTCCACTT-3′) and reverse (5′-CATTTTCTACTGGGCACATC-3′) primers were used as per reaction conditions described previously. The cycling conditions were as follows: heat denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, elongation at 72°C for 30 sec, and a final elongation step at 72°C for 10 min. The PCR amplicon ($\sim$200 ng) was digested with 0.2unit *Ase I* (Bangalore Genei) enzyme, 1 X assay buffer E (Bangalore Genei) containing 33 mM Tris-acetate (pH 7.9), 10 mM Mg-acetate, 66 mM K-acetate and 0.5 mM dithiothreitol (Bangalore Genei) in a 20 μl reaction volume at 37°C for 16 h. For 1173(C/T) polymorphism, the PCR amplification was

performed with 15 pmol each of forward primer (5′-CAGCCACATTTCTTCTTGTCC-3′) and mutagenic reverse primer (5′-CCTTCCTCTTTGAGACTAATG-AT-3′) [14]. All the reaction and cycling conditions were same as described previously. The PCR product (500ng) was subjected to restriction enzyme digestion with 0.5 unit *EcoR V* (Bangalore Genei) enzyme, 1Xassay buffer A (Bangalore Genei) containing 10 mM Tris-Cl (pH 7.9), 150 mM NaCl, 7 mM $MgCl_2$, 1 mM dithiothreitol (Bangalore Genei) and 1X nuclease free BSA (Bangalore Genei) in a 20 μl reaction volume at 37° for 16 h. Restriction fragments and size standards were resolved in 16% polyacrylamide gels containing 5% glycerol. Electrophoresis was carried out at 90 V (constant voltage) for ∼14 h overnight. Size discrimination of bands was performed by means of Quantity One software (BioRad, CA).

2.4. Statistical analysis

Analysis of allele and genotype frequency, heterozygosity and tests for Hardy-Weinberg equilibrium (HWE) were performed using the TFPGA v1.3 software program [24]. The Likelihood Ratio test [10] was performed to ascertain HWE proportions. The EH+ program v.1.2 [18] was used to estimate the haplotype frequencies. Subsequently, we computed linkage disequilibrium values using the 2LD program v.1.00 [9] and JLIN: Java linkage disequilibrium plotter v.1.50 [21] software.

3. Resullts

GluK1 polymorphisms were characterized by PCR-coupled RFLP assays. Genotyping of the 522(A/C) SNP was performed with *Btg I* that cleaves the sequence C↓CRYGG but not CARYGG. Accordingly, enzymatic digestion of a 167 bp PCR-amplicon yields retriction fragments of 132 bp and 35 bp. The 32 bp band was run-off to optimize resolution and, as shown in Fig. 2, the A/A genotype is identified by an uncut 167 bp band (lane 1), the A/C heterozygote yields two bands corresponding to 167 bp and 132 bp (lane 2) and the C/C genotype is denoted by a single band of 132 bp (lane 3). Out of 510 chromosomes analyzed, only 1 individual was identified with C/C genotype (132 bp) (lane 3).

Genotyping of the 1173(C/T) SNP was performed with *EcoR V* that cleaves the sequence GAT↓ATC but not GACATC. Enzymatic digestion of a 121 bp PCR-amplicon yields restriction fragments of 98 bp and 23 bp. The 23 bp band was run-off to optimize resolution. As shown in Fig. 2, the C/C genotype is identified by an uncut 121 bp band (lane 5), C/T heterozygotes yield two bands corresponding to 121 bp and 98 bp (lane 6) while the T/T genotype is denoted by a 98 bp band (lane 7).

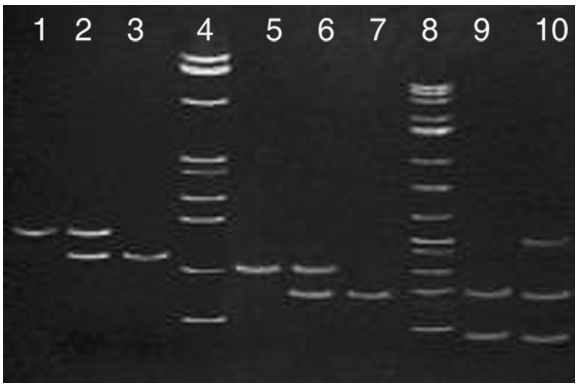

Fig. 2. Genotypes identified by PCR-coupled RFLP analyses of various polymorphic loci: 522(A/C)[lane 1: A/A, lane 2: A/C, lane 3: C/C]; 1173(C/T)[lane 5: C/C, lane 6: C/T, lane 7: T/T] and 2705(T/C)[lane 9: T/T, lane 10: T/C]; lane 4: size standards obtained from Φ x174DNA digested with *Hae III*; lane 8: size standards obtained from Φ x174DNA digested with *Hinf I*.

Genotyping of the 2705(T/C) SNP was performed using *Ase I* that cleaves A↓TTAAT but not ACTAAT. Enzymatic cleavage of a 153 bp PCR-amplicon yields restriction fragments of 96 bp and 57 bp. As shown in Fig. 2, the T/T genotype is identified by bands of 96 bp and 57 bp (lane 9), the heterozygous T/C genotype is denoted by three bands of 153 bp, 96 bp and 57 bp (lane 10) and the C/C genotype corresponds to the uncut band of 153 bp.

The distributions of allele and genotype frequencies for all three loci under study are shown in Table 1. Of the three polymorphisms studied, the 1173(C/T) marker is the most informative due to moderate heterozygosity (0.339) (Table 1). For the 522(A/C) and 2705(T/C) polymorphisms, we determined that the C allele is the minor allele as indicated by allele frequencies of 0.086 and 0.078, respectively (Table 1). Not surprisingly, the rare allele is commonly manifested in the heterozygote in case of both polymorphisms. In our sample, we detected only one individual who was homozygous for 522(C/C) and none with the 2705(C/C) genotype (Table 1) and that may be the reason why distribution of allele frequencies at the 2705(T/C) locus is only marginally in Hardy-Weinberg equilibrium ($p = 0.065$ at C.I. = 95%; Table 1). Overall, there is no significant deviation in allele frequencies from that expected ac-

Table 1
Determination of Hardy-Weinberg equilibrium proportions

N	Polymorphism	Allele	Frequency	Genotype	Frequency	Heterozygosity (unbiased)	G	P
255	522(A/C)	A-522	0.914	AA	0.831 (212)	0.158	0.597	0.44
		C-522	0.086	AC	0.168 (42)			
				CC	0.004 (01)			
255	1173(C/T)	C-1173	0.784	CC	0.600 (153)	0.339	2.190	0.14
		T-1173	0.216	CT	0.369 (94)			
				TT	0.031 (08)			
255	2705(T/C)	T-2705	0.922	TT	0.843 (215)	0.145	3.408	0.07
		C-2705	0.078	TC	0.157 (40)			
				CC	0.000 (00)			

Figures in parentheses and under column N denote number of individuals; G ($\log_{10}$ of likelihood ratio), and P(probability) values derived at 95%C.I. using the maximum likelihood ratio test of HWE (10); data pertain to analysis of 255 control samples.

Table 2
Pair-wise values of raw (D) and normalized (D′) linkage disequilibrium coefficients for allelic combinations of human kainate receptor [522(A/C), 1173(C/T) and 2705(T/C)] polymorphisms in 255 controls

N	Haplotype	Frequency	D	D′	$\chi^2(df=1)$	Significance $P(D=0)$
255	A-522-C-1173	0.75	0.029	0.422	31.18	0.0001
	A-522-T-1173	0.17	−0.029	−0.422		
	C-522-C-1173	0.04	−0.029	−0.422		
	C-522-T-1173	0.05	0.029	0.422		
255	C-1173-T-2705	0.74	0.021	0.340	18.30	0.0001
	C-1173-C-2705	0.04	−0.021	−0.340		
	T-1173-T-2705	0.18	−0.021	−0.340		
	T-1173-C-2705	0.04	0.021	0.340		
255	A-522-T-2705	0.84	0.003	0.037	0.62	0.43
	A-522-C-2705	0.07	−0.003	−0.037		
	C-522-T-2705	0.08	−0.003	−0.037		
	C-522-C-2705	0.009	0.003	0.037		

cording to HWE proportions. The marker loci are not subject to evolutionary forces and the identified alleles are segregating stably in the sample population. Furthermore, parental transmission of these alleles may be conveniently studied in a family-based study design.

We next estimated linkage disequilibrium (LD) values between pair-wise combinations of alleles comprising the 522(A/C), 1173(C/T) and 2705(T/C) polymorphic systems by using data from control samples. By computing the normalized disequilibrium coefficient (D′), we determined that significant ($\chi^2 = 31.18$, $df = 1$, $p = 0.0001$) LD of weak magnitude ($D' = 0.422$; $r^2 = 0.061$) exists between 522(A/C)-1173(C/T), the rank order of estimated haplotype frequencies being A522-C1173 > A522-T1173 > C522-T1173 > C522-C1173 (Table 2). The very low value for the latter two-haplotype combinations is due to the low frequencies of the C522 and T1173 minor alleles (Table 1). Similarly, 1173(C/T)-2705(T/C) markers are also in significant LD ($\chi^2 = 18.30$, $df = 1$, $p = 0.0001$) LD, albeit of lower magnitude ($D' = 0.340$; $r^2 = 0.036$). A positive D′ value and the magnitude of estimated haplotype frequencies indicates that A522-C1173 and C1173-T2705 haplotypes tend to segregate on the same chromosome more often than expected by mere chance (Table 2). Of 255 individuals in the total sample, we detected only 1 individual who was homozygous for the rare C522 allele (Table 1) and none with the 2705(C/C) genotype. Not surprisingly, the estimated C522-C2705 haplotype frequency is negligible (0.009) (Table 2) and no LD exists between these markers.

From a total sample of 58 trios, we excluded 25 trios as being non-informative with respect to the 1173(C/T) marker polymorphism since their genotypes were either all homozygous (lanes 1, 2, 3) or heterozygous (lanes 5, 6, 7) (Fig. 3A). A total of 4 trios showed parental genotypes that were heterozygous (lanes 9 and 10) while corresponding offspring genotypes were homozygous for either the C-1173 allele (lane 11) or for the T-1173 allele (lane 12) (Fig. 3A). We infer that either parent in these trios could have contributed the non-disjoining allele (C or T) during meiosis-II (Table 3a).

Table 3a
Determination of parent/stage of origin of non-disjunction error in trio/duo families genotyped for 1173(C/T) polymorphism

NDJP genotype	CDJP genotype	child genotype	marker status	stage of origin	# trios (T) /duos (D)
CT (♀/♂)	CT(♂/♀)	CCC	R	M-II	3 (T)
CT (♀/♂)	CT(♂/♀)	TTT	R	M-II	1 (T)
CC (♀)	CT (♂)	CCC	N	M-I	10 (T)
CC (♂)	CT (♀)	CCC	N	M-I	4 (T)
CT (♂)	CC (♀)	CCT/CTT	N/R	M-I/M-II	4 (T)
CT (♀)	CC (♂)	CCT/CTT	N/R	M-I/M-II	8 (T)
CT (♀)	TT (♂)	CCT/CTT	R/N	M-II/M-I	3 (T)
missing (♂)	CT (♀)	CCC	N	M-I	2 (D)
missing (♂)	CT (♀)	TTT	N	M-I	1 (D)
missing (♀)	CT (♂)	CCC	N	M-I	2 (D)
missing (♂)	CC (♀)	CCT/CTT	N/R	M-I/M-II	4 (D)
missing (♂)	TT (♀)	CCT/CTT	R/N	M-II/M-I	1 (D)

R = marker status reduced to homozygosity, N = marker status not-reduced to homozygosity, M-I = meiosis-I, M-II = meiosis-II.

Of the remaining 29 informative trios, analyses of parental genotypes revealed that 14 trios comprised of one parent heterozygous for the 1173(C/T) marker, whereas, the other parent and the proband were homozygous. In such families, the heterozygous parent was the assumed to be the informative or correctly disjoining parent (CDJP) while the other parent was deemed to be the non-disjoining parent (NDJP) in whom the error occurred in meiosis I. As shown in Table 3a and Fig. 3B, non-disjunction occurred during maternal meiosis I (lane 1, 2, 3) in 10 out of 14 such trios while the error occurred in paternal meiosis I (lane 5, 6, 7) in 4 trios.

Analysis of the remaining 15 trios reveals that one parent and the proband are both heterozygous for 1173(C/T). In such families, the homozygous parent is deemed informative (CDJP) as the offspring/proband genotype is recombinant; the heterozygous parent is therefore the NDJP. Although the parent-of-origin with respect to the non-disjoining allele can be readily verified in such families, determining the stage at which the error occurred remains equivocal due to inherent limitations of RFLP-based genotyping of trisomic individuals. As shown in Fig. 3C, the error can occur either during paternal meiosis I or II if the proband genotype is CCT or CTT, as observed in 4 out of 15 trios (lanes 1, 2, 3). Similarly, the error may occur either in maternal meiosis I or during maternal meiosis II, given that offspring genotype is CCT or CTT in 8 out of 15 families (lane 5, 6, 7). In the preceding 12 trios, the informative parent had the C/C genotype. Interestingly, the rare allele also manifests as the informative T/T genotype in 3 out of 15 trios and the error could correspondingly occur in maternal meiosis II or I as offspring genotypes are CCT or CTT (lanes 9, 10, 11)(Table 3a).

From a total sample of 28 duos we excluded 18 pairs since the genotypes were either all homozygous or heterozygous for the 1173(C/T) marker. We observed that 10 duos possessed offspring genotypes that were recombinant with respect to the available parental genotype and were deemed informative as the CDJP was identified (Fig. 3D) and the missing parental genotype corresponds to the NDJP. However, determining the stage at which the non-disjoining allele originates remains equivocal (Table 3a). Taken together, results indicate that C-1173 is the non-disjoining allele in 22 cases, a total of 12 cases originating in maternal meiosis-I, 7 cases in paternal meiosis-I and 3 cases in which the error is likely at the level of meiosis-II with unknown parent-of-origin. An error in meiosis-II accounts for non-disjunction of the rare T-1173 allele in single case, the parent-of-origin being unresolved. The non-disjoining allele was not ascertained in 20 cases i.e., in 13 cases of maternal origin and 7 cases of paternal origin. When we analyzed the genotypes of 13 DS singletons and found them to be CCC we inferred that the C-1173 allele is the non-disjoining allele in all these cases. This is in agreement with our finding that the major allele frequency for C-1173 is $\sim$0.78 (Table 1). The estimated ratio of meiosis-I to meiosis-II errors arising from allelic non-disjunction of 1173(C/T) is 4:1 in maternal cases and 2:1 in paternal cases (Table 3a).

With respect to 522(A/C), 42 trios were excluded from a total sample of 58 trios, as they are non-informative. As shown in Table 3b, non-disjunction originated in the mother for 10 trios and in the father for 5 trios. However, the parent-of-origin could not be ascertained in the case of 1 trio. The stage of non-disjunction was attributable to meiosis-I in 9 trios and

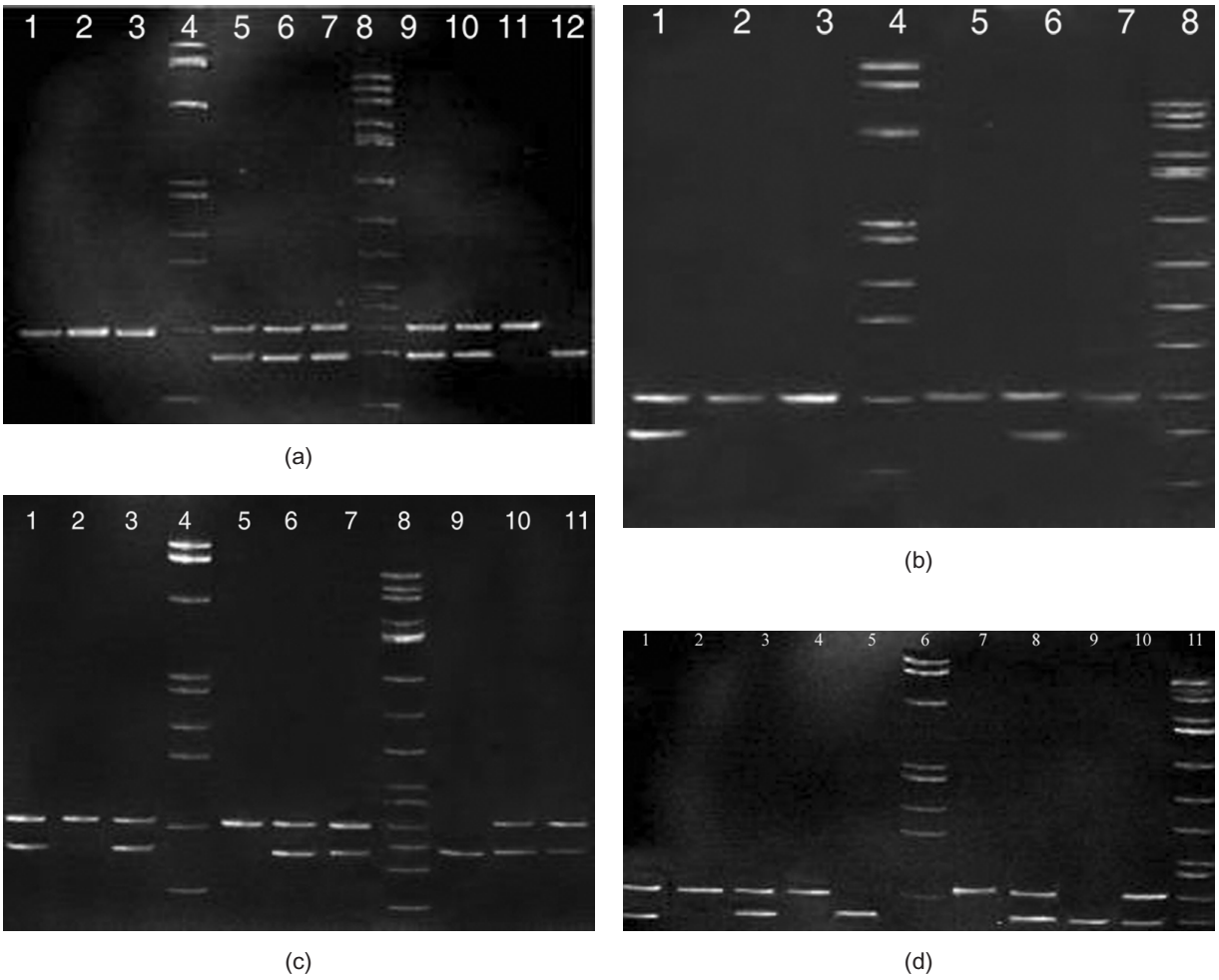

Fig. 3. A: Indicates trio patient family genotyped for 1173(C/T) polymorphism: lane 1: ♂:C/C, lane 2: ♀ C/C, lane 3: proband C/C/C, lane 4: size standards obtained from Φ x174DNA digested with *Hae III*, lane 5: ♂ C/T, lane 6: ♀ C/T, lane 7: proband C/C/T or C/T/T, lane 8: size standards obtained from Φ x174DNA digested with *Hinf I*, lane 9: ♂ C/T, lane 10: ♀ C/T, lane 11: proband C/C/C, lane 12: proband T/T/T. B: Indicates trio patient family genotyped for 1173(C/T) polymorphism: lane 1: father C/T, lane 2: mother C/C, lane 3: C/C/C, lane 4: size standards obtained from Φ x174DNA digested with *Hae III*, lane 5: father C/C, lane 6: mother C/T, lane 7: proband C/C/C, lane 8: size standards obtained from Φ x174DNA digested with *Hinf I*. C: Indicates trio patient family genotyped for 1173(C/T) polymorphism: lane 1: father C/T, lane 2: mother C/C, lane 3: proband C/C/T, lane 4: size standards obtained from Φ x174DNA digested with *Hae III*, lane 5: father C/C, lane 6: mother C/T, lane 7: C/C/T or C/T/T, lane 8: size standards obtained from Φ x174DNA digested with *Hinf I*, lane 9: father T/T, lane 10: mother C/T, lane 11:proband C/C/T or C/T/T. D: Indicates duo family genotyped for 1173(C/T) polymorphism: lane 1: father C/T, lane 2: proband C/C/C, lane 3: mother C/T, lane 4: proband C/C/C, lane 5: proband T/T/T, lane 6: size standards obtained from Φ x174DNA digested with *Hinf I*, lane 7: mother C/C, lane 8: C/C/T or C/T/T, lane 9: mother T/T, lane 10: proband C/C/T or C/T/T, lane 11: size standards obtained from Φ x174DNA digested with *Hinf I*.

meiosis-II in 1 trio whereas it could not be ascertained in 6 trios (Table 3b). We observed that 4 duos (Table 3b) possessed offspring genotypes that were recombinant with respect to the available parental genotype (CDJP) and the NDJP was inferred. Accordingly, non-disjunction occurred in meiosis-I in a single trio while parental origin could not be determined in 3 trios. The A-522 allele was identified as the non-disjoining allele in 14 cases, a total of 9 cases originating in maternal meiosis-I, 4 cases in paternal meiosis-I and 1 case in meiosis-II with equivocal parent-of-origin. Although the PCR-RFLP technique did not permit identification of the non-disjoining allele in 6 families, the parent-of-origin in 5 cases is the mother and in 1 case corresponds to the father. We analyzed the genotypes of 13 DS singletons and found them to be all AAA. Accordingly, we infer that the A-522 allele is the non-disjoining allele in all these cases.

Table 3b
Determination of parent/stage of origin of non-disjunction error in trio/duo families genotyped for 522(A/C) polymorphism

NDJP genotype	CDJP genotype	child genotype	marker status	stage of origin	# trios (T) /duos (D)
AC (♀/♂)	AC (♂/♀)	AAA	R	M-II	1 (T)
AA (♀)	AC (♂)	AAA	N	M-I	5 (T)
AA (♂)	AC (♀)	AAA	N	M-I	4 (T)
AC (♂)	AA (♀)	AAC/ACC	N/R	M-I/M-II	1 (T)
AC (♀)	AA (♂)	AAC/ACC	N/R	M-I/M-II	5 (T)
missing (♀)	AC (♂)	AAA	N	M-I	1 (D)
missing (♂)	AA (♀)	AAC/ACC	N/R	M-I/M-II	2 (D)
missing (♀)	AA (♂)	AAC/ACC	N/R	M-I/M-II	1 (D)

R = marker status reduced to homozygosity, N = marker status not-reduced to homozygosity, M-I = meiosis-I, M-II = meiosis-II.

Table 3c
Determination of parent/stage of origin of non-disjunction error in trio/duo families genotyped for 2705(T/C) polymorphism

NDJP genotype	CDJP genotype	child genotype	marker status	stage of origin	# trios (T) /duos (D)
TT (♀)	TC (♂)	TTT	N	M-I	2 (T)
TT (♂)	TC (♀)	TTT	N	M-I	3 (T)
TC (♂)	TT (♀)	TTC/TCC	N/R	M-I/M-II	4 (T)
TC (♀)	TT (♂)	TTC/TCC	N/R	M-I/M-II	2 (T)
missing (♀)	TC (♂)	TTT	N	M-I	1 (D)
missing (♂)	TC (♀)	TTT	N	M-I	2 (D)
missing (♂)	TT (♀)	TTC/TCC	N/R	M-I/M-II	3 (D)
missing (♀)	TT (♂)	TTC/TCC	N/R	M-I/M-II	2 (D)

R = marker status reduced to homozygosity, N = marker status not-reduced to homozygosity, M-I = meiosis-I, M-II = meiosis-II

From a total of 58 trios, 47 trios where excluded as being non-informative with respect to the 2705(T/C) marker. As shown in Table 3c, non-disjunction originates in the mother for 4 trios and in the father for 7 trios. The stage of non-disjunction was attributable to meiosis-I in 5 trios and remained equivocal in 6 trios (Table 3c). We observed that 8 duos possessed offspring genotypes that were recombinant with respect to the available parental genotype (CDJP) and the NDJP was thus inferred. Accordingly, in 3 duos, the non-disjunction error occurred in meiosis-I, whereas the parent of origin remained equivocal in the remaining 5 duos. Results show that T-2705 is the non-disjoining allele in 8 cases, with 3 cases arising in maternal meiosis-I and 5 other cases arising in paternal meiosis-I (Table 3c). Though the PCR-RFLP assay did not permit identification of non-disjoining allele in 11 families, maternal non-disjunction was identified in 4 cases and with 7 cases being paternal in origin (Table 3c).

We found one trio in which the non-disjoining allele, the parent and stage-of-origin of non-disjunction could be ascertained unequivocally for all three [522(A/C), 1173(C/T) and 2705(T/C)] markers. The non-disjoining alleles were independently identified as A-522, C-1173 and T-2705, respectively. Non-disjunction during maternal meiosis-I accounts for the observed genotype of this DS patient. Data from this family thus furnishes indirect evidence of triplication of the GluK1 gene in the patient since A522-C1173-T2705 alleles are predicted to lie on the same chromosome (Table 2).

4. Discussion

This study presents evidence for allelic non-disjunction at 1173(C/T) in the GluK1 gene that is an important candidate for genetic studies on DS-related MR. Moderate heterozygosity (0.339) and a major allele frequency of 0.78 renders the 1173(C/T) polymorphism informative (Table 1) and the estimated proportion of meiosis-I to meiosis-II errors in the sample is 4:1 in maternal cases, whereas, in paternal cases the ratio is 2:1 (Table 3a). Therefore, we infer that varying risk for allelic non-disjunction derives from cellular environment during chromosome segregation. Despite lower heterozygosity values estimated for the other polymor-

phisms studied (Table 1), we report that the distribution of allele frequencies is in Hardy-Weinberg equilibrium and that the 522(A/C)-1173(C/T) and 1173(C/T)-2705(T/C) polymorphic systems are in significant linkage disequilibrium of weak magnitude (Table 2).

For the 1173(C/T) polymorphism, the estimated major allele (C-1173) frequency (Table 1) is similar to reports from German (0.82) [8] but different from Japanese (0.65) populations [14]. Furthermore, the C-522 and C-2705 alleles are rare in our sample (Table 1). Though the estimated frequencies for A522-C1173 and C1173-T2705 haplotypes are similar in magnitude (Table 2), the low D' and r^2 values are indicative of weak linkage disequilibrium (Table 2). No LD exists between the 522(A/C) and 2705(T/C) markers (Table 2) that are $\sim$136 kb apart and it is likely that LD erodes as a function of distance between markers [22] and/or due to their position in the medial portion of the meiotic recombination map [5,33]. Accordingly, it precludes the prediction of a robust risk haplotype although our sample included one informative trio in which the non-disjoining alleles were independently identified as A-522, C-1173 and T-2705, respectively. Further studies with additional genetic markers and more patient families are warranted to substantiate the present findings.

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