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SHORT REPORT

Failure to detect DUP25 in lymphoblastoid cells derived from patients with panic disorder and control individuals representing European and American populations

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Investigation of the co-occurrence of panic and phobic disorders with joint laxity led to the identification of interstitial duplications involving human chromosome 15q24–26 (named 'DUP25') in a Spanish population. DUP25 was observed in 97% of patients and in 7% of control individuals. In the present study, we used two different methods to detect DUP25: high-throughput molecular gene dosage analysis and fluorescence *in situ* hybridization (FISH). We evaluated 56 lymphoblastoid cell lines derived from 26 unrelated patients with panic disorder obtained from several European and American populations and 30 normal controls. We could not find any cell line showing a result consistent with DUP25. These data do not support any association of DUP25 with panic disorder.
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Introduction

Gratacòs *et al*[1] reported that a polymorphic genomic interstitial duplication of 15q24–q26 ('DUP25') was associated with both familial and nonfamilial panic and phobic disorders. The chromosomal analyses were performed in both peripheral blood lymphocytes and sperm. In 70 unrelated patients with an anxiety disorder, DUP25 was found in 68. Among 189 controls, DUP25 was seen in only 14. The DUP25-positive cells were almost always in the majority, with 59% being the average proportion among positive individuals. These observations are extremely interesting because they represent an association between a genetic difference and common psychiatric disorders. Second, the pattern of DUP25 distribution in families was that of a very common, *de novo* mutation, with mosaicism in affected family members with panic or phobic disorders.

We used two different methods to detect DUP25: high-throughput molecular gene dosage analysis and fluorescence *in situ* hybridization (FISH). We studied 56 lymphoblastoid cell lines derived from 26 unrelated patients with panic disorder and 30 normal controls. Such cell lines are polyclonal and derived from B-lymphocyte

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precursors. We found no cell line showing a result consistent with DUP25.

Materials and methods

Lymphoblastoid cell lines

Lymphoblastoid cell lines were prepared using a standard Epstein–Barr virus transformation protocol and stored at −140°C until use. A total of 56 lymphoblastoid cell lines were examined for DUP25, including 26 derived from unrelated patients with panic disorder. The panic disorder sample consisted of 10 Italian Caucasians, seven American Caucasians, five Finnish Caucasians and four American Indians (16 female and, 10 male subjects, aged from 20 to 68 years, Table 1). A total of 30 unrelated normal controls included 10 Italian Caucasians and 20 American Caucasians (16 female and, 14 male subjects, aged from 15 to 80 years). Subjects received a structured psychiatric interview: either the SADS-L (American Indians) or the SCID (all others). Diagnoses were by DSM IIIR criteria. All patients gave written informed consent to participate in the study. Human research protocols were approved by the Institutional Review Boards of the National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, the National Institute of Mental Health, Bethesda, MD, the University of Helsinki, Helsinki, the University of Pisa, Pisa, and by the Office for Protection From Research Risks, Bethesda, MD. DNA was isolated for gene dosage analysis and chromosomes were prepared for FISH.

Real-time quantitative PCR for gene dosage analysis

Gene dosage analysis of *NTRK3*, a candidate gene identified within DUP25[1] was performed using real-time quantitative PCR, by modification of a previously reported method.[2] Fluorescent signals for a representative DUP25 sequence and a control sequence were generated using specific probes labeled with a signal dye and a quencher dye – with liberation of signal by the 5′ nuclease cleavage of the nucleotide coupled to the signal dye. *NTRK3* and a control sequence (serotonin transporter gene, *5-HTT*) located on chromosome 17 were coamplified, and amplification of both loci was simultaneously monitored in the same reaction well in real time. Primers and probe for *NTRK3* were: forward, 5′-CTGGAGCACTGCATCGAGTTT-3′; reverse, 5′-GAAATCTCTCCCTCTTGGTAGTATTCC-3′; and probe, 5′-(VIC) CGCTGCACTGGCTGCACAATG (TAMRA)-3′. Primers and probe for *5-HTT* were: forward, 5′-TGGC-CTGGGCGC TATACTAC-3′; reverse, 5′-GTTGTCCTCGGAGAAGTAATT GGT-3′; and probe, 5′-(FAM) ACCAGCTGCAAGAACTCC TGGAACA (TAMRA)-3′. The 25-μl reaction volume contained 2 μl (100ng) genomic DNA, 1 μM of each primer, 0.2 μM of each dual-labeled probe, and further components supplied in TaqMan Universal PCR Master Mix (Perkin–Elmer), corresponding to 3.5 mM $MgCl_2$, 100 μM dNTPs, 0.025 U/μl AmpliTaq Gold, and 0.01 U/μl Amp Erase. Cycling conditions were as follows: incubation for 2 min at 50°C to permit Amp Erase activity, and for 10 min at

Table 1 Patients with panic disorder

Patient	*Population*	*Age (year)*	*Sex*	*DSM-IIIR Diagnoses*
1	Italian	37	F	Panic disorder with agoraphobia, major depression
2	Italian	63	M	Panic disorder with agoraphobia
3	Italian	46	F	Panic disorder without agoraphobia, social phobia, obsessive–compulsive disorder, dysthymia
4	Italian	43	F	Panic disorder with agoraphobia, major depression
5	Italian	20	F	Panic disorder, social phobia, major depression
6	Italian	62	M	Panic disorder with agoraphobia, simple phobia
7	Italian	33	F	Panic disorder, major depression
8	Italian	42	F	Panic disorder without agoraphobia, bipolar II disorder
9	Italian	55	M	Panic disorder without agoraphobia, major depression
10	Italian	24	M	Panic disorder without agoraphobia, simple phobia
11	American	26	F	Panic disorder, phobia
12	American	30	F	Panic disorder, major depression
13	American	41	F	Panic disorder, major depression
14	American	40	F	Panic disorder, major depression
15	American	23	F	Panic disorder, major depression, obsessive compulsive disorder
16	American	42	F	Panic disorder, major depression
17	American	36	M	Panic disorder, major depression
18	Finnish	39	M	Panic disorder
19	Finnish	25	M	Panic disorder
20	Finnish	45	F	Panic disorder, alcohol dependence
21	Finnish	41	M	Panic disorder
22	Finnish	56	M	Panic disorder, major depression, simple phobia, alcohol dependence
23	Am Indian	51	F	Panic disorder, major depression, phobia
24	Am Indian	68	M	Panic disorder
25	Am Indian	48	F	Panic disorder, major depression, phobia
26	Am Indian	27	F	Panic disorder, major depression, phobia, post-traumatic stress disorder

95°C for AmpliTaq Gold activation and DNA denaturation, followed by 40 cycles of 1 min at 60°C and 15 s at 95°C. The ratio of the target *vs* control sequence was determined by the difference in threshold cycle value (ΔCt) at fluorescence intensity threshold ΔRn = 0.03. Triplicate assays were performed for each sample.

FISH testing

FISH was performed on all Italian patients (10) and controls (10), and on 16 other panic disorder patients. Slides for FISH were prepared using standard procedures. A 640 kb YAC (957b4) which maps to the DUP25 region[1] was selected as probe for FISH. The YAC DNA was processed as described elsewhere.[3,4] YAC DNA was amplified using a degenerate oligonucleotide primed PCR (DOP-PCR) protocol: 25 μl reaction mix contained 1 μl template DNA (about 50 ng), 2.5 μl 10 × Buffer, 3.5 mmol/l $MgCl_2$, 0.25 mmol/l dNTP, 0.05 U/μl *Taq* polymerase, 2 μmol/l primer (5′-CCGACTCGAGNNNNNNATGTGG-3′). The PCR was performed for 35 cycles with a predenaturation temperature of 94°C for 1 min, a denaturation temperature of 94°C for 1 min, an annealing temperature of 56°C for 1 min, and an extension temperature of 72°C for 4 min for every single cycle, and ending at 72°C for 10 min. Probe labeling with tetramethylrhodamine (TMR) was performed by DOP-PCR: 10 μl reaction mix consisted of 1 μl product from the PCR above, 1 μl 10 × Buffer, 4 mmol/l $MgCl_2$, 0.2 mmol/l d(ACG)TP, 0.15 mmol/l dTTP, 0.05 mmol/l TMR-6-dUTP, 0.1 U/μl *Taq* polymerase, and 6 μmol/l degenerate oligonucleotide primer with the same sequence as above. The PCR was performed for 25 cycles with a predenaturation temperature of 95°C for 3 min, a denaturation temperature of 94°C for 1 min, an annealing temperature of 56°C for 1 min, and an extension temperature of 72°C for 2 min for every single cycle, and ending at 72°C for 10 min. Products were cut with DNase I (0.2 U/ml, 37°C 3 min) and mixed with Cot-1 DNA (20 μg). Labeled probes were purified by cold ethanol precipitation, and dissolved in 8 μl 1 × TE. The additional FISH experiment on patient No. 25 who showed DUP25 look-alike signals with YAC 957b4 in a subset of metaphases was performed using cosmids c251-3 and t216-1 from Gratacòs *et al*[1] with a previously described amplification and labeling procedure.[4]

FISH: A mixture of 1.6 μl probe solution and 5.4 μl Hybrisol VI (Oncor, Gaithersburg, MD, USA) was pipetted onto the slide containing target metaphases. The slide was covered with a coverslip and rubber cement and heated at 72°C for 5 min, incubated at 37°C for 48 h, washed with 50°C formamide/2 × SSC solution at 42°C for 7 min, washed with 0.4 × SSC solution at 42°C for 10 min, and stained with DAPI. Microscopy was performed with an Axiophot epifluorescence microscope (Carl Zeiss, Göttingen, Germany) and the ISIS digital imaging system (MetaSystems, Altlussheim, Germany).

Results

By quantitative gene dosage analysis, we observed ΔCt = 0.03 ± 0.16 for control samples and ΔCt = 0.00 ± 0.15 for patient samples, indicating no difference for the fluorescent signal for the DUP25 locus and the control locus on chromosome 17. The highest ΔCt was observed in Patient No. 25 with ΔCt = 0.3 (from two independent triplicate measurements), yielding a ratio of 1.23:1 for *NTRK3* to the control locus (Figure 1).

For FISH of 26 patients and 10 controls, 20–49 metaphases (mean = 28 ± 5, median = 30) from each cell line were analyzed for DUP25 by one cytogeneticist (CS) and confirmed by a well-experienced molecular cytogeneticist (OB). One patient (No. 25) showed DUP25 look-alike signals. In this individual, 19/49 (37%) analyzed cells seemed to show four-spot or three-spot hybridization signals on one chromosome 15 homologue and a normal hybridization signal on the other chromosome 15 homologue, the other 30 analyzed cells showed the usual pattern of positive hybridization to both chromosome 15 homologues and no evidence of duplication of the region. In other patients and in all controls, the number of cells that were abnormal was few or none. In total, 11 patients and four controls showed a few (1, 2 or 3) cells with four hybridization spots on one chromosome 15 homologue and a normal twin-spot signal on the other homologue. However, based on the low frequency of 4-spot-cells per individual, we interpreted these observations as split-signal artefacts (normal cells). Possible explanations for the observed split-signals include preparation artefacts such as DNA fibers leaking out of their chromosomal position and/or the distribution of single-copy *vs* repetitive sequences within the YAC probe used. Since patient No. 25 was also the subject in which the highest ΔCt was observed by repeated quantitative gene dosage analysis, we repeated FISH experiments on this particular case using two cosmid

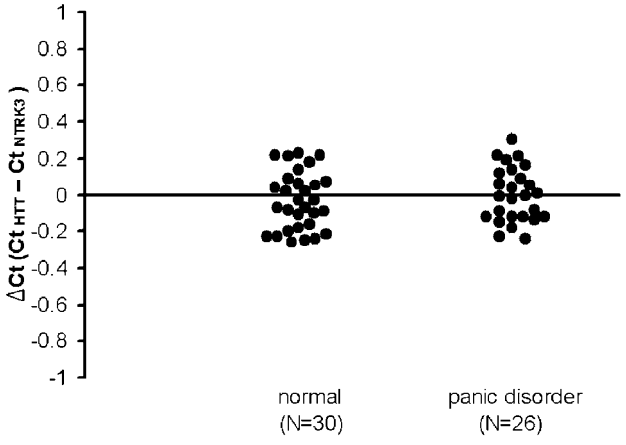

Figure 1 Ratios of a DUP25-specific locus to a control locus in patients with panic disorder and normal controls.

probes, c251-3 and t216-1, that have been used by Gratacòs *et al*.[1] A total of 20 metaphases were evaluated for each probe. Only one cell hybridized with probe c251-3 showed a pattern compatible with DUP25 and the other 39 cells showed normal signals. This result substantially excluded the existence of DUP25 in any individual of our sample.

Discussion

The results of this study do not support the presence of DUP25 in patients with panic disorder. [1] Our findings are consistent with Tabiner *et al*,[5] who detected DUP25 in none of 16 patients with panic disorder and 40 control individuals. In contrast, Gratacòs *et al*[1] had found that 97% of panic disorder patients had DUP25 and the average percentage of metaphases with DUP25 was 59%.

Three of the lymphoblastoid cell lines reported by Gratacòs *et al*[1] as positive for DUP25 have been studied for a second time: independently by Tabiner *et al*[5] and again by Gratacòs *et al*.[1] Tabiner *et al*[5] were not able to detect DUP25 in these cell lines, but Gratacòs *et al*[1] again did. More recently, Weiland *et al*[6] used multicolor FISH for confirmation of two slides supposed to be DUP25 positive by Gratacòs *et al*,[1] one made from a lymphoblastoid cell line and the other made directly from lymphocytes. Again, the results were negative. Taken together with our results, it is highly likely that technical problems led Gratacòs *et al*[1] to overestimate the abundance of DUP25. In our FISH study, we also observed a few DUP25 look-alike cells in about half of individuals, but none with the frequency of DUP25 metaphases reported by Gratacòs *et al*.[1] These FISH results were fully congruent with the quantitative PCR.

Many factors can critically influence FISH. These include chromosome preparation, probe size and content of repetitive DNA sequences within the probe. A short distance between signals in small duplications as occurs with DUP25 makes it most difficult to obtain a clear result with metaphase FISH, unaided by a reliable gene dosage method as used in this study. Up to now, the observation of Gratacòs *et al*[1] has not been confirmed by any other group. Instead, accumulating evidence indicates that DUP25 may not actually exist. Further studies combining FISH and gene dosage methods are needed to clarify the existence of DUP25 in different populations and the relationship between DUP25 and panic disorders, or other phenotypes. It would be of great interest if this kind of study could be replicated in the Spanish population.

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