TITLE

Identification of a recurrent breakpoint within the SHANK3 gene in the 22q13.3 deletion syndrome.

AUTHORS

Maria Clara Bonaglia (1,*), Roberto Giorda (1,*), Elisa Mani (1), Giuseppe Aceti (1), Britt-Marie Anderlid (2) Anna Baroncini (3), Tiziano Pramparo (4) and Orsetta Zuffardi (4,5)

* The first two authors contributed equally to this work

AUTHORS’ AFFILIATIONS

1- Scientific Institute Eugenio Medea, Bosisio Parini LC, Italy
2- Department of Molecular Medicine, Clinical Genetic Unit, Karolinska Hospital, Stockholm, Sweden
3- U.O Genetica Medica, AUSL Imola, Italy
4- Biologia Generale e Genetica Medica, Università di Pavia, Pavia, Italy
5- IRCCS Policlinico San Matteo, Pavia, Italy

KEYWORDS

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CORRESPONDING AUTHOR

Maria Clara Bonaglia, Scientific Institute E. Medea, via don Luigi Monza 20, 23842 Bosisio Parini, Lecco, Italy.
E-mail: bonaglia@bp.lnf.it
ABSTRACT

Introduction
The 22q13.3 deletion syndrome (MIM 606232) is characterized by neonatal hypotonia, normal to accelerated growth, absent to severely delayed speech, global developmental delay, and minor dysmorphic facial features. We report the molecular characterization of the deletion breakpoint in two unrelated chromosome 22q13.3 deletion cases.

Material and Methods
The deletions were characterized by Fish, checked for other abnormalities by Array-CGH, confirmed by Real-Time PCR, and finally the breakpoints were cloned, sequenced and compared.

Results
Both cases show the cardinal features of the 22q13.3 deletion syndrome associated with a deletion involving the last 100 Kb of chromosome 22q13.3. The cases show a breakpoint within the same 15 base-pair repeat unit overlapping the results obtained by Wong and colleagues in 1997 and suggesting that a recurrent deletion breakpoint exists within the SHANK3 gene. The direct repeat involved in these 22q13 deletion cases is presumably able to form slipped (hairpin) structures, but it also has a strong potential for forming tetraplex structures.

Discussion
The three cases with the common breakpoint within SHANK3 share a number of common phenotypic features, such as mental retardation and developmental delay with severely delayed or absent expressive speech.

The two cases presented here, having a deletion partially overlapping the commercial subtelomeric probe, highlight the difficulties in interpreting FISH results and suggest that many similar cases may be overlooked.
INTRODUCTION

Cryptic deletions involving the distal portion of chromosomes have been demonstrated to contribute to mental retardation, dysmorphisms and/or congenital anomalies accounting for approximately 5% of these cases.[1] The introduction of different molecular techniques aimed to detect subtelomeric regions led to the definition of new genetic syndromes resulting from distal microdeletions. At the moment, the best known distal deletion syndromes involve 22q13.3,[2] 1p36,[3] 2q37.3,[4] and 3q29.[5] Identification of the 22qter deletion has been serendipitous in some patients referred for VCF/DGS.[6] About 75% of individuals with the syndrome have simple terminal or interstitial deletions, 25% have deletions resulting from an unbalanced translocation or ring 22q syndrome.[6][7][8] Previously, we have shown that the disruption of SHANK3/ProSAP2 gene in a subject with a de novo balanced translocation t(12;22)(q24.1;q13.3) results in the same features of the 22q13.3 deletion syndrome.[9] Now we report the molecular characterization of the deletion breakpoints in two unrelated cases, a new one and the one reported by Anderlid et al.[10] both showing the cardinal features of the 22q13.3 deletion syndrome and associated with the smallest deletion up to now reported involving the last 100 kb of 22q13.3. Our results overlap those obtained by Wong.[2] These three unrelated cases have essentially coincident breakpoints defined at the base-pair level and suggest that a deletion hot spot exists within the SHANK3. To our knowledge, this is the first instance of terminal deletions having a recurrent breakpoint.

SUBJECTS AND METHODS

Case History

Medical history

Our new patient was the first child of healthy unrelated parents whose family history was unremarkable. Her younger brother was healthy. She was born at 40 weeks after an uneventful pregnancy. Her birth weight was 3360 g (between 50th and 75th centile), birth length 51 cm (75th centile), and occipital-frontal circumference (OFC) 35 cm (10th centile). The perinatal period was uneventful and hypotonia was not recorded. Until the age of 1 year her developmental milestones were normal. She started walking at the age of 11-12 months. Her language development was severely impaired: she spoke her first 2-3 words at the age of 1 year, but then failed to develop spoken language. At the age of two years because of recurrent otitis she underwent hearing evaluation (including also brainstem auditory evoked responses) showing no hearing impairment. Southern analysis for Fragile X and methylation-specific PCR analysis for Angelman syndrome were negative.

Physical Examination

At age 17 years her stature is 164.5 cm (50th centile), her weight 93 Kg (> 97th centile) and head circumference 56 cm (between 75th and 90th centile). Obesity (with a present Body Mass Index of 34,2) was related to hyperphagia. She has subtle facial dysmorphisms including long and flat face, brachycephaly (cephalic index of 85,6 %), deep-set eyes, short phyltrum, mild prognatism, hypoplastic ear lobules and macrostomia (intercommissural distance of 5,6 cm, > 97th centile). 2-3 syndactyly of the toes with toenail hypoplasia is also apparent. Neurological examination is normal. Menarche occurred at age 13 and since then menses are regular with a 28-day cycle.

Psychiatric Examination

Since the age of two years her cognitive function was severely impaired; she showed severe mental retardation, language was absent and autistic traits were noticed. From early infancy she displayed sleep disturbances, anxiety and poor eye contact. Later she developed balance problems and stereotypic hand movements. She has impaired fine and gross motor skills. She shows social interactions limited to expressing pleasure and needs, scarce respect for rules but no aggressive behavior. She is partially able to feed and clean herself autonomously and to perform simple tasks. At present, she has no bowel control, while diurnal enuresis stopped at 13 years of age.
Psychiatric Evaluation
At age 13 years,1 month a formal psychiatric evaluation was undertaken. Overall, on the Psycho-Educational Profile-Revised (PEP-R) [11] the girl achieved an age equivalent (AE) of about 16 months, indicating a profound delay in all developmental milestones. The Vineland Adaptive Behaviour Scale (VABS) [12] was administered to both parents. Her global AE was less than 18 months, corresponding to severe mental deficiency. Receptive language and expressive skills scores were below the bottom of the scale; socialization AE was less than 18 months; daily living skills AE was 32 months; motor skills AE was 23 months.

Cytogenetic and FISH analysis
Routine cytogenetic analysis (500-550 band level) was performed on the proposita and her parents’ blood using standard high-resolution techniques. The Chromoprobe –T kit with telomere specific probes (Cytocell) and the ARSA probe (LSI DiGeorge/VCFS regions probe, Vysis) were used according to suppliers’ instructions. The 22q AquariusT probe (Cytocell) was also used to re-test proposita and parents. Cosmids n66c4 (AC000050), n85a3 (AC000036), n94h12 (AC002056) and n1g3 (AC002055) were labeled with biotin-dUTP (Vector Laboratories, Burlingame, CA) using standard nick translation reactions. The biotin-dUTP labeled probes were visualized with FITC-avidin (Vector) and the chromosomes were counterstained with DAPI (Sigma Aldrich, Milano, Italy). Hybridizations were analyzed with an Olympus BX61 epifluorescence microscope and images were captured with the Power Gene FISH System (PSI, Newcastle upon Tyne, UK).

Array-CGH analysis
Array-CGH was performed using the Agilent Human Genome CGH Microarray Kit 44B (Agilent Technologies). This platform is a high-resolution 60-mer oligonucleotide-based microarray that allows genome-wide survey and molecular profiling of genomic aberrations with a resolution of ~75 kb. Labelling and hybridization were performed following the protocols provided by Agilent. Briefly, 4µg of purified DNA of the patient and of a female control (Promega) were double-digested with Rsal and AluI for two hours at 37 degrees. After columns purification, 1µg of each digested sample was labelled by random priming (Invitrogen) for two hours using Cy5-dUTP for the patient DNA and Cy3-dUTP for the control DNA. Labelled products were column purified and prepared according to the Agilent protocol. After probe denaturation and pre-annealing with 50µg of Cot-1 DNA, hybridization was performed at 65 degrees with rotation for 40 hours. After two washing steps, the array was analysed with the Agilent scanner and the Feature Extraction software (v8.0). A graphical overview was obtained using the CGH analytics software (v3.1).

Real-Time PCR analysis.
The annotated genomic sequence of chromosome 22 is available through the UCSC Human Genome Browser (May 2004 assembly) (http://genome.ucsc.edu/cgi-bin/Gateway). The genomic structure of SHANK3 is based in part on the data from [9] and [13] and on the sequence of murine cDNAs (NM_021423 and NM_021676). Exons were numbered according to the murine orthologs. Seven chromosome 22-specific target sequences for Real-Time PCR analysis (amplicons 22-1 to 22-7) were selected at approximately 5 Kb intervals within non-repeated portions of the SHANK3 gene using Primer Express software (Applied Biosystems); a control amplicon was selected with the same parameters in the MAPK1 gene on 22q11.2; size (approximately 60 nt) and TM (58°C) were the same for all amplicons. Primer sequences are shown on Table I.
Table I. Chromosome 22 Real-Time PCR primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>22-1F</td>
<td>AGGCGGGTGATGTTCAGATG</td>
<td>22-1R</td>
<td>AACACCGGCCGTCAGTCA</td>
</tr>
<tr>
<td>22-2F</td>
<td>CCTGCGCACGCCATGT</td>
<td>22-2R</td>
<td>GAGACCATTGGCGGACAACAG</td>
</tr>
<tr>
<td>22-3F</td>
<td>CAGCGGGCAGCAAACG</td>
<td>22-3R</td>
<td>CACATTGAACCAAGTGGAGTCCA</td>
</tr>
<tr>
<td>22-4F</td>
<td>AACATCAATGGGCCCTTAGCA</td>
<td>22-4R</td>
<td>CAGCCCCCTCCTACCTGATC</td>
</tr>
<tr>
<td>22-5F</td>
<td>GACACACGGCCTGTTGAGT</td>
<td>22-5R</td>
<td>ATTTAGCACAAGAGGAGAACAAGG</td>
</tr>
<tr>
<td>22-6F</td>
<td>CGCCTCGTCATGAGGTTGT</td>
<td>22-6R</td>
<td>CGAGCCCCGTCTCTTTT</td>
</tr>
<tr>
<td>22-7F</td>
<td>GCTGTCTCTGCCCTAAAATG</td>
<td>22-7R</td>
<td>GCACAAAAAGCTACAAGAGCA</td>
</tr>
<tr>
<td>MAPK-F</td>
<td>CGAAATAACATTACCTTTGGAGTCA</td>
<td>MAPK-R</td>
<td>CACAAAAATAAAGGTGCGATGGA</td>
</tr>
</tbody>
</table>

Amplification and detection were performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems); thermal cycling conditions were 50°C, 2 minutes, 95°C, 10 minutes, followed by 40 cycles of 95°C, 15 seconds, 60°C, 1 minute; all samples were amplified in quadruplicate.

Validation experiments demonstrated that amplification efficiencies of the control and all target amplicons were approximately equal (not shown); accordingly, relative quantification of DNA amount was obtained using the Comparative CT method (described in Applied Biosystems User Bulletin #2, December 11, 1997: ABI PRISM 7700 Sequence Detection System).

**Breakpoint cloning.**

Three chromosome 22-specific primers adjacent to the deletion breakpoints (primers F1 to F3) and a telomere-specific Annealing Control Primer (Tel-ACP) (5’ TCA CAG AAG TAT GCC AAG CGA III IIA ACC CTA ACC CT 3’) were designed. The telomere-specific primer contained two copies of the telomeric AACCT repeat and a polydeoxyinosine linker between the 3’ end target core sequence and the 5’ end non-target universal sequence;[14] a universal primer (Un: 5’ TCACAGAAGTATGCCAAGCGA 3’) was also designed. The amplification strategy calls for a PCR with primers del22-1+Tel-ACP, followed by two nested PCRs with primers del22-2+Un and del22-3+Un, respectively. The first PCR (PCR1) features two cycles with low-stringency annealing (30 sec at 95°C, 1 min at 50°C, 2 min at 68°C) followed by 30 cycles (30 sec at 94°C, 30 sec at 58°C, 3 min at 68°C); the second PCR (PCR2) has 35 cycles of (30 sec at 94°C, 30 sec at 58°C, 3 min at 68°C), the third (PCR3) has 30 cycles of (30 sec at 94°C, 30 sec at 60°C, 3 min at 68°C). JumpStart Red AccuTaq DNA polymerase (Sigma) was used in all amplifications. PCR3 products were both directly sequenced and cloned with a TOPO TA cloning kit (Invitrogen), followed by sequencing of individual clones. Sequencing reactions were performed with the BigDye Cycle Terminator Sequencing kit (Applied Biosystems) and run on an ABI Prism 3100 AV Genetic Analyzer.

**RESULTS**

**Molecular characterization of the deletion**

FISH tests with subtelomeric probes (Multi-T Cytocell) revealed a slight difference in intensity between the signals detected on the two 22q homologous chromosomes, which led us to suspect a partial 22qter deletion. The patient was re-tested with a single tel22q probe (Cytocell) and FISH analysis confirmed the previous results (Figure 1A). FISH with the ARSA probe (Cytocell) showed two equally strong hybridization signals (Figure 1C). To rule out a possible polymorphism, FISH
analysis with probe Tel 22q was extended to the parents, and a normal hybridization pattern in all 50 metaphases analyzed was observed (Figure 1B shows the father’s Tel22q Fish analysis). FISH analysis with cosmid probe n85a3, partially overlapping the Tel22q probe, confirmed the deletion (Figure 1E). The deletion breakpoint was refined using the following cosmid clones: n66c4, partially overlapping n85a3, gave normal hybridization pattern (Figure 1D), while cosmid clones n85a3 (Figure 1E), n94h12 (Figure 1F), and n1g3 (not shown) were deleted. Thus, the breakpoint was localized in the proximal part of n85a3, which contains the SHANK3 gene, and the size of the deletion was estimated to be 100 Kb (Fig. 1, map). These data overlap those obtained by Anderlid et al.[10] FISH analysis on the parents with probes 22q (Cytocell), n66c4, n85a3, 92h12 and n1g3 gave normal results in all 50 metaphases analyzed (not shown).

To exclude the presence of cryptic imbalances (microdeletion e/o microduplication) in other locations of the genome, we performed genome-wide array-CGH with an average resolution of 75 kb (Agilent). The array contains oligomers for the ARSA and ACR genes, whereas SHANK3 is not represented. The analysis resulted normal apart from the two ACR targets at distal 22q that were deleted (data not shown).

**Molecular analysis of our case and the case previously reported in Anderlid 2002.**

Real-Time PCR quantification showed that a normal control and a deleted control could be easily differentiated at all seven amplicons; both subjects under study appeared to have normal copy number for 22-1 and 22-2, and to be deleted for 22-3 to 22-7 (Figure 2A). We used ACP technology [14] to clone the deletion breakpoints, selecting chromosome 22-specific primers in the L2 region, immediately adjacent to the D22S163 polymorphism (Figure 3).[15] Nested amplification generated specific fragments of approximately 500 bp in the two patients only; patients and normal control shared a non-specific 1.6 Kb fragment (Figure 2B). Sequencing of the 500 bp fragments showed truncation of the chromosome 22 sequence and healing by the addition of a telomere repeat array (Figure 2C). The two breakpoints are at a distance of no more than 15 bases, within a short simple repeat located between exons 8 and 9 of SHANK3, 900 bp distal to MS607A and approximately 2 Kb proximal to MS607B.[15] Small variations in the sequence of the terminal nucleotides suggest artifactual recombination between copies of the simple repeat during PCR or telomerase “stuttering” at the start of repeat addition (Figure 2C). The overall structure of the SHANK3 gene, together with the position of all known breakpoints, is shown in Figure 3.

**DISCUSSION**

**Breakpoint analysis**

The molecular basis of the mechanisms leading to terminal deletions is poorly defined, essentially for two reasons: first, the breakpoints have been determined at the base-pair level only in few cases; second, the observed breakpoints could be different from the original breakpoints as a consequence of the action of repair mechanisms activated by DNA double strand breakages (DSBs).[16] Molecular analysis of a large cohort of monosomy 1p36 subjects demonstrated that deletion sizes vary widely from about 1 Mb to more than 10.5 Mb in the most distal portion of 1p36 with no single common breakpoint.[3] This is also the case with the majority of 22q13 deletions, but in the two cases analyzed here the deletion breakpoint falls within SHANK3 at a distance of no more than 15 base pairs, inside a short simple repeat located between exons 8 and 9 of the gene. The breakpoints of two additional rearrangements involving SHANK3 have been determined so far; in a 22q deletion [2] the breakpoint was located within the same 15 base pair repeat, while in a t(12;22) translocation [9] it was located in exon 21 (see map in Figure 3). Our data demonstrate that the region containing the D22S163 polymorphism [15] and its flanking repeats constitutes a tight deletion hotspot within the SHANK3 gene. The highly polymorphic locus detected by microsatellite clone cMS607 (D22S163) in MboI digested DNA was described as being composed of two distinct microsatellite arrays, one with moderate heterozygosity (607A) contained in the cMS607 plasmid,
the other, contributing to the greatest part of the variability, (697B) not contained in cMS607.[15]
Our re-analysis based on the published chromosome 22 sequence shows that D22S163 contains
only a minisatellite repeat with an 88 base pair period (Chr. 22: 49408021-49411525) and moderate
size variability. Most of its heterozygosity is due to variations in the number and location of MboI
restriction sites. The shorter simple repeat involved in 22q13.3 recurrent breakpoints lies distally
approximately 800 base pairs from D22S163, while a third, apparently non-polymorphic,
minisatellite repeat (Chr.22: 49413872-49415514) is located 1.4 Kbase pairs downstream of the
second repeat. No recombination-associated motifs [17] were identified in or around the breakpoint
region. In addition, MFOld analysis [18] did not reveal any structural feature that could explain
the hotspot region’s extreme narrowness. An answer may lie in the sequence itself, an imperfect
(AGAGGGGGGTGGTGG)10 repeat with a >75% G content. The repeat does not appear to be
variable in size, since Southern blot analysis revealed a PvuII band of less than 1 Kb in all subjects
tested, a size consistent with the 930 bp expected from the chromosome 22 genomic sequence (not
shown). However, it is quite refractory to amplification, yielding fragments of variable size and
repeat copy number, and sequencing, making it hard to determine with absolute certainty the
parental sequence. Thus, although variations in the sequence of the terminal nucleotides (Figure 2C)
are probably due to recombination between copies of the simple repeat, there is still a chance that
other mechanisms, such as errors in sequence repair by telomerase at the start of repeat addition,
may be involved.
Telomere healing has been demonstrated in a series of non-recurrent terminal deletions of
chromosomes 1p,[16] 7q,[19] 16p,[20][21][22] and 22q.[19] For the first time, we demonstrate the
presence of a hotspot for a recurrent terminal deletion healed by de novo telomere addition.
Genomic rearrangements, such as translocations and gross deletions, have been associated with the
presence of non-B DNA conformations at or near the breakpoints.[23][24][25] Some of the
sequences involved in the formation of non-B structures are inverted, mirror and direct repeats, left-
headed Z-DNA and tetraplex-forming sequences.[24] The direct repeat involved in the 22q13
deletion is presumably able to form slipped (hairpin) structures, but it also has a strong potential for
forming tetraplexes. In fact, using computational methods [26], we can predict that the SHANK3
repeat is about four times more likely to form a tetraplex structure (G-quartet score: 0.666) than the
telomeric (T2AG3)n repeat (score: 0.166), where tetraplex formation is well documented [27]. In this
respect, it is intriguing to speculate that telomerase could be more efficiently recruited to breakpoint
sites with a telomere-like structure.

The 22q13.3 syndrome
This deletion syndrome is characterized by neonatal hypotonia, normal to accelerated growth,
absent to severely delayed speech, global developmental delay, and minor dysmorphic facial
features.[6] Additional clinical features suggestive of 22q13.3 deletion include relatively large and
fleshy hands, dysplastic toenails, sacral dimple, decreased perspiration,[7] and behavioural
characteristics consisting in mouthing or chewing non-food items, increased tolerance to pain, and
autistic-like behavior.[28] The increasing number of patients being reported supports the hypothesis
that this syndrome may be a common source of mental retardation and be considered the second
most common subtelomeric deletion, after the 1p36.6 deletion.[3]
All three cases with a common breakpoint within SHANK3 [2][10] (this report) share a number of
common phenotypic features, such as mental retardation and developmental delay with severely
delayed or absent expressive speech. These features have been described in all cases with larger
deletions or r(22) chromosomes and in a subject with t(12;22) balanced translocation involving the
SHANK3 gene.[9] In addition, a characteristic autistic-like behavior was present in the case
described by Anderlid and colleagues and in our case. Neonatal hypotonia was not present in our
case as well as in the cases reported by Anderlid [10] and Wong [2]. As already noted,[9][13]
SHANK3 haploinsufficiency is almost certainly responsible for the major neurological and
psychiatric features of the 22q13 syndrome, as well as for the regression of skills experienced by
many patients. On the other hand, there are some obvious differences between the cases. In particular, mental retardation was severe in our case while in the other two small deletions and in the translocation mental retardation was always mild. In addition, autistic behaviour was first seen in our subject at the age of two years and became more noticeable with time, while the case described by Anderlid and colleagues [10] exhibited abnormal behavior with autistic features (lack in contact, stereotypic movement) at a later age (late teens). In the case described by Bonaglia et al [9] the VABS test administered at the age of 7 years and 6 months to both parents demonstrated an overall AE of 39 months. Specifically, the boy showed a relative strength in Daily Living Skills and Socialization domain with AEs of 46 and 41 months respectively, while Motor Skills AE was 37 months and Receptive Language and Expressive Skills AE was 22 months and . His behavioural phenotype has some features also found in autistic subjects (stereotypic movements, hyperactivity, hyperkinesia) but the pattern of VABS is not characteristic of autistic disorder.

Thus, subjects with the same kind of SHANK3 disruption can exhibit different degrees of severity in their phenotype.

**SHANK3 and neurological deficits**

The minimum region of overlap of rearrangements leading to the 22q13 syndrome is a 100 Kb region between cosmid n66c4 proximally and cosmid n94h12 distally. The clone n66c4 is distal to the ARSA locus and overlaps the 5’ half of SHANK3, while clones n85a3 and n94h12 overlap the 3’ end of SHANK3 and ACR, respectively (Figure 1). SHANK3 belongs to a family of proteins that interact with receptors and structural proteins of the post-synaptic membrane, and is the central link between receptors and the actin cytoskeleton. These proteins are important scaffolding molecules in the post-synaptic density (PSD) and function to receive and integrate synaptic signals and transduce them into the post-synaptic cells. In addition to their role to assemble the PSD during synaptogenesis, they may play a role in synaptic plasticity and in the regulation of dendritic spine morphology. [29][30][31] SHANK3 is the best candidate gene for the neurological deficits (developmental delay and absent speech) in the 22q13.3 syndrome since it is located in the critical region, it is always deleted in all reported cases with 22q13.3 syndrome, it encodes a structural protein located in the post synaptic density (PSD), and is involved in spine maintenance in hippocampal neurons. [31]

**Overlooking the 22q13.3 deletion syndrome**

Approximately 75% of subjects with the 22q13.3 deletion syndrome have pure 22q deletions, [13] either terminal or interstitial, and about 25% have deletions resulting from an unbalanced translocation [32] or other structural rearrangement [13] such as r(22) [8] or reciprocal translocations interrupting the SHANK3 gene. [9][10] In spite of the increasing number of reported cases, the deletion 22q13.3 remains under-diagnosed due to failure to detect the 22qter deletion in routine chromosome analysis and to recognize the phenotype on clinical examination. As a consequence, its incidence is not yet established. [7] Since the deletions have sizes ranging from 100 Kb [10] to more then 9 Mb, [13] high resolution karyotype analysis will miss many of them. FISH analysis with specific subtelomeric probes can also give ambiguous results, and requires careful evaluation of the hybridization signal intensity, not just discriminating between the presence or absence of a signal. The two cases we presented here, having a deletion partially overlapping the commercial subtelomeric probe, highlight the difficulties in interpreting the results and indeed suggest that many similar cases may be overlooked. Considering the non-specificity of the phenotype, all subjects presenting global developmental delay and severe speech delay should undergo appropriate tests (FISH, MLPA) in order to search for a cryptic 22q13 deletion. Obviously, the recruitment of additional cases will lead to a better characterization of the syndrome and to a definition of its incidence.
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COMPETING INTERESTS

The authors state that they have no competing interests that could interfere or influence the publication of this manuscript.

FIGURE LEGENDS

Figure 1

*Molecular cytogenetic characterisation of the 22q13.3 deletion.*

A specific subtelomeric probe for chromosome 22q (Cytocell). Abnormal chromosome 22 (dotted arrow) shows on 22qtel a signal smaller than the one detected on the normal homologue (arrow).

B normal control FISH for probe Tel22q (Cytocell). The two chromosome 22 homologues (arrow) show equal hybridization signal intensity.

C specific commercial probe for the DiGeorge/VCFS syndrome (Vysis). Proximal signals at 22q11.2 (red) indicate hybridization of LS1 Tuple 1 and distal signals (green) indicate hybridization at the LS1 ARSA (Arylsulfatase A) locus. Arrows indicate the presence of signals on the distal end of both chromosomes 22.

D cosmid probe n66C4. Both chromosome 22 homologues (arrow) show the presence of hybridization signals.

E,F distal cosmid probes n85a3 and n94h12, respectively, show the absence of hybridization signals on one chromosome 22 (arrowhead).

The map of the 22q13.3 deleted region is shown in the center of the Figure: deleted clones (n85a3 and n94h12) are depicted as green empty bars, the retained clones (LS1 ARSA and n66c4) and the Tel22q probe (Cytocell) as green and red bars, respectively. Gene are shown as horizontal gray bars (bottom). The map was constructed according to the UCSC Genome Browser; the localization of the Cytocell probe is shown according to Anderlid et al (2002).

Figure 2

*Breakpoint identification.* A. Real-Time PCR analysis at seven locations (22-1 to 22-7, on the X axis) in the SHANK3 gene. 2DDct values and their standard deviation are shown on the Y axis; normal control (Control, blue diamonds), deleted control (Deleted, purple squares), the subject described in Anderlid 2002 (Subject 1, yellow triangles), and the subject described in this paper (Subject 2, light blue circles) are shown. B. Breakpoint amplification from Subject 1 (1), Subject 2 (2), a normal control (C), and water control (N) were analyzed on a 1% agarose/ 1x TAE gel; the molecular weight marker (M) is Marker X (Roche Diagnostics). C. Comparison between the chromosome 22 breakpoint sequences. Only a portion of the repeat is shown, and single repeat units
are divided by spaces. The normal chromosome 22 sequence (wt chr. 22) is on the first line; the sequence reported in Wong 1997, two types of clones obtained from Subject 1 (Subj. 1 A and B), and two types of clones obtained from Subject 2 (Subj. 2 A and B) were aligned with the wt sequence. A larger spacing was used for Subject 2 in order to optimize the alignment. The telomeric repeats found in all breakpoint sequences are shown in lowercase lettering. Base differences with the wt sequence are typed in red.

**Figure 3**

*Genomic structure of the human SHANK3 gene.* The locations of selected FISH probes, the D22S163 polymorphism, Real-Time PCR probes, selected structural motifs and all known breakpoints are indicated.
BIBLIOGRAPHY


A

Locus SHANK3

Amplified probe

B

PCR2

PCR3

M 1 2 C N 1 2 C N

C

wt chr. 22 AGAGGGGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGATGGTGG AGAGGGGGATGGTGG

Wong 1997 AGAGGGGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG

Subj. 1 A AGAGGGGGTGG AGAGGGGGATGGTGG AGAGGGGTAaggggtt aggggtt aggggtt aggggtt

Subj. 1 B AGAGGGGGTGG AGAGGGGGATGGTGG AGAGGGGGTGGTGG AGAGGGGGATGGTGG AGAGGGGGAaggggtt aggggtt aggggtt aggggtt

Subj. 2 A AGAGGGGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG

Subj. 2 B AGAGGGGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG AGAGGGGGTGGTGG