The human genome; you gain some, you lose some

Kriek, M.

Chapter III-3

Telomeric deletions of 16p causing alpha-thalassemia and mental retardation characterized by multiplex ligation-dependent probe amplification

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Abstract

Alpha thalassemia retardation associated with chromosome16 (ATR-16 syndrome) is defined as a contiguous gene syndrome resulting from haploinsufficiency of the α-globin gene cluster and genes involved in mental retardation (MR). To date, only few cases have been described which result from pure monosomy for a deletion of 16p. In most of these cases the deletion was identified by densitometric analysis of Southern blot results or by Fluorescent In Situ Hybridization analysis, and these alterations have not been mapped in detail. In this study, we have fine mapped deletions causing α-thalassemia within 2 Mb from the telomere of 16p by multiplex ligation-dependent probe amplification (MLPA). We have developed a rapid and simple test for high resolution mapping of rearrangements involving the tip of the short arm of chromosome 16 by incorporating 62 MLPA probes spaced approximately 10–200 kb over a region of 2 Mb from the telomere. One deletion of approximately 900 kb without MR was identified in addition to three *de novo* deletions varying between 1.5 and 2 Mb causing ATR-16 in three patients having mild MR and α-thalassemia. Two were found by chance to be ATR-16 because they were included in a study to search for telomeric loss in MR and not by hematological analysis. This would plead for more alertness when a persistent microcytic hypochromic anemia at normal ferritin levels is observed as suggestive for the ATR-16 syndrome. The region on chromosome 16p for which haploinsufficiency leads to the dysmorphic features and MR typical for ATR-16, has been narrowed down to a 800 kb region localized between 0.9 and 1.7 Mb from the telomere.

Introduction

Genomic deletions involving the α-globin gene cluster on chromosome 16p13.3 are the most common molecular cause of α-thalassemia (approximately 80–90% of cases) (Bernini and Harteveld 1998; Higgs 1993). Due to selective advantage, α-thalassemia carrier frequencies are high in areas endemic for malaria tropica. Less frequently α-thalassemia is found in North-European Caucasians. Even more rare are mental retardation (MR) syndromes in which the occurrence of α-thalassemia is merely a marker of the genomic defect. These syndromes are known as α-thalassemia MR syndromes ATR-X and ATR-16 (depending on the respective chromosomes involved) (Weatherall *et al.* 1981; Wilkie *et al.* 1990a, b).

ATR-X involves mutations of the X-linked *ATRX* gene, coding for helicase-2, a putative global transcriptional regulator (Cardoso *et al.* 2000, 1998; Gibbons and Higgs 2000; Gibbons *et al.* 1992; Villard *et al.* 1997; Wilkie *et al.* 1991; Wilkie 1990b; Yntema *et al.* 2002). ATR-16 is characterized by the occurrence of large deletions involving the α-globin gene cluster on chromosome 16p and is most likely a contiguous gene syndrome (Daniels *et al.* 2001; Flint *et al.* 1996; Horsley *et al.* 2001; Lamb *et al.* 1993; Wilkie *et al.* 1991). At present molecular tests commonly used to identify deletion types of α-thalassemia and ATR-16 are gap-PCR, Southern blot or fluorescent in situ hybridization (FISH) analysis (Daniels *et al.* 2001; Flint 1996; Gallego *et al.* 2005; Lindor *et al.* 1997). However, the applicability of these techniques is limited to known deletions, may involve radio-activity, is dependent upon the hybridization probes available and may require time consuming and laborious cell culture to generate metaphase chromosome spreads.

Recently, we have developed an multiplex ligation-dependent probe amplification (MLPA) based assay to perform high resolution screening for unknown rearrangements on chromosome 11p15.4 and in a 700 kb telomeric region of the short arm of chromosome 16 (16p13.3) causing β-and α-thalassemia, respectively. During the examination of 38 putative α-thalassemia carriers, we identified a single patient showing a telomeric deletion without MR, for which the 3¢breakpoint could not be determined (- $-^{GZ}$) (Harteveld *et al.* 2005). During this study we extended the MLPA assay to investigate a region of approximately 2 Mb involved in α-thalassemia and MR. A second patient was brought to our attention because of a persistent microcytic hypochromic anemia without iron depletion. The patient showed MR and the parents were normal. Two patients were detected using multiplex amplifiable probe hybridization (MAPH) for the screening of genomic imbalances in the subtelomeric region among

mentally retarded patients. Only the telomeric probe associated with the telomere of 16p appeared to be deleted, excluding unbalanced translocation in these patients. The results of this screening were verified by MLPA and FISH analysis. One of these patients has been previously described (P.V.) (Kriek *et al.* 2004).

Materials and methods

Appropriate informed consent was obtained from all human subjects studied.

Clinical report

Case G.Z.

Shortly after birth the patient had surgery for pylorus stenosis. He was regularly seen until the age of 4 because of persisting microcytic hypochromic anemia with normal iron levels. There was no developmental delay nor any other abnormalities related to the ATR-16 syndrome. The propositus was investigated for the first time at the age of 5 at the hematological and biochemical level together with his parents because of a suspected α-thalassemia. The father presented with normal hematological parameters, the mother and the patient both showed hematological abnormalities and an unbalanced hemoglobin chain synthesis ratio typical of α^0-thalassemia carriership. At that time DNA analysis was not feasible. The patient was re-investigated in 2002 at the age of 30, when microcytic hypochromic anemia at normal ferritin was still present. The seven most common α-thalassemia deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $-(\alpha)^{20.5}$, $--^{MedI}$, $--^{SEA}$, $--^{THAI}$, $--^{FIL}$) were excluded at the molecular level by multiplex PCR (Chong *et al.* 2000; Liu *et al.* 2000). Both mother and son had a normal school education and there was no indication for MR in these family members.

Case H.N.

H.N. is the third child of healthy, non-consanguineous parents. He was born after an uncomplicated pregnancy and delivery, with a birth weight of 3.1 kg. Directly after birth a short period of asphyxia was recorded, and was attributed to meconium-stained amniotic fluid (Apgar scores 3/6/9 after resp. 1, 5 and 10 min; pH cordblood 7.13). He made a quick recovery with an oxygen mask. He had a clubfoot on the left, for which he was operated on at the age of 1 year (lengthening of achilles tendon).

In infancy, he suffered from recurrent chest infections and asthma. His motor development was delayed: sitting at the age of 10 months, crawling at the age of 18 months

and walking at the age of 23 months. He spoke his first words at the age of 18 months. At the age of 2 years and 6 months he was referred to the pediatric neurologist because of hypotonia, psychomotor retardation and speech delay. At the age of 3 years and 8 months a severe delay in active language ability was reported. Subsequent testing showed mild MR (SON-IQ 58).

We first examined him at the age of 4 years and 8 months. He was able to construct short sentences (three words) and went to a special school with an individual teaching program. By that time, mild anemia had become apparent. Physical examination showed the following: height 104 cm (–1 SDS), weight 17.5 kg (+1 SDS), head circumference 53 cm (0 SDS). He had nasal speech. His facial features showed downslanted palpebral fissures, mild hypertelorism, a broad nasal tip, small posteriorly rotated ears, a short neck with webbing, and a low trident posterior hairline. Apart from pectus carinatum, an operated clubfoot on the left, and a flat foot on the right, no other anomalies were noted (Fig. 1a).

Additional investigations

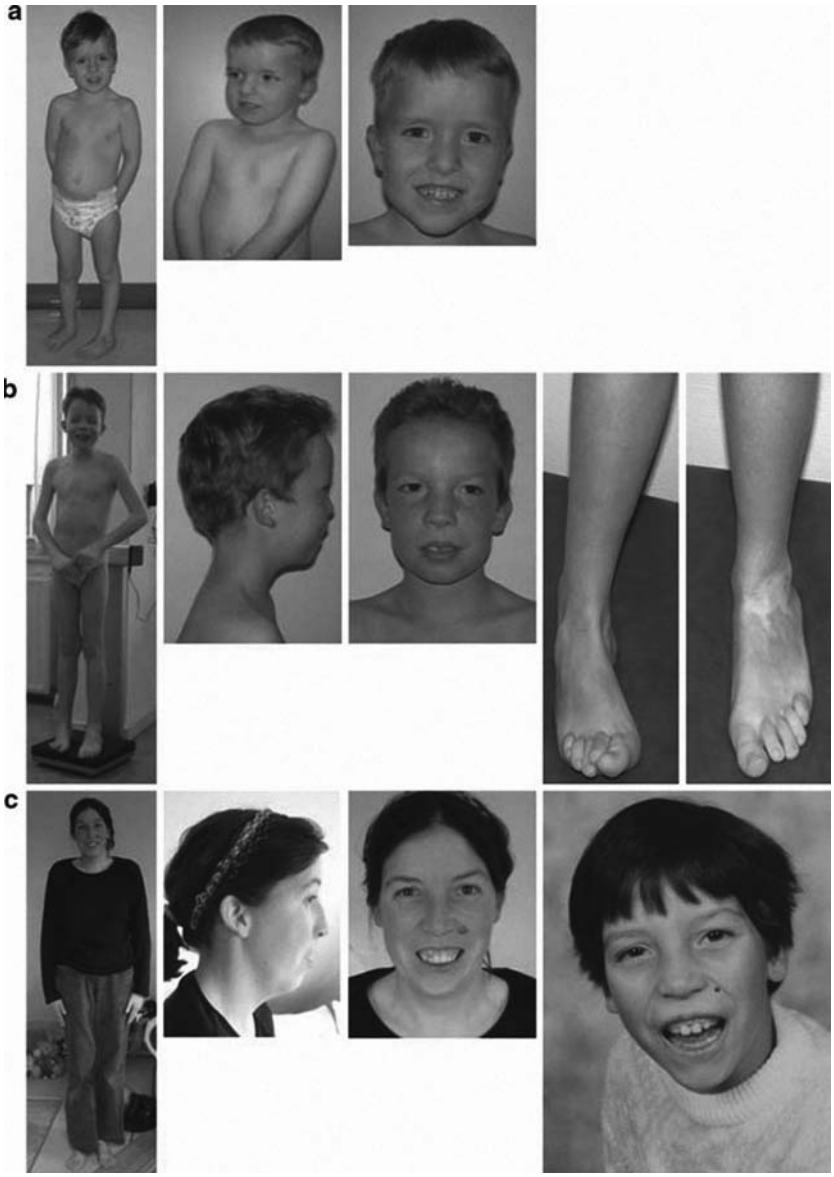
Conventional chromosome analysis showed a normal male karyotype. Metabolic screen was negative. MRI of the brain showed an arachnoidal cyst in the right temporal lobe. As some features were consistent with Noonan syndrome, *PTPN11* mutation analysis was performed, which resulted negative.

Case P.V.

This male patient was born after an uneventful pregnancy and delivery. He is the youngest of a family of three children to non-consanguineous parents. His sister died 3 days postpartum due to severe complications at delivery resulting in asphyxia. There was no family history of either developmental delay or congenital malformations. In early childhood, the patient had several episodes of pneumonia, and was diagnosed with asthma at a later stage. Neuropsychological testing was performed at the age of 2, because of developmental delay and because his overall behavior was far from consistent with his age. The patient started walking at the age of 30 months. He started to actively use language at the age of 5. He suffered from recurrent epileptic seizures at the age of seven that were treated successfully with Depakine. In addition, mild anemia was detected.

A physical examination at age 11, revealed a very outgoing boy. His height was 144.5 cm (–1 SDS), his weight 29 kg (–2 SDS) and head circumference 52.3 cm (–1 SDS). He showed mild dysmorphic features including high forehead, some periorbital

Figure 1. Three unrelated patients.



H.N. (a), P.V. (b) and F.T. (c) showing a mild mental retardation (IQ 50–60), a severe delay in active language ability, some typical facial features like downslanted palpebral fissures, mild hypertelorism, a broad nasal tip and small ears and a short neck with webbing, which is most pronounced in a and b. Patient H.N. and P.V. both show pectus carinatum. This was also observed for patient F.T. (not shown). H.N. also has an operated clubfoot on the left, while patient P.V.'s right foot is turned inside, the other foot showing a cafe´-au-lait spot. c Patient F.T. has a short neck and small ears. On the outer right a photograph is shown of the patient at age 11. The karyotype was normal in all patients and hematological analysis showed a persistent microcytic hypochromic anemia without iron deficiency [See appendix: colour figures.]

fullness, microphthalmia, telecanthus, broad nasal bridge, posterior rotated ears, a flat, rather long philtrum, full lips and micrognatia (Fig. 1b.). He had an extra incisor. His trunk showed a mild pectus excavatum and two café au lait spots. Auscultation of the heart was normal. Hyperlaxity of the joints was observed.
Conventional chromosome analysis at a resolution of 500 bands showed a normal male karyotype. Due to his behavior and some of the facial dysmorphism, FISH analysis for the Williams Syndrome Critical region was performed. No deletion was detected on chromosome band 7q11.23. No metabolic abnormalities were observed.

Case F.T.
F.T. was referred at the age of 30 years because of mild and persistent microcytic hypochromic anemia. She was born at term after an uneventful pregnancy. Her birth weight was 2,600 g. APGAR score was reported as 'low' after 1 min but recovered to ten after 5 min (no direct data, home delivery). In infancy, it was noted that her development was retarded in comparison to other children (e.g. first words after 2 years of age), but the parents declined further investigation at the time. Her behavior was reported as shy and dependant. She attended special education at the age of 6 and she is now employed in a program for people with a mental handicap. She lives in a support home. Furthermore, she plays the drums and enjoys horseback riding. She has no problems with her general health. Hearing and vision were normal. Family history was unremarkable, she had one healthy sister.

Physical examination at the age of 31 showed a height of 162 cm (–1.3 SD), head circumference 52.5 cm (–1.7 SD), elongated face with a flat midface and a prominent nasal bridge. The palpebral fissures of her left eye showed slight upslant. Her ears were rather small, and had a slight question mark configuration. In addition, she had some irregularity of teeth, marked micrognathia and retrognatia (Fig. 1c). On her palatum durum, two small bulbous lumps were present and she had hypertrophic gums. Her neck was short. A pectus excavatum was present. Internal screen was normal. Her hands are rather short with slight tapering of the fingers and bilateral fifth finger clinodactyly. On the feet, both halluces showed laterial deviation, the second digits were short and the fourth digits showed medial deviation. Her joint were rather stiff. She did not have any skin pigment aberrations. She has scarce body hair with normal scalp hair.

Hematological analysis
Blood samples of patients and parents were collected in vacutainers with EDTA as anticoagulant. Hematological analysis was carried out according to standard methods

(Dacie and Lewis 1991). The red cell indices were measured with a standard cell counter (Micros 60; ABX Diagnostics, Montpellier, France). A Brilliant Cresyl Blue staining was performed on the blood smear to identify HbH inclusion bodies according to a standard method (Dacie and Lewis 1991). Globin chain synthesis was performed for patient GZ and his parents according to standard procedures (Giordano *et al.* 1999). DNA was isolated according to standard procedures (Miller *et al.* 1988).

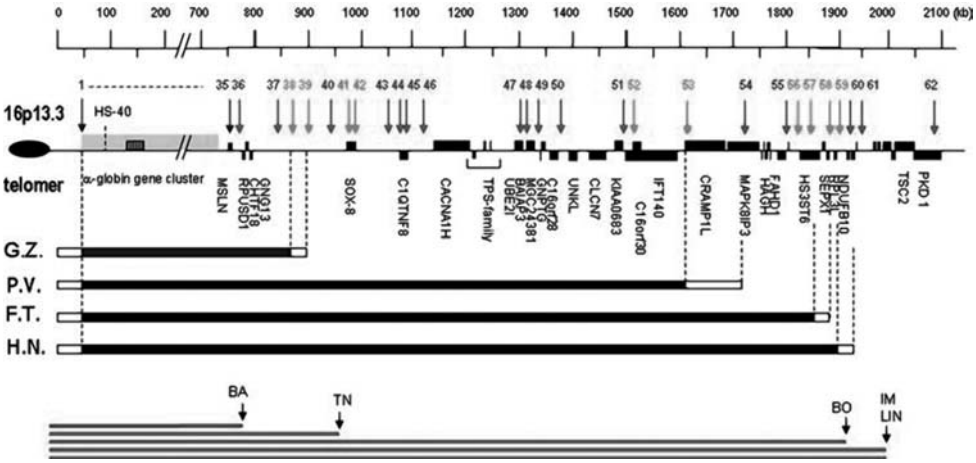
Design of the MLPA assay

In total, 62 MLPA probe pairs were designed covering a region of 2 Mb from the telomer of 16p13.3 to the *PKD*-gene. Of these, 35 were previously reported to detect (unknown) α-thalassemia deletions and rearrangements in a 700 kb region from the telomer of 16p to the *MSLN* gene (Harteveld *et al.* 2005). An additional 17 probe pairs were designed to extend the region covered by MLPA from the *MSLN* gene towards the *PKD* gene to screen for even larger rearrangements (Table 1, Fig. 2). Each primer pair contained common ends corresponding to either a HEX-labeled amplification primer (detection in green), a FAM labeled primer (detection in blue) or a ROX labeled primer (detection in red) to be analyzed simultaneously in the same fragment run on the ABI3730. Finally, ten primer pairs were designed for high resolution mapping of the breakpoints after initial screening for large deletions in three colors. The data were collected and ratios between normalized peak heights of the patient and the normal controls were presented in a single scatter plot for each patient.

The oligonucleotides were ordered from Illumina, Inc. (San Diego, CA, USA), synthesized in a salt-free environment (50 nmol scale) and used without further purification. For each probe pair the downstream primer was 5'phosphorylated to allow ligation.

The MLPA reactions were performed as described by Schouten *et al.* (2002) and White *et al.* (2004) in brief, approximately 200 ng of genomic DNA in a final volume of 5 µl was heated for 5 min at 98°C. After cooling to room temperature, 1.5 µl of the probe mix and 1.5 µl SALSA hybridization buffer (MRC-Holland, Amsterdam, The Netherlands) were added to each sample, followed by heat denaturation (2 min at 95°C), hybridization (16 h at 60°C). Ligation was performed by adding 32 µl of ligation mix at 54°C for 10 min, the reaction was terminated by 5 min incubation at 95°C. PCR amplification was carried out for 33 cycles in a final volume of 25 µl, adding the 5'ROX-labeled M13-Forward and M13-Reverse primer set to a final concentration of 100 nM. The second common primer set designed to fine map the deletion breakpoint region between two MLPA probes of the first set, were amplified by

Figure 2 Schematic presentation of short arm of chromosome 16 (16p13.3), showing a 2 Mb region from the telomere containing the α-globin gene cluster up to the *TSC* and *PKD* genes.



The *arrows* and *numbers* represent the location of the probes. The deletions found by MLPA are shown as *bars* below the figure. Large deletions previously described are indicated as *red bars* [See appendix: colour figures.]

adding the 5'HEX-labeled MAPH-Forward and MAPH-Reverse primers to a final concentration of 100 nM. Products were separated by capillary electrophoresis on the ABI 3730 (Applied Biosystems) and data analyzed as described previously (Harteveld *et al*. 2005).

Results

Hematological analysis

All patients presented with a microcytic hypochromic anemia without iron deficiency (Table 2). Two were brought to our attention because of a suspected α-thalassemia (G.Z. and F.T.) after routine hematological investigation. The other two (H.N. and P.V.) were investigated at the hematological level after identifying the telomeric loss of 16p as the only chromosomal abnormality causative for the observed MR. The patient G.Z. without MR and his parents were investigated at the hematological and biochemical level. He and his mother showed microcytic hypochromic parameters and an unbalanced α/β-globin chain synthesis ratio indicative for α^0-thalassemia carrier-ship

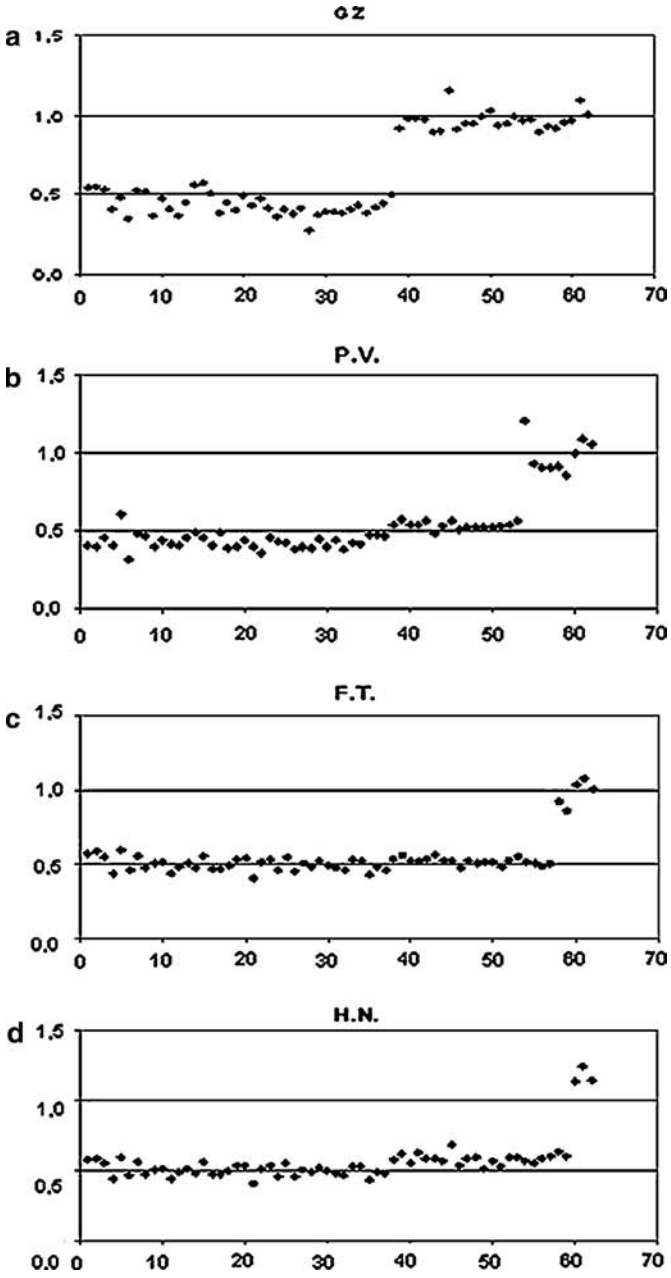
Table 1. Names and sequences of probes used for MLPA located between 750 kb and 2 Mb from the telomere of chromosome 16p.

No.	Name	Upstream hybridising sequence	Downstream hybridising sequence	Positions[a]
36	hs335h7	**GGCGATTAAGTTGGGTAAC**GAAGAGAGC TAGGAAAGGTCTGGGTGGCC	AGACCTGTCCCTTGGAAGCCCCAC T**GCTGTTTCCTGTGTGAAC**	774,902–774,930
37	hs.58362	**GGCGATTAAGTTGGGTAAC**GAAGGGCAAA CGTTGCTGAGCCG	CCGAGGGGCTGGGTCTTCGCCTTTATTTC **GCTGTTTCCTGTGTGAAC**	844,471–844,493
38	FLJ12681(2)	**GGCCGCGGGAATTCGATT**GAAGGGCACCA GGAGCTCGATGAAGTGGTTGCTGAGC	GTCTCGAAGCGATGGAACCACCAGGGTGAG T**CACTAGTGAATTCGCGGC**	869,631–869,698
39	FLJ12681(1)	**GGCCGCGGGAATTCGATT**CACCACGCAGA AGAGCTCCACTTACTGCTC	CAAGCATGATCCTGAAGATCAGCCACCGGA **CACTAGTGAATTCGCGGC**	900,907–900,966
40	hs.58362'	**GGCGATTAAGTTGGGTAAC**CTGGCTGCATCTCGGCCT GGCAGGCTCCCCTCACTTCCATACTCATTTGAGAGCCT	CACTTGAGGTTCGTAGCTCCTGAC**GCTGTT TCCTGTGTGAAC**	959,724–959,779
41	Sox-8	**GGCCGCGGGAATTCGATT**GCTCAAAGCC AAGCCGCATGT	GAAGCGGCCCATGAACGCATTCATG **CACTAGTGA ATTCGCGGC**	972,208–972,253
42	Sox-8(2)	**GGCCGCGGGAATTCGATT**GGGCCTCAG TTCTAGACGAGTCAT	ACCTGATTCACCTGCACTGCTTCCCC **CACTAGTGAA TTCGCGGC**	976,591–976,640
43	hs394h11	**GGCGATTAAGTTGGGTAAC**GTCCTTTGAC GAGGAGTTTGCGCCTC	ATTCAAGTCCCCTACCTGCATCCCTGGCG**GGCTGTT TCCTGTGTGAAC**	1,053,683–1,053,708
44	hs349e11'	**GGCGATTAAGTTGGGTAAC**CAATTTGCTTAA CGTGATTCCCGGCCAAGCTAAACATGACT	AATCGTGTTTACCCGGTGATCCCGC**GCTGTTT CCTGTGTGAAC**	1,068,678–1,068,718
45	hs394e11'	**GGCCGCGGGAATTCGATT**GTCGGGATCCTC AATATTCCC	TGAAGATGGGCTCTGCTGGAC**CACTAG TGAATTCGCGGC**	1,085,683–1,085,724
46	hs344f5	**GGCGATTAAGTTGGGTAAC**GAAGGGGCTGGTG GCTCATCTTCTCCTTGGGAGAG	CACGGTGAGGGATGGTGTCTCTGAGG**CTGTTT CCTGTGTGAAC**	1,124,629–1,124,663
47	hs.84285	**GGCGATTAAGTTGGGTAAC**GGTTGTTTTCTTTT CTCTCAGGGTTTCGTGGCTGTCCCAA	CAAAAAATCCCGATGGCACGATGAACCTCA**GCTGT TTCCTGTGTGAAC**	1,304,274–1,304,313
48	hs349e11	**GGCGATTAAGTTGGGTAAC**CGAATAAGGCAAG TTCCCACTCCTC	GATGCGGGCTGTGCCTTATTTAT**GCTGTTTC CTGTGTGAAC**	1,318,158–1,318,182
49	hs.134846	**GGCGATTAAGTTGGGTAAC**GCTGCGGCTGCACA AATGTTCCACCCGCGAGTGCATCGAGCAGTT	CTACCTGGACAAGCTCAAACAGGTAGGGA**GCTGT TTCCTGTGTGAAC**	1,337,561–1,337,605

No.	Name	Upstream hybridising sequence	Downstream hybridising sequence	Positions[a]
50	hs.118261	**GGCGATTAAGTTGGGTAAC**GGTGCACAACAGAGC CCACATAGGAACACCAGGGCTGCGAGGGGGAGAT	GCACACGGCCACAAAAGTTCCCA**GCTGTT TCCTGTGTGAAC**	1,372,574–1,372,622
51	hs305c8'	**GGCGATTAAGTTGGGTAAC**GGTGCCTTTGCCGGG ATTCCTGAAAGGCAGGGTCCATGGT	TTGCACCGAGGAACTGGATTTTGG**GCTGTT TCCTGTGTGAAC**	1,495,719–1,495,758
52	WDTC2 (3)	**GGCCGCGGGAATTCGATT**GCTGCTCCGACTT CTTCATCGAGC	ACAGTCAGTACGAGAGGGCGGTAG**CACTAG TGAATTCGCGGC**	1,513,567–1,513,566
53	CRAMP1L	**GGCCGCGGGAATTCGATT**CCGCCAGCTTTCTGG AACAGATCAC	TGTACGGTGCTTTCTCTCCAAGGAGCT**CACTA GTGAATTCGCGGC**	1,615,928–1,615,979
54	hs.88500	**GGCGATTAAGTTGGGTAAC**GCCCACACCCTCTA ACACCTGTCTC	CTTATCGTCACATGCTGACCGTCCG**GCTGTTT CCTGTGTGAAC**	1,730,426–1,730,450
55	hs.155482	**GGCGATTAAGTTGGGTAAC**CTGCTCAGAATGTG GGAACAGGC	TGGGCGCTTTTTACACACGCAGC**GCTGTTT CCTGTGTGAAC**	1,806,637–1,806,659
56	FAHD1	**GGCCGCGGGAATTCGATT**GGAGATATTATCTTG ACTGGGACGCCAAAGG	GAGTTGGACCGGTTAAAGAAAACGATGAGAT**C ACTAGTGAATTCGCGGC**	1,817,784–1,817,845
57	HSAC76P10	**GGCCGCGGGAATTCGATT**CATTGTTGCAAATGG ACACAGTCTTAATGG	GAGGATTATTAACGTGCTTGCAGCTGTGAAATCG**CA CTAGTGAATTCGCGGC**	1,847,771–1,847,770
58	HSAC76P10 (2)	**GGCCGCGGGAATTCGATT**CAAGGAGAACGCTTGGC GTGTCCTCCTTTGGTCCAGC	CTGGGTTGCAGCTTCGATGCTCCCTCTGT**CACTAGT GAATTCGCGGC**	1,880,212–1,880,277
59	HS3ST6	**GGCCGCGGGAATTCGATT**CCACTTCAGATGCCTGA TTTGCCCGAGCT	CATAAAGGATGTGGTTCCCTCCCAGGGAG**CACTAG TGAATTCGCGGC**	1,913,866–1,913,923
60	hs.48384	**GGCGATTAAGTTGGGTAAC**GGGAGTGTCCTGATGTTT CAACCACTGCGCCCTGCCTTCCTGTCTCGACG	CCCAGGGTTCCAACTCCAAGGTGGAAT**GGCTGTTT CCTGTGTGAAC**	1,928,982–1,929,031
61	hs.198274	**GGCGATTAAGTTGGGTAAC**CCAATCCCATCGTCTACA TGATGAAAGCGTTCGACCTCATCGTGGACCG	ACCCGTGACCCTCGTGAGAGGTACGAA**GCTGTTT CCTGTGTGAAC**	1,949,688–1,949,736
62	PKD 1	**GGCGATTAAGTTGGGTAAC**GGTCATATAGAGGTTAC CTTGTATGTAGTCACG	CACATAGTCACGCACATGGCAGCCG**GCTGTTTC CTGTGTGAAC**	2,086,449–2,086,481

Letters in bold signify the 5' and 3' universal tags for amplification in the MLPA reaction
a UCSC Genome Browser (May 2004) chromosome 16p13.3

Figure 3. Scatterplots showing the MLPA results of the patients G.Z.



(a), P.V. (b), H.N. (c) and F.T. (d). The *Y*-axis represents the ratio peak height of patient divided by that of the normal control, the *X*-axis shows the chronological position of MLPA probes along the p-arm of chromosome 16.

Table 2. Hematological parameters of patients G.Z., P.V., N.M. and F.T. and family members.

Case	Sex–age (years)	Hb (g/dl)	RBC ($\times 10^{12}$/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	ZPP (umol zp/mol heme)	A_2 (%)	I.B.	α Globin genotype
Propositus G.Z.	M–31	12.7	5.84	70	22.1	31.6	53	2.8	+	aa/-GZ
Mother of G.Z.	F–55	11.6	5.50	67	21.1	31.7	ND	ND	ND	aa/-GZ
Propositus P.V.	M–11	10.5	4.66	69	22.4	32.2	29	2.9	+	aa/-PV
Father of P.V.	M–49	15.1	5.05	89	29.8	33.3	20	3.1	–	aa/aa
Mother of P.V.	F–48	13.7	4.34	91	31.6	34.6	13	2.7	–	aa/aa
Propositus H.N.	M–5	10.3	4.75	71	21.7	30.6	74	2.7	+	aa/-HN
Father of H.N.	M–41	14.0	4.90	89	28.5	32.0	35	2.7	–	aa/aa
Mother of H.N.	F–40	12.9	4.62	87	28.0	32.0	50	2.9	–	aa/aa
Propositus F.T.	F–30	10.6	5.14	74	20.8	28.2	56	2.6	ND	aa/-FT
Father of F.T.	M–60	14.8	4.84	93	30.8	33.0	32	2.6	ND	aa/aa
Mother of F.T.	F–60	15.0	4.72	95	31.9	33.6	39	2.6	ND	aa/aa
Sister of F.T.	F–27	13.8	4.67	91	29.8	32.7	30	3.0	ND	aa/aa

ZPP Zinc Proto Porphyrin (lmol ZP/mol Heme), *ND* not determined
+/– = Positive/negative Inclusion Bodies test

(α/β = 0.6). The father showed a completely normal hematology and balanced chain synthesis (α/β = 1.1).

The three patients with MR and α-thalassemia all have healthy parents presenting with normal hematological parameters (Table 2), supporting a *de novo* event.

Molecular analysis

Case G.Z.

The MLPA probes 1–35 appeared to be deleted in this patient as shown in the previous study (Harteveld *et al*. 2005), indicating that the 3'breakpoint was located somewhere between the *MSLN* gene and the *TSC* gene, approximately 2 Mb from the telomere of 16p. Additional MLPA primer pairs designed in this study, revealed the deletion breakpoint to be localized between probe no. 37 and 40, which spans a region of approximately 100 kb (Fig. 3a). Two probes, no. 38 and 39 were designed to narrow the breakpoint region to 31 kb between positions 869,698 and 900,907. The deletion involves the telomeric region including the complete α-globin gene cluster but leaving the *SOX8*-gene intact. According to the hematological analysis of the parents, the deletion is inherited from the mother, who presented with microcytic hypochromic

anemia, similar to the propositus. Unfortunately, no DNA of the parents was available for MLPA analysis. No clinical phenotype other than α^0-thalassemia seems to be associated with this deletion.

Case P.V.
During an initial screening, a telomeric deletion was observed extending to *KIAA0683* at position 1,495,758 (probe no. 51). Two probe pairs (nos. 52 and 53) were designed between the last MLPA probe pair deleted in this patient and the first still present (probe no. 54) to reduce the breakpoint region. Both probes 52 and 53 appeared to be involved in the deletion reducing the breakpoint region to 114 kb between positions 1,615,979 and 1,730,426 (Figs. 2, 3b). This deletion of approximately 1.62–1.73 Mb causes monosomy for several genes including the *SOX8*-gene. No other chromosomal aberrations were found in this patient, neither at the cytogenetic level, by FISH analysis, nor by MAPH analysis (Kriek *et al*. 2004).

Case H.N.
After an initial screening the deletion was found to involve a 1.8–1.93 Mb region from the telomere. An additional MLPA analysis using newly designed probe pairs 56–59 identified the deletion breakpoint between probes 59 and 60, which limits the breakpoint region to 15 kb between positions 1,913,923–1,928,982 (Figs. 2, 3c).

Case F.T.
Initially, the deletion length appeared similar to that of patient H.N.. Fine mapping using MLPA probe pairs 56–59 revealed the 3'breakpoint to be different. The breakpoint is localized between probe pairs 58 and 59, which equal a deletion length of 1.9 Mb from the telomere to position 1,880,277–1,913,866 (Figs. 2, 3d). This region is extremely rich in Alu repeats, which may have played a role in the mechanism leading to these large deletions. This also might explain the observed clustering of 3¢breakpoints in patients F.T. and H.N.

Discussion

In contrast to the ATR-X syndrome, ATR-16 does not present with a clearly defined phenotype. Sixteen cases have been described in literature and in most cases it was not clear whether the dysmorphic features were attributable to the monosomy for 16p

or to the additional chromosomal aberrations found in these patients (Brown *et al*. 2000; Eussen *et al*. 2000; Gallego *et al*. 2005; Warburton *et al*. 2000; Wilkie 1990a). Only five patients (indicated as BA, TN, BO, IM and LIN in Fig. 2) were described with a clear monosomy for a telomeric deletion of 16p (Daniels *et al*. 2001; Fei *et al*. 1992; Lamb *et al*. 1993; Lindor *et al*. 1997; Wilkie *et al*. 1990a). We present the high resolution mapping by MLPA of ATR-16 deletions in four Caucasian patients affected with microcytic hypochromic anemia without iron deficiency, due to a large deletion including the complete a-gene cluster. One patient (G.Z.) with a deletion of 870–900 kb presented no dysmorphic features or MR, while three patients showing deletions ranging from 1.7 to 1.9 Mb presented with mild MR (IQ 50–60) and a variety of dysmorphic features. The samples have been checked for unbalanced translocations and partly for deletions/duplications in other parts of the genome, either using telomere MLPA (Schouten *et al.* 2002; Kriek *et al*. 2004) for samples H.N. and P.V.) or a 1420-plex bead-based MLPA (for samples H.N. and F.T., Fan *et al*. 2006; Aten *et al*., in preparation). No other deletions/ duplications or unbalanced translocations were found in H.N., F.T. and P.V. other than the deletions at the tip of chromosome 16p13.3 confirmed by MLPA analysis using the probes described in this manuscript.

The two patients, G.Z. and P.V., showing respectively the largest deletion with only α^0-thalassemia and the smallest deletion clearly associated with the classical ATR16 features, might give a better insight into the genes for which haploinsufficiency contributes to the syndrome. Three cases - $-^{BO}$,- $-^{IM}$ and - $-^{LIN}$ are known from the literature without additional chromosomal rearrangements besides the deletion causative of ATR-16 (Daniels *et al*. 2001; Fei *et al.* 1992; Lindor *et al*. 1997). These deletions are larger than the presently described case, which restricts the region for which monosomy seems to contribute to the ATR-16 associated phenotype to an approximately 800 kb region between 0.9 and 1.7 Mb from the telomere of 16p. Approximately 14 genes and gene families of known function are located in this area.

The *SOX8* gene is a member of the *SOX* (*SRY*-related HMG-box) family and encodes for a transcription factor involved in regulation of embryonic development and in determination of cell fate. The *SOX8*-protein is suggested to be involved in brain development and function and is strongly expressed in brain and less abundant in other tissues. Therefore *SOX8* is considered to be a good candidate gene for which haploinsufficiency may contribute to the MR phenotype seen in ATR-16 patients (Holinski-Feder *et al*. 2000; Pfeifer *et al*. 2000). However, MLPA analysis in several members of a Brazilian family without MR or dysmorphic features using the 62 probes showed a deletion of the tip of the short arm of chromosome 16 including *SOX8* (manuscript in preparation).

Other disease genes located in the 800 kb region deleted between G.Z. and P.V. are *CACNA1H*, *GNPTG* and *CLCN7*. These genes are associated respectively with childhood absence epilepsy, autosomal recessive pseudo Hurler polydystrophy and autosomal dominant Albers-Schonberg osteopetrosis type II (Cleiren *et al.* 2001; Liang *et al.* 2007; Perez-Reyes 2006; Tiede *et al.* 2006). It is not clear, however, how haploinsufficiency would lead to the phenotypic features seen in ATR-16. Of the three patients described in this report, only one (P.V.) suffers from epilepsy, while the others do not. No other features typical for pseudo-Hurler or Albers-Schonberg disease are seen in the ATR-16 patients described. Members of a Tryptase precursor gene family (*TPSG1, AB1, B2* and *D1*) are located between 1.21 and 1.26 Mb, and are believed to play a role in the pathophysiology of the polygenic disorder of asthma (Pallaoro *et al.* 1999). Also in P.V. and H.N. asthma was reported, supporting the assumed involvement of the Tryptase family genes. Of the other genes in this region, such as *C1QTNF8, UBE2I, BAIAP3, IFT140, C16orf30* and *CRAMP1L*, only *BAIAP3* is highly expressed in brain. This gene encodes a transmembrane protein, a member of the secretin receptor family, which interacts with the cytoplasma specific angiogenesis inhibitor1 and may be involved in synaptic functions (Shiratsuchi *et al.* 1998). To determine how haploinsufficiency for these genes may be of influence on the intellectual development and variability of dysmorphic features seen in these patients, more deletions should be studied.

Some common features associated with ATR-16 include a severe delay in active language ability, downslant of the palpebral fissures, mild hypertelorism, a broad nasal bridge and small ears and a short neck with webbing. Most of these features are seen in two of our patients (P.V. and H.N.), who are considered monosomic for the telomeric deletion on chromosome 16p. On the other hand, patient F.T. who has a deletion length similar to H.N. shows much less pronounced dysmorphic features presumed to be characteristic for ATR-16, which subscribes the variability in expression of this syndrome. All patients have at least one common feature measurable at the hematological level, i.e. α^0-thalassemia. Nevertheless only F.T. was recognized as a possible ATR-16 syndrome at the hematological level, because of a persistent microcytic hypochromic anemia at normal ferritin levels. The other two patients (P.V. and H.N.) were identified by MAPH screening using subtelomeric probes and mapped in detail by MLPA in the present study. Because dysmorphic features associated with ATR16 are not always very specific, we would like to plead for incorporating a simple hematological test if ATR-16 is suspected and, when positive, recommend a molecular screening using the 62 MLPA probes as described in this study.

Ultimately cloning of the breakpoint and subsequent sequence analysis is the only way to determine the nature of the deletion found, e.g. healed telomere break, interstitial deletion or translocation to another non-coding subtelomeric region (with the loss of the area involved in ATR16) and this will be subject for future study. On the other hand MLPA is a strong diagnostic tool in determining whether a genomic region is deleted, the extent of the deletion and the location of breakpoints. Because MLPA employs standard technology operational in most diagnostic laboratories, it is highly suitable for rapid testing for these disorders, which are believed to be under diagnosed (Daniels *et al*. 2001; Wilkie *et al*. 1990a).

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References

Bernini LF, Harteveld CL (1998) Alpha-thalassaemia. *Baillieres Clin Haematol* **11**:53–90

Brown J, Horsley SW, Jung C, Saracoglu K, Janssen B, Brough M, Daschner M, Beedgen B, Kerkhoffs G, Eils R, Harris PC, Jauch A, Kearney L (2000) Identification of a subtle t(16;19)(p13.3;p13.3) in an infant with multiple congenital abnormalities using a 12-colour multiplex FISH telomere assay, M-TEL. *Eur J Hum Genet* **8**:903–910

Cardoso C, Timsit S, Villard L, Khrestchatisky M, Fontes M, Colleaux L (1998) Specific interaction between the XNP/ATR-X gene product and the SET domain of the human EZH2 protein. *Hum Mol Genet* 7:679–684

Cardoso C, Lutz Y, Mignon C, Compe E, Depetris D, Mattei MG, Fontes M, Colleaux L (2000) ATR-X mutations cause impaired nuclear location and altered DNA binding properties of the XNP/ATR-X protein. *J Med Genet* **37**:746–751

Chong SS, Boehm CD, Higgs DR, Cutting GR (2000) Single-tube multiplex-PCR screen for common deletional determinants of alpha-thalassemia. *Blood* **95**:360–362

Cleiren E, Benichou O, Van Hul E, Gram J, Bollerslev J, Singer FR, Beaverson K, Aledo A, Whyte MP, Yoneyama T, deVernejoul MC, Van Hul W (2001) Albers-Schonberg disease (autosomal dominant osteopetrosis, type II) results from mutations in the ClCN7 chloride channel gene. *Hum Mol Genet* **10**:2861–2867

Dacie JV, Lewis SM (1991) Practical haematology, 7th edn. Churchill Livingstone, Edinburgh

Daniels RJ, Peden JF, Lloyd C, Horsley SW, Clark K, Tufarelli C, Kearney L, Buckle VJ, Doggett NA, Flint J, Higgs DR (2001) Sequence, structure and pathology of the fully annotated terminal 2 Mb of the short arm of human chromosome 16. *Hum Mol Genet* **10**:339–352

Eussen BH, Bartalini G, Bakker L, Balestri P, Di Lucca C, Van Hemel JO, Dauwerse H, van Den Ouweland AM, Ris-Stalpers C, Verhoef S, Halley DJ, Fois A (2000) An unbalanced submicroscopic translocation t(8;16)(q24.3;p13.3)pat associated with tuberous sclerosis complex, adult polycystic kidney disease, and hypomelanosis of Ito. *J Med Genet* **37**:287–291

Fan J, White SJ, Bibikova M, Zhou L, Chen J, Wickham-Garcia E, Kalf M, van Ommen GJ, Breuning MH, Guo L, Lu S-H, Zhan Q, Jiang W, Chan O, Wang-Rodriguez J, Barker DL, den Dunnen JT (2006) Screening of deletions and duplications in 1500 genomic loci in a single assay. *Am J Hum Genet* (56th Annual meeting, New Orleans, Louisiana), **25**, 10 September 2006

Fei YJ, Liu JC, McKie VC, Huisman TH (1992) Hb H disease and mild mental retardation in a black girl with a Hb S heterozygosity. *Hemoglobin* **16**:431–434

Flint J, Rochette J, Craddock CF, Dode C, Vignes B, Horsley SW, Kearney L, Buckle VJ, Ayyub H, Higgs DR (1996) Chromosomal stabilisation by a subtelomeric rearrangement involving two closely related Alu elements. *Hum Mol Genet* **5**:1163–1169

Gallego MS, Zelaya G, Feliu AS, Rossetti L, Shaffer LG, Bailey KA, Bacino CA, Barreiro CZ (2005) ATR-16 due to a *de novo* complex rearrangement of chromosome 16. *Hemoglobin* **29**:141–150

Gibbons RJ, Higgs DR (2000) Molecular-clinical spectrum of the ATR-X syndrome. *Am J Med Genet* **97**:204–212

Gibbons RJ, Suthers GK, Wilkie AO, Buckle VJ, Higgs DR (1992) X-linked alpha-thalassemia/mental retardation (ATR-X) syndrome: localization to Xq12-q21.31 by X inactivation and linkage analysis. *Am J Hum Genet* **51**:1136–1149

Giordano PC, van Delft P, Batelaan D, Harteveld CL, Bernini LF (1999) Haemoglobinopathy analyses in

the Netherlands: a report of an in vitro globin chain biosynthesis survey using a rapid, modified method. *Clin Lab Haematol* **21**:247–256

Harteveld CL, Voskamp A, Phylipsen M, Akkermans N, den Dunnen JT, White SJ, Giordano PC (2005) Nine unknown rearrangements in 16p13.3 and 11p15.4 causing alpha-and beta-thalassaemia characterised by high resolution multiplex ligation-dependent probe amplification. *J Med Genet* **42**:922–931

Higgs DR (1993) alpha-Thalassaemia. *Baillieres Clin Haematol* **6**:117–150

Holinski-Feder E, Reyniers E, Uhrig S, Golla A, Wauters J, Kroisel P, Bossuyt P, Rost I, Jedele K, Zierler H, Schwab S, Wildenauer D, Speicher MR, Willems PJ, Meitinger T, Kooy RF (2000) Familial mental retardation syndrome ATR-16 due to an inherited cryptic subtelomeric translocation, t (3;16) (q29;p13.3). *Am J Hum Genet* **66**:16–25

Horsley SW, Daniels RJ, Anguita E, Raynham HA, Peden JF, Villegas A, Vickers MA, Green S, Waye JS, Chui DH, Ayyub H, MacCarthy AB, Buckle VJ, Gibbons RJ, Kearney L, Higgs DR (2001) Monosomy for the most telomeric, gene-rich region of the short arm of human chromosome 16 causes minimal phenotypic effects. *Eur J Hum Genet* **9**:217–225

Kriek M, White SJ, Bouma MC, Dauwerse HG, Hansson KB, Nijhuis JV, Bakker B, van Ommen GJ, den Dunnen JT, Breuning MH (2004) Genomic imbalances in mental retardation. *J Med Genet* **41**:249–255

Lamb J, Harris PC, Wilkie AO, Wood WG, Dauwerse JG, Higgs DR (1993) *De novo* truncation of chromosome 16p and healing with (TTAGGG)n in the alpha-thalassemia/mental retardation syndrome (ATR-16). *Am J Hum Genet* **52**:668–676

Liang J, Zhang Y, Chen Y, Wang J, Pan H, Wu H, Xu K, Liu X, Jiang Y, Shen Y, Wu X (2007) Common polymorphisms in the CACNA1H gene associated with childhood absence epilepsy in Chinese Han population. *Ann Hum Genet* **71**:325–335

Lindor NM, Valdes MG, Wick M, Thibodeau SN, Jalal S (1997) *De novo* 16p deletion: ATR-16 syndrome. *Am J Med Genet* **72**:451– 454

Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB (2000) Rapid detection of alpha-thalassaemia deletions and alpha-globin gene triplication by multiplex polymerase chain reactions. *Br J Haematol* **108**:295–299

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**:1215

Pallaoro M, Fejzo MS, Shayesteh L, Blount JL, Caughey GH (1999) Characterization of genes encoding known and novel human mast cell tryptases on chromosome 16p13.3. *J Biol Chem* **274**:3355–3362

Perez-Reyes E (2006) Molecular characterization of T-type calcium channels. *Cell Calcium* **40**:89–96

Pfeifer D, Poulat F, Holinski-Feder E, Kooy F, Scherer G (2000) The SOX8 gene is located within 700 kb of the tip of chromosome 16p and is deleted in a patient with ATR-16 syndrome. *Genomics* **63**:108–116

Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res* **30**:e57

Shiratsuchi T, Oda K, Nishimori H, Suzuki M, Takahashi E, Tokino T, Nakamura Y (1998) Cloning

and characterization of BAP3 (BAI-associated protein 3), a C2 domain-containing protein that interacts with BAI1. *Biochem Biophys Res Commun* **251**:158– 165

Tiede S, Cantz M, Spranger J, Braulke T (2006) Missense mutation in the *N*-acetylglucosamine-1-phosphotransferase gene (GNPTA) in a patient with mucolipidosis II induces changes in the size and cellular distribution of GNPTG. *Hum Mutat* **27**:830–831

Villard L, Lossi AM, Cardoso C, Proud V, Chiaroni P, Colleaux L, Schwartz C, Fontes M (1997) Determination of the genomic structure of the XNP/ATRX gene encoding a potential zinc finger helicase. *Genomics* **43**:149–155

Warburton P, Mohammed S, Ogilvie CM (2000) Detection of submicroscopic subtelomeric chromosome translocations: a new case study. *Am J Med Genet* **91**:51–55

Weatherall DJ, Higgs DR, Bunch C, Old JM, Hunt DM, Pressley L, Clegg JB, Bethlenfalvay NC, Sjolin S, Koler RD, Magenis E, Francis JL, Bebbington D (1981) Hemoglobin H disease and mental retardation: a new syndrome or a remarkable coincidence? *N Engl J Med* **305**:607–612

White SJ, Vink GR, Kriek M, Wuyts W, Schouten J, Bakker B, Breuning MH, den Dunnen JT (2004) Two-color multiplex ligation-dependent probe amplification: detecting genomic rearrangements in hereditary multiple exostoses. *Hum Mutat* **24**:86–92

Wilkie AO, Buckle VJ, Harris PC, Lamb J, Barton NJ, Reeders ST, Lindenbaum RH, Nicholls RD, Barrow M, Bethlenfalvay NC (1990a) Clinical features and molecular analysis of the alpha thalassemia/mental retardation syndromes. I. Cases due to deletions involving chromosome band 16p13.3. *Am J Hum Genet* **46**:1112–1126

Wilkie AO, Zeitlin HC, Lindenbaum RH, Buckle VJ, Fischel-Ghodsian N, Chui DH, Gardner-Medwin D, MacGillivray MH, Weatherall DJ, Higgs DR (1990b) Clinical features and molecular analysis of the alpha thalassemia/mental retardation syndromes. II. Cases without detectable abnormality of the alpha globin complex. *Am J Hum Genet* **46**:1127–1140

Wilkie AO, Gibbons RJ, Higgs DR, Pembrey ME (1991) X linked alpha thalassaemia/mental retardation: spectrum of clinical features in three related males. *J Med Genet* **28**:738–741

Yntema HG, Poppelaars FA, Derksen E, Oudakker AR, van Roosmalen T, Jacobs A, Obbema H, Brunner HG, Hamel BC, van Bokhoven H (2002) Expanding phenotype of XNP mutations: mild to moderate mental retardation. *Am J Med Genet* **110**:243–247