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SHORT REPORT

Identification of non-recurrent submicroscopic genome imbalances: the advantage of genome-wide microarrays over targeted approaches

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Genome-wide analysis of DNA copy-number changes using microarray-based technologies has enabled the detection of *de novo* cryptic chromosome imbalances in approximately 10% of individuals with mental retardation. So far, the majority of these submicroscopic microdeletions/duplications appear to be unique, hampering clinical interpretation and genetic counselling. We hypothesised that the genomic regions involved in these *de novo* submicroscopic aberrations would be candidates for recurrent copy-number changes in individuals with mental retardation. To test this hypothesis, we used multiplex ligation-dependent probe amplification (MLPA) to screen for copy number changes at eight genomic candidate regions in a European cohort of 710 individuals with idiopathic mental retardation. By doing so, we failed to detect additional submicroscopic rearrangements, indicating that the anomalies tested are non-recurrent in this cohort of patients. The break points flanking the candidate regions did not contain low copy repeats and/or sequence similarities, thus providing an explanation for its non-recurrent nature. On the basis of these data, we propose that the use of genome-wide microarrays is indicated when testing for copy-number changes in individuals with idiopathic mental retardation.
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Introduction

Genome-scanning array technologies, such as microarray-based comparative genomic hybridisation (array CGH), enable the detection of interstitial submicroscopic DNA copy-number alterations in individuals with mental retardation (MR) of unknown aetiology. *De novo* submicroscopic alterations have been identified in approximately 10% of individuals with MR using both bacterial artificial

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chromosome (BAC) microarrays[1–8] and single-nucleotide polymorphism-based microarrays.[9]

Using genome-wide microarray strategies, novel recurrent interstitial submicroscopic aberrations have only been reported sparsely in individuals with MR.[10–17] So far, the vast majority of the cryptic microdeletions/duplications identified appear to be unique, which hampers its clinical interpretation and counselling of the families. However, because these genomic imbalances are likely to harbour dosage sensitive genes related to the pathogenesis of MR, we hypothesised that the genomic regions involved in *de novo* submicroscopic aberrations are candidates for recurrent copy-number changes in individuals with idiopathic MR. To test this hypothesis, we subjected eight pre-selected regions to targeted copy-number analysis using multiplex ligation-dependent probe amplification (MLPA) in a cohort of 710 individuals with idiopathic MR and compared the efficacy of our targeted MLPA-based approach to genome-wide scanning strategies.

Materials and methods

Subjects

In total, 710 mentally retarded individuals with or without facial dysmorphisms or congenital malformations were included in this study. All individuals exhibited normal G-banded karyotypes at 550-band resolution. Genomic DNA was prepared from blood lymphocytes by standard procedures. The DNA samples were derived from Nijmegen, The Netherlands ($n=200$), Oxford, UK ($n=200$), Schwerzenbach, Switzerland ($n=100$), Stockholm, Sweden ($n=80$), Antwerp, Belgium ($n=80$), and Troina, Italy ($n=50$).

Selection of novel submicroscopic aberrations and *in silico* LCR analysis

We selected eight *de novo* submicroscopic copy aberrations for testing among individuals with MR. The aberrations varied in size from 480 kb to 12.4 Mb and were dispersed throughout the genome (Table 1). The aberrations were previously identified in a cohort of 100 mentally retarded individuals using genome-wide tiling path resolution array CGH.[1] The 2q23.1q23.2 microdeletion partly overlapped with a microdeletion previously reported by our group.[8,18]

The flanking 400 kb break point regions of the eight candidate regions were screened for the presence of homologous low copy repeats (LCRs) using the Segmental Duplication Database (http://humanparalogy.gs.washington.edu) and BLAST2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) analyses. The break points were defined by the average start and end positions, respectively, of the first and last flanking BAC clone that identified the genome imbalance (based on NCBI, Build 35, May 2004).

Table 1 Candidate regions screened for DNA copy-number changes

	Location	*Start (Mb)*[a]	*End (Mb)*[a]	*Size (Mb)*
1	1p34.3p34.2	39.2	43.1	3.85
2	2q23.1q23.2	149.2	150.1	0.92
3	3q27.1q29	184.3	196.7	12.42
4	5q35.1	170.5	171.5	0.97
5	9q31.1	99.7	102.6	2.85
6	9q33.1	115.3	115.8	0.48
7	11q14.1q14.2	77.8	85.1	7.28
8	12q24.21q24.23	114.9	116.9	1.98

[a]On the basis of 32k BAC microarray data (NCBI, Build 35, May 2004).[6]

Multiplex ligation-dependent probe amplification

For the MLPA screening two to seven probes were designed within exonic sequences in the genomic regions of interest (Table 2) according to a protocol provided by MRC-Holland (http://www.mlpa.com/pages/support_desing_synthetic_probespag.html). The same probes had also been used for the confirmation of the *de novo* aberrations, previously identified by genome-wide tiling resolution array CGH (Figure 1).[1] The MLPA probes were combined in one MLPA assay in conjunction with four standard control probes in three different genes, *VIPR2*, *MRPL41* and *KIAA0056*. MLPA reactions using 200 ng genomic DNA were performed as described previously.[19,20] All MLPA reagents were obtained from MRC-Holland, Amsterdam, The Netherlands. Amplification products were identified and quantified by electrophoresis on a capillary sequencer (ABI 310, ABI 3100, ABI 3130 or ABI 3730), using GeneMapper software (Applied Biosystems, Foster City, USA). For copy-number quantification data were normalised by dividing each probe's peak area by the average peak area of the control probes of the sample. The normalised peak patterns were divided by the average peak area of all the samples in the same experiment. For all DNA samples, we computed the coefficient of variation (c.v.) of the normalised signal strength over the controls. If a particular sample had a c.v. of more than 15, the result of the analysis for that particular sample was discarded. If a particular probe had a c.v. of more than 15 over all samples tested, the analysis was repeated. Copy-number change detection was based on thresholds for gains and losses of 1.30 and 0.70, respectively. The MLPA analyses were repeated for all samples in which an aberration was identified. For these confirmation experiments, DNA samples of healthy controls were used for the normalisation. If available, DNA of positive controls were included in the MLPA assays.

Results and discussion

We used MLPA to look for copy-number changes at eight pre-selected genomic regions in a European cohort comprising 710 individuals with idiopathic MR with or without associated dysmorphisms or congenital anomalies.

Table 2 MLPA probe information

Gene	*Band*	*Size*	*5′ hybridisation sequence*[a]	*3′ hybridisation sequence*[b]
KCNQ4	1p34.2	100	CAGGGCAACATCTTCGCCACGTCCGCGCT	GCGCAGCATGCGCTTCCTGCAGATCCTGC
EDN2	1p34.2	124	GCGGGAGCCTCGGTCCACACATTCCAGGTGGAGGAAGAGAT	AGTGTCGTGAGCTGGAGGAACATTGGGAAGGAAGCCCGCGG
ZMYND12	1p34.2	128	CCATGTTTGACCCTTACCGGCCACTGTACGGGCCTTTCTGGAC	TTGGACTCCTTGGGAGTCGTTTCTCGGCCATTTGACCCGTGGG
MACF1	1p34.3	92	GTGGAATGTTTCACTGCTCCCAAGG	AGCGGGTAATGAGAGTGGCACTTAG
MBD5	2q23.1	84	CAAAGAGTGTGACGGAGGGGA	CAAGGAAGGAGGTCTTCCAGC
EPC2	2q23.1	92	GAGGTGGAAGGTGAAGTATTTGTTT	TCACCTGGTTTTTGTTTGCTATCTG
MBD5	2q23.1	100	CACTAACAGAAGGTTTGGAAGCCTACAGC	CGTGTCCGGAAAAGGAACAGAAAGTAAGC
EPC2	2q23.1	108	CAGTTAGTTCAGATGCAAAGGCAGCAACTTGCC	CAGCTTCAGCAGAAACAGCAATCTCAGCATTCC
KIF5C	2q23.1	112	CCGTGTTTGTATTTTCGCCCACTAGGGGAAGCTGC	ATGACCCCCAGCTCATGGGGATCATCCCACGAATT
MBD5	2q23.1	120	CAGAGTCGGGGATTTGGAGAGCTGCTAAGCACTGCAAAG	CAAGACCTGGTCCTAGAGGAGCAGTCTCCAAGTTCCTCA
MBD5	2q23.1	124	CTAAATACCCCAAGCAGTGCAGCTTTTCCTACTGCATCTGC	CGGAAGTAGTTCTGTAAAGAGTCAGCCTGGTTTGCTGGGAA
MAP3K13	3q27.2	84	GCACGATGGCCAACTTTCAGG	AGCACCTGAGCTGCTCCTCTT
LPP	3q28	88	GACGCTGAGATTGACTCCTTGAC	CAGCATCTTGGCTGACCTTGAGT
TP723L	3q28	92	GGAGAATGGGGTGATATTGGAGAAG	CTGCATGATAAGACCTGTGACCTTC
HRASLS	3q29	100	CCTTTACAAGCGCCAAGTCTGTATTCAGC	AGTAAGGCCCTGGTGAAAATGCAGCTCTT
FGF18	5q35.1	108	CCTCAGGTCCCACTGACCGCTTCTCCATCTGTT	TCCCGCAGGTGTTTACACTTCCTGCTGCTGTGC
STK10	5q35.1	112	CTCGCCCTGTGCCACCCCAACTGTGCCTGATAGAC	CTGCCCCAGCGTTCCTGACTTCTTGCTGGCCTGTG
DC-UbP	5q35.1	120	CTTGGCACCGCCAATCAACATGATAGAGGAAAAGAGCGA	CATAGAGACTCTGGATATTCCTGAGCCACCACCCAATTC
STX17	9q31.1	92	CAGGTGCACTCATCGGGGGAATGGT	AGGGGGTCCTATTGGCCTCCTTGCA
PRG-3	9q31.1	104	CGAATGCACTGACACTTTTCAGGTGCATATC	CAAGGATTCTTCTGTCAGGACGGAGACTTAA
GRIN3A	9q31.1	108	GCCACAGCCACCATGATCCAACCAGAACTTGCT	CTCATTCCCAGCACGATGAACTGCATGGAGGTG
C9orf27	9q33.1	92	CAAAGGCTCTGTGTTAGTGGATTCA	CAAGCAACCTGTACTCCTCAAAGAA
C9orf27	9q33.1	100	GTATGCAGACTCATCCATCCATGAATCTA	GATTGCAGGGATGTGACCTATGTAATGAA
GAB2	11q14.1	84	CTCCAGCCAGCCAACTCTGTT	CACGTTTGAACCCCCTGTGTC
DLG2	11q14.1	112	CTGACGGGCTTTCTGCCCAAGAGATGAGATGAGAG	CCTCCTCACCCCAGCAGATGTCCAGAGCTGATTTA
PICAM	11q14.2	120	CACAGTGTCACCGGCTCTGCCGTATCCAAGACAGTATGC	AAGGCCACGACCCACGAGATCATGGGGCCCAAGAAAAAG
NOS1	12q24.22	112	CACATGTTCGGTGTTCAGCAAATCCAGCCCAATGT	CATTTCTGTTCGTCTCTTCAAGCGCAAAGTTGGGG
KSR2	12q24.23	120	CTGGCTTTCTCATGGCTTACCCATTGTCTCTGCTCTTCT	GTTCCAACTCAGGAGGCAACCTTTCCAAACAAGACTGGA

[a]The 5′ half-probe is preceded by the 5′ universal primer tag.
[b]The 3′ half-probe is preceded by a 5′ phosphate group and followed by the 3′ universal primer tag.

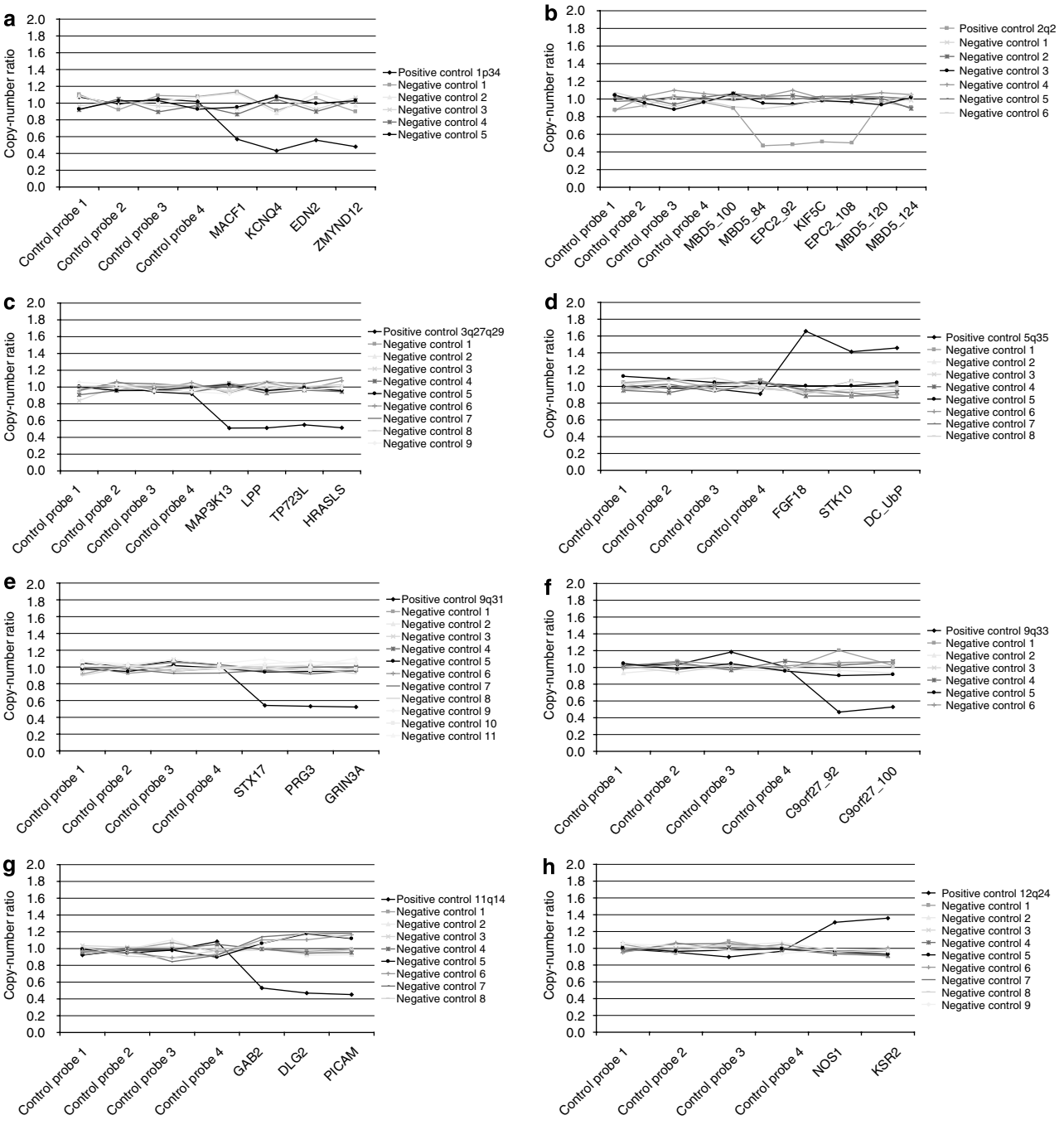

Figure 1 MLPA validation of submicroscopic genome imbalances. (**a**) Loss 1p34.3p34.2, (**b**) loss 2q23.1q23.2, (**c**) loss 3q27q29, (**d**) gain 5q35, (**e**) loss 9q31.1, (**f**) loss 9q33.1, (**g**) loss 11q14.1q14.2, (**h**) gain 12q24.21q24.23.

Causative copy-number changes in these eight regions were previously described in single individuals with MR.[1] The eight regions were based upon the unique and *de novo* aberrations that had been found in our initial study among a cohort of 100 MR patients using genome-wide tiling path resolution array CGH.[1] Through the MLPA assay, we failed to detect additional submicroscopic rearrangements at all candidate regions in this patient cohort. Figure 2 shows an example of the data obtained by the MLPA copy-number screening of the candidate regions. The loss of the 11q14.1 segment is clearly demonstrated in the positive control sample, whereas in the remaining test samples in the assay,

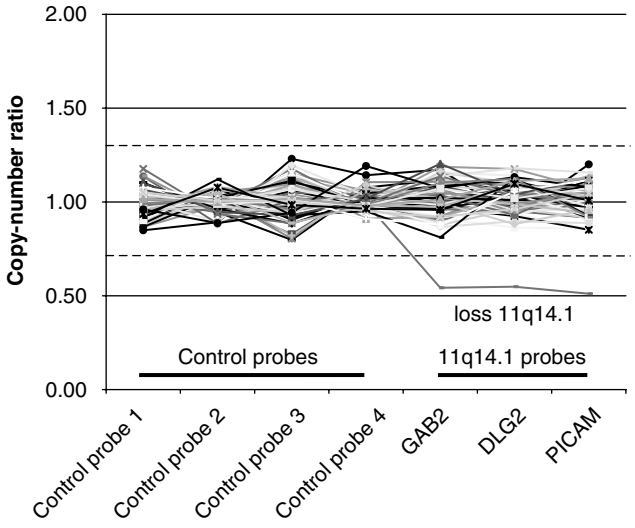

Figure 2 Copy-number screening at 11q14.1 using multiplex ligation-dependent probe amplification. *x*-axis, standard control probes and three probes hybridising to 11q14.1 (for a complete list of probe sequences, see Table 2). *y*-axis, normalised copy-number ratios. Copy-number change detection is based on thresholds for gains and losses of 1.30 and 0.70, respectively. The loss of the 11q14.1 segment is clearly demonstrated in the positive control sample, whereas in the remaining test samples in the same assay, no copy-number changes could be identified.

no copy-number changes are found. Subsequently, we screened the flanking break point regions of the pre-selected candidate regions for the presence of LCRs and/or sequence similarities that might predispose for the occurrence of non-allelic homologous recombination events leading to loss or gain of the intervening DNA sequence.[21] However, no significant LCRs and/or sequence similarities could be identified. The present study is the first report of a comprehensive screen for interstitial submicroscopic aberrations in a large cohort of individuals with MR using MLPA. Of course, the results might have been different if other regions, such as subtelomeric regions, had been analysed in this cohort. Although copy-number changes in these latter regions are usually not mediated by LCRs, which is similar to our eight selected regions, they are in regions that have already been associated with recurrent aberrations.

Others have employed a variety of microarray-based targeted approaches to detect recurrent submicroscopic aberrations. Sharp *et al*,[15] for example, generated a segmental duplication BAC microarray targeted to 130 potential rearrangement hot spots in the human genome. By using this targeted approach, they tested 290 individuals with MR and identified 16 pathogenic rearrangements, including four microdeletions in 17q21.31.[22] The phenotypic similarities between the individuals with an overlapping 17q21.31 deletion subsequently pointed to a new microdeletion syndrome.[10,17,22] More recently, several other novel recurrent microdeletions that are mediated by flanking LCRs have been identified. These recurrent aberrations may give rise to new genomic disorders, such as the 15q24 microdeletion syndrome[16] and the 10q22q23 microdeletion syndrome.[23] Targeted microarrays have been developed with target sequences corresponding to genomic regions of known clinical significance, such as the chromosome subtelomeres and regions implicated in well-known human genomic disorders.[24–27] Using these targeted microarrays, Shaffer *et al*[28] found clinically relevant genomic alterations in 5.6% of 1500 consecutive cases referred to the clinic for a variety of developmental problems. Indeed, these targeted microarrays have some advantages over genome-wide microarray scanning technologies, especially in a diagnostic setting, as parental samples are not requisite for the clinical interpretation of the array CGH findings.[29] In addition, in most cases there is ample information available about the clinical consequences of these submicroscopic copy-number alterations, thus facilitating the genetic counselling of families. However, most known microdeletion syndromes, will be clinically recognised by experienced clinical geneticists and can be confirmed by specifically designed FISH tests. Therefore, patients with a recognisable microdeletion syndrome will only rarely be sent in for microarray analysis. Moreover, in contrast to genome-wide microarray approaches, targeted approaches will miss sporadic DNA copy-number changes in MR, as these regions will not be represented on such microarrays.[30] The latter might be overcome if the targeted array is up-dated, regularly, by including all newly and uniquely reported microaberrations. By doing so, one might eventually end up with a whole genome-wide array.

Our study indicates that *de novo* submicroscopic aberrations that are not flanked by genomic architectural features conferring susceptibility to rearrangements appear to be non-recurrent in a large cohort of patients. In the future it is conceivable that advanced technologies and results from large numbers of patient studies will help unravel the majority of genes involved in MR, thereby making targeted testing approaches more viable. However, in the meantime, we recommend the use of genome-wide microarrays when testing idiopathic MR patients for genome imbalance.

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