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Research Letter

Investigation of Patients With Mental Retardation and Dysmorphic Features Using Comparative Genomic Hybridization and Subtelomeric Multiplex Ligation Dependent Probe Amplication

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To the Editor:

For a number of years we have used high resolution metaphase comparative genomic hybridization (CGH, referred to as HR-CGH in our prior papers) for detection of cryptic chromosomal imbalances in patients with mental retardation and dysmorphic features. Although this technique detects imbalances in as many as 12% of patients, additional diagnoses can be obtained if CGH is combined with subtelomeric FISH analysis [Kirchhoff et al., 2004]. In this study, we set out to test the diagnostic yield of subtelomeric multiplex ligation-dependent probe amplification (MLPA) in patients already investigated by CGH.

In this study, we present the CGH and subtelomeric MLPA results of 258 patients with mental retardation and dysmorphic features. Introduction of a new method in a diagnostic routine setting requires careful consideration regarding evaluation of results. Thus, the diagnostic criteria for the MLPA analysis and the strategy used for exclusion of putative polymorphisms are discussed.

The MLPA assay was performed using the SALSA P019, P020, and P036 human subtelomeric probe sets (MRC-Holland, Amsterdam, The Netherlands). Samples of DNA from 20 normal individuals were used to create reference values for each MLPA probe set. The references for P019, P020, and P036 were based on 86, 81, and 92 analyses respectively. Henceforth, P019 and P020 are merged and referred to as a single probe set as combination of these two sets interrogate all subtelomeric regions (except the acrocentric p-arms) as does the P036 probe set. Following capillary electrophoresis on an ABI Prism 3100, peak areas were normalized in relation to the mean of neighboring peaks. The ratio of each peak and the corresponding mean peak area of the reference were computed, and it was measured in SDs how far each peak area was from the corresponding mean area of the reference. The "area distance in SDs" reflects the individual probe reliability, because a peak ratio indicating an imbalance get a low area distance in SDs, if the probe shows extensive variation (i.e., high SD value) in the reference data set; hereby false positives may be spotted (for normally distributed data the probability for observing an area outside ± 3 SD is less than 1%) [Gerdes et al., 2005; Gerdes et al., in press] (details of data analysis and downloads of the software are available on www.chromosomelab.dk).

Thirteen cases with 20 known aberrations in subtelomeric regions (12 deletions and eight duplications) were used to set up a diagnostic threshold, which was based on both peak area ratios and the area distance in SDs. The average ratio of the deletion probes to the reference was 0.50 with a range of 0.40–0.60. The average area distance in SDs was 7.4 with a range of 4.1–11.8. The average ratio of the duplication probes to the reference was 1.46 with a range of 1.35–1.64. The average area distance in SDs was 7.7 with a range of 4.4–10.2. On the basis of these values, a diagnostic threshold was set: a peak was considered abnormal when the peak area was more than 4.0 SDs from the reference mean and the ratio was <0.60 or >1.35.

The diagnostic threshold was tested on samples from 258 consecutive patients with mental retardation and dysmorphic features who were referred for CGH analysis. The results are summarized in Table I. Abnormalities were detected in 29 (11.2%) patients by subtelomeric MLPA. In 14 of those 29 cases (5.4% of 258), the imbalances were detected by both probe sets (in cases 8, 11, and 29 one of the ratio values or one of the area distances in SDs values were close to, but did not reach, the diagnostic threshold) and in remaining 15 cases (5.8% of 258) the imbalances were detected by one probe set.

In two cases, the P019/P020 probe set showed a deletion of 3pter. Sequencing analysis showed that these patients had a G instead of an A in the splice-site of the probe. This probe is now recognized to detect a polymorphic target sequence (J. Schouten, MRC-Holland, personal communication), and has been replaced in a new generation of subtelomeric probe sets (www. MRC-Holland.com). Thus, the two 3pter deletions are considered to be false positive results caused by a single nucleotide polymorphism (SNP).

Recent studies have shown that there is considerable structural variation in the genomes of normal individuals [Iafrate et al., 2004; Sebat et al., 2004]. In the design of subtelomeric MLPA probe sets, a compromise is made between avoiding polymorphisms and selecting target sequences close to the telomeres, in order to maximize the sensitivity of the screen. In order to reduce the risk of detecting polymorphisms, imbalances detected by only a single probe set were assessed as clinically insignificant. We made this decision for two reasons. First, with regard to small duplications/deletions there is a risk that the clinical significance remains unresolved or a false diagnosis is made. This is distressing for both the family and the medical professionals and genetic counseling becomes inadequate. Second, when submicroscopic imbalances are detected, follow-up is often labor-intensive and costly. In the present study, 15 duplications were detected by one probe set only. Apart from two duplications, all were detected in two, three, or five patients, which is consistent with the assumption that they represent polymorphisms. Indeed, a duplication of

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TABLE I. Summary of CGH and MLPA Results

Case	Imbalance	Detected by CGH[a]	Detected by MLPA		Supplementary confirmative investigation[b]	Origin	Assessment of clinical significance[c]
			P019/P020	P036			
1	del(1)(p36.3 → pter)	Yes	Yes	Yes	FISH	De Novo	+
2	del(1)(p36.1p36.1)	Yes	No	No	QPCR	De Novo	+
3	dup(1)(p32.3p34.1)	Yes	No	No	—	Paternal	?
4	del(1)(q43 → qter)	Yes	Yes	Yes	G-banding/FISH	De Novo	+
5	del(2)(q13q13)	Yes	No	No	—	Maternal	?
6	del(2)(q23q24)	Yes	No	No	G-banding/QPCR	De Novo	+
7	dup(3)(p26 → pter)	Yes	Yes	Yes	FISH	Paternal	+
	del(9)(q34)	No	Yes	Yes			
8	dup(3)(q29)	No	Yes	Yes	FISH	De Novo	+
	del(6)(p23 → pter)	Yes	Yes	Yes			
9	del(4)(p15.3 → pter)	Yes	Yes	Yes	G-banding/FISH	De Novo	+
10	del(4)(p16)	No	Yes	Yes	QPCR	Nd	+
11	dup(4)(q35)	No	Yes	Yes	QPCR	Paternal	?
	del(18)(q12q21)	Yes	No	No			
12	del(7)(p21p21)	Yes	No	No	QPCR	De Novo	+
13	del(7)(q22q22)	Yes	No	No	QPCR	De Novo	+
14	del(7)(q32q36)	Yes	No	No	G-banding	De Novo	+
15	del(8)(q12q13)	Yes	No	No	QPCR	De Novo	+
16	dup(9)(p24)	No	Yes	No	QPCR	Nd	−
17	dup(9)(p24)	No	Yes	No	QPCR	Nd	−
18	dup(9)(p24)	No	Yes	No	QPCR	Nd	−
19	del(9)(q34)	No	Yes	Yes	FISH	De Novo	+
20	del(10)(q11.1q21.1)	Yes	No	No	G-banding	Maternal	?
21	del(10)(q22q22)	Yes	No	No	QPCR	De Novo	+
22	del(10)(q24q24)	Yes	No	No	QPCR	De Novo	+
23	dup(10)(q26)	No	No	Yes	QPCR	Nd	−
24	dup(10)(q26)	No	No	Yes	QPCR	Nd	−
25	dup(10)(q26)	No	No	Yes	QPCR	Nd	−
26	dup(10)(q26)	No	No	Yes	QPCR	Nd	−
27	dup(10)(q26)	No	No	Yes	QPCR	Nd	−
28	del(12)(p13.3 → pter)	Yes	Yes	Yes	FISH/QPCR	De Novo	+
	dup(17)(p13.3 → pter)	Yes	Yes	Yes			
29	dup(12)(p11.1 → pter)	Yes	Yes	Yes	FISH	De Novo	+
30	del(13)(q32 → qter)	Yes	Yes	Yes	QPCR	De Novo	+
31	dup(14)(q11.1q13)	Yes	No	No	QPCR	De Novo	+
32	dup(14)(q31 → qter)	Yes	Yes	Yes	FISH	De Novo	+
33	del(15)(q11.1q15)	Yes	No	No	FISH	De Novo	+
34	del(16)(p13.3)	No	Yes	Yes	QPCR/FISH	De Novo	+
35	dup(16)(p11.1p13.1)	Yes	No	No	G-banding	De Novo	+
36	dup(16)(q24)	No	Yes	No	QPCR	Nd	?
37	del(17)(p12p12)	Yes	No	No	QPCR	De Novo	+
38	del(17)(p11.2p12)	Yes	No	No	FISH	De Novo	+
39	dup(17)(p11.2p12)	Yes	No	No	QPCR	De Novo	+
40	dup(17)(q25)	No	Yes	No	QPCR	Nd	?
41	del(18)(p13.3 → pter)	Yes	Yes	Yes	FISH	Maternal	+
	dup(21)(q22 → qter)	Yes	Yes	Yes			
42	dup(X)(p22.3)/(Y)(p11.3)	No	No	Yes	QPCR	Nd	−
43	dup(X)(p22.3)/(Y)(p11.3)	No	No	Yes	QPCR	Nd	−
44	dup(X)(p22.3)/(Y)(p11.3)	No	No	Yes	QPCR	Nd	−
45	dup(X)(q28)/(Y)(q12)	No	Yes	Yes	QPCR	Nd	−
46	dup(X)(q28)/(Y)(q12)	No	Yes	Yes	QPCR	Nd	−
47	dup(Y)(q11.2q11.2)	Yes	No	No	—	Paternal	?
48	dup(Y)(q11.2q11.2)	Yes	No	No	—	Nd	?

nd, not done.

[a]A confidence level of 99.5% was used as diagnostic threshold.

[b]FISH analyses were performed with subtelomeric or locus specific probes for the involved chromosomal regions. Quantitative PCR using SYBR Green was performed by an ABI Prism 700 sequence detection system (Applied Biosystems, Foster City, CA). In each case, primer sets were designed in DNA sequences overlapping or close to MLPA probe sequences.

[c]Assessment of clinical significance is based on personal judgement. +,clinically significant; −, clinically insignificant; ?, clinical significance is questionable.

10qter, which was detected in 5/258 patients in this study, was found in 5/210 patients in a similar study by Koolen et al. [2004]. As the patients in the study by Koolen et al., did not show clinical resemblance and parental analysis of two of the cases also showed duplication of 10qter, the authors concluded that the duplication was likely to be a polymorphism.

We conclude that the application of two independent subtelomeric probe sets increases the chance of detecting clinically significant terminal imbalances, and this approach is well suited for high throughput routine diagnostics. Yet, we recognize that this approach reduces the sensitivity of the screen. For some subtelomeres the probes in the two sets are

relatively far apart and clinically significant imbalances may involve the target regions of a single probe.

The CGH analysis of the 258 patients showed imbalances in 30 (11.6%) patients (Table I). Interstitial imbalances were found in 20/30 patients while terminal imbalances were found in 9/30 patients. The remaining patient had an i(12p) (case 29, Table I).

Five of the inherited abnormalities detected by CGH were also unbalanced in the apparently phenotypically normal parent (cases 3, 5, 11, 20, and 47). In these cases, casuality cannot be determined and it is possible that they represent structural polymorphisms. Alternatively, they may be causative and involve genomic imprinting, mosaicism, intrafamilial variance of the imbalances at the molecular level, variability in "penetrance," or incomplete clinical details. We have assigned the clinical significance of these imbalances as questionable in Table I.

In the present study subtelomeric MLPA and CGH were complementary. Terminal imbalances were detected in four patients by both subtelomeric probe sets where the CGH result was normal. In two cases MLPA detected two terminal imbalances, where only one of the two were identified by CGH. As expected, none of the 20 interstitial imbalances detected by CGH were detected by subtelomeric MLPA. If only imbalances assessed to have clinical significance are considered (Table I), subtelomeric MLPA detected abnormalities in 13/258 (5.0 %) patients, while CGH detected abnormalities in 24/258 (9.3%) patients. Neither CGH nor subtelomeric MLPA alone is highly sensitive, and it appears necessary to apply both techniques to maximize the detection of aberrations. MLPA is less expensive in our hands, which may be important when designing a clinical testing algorithm.

In the MLPA analyses of the present study, calculations were made relatively to a reference, which renders inclusion or normal samples in each run superfluous. These calculations also allow a diagnostic threshold that is based on both peak area ratio values and area distances in SDs. This approach is highly valuable as it takes into account that ratios for individual probes occasionally do not reach expected values in addition to showing different degrees of variation. All imbalances detected by the selected diagnostic threshold were either verified by a second technique or shown to be caused by a SNP in the splice site of the probe. Thus, no true false positive results were present.

Improved MLPA probe sets are now available and future applications of the technique are likely to show improvement with regards to both selection of targets for some probes and substitution of other probes shown to target polymorphisms. We suspect, however, that variation in probe performance and the risk of detecting polymorhisms will remain. Data calculations relative to a reference and the application of the "two probe set rule" take these circumstances into account.

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