Detecting copy number changes in genomic DNA - MAPH and MLPA

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Chapter 2.3

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ELECTRONIC LETTER

An alternative to FISH: detecting deletion and duplication carriers within 24 hours

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A range of genetic disorders has been revealed to be caused by deletions and duplications within the genome.[1–3] In addition, computational analysis of the recently completed human genome sequence[4] suggests that many more rearrangements might exist. Such rearrangements are either directly involved in genetic disease or may play an important, but yet to be determined, role in human variation and multifactorial diseases. Efficient methods are thus required to screen for and detect such rearrangements.

While changes of several megabases are usually cytogenetically visible, smaller changes require other methods of analysis. Many techniques have been applied, including dinucleotide repeat polymorphism analysis,[5] array comparative genomic hybridisation,[6] fluorescent in situ hybridisation (FISH),[7 8] quantitative multiplex PCR,[9 10] and Southern blotting.[11 12] The last three mentioned are the most commonly applied techniques,[13] with FISH analysis preferred as the method of choice in many clinical centres. FISH has the advantage that the analysis is visual, with the number of fluorescent signals determining the copy number of the region examined. However, the method is rather laborious, with cell culturing and preparation of metaphase spreads being necessary, but difficult and time consuming steps. FISH is thus expensive and not suitable for high throughput analysis. In addition, as FISH probes are usually artificial chromosomes or cosmids, it precludes the analysis of small rearrangements, and duplications can be difficult to detect.

Quantitative multiplex PCR seems an attractive alternative. It can co-amplify up to 15 products per sample, with the amount of each product corresponding to the copy number of the locus. However, achieving consistent results has proven to be technically challenging, and the method requires fluorescent labels and sophisticated equipment.

Southern blotting is more flexible and does not require sophisticated equipment. Its disadvantages are that it is laborious, requiring several blots if multiple loci are to be examined, and its accuracy critically depends on the quality of the blot, with duplications being particularly difficult to detect.

We have applied an alternative method, based on multiplex amplifiable probe hybridisation (MAPH).[14] MAPH facilitates the quantitative recovery of probes hybridised to immobilised genomic DNA, and thus the detection of deletions and duplications. Previous studies have separated the resultant PCR products on acrylamide gels or with a capillary sequencer, using a radioactively[14] or fluorescently[15] labelled primer respectively. To speed up the analysis, we used a chip based gel electrophoresis system (Lab-on-a-chip; Agilent, Palo Alto, CA, USA) to analyse and quantify the reaction products. This system analyses 12 unlabelled samples in ~30 min, with quantitative data being generated automatically by the accompanying software.

We have tested the efficacy and reliability of this methodology by performing carrier detection in Duchenne muscular dystrophy (DMD). This lethal disease is caused by a deletion or duplication of one or more of the 79 exons of the DMD gene in ~70% of cases.[11 16] As the DMD gene is located on the X chromosome, deletion screening in male DMD patients is relatively simple.[17 18] Detecting duplications or carrier status in females, however, requires a quantitative method of analysis. By selecting probes for exons within and outside the rearranged regions, it is possible to compare the relative ratios for the two groups. As multiple probes in parallel hybridisations are used, a high level of redundancy, and thus reliability, can be obtained.

In this paper, we show the validity of this approach by analysing 17 potential carriers for deletion/duplication mutations.

Key points

- When a deletion or duplication mutation has been detected in an index case, relatives may wish to be analysed for carrier status. Methods currently applied are either technically demanding, time consuming or not always applicable.
- We have previously described multiplex amplifiable probe hybridisation (MAPH) as a versatile method for the detection of deletions and duplications, applied to the analysis of Duchenne muscular dystrophy patients.
- Here we show that MAPH is a reliable, quick, and inexpensive alternative for fluorescent in situ hybridisation as a method for carrier detection of deletion/duplication mutations. Following MAPH-based hybridisation and PCR, the amplification products are separated using ''Lab-on-a-chip'' electrophoresis, which quantitatively processes 12 samples in 30 minutes.
- The method is very rapid, taking less than 24 h. Moreover, as several independent probes and duplicates can be run in parallel, it is also very reliable. This approach is an attractive alternative for current FISH-based screens, and should especially facilitate genetic counselling in situations where a rapid diagnosis is important.

METHODS

Probe preparation and the MAPH protocol used have been described previously.[15] Based on the mutation to be tested, a specific set of probes were selected. Where possible, at least

Abbreviations: FISH, fluorescent in situ hybridisation; MAPH, multiplex amplifiable probe hybridisation; MLPA, multiplex ligation dependant probe amplification

two probes within the rearrangement were included, with a minimum of 1 exon from an unaffected region of the gene. In addition, at least two control probes were chosen from a set of autosomal probes. A minimum of two hybridisations were performed on each sample; if the mutation was of a single exon, then three separate hybridisations with the specific probe were carried out.

Following hybridisation and washing, the PCR reaction was performed as previously described,[15] with both primers being unlabelled. Bioanalyzer 2100 (Agilent) analysis was carried out according to the manufacturer's instructions (http://www.chem.agilent.com). Briefly, the DNA500 chip was preloaded with a gel matrix containing a DNA dye. From each PCR sample, 1 µl (~10 ng) of product was added, with a maximum of 12 samples loaded per chip. The samples were then separated, with the data being subsequently exported to Excel (Microsoft Corp.).

Exon specific peaks were normalised within each sample to unlinked probes, with each exon subsequently being normalised to 1.0 based on those samples known to be unaffected at the respective loci.

Ratios derived from probes outside the rearranged regions were compared with those from probes within the rearranged regions with an independent samples Student's *t* test. An individual was considered to be a carrier of the mutation if the difference between the two groups was statistically significant ($p<0.01$). Confidence intervals of 99% were calculated, giving a predicted error rate of 1%. Statistical analysis was performed using SPSS 10.0.7 (SPSS Inc., Palo Alto, CA, USA).

RESULTS

Analysis started with the selection of the probes to be tested. After hybridisation and subsequent amplification, the PCR products were separated on the Lab-on-a-chip. In the resulting trace pattern, each peak corresponded to a specific probe. As shown in fig 1, changes in peak height and area correspond to a deletion or duplication at that specific locus. Although most mutations could be detected visually, quantitative analysis was always performed. The area underneath each peak was calculated by the Bioanalyzer software and subsequently tabulated in Excel. A typical example is shown in table 1. In this analysis, six samples were tested: two deletion and four duplication carriers. Based on the exons known to be affected, four DMD exon probes were chosen, ensuring that for each sample at least one exon gave a normalised ratio of ~1.0. This probe represents the control for hybridisation quality. As can be seen in this example, deletions and duplications could be detected as ratios of around 0.5 and 1.5 respectively. All samples were screened at least twice, with the data from each sample being collated.

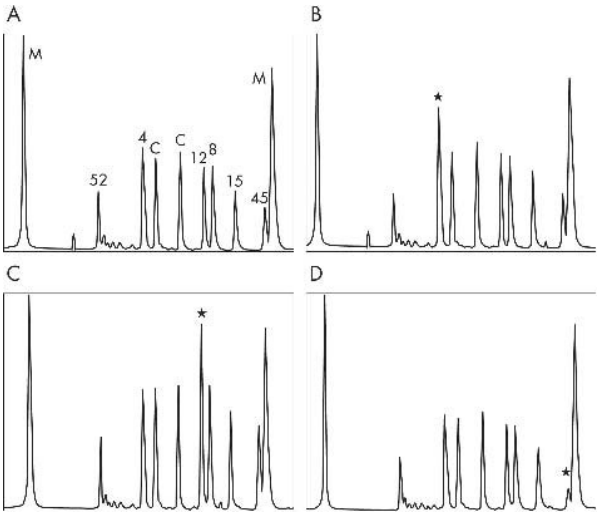

Figure 1 An example of the trace patterns obtained from the Bioanalyzer software. Changes in the peak height and area correspond to changes in copy number of the specific probe. The numbers refer to DMD exons, with autosomal control probes indicated with C. M indicates the two marker alignment peaks, at 15 and 600 bp. These are used by the software for lane to lane alignment. Four different cases are shown here: A, no mutation; B, duplication exon 4; C, duplication exon 12; D, deletion exon 45. In each case, the affected exon is indicated with an asterisk.

Table 1 Lab-on-a-chip analysis

	Sample number						
	1	2	3	4	5	6	Mean
A							
Exon 52	5.31	6.86	8.08	14.81	7.27	6.40	—
Exon 62	12.66	8.14	13.68	12.16	5.71	5.08	—
C1	16.77	12.11	14.50	18.43	8.84	7.06	—
C2	16.12	8.86	11.54	13.42	7.03	5.69	—
C3	17.74	12.14	13.73	18.08	8.61	6.82	—
Exon 54	6.68	8.71	10.93	18.75	9.51	6.76	—
Exon 49	7.32	4.34	10.18	12.46	6.60	4.88	—
B							
Exon 52	0.10	0.21	0.20	0.30	0.30	0.33	0.21
Exon 62	0.25	0.25	0.34	0.24	0.23	0.26	0.25
Exon 54	0.13	0.26	0.27	0.38	0.39	0.35	0.27
Exon 49	0.14	0.13	0.26	0.25	0.27	0.25	0.25
C							
Exon 52	*0.48*	1.00	0.95	**1.43**	**1.43**	**1.57**	—
Exon 62	1.00	1.00	**1.40**	0.96	0.92	1.04	—
Exon 54	*0.48*	0.96	1.00	**1.41**	**1.44**	**1.30**	—
Exon 49	*0.56*	*0.52*	1.04	1.00	1.08	1.00	—

Following electrophoresis, the peak data areas from six samples (1–6) were imported into Excel from the Bioanalyzer software (Section A). By dividing the area under each exon (specific peak is divided by the sum of the area of the control peaks), a ratio for each exon was obtained (Section B).
These ratios are were then normalised to 1.0 based on the mean ratio of samples known to be unaffected at that specific locus (Section C).
The normalised ratios of the exons that are duplicated are shown in **bold**, those of the deleted exons are in *italics*.

In total, 17 potential DMD carriers were analysed, with the results summarised in table 2. The extent of the mutations varied, ranging from a deletion or duplication of a single exon to a deletion of 37 exons. Of the 17 samples tested, 13 were shown to be mutation carriers. This agreed completely with the results found with other methods, namely FISH, Southern blotting or by MAPH analysed by capillary electrophoresis.

Although duplications are known to be more difficult to detect than deletions, the results were unequivocal in all cases. All carriers had a p value of <0.001, whereas the four non-carriers had p values $\geqslant 0.10$.

DISCUSSION

We describe a novel method for the clinical diagnosis of deletion/duplication mutations, which we consider an attractive alternative for FISH analysis. Based on prior knowledge as to where a mutation might be (index patient), a set of probes is selected, of which some are located inside the rearranged region, some directly flanking and some from other, unrelated regions in the genome. Rapid, quantitative analysis of the reaction products is possible using the Lab-on-a-chip from Agilent. This chip allows the electrophoretic separation of 12 samples in ~30 min, providing a detailed analysis of each peak.

Unless the suspected mutation was of a single exon, at least two probes within the region of interest were chosen,

Table 2 The 17 samples examined

Case no.	Mutation in son	Mean ratio within rearrangement (n)	Mean ratio outside rearrangement (n)	99% CI of the difference	p Value	Carrier?
1	dup 58-63	**1.44** (3)	1.01 (11)	−0.58 to −0.28	<0.001	Yes
2	del 10-46	*0.47* (10)	0.97 (13)	0.30 to 0.70	<0.001	Yes
3	dup 44-57	**1.51** (13)	1.07 (24)	−0.58 to −0.31	<0.001	Yes
4	dup 50-55	**1.39** (6)	0.98 (19)	−0.51 to −0.30	<0.001	Yes
5	dup 52-55	**1.48** (6)	1.03 (13)	−0.61 to −0.29	<0.001	Yes
6	dup 51-55	**1.60** (7)	0.99 (18)	−0.94 to −0.26	<0.001	Yes
7	del 45	*0.39* (3)	1.02 (15)	0.46 to 0.80	<0.001	Yes
8	del 49-54	*0.51* (10)	1.00 (19)	0.39 to 0.59	<0.001	Yes
9	del 48-50	*0.53* (5)	1.01 (12)	0.41 to 0.55	<0.001	Yes
10	dup 2-9	1.01 (4)	0.98 (11)	−0.16 to 0.11	0.63	No
11	dup 3-7	**1.43** (6)	0.94 (20)	−0.65 to −0.32	<0.001	Yes
12	dup 12-13	**1.47** (4)	1.03 (17)	−0.64 to −0.23	<0.001	Yes
13	dup 2-6	**1.28** (4)	1.01 (17)	−0.42 to −0.11	<0.001	Yes
14	dup 2-7	1.07 (4)	0.94 (8)	−0.35 to 0.12	0.13	No
15	del 52	*0.55* (3)	0.96 (12)	0.10 to 0.63	<0.001	Yes
16	del 8-43	1.00 (4)	0.96 (6)	−0.24 to 0.15	0.47	No
17	dup 12	1.10 (3)	1.00 (12)	−0.28 to 0.07	0.10	No

Listed are the ratios derived from probes within and outside the rearrangements.
The mean ratio for each sample is given (duplicated in **bold**, deleted in *italics*), with the figure in brackets being the number of probes tested.
The p values were determined with Student's *t* test, and the associated 99% confidence intervals (CI) of the differences are also shown.

and all samples were tested in at least two hybridisations (three hybridisations for single exon mutations). Due to the simplicity of the technique, it is little extra effort to perform these hybridisations in parallel, and no time is lost. Data derived from the different hybridisations for each sample were collated, and the ratios were separated into two groups based on whether the probes were localised within or outside the potential breakpoints. By combining the data, the potential influence of any false positives and negatives was minimised. Previous studies have used different methods of assessing a positive result, ranging from setting arbitrary boundaries of 0.75 and 1.25,[19] to bivariate analysis for each affected probe.[20] We have taken advantage of the fact that the potential mutation was already known, by comparing the ratios derived from probes within and outside the rearranged region. If the difference was not statistically significant ($p>0.01$) then it was assumed that the individual was not a carrier. Conversely, a significant difference was taken to indicate the presence of the suspected mutation. This was confirmed by the results obtained. As can be seen by the 99% confidence intervals, the actual error rate will be considerably lower than the 1% predicted.

In some cases, the mother may be a mosaic, meaning that the mutation will not be present in all cells. This makes the analysis more difficult. Whether such cases would be detected by the described method depends on several factors, including the standard deviation of the probes, the number of different probes that can be used, and the degree of mosaicism. Due to the influence of the unaffected cells, a p value between 0.01 and 0.1 may occur, prompting further analysis.

There are several advantages to using MAPH in combination with the Lab-on-a-chip. It can be broadly applied, as a variety of probes can be chosen and all can be used under identical PCR conditions. The resolution is limited only by the size of the probes, which can be as short as 100 base pairs. Analysis is rapid, simple and can be readily automated, as data can be exported to Excel. The DNA chip can measure DNA fragments at less than 1 ng, meaning that unlabelled samples can be directly loaded on the chip without any prior concentration.

The advantages described here for MAPH based analysis also apply to a similar technique, multiplex ligation dependant probe amplification (MLPA).[21] MLPA is based on the specific hybridisation and subsequent ligation of two oligonucleotides, with only ligated end products generating a target for PCR amplification. MLPA has the advantage of being a ''single tube'' assay, and requiring less input DNA. However, compared to MAPH, probe preparation for MLPA is more time consuming. The method of choice would be based on the exact goal and probe availability.

Many probes for MAPH/MLPA have already been developed[15 19–22] and as more probes become available, the possibility of screening other regions of the genome increases (Kriek *et al*, manuscript in preparation). The combination of these techniques with a rapid and simple method of analysis should allow diagnostic laboratories to implement this as a broadly applicable, robust, and readily automated method for high resolution copy number determination.

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