Detecting copy number changes in genomic DNA - MAPH and MLPA

White, S.J.

Chapter 5.1

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(Colour images from this chapter can be seen in the appendix)

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METHODS

Two-Color Multiplex Ligation-Dependent Probe Amplification: Detecting Genomic Rearrangements in Hereditary Multiple Exostoses

Stefan J. White,[1]* Geraldine R. Vink,[1] Marjolein Kriek,[1] Wim Wuyts,[2] Jan Schouten,[3] Bert Bakker,[1] Martijn H. Breuning,[1] and Johan T. den Dunnen[1]

[1]Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands; [2]Department of Medical Genetics, University of Antwerp, Antwerp, Belgium; [3]MRC-Holland, Amsterdam, The Netherlands

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Genomic deletions and duplications play an important role in the etiology of human disease. Versatile tests are required to detect these rearrangements, both in research and diagnostic settings. Multiplex ligation-dependent probe amplification (MLPA) is such a technique, allowing the rapid and precise quantification of up to 40 sequences within a nucleic acid sample using a one-tube assay. Current MLPA probe design, however, involves time-consuming and costly steps for probe generation. To bypass these limitations we set out to use chemically synthesized oligonucleotide probes only. The inherent limitations of this approach are related to oligonucleotide length, and thus the number of probes that can be combined in one assay is also limited. This problem was tackled by designing a two-color assay, combining two sets of probes, each amplified by primers labeled with a different fluorophore. In this way we successfully combined 28 probes in a single reaction. The assay designed was used to screen for the presence of deletions and duplications in patients with hereditary multiple exostoses (HME). Screening 18 patients without detectable point mutations in the EXT1 and EXT2 genes revealed five cases with deletions of one or more exons: four in EXT1 and one in EXT2. Our results show that a two-color MLPA assay using only synthetic oligonucleotides provides an attractive alternative for probe design. The approach is especially suited for cases in which the number of patients to be tested is limited, making it financially unattractive to invest in cloning. Hum Mutat 24:86–92, 2004.

KEY WORDS: MLPA; EXT1; EXT2; hereditary multiple exostoses; HME; mutation detection

DATABASES:
EXT1 – OMIM: 608177; GenBank: NM_000127.1
EXT2 – OMIM: 608210; GenBank: NM_000401.1

INTRODUCTION

Intragenic rearrangements are a common cause of human disease. As mutation screening is usually based on sequence analysis of PCR-amplified fragments, deletions and duplications of complete exons will be missed unless quantitative methods are applied. Many alternatives have been described [reviewed in Armour et al., 2002]. Southern blotting [Den Dunnen et al., 1989], quantitative multiplex PCR [Yau et al., 1996], and fluorescent in situ hybridization (FISH) [Petrij et al., 2000] have been most commonly used, but all have limitations that hinder routine implementation in a flexible and high-throughput manner.

A quick and simple technique for quantitative analysis has recently been described, termed multiplex ligation-dependent probe amplification (MLPA) [Schouten et al., 2002]. This method is based around the hybridization and ligation of two adjacently-annealing probes. Only if these half-probes are ligated can they serve as a template for PCR amplification. The different probes in a set are designed to have common ends, meaning all can be simultaneously amplified with one primer pair. By using a fluorescently-labeled primer, the resulting products can be separated according to size and quantified. This method, which can be performed in a one-tube format, has been successfully applied to several genes in which deletions and duplications are known to frequently occur [Gille et al., 2002; Hogervorst et al., 2003; Taylor et al., 2003].

A significant drawback of the method is the time-consuming nature of probe production. Following the original protocol [Schouten et al., 2002], the generation

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*Correspondence to: S. J. White, Center for Human and Clinical Genetics, Leiden University Medical Center, Wassenaarseweg 72, 2333AL Leiden, The Netherlands. E-mail: s.white@lumc.nl

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of single-stranded DNA fragments of several hundred nucleotides requires cloning into, and subsequent isolation from, a specifically modified M13 vector.

Theoretically, it is possible to use chemically-synthesized oligonucleotides for both of the half-probes, but length limitations mean that relatively fewer probes can be used within the size range available ($2 \times \sim 40$–60 nt). This can be partially circumvented by making use of the increased resolution of capillary electrophoresis, as well as the ability to use multiple colors for detection. By designing the probes such that two different fluorophores can be used simultaneously, it is possible to combine twice as many probes within a single reaction.

To test the efficacy of this approach we designed probe sets to screen for deletions and duplications in the EXT1 (MIM# 608177) and EXT2 (MIM# 608210) genes, in which mutations cause hereditary multiple exostoses (HME). This is a genetically heterogeneous disorder, characterized by multiple bony outgrowths (osteochondromas) on the ends of the long bones, having an incidence of ~ 1 out of 50,000. EXT1 is found on chromosome 8q24 [Ahn et al., 1995], and is an 11-exon gene, spanning 250 kb. EXT2 is a smaller gene, composed of 14 exons and covering 110 kb on 11p11.2 [Stickens et al., 1996]. Both genes code for glycosyltransferases, which are involved in heparan sulfate synthesis.

The disease shows a dominant pattern of inheritance, and mutations are found in either EXT1 or EXT2 in 70 to 80% of all cases [Wuyts et al., 1998; Wuyts and Van Hul, 2000]. The mutations found to date have been mostly truncating mutations or missense mutations, most probably leading to loss of EXT function. Linkage analysis has implicated a third region (on chromosome 19p) in this disease [Le Merrer et al., 1994], but no gene has been identified to date.

Several cases remain, however, where no mutations could be found. As mutation screening was performed almost exclusively at the sequence level, quantitative (deletions, duplications), and positional (inversions, translocations) changes will not have been detected. Entire gene deletions have been seen involving EXT1 (in Langer Gideon syndrome [Ludecke et al., 1995]) and EXT2 (in P11pDS [Bartsch et al., 1996; Wuyts et al., 2001]) as part of contiguous gene syndromes, but to date there has only been one suggestion of a partial gene deletion, in EXT2 [Stickens et al., 1996]. Using the two-color MLPA assay, we detected single- and multi-exon deletions in 5 out of 18 HME cases, with exon 1 of EXT1 being deleted in three unrelated cases.

MATERIALS AND METHODS

Patients

The DNA of 18 unrelated HME patients was studied to identify mutations in the EXT1 or EXT2 genes. The entire coding sequence of the EXT1 and EXT2 genes had been previously analyzed with direct sequence analysis, with no mutations being detected. All patients showed multiple osteochondromas, and 11 were known to have no family history of HME.

Probe Design

Probes were designed for each coding exon of EXT1 and EXT2 (Table 1). To allow simultaneous probe amplification, each set of probes were designed to allow amplification with one pair of primers. For each EXT1 probe, the common ends corresponded to the MLPA primers described in Schouten et al. [2002], and the EXT2 probes used the multiplex amplifiable probe hybridization (MAPH) amplification primers described in White et al. [2002]. To ensure specific hybridization, the presence of repetitive sequences was excluded using the BLAT program from the University of California Santa Cruz (UCSC) website (http://genome.ucsc.edu) [Kent, 2002]. Probes within each set were designed to produce PCR products with a minimum separation of 2 bp, with the products ranging in size from 80 to 125 bp. The hybridizing regions of the probes had a Tm of at least 65°C (defined using the RAW program (MRC-Holland, Amsterdam, The Netherlands), with a GC% between 35 and 60%.

Oligonucleotides were ordered from either Sigma Genosys (UK, www.sigma-genosys.com) or Illumina, Inc. (San Diego, CA). The oligonucleotides from Sigma Genosys were desalted without further purification, whereas the oligonucleotides from Illumina were synthesized in a salt-free environment and were unpurified. All oligonucleotides were synthesized at a starting scale of 50 nmol. The downstream oligonucleotide of each pair was 5′ phosphorylated to allow ligation to occur.

Probe mixes were prepared by combining each oligonucleotide so that all were present at a final concentration of 4 fmol/μl. The EXT MLPA mixes are available on request (www.LGTC.nl).

MLPA Reaction

All reagents for the MLPA reaction and subsequent PCR amplification were purchased from MRC-Holland (Amsterdam, The Netherlands), with the exception of the MAPH-F and MAPH-R primers (Sigma Genosys). The MLPA reactions were performed essentially as described in Schouten et al. [2002]. Briefly, 50–200 ng of genomic DNA (concentration determined using a UV spectrophotometer) in a final volume of 5 μl was heated at 98°C for 5 minutes. After cooling to room temperature, 1.5 μl probe mix and 1.5 μl SALSA hybridization buffer were added to each sample, heat denatured at 95°C for 2 minutes, followed by hybridization for 16 hr at 60°C.

Ligation was performed at 54°C by adding 32 μl ligation mix. After 10–15 minutes, the reaction was stopped by heat inactivation at 95°C for 5 minutes.

PCR amplification was carried out for 30–33 cycles in a final volume of either 25 μl or 50 μl. In addition to the reagents described [Schouten et al., 2002], MAPH-F and MAPH-R were added to each PCR reaction to a final concentration of 100–200 nM, with MAPH-F being fluorescently labeled with either HEX or ROX. The MLPA primers were labeled with FAM. From each PCR reaction, 1–2 μl of product was mixed with 10 μl (Hi Di) formamide in a 96 well plate. For reactions performed with HEX labeled MAPH-F, 0.1 μl ROX 500 size standard (Applied Biosystems, www.appliedbiosystems.com) was also added to each well. Product separation was performed using capillary electrophoresis on the ABI 3700 (Applied Biosystems).

Data Analysis

For quantitative analysis, trace data were retrieved using the accompanying software (GeneScan; Applied Biosystems). These data were then exported to Excel (Microsoft; www.microsoft.com) for further calculations. Within each probe set, two probes for unlinked loci were included as a reference, and all calculations were performed within one probe set. The height of each exon-specific peak was divided by the sum of the heights of the two reference peaks, to give a ratio. The median ratio for each probe across all samples was calculated, and this value was used for

TABLE 1. The EXT1 and EXT2 Exonic Probes Used in This Analysis*

Probe	Standard deviation (range)	Upstream hybridising sequence	Downstream hybridising sequence
EXT1 exon 1	0.06 (0.88–1.04)	GCATGGCAAAGACTGGCAAAAGCACAAGGAT	TCTCGCTGTGACAGAGACAACACCGAGTATGAGAAGTAA
EXT1 exon 2	0.04 (0.92–1.07)	GTATGATTATCGGGAAATGCTGCACAAT	GCCACTTTCTGTCTGGTTCCTCGTGGTCGC
EXT1 exon 3	0.03 (0.93–1.03)	CCCTGTGATGCTCAGCAATGGATGGGAGTTGCCATTCTCT	GAAGTGATTAATTGGAACCAAGCTGCCGTCATAGGCGATG
EXT1 exon 4	0.05 (0.95–1.08)	GGTCTATTCATCAGGATAAAATCCTAGCACTTAGACA	GCAGACACAATTCTTGTGGGAGGCTTATT
EXT1 exon 5	0.03 (0.92–1.04)	CCACAGTATTCATCTTATCTGGGAGATTTTCCTT	ACTACTATGCTAATTTAGGTAAGTGAATTTCCTCCAGGG
EXT1 exon 6	0.02 (0.94–1.02)	GTACTGTGCCCAGGTGAGCGGGAAGT	TGACAGAGAAGCCCCTGCCTGCT
EXT1 exon 7	0.06 (0.94–1.13)	CAGCCATCTAATGAGCCCCATCCCTTTCAGATCATAGTTCT	ATGGAATTGTGACAAGCCCCTACCAGCCAAACACCG
EXT1 exon 8	0.05 (0.96–1.10)	CGAGGACACGGTGCTTTCAACAACAG	AGGTAAGAACCCATGCCTGAGGAGCA
EXT1 exon 9	0.05 (0.95–1.14)	CTCCATGGTGTTGACAGGAGCTGCTATT	TACCACAAGTGAGGAATCTGGACATGT
EXT1 exon 10	0.03 (0.93–1.03)	CCTGAAGAACATGGTGGACCAATTGGCCAATTGTGAGGACAT	TCTCATGAACTTCCTGGTGTCTGCTGTGACAAAATTGCCTC
EXT1 exon 11	0.04 (0.96–1.02)	GAGCTGCATGAATACGTTTGCCA	GCTGGTTTGGCTACATGCCGCTG
EXT2 exon 2	0.04 (0.96–1.08)	CAGTTGCAGAATGCACACGTGTTTTG	ATGTCTATCGCTGTGGCTTCAAC
EXT2 exon 3	0.03 (0.94–1.05)	CTTGACAGGTGGGATCGAGGT	ACGAATCACCTGTTGTTCAACATGTTGCCTG
EXT2 exon 4	0.03 (0.93–1.04)	CTATAGTCCACTGTCAGCTGAGGTGGATCTTCCA	GAGAAAGGACCAGGGTAAGGTACATTCATCCCA
EXT2 exon 5	0.06 (0.94–1.14)	CAAACATGGAGAGTCAGTGTTAGTACTCGAT	AAATGCACCAACCTCTCAGAGGGTGTCCTTT
EXT2 exon 6	0.05 (0.96–1.12)	GCAGTATTGAGCGATGTGTTACAAGCTGGCTGT	GTCCCGGTTGTCATTGCAGACTCCTATATTTT
EXT2 exon 7	0.12 (0.82–1.21)	CAGAGCATCTGTGGTTGTACCAGAAGAAAAGATGTCAGATGT	GTACAGTATTTTGCAGAGCATCCCCCAAAGACAGATT
EXT2 exon 8	0.03 (0.96–1.06)	GCTGCCATCTCCTATGAAGAATGGAATGACCCTCCT	GCTGTGGTAAGTGAATTCCAGTGCTAGCCACATGA
EXT2 exon 9	0.03 (0.94–1.02)	GTTCACCGCCATAGTCCTCACCT	ACGACCGAGTAGAGAGCCTCTTC
EXT2 exon 10	0.03 (0.96–1.05)	CCAGATTCTCTCTGGCCCAAAATCCGGGT	TCCATTAAAAGTTGTGAGGACTGCTGAA
EXT2 exon 11	0.04 (0.95–1.10)	GCATCTCTGGGACCATGAGATGAATAAGTGGAAGT	ATGAGTCTGAGTGGACGAATGAAGTGTCCATGGT
EXT2 exon 12	0.04 (0.95–1.08)	GGTGGCCAACGTCACGGGAAAAGCAGTT	ATCAAGGTAGGAGGCTCTGCCACTCAC
EXT2 exon 13	0.19 (0.69–1.32)	CAGCCATAGATGGGCTTTCACT	AGACCAAACACACATGGTGGAG
EXT 2 exon 14	0.03 (0.94–1.05)	GTTAAGGGTGGAAGGTTGACCTACTTGGATCTTGGCAT	GCACCCACCTAACCCACTTTCTCAAGAACAAGAACCTA

*For each exon the unique sequence of the two half-probes is given, along with the SD and range of normalized ratios obtained when analyzing 12 unaffected control samples.
Mixes of these probes are available upon request (www.LGTC.nl).

normalizing each probe to 1.0 (corresponding to a copy number of two). Thresholds for deletions and duplications were set at 0.75 and 1.25, respectively, meaning that the adjusted ratios within each sample needed to be normalized to 1.0. The normalizing factor was calculated by determining the mean value of the unaffected probes within a sample (defined as falling between 0.8 and 1.2), and dividing all values within that sample by this value. All samples were tested at least twice.

Confirmation of Single-Exon Mutations

Because sequence changes at the ligation site of the two half-probes can also appear as deletions, all single-exon changes were confirmed using another technique. Two of the EXT1 exon 1 deletions were confirmed using MAPH. The sequences for amplification of the probe were forward; AGATGCAGG-GATTTGTGAGG, reverse; CATCTTTGGGTTGCACAATG. Further probe preparation and MAPH was carried out as previously described [White et al., 2002].

The third EXT1 exon 1 deletion was confirmed by FISH analysis. This was performed using standard protocols, with the following probes: D822 (orange), 90D8 (red), and 46F10 (green). D822 is the reference probe for chromosome 8, 90D8 matches exon 1 and the 5′ upstream region of EXT1, and 46F10 covers exons 6–11 of EXT1 [Bernard et al., 2001].

The exon 2 deletion in EXT2 was confirmed by long-range PCR and sequencing across the breakpoints. The PCR reaction was performed using the Expand Long Template PCR System (Roche, www.roche-applied-science.com), with the primers used being forward; CATGATGGGTGCTCAATAATGGTTT, reverse; GCTGTGTTATAATCTGGGGGACCTC. The sequencing reaction used the nested primer, ATTATGTAAGTGCTACGAG-GAGGTG, and was analyzed by the Leiden Genome Technology Center on an ABI 3730 capillary sequencer.

RESULTS

To maximize the number of loci that can be analyzed in a single MLPA assay, we chose to test whether different primer sets can be efficiently coamplified under the same PCR conditions. Testing showed that the primer sequences we used for MAPH analysis [White et al., 2002] were also effective under the MLPA conditions. The probes for EXT1 were designed with the MLPA primer sequences attached, and the probes for EXT2 used MAPH primer sequences. To circumvent the laborious cloning step, we decided to use synthetic oligonucleotides, ranging in size from 39–64 nt, including amplification sequence. The probes were tested on 12 control samples to assess their reliability and consistency, as well as to determine the influence of the two primer pairs on the amplification. The signal strength between the two colors was not always equal, which complicated analysis. Titration experiments showed that adding the MAPH primers at half the concentration of the MLPA primers resolved this issue, usually yielding similar peak heights for both probe sets. The accuracy of analysis, however, was not affected when equimolar amounts of MLPA and MAPH primers were added, even though up to a 10-fold difference in peak height between the two probe sets was occasionally observed.

Of the 24 exonic probes tested, two (EXT2 exon 7 and EXT2 exon 13) gave a standard deviation of greater than 10% (Table 1). These probes were considered to be unreliable, and were not included in further calculations. Of note, the smallest standard deviations were obtained when comparisons were only performed between samples from the same source.

To see if any deletion or duplication mutations could be detected in patients suffering from HME, a total of 18 samples were examined, in which previous sequence analysis was unable to identify any mutations. We identified five rearrangements (Table 2; Figs. 1 and 2)—four in EXT1 and one in EXT2. These mutations were seen irrespective of whether the two probe sets were used separately or combined. The most common deletion was exon 1 of EXT1, which was seen in three unrelated individuals. The deletion was confirmed in one of the samples using FISH (Fig. 3). A probe covering exon 1 and the 5′ upstream region was deleted on one copy of chromosome 8. A probe covering exon 6–11 was present on both copies. In addition, heterozygosity for a single nucleotide polymorphism (SNP) in exon 3 confirmed that the deletion did not extend past exon 2 (data not shown).

Additional analysis of the sample with the deletion of exon 2 of EXT2 showed that the deletion did not include exon 1 (data not shown). Long-range PCR and sequencing defined the deletion to be 422 bp, with one of the breakpoints being in exon 2. The last five nucleotides before the upstream breakpoint (ctccc) are also the last five nucleotides of the deleted sequence, but no further sequence homology was seen.

DISCUSSION

We describe here a further development of MLPA, using synthetic oligonucleotides and two colors. In the original description, one of the two half-probes was generated by cloning into an M13 vector. This approach allows the generation of single-stranded DNA molecules several hundred nucleotides long. The cloning and subsequent restriction digestion, however, is time-consuming and expensive. Using chemically synthesized

TABLE 2. A Summary of the Mutations Found*

Sample	Gene	Mutation at DNA-level	Description of mutation	Confirmed by
1	EXT1	c.-772-?_962+?del	EX1del	MAPH
2	EXT1	c.-772-?_962+?del	EX1del	MAPH
3	EXT1	c.-772-?_962+?del	EX1del	FISH
4	EXT1	c.963-?_3287+?del	EX2_EX11del	Multiple exons
5	EXT2	c.-30-10_441del	EX2del	Long range PCR and sequencing

*The cDNA reference sequences used are NM_000127.1 for EXT1 and NM_000401.1 for EXT2. Nucleotide numbering uses the A of the ATG-translation initiation codon as nucleotide +1.

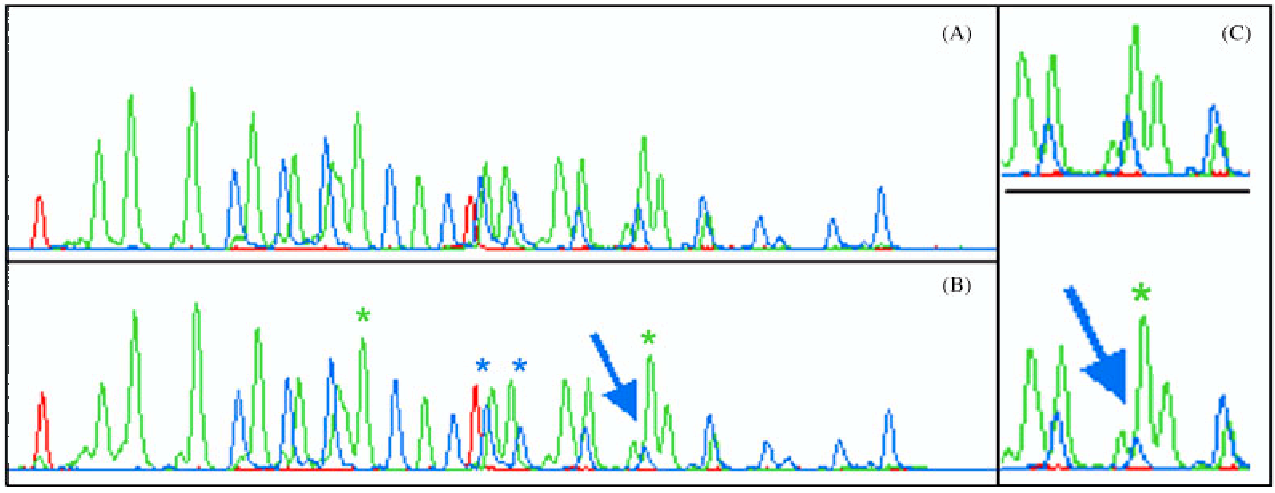

FIGURE 1. **Traces showing the peaks from the two probe sets for EXT1 exons (blue) and EXT2 exons (green). Each set contains two control probes for normalization purposes (marked with *). A: A normal trace. B: An ~50% reduction in the height of the peak corresponding to exon 1 of EXT1 (indicated by arrow). The red peaks are size standard peaks (from left to right; 75 bp and 100 bp). C: An enlargement around the EXT1 exon 1 peak.**

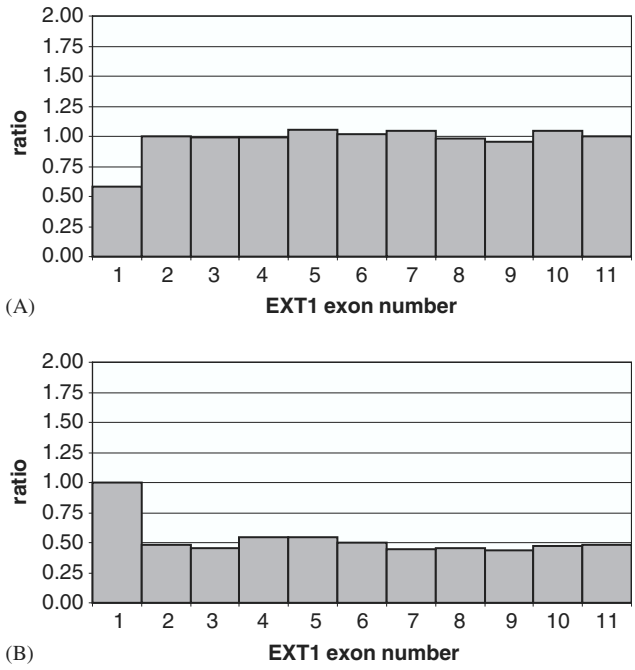

FIGURE 2. **Graphs showing two of the mutations found. A: EXT1 exon 1 deletion. B: EXT1 exons 2–11 deletion.**

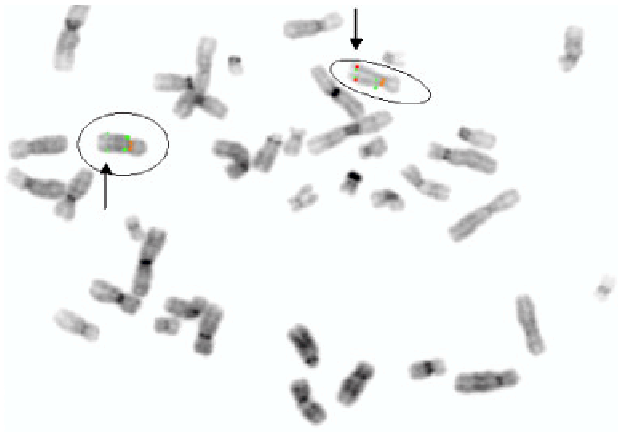

FIGURE 3. **FISH analysis showing a deletion of EXT1 exon 1. The following probes have been hybridized: D822 (orange), 90D8 (red), and 46F10 (green). D822 is the reference probe for chromosome 8, 90D8 matches exon 1 and the 5′ upstream region of EXT1, and 46F10 covers exons 6–11 of EXT1 [Bernard et al., 2001]. The two copies of chromosome 8 are circled, and the EXT1 region is indicated with an arrow. There is no red signal on one of the chromosomes, indicating a deletion of the region corresponding to 90D8.**

oligonucleotides allows rapid and cheap probe development. Furthermore, as each test is performed with only 6 fmol of each oligonucleotide, a synthesis yield of 6 nmol would be sufficient for 1 million reactions.

A size range of 80–125 bp was used for the different probes, with up to 15 probes being combined within a single probe mix. The use of synthetic oligonucleotides limits the length of the probes that can be used. We partially compensated for this by combining two probe sets, each labeled with a different fluorophore. Using two colors effectively doubles the number of probes that can be used in this size range, and in this report 28 probes were used. The size range available is dependent on the maximum length of oligonucleotide synthesis that can be achieved. Our observations with other probe sets are that individual oligonucleotides of up to 75 nt in length can be effectively used, meaning that products of up to 150 bp can be generated. Work is in progress regarding the possibility of using a third primer pair, labeled with a different fluorophore. Together, these factors could allow up to 75 probes to be combined in a single reaction.

We observed that, as previously reported [Schouten et al., 2002], the reliability and reproducibility of the technique is primarily dependent on the quality of the

genomic DNA. We noticed that comparisons made between DNA samples from different sources lead to larger standard deviations than when the same data were normalized only within samples from one source. This was presumably due to different methods of DNA isolation. This observation may have implications when analyzing a series of samples from different laboratories.

The ability to multiplex allows much greater flexibility with regard to future applications. We previously described the use of MAPH as an alternative to FISH with regards to confirming the presence or absence of a rearrangement [White et al., 2003]. Although this multicolor approach should be equally applicable to MAPH, MLPA is perhaps more attractive. The ligation step means that it is not necessary to immobilize the genomic DNA on a filter, and consequently the washing steps can be omitted.

We detected exonic deletions in 5 out of 18 HME samples (28%), four in EXT1 and one in EXT2. The mutations found in EXT1 all have one of the breakpoints within intron 1 of the gene. Notably, this intron makes up ~85% of the total size of the gene. There has recently been a report of a familial translocation, also within intron 1 [Pramparo et al., 2003]. Further work needs to be performed on these samples to characterize the breakpoints and to see if there is a common mechanism involved. Additionally, haplotype analysis could be performed on the patients with the EXT1 exon 1 deletion to see if a common ancestor might be involved. This, however, is unlikely, as the three DNA samples are from three different countries (Spain, the Netherlands, and the United States). In addition, the patient from the Netherlands has no previous family history of HME.

As point mutations are found in EXT1 and EXT2 in 70 to 80% of HME patients, our findings suggest that deletions of one or more exons occur in 5 to 8% of all cases. There are several possible reasons mutations were not found in the remaining samples. The methods applied so far will not detect positional changes (i.e., translocations, inversions, insertions, or transpositions) that affect the structure of the gene without changing the sequence or dosage of any of the exons. This kind of rearrangement will not usually be detected by either MLPA or sequencing. To detect such mutations, analysis at the RNA level may be appropriate [Gardner et al., 1995; Beroud et al., 2004]. Another possibility is that the causative mutation lies not in EXT1 or EXT2, but in another gene. The existence of a third gene (EXT3) on 19p has been postulated [Le Merrer et al., 1994], but to date no specific gene has been identified. Both EXT1 and EXT2 belong to the EXT gene family [Duncan et al., 2001], whose other members also show glycosyltransferase activity. No mutations, however, have been reported in any of the genes (EXTL1, EXTL2, or EXTL3) in HME individuals [Wuyts and Van Hul, 2000]. These genes are potential targets for future copy number analysis.

In summary, we show that MLPA is compatible with the use of synthetic oligonucleotides and a two-color analysis. This combination should facilitate quick and inexpensive probe set development, allowing any gene or region of interest to be rapidly scanned for changes in copy number. In total, design, testing, and application should be feasible within two weeks, with most of the time taken up by oligonucleotide ordering and delivery.

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