existing panels (or replace current markers) as a way to increase the detection rate and/or decrease the false-positive rate. However, before ITA is included in such a panel, these findings must be confirmed in a larger study.

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Direct Detection of Exon Deletions/Duplications in Female Carriers of and Male Patients with Duchenne/Becker Muscular Dystrophy, *Giulia Frisso, Antonella Carsana, Nadia Tinto, Giuseppe Calcagno, Francesco Salvatore, and Lucia Sacchetti** (Dipartimento di Biochimica e Biotecnologie Mediche and CEINGE Biotecnologie Avanzate, Università Federico II, Naples, Italy; * address correspondence to this author at: Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, and CEINGE Biotecnologie Avanzate, Via S Pansini 5, 80131 Napoli, Italy; fax 39-81-7462404, e-mail sacchetti@dbbm.unina.it)

Duchenne and Becker muscular dystrophies (DMD/BMD) are X-linked allelic neuromuscular disorders that have a prevalence of 1 in 3500 live-born males. The genetic defect is the result of mutations of the dystrophin gene, which encodes a 427-kDa rod-shaped cytoskeletal protein *(1)*. The locus is very unstable: one-third of all DMD/BMD cases represent new mutations without a family history of the disease *(1)*. Approximately 50–70% of DMD/BMD cases are the result of macrodeletions, and partial gene duplications have been reported in ~6% of patients *(2)*. Both macrodeletions and macroduplications are preferentially clustered in two areas, the amino-terminal (exons 3–7) and the central (exons 44–55) regions *(1)*. The remaining cases are presumably attributable to point mutations or small insertions/deletions *(2)* scattered along the entire gene. PCR detection of macrodeletions is very useful in the analysis of affected males *(3, 4)*, but it provides no information about the carrier status of at-risk women. Carrier status within families is usually assessed by haplotype analysis *(5)*, fluorescence in situ hybridization *(6)*, amplification of ectopic transcripts *(7, 8)*, dosage analysis on Southern blots *(9)*, or separation of quantitative PCR products by gel electrophoresis *(10)*. Semiquantitative methods, based on the separation of fluorescently labeled amplified exons by gel or capillary electrophoresis, are also available *(11–15)*.

Here we report a quantitative PCR method, followed by separation by capillary gel electrophoresis of the fluorescently labeled amplified exons of hot spot regions of the dystrophin gene, which allowed us to detect ~99% patients (affected males and female carriers) with macrodeletions and 89% with macroduplications, and to identify small insertions or deletions in those regions. This method is rapid, and unlike other procedures, it includes an internal standard to normalize amplification efficiency and to calculate a diagnostic index. We validated the method by screening 135 DMD/BMD patients previously

diagnosed in our laboratory [103 male DMD/BMD patients, 30 unrelated female carriers of deletions in the dystrophin gene, and a mother/daughter pair under investigation for DMD/BMD carrier status because the mother had aborted a DMD male bearing a macroduplication] and 30 controls [non-DMD/BMD males (n = 15) and noncarrier females (n = 15)]. All samples came from the biological sample bank of our Department. Informed consent was obtained for each patient according to the procedure established by the local Institutional Bioethics Committee.

Genomic DNA was extracted with the Nucleon reagent set (Amersham). Four fluorescently labeled multiplex PCR reactions were set up to amplify 24 dystrophin gene exons: exons 4, 8, 12, 17, 19, 44, and 45 in multiplex A; muscle promoter and exons 13, 43, 49, 50, and 52 in multiplex B; exons 41, 42, 46–48, 51, and 53 in multiplex C; and exons 2, 3, 5, and 6 in multiplex D. The primers for exons 3, 4, 6, 8, 12, 13, 17, 19, 43–45, and 47–52 and for the muscle promoter are reported elsewhere (3, 4). We used the following forward (F) and reverse (R) primers for exons 2, 5, 41, 42, 46, and 53:

2F: 5′-AGATGAAAGAGAAGATGTTCAAAA-3′
2R: 5′-AATGACACTATGAGAGAAATAAAACGG-3′
5F: 5′-GTTGATTTAGTGAATATTGGAAGTAC-3′
5R: 5′-CTGCCAGTGGAGGATTATATTCCAAA-3′
41F: 5′-GTTAGCTAACTGCCCTGGGCCCTGTATTG-3′
41R: 5′-TAGAGTAGTAGTTGCAAACACATACGTGG-3′
42F: 5′-CACACTGTCCGTGAAGAAACGATGATGG-3′
42R: 5′-CTTCAGAGACTCCTCTTGCTTAAAGAGAT-3′
46F: 5′-TGTGTCCCAGTTTGCATTAACAA-3′
46R: 5′-CTTGACTTGCTCAAGCTTTTCTTTTAG-3′
53F: 5′-TTGAAAGAATTCAGAATCAGTGGGATG-3′
53R: 5′-CTTGGTTTCTGTGATTTTCTTTTGGATTG-3′

The forward primers were labeled with the fluorochromes 6-carboxyfluorescein (FAM), tetrachloro-6-carboxyfluorescein (TET), or hexachloro-6-carboxyfluorescein (HEX). Exon 10 of the pyruvate kinase (PK) gene (labeled with TET in reactions A and B and with FAM in reactions C and D) was coamplified (16) as an internal standard reference for the double-copy gene in each multiplex PCR; DNA from a noncarrier female was also amplified in each multiplex PCR as a control of the double dosage of the dystrophin gene. The linearity of the PCR reaction was checked for each dystrophin exon tested and for the control exon (PK exon 10) and was constant up to 25 cycles with a DNA concentration of 100–300 ng for male samples and 50–300 ng for female samples. We therefore used a DNA sample concentration of 200 ng and 22 amplification cycles in the quantitative PCR reactions. Multiplex PCR mixtures (50 μL) contained 0.5 mM deoxynucleotide triphosphate mixture, 67 mM Tris-HCl (pH 8), 6.7 mM $MgCl_2$ (7.2 mM $MgCl_2$ in multiplex PCR reaction A), 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 6.7 μM EDTA, 100 nL/μL glycerol (or 100 nL/μL dimethyl sulfoxide in multiplex PCR reaction A), and 2.5 U of Taq polymerase. Amplification reactions (denaturation at 94 °C for 7 min; 22 cycles with denaturation at

94 °C for 30 s, annealing at 50 °C for 30 s, and polymerization at 65 °C for 4 min; and final extension at 65 °C for 7 min) were performed with a thermal cycler PCR System 480 (Applied Biosystems).

PCR products (1.5 μL) were mixed (A + C or B + D) and separated by gel electrophoresis (15 KV at 60 °C for 30 min) on the ABI Prism 310 Genetic Analyzer, using the POP-4™ polymer. Peak heights were always within the linear range of the photomultiplier [100–8000 relative fluorescence units (RFU)] and never exceeded 4000 RFU. We used the Genotyper 2.0 (Applera) software for data analysis and created a macro that allowed us to label the peaks automatically. Data were exported to Excel (Microsoft), and the R values were calculated. To evaluate the gene copy number for each exon, we measured the peaks of the dystrophin gene exons (dys exons) and of PK exon 10 (double gene copy) and calculated the ratio between the value of each peak for each patient (R_x, where x represents individual dystrophin exons) and for a female control (R_c):

$$R_x = \frac{\text{dys exon } x \text{ (peak) in patient}}{\text{PK exon 10 (peak) in patient}}$$

$$R_c = \frac{\text{dys exon } x \text{ (peak) in control}}{\text{PK exon 10 (peak) in control}}$$

The diagnostic index (DI), i.e., the ratio between R_x and R_c for each exon, was calculated. Theoretically, the DI should be 0.5 for a single gene copy and 1 for a double gene copy. The DI values obtained for a single and a double gene copy (mean values = 0.499 and 0.982, respectively) in our controls never overlapped, and the experimental CV was <10% (interindividual variability). The between-run imprecision (CVs) of the DIs for a non-DMD/BMD male sample and a noncarrier female sample were <4% (n = 5). Shown in Fig. 1 are the electropherograms of a control noncarrier female (Fig. 1A), a control non-DMD/BMD male (Fig. 1B), a male with a duplicate exon 4 (Fig. 1C), a female with a macrodeletion (Fig. 1D), and a female with a macroduplication (Fig. 1E).

We examined 103 previously diagnosed DMD/BMD males with this method and identified 86 with macrodeletions and 1 with a macroduplication; the others probably had unidentified mutations. The DIs for deleted exons of all 30 female carriers with macrodeletions were between 0.4 and 0.59, and the previous diagnosis (10) was confirmed in these carriers. Consequently, the diagnostic sensitivity of our method for detecting female carriers with macrodeletions was 100%. We also tested a mother/daughter pair suspected of being DMD/BMD carriers and detected in both a macroduplication spanning exons 45–53 (Fig. 1E) with DIs of 1.3 and 1.53. We thus used this range for duplicated exons in females in our standard experimental conditions.

Our method also identified small insertions/deletions. In fact, we amplified (primer 7F, 5′-FAM-TGACTG-GAATAGTGTGGTTTGC-3′; primer 7R, 5′-CTTCAG-GATCGAGTAGTTTCTCTA-3′) the DNA from a male carrying the insertion of one nucleotide in exon 7 of the

dystrophin gene (kindly provided by Prof. V. Nigro, Second University of Naples, Naples, Italy). We also synthesized five additional reverse primers, each of them one nucleotide smaller at the 3′ end, to obtain six different PCR products (102–107 bp). In all cases we were able to separate DNA fragments that differed 1 bp in length (data not shown). Consistent with the latter finding, in 2 of 103 male patients, we identified a 1-bp insertion in the PCR product of exon 3, which at DNA sequencing was found to be a 94-9dupT polymorphism (data not shown).

We have described a quantitative PCR-based method for the direct detection, in DMD/BMD-affected males and female carriers, of macrodeletions, macroduplications, and small insertions or deletions. Unlike Yau et al. *(11)*,

we calculated the DI value with respect to an internal standard, i.e., PK exon 10. The DI value can be compared with other results and is easily understood by patients and their families. Furthermore, the annealing temperature of our amplification reaction is sufficiently permissive (50 °C) to also amplify the 1483-110A>G and 6118-193C>A substitutions in introns 12 and 42, respectively *(11, 17)*. All other reported polymorphisms *(2)* are located outside the primer binding sites or near the 5′ end of oligonucleotides. The multiplex amplifiable probe hybridization method *(15)* is effective in detecting macroduplications and macrodeletions in the dystrophin gene, but it is rather labor-intensive and lengthy (1–2 days). Although our method does not screen the entire dystrophin gene, it
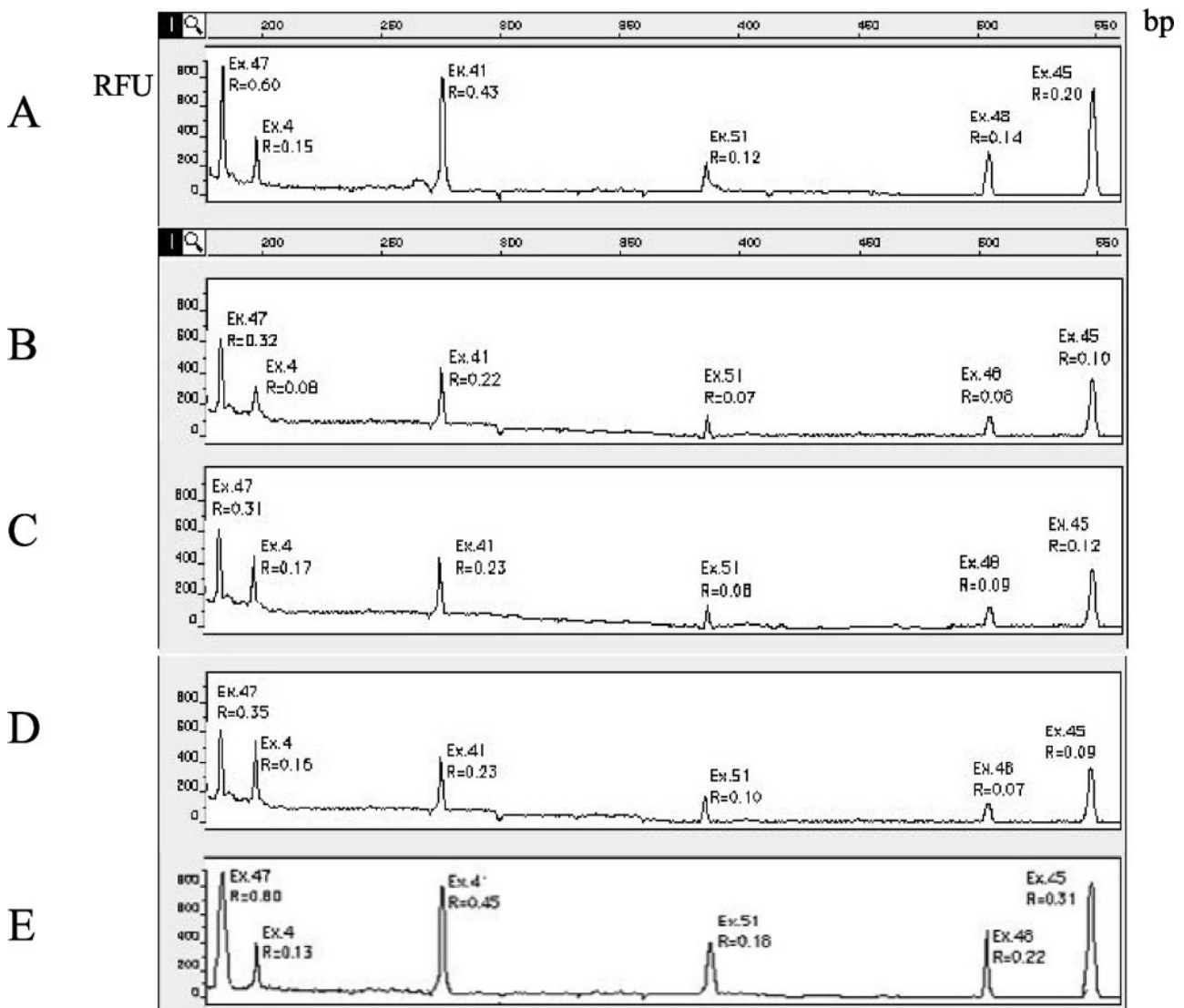
Fig. 1. Sample electropherograms of dystrophin exons 4, 41, 45, 47, 48, and 51, labeled with HEX, obtained with the ABI-Prism apparatus and Genotyper 2.0 software.

The PK exon 10 coamplified in each multiplex PCR was labeled with TET in reaction A and with FAM in reaction C and was detected in other channels. The R values measured for each dystrophin gene exon with respect to PK exon 10 are given to facilitate comparison of the results. (*A*), control noncarrier female; (*B*), non-DMD/BMD male; (*C*), male with duplicated exon 4; (*D*), female with deleted exons 41 and 45–47; (*E*), female with duplicated exons 45–47 and 51. R = DMD exon peak/PK exon 10 peak. *Ex*, exon.

is rapid (all multiplex PCRs are amplified in ~2.5 h, and the capillary gel electrophoresis of the pooled PCR products takes ~30 min), easy to perform, detects ~99% patients with macrodeletions and 89% with macroduplications, and identifies small insertions or deletions.

In conclusion, this method detects >70% of mutations in DMD/BMD families and can be easily applied to the screening of at-risk female carriers, i.e., symptomatic females and females with increased serum creatine kinase and no clinical evidence of muscle disease *(18)*, or when the sample of an affected male is not available.

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Protein Profiling in Urine for the Diagnosis of Bladder Cancer,

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At present, the most reliable means of diagnosis and surveillance of bladder cancer are cystoscopic examination and bladder biopsy for histologic confirmation. The invasive and labor-intensive nature of this procedure underscores the need to develop better, less costly, and nonsurgical diagnostic tools *(1, 2)*. Use of surface-enhanced laser desorption/ionization (SELDI) time-of-flight mass spectrometry has been successful in facilitating protein profiling of complex biological mixtures. This technology uses chemical affinity platforms to capture protein molecules from various biological sources. Retained proteins are subsequently analyzed by mass spectrometry [reviewed in Ref. *(3)*]. Recent reports provide evidence that analysis of SELDI data by "learning" algorithms can lead to the identification of serum protein "fingerprints" for prostate, ovarian, and breast cancers *(4–9)* and urinary fingerprints for kidney cancer *(10)*. We recently reported the application of the SELDI system for detection of potential bladder cancer-associated biomarkers in urine *(11)*. In this earlier study, we showed that combination of five transitional cell carcinoma (TCC)-associated protein peaks by simple statistical methods provided 87% sensitivity and 66% specificity in disease detection. The objectives of the current study were *(a)* to evaluate a commercial available data-mining classification algorithm for the analysis of the SELDI mass spectral data, and *(b)* assessing the clinical utility of this assay in detecting bladder cancer from a geographically and clinically mixed population.

Fresh spot-voided urine specimens from 230 individuals were included in the study. Specimens were collected from patients seen in the Departments of Urology at Eastern Virginia Medical School (Norfolk, VA) and Laikon Hospital (Athens Greece). In all cases, patients gave consent according to the regulations for human