Molecular Diagnostics of Genetic Diseases: Experience from Studies of *DMD*, *APC*, *TSC1*, and *OPG* Genes. Part 1

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Summary Over the past decade geneticists have searched for rapid and efficient means of identification of genetic diseases. Nucleic acid techniques, such as restriction fragment length polymorphisms, polymerase chain reaction, and sequence analysis of amplified DNA are making increasing inroads as tools in diagnostic laboratories. Our laboratory is involved in the molecular diagnostics of several genetic diseases including Duchenne muscular dystrophy, familial adenomatous polyposis coli, tuberous sclerosis, and osteoporosis. Each disease varies in type of molecular lesions and different approaches are applied to achieve the best mutation detection rate. Duchenne/Becker muscular dystrophy (DMD/BMD) is a lethal X-linked recessive disease caused by mutations within the dystrophin gene (*DMD* gene). The *DMD* gene is the largest known human gene, spanning about 2,500 kb and consisting of 79 exons. In approximately 60% of DMD/BMD patients, deletions of one or more exons are detected. Carrier detection still causes problems for many cases, because of the enormous size of the *DMD* gene and the high intragenic recombination frequency. Familial adenomatous polyposis coli (FAP) is a dominantly inherited autosomal disorder caused by germ line mutation in the adenomatous polyposis coli (*APC*) gene and is characterized by early onset of multiple adenomatous polyps, which leads to the development of colorectal carcinoma. The development of colon carcinoma is associated with loss of heterozygosity (LOH) in the *APC* gene and accumulation of mutations in a number of tumor suppressor genes. Detection of mutation carriers in FAP families before pathological symptoms occur is very important and makes possible clinical treatment.

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Molecular changes in the *APC* gene were found in 30% of the studied patients with familial adenomatous polyposis coli, with a similar frequency for the common delta1309 mutation as in other populations. In a Polish population of FAP patients, most mutations were localized in a region of the *APC* gene encompassing codons 1040–1309.

Key Words: molecular genetics, Duchenne muscular dystrophy, familial adenomatous polyposis coli

Duchenne muscular dystrophy (DMD) is one of the most common of the progressive lethal X-linked recessive diseases and affects 1 in 3,500 live newborn males. Allelic to DMD is the clinically less severe Becker muscular dystrophy (BMD). The gene responsible for Duchenne/Becker muscular dystrophy (*DMD* gene) has been cloned [1], and the gene product was named dystrophin. The entire 14-kb cDNA of the Xp21–23 DMD locus consists of 79 exons spanning approximately 2,500 kb of genomic DNA. Thus *DMD* gene is the largest known human gene representing nearly 0.1% of the human genome [2]. The majority of the detectable mutations in the *DMD* gene are deletions (near 60% of all mutation) and duplications (near 6% of all mutations) located around two specific regions of the gene at a distance of 0.5 and 1.2 kb from the promoter [3]. Point mutations are responsible for approximately 30–35% of all mutations [4–7]. In the absence of efficient treatment and rehabilitation for progressive muscular dystrophy, genetic counseling and molecular diagnostics is the solution that medical genetics can offer. Deletions are usually detected by hybridization with cDNA probes or PCR amplification of fragments in the deletion hot spots [8]. Duplications can be detected after pulse field electrophoresis. The most efficient method of deletion screening is the multiplex PCR [9]. This reaction initially enabled the detection of approximately 80% of all deletions that could be detected with cDNA probes; but after the further addition of nine primer sets as described by Beggs *et al.* [10], the deletion detection efficiency increased to 98% [3].

Approximately 30% of affected individuals with no detectable deletions or duplications have most likely subtle mutations that are more difficult to detect. Until recently mutations in promoter elements or point mutations and microdeletions in coding sequences could not be detected, leaving families at risk only indirect approaches.

The detection of mutations in the *DMD* gene is also possible by analysis of ectopic (illegitimate) transcripts [11]. Using ten sets of overlapping primers and total RNA from peripheral blood lymphocytes as a template for specific reverse transcription, Roberts *et al.* [12] amplified the entire dystrophin coding sequence. Products of cDNA-PCR, and similarly genomic products, could be conveniently visualized by gel electrophoresis and ethidium bromide staining. Analysis of ectopic transcripts found immediate practical application for the determination of the carrier status [3, 13–15].

In recent years analysis of microsatellite repeats $(CA)_n$ has found application in tracking of defective genes and carrier detection. The $(CA)_n$ repeats located in the deletion-prone or untranslated region of the *DMD* gene provide also direct information for detection of carriers [16].

Familial adenomatous polyposis coli (FAP) is a dominantly inherited autosomal disorder characterized by early onset of multiple adenomatous polyps in colon and rectum, which leads to the development of colorectal carcinoma. FAP is caused by germ line mutation in the adenomatous polyposis coli (*APC*) gene localized on chromosome 5q21 [17]. The other symptoms caused by the mutations of the *APC* gene are osteomata of the jaw, sebaceous cysts, and lesions of the pigmented retina. FAP is the second in frequency to hereditary non-polyposis colon cancer (HNPCC) among hereditary colon cancers [18]. A distinct feature of FAP as compared to HNPCC and most other forms of hereditary cancer is the development of multiple polyps in the colonic mucosa result in the malignant transformation by years [19], which made possible the diagnosis of the FAP-inducing mutations and related susceptibility to colon cancer in a period before the advent of molecular studies.

The *APC* gene is 8,532 bp in length, consists of 15 exons, and encodes a protein of 2,844 amino acids [20]. The gene is expressed in a tissue-specific manner, and numerous alternatively spliced forms are observed [21]. The development of colon carcinoma in FAP patients is associated with the loss of the normal allele of *APC* gene (LOH) [22]. Furthermore, introduction of the *APC* gene by transfection into human colon carcinoma cell lines suppressed the tumorigenicity of these cells in nude mice, proving that the *APC* gene is a tumor suppressor gene. Accumulation of mutations in a number of other tumor suppressor genes is a prerequisite for malignant transformation [23]. The *APC* gene is expressed in most of the tissues, but only some of them, such as duodenum, pancreas, and stomach, are susceptible to malignant transformation as a consequence of its mutation. The molecular weight of the APC protein is approximately 300 kDa, and the protein is located in the cytoplasm. The *APC* gene product is involved in the regulation of the β -catenin level in cells and, in this manner, can inhibit the c-myc oncogene [24, 25].

Most of the mutations causing FAP are clustered at the 5' part of exon 15. Common types of the FAP-inducing APC mutations are small deletions and insertions, which produce reading frame shifts, generating stop codons and, consequently, truncation of the gene product [26–28].

In the present study scanning of *DMD* and *APC* genes for the presence of mutations is described.

PATIENTS

Duchenne muscular dystrophy. Molecular genetic analysis was performed on DNA obtained from 123 DMD and 2 BMD patients. Together, 52 families with

at least one affected child were directed to us from various clinics in Poznan, Katowice or Olsztyn. Twenty DNA samples of DMD patients were obtained from the Institute of Psychiatry and Neurology in Warsaw. Patients were selected on the basis of clinical findings and elevated creatine phosphokinase (CK) level.

Familial adenomatous polyposis coli. Analysis was performed on DNA isolated from 82 FAP patients, 119 FAP family members (64 families), and 50 healthy controls. Patients and their family members, in most cases, were directed for analysis to Department of Surgery at University School of Medical Sciences in Poznan. Patients were selected on the basis of clinical findings and familial history of cancer. From all FAP patients 8.5% had features of Gardner's syndrome. In 3 other cases symptoms of attenuated adenomatous polyposis of the colon (AAPC) were observed.

MATERIALS AND METHODS

Genomic DNA was isolated from 10 ml of peripheral blood lymphocytes by the salting out [18] or phenol chloroform method. PCR amplifications of genomic DNA samples were performed in 20- μ l reaction mixtures containing 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.25 mM dNTP, 30 ng of each primer, and 0.6 unit of Taq DNA polymerase. The reactions were performed using general cycling protocol with annealing temperatures adjusted to primer requirements. Prior to the first cycle there was an initial denaturation step of 95°C for 3 min and, after the last cycle, an extension step of 5 min at 72°C. Primers for DMD and FAP were synthesized as described [3, 20, 29].

Heteroduplexes (HD) and single-strand conformation polymorphism (SSCP) analyses were used to detect mutations in all studied exons of the *DMD* and *APC* genes. HD analysis was performed by using weak denaturing gels [30]. PCR products were sequentially heated to 95°C for 5 min, incubated at 65°C for 1 h, mixed with 30% formamide and 20% ethylene glycol, and immediately thereafter loaded on 20×20×0.15-cm 10% polyacrylamide gels (49:1, acrylamide:bis) containing 15% formamide and 10% ethylene glycol, and then electrophoresed in 89 mM Tris, 15 mM taurine, 0.5 mM EDTA-gradient buffer. After electrophoresis the gels were silver stained. DNA products with mobility shifts were subjected to sequence analysis. SSCP gels, which were 20×20×0.15-cm 10% polyacrylamide gels (49:1, acrylamide:bis), were run in 0.5×TBE buffer at 20°C at 70 V for 10–16 h. DNA fragments were visualized by silver staining, and PCR products with abnormally migrating bands were subjected to sequence analysis.

Sequencing was performed by using Taq polymerase in cycle sequencing with labeling of primers with ³²P (Amersham, Warsaw, Poland) or Cy5 (TIBMOBOL, Poznan, Poland) followed by autoradiography or laser fluorescence detection (ALFExpress, Pharmacia Biotech, Uppsala, Sweden).

The indirect methods of detection of carrier status in families at risk of developing disease were performed for *DMD* and *APC* genes. Several (CA)_n

repeat markers were used. PCR products were analyzed in 6% polyacrylamide gel containing 7 M urea and 1×TBE buffer by use of an ALFExpress Sequencer. One microliter of the PCR product was mixed with 4 μ l of stop solution containing two internal standards (113 and 268 bp). Additional size markers (100–500 bp) were used as an external standard. The mixture was denatured at 95°C for 5 min, loaded on the gel, and electrophoresed for 2 h at 25 W, 1,500 V, 60°C. Allele size was quantified by Fragment Manager (Pharmacia Biotech) software.

RESULTS

Over the past years we have analyzed 123 patients with DMD. For deletion screening we recombined the amplification primer sets designed previously [3, 8–10] in 6 multiplex PCR reactions, enabling us to achieve satisfactory resolution of PCR products in agarose gels (Fig. 1). Reactions were performed for brain promoter, muscle promoter, and exons 3, 4, 6, 8, 12, 13, 17, 19, 22, 43–52, 55, 60, and 74. The overall deletion rate among Polish DMD patients is approximately 51%. The result of deletion screening among our patients, showing the extent and location of the deletions, is presented in Fig. 2. Over 86% of the deletions occurred in the central part of the *DMD* gene, between exons 43 and 60, and the deletions varied from 1 to over 15 exons in length. Eight cases (13%) had proximal deletions spanning promoter region exons 3 to 22 and involving 1 to 17 exons, and one case

Fig. 1. Detection of deletions in the *DMD* gene by multiplex PCR. Multiplex PCR was performed for 5 exons, and products were separated in 1% agarose gel. Lanes 1–6, exons: 45 (547 bp); 49 (439 bp); 8 (380 bp); 50 (271 bp), and 4 (196 bp). Lanes 8–13, exons: 48 (506 bp); 3 (410 bp); 12 (331 bp); 13 (238 bp), and 60 (139 bp). Lanes 1 and 8, patient 1092, deletion of exons 45, 48, 49, and 50. Lanes 2 and 9, patient 1096, deletion of exons 8, 12, and 13. Lanes 3 and 10, patient 1123, no deletion observed. Lanes 4 and 11, patient 1120, deletion of exon 45. Lanes 5 and 12, positive control. Lanes 6 and 13, negative control. Lane 7, size marker (KBL).

Fig. 3. Identification of point mutation in exon 6 of the *DMD* gene. A. Exon 6 of DNA samples from 8 patients was amplified by PCR. No deletion was observed in DNA samples from these patients after multiplex PCR screening. PCR product ($4\ \mu\text{l}$) was mixed with $6\ \mu\text{l}$ of loading buffer containing formamide, denatured at 95°C for 5 min, and ice chilled. Samples ($8\ \mu\text{l}$) were subjected to electrophoresis in 10% polyacrylamide gels, $20 \times 20 \times 0.15\ \text{cm}$, at 4°C , 100 V, for 17 h. After electrophoresis, gels were silver stained. Sample 1004 (lane 5, marked with arrow) was sequenced, and a $641\text{C} \rightarrow \text{T}$ transition was observed. This transition creates a new stop codon. B. Pedigree of family 1004 and segregation of the mutant allele in the family.

sible by both radioactive and nonradioactive methods. Analysis was performed on 38 families. In 7 cases deletion of one locus was observed, which made possible direct analysis for other family members. For all families an informative result was obtained.

In analysis of the *APC* gene in a Polish population, exons 10, 11, 12, 13, 14, and the 5' region of exon 15 were scanned for mutations by PCR-HD (Fig. 5), PCR-SSCP, and partially by using protein truncation test (PTT). All differential SSCP patterns and heteroduplex bands were analyzed by direct sequencing of PCR products (Fig. 6). Premature termination of translation was found in 2 families (a total of 17 families were studied for segment 3 of exon 15). Mutations of the *APC* gene in the Polish population are heterozygous, but most of them (70%) were observed between codons 1040–1309. The frequency of the common 5-bp deletion at codon 1309 is the same as that reported for other groups of FAP patients [27, 28, 31].

Results from scanning for mutation were subsequently used for carrier detection in FAP families (Fig. 5). In 9 families comprising 36 persons at mutation risk but without clinical symptoms we found 11 persons with an *APC* gene mutation.

Fig. 4. Identification of 5-bp deletion in exon 45 of the *DMD* gene. A. No deletion was observed in multiplex PCR for a total of 24 exons, and a search for point mutations was performed. Exon 45 from 14 patients was amplified by PCR. PCR products ($2.5 \mu\text{l}$) were mixed with $2.5 \mu\text{l}$ of control DNA, denatured at 95°C for 5 min, and renatured at 60°C for 1 h. Loading buffer ($5 \mu\text{l}$) was added, and $7 \mu\text{l}$ were separated in 10% polyacrylamide gel ($20 \times 20 \times 0.15 \text{ cm}$ at 250 V for 6 h). Patient 1105 in lane 12 showed presence of heteroduplex bands. After sequencing delTCGGA was observed. This deletion creates a new stop codon. B. Pedigree of family 1105 and segregation of the mutant allele.

Thus our study excluded 25 persons from standard clinical treatment used for group at risk of heredity mutation in the *APC* gene. Direct analysis of mutation heredity is the most effective, but indirect methods of carrier detection are also useful. However, genetic markers selected for this analysis should be characterized by high PIC (polymorphic information content) and heterozygosity. We studied frequencies of polymorphic sequences within the *APC* gene (Fig. 7). For indirect detection of carrier status we used three highly polymorphic $(\text{CA})_n$ markers closely linked to the *APC* gene [32, 33]. One hundred chromosomes from unrelated healthy individuals and 128 chromosomes from unrelated FAP patients were subjected to analysis of allele frequencies at D5S299, D5S346, and D5S82 loci. In our studies significantly higher frequency of allele A4 of D5S299 was observed among FAP cancer patients. We also observed new alleles in the examined loci in our Polish population: in locus D5S299, allele A 6.1 spanning 158 bp; in locus D5S346, allele A14 (94 bp); and in locus D5S82, allele C6.1 (167 bp). In four cases

Fig. 5. Detection of deletion in exon 15 of the *APC* gene by heteroduplex analysis. Fragment E of exon 15 was amplified by PCR in a 20- μ l reaction volume, and 5 μ l was subjected to heteroduplex formation by denaturation at 95°C followed by incubation at 65°C for 60 min. Samples were separated in 10% polyacrylamide gel (20 $\times$ 20 $\times$ 0.15 cm) and silver stained. Arrows indicate heteroduplexes. Sequencing revealed delAGAAA at codon 1309, which creates a new stop codon at position 1314. Patient 9065.1, who at the time of analysis had no symptoms of disease, inherited the mutant allele.

Fig. 6. Detection of point mutation in exon 15 of the *APC* gene. Fragment C of exon 15 was amplified by PCR in a 20- μ l volume, column purified, and subjected to direct sequencing using the cycling protocol. Three microliters of sequenced samples were separated in 6% polyacrylamide sequencing gel containing 7% urea by using an automated sequence analyzer. The arrow indicates C $\rightarrow$ T transition, creating a new stop codon.

Fig. 7. Polymorphism of the *APC* gene. Mutation in the *APC* gene was not found and indirect diagnostics was used in familial cases based on DNA polymorphism. Fragment K of the gene was amplified by PCR and sequenced. Arrows show polymorphic site where change of nucleotide does not result in amino acid substitution. (A) 9007, homozygote, codon 1756 TCG; (B) 9006, heterozygote, codon 1756 TCK; (C) 9054, homozygote, codon 1756 TCT.

Table 1. Results of carrier status detection by using direct and indirect methods.

No.	Family	Group of risk (number of persons)	Persons without mutation	Used method
1.	9001	10	9	D, ID
2.	9026	3	2	ID
3.	9028	4	3	D
4.	9030	3	3	D
5.	9041	2	1	D
6.	9043	2	2	D
7.	9051	10	9	D
8.	9059	1	0	D
9.	9064	1	0	ID
10.	9065	3	1	D
11.	9067	11	7	D, ID
Total		49	37	

(D) direct method, (ID) indirect method.

polymorphic molecular markers were used for presymptomatic diagnostics. Our strategy, involving direct and indirect methods, permitted us to diagnose 49 persons in group at risk in 11 Polish FAP families by using both direct and indirect methods (Table 1).

DISCUSSION

From available methods of scanning and screening for mutations in *DMD* and *APC* genes as diagnostics, physical separation methods, enzymatic cleavage methods, and DNA sequence analysis were applied. PCR procedures have now been routinely applied for deletion screening of *DMD*, an X-linked disease. However, we noted lower deletion rate among Polish patients, which did not exceed 51%. Dystrophin, like a large rod, binds F-actin, β -dystroglycan, and syntrophins proteins. β -Dystroglycan is associated with α , β , γ , δ -sarcoglycans, α -dystroglycan, and merosin [34]. Mutations in genes encoding dystrophin-associated proteins may result in heterogenous Duchenne-like dystrophies and were not distinguished in our studies [35].

Eighty-six deletions occurred in the central part of the *DMD* gene in the studied cases, and the remaining cases had proximal deletions, indicating an uneven distribution of deletions in the studied group. Approximately half of all deletions involved exons 45 to 50. Deletion sizes were in agreement with those previously reported by others and ranged between a minimum of one exon and a maximum exceeding 30 exons. Deletions within the muscle promoter region were observed only once in our group of patients. RNA analysis reported by us previously [13–15, 36] might resolve difficult cases in which point mutations at the genomic DNA level could not be found. Ectopic transcripts truly reflect the mRNA structure in specifically expressing tissues, and the procedure is especially useful for studying complex human genes such as the *DMD* [11, 12], proacrosin [14], or *CFTR* gene [15].

The scanning method for detection of point mutations/polymorphism in the *DMD* gene used in this study, PCR-SSCP is very simple; and the deletion rate seems to be satisfactory when the enormous size of the gene is taken into account. It is expected that mutations in the rod part of dystrophin protein would have little consequence at the clinical level, since the described cases of mild dystrophies involve in-frame deletions or duplications in the rod [37].

The diagnosis of carrier status in female relatives of *DMD* patients, which is of fundamental importance for genetic counseling and prevention, used to face many problems involving technical difficulties and problems with choosing the proper method. The dystrophin gene is more than 2,500 kb long, and searching for intragenic markers suitable for carrier detection is extremely important. Our routine work involves currently three microsatellite (CA)_n repeats and fluorescence laser detection.

Though the molecular and biochemical bases of *DMD*/*BMD* muscular

dystrophy are known, many questions still remain unanswered. They range from the nature and cause of the deletions of the gene to the relation between *DMD* gene defects and the observed clinical phenotype, in both affected males as well as female carriers. Answers to these questions may not only give rise to the elucidation of pathogenesis of muscular dystrophies in general, but could perhaps also suggest novel approaches to the treatment of DMD.

In our DNA bank of FAP, the DNA from 64 FAP families (82 FAP patients and 119 FAP families members) was collected. All data including disease case report and genetic study result are stored in a standard computer program. Using PCR-HD and PCR-SSCP methods, we observed genetic changes in 33% of FAP patients but only part of the *APC* gene has been studied. An analysis of additional regions of the gene may increase the percent of the detected mutations [38]. Study of allele-specific expression of the *APC* gene may also increase the rate of detection of mutations [39]. The methods used in this paper for visualization of mutation (PCR-HD and PCR-SSCP) differed in relative effectiveness, depending on the location of the mutation within the PCR product. In our studies PCR-HD was more sensitive than PCR-SSCP; however, use of both methods further increased the rate of detection of molecular changes. Detection of mutation made possible by the use of direct molecular approach allowed for carrier detection in the risk group of FAP. Highly polymorphic molecular markers permitted us to recognize mutation carriers in four families. This simple and easy method is not useful for families where first germ line mutation is observed. In our studies of allele frequency in Polish population and the patient group with FAP, significantly higher frequency of allele A4 at locus D5S299 was observed among FAP cancer patients; however, the presence of this allele is not useful for making individual prognosis.

Molecular diagnostics of DMD and FAP, although performed by using similar methods, differ in many details. In the case of DMD, the disease involves only male patients because of X-linkage. Affected persons are hemizygotes, which simplified detection of mutations. In diagnostics of changes in the *APC* gene, normal and mutant DNA sequence overlapped. Both genes differ also in the nature of mutations, which in the case of DMD is mostly restricted to large deletions, whereas in the *APC* gene the prevalence of small deletions, insertions, and substitutions is observed. Minor molecular changes in the *DMD* gene are detected by PCR-SSCP and in the case of the *APC* gene, by PCR-HD. In the latter a hot spot of gene mutation was identified in exon 15. Unusual size of this exon permits application of methods typical for RNA studies, such as the protein truncation test.

For both genes indirect genetic diagnostics can be performed based on inheritance of polymorphic markers located within gene sequences or gene flanking sequences. This kind of analysis is used in cases when mutation cannot be found due to the enormous size of the *DMD* gene or location outside of the mutation hot spot of the *APC* gene.

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