Detection of heterozygous large deletions in the antithrombin gene using multiplex polymerase chain reaction and denatured high performance liquid chromatography

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The present study reports a method for the easy, rapid and cost effective detection of heterozygous large deletions. As a model gene all exons of the antithrombin gene were amplified in a one tube multiplex polymerase chain reaction (PCR) and the products separated according to their size by reverse-phase ion-pair high performance liquid chromatography. A significant reduction in the height of a peak in the proband's sample compared to in the control indicates the presence of a large deletion of the corresponding allele. Using this approach we identified heterozygous deletions in four patients: the deletions affected exons 1 and 2, exon 7 and the whole antithrombin gene.

Key words: large deletion, detection of heterozygous deletion, antithrombin, DHPLC.

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Large gene rearrangements such as deletions, duplications and inversions represent a considerable proportion of mutations causing hereditary disorders. Most methods applied for screening of disease-causing mutations are polymerase chain reaction (PCR)-based and enable the easy detection of sequence alterations such as point mutations and small deletions/insertions. However, these techniques are not capable of detecting large heterozygous autosomal deletions because of the presence of a second normal allele. Non-PCR-based methods such as Southern blotting, fluorescent *in situ* hybridization (FISH), or comparative genomic hybridization (CGH)[1,2] are labor-intensive, require large amounts of DNA and have a low throughput. Other methods based on quantitative differentiation such as real-time PCR, multiplex amplifiable probe hybridization (MAPH), multiplex ligation-dependent probe amplification (MLPA) or quantitative multiplex PCR of short fluorescent fragments (QMPSF)[3-7] are limited by the requirement of multiple sets of specific primers, probes and dyes that greatly increase costs. Furthermore, polymorphisms or rare variants at the probe target site may prevent probe hybridization and ligation and can lead to false positive results.

To overcome these restrictions we established a new approach for the detection of heterozygous large deletions based on a multiplex PCR followed by denatured high performance liquid chromatography (DHPLC) analysis.[8-10] We used the autosomal antithrombin gene (*AT*) as a model for testing the new approach. Antithrombin (AT) is a major physiological inhibitor of blood coagulation and its deficiency greatly increases the risk of recurrent thromboembolism.[11]

AT is located on the long arm of chromosome 1 (q23-25) and comprises seven exons spanning 13,477 bp of genomic DNA. The mutation profile in *AT* is heterogeneous, including nonsense and missense mutations as well as small deletions/insertions,[12,13] while large deletions are rare.[14-16] In our cohort of 24 AT deficient patients we were unable to find a mutation in seven patients by standard mutation screening. We, therefore, proposed that at least some of these patients might carry heterozygous large deletions of one or more exons. Analyzing these patients with the new multiplex PCR/DHPLC-based technique we identified four large deletions involving exons 1, 2, 7 and the whole *AT* gene.

Design and Methods

Patients

Twenty-four unrelated patients with type I AT deficiency (low AT antigen level and activity; mean values of 13.08 mg/dL and 48.97%, respectively) were included in this study. All patients had suffered from thromboembolic events. AT deficiency was confirmed by repeated measurement of both AT antigen by

radial immunodiffusion (NOR-Partigen Immundiffusionsplatte Antithrombin, Dade Behring, Marburg, Germany) and activity by factor Xa-based chromogenic substrate assay (Coamatic LR Antithrombin, Haemochrom Diagnostika, Essen, Germany) according to the manufacturers' recommendations. Patients with reduced AT activity potentially due to confounding liver disease or acute thrombosis were excluded from the study (four patients). Informed consent was obtained from all index patients and their family members. In 17 patients nonsense or missense mutations, small deletions or insertions were identified by direct sequencing of *AT* (*data not shown*). In the remaining seven patients, in whom no mutations were identified by conventional sequencing approach, large gene rearrangements were proposed to be the cause of the deficiency.

Antithrombin gene analysis

DNA isolation: High molecular genomic DNA was isolated from peripheral whole blood by the standard salting out procedure described by Miller *et al.*[17] DNA concentrations were standardized to 100 ng/µL.

Multiplex PCR: All seven exons and flanking introns of *AT* were amplified by semi-quantitative multiplex PCR. The primers (Table 1) were designed to allow amplification at the same annealing temperature (55°C) and giving rise to amplicons which differed by at least 17 bp. An *HGH* fragment (exons 2 and 3) 434bp long was amplified together with *AT* as an internal PCR control. A common master mix was used to achieve maximal equivalent amplification conditions. Amplifications were performed using 100 ng of genomic DNA, 20 pmol of each primer, 50 µM of each of the four types of dNTP, and 2.5 U of Ampli*Taq*-Gold DNA polymerase (PE Applied Biosystems) in a total volume of 50 µL containing 1×GeneAmp PCR buffer with 1.5 mM $MgCl_2$ (PE Applied Biosystems). To keep the PCR reaction in the linear exponential phase, 25 cycles of touch-down PCR were performed under the following conditions: initial denaturation at 95°C for 5 min followed by denaturation at 95°C for 30 sec., annealing under reduction of temperature from 61°C to 55°C by 3°C every 3 cycles for 30 sec, extension at 72°C for 30 sec and final extension for 5 min at 72°C.

DHPLC

Aliquots of 10 µL of the multiplex PCR product were injected in a semi-automated high throughput DHPLC system WAVE (Transgenomic Ltd., Omaha, USA). Separation was performed on a DNAsep cartridge (Ion pair reverse phase C18 column - Transgenomic Ltd.) at a constant oven temperature of 50°C and with UV detection at 260 nm. The mobile phase was 0.1 M triethylammonium acetate (TEAA) solution in water, pH7 (buffer A) and 0.1 M TEAA and 25% acetonitrile (buffer B). The PCR product was eluted at a flow rate of 0.9 mL/min with a gradient of 40 - 72% for buffer B over 20 min. Navigator 1.5.3. Software (Transgenomic Ltd.) was used for data analyses. The chromatograms of investigated patients' samples were superposed on those of the normal control and normalized according to the internal *HGH* control peak. The ratio for each peak was calculated (peak height of the sample/ peak height of control). Ratios between 0.4 and 0.6 indicated the presence of a large gene deletion.

Table 1. Amplification primer sequences for *AT* and *HGH*.

PCR region	*Primer sequence (5' to 3')*	*Annealing temperature (°C)*	*Product Size (bp)*
AT-Exon1F	ctg tcc tct gga acc tct gcg	55	273
AT-Exon1R	ttt gac tgt aac tac cag gga ga	55	
AT-Exon2F	ctc tgc ttt act ggg gca ac	55	580
AT-Exon2R	agc cca aag gtg ctc cta ac	55	
AT-Exon3F	act gag gtg gct att agt cag ag	55	373
AT-Exon3R	aat att gag tgg aga gga aga a	55	
AT-Exon4F	tga ata gca cag gtg agt agg t	55	236
AT-Exon4R	gta agc tga aga gca aga gga a	55	
AT-Exon5F	tgt ctg tgt caa taa cta tcc tcc t	55	607
AT-Exon5R	tcc aac tct tcc act ttt ggt c	55	
AT-Exon6F	agc caa ctt tct ccc atc tc	55	253
AT-Exon6R	ggt ttt gga gag ggc tgt at	55	
AT-Exon7F	ggc aga gtg gct aat tta gtt tta	55	327
AT-Exon7R	att tca aat gca gag tcc att tat	55	
HGH-Exon2-3F	tgc ctt ccc aac cat tcc ctt	55	434
HGH-Exon2-3R	cca ctc acg gat ttc tgt tgt gtt tc	55	

Primer sequences do not overlap with any known single nucleotide polymorphisms as screened against the SNP database (http://www.ncbi.nlm.nih.gov/SNP).

Results and Discussion

In four patients (numbers 1, 2, 6 and 7) the ratio of the peaks' height for at least one peak was in the range of 0.4–0.6, indicating a heterozygous deletion in the corresponding exon(s) (Table 2). In patient 1, a ratio of 0.53 was observed in exon 7, pointing to a deletion of this exon (Figure 1A). This result was confirmed in three other members of the family. Patient 7 exhibited a deletion of exons 1 and 2 producing ratios of 0.57 and 0.60, respectively (Figure 1C). In patients 2 and 6, an approximately 50% reduction of the height in all peaks was found (ratios 0.42–0.60) reflecting a deletion of the whole gene (Figure 1B, D). This deletion was confirmed in another AT-deficient relative of patient 2. In the remaining three samples (numbers 3, 4 and 5) the ratios of the peaks' height for all fragments were in the range between 0.85 and 1.00 and excluded a large deletion as a cause of the AT deficiency.

To validate the sensitivity of the method we examined all 17-AT deficient patients in whom point mutations or small deletions and insertions had been found to be causative for the disorder and 20 healthy donors.

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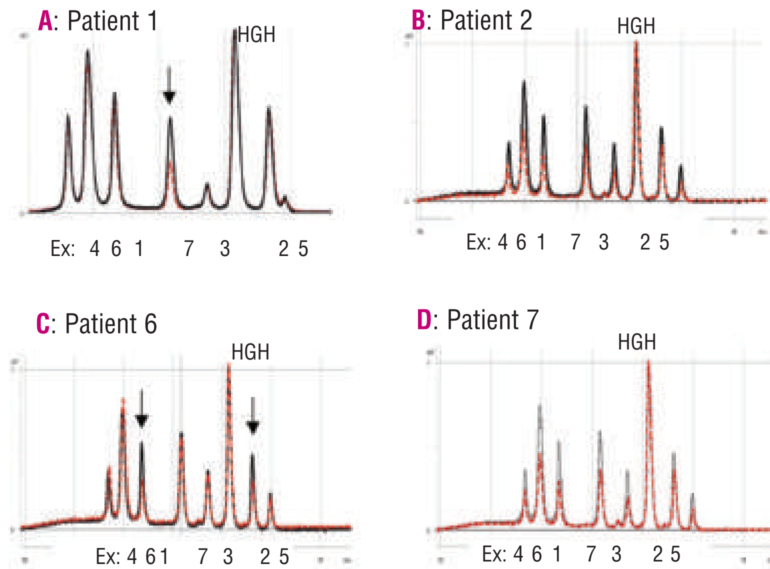

Figure 1. DHPLC chromatograms of four-AT deficient patients with partial and whole gene deletions. The solid line (in black) represents the normal control. The dotted line (in red) represents the examined sample. All samples are superposed and normalized according to the internal control peak of *HGH*. **A.** In sample 1 the height of only exon 7 is half that of the normal control, indicating a deletion of this exon. **B.** Sample 2 shows deletion of the whole gene; the heights of all exon peaks are half those of the control peaks. **C.** Deletion of exons 1 and 2 were found in sample 6. **D.** Deletion of the whole gene was also found in sample 7. Vertical arrows indicate the reduced peak corresponding to a deletion.

The ratio in all samples for all exons was in the range between 0.9 and 1.0, indicating the absence of large deletions (*data not shown*). Large deletions were detected only in patients without point mutations. The reproducibility of the technique was controlled by repeating the multiplex amplification of samples from a normal control and a patient with whole gene deletion (patient number 2) ten times. This showed high reproducibility with a standard deviation ranging from 0.0049 to 0.0087. In addition the detected deletion in family 2 (Table 2) segregated well with the clinical phenotype; in particular the brother who does not have a reduced AT level does not carry the deletion. The large deletions represent 16.6% of the mutations in our cohort of patients with AT deficiency, which differs from the 9.2% reported in the AT mutation database[13,16] or that estimated to be about 5.5.% in the Human Gene Mutation database. This fact poses the question of whether the proportion of large *AT* deletions may be more common than currently thought, but remain underestimated because of the difficulties in their detection. Gene alterations can occur through many mechanisms, one of which is homologous recombination between *Alu* repeats or longer repetitive L1 fragments. The high number of ten such elements in *AT* gene[18,19] may contribute to the formation of large deletions in this gene. In the patients with no detectable disease-causing mutations in *AT*, we cannot exclude mutations located deeply within the introns and affecting splicing since the RNA was not analyzed. It must also be considered that despite the stringent inclusion criteria these patients may have acquired rather than inherited AT

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Table 2. AT antigen and AT activity values and the ratio of peak heights compared to the normal control in patients with and without deletions, after normalization to 1.00.

Family #	*Patients*	*AT:Ag (21-30 mg/dL)*	*AT Activity (86-122%)*	*Exon 1*	*Exon 2*	*Exon 3*	*Exon 4*	*Exon 5*	*Exon 6*	*Exon 7*	*Results*
Family 1	Index patient	17,5	47	0.99	0.98	1.00	0.98	1.00	1.00	0.53	Exon 7 del.
	Grand father	15,3	42	0.97	0.98	0.96	0.99	0.92	0.98	0.49	Exon 7 del.
	Mother	16,2	44	1.00	0.95	0.99	0.85	0.96	0.99	0.54	Exon 7 del.
	Brother of Mother	15,8	40	0.99	0.92	0.98	0.96	0.90	0.97	0.50	Exon 7 del.
	Brother	28.1	98	0.98	0.99	1.00	0.97	1.00	0.99	0.98	No del.
Family 2	Index patient	13,3	67	0.53	0.52	0.42	0.56	0.54	0.56	0.51	Whole gene del.
	Mother	12.0	44	0.54	0.47	0.42	0.52	0.50	0.57	0.50	Whole gene del.
Family 3	Index patient	17,3	60	1.00	1.00	0.98	0.98	1.00	0.99	0.98	No del.
Family 4	Index patient	11,2	30	0.85	0.98	1.00	0.90	0.99	0.96	1.00	No del.
Family 5	Index patient	12.8	67	0.98	0.99	0.99	1.00	0.91	0.98	1.00	No del.
Family 6	Index patient	14,8	43	0.57	0.59	0.98	1.00	0.98	1.00	0.86	Exons 1-2 del.
Family 7	Index patient	11,4	39	0.52	0.61	0.50	0.53	0.54	0.56	0.56	Whole gene del.

Normal values of AT antigen and activity are shown in brackets. AT:Ag : antithrombin antigen; del. :deletion.

deficiency. In conclusion, screening for large deletions in *AT* will complete the genetic analysis of *AT*, thus improving differentiation between inherited and acquired AT deficiency and also aiding genetic counseling of affected families. The method described in this study is a rapid, simple and sensitive screening test for *AT* rearrangements.

JO, AP, OE-M: initiation and design of the study, writing of the manuscript; AP, LK, DD, MW: experimental work on the molecular characterization of the patients with antithrombin deficiency, development of the novel method for the detection of heterozygous large deletions; BL, EL-L, HB, ES: diagnosing and clinical characterization of the patients/families with antithrombin deficiency.

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