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A novel mutation in the *DNASE1* gene is related with protein instability and decreased enzyme activity in thyroid autoimmunity

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ABSTRACT

A deficiency in the DNase enzyme, and thereby, a failure to remove DNA from nuclear antigens promotes disease susceptibility to autoimmune disorders. This study examined in patients with autoimmune thyroid disease (AITD) whether a reduced DNase activity is associated with sequence variations in the *DNASE1* gene. The study included 18 patients with AITD, their 10 relatives, and 111 unrelated healthy controls. Serum DNase activity was determined with a validated, standardized enzyme-linked-immunosorbent assay. The promoter and all nine exons of the *DNASE1* gene were sequenced. Heat stability of DNase enzyme was tested. In patients with AITD, a novel mutation (1218G > A, exon 5) and multiple polymorphisms were identified in the *DNASE1* gene. The allele frequency of the mutation was increased in patients *vs* controls ($P = 0.001$). In contrast to controls, the novel mutation was present in all five members of a family with AITD showing decreased DNase activity. The mutation resulted in the replacement of highly conserved valine with methionine at amino acid position 89 of the DNase enzyme. It was related to lowered heat stability and lowered activity of the enzyme. The identified new mutation and numerous polymorphisms, noted for the first time in AITD patients, may alter transcription and translation of the *DNASE1* gene, thereby decreasing the stability and activity of the corresponding enzyme.

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1. Introduction

The enzyme deoxyribonuclease 1 (DNase 1, EC 3.1.21.1) is the major endonuclease in human serum, urine, and secreta. It catalyzes the hydrolysis of double-stranded DNA [1] and cleaves endonucleolytically DNA to 5′-phosphodinucleotide and 5′-phosphooligonucleotide end-products. A deficiency in DNase 1, and thereby, a failure to remove DNA from nuclear antigens promotes disease susceptibility to autoimmune disorders [2]. The DNase 1 protein comprises 282 amino acids. The first 22 N-terminal amino acids form a signal peptide which directs the posttranslational transport of the protein into the rough endoplasmic reticulum. After the protein is transported, the signal peptide is cleaved from the protein by signal peptidase. The mature DNase protein is normally secreted outside of the cell but may also be able to gain access to the nucleus where it is involved in cell death by apoptosis [3].

The *DNASE1* gene (NCBI accession no. D83195) maps on chromosome 16p13.3 [4]. It is approximately 4.11 kb long with nine exons, separated by eight introns [5], and has two transcriptional-starting exons [6]. Six autosomal codominant alleles (*DNASE1*1* through *DNASE1*6*) have been described in Refs. [7–12], caused by single nucleotide polymorphisms (SNPs) resulting in amino acid substitutions [4,5,8,11,12]. They possibly do not affect the specific function of the DNase enzyme except for the gene product of *DNASE1*6*, which had reduced enzyme activity and heat stability [12]. All six alleles were observed in Japanese, whereas in Germans only the alleles *DNASE1*1* (0.252) and 1*2 (0.748) have been found [13].

Additional mutations in the *DNASE1* gene have been associated with reduced DNase activity in systemic lupus erythematosus (SLE) [2]. In particular, a heterozygous nonsense mutation at position 172 in exon 2 of the *DNASE1* coding sequence resulted in the replacement of lysine with a stop signal at amino acid position 5 leading to reduced levels of serum DNase activity. Further, a 36 base pair deletion in exon 2 and a varied pattern of alternatively spliced *DNASE1* transcripts have been found in patients with SLE [14,15].

Beside genetic factors, also serological factors are under discussion as possible causes for a lowered serum activity of DNase. On the one hand, mutations in the *DNASE1* gene may lead to a reduced expression of the gene. On the other hand, DNase inhibitors in the serum may reduce activity of normally expressed DNase 1 enzyme. G-actin has been identified as a potent inhibitor of DNase 1 in the presence of ATP [16–18]. Increased serum

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Table 1
Primers used for amplifying and sequencing of the promoter and 9 exons of the *DNASE1* gene.

Region	Forward primer	Reverse primer	Product size (bp)
Promoter	5′-TGGGCCTAAACACACAGCCTAT	5′-GACAAAGAGGCCAAAGCCACAT	284
Promoter/exon 1	5′-GGGACACGGGATAGGAACCTTTGGCCT	5′-CCTCACTCGCTGCTGGAGGATGAGACT	515
Exon 2	5′-GCTGTTTGGCTTTCTGGACGTTGT	5′-CTCATGTTTCTGCCTGGGCTCCT	526
Exon 3/exon 4	5′-ATCAGCTGTGGCTCCCTTTGT	5′-GGACAAACTACTTCCCTGCTCTGT	495
Exon 5	5′-TGCCTCCTGGGAAGCAGGAGT	5′-TGTGACACAGGCATTCCAGGT	274
Exon 6/exon 7	5′-TTCCAGCTGACATGGTGACTGA	5′-GTTGGGTGAGTGTTCAAAGGCT	518
Exon 8/exon 9	5′-TGCTGAGCCAGGCCCATGTGTGAAAG	5′-AGCTGCTTGTCAAGGCCCTGGAGCGA	500

All primers were intronic.

concentrations of actin correlated with lowered DNase activity in patients with SLE [19].

Recently, we have shown impaired DNase activity in patients with endocrine autoimmunity [20]. But so far, no molecular studies were performed in the *DNASE1* gene in these patients. Therefore, our hypothesis was that the reduced DNase activity in these patients with AITD is associated with sequence alterations in the *DNASE1* gene.

2. Subjects and methods

2.1. Subjects

18 patients with autoimmune thyroid disease, their 10 healthy relatives, and a total of 111 unrelated healthy controls were examined. DNase activity was analyzed in all patients, relatives, and 61 of 111 controls. Genotyping was done in all patients, relatives, and 50 of 111 controls. Graves' disease was defined as hyperthyroidism with the presence of TSH receptor autoantibodies. Hashimoto's thyroiditis was defined as hypothyroidism, presence of high TPO autoantibodies, and a typical pattern in thyroid ultrasound. The study has been approved by the Ethics Committee of Rhineland-Palatinate, Germany, and informed consent has been obtained by the subjects.

2.2. Measurement of DNase activity

Blood samples were drawn in the morning and serum samples were immediately frozen at −20 °C. Serum DNase activity was determined with a standardized, validated, and reproducible enzyme-linked-immunosorbent assay, as described earlier [20]. In brief, DNase substrate was used as a solid phase and the serum DNase degraded the DNase substrate. For this, 10 μl of subject's serum and of calibrators were diluted, pipetted into a 96 well microplate with immobilized DNase substrate, incubated for 60 min at 37 °C, and washed. DNase substrate horseradish peroxidase conjugate was added recognizing the remaining DNase substrate, incubated for 15 min at room temperature, and washed. A chromogenic substrate solution was added and 15 min incubated at room temperature. A stop solution was added in order to finish color development. The amount of color is inversely proportional to DNase activity. Optical density of the probes was measured at 450 nm. Bi-chromatic measurement with a 620 nm reference was done. DNase activity was calibrated with defined concentrations of DNase enzyme. The lower detection limit is 0.3 pg/ml DNase. A DNase activity lower than 5 ng/ml was pathological.

2.3. Amplification and sequencing of the DNASE1 gene

Genomic DNA was prepared from peripheral EDTA venous blood with QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) protocol. The promoter, all nine exons, and flanking intron regions of the *DNASE1* gene were amplified and sequenced in the 18 patients and their 10 healthy relatives using 14 primers as given in Table 1. Primers were designed with PrimerQuest (Biotools, http://biotools.idtdna.com/). PCR reaction was done with 2 μl DNA (75 ng/μl), 1 μl of each primer (10 μM), 0.5 μl dNTP (25 mM each base), 5 μl 10× PCR buffer, 0.4 μl (5 U/μl) Taq polymerase (Roche, Mannheim, Germany), and 40.1 μl HPLC water. DNA amplification conditions were 94 °C for 5 min, 36 cycles of 94 °C for 1 min, 61 °C (fragments 1,4,5)/62 °C (fragment 7)/63 °C (fragments 2,6)/67 °C (fragment 3) for 10 s, 72 °C for 1 min, finished by a posttreatment of 72 °C for 10 min. The PCR products were electrophoresed on a 2% agarose gel, purified by isopropanol precipitation, and sequenced by cycle sequencing, using same primers as for PCR. The sequencing reaction (10 μl) contained 1 μl 5× sequencing buffer, 1 μl forward or reverse primer (5 μM), 0.5–6.5 μl PCR product, 1 μl Dye® Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Darmstadt, Germany), and HPLC water. Sequencing conditions were pre-treatment of 94 °C for 2 min, 25 cycles of denaturation at 94 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min. The products were purified by ethanol precipitation. 12 μl of product were sequenced in both directions on a four capillaries ABI PRISM 3100-Avant Genetic Analyzer.

2.4. Preparation, amplification and sequencing of cDNA

RNA was extracted from peripheral EDTA venous blood with the QIAamp® RNA Blood Mini Kit (Qiagen, Hilden, Germany) following the protocol. 1 μl RNase inhibitor (RiboLock Ribonuclease inhibitor,

Table 2
Restriction fragment length analysis primers and enzymes used for genotyping of the *DNASE1* single nucleotide polymorphisms (SNPs) identified. For SNPs G1218A and A1237C, the whole DNA fragment was sequenced.

SNP	Forward primer[a]	Reverse primer[a]	Product size (bp)	Restriction enzyme	Genotype	Digestion products (bp)
G6T	5′-CCCTGTGCTCTCCCTGGATGAG	5′-CTCATGTTTCTGCCTGGGCTCCT	379	*Eco*NI	GG	17,49,313
					GT	17,49,66,313
G105C	5′-GCTGTTTGGCTTTCTGGACGTTGT	5′-GAGGGTGGCATTGGACATCTTGAT	293	*Mbo*I	GG	25,33,235
					CG	25,33,58,235
C1660G	5′-TTCCAGCTGACATGGTGACTGA	5′-GCATTGAAGTCGCCCATCAACA	286	*Hpy*CH4V	CC	98,188
					CG	63,98,125,188
G-637A	5′-GGGACACGGGATAGGAACCTTTGGCCT	5′-CCTCACTCGCTGCTGGAGGATGAGACT	515	BbrPI	GG	118,397
					AG	118,397,515

[a] Sequence motifs either contained a restriction enzyme cutting site or a cutting site via modified PCR primer was introduced (nucleotides, which were modified as compared to the gene sequence, are underlined).

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