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Identification of a new *SERPINC1* mutation in a Kazak family that alters the heparin binding capacity of antithrombin ☆

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ABSTRACT

Introduction: Given its central role in mediating heparin-induced anti-coagulation, *antithrombin* (*AT*) gene mutations may result in heparin resistance. This study investigates the relationship between familial *AT* gene mutations and tolerance to heparin.
Methods: The medical history of a male patient with heparin resistance who received heart surgery and six of his family members was reviewed. Activated partial thromboplastin time (APTT), prothrombin time (PT), fibrinogen (Fib), D-dimer (D = D), and platelet count were determined to assess coagulation function. AT activity and the *AT* gene were also analyzed. For the newly identified gene mutations, polymorphisms were excluded in 120 healthy Kazak controls.
Results: Two mutations were identified in exon 7 of the *AT* gene, *SERPINC1*: g.1267G > A (p.A391T) found in five participants, including the index patient, and g.1334G > A, a silent mutation, in two family members. The g.1267G > A mutation may alter focal AT protein conformation. Neither of these mutations was observed in the healthy Kazak controls. Although all coagulation parameters and AT activity were within the normal ranges for the index patient and his family members, the platelet levels were significantly lower than that observed for the healthy Kazak controls ($p = 0.001$). There was no significant difference in AT antigen levels between the groups; however, participants with the g.1267G > A mutation had a 44.25% reduction in heparin binding compared to the control group ($p < 0.001$).
Conclusion: We identified a novel hereditary mutation, g.1267G > A (p.A391T), in the *AT* gene, which reduces its heparin binding capacity and might be associated with resistance to heparin.

Introduction

Anti-coagulation with heparin is one of prerequisites for successful open heart surgery in the presence of cardiopulmonary bypass (CPB) as insufficient anti-coagulation can result in life-threatening thrombosis. However, heterogeneity in antithrombin (AT) activity has been noted in the general population [1]. Resistance to heparin or insufficient anti-coagulation during cardiac bypass procedures is currently defined as an activated clotting time (ACT) <400 s after administration of heparin and/or administration of exogenous AT [2]. In addition to hyperpyrexia, infection, high platelet count and the presence of a concomitant tumor, such as atrial myxoma, abnormal AT activity and function secondary to *AT* gene (*SERPINC1*) mutation are also important causes of heparin resistance [3]. However, resistance to heparin is seldom diagnosed before surgery [4], suggesting that the tested parameters are not always predictive of heparin resistance. Therefore, analyzing the mechanisms underlying heparin resistance is crucial for its identification and clinical treatment.

Heparin exerts its anti-coagulant effect via binding to AT, a glycoprotein of 432 amino acids produced in the liver with a half life of 2-3 days, and inducing a conformational change that permits interaction with its targets [5]. AT exerts its anti-coagulation function through inactivating enzymes in the coagulation cascade, including thrombin and factors IXa, Xa, and XIIa [6]. Under normal conditions, AT has a weak anticoagulant activity; however, interaction with pharmacologic heparin or heparan sulfate from the vascular wall exposes the reaction center loop of AT [5,7], increasing its anti-coagulant effect by several thousand-fold [8].

Mutations in the genes associated with coagulation cascade are thought to confer AT resistance. For example, a rare Arg596Leu mutation in thrombin reduced the formation of a thrombin-AT complex, resulting in AT resistance [9]. The *AT* gene is comprised of 7 exons and 6 introns, and the AT protein contains three disulfide bonds and four potential glycosylation sites [10] as well as two important functional regions, including a heparin-binding domain at the N-terminus and a reaction site at the C-terminus. To date, over 200 mutations in the *AT* gene (*SERPINC1*) have been identified [11–17]. Given its central role in mediating heparin-induced anti-coagulation, we hypothesized *AT*

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mutations may result in heparin resistance and that novel *AT* mutations may be identified in patients with heparin resistance. Therefore, the *AT* gene was analyzed in a Kazak patient with heparin resistance who received heart surgery as well as in six of his family members. In addition, 120 healthy controls were also recruited to exclude potential polymorphisms of the *AT* gene. This data may provide evidence of the relationship between the *AT* gene and familial heparin resistance and form the basis for developing new methods to identify patients likely to be heparin resistant prior to heart surgery.

Materials and methods

Study participants

A 35 year-old male Kazak patient was admitted into the First Affiliated Hospital of Xinjiang Medical University and received mitral valve replacement due to severe mitral regurgitation and mild mitral stenosis. Routine examination did not identify other abnormalities beyond the presence of mitral lesions. Following administration of general anesthesia and thoracotomy, heparin was intravenously administered at 400 U/kg. After 5 min, the activated coagulation time (ACT), as measured using an ACT Plus Automated Coagulation Timer System (Medtronic, Minneapolis, MN, USA), was 300 s. Because the target therapeutic range of ACT for surgery was 480 s, 100 U/kg additional heparin was administered, resulting in an ACT of 380 s after 5 min. With an additional 100 U/kg heparin, the ACT increased to 520 s after 5 min. The surgery was subsequently performed without incidence; patient recovered normally and was discharged. Follow-up analysis did not reveal the presence of any abnormality within 6 months after surgery.

In addition to the original patient, who was Kazak, six members of the patient's family spanning three generations were recruited, including an aunt, a brother, three sisters and a nephew with ages ranging from 23 to 67 y. The patient's grandparents and parents had died and therefore were not included. Although three members had a history of hypertension, they did not have a history of thrombosis and other hereditary diseases. Furthermore, Kazak healthy control patients (n = 120) were also recruited to participate in this study. Informed consent was obtained from all study participants, and their medical history was reviewed. This study was approved by the institutional review board of the First Affiliated Hospital of Xinjiang Medical University and patients' informed consents were obtained in advance.

Analysis of coagulation function

Except the index male Kazak patient, whose blood was collected during surgery, blood from all other study participants were collected without surgery treatment for the analysis of coagulation function. Briefly, blood (3 mL) was collected, anti-coagulated with sodium citrate at a ratio of 1:9 and divided into two parts (i) for the detection of AT activity and (ii) for the detection of coagulation functions, including activated partial thromboplastin time (APTT), plasma prothrombin time (PT), fibrinogen (Fib), and D = dimer (D = D) using an ACL-TOP Coagulation Analyzer (N0415800; Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. D = D was detected by immunonephelometry. Platelet count was determined using a Beckman Coulter LH 750 (Beckman Coulter, Brea, CA, USA).

AT activity

AT activity was detected using the Coatest AT kit (Chromogenix Instrumentation Laboratory, Milan, Italy) as described by Beeck et al. [18]. Briefly, after diluting the plasma with saline, it was incubated with excess human factor IIa in the presence of heparin for 90 s, forming an AT-Heparin-factor IIa complex. Excess factor IIa catalyzes the release of p-nitroanaline (pNa) from a chromogenic substrate. The absorbance measured at 405 nm is inversely proportional to the AT activity concentration in the sample.

Analysis of AT antigen levels

For evaluation of AT antigen levels, a thrombin-antithrombin complex-derived human enzyme-linked immunosorbent assay kit (ab108907; AbCam China, Shanghai, China) was used in accordance with manufacturer's instructions. The absorbance was measured at 450 nm within 5 min after stopping the reaction using an xMark™ microplate spectrophotometer (Bio-Rad, Hercules, CA, USA).

PCR amplification and sequencing of the AT gene

An additional 3 mL of blood was collected from all study participants and stored with EDTA-K_2 at -80 °C for subsequent sequencing analysis. All seven exons of the *AT* gene of the initial patient were sequenced. Once mutations were noted, sequencing at the specific sites was performed in the participating family members and healthy controls.

In brief, 200 μL of samples was used for genomic DNA extraction according to the manufacturer's instructions, Whole Blood Genomic DNA extraction kit (Ou Yi Biotechnology company, Shanghai); the resultant genomic DNA served as templates for PCR analysis of the *AT* gene. The PCR reaction mixture included 10 × EX Buffer (2 μL), 2.5 mM dNTPs (1.6 μL), forward primer (1 μL), reverse primer (1 μL), 5 U/μL Ex Taq polymerase (1 μL), DNA (1 μL) and ddH2O (up to 20 μL). The primers were designed as previously reported [16] and are shown in Table 1. The reaction was subjected to one cycle at 95 °C (denaturing) for 10 min, then 35 cycles at 60 °C (anneling) for 30 s and 72 °C (extension) for 45 s. After 35 cycles of annealing and extensions the reaction was subjected one cycle at 72 °C for 10 min. Products were stored at 4 °C and then subjected to 2% agarose gel electrophoresis to confirm the quality and product size.

The PCR products were sequenced in the Beijing Microread Genetics Co., Ltd.(Beijing, China). Once an *AT* gene mutation was identified, the products underwent sequencing in another company to confirm the initial results (Shanghai Oebiotech Co., Ltd; Shanghai, China). Sequence comparisons between the initial patient and the family members and healthy controls were performed with DNAMAN 6.0 software.

Heparin binding assay

A heparin binding assay was performed to detect the binding of wild-type and mutant AT protein to low molecular weight heparin. Low molecular weight heparin (Sigma, St. Louis, MO, USA) was dissolved in 0.01 M PBS (pH 7.4) at a final concentration of 25 μg/mL, and 200 μL was added to each well of a 96-well plate and incubated overnight. After three washes in PBS, the wells were blocked with

Table 1
Primers used to amplify all seven exons of the *AT* gene.

Exons	Name	Sequences	Product size (bp)
1	AT-1-F	ACTGGGCTCTACACTTTGCTTA	308
	AT-1-R	TCATTCCTGTGAGTCCTTTGG	
2	AT-2-F	TGGAATCCTCTGCTTTACTGG	545
	AT-2-R	GTTGGTTGAGGAATCATTGGA	
3	AT-3-F	TGTCCCAGGTACTGTGCTTGA	490
	AT-3-R3	GCAGGGGTTCTAACTTTTAGTCA	
4	AT-4-F	AGGACAGAGGGATGGTGAGAA	345
	AT-4-R	CAGTCCATTTGCCCTCTCAG	
5	AT-5-F	CCATCATTCTGACACAGCCAT	673
	AT-5-R	CTAGGATCAGTATCCAGGAGTCC	
6	AT-6-F	CCAAAGGATCTCTTAATCCAAAC	299
	AT-6-R	GGTTTTGGAGAGGGCTGTATTA	
7	AT-7-F	ATTGCTGTGTCTGTGGATGATT	402
	AT-7-R	GCCCCAATAGCATGTTTCC	

0.2% gelatin (Sigma) dissolved in PBS (250 μL/well) and incubated at 37 °C for 1 h. After washing in PBS, serum containing wild-type or mutant AT protein was added (100 μL/well), followed by incubation at 37 °C for 2 h. Following washing in PBS, a rabbit anti-human AT polyclonal antibody (Sigma) was added for 1 h at 37 °C followed by an HRP-conjugated goat anti-rabbit IgG antibody (Sigma) for 1 h at 37 °C. After five washes with PBS, tetramethylbenzidine (TMB) was used for visualization, followed by addition of 2 M H_2SO_4 to stop the reaction. Absorbance was measured at 450 nm. The experiment was performed three times with each sample in duplicate. The wild-type AT protein was subjected to a serial 2-fold dilution for the delineation of a standard curve. The binding activity of the mutant AT protein was normalized to the binding activity of wild-type AT protein and expressed as percentages.

Analysis of the AT protein structure

The effects of all identified mutants on AT protein structure were analyzed using the Swiss-PdbViewer (SPDBV) software, which is linked with the SWISS-MODEL for homology modeling. SPDBV permits users to analyze superimpose proteins for detailed analysis of structural changes.

Statistical analysis

Continuous data were presented by median and full range due to the small sample size; categorical data were presented by numbers (percentages). The nonparametric Mann-Whiney t test and Fisher's exact test were performed to compare the difference between the healthy controls and the index case's family using continuous and categorical data, respectively. The one-sample t-test was performed to evaluate the heparin binding of mutated group relative to control. All statistical assessments were two-tailed and evaluated at the 0.05 level of difference. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS Inc, Chicago, Illinois, USA).

Results

Identification of AT gene mutations in the index case's family

In addition to the index patient, six family members were enrolled in the study with a median age of 50 y (ranged in 23 – 67 y). Although a history of hypertension was revealed, a history of thrombosis and other hereditary diseases was not observed in these members and their relatives within three generations.

In exon 7, g.1267G > A (p.A391T) was found in five family members, including the index patient; the remaining two family members were wild-type homozygotes (Table 2). This mutation was not identified in the *AT* gene of healthy Kazak controls; therefore, the mutation rate in the index case family was significantly higher than that observed in the control population ($p < 0.001$, Table 2). g.1267G > A resulted in an A391T substitution (Figs. 1 and 2). Analysis of the influence of this mutation on the structure of the AT protein suggests that it would not influence AT protein structure (Fig. 2). However, in normal AT, Ala, which is a small, non-polar hydrophobic amino acid, may form a hydrogen bond with the 192Gln in the secondary structure. In the A391T mutation, the polar T may form two hydrogen bonds with 192E, thereby increasing the steric hindrance and changing the focal conformation (Fig. 2).

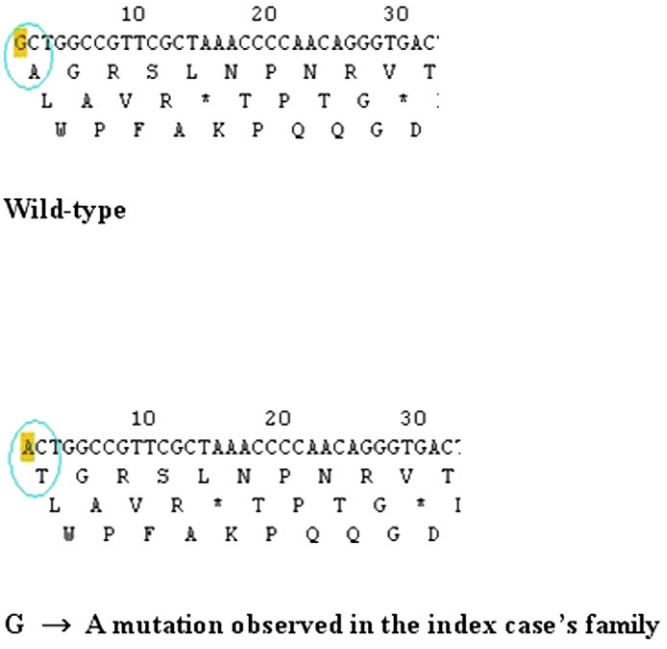

Fig. 1. The g.1267G > A (p.A391T) mutation in exon 7 induces an A391T substitution. This mutation was observed in some members of the index cases' family but not in the normal healthy Kazak controls.

Another silent mutation, g.1334G > A, was found in three family members, but not the index patient. This mutation was not identified in the *AT* gene of healthy Kazak controls.

Coagulation function and AT activity

As shown in Table 2, the platelet count of the index case's family was significantly lower than that observed in the healthy control group (median 139.0 vs. 195.0, $p = 0.001$; Table 2). Only one index patients' family member had AT level of 70% but no significant difference in AT activity was noted between the index case's family group and the healthy control group. In addition, no difference in the median AT antigen level was observed between the healthy Kazak control group and the index case's family (87.3 [IQR: 64.7-104.7] and 87.6 [IQR: 59.4-

Table 2
Coagulation function and AT SNP and activity in the index case's family and in healthy Kazak controls.

		Healthy Kazak controls (n = 120)	Index case's family (n = 7)	*P*-value
g.1267G > A (p.A391T)[b]	G/A	0 (0.0%)	5 (71.4%)	<0.001*
	G/G	120 (100.0%)	2 (28.6%)	
Platelet count (10^9/L)[a]		195.0 (124.0, 361.0)	139.0 (128.0, 172.0)	0.001*
AT activity (%)[a]		102.0 (72.0, 129.0)	110.0 (70.0, 117.0)	0.345
AT antigen level (μg/mL)		87.3 (64.7, 104.7)	87.6 (59.4, 103.6)	0.962
Heparin binding assay (relative to control, %)		100	55.75 (59.3-52.2)[c]	<0.001*

Normal ranges: platelet levels, 150-400 × 10^9/L; AT activity, 70-130% [29].
[a] Data are presented as median and full range.
[b] Data are presented as number and percentage.
[c] Data are presented as the mean of percentages relative to control with 95% CI.
* indicates a significant difference between the groups.

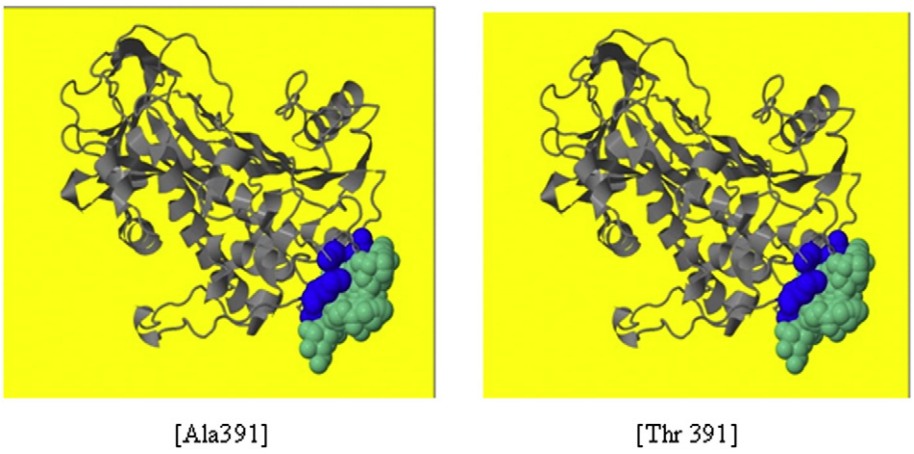

Fig. 2. Effects of the g.1267G > A (p.A391T) mutation on AT protein conformation.

103.6], respectively; p = 0.962). Analysis of the heparin binding capacity of the serum AT from the participants with the A391T mutation at g.1267G > A had revealed 55.75% (95% CI of 55.75 (59.3-52.2) in antigen binding in comparison with the control group (p < 0.001). It is a 44.25% reduction in antigen binding. The index case's family was further divided into two subgroups, including two without any mutation at exon 7 or with only the silent mutation at g.1334G > A and five with the relevant A391T mutation at g.1267G > A. As shown in Fig. 3, the subjects with the A391T mutation at g.1267G > A had significantly lower platelet levels than the general population (median 134.0×10^9/L vs. 195.0×10^9/L, p = 0.002). However, no significant difference in AT activity was noted between the three groups (Fig. 3).

Discussion

The maintenance and management of coagulation status using heparin is a key determinant of success during open heart surgery with CPB. Anti-coagulation induced by heparin is mediated by its interaction and activation of plasma AT; it interacts with amino acids 41-49 and 107-156 of AT with Arg47, Lys114, Lys125 and Arg129 playing important roles [9]. Given the central role of AT in mediating the anti-coagulation effects of heparin, we hypothesized that *AT* gene mutations may contribute to heparin resistance or anti-coagulation insufficiency. In the present study, analysis of a Kazak patient with heparin resistance and six additional family members identified two *AT* gene mutations in exon 7: g.1267G > A (p.A391T) and g.1334G > A. Further gene analysis in healthy Kazak controls excluded the possibility of gene polymorphism of *AT*. Both mutations have been never reported in previous studies after searching the NCBI database.

More than 200 *AT* mutations have been identified to date [11–17]. For example, in a cohort of 272 AT-deficient patients, 87 mutations were identified of which nine were thought to induce conformational changes that prevented AT activation [16]. In addition, two *AT* SNPs have been identified in the human *AT* gene, *SERPINCl*, in Spanish Caucasian blood donors, which might contribute to inter-individual differences in AT activity, and include rs3138521 in the promoter and rs2227589 in intron 1 [17]. rs2227589 was associated with anti-factor Xa activity and AT levels as well as reduced anti-coagulant activity [17]. Furthermore, three new missense polymorphisms in *AT* have recently been identified in five patients with a history of thromboembolism [11].

In the present study, whereas the g.1334G > A was a silent mutation, g.1267G > A resulted in an A391T missense mutation, which is in close proximity to the P1-P1' cleavage site of AT. In addition, a significant reduction in heparin binding capacity was noted in the family members with the g.1267G > A mutation as compared the controls. P1 interacts with the Ser at the active center of thrombin, resulting in inactivation of thrombin [19–21]. Although analysis with SPDBV software indicated that this mutation did not significantly influence the protein structure,

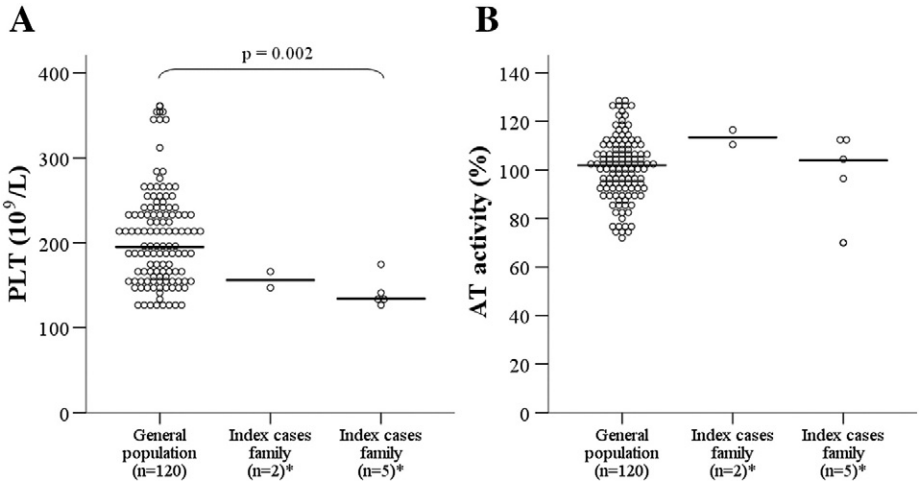

Fig. 3. Comparisons for platelet (A) and AT activity (B) between normal healthy Kazak controls (n = 120) and two subgroups of index case's family. *The subgroups of index case's family included two with silent mutation (g.1334G > A) in exon 7 and five with the g.1267G > A (p.A391T) mutation in exon 7.

changes in focal conformation may occur, which could influence AT function. This is consistent with a previous study that reported the susceptibility of AT protein to minor changes in the primary structure [22]. It is important to note that this mutation was found in the index patient that had heparin resistance although no significant differences in AT activity were noted between the index case's family and that of normal healthy Kazak controls. Analysis of *AT* gene mutations in patients with thrombotic events also revealed AT activity within the normal range [11]. Therefore, additional studies will assess the impact of this particular mutation on AT activity in detail.

Mutations at amino acids 392, 393, and 394 may compromise AT-induced inactivation of thrombin [9]. In addition, Chuang et al. [23] reported that of all the serine protease inhibitors, including AT, P1 (R393) is an important site determining anti-coagulation function. In addition, two hydrogen bonds at A391, which interact with the side chain of 192E and the side chain of 216G of factor X, were involved in the inhibition of factor X [23]. Thus, the A391 mutation may alter AT protein function, which will be investigated in further studies.

Heparin may also inactivate coagulation factors with proteolytic activities, which then increases its inhibitory effects on factor Xa and thrombin [24]. In addition, heparin may also mediate the allosteric activation of AT to exert its anti-coagulation effect. In the presence of heparin, the binding resistance between AT and Fxa / FIXa reduces, which promotes the binding of exosite at the reaction center of AT to Fxa and FIXa [25]. Furthermore, in the presence of heparin, AT interaction with factor IXa is increased by one million-fold [26]. Heparin also promotes the binding of exosite at the reaction center loop of AT to factor Xa and factor IXa [27]. The effects of this mutation on the interaction of AT with its target proteins will be assessed in further studies.

The endogenous and exogenous coagulation function, as determined by APTT and PT, respectively, as well as the extent of fibrinolysis (i.e., D = D values) and AT activity of the index case's family members were within normal ranges. In addition, no differences in AT antigen level were detected, suggesting the absence of type II deficiency. Although no differences were noted in AT activity, the identified mutation might alter AT-mediated inhibition of thrombin and factor X, resulting in resistance to heparin that may be overcome by increasing the dose of heparin as with the index patient to achieve the target ACT for CPB. Although a heparin resistance test was not performed in the family members, a review of their medical records showed no history of thrombosis, suggesting that the influence of the A391T mutation on AT function is small. Further analysis of the actual influence of this mutation is required.

In the present study, the platelet levels were significantly lower in the index patient's family with the A391T mutation as compared to the healthy Kazak controls. The reduced platelet count as compared to the control group might be ascribed to the mismatched sample sizes between the two groups; however, the results were not biased. Given that the platelet levels were below the range considered normal, this suggests the presence of familial thrombocytopaenia, which will be investigated in further studies. Increased platelet levels are closely related to heparin resistance given their important roles in hemostasis, coagulation and thrombosis [28]. Although heparin has little effect on the inhibition of platelet aggregation or even platelet function, it may promote the aggregation of platelets in the citrate anti-coagulated plasma rich in platelets. In addition, heparin may also increase adenosine diphosphate- or epinephrine-induced aggregation of platelets. Thus, after heparinization, cardiopulmonary bypass (CBP) patients may also have abnormal platelet activity, resulting in release of a large amount of procoagulants, such as platelet factor IV (PF4), β-thromboglobulin (B-TG), and thromboxane A2 (TXA2). Of these factors, PF4 is an antagonist of heparin and may neutralize the anti-coagulation of heparin.

There were several limitations to the present study. First, the presence of AT mutations was only detected in one family of a single patient with heparin resistance. Furthermore, the heparin resistance test was not performed in the other family members, which prevented analysis of the association of the mutations with resistance to heparin. In addition, the ACT was not evaluated in the study participants; therefore, further studies are required to evaluate the role of the g.1267G > A mutation on clotting time.

In conclusion, analysis of a Kazak patient with heparin resistance and six of his family members identified two novel mutations in exon 7 of the *AT* gene with familial inheritance. The g.1267G > A mutation was responsible for an A391T substitution that reduced heparin binding and therefore may have a role in heparin resistance. Further studies will characterize the full impact of this mutation as well as the clinical significance of identifying patients with AT mutations in an effort to diagnose patients at risk of thrombotic events prior to surgery.

Conflict of interest

None.

Acknowledgements

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