中文題目：確認兩個全新點突變於先天遺傳性抗凝血酶缺乏症的相關分子生物學效應

英文題目：Identification of two novel point mutations in inherited antithrombin deficiency related molecular biological effects

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Background: Antithrombin (AT) is a serine protease inhibitor which plays vital role in anticoagulation through binding to the serine residues in the active centers of serine proteases such as thrombin. Inherited AT deficiency (IAD) is associated with high risk of venous thrombosis

Methods: A total of 9 patients with IAD identified by chromogenic assay (Liquid Antithrombin, HemosIL) were collected. Each patient's peripheral blood DNA was extracted. 7 encoded exons including the boundary of intron/exon of SERPINC1 gene were amplified by polymerase chain reaction (PCR), followed by direct sequencing. Enzyme-linked immunosorbent assay (ELISA) and HITRAP HEPARIN column experiment were applied to analyze the secretion and purification efficiency of AT. The mutant AT models were generated based on the AT-thrombin-heparin mimetic ternary complex using the Built Mutants protocol in the program of Discovery Studio (version 4.5, Accelrys Inc, San Diego, CA). The geometries of the models were optimized using the algorithm of smart minimization in CHARMm force field including the implicit solvent model of generalized born in the calculation.

Results: Among the 9 patients with IAD, 8 patients had type I AT deficiency and 1 patient had type II AT deficiency in subtype of reactive site mutation (type II RS). Seven of them (77.8 %) experienced venous thrombotic events and all patients were found genetic aberrations including deletion (n=2), insertion (n=1) and missense (n=6). There were four novel mutations identified, including frameshifts of c.539delG、c.1148insC、c.992delTCAC；missense point mutation of c.663G>T (W221C) and c.851T>G (M284R). Using mimetic ternary complex survey, the mutants of M284R (for type I) and W221C (for type II RS) were hypothesized to destabilize the central β-sheet, leading to loss of function. By immunoassays and heparin purification, we found W221C mutant impaired AT secretion to medium without impact on total production, whereas M284R mutant decreased the total AT production (1144.52 ng/ml versus 4758.24 ng/ml, p=0.0001). Both mutants delayed the peak of AT release from the 9th-11th column to 17th-19th column in heparin affinity chromatography.

Conclusion: This is the first study showing that mutations in SERPINC1 alters the structure of AT through in vitro protein expression and functional studies. The results of ELISA and Western blotting indicated that W221C mutant impaired AT secretion and M284R mutant decreased the total AT production. Both mutants may affect the binding ability to heparin and delay the peak of AT release. These results will help us understand the potential molecular mechanism and may provide novel treatment strategies for IAD.

Table 1. Primer pair names, sequences and references for the primers

Primer Name	Primer Sequence (5' to 3')
c.663G>T (W221C)	5'-gcggccatcaacaaatgtgtgtccaataagaccga-3'
c.663G>T (W221C)_antisense	5'-tcggtcttattggacacacatttgttgatggccgc-3'
c.851T>G(M284R)	5'-gtgttcagcatctatgaggtaccaggaaggcaagt-3'
c.851T>G(M284R)_antisense	5'-acttgccttcctggtacctcatagatgctgaacac-3'

Table 2 Genetic variants of the SERPINC1 gene and thrombotic manifestations in 9 unrelated Chinese patients with antithrombin deficiency.

No.	Age/ gender	Activity (%)	Antigen (ug/mL)	Progressive Activity(%)	Type of deficiency	Nucleotide variation (cDNA)	Amino acid variation	Thrombotic episode (onset age)
1	M/43	49.2	235.2	47.0	I	c.1301T>C	p.Phe434Ser	PE (43)
2	M/42	40.4	202.8	61.8	I	c.539delG	Framshift	DVT (25)
3	M/40	61.8	383.4	78.7	II RS	c.663G>T	p.Trp221Cys	DVT (40)
4	M/38	48.5	224.1	41.0	I	c.1148insC	Framshift	DVT (20)
5	M/23	41	202.5	46.0	I	c.851T>G	p.Met284Arg	CVT (23)
6	M/16	47.9	202.5	55.5	I	c.1301T>C	p.Phe434Ser	ND
7	M/15	45.2	216.6	51.8	I	c.1301T>C	p.Phe434Ser	ND
8	M/36	53.2	202.5	46.5	I	c.992delTCAC	Framshift	PE (36)
9	F/46	52.1	250.2	57.5	I	c.374G>A	p.Gly125Asp	MVT (24)

RS, reactive site; PE, pulmonary embolism; DVT, deep vein thrombosis; CVT, cerebral venous sinus thrombosis; ND, no detection; MVT, mesenteric venous thrombosis

Figure legend:

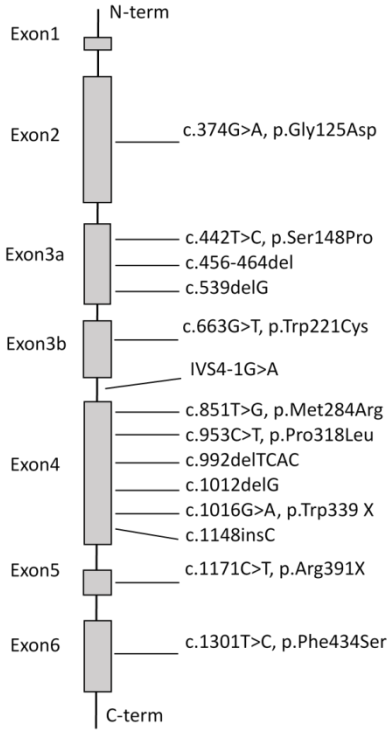


Fig. 1. Structure of the antithrombin-thrombin-heparin mimetic ternary complex (pdb code: 1TB6). The proteins were present as ribbon model. Thrombin was shown as green color. The β-sheet, α-helix and loop in antithrombin were colored as magenta, cyan and brown, respectively. The heparin mimetic was shown as stick model and colored as white for the carbon atom. The residues of mutations were highlighted as space-filling model and colored as yellow for the carbon atom.

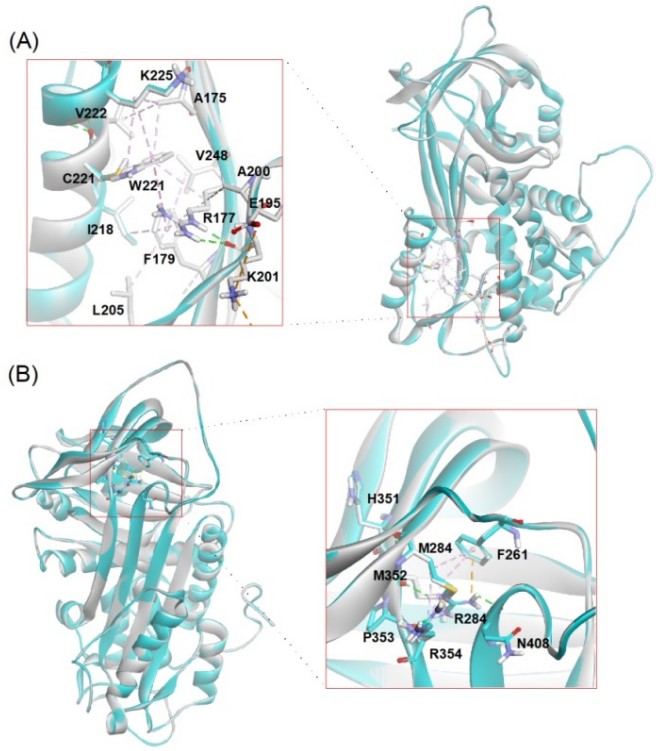


Fig. 2. Superimposition of the wild type (white) and mutant (cyan) antithrombin models. (A) The W221C mutant model. (B) The M284R mutant model. The red rectangular highlighted the mutation site which was shown as a close-up view in the right or left side. The hydrogen bond, carbon hydrogen bond, electrostatic and hydrophobic interactions were shown as dashed green, teal, orange and pink lines, respectively.

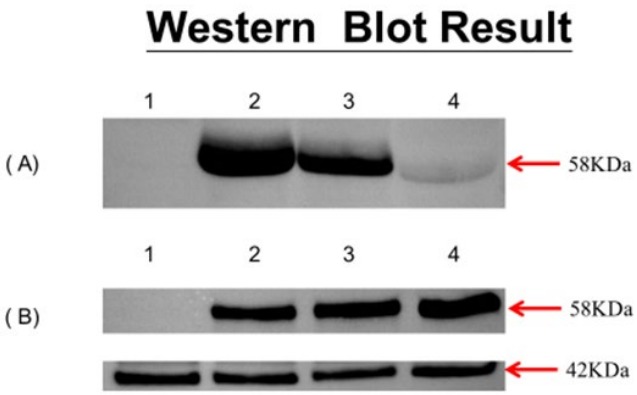


Fig 3. Result of western blot analysis of the recombinant AT molecules. Wild type and mutant ATs were transiently expressed in HEK293 cells, and the culture media and cell lysates were analyzed by Western blotting. We loaded 10 ug of each sample normalized for total protein. (A) Culture media, (B) cell lysates. Lane1, HEK293 cell line; lane 2, wild type-AT; lane 3, mutant AT-g663t; lane 4, mutant AT-t851g.

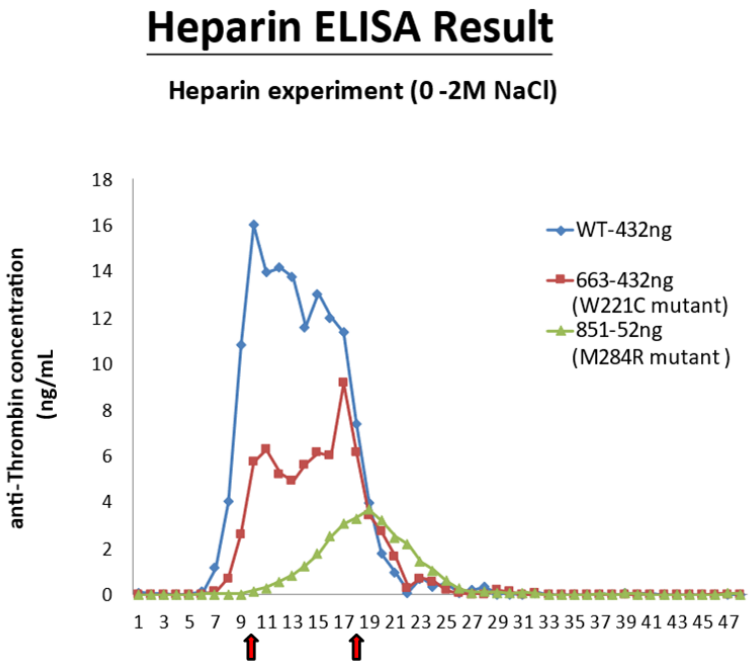
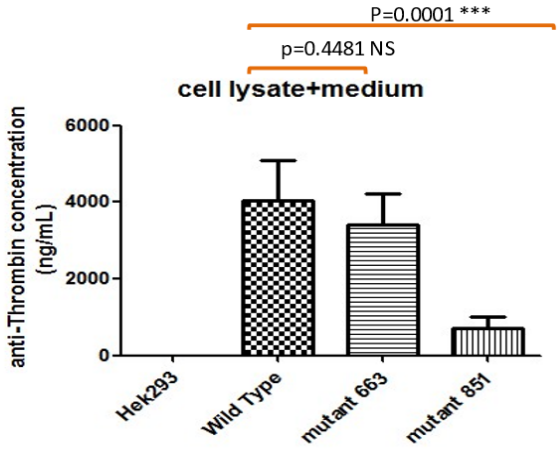


Fig 4. M284R mutants delay the peak of anti-thrombin release from the 9th-11th column to 17th-19th column.



**1 way ANOVA analysis : p=0.0045, * *

Fig 5. The total concentration of anti-thrombin in cell lysate and medium. The mutant 663 (W221C) and mutant 851 (M284R) were lower than the wild type (p=0.0045).

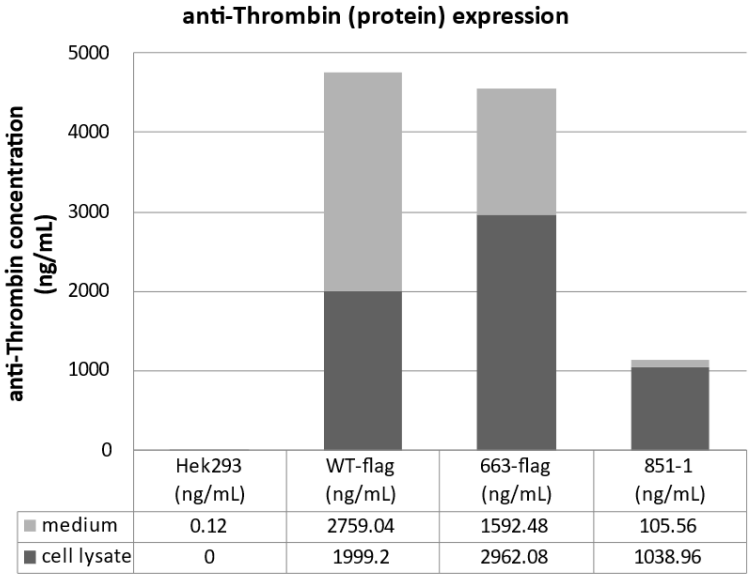


Fig 6. The total concentration of anti-thrombin (AT) in cell lysate and medium separately. The cell lysate AT concentration of mutant 663 significantly (W221C) was higher than the wild type (2962.08 ng/ml vs 1999.20 ng/ml).