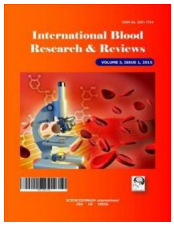

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Detection of Prethrombin 1 in Human Blood Plasma

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Authors' contributions

Author DK purified and characterized ecamulin from the venom of Echis multisquamatis, developed the model systems for study. Author VC performed SDS-PAGE and Western Blotting. Authors TP and TC performed the diagnostic tests. Author EL developed an ideology of experiments and interpreted the data obtained. All authors substantially contributed to the analysis and interpretation of data, as well as the drafting of the article. All authors approved the final version of the article to be published.

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ABSTRACT

Background: Prethrombin 1 is one of prothrombin derivatives which appears in the presence of thrombin in vitro. However, existence of prethrombin 1 *in vivo* remained questionable. The aim of present work was to detect of prethrombin 1 *in vivo* at abdominal aortic aneurysm, hip replacement after fracture and stroke.
Methodology: Blood plasma samples of patients with abdominal aortic aneurysm, hip replacement after fracture, stroke, and patients which were treated with warfarine were collected. Detection of prethrombin 1 was completed using three independent approaches: combination of prothrombin index and ecamulin (ecarin) index; the APTT-test modified by addition of exogenous prothrombin; Western-blotting of blood plasma using polyclonal antibody to prothrombin.
Results: Prethrombin 1 presence in patients' blood plasma at studied pathologies was proven. Inactive prothrombin (descarboxy-prothrombin), but not prethrombin 1 was found in blood plasma of patients which were treated with warfarine. Developed approach can be used for testing the prethrombin 1 in clinical diagnostics.
Conclusion: Prethrombin 1 at different pathologies, accompanied by coagulation disorders was detected in patients' blood plasma directly.

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Keywords: Prethrombin 1; prothrombin; echis multisquamatis, hip-replacement therapy; blood coagulation.

1. INTRODUCTION

Prothrombin is an inactive precursor of thrombin, which is a multifunctional enzyme and plays a crucial role in haemostasis: converts fibrinogen into fibrin, activates platelets, endothelium and a number of clotting factors, protein C, TAFI etc [1]. It is consisted of of N-terminal Gla-domain, two kringle domains and C-terminal catalytic domain. The prothrombinase complex (composed by factors Xa and Va assembled on phospholipid membranes in the presence of Ca++ [2]) converts prothrombin to thrombin by cleaving sequentially at Arg271-Thr272 and Arg320-Ile321 [3].

It was shown that prothrombin can be cleaved by thrombin with the formation of prethrombin 1 [4] that is composed of kringle domain and inactive catalytic domain and has no enzymatic activity [5]. Previously it was shown that prethrombin 1 is present in blood plasma at some diseases [6], later studies of prothrombin cleavage in different models confirmed the possibility of prethrombin 1 formation during prothrombin activation on platelets membrane [7], but prethrombin 1 formation *in vivo* was uncertain.

The aim of present study was to detect prethrombin 1 as the marker of activation of blood coagulation system *in vivo* at abdominal aortic aneurysm, hip replacement after fracture, and stroke.

2. MATERIALS AND METHODS

2.1 Materials

Goat anti-mouse-HRP were purchased from Sigma-Aldrich (US). Chromogenic substrates S2238 (H-D-Phe-Pip-Arg-pNA) and S2236 (p-Glu-Pro-Arg-pNa) were purchased from BIOPHEN, thromboplastin (INR=1,12), APTT-reagent were from Renam (Russia). Polyclonal anti-prothrombin antibody was generously donated to us by Dr Lyuba Mikhalovska, Brighton University, UK. Prothrombin activator from *Echis multisqumatis* venom (ecamulin) also known as multisquamase [8] was purified from the crude venom by the method of Solov'ev et al. [9]. Prothrombin was isolated from human blood plasma by following procedure [10]. Prethrombin 1 was prepared by modified procedure of Mann [10], as follows. Prothrombin in 0.05 M Tris-HCl, 0.13 M NaCl, pH 7.4 (TBS) was incubated at 37℃ with thrombin for 1.5 hours (prothrombin-thrombin ratio was 1:1). Obtained prethrombin 1 was purified on Q-Sepharose in 0.02 M Tris-HCl, pH 7.4; prethrombin 1 was eluted by 0.4 M NaCl. Purified prethrombin 1 had no enzymatic activity towards S2238 and fibrinogen.

2.2 Methods

2.2.1 Immunological determination of soluble fibrin and D-dimer

Soluble Fibrin (SF) and D-dimer in blood plasma were quantificated using immunodiagnostic test-systems DiaProph-med© created on the base of monoclonal antibodies produced in Palladin Institute of biochemistry.

In this analysis monoclonal DD-specific antibody MonAB III-3d was used as the catch-agent. 100 µl of analyzed blood plasma was dissolved 1:10 in PBS. Another DD-specific monoclonal antibody MonABII-4d was used as tag-agent. Concentration of D-dimer was measured using calibration curve obtained for purified D-dimer [11].

For the Soluble Fibrin (SF) quantification, MonAB IA-5C specific to fibrin desA was used as catch-agent and MonAB II-4d as tag-agent [11,12]. Concentration of SF was measured using calibration curve obtained for purified fibrin desA [12].

Normal parameters in developed test were calculated as 3 µg/ml for SF and 0.07 µg/ml for D-dimer.

2.2.2 Protein C activity determination

Protein C activity in studied blood plasma was measured using specific activator [13] and chromogenic substrate S2236 according to recommendations of Renam©.

2.2.3 Kinetic constants

The kinetic constants were determined by linear Lineweaver-Burk plot of initial reaction velocity to substrate concentration. Products of ecamulin hydrolysis of prothrombin and prethrombin 1 were determined by SDS-PAGE [14].

Concentrations of the products of each reaction after 5, 10 and 15 min were calculated using densitometry of scanned electrophoregrams with Totallab TL100 software [15].

2.2.4 Prothrombin activation

Prothrombin activation was studied using either thromboplastin or ecamulin. Ecamulin (prothrombin activator from *Echis multisqumatis* venom) is an enzyme that activates prothrombin, descarboxy-prothrombin and prethrombin 1. So activation by ecamulin allowed us to determine total prothrombin level [16]. Thromboplastin acts through tissue factor pathway of coagulation and activates only carboxylated and uncleaved forms of prothrombin as it is in "prothrombin index" test. Thrombin generation was measured by chromogenic substrate assay [17] using thrombin-specific S2238. Results were presented as prothrombin (PI) and ecamulin (EI) indexes, that were calculated by formula: EI = An/Ap; PI= An/Ap, An – donor's blood plasma thrombin activity; Ap – patient's blood plasma thrombin activity.

2.2.5 APTT-test

Thrombin generation induced by APTT-reagent [18] was also measured by chromogenic substrate assay [17] using thrombin-specific S2238. Such modification of APTT-test allowed us to avoid the influence of fibrin clotting inhibitors on the test results and detect thrombin generation directly instead of evaluation of fibrin clotting time. Results were presented as APTT ratio, calculated using formula: APTT ratio = APTTp/APTTn x 100%; APTTp – thrombin activity of patient's blood plasma; APTTn – thrombin activity of donor's blood plasma.

APTT-test was also done with the addition of exogenous prothrombin. APTT ratio in the presence of exogenous prothrombin was calculated according to the mentioned above formula.

2.2.6 Western blotting

Western blotting was used for direct identification of prothrombin derivatives in blood plasma. Electrophoretically separated proteins of blood plasma [14] were further transferred to a PVDF-membrane in order to specify the bands by immunoprobing. The membrane was blocked with 5% milk in PBS for an hour, incubated with rabbit polyclonal anti-prothrombin antibody for another hour and then with secondary HRP-labelled goat anti-rabbit antibody. The bands were visualized using 0,001 M 4-chloro-1-naphtol solution in 0.5 M Tris pH 7.5 and 0.03% H_2O_2.

2.2.7 Statistic data analysis

Statistical data analysis was performed using Microsoft Excel. All assays were performed in series of three replicates and the data were fitted with standard errors using "Statistica 7".

3. RESULTS

3.1 Characteristics of the Patients

We analyzed blood plasma of 72 patients with different pathologies, accompanied by coagulation disorders: abdominal aortic aneurysm, hip replacement after fracture and stroke. For the summarized group of patients with hip replacement after fracture and aortic aneurism the levels of SF and D-dimer in blood plasma were ranged from 2 to 83 μg/ml and 0.16 to 0.9 μg/ml respectively; for stroke patients SF level achieved 7-10 μg/ml and D-dimer was ranged from 0.05 to 0.150 μg/ml. Protein C activity in blood plasma of all patients was ranged from 110 to 60%. For further investigations we selected 21 patients with the accumulation of SF above 30 μg/ml. Most of them also have decreased protein C activity. Control plasma samples were obtained from the blood of healthy donors (n = 20). Volunteers signed informed consent prior to blood sampling according to the Helsinki declaration. This study was approved by the institution's Ethics Committee (23.08.2015, N6).

3.2 Ecamulin Characterisation and Test Development

Previously it was shown that ecamulin as well as ecarin from the venom of *Echis carinatus* cleaves prothrombin at Arg320-Ile321 [19,20]. Thus activation of prothrombin by ecamulin leads to the formation of meizothrombin. Ecamulin also activates prethrombin 1 but slower then the prothrombin (Table 1) and doesn't hydrolyze thrombin-specific chromogenic substrate (S2238) and fibrinogen [9]. Therefore ecamulin can be used for testing in blood plasma.

Table 1. Kinetic of prothrombin and prethrombin 1 cleavage by ecamulin

Substrate	K_m, μM	V_{max}, nmole/min	$k_{cat}= V_{max}/[E]$ min^{-1}	$K_{ef}= k_{cat}/K_m$ $(min \cdot \mu M)^{-1}$	Product
Prothrombin	3.4	0.23	575.0	169.1	Meizothrombin
Prethrombin 1	3.5	0.05	83.3	23.8	Meizothrombin 1

As it is shown on Fig. 1 the addition of prethrombin 1 to healthy donor's blood plasma significantly decreased EI but not PI determined with chromogenic substrate of thrombin (S2238). This fact allowed us to assume that the difference of PI and EI could be an evidence of accumulation of the functional inactive forms of prothrombin in blood plasma. So we used this method for preliminary screening and selection samples of blood plasma for further investigation.

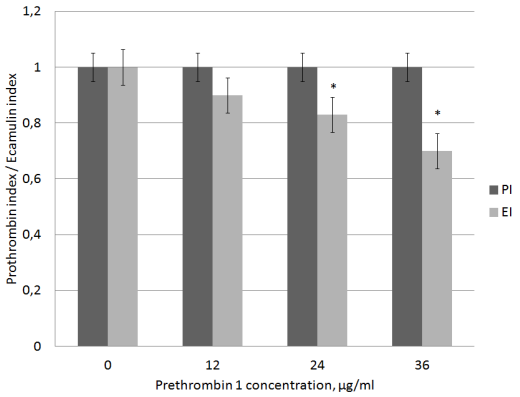

Fig. 1. Prothrombin (PI) and ecamulin (EI) indexes of blood plasma in the presence of prethrombin 1

** - Significant at p < 0.005, n = 3*

3.3 Express Determination of Inactive Forms of Prothrombin

For the determination of functionally inactive forms of prothrombin in the selected blood plasma samples we used the combination of commonly used prothrombin index (PI) and the test with use of ecamulin (prothrombin activator from the *Echis multisquamatis* snake venom) [8,9].

Ecamulin induces the generation of thrombin activity and allows detecting total level of prothrombin (intact molecule and its derivatives). From the other hand, PI based on the activation of coagulation cascade by thromboplastin was used to detect the level of intact prothrombin (uncleaved and carboxylated) [21]. Results were presented as prothrombin index (PI) for prothrombin activation by thromboplastin, and ecamulin index (EI) for prothrombin activation by ecamulin (Fig. 2).

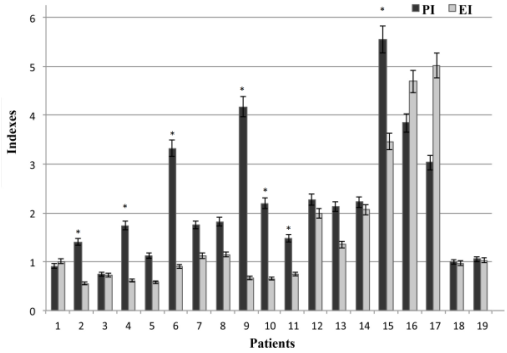

Fig. 2. Prothrombin (PI) and ecamulin (EI) indexes of patients' blood plasma

** - Significant at p < 0.005, n = 3*

The distinct difference in the ecamulin and prothrombin indexes was observed for patients 2, 4, 6, 9, 10, 11, 15. This was an evidence for the drop-in of active prothrombin quantity caused either by decarboxylation of all vitamin K dependent coagulation proteins (descarboxy-proteins formation [21-23]) or prothrombin proteolytical digestion.

3.4 Modified APTT-test for Prethrombin Detection

To distinguish the coagulation affection caused by prethrombin 1 formation from descarboxy-proteins accumulation we used modified activated partial thromboplastin time test (APTT). APTT is a laboratory test that characterizes the intrinsic coagulation pathway. Hence prolongation of coagulation or deceleration of the chromogenic substrate cleavage in APTT-test could be a consequence of decarboxylation of clotting cascade factors (prothrombin, factors X, IX and VII) as well as prothrombin deficit in the case of transforming prothrombin to prethrombin 1.

That is why the addition of exogenous prothrombin to the blood plasma of patients with descarboxy-proteins accumulation did not

influence APTT test. From the other hand, the restoration of native prothrombin in the blood plasma of patients with proteolytically digested prothrombin (prethrombin 1) normalized APTT test parameters.

To prove this hypothesis we developed model system with donor's blood plasma. It was shown that the addition of intact prothrombin to blood plasma caused increasing the chromogenic substrate cleavage in APTT-test. The addition of prethrombin 1 (in contrast to prothrombin) did not influence APTT test results (Table 2). Results are presented as ratio of APTT in the presence of prothrombin to APTT (APTT ratio) of blood plasma.

Table 2. APTT ratio of human blood plasma determined after the addition of exogenous prothrombin or prethrombin 1

Substrate	APTT ratio
Healthy donor's blood plasma	1.00±0.05
Donor's blood plasma in the presence of prothrombin (2 μg/ml)	1.35±0.05
Donor's blood plasma in the presence of prethrombin 1 (2 μg/ml)	1.00±0.05

So we used modified activated partial thromboplastin time test (APTT) to detect prethrombin 1 in blood plasma samples selected according to EI/PI results. All patients had the deceleration of the chromogenic substrate cleavage in APTT-test. However the addition of intact prothrombin to blood plasma caused the acceleration in the chromogenic substrate cleavage in APTT test (Fig. 3, black lines).

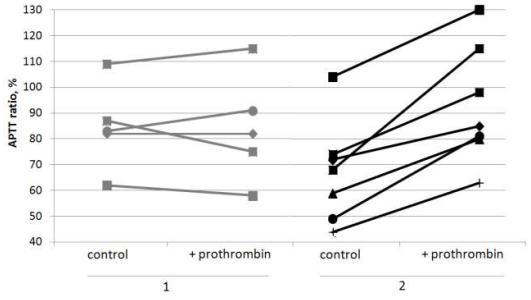

Fig. 3. The effect of the addition of exogenous prothrombin to the samples of blood plasma on their APTT-ratio. 1 – patients with descarboxy-proteins accumulation. 2 – patients without descarboxy-proteins

Blood plasma samples of patients treated with warfarine were selected as a negative control as those which contain decarboxylated proteins [24]. As it is shown on Fig. 3 (grey lanes) there was no acceleration in the chromogenic substrate cleavage in APTT-test was shown for these patients.

3.5 Direct Detection of Prethrombin 1 in Blood Plasma

We also had to directly confirm the accumulation of prethrombin 1 in the blood plasma of patients. For this purpose we studied patients' blood plasma by SDS-PAGE followed by western-blot immunoprobing using prothrombin-specific polyclonal antibody that interacts with prothrombin and all its derivatives. Sample containing prethrombin 1, thrombin and prothrombin was used as a positive control (Fig. 4, track 4). Two protein bands with molecular weight 72 kDa and 55 kDa (prothrombin and prethrombin 1 respectively) were detected in the blood plasma of selected patients (Fig. 4, tracks 6-12). However in the blood plasma of warfarine-treated patients (Fig. 4, tracks 1-3, 5) we observed only 72 kDa protein (prothrombin) and no prethrombin 1.

4. DISCUSSION

The prethrombin 1 – inactive derivative of prothrombin – was detected in human blood plasma at severe pathologies. Previously there were no reports about it's formation *in vivo*. Detection of prethrombin 1 seems to be an important clinical diagnosis. Its relevance is much more meaningful than prothrombin monitoring and indicates thrombin existence.

We developed and applied a method of detection of prethrombin 1 in human blood plasma in severe pathologies based on the combination of PI and EI test followed by modified APTT test. This method was verified with Western-blot immunoassay using polyclonal anti-prothrombin antibody and can be used in clinical trials.

Nowadays the main diagnostic test used for the detection of thrombin formation is direct estimation of prothrombin fragment F1+2 (F1+2) [25]. F1+2 is generated *in vivo* cleavage of prothrombin by factor Xa and this test considered to be useful for diagnosis of thrombosis. At the same time detection of F1+2 show the number of activated prothrombin molecules but does not provide any information on the activity of thrombin that is formed during this activation.

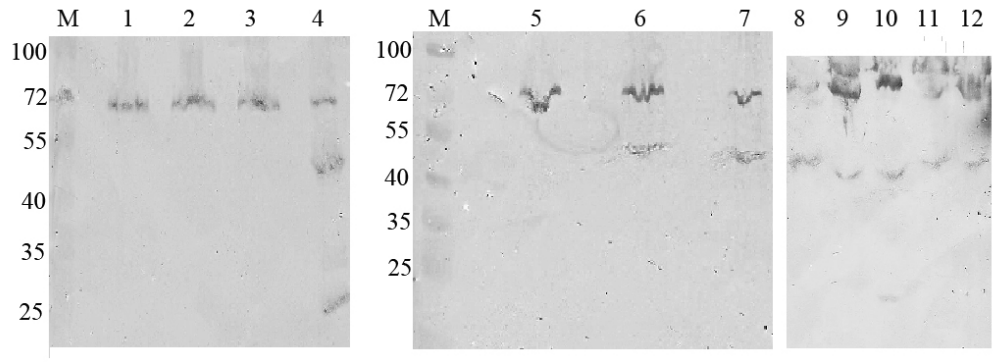

Fig. 4. Western blot analysis of patients' blood plasma with prothrombin-specific antibody M – molecular weight markers (100, 72; 55; 40; 35; 25 kDa). 1-3, 5 – blood plasma of warfarine-treated patients; 6-12 – blood plasma of patients with acute haemostatic disorders (selected by modified APTT-test); 4 – the mixture of prothrombin, prethrombin 1 and thrombin

From the other hand the detection of prethrombin 1 in blood plasma shows the appearance in bloodstream of thrombin that remains enzymaticly active and not inhibited by natural inhibitors or used drugs. Detection of fibrinopeptides (that are direct products of thrombin action on fibrinogen) has similar diagnostic relevance [26].

5. CONCLUSION

Up to 10% of patients with abdominal aortic aneurysm, hip replacement after fracture and stroke had prethrombin 1 generated in blood plasma. The accumulation of prethrombin 1 was shown by combination of common diagnostic tests (prothrombin index, ecamulin/ecarin index and modified APTT) and confirmed by Western-blot analysis using polyclonal anti-prothrombin antibody.

ETHICAL APPROVAL

The volunteers and patients signed informed consent prior to blood sampling according to the Helsinki declaration. This study was approved by the institution's Ethics Committee (23.08.2015, N6).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Di Cera. Thrombin interactions. Chest. 2003;124:11S–17S.
2. Rosing J, Tans G, Govers-Riemslag JW, Zwaal RF, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem. 1980;255(1):274-83.
3. Mann KG, Butenas S, Brummel K. The dynamics of thrombin formation. Arterioscler Thromb Vasc Biol. 2003;23: 17–25.
4. Wood JP, Silveira JR, Maille NM, Haynes LM, Tracy PB. Prothrombin activation on the activated platelet surface optimizes expression of procoagulant activity. Blood. 2011;117:1710-1718.
5. Chen Z, Pelc LA, Di Cera E. Crystal structure of prethrombin-1. PNAS. 2010; 107:19278-19283.
6. McDuffie FC, Giffin C, Niedringhaus R, Mann KG, Owen CA Jr, Bowie EJ, Peterson J, Clark G, Hunder GG. Prothrombin, thrombin and prothrombin fragments in plasma of normal individuals and of patients with laboratory evidence of disseminated intravascular coagulation. Thromb. Res. 1979;16:759-773.
7. Haynes LM, Bouchard BA, Tracy PB, Mann KG. Prothrombin activation by platelet-associated prothrombinase proceeds through the prethrombin-2 pathway via a concerted mechanism. J Biol Chem. 2012;287:38647-38655. DOI: 10.1074/jbc.M112.407791
8. Petrovan RJ, Govers-Riemslag JW, Nowak G, Hemker HC, Rosing J, Tans G. Purification and characterization of multisquamase, the prothrombin activator present in *Echis multisquamatus* venom. Thromb Res. 1997;88(3):309-316.

9. Solov'ev DA, Platonova TN, Ugarova TP. Isolation and characteristics of ecamulin - a prothrombin activator from multiscaled viper (*Echis multisquamatus*) venom. Biokhimiia. 1996;61:1094-1105.
10. Mann KG. Prothrombin. Methods Enzymol. 1976;45:123-156.
11. Lugovskoi EV, Kolesnikova IN, Lugovskaia NE, Litvinova LM, Gritsenko PG, Gogolinskaia GK, Liashko ED, Kostiuchenko EP, Remizovskiĭ GA, Pedchenko VN, Komisarenko SV. Quantification of D-dimer and soluble fibrin in blood plasma of people with ischemic heart disease and hypertension. Ukr Biokhim Zh. 1999;76(6):136-141.
12. Lugovskoy EV, Kolesnikova IN, Platonova TN, Lugovskaia NE, Litvinova LM, Kostiuchenko EP, Chernyshenko TM, Ganova LA, Spivak NIA, Komisarenko SV. Simultaneous quantification of soluble fibrin and D-dimer in blood plasma for the assessment of the threat of thrombosis. Klin Med (Mosk). 2013;91(11):38-44.
13. Gornitskaia OV, Platonova TN. Isolation and properties of the protein C activator from Agkistrodon halys halys venom. Biomed Khim. 2003;49(5):470-8.
14. Laemli RV. Cleavage of structural proteins during of bacteriophage T4. Nature. 1970;227:680-685.
15. Wu HC, Yen CC, Tsui WH, Chen HM. A red line not to cross: Evaluating the limitation and properness of gel image tuning procedures. Anal Biochem. 2010; 396(1):42-50.
16. Korolova DS, Chernyshenko TM, Gornytska OV, Chernyshenko VO, Platonova TN. Meizothrombin preparation and its role in fibrin formation and platelet aggregation. Advances in Bioscience and Biotechnology. 2014;5:588-595.
17. Gershkovich AA, Kibirev VK. Chromogenic and fluorogenic peptide substrates of proteolytic enzymes. Bioorg Khim. 1988; 14:1461-1488.
18. Toh CH, Giles AR. Waveform analysis of clotting test optical profiles in the diagnosis and management of disseminated intra-vascular coagulation (DIC). Clin Lab Haematol. 2002;24:321-327.
19. Kornalik F, Blombäck B. Prothrombin activation induced by Ecarin - A prothrombin converting enzyme from Echis carinatus venom. Thromb Res. 1975;6:57-63.
20. Nowak G. The ecarin clotting time, a universal method to quantify direct thrombin inhibitors. Pathophysiol Haemost Thromb. 2003;33:173-183.
21. Hryschenko VA, Tomchuk VA, Lytvynenko OM, Chernyshenko VO, Gryschuk VI, Platonova TM. An estimate of protein synthesis in liver under induced hepatitis. Ukr Biokhim Zh. 2011;83:63-68.
22. Suttie JW. Vitamin K in health and disease. Boca Raton: CRC Press. 2009;231.
23. Cavallari LH, Nutescu EA. Warfarin pharmacogenetics: To genotype or not to genotype, that is the question. Clin Pharmacol Ther. 2014;96:22-24.
24. Suttie JW. The biochemical basis of warfarin therapy. Adv Exp Med Biol. 1987;214:3-16.
25. Páramo JA. Prothrombin fragments in cardiovascular disease. Adv Clin Chem. 2010;51:1-23.
26. Lippi G1, Cervellin G, Franchini M, Favaloro EJ. Biochemical markers for the diagnosis of venous thromboembolism: The past, present and future. J Thromb Thrombolysis. 2010;30:459-471.

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