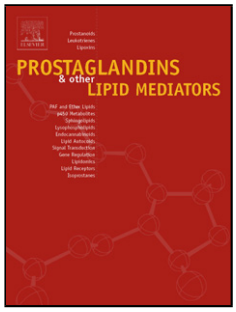

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Differential TBXA2 receptor transcript stability is dependent on the C924T polymorphism

Vincenzo De Iuliis[1], Sebastiano Ursi[1], Alfonso Pennelli[2], Marika Caruso[2], Angela Nunziata[2], Antonio Marino[1], Vincenzo Flati[3], Francesco Cipollone[4], Maria Adele Giamberardino[4], Gianfranco Vitullo[1], Elena Toniato[2,*], Pio Conti[5], Stefano Martinotti[1,2]

[1] SS Annunziata University Hospital, Unit of Clinical Molecular Biology and Predictive Medicine, University of Chieti, ASL Lanciano-Vasto-Chieti, Chieti, Italy

[2] Department of Medical, Oral and Biotechnological Sciences, Via dei Vestini 31, University of Chieti, Italy

[3] Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, L'Aquila, Italy

[4] Department of Medicine and Science of aging, Via dei Vestini 31, University of Chieti, Italy

[5] Immunology Division, Postgraduate Medical School, University of Chieti, Viale Unità d'Italia 73, Chieti, Italy

***Corresponding Author**

Elena Toniato

Department of Medical, Oral and Biotechnological Sciences, University of Chieti, Italy

Via dei Vestini 31, Chieti, Italy

Telephone: +3908713554161

Fax: +390871357340

e-mail: e.toniato@unich.it

Highlights

- role of the C924T polymorphism of the TBXA2R gene
- effect on platelet aggregation linked to C924T polymorphism
- protective role for the TBXA2R TT genotype against atherothrombosis

Abstract

Background. In order to better characterize the molecular mechanisms involved in processing mutated transcripts, we investigated the post-transcriptional role of the C924T polymorphism (rs4523) located in the 3' region of the TBXA2R gene.

Methods and Results. Experiments of dose response with Actinomycin D on MEG-01 human cell line showed a significant decrease on cell viability that was more evident on cells treated for 24 h. In addition, we showed that treatments with 5-10 μM, 15 μM and 20 μM of actinomycin D reduced cell viability by 44%, 72% and 75%, respectively, compared to the control group. Conversely, the samples treated with 1 μM of actinomycin D did not show significant difference on cell viability as compared to the control group. Analysis of the steady state mRNA level of TBXA2R by qRT-PCR evidenced an increase in mRNA stability for the wild type (C) compared to the mutant (T) allele. Furthermore, the expression levels of TBXA2R on wild type (CC) and mutant type (TT) patients, based on C924T polymorphism, were analyzed. The wild type showed a higher expression of TBXA2 receptor also with two different degrees of glycosylation (55 and 64 kDa), when compared to the mutant. These observations correlated with platelet aggregation, which was reduced in TT, independently of the platelet aggregation stimuli.

Conclusions. The instability of the TBXA2R transcript and the lack of effect on platelet aggregation might suggest a protective role for the TBXA2R TT genotype against atherothrombosis and its complications in high-risk aspirin-treated patients.

Key Words: gene expression ● platelet aggregation ● single nucleotide polymorphism

Introduction

Interactions between the vascular system and inflammatory cells play a major role in the pathogenesis and development of atherosclerosis [1, 2]. Several studies have shown the involvement of several pro-inflammatory mediators in plaque formation and lesion progression [3]. As widely debated, Thromboxane A_2 (TXA_2) biosynthesis (detected as Thromboxane B_2) is increased during atherosclerosis, and the initial progression of this process is promoted by TXA_2. This regulator exerts its physiological and pathophysiological responses by binding to the TXA_2 receptor (TBXA2R), inducing activation of platelet cells, controlling endothelial integrity and leukocyte-endothelial cell interaction [4]. As a matter of fact, several studies demonstrated an increased expression of TXA_2 and thromboxane A_2 receptor (TBXA2R) during the atherogenesis progression, indicating that the TBXA2R signaling pathway plays a key role in advanced atherosclerosis [5]. Interestingly, in the ApoE knockout mouse model, the pharmacological inhibition of TBXA2R was effective in reducing the development of atherosclerotic plaques rather than the systemic depletion of TXA_2 levels [6]. Moreover, clinical and experimental data pointed out a critical role of the TBXA2R in ischemia and myocardial infarction. Inhibition of TBXA2R by receptor antagonists prevented the extension of ischemic damage in myocardial ischemia and improved early survival following permanent coronary artery ligation [7].

Based on several clinical and physiological observations, TXA_2 is a potent mediator of the vascular activity, such as platelet activation, thrombosis, angiogenesis, inflammation and atherogenesis [8, 9].

TXA_2 is a member of the eicosanoids, synthesized through the sequential metabolism of arachidonic acid by several enzymes. Firstly, the cytosolic phospholipase A_2 (PLA_2) catalyzes the release of arachidonic acid from membrane phospholipids, which are further converted into the prostaglandin endoperoxides, PGG2 and PGH2, via cyclooxygenase (COX 1 and COX 2) activity [10]. In a further step, these endoperoxides are converted into TXA_2 by the thromboxane synthase, an enzyme abundantly expressed in a wide variety of different tissues. TXA_2 exerts its activity by binding to thromboxane receptor (TBXA2R), while the residual TXA_2 is non-enzymatically degraded into biologically inactive TXB_2 [11].

TBXA2R is a member of the superfamily of G-protein-coupled seven-transmembrane receptors [12]. In humans, its gene is encoded on chromosome 19p13.3, and spans 15 kbps containing three exons divided by two introns. TBXA2R is widely expressed and localized either on the cell membranes or on intracellular structures [13]. Two isoforms of TBXA2R have been described. A human cDNA was cloned from human placenta, based on the sequence of the protein from platelets, composed by seven transmembrane regions (TBXA2R-α). A second isoform was described on endothelial cells, named TBXA2R-β, resulting from an alternative splicing of the gene at the cytoplasmic carboxyl tail [14].

SNPs of the human TBXA2R gene are associated to a variety of pathologic traits. Hirata *et al*. identified a missense mutation (Arg60 to Leu) in patients with a dominantly inherited bleeding disorder characterized by defective platelet response to TXA2. The substitution of amino acid is located in the first cytoplasmic loop of the TBXA2R protein. This mutation is thought to be a very rare mutation, since it is observed only in few family members [15]. Moreover, Unoki *et al*. showed that a synonymous mutation (T924C; codon TAT>TAC Tyrosine; rs4523) in the human TBXA2R gene correlated with bronchial asthma [16], despite the fact that no amino-acid substitution was generated by such polymorphism. In order to better characterize the molecular mechanisms involved in the processing of mutated transcripts, we investigated the post-transcriptional role of C924T polymorphism (rs4523) located at the 3' region of the TBXA2R gene.

Methods

Patients' genotyping

DNA was extracted from whole blood of 84 healthy volunteers, recruited using an informed consent for evaluation of the C924T genotype. The TBXA2 gene portion of 539 bps containing the C924T mutation was amplified using the following PCR primers: forward 5'-CTTTGCAGGTCTTCATCGC - 3' and reverse 5'- CCTCTTCCAATGTCTGCATG – 3' (Diatech pharmacogenetics, Jesi, Ancona, Italy). In order to identify the wild type and/or mutant genotype, we used 5 U/µl RsaI (New England biolabs, Ipswich, MA, USA) restriction enzyme analysis on agarose gel (Fig. 1A) and further we confirmed the results sequencing the TBXA2 gene (Fig. 1B).

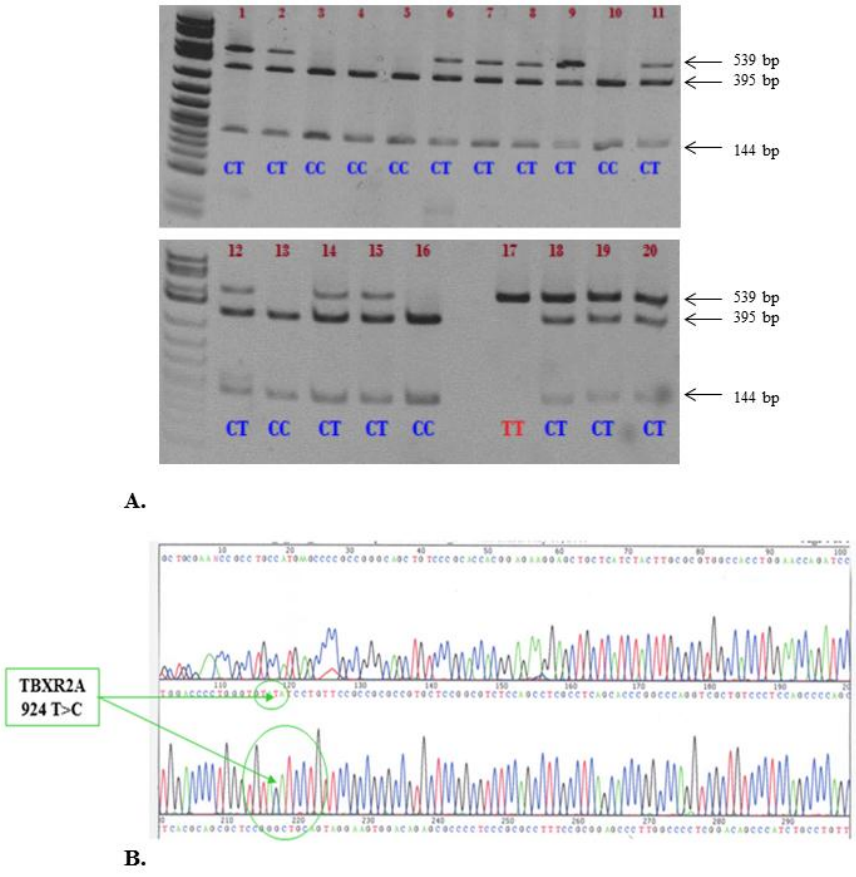


Fig. 1. A. Restriction enzyme analysis on agarose gel. B. The human TBXA2R gene is located on 19p13.3, which spans 15 kbps containing three exons divided by two introns. The two variants of the TBXA2R receptor have been described, based on the presence or absence of C924T polymorphism. This polymorphism, defined by RsaI digestion on PCR product, has been confirmed by sequence analysis.

Western Blot

For Western blot analysis, total proteins were extracted from lymphomonocyte isolated from whole blood of selected patients using a Ficoll-Paque [17]. The cell pellets were lysed in a buffer containing 50 mM Tris.Cl pH 7.8, 1% Triton X100, 0.1% SDS, 250 mM NaCl, 5 mM EDTA, 100 mM NaF, 2 mM NaPPi, 2 mM Na3VO4, 1 mM PMSF. Cell lysates were loaded onto a 12% SDS-PAGE and the separated proteins electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight at 4°C with a specific polyclonal anti-TBXA2R Ab (Cayman Chemical, Ann Arbor, MI, USA). Membranes were then incubated with a secondary anti-rabbit Ab (Cayman Chemical, Ann Arbor, MI, USA) for 1 h at room temperature. In order to confirm that equal amounts of protein were loaded in each lane, the membranes were incubated with an anti-actin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) [18]. Immunocomplexes were visualized using the ECL detection system (GE Healthcare Life Sciences, UK). Densitometric analysis was performed for the quantification of the immunoblots, using the Molecular Analyst System (Bio Rad, Hercules, CA, USA).

Platelet aggregation

All patients were stratified on the basis of their genotype profile (CC, CT and TT) for the SNP C924T and analyzed for platelet aggregation test. Blood samples were collected without stasis from the antecubital vein with a 19 G butterfly needle, after 12–14 hrs of fasting. Each sample was anti-coagulated with a 3.8% sodium citrate solution (1:10 v/v). The platelet-rich plasma (PRP) was obtained by centrifugation at 160g for 10 min at room temperature. Platelet-poor plasma (PPP) was prepared by further centrifugation of the remaining blood at 2,000g for 20 min. PRP was adjusted with PPP to reach a platelet count of 250,000–300,000/ml. Platelet aggregation was induced by challenging with Adenosine diphosphate (ADP) at 2 and 6 μM, epinephrine at 0.6 and 10 μg/ml and collagen at 1 and 9 mg/ml final concentrations. To evaluate platelet aggregation, according to the Born method [19], a multichannel aggregation analyzer (ARKRAY) was used. Optical density for PRP was set at 0% and for PPP at 100%. In addition, platelet aggregation induced by ADP stimulation was also evaluated as closure time using a PFA-100 analyzer (Siemens Medical Solutions USA, Inc., PA, USA).

Cell culture

The EBV-producing MEG-01 human cell line (ATCC) was grown in RPMI 1640 medium, supplemented with 10% heat- inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% Pen-Strep at 37 °C in 5% CO_2. The cells were seeded at 2.0 x 10^5/ml and the experiment was performed in triplicate. This cell clone was genotyped for heterozygosity at the T924C site.

Dose response with Actinomycin D on MEG-01 cell line

MEG-01 cells were seeded on six-well plates (2 x10^5 cells/well) and treated with increasing doses (1 µM – 5 µM – 10 µM – 15 µM – 20 µM) of the transcription inhibitor Actinomycin D (Sigma Aldrich, Saint Louis, MO, USA) at different exposure times (0, 1, 6, 12, 24 hours). After treatment, the cells were further incubated at 37 °C in 5% CO_2. Cell pellets were washed three times in PBS, centrifuged (1,000 rpm for five minutes), the supernatant was removed and finally the cells were re-suspended and counted using a Burker chamber and Trypan blue stain, for testing the viability.

Analysis of the steady state mRNA level of TBXA2R by qRT-PCR after Actinomycin D treatment on MEG-01 cell line

MEG-01 cells were seeded on Petri dishes (2x10^5 cells/ml) and treated with 10 µM of Actinomycin D at different exposure times (0, 1, 6, 12, 24 hours), as reported in several studies [20]. After treatment, the cells were incubated at 37 °C in 5% CO_2, washed two times with PBS and then lysed directly in culture dishes using 1 mL of TRIZOL® Reagent. RNA isolation and extraction was performed using the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The PCR primers for TBXA2R (+924C/T) allele-specific were respectively: forward for "C" allele (F: 5'- CTGGACCCCTGGGTGTAT - 3'); forward for "T" allele (F: - CTGGACCCCTGGGTGTAC); reverse (R: 5'- GGAGAGTGCTTGGTAAAAGGATCA- 3'). The PCR primers for the TBXA2R preserved region outside of the C924T polymorphism, used as a reference gene, were: forward (F: 5'- CCCAATCCAACCCGGG) and reverse (R: 5'- GGAGAGTGCTTGGTAAAAGGATCA) (Operon molecules for life, Mountain View, CA, USA). Subsequently, we performed a qRT-PCR (Rotor-Gene 6000 Series 5 plex, Corbett Research, Mortlake, NSW, AU) of the 5' of the gene using specific

probes for the TBXA2R (+924C/T): for "T" allele VIC-AGACACCCAGGGGTCCAG[MGB:minor groove binding]; for "C" allele FAM-GGACACCCAGGGGTCCAG[MGB]; for the TBXA2R preserved region were: VIC-TCCAGGATCTGGTTCCA[MGB] and FAM-ACCCCCAACTCCTC [MGB]). In addition, we used the GAPDH, as a qPCR reference gene, in order to evaluate the efficiency of the assay (Supplementary Fig. S1).

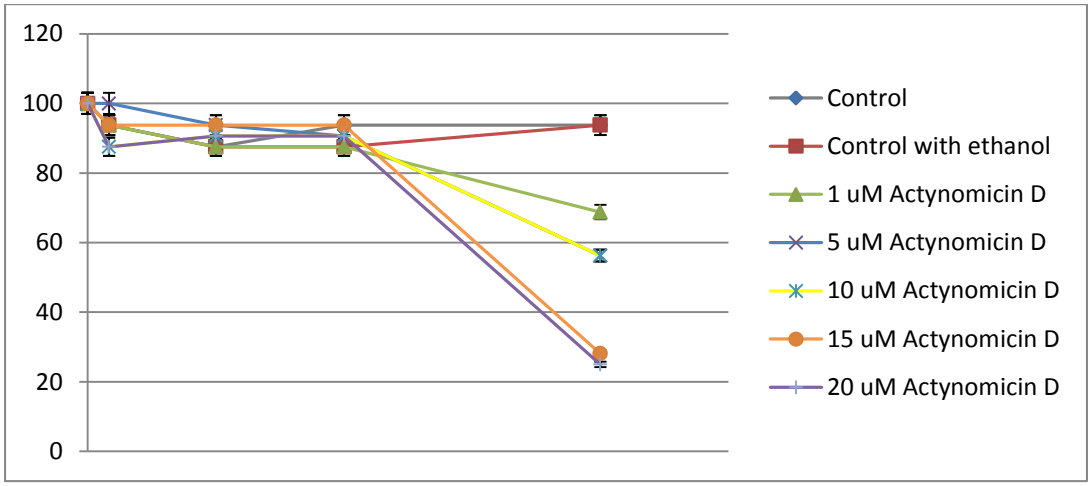
Statistical analysis

Data are expressed as means ± SEM. Student t test or ANOVA followed by the appropriate post-hoc test was used (Stat View 4.0 software, Abacus Concepts, Berkeley, CA, USA). P values < 0.05 were considered statistically significant, with a confidence interval of 95%.

Results

Dose response with Actinomycin D on MEG-01 cell line

As shown in Fig. 2, a concentration of Actinomycin D of at least 5 μM had a greater effect on cell viability. The decrease on cell viability was more evident on cells treated for 24 hr. The samples treated with 5-10 μM, 15 μM and 20 μM of Actinomycin D showed, a 44%, 72% and 75% in reduction of cell viability compared to a control group ($p< 0.05$), respectively. The samples treated with 1 μM of Actinomycin D didn't show significant difference on cell viability as compared to the control group.



	time course of Actinomycin D treatment				
	0	1	6	12	24
Control	100	93.75	87.5	93.75	93.75
Control with ethanol	100	93.75	87.5	87.5	93.75
1 uM Actynomicin D	100	93.75	87.5	87.5	68.75
5 uM Actynomicin D	100	100	93.75	90.625	56.25
10 uM Actynomicin D	100	87.5	90.63	90.625	5625
15 uM Actynomicin D	100	93.75	93.75	93.75	28.13
20 uM Actynomicin D	100	87.5	90.63	90.63	25

Fig. 2 Effect of Actinomycin D on cell viability. The decrease on cell viability is highly evident on cells treated for 24 h, whereas samples treated with 5-10 μM, 15 μM and 20 μM of Actinomycin D show a 44%, 72% and 75% in reduction of cell viability compared to the controls ($p < 0.05$), respectively. Decrease in cell viability is more relevant for doses higher than 5 μM and for exposure times longer than 24 h. The samples treated with 1 μM of Actinomycin D didn't show significant difference on cell viability as compared to the control group. The experiment was performed in triplicate.

Analysis of the steady state mRNA level of TBXA2R by qRT-PCR after Actinomycin D treatment on MEG-01 cell line

The influence of the C924T polymorphism on MEG-01 cells, after treatment with 10 μM Actinomycin D, at different times, was analyzed. As shown in fig. 3 the TBXA2R polymorphism expression was higher for the C allele compared to the T allele. We observed a significant decreased expression of both alleles, when compared to the respective controls and lower levels of transcript after 6 hours of treatment ($p< 0.05$). In addition the statistical analysis of the fold changes between the C and T treated groups compared to the control ones showed that the mRNA stability was greater for the C allele compared to the T allele, which instead evidenced a high degree of instability (Fig. 3).

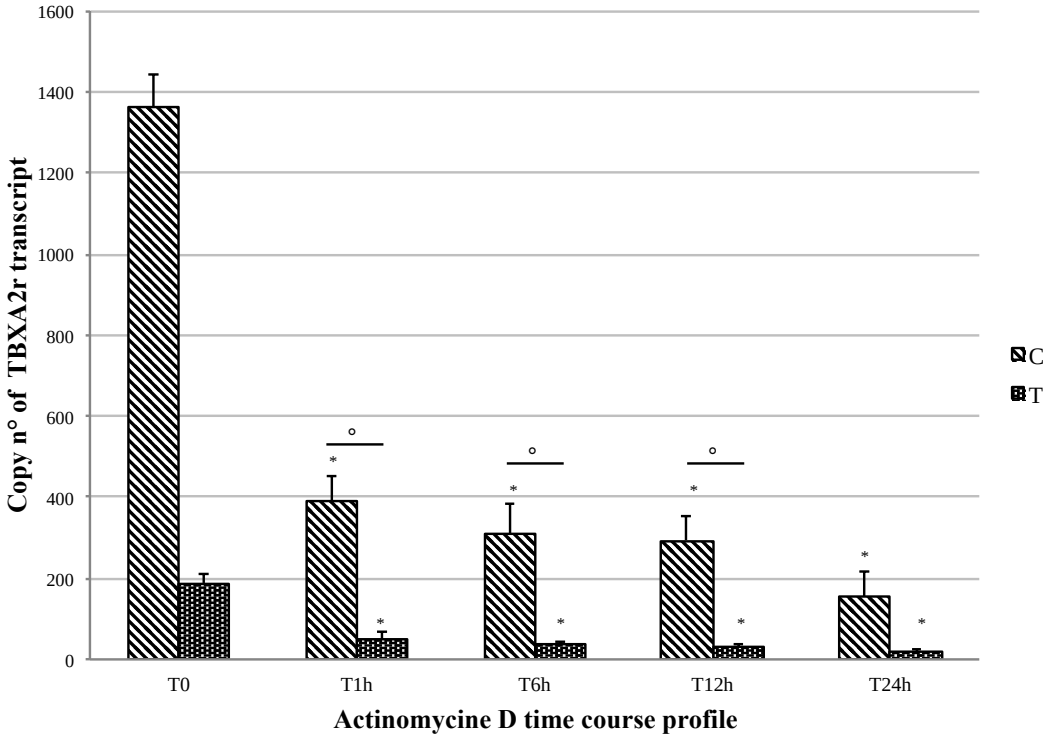


Fig. 3. A significant decreased expression of C and T alleles was observed on MEG-01 cells treated with 10 μM of Actinomycin D at different exposure times when compared to the respective controls. *: $p< 0.05$ compared to the respective controls. The C and the T allele were down-regulated 3.27 and 3.87 fold (°: $p= 0.013$) in T1h groups, 4.62 and 5.33 fold (°: $p= 0.023$) in T6h, 4.89 and 6.86 fold (°: $p< 0.001$) in T12h, 9.92 and 9.85 fold ($p= 0.9$) in T24h, when compared to the respective control groups. This data suggests a higher instability of the mutant type transcript and an exponential

decrease of TBXA2R mRNA as early as 1 hour after treatment. Fold change = $2^{-\Delta\Delta Ct}$. The experiment was performed in triplicate.

C924T allele frequency and patients' genotype profile

The table 1 showed the allele frequency and the patients' genotype profile of the C924T polymorphism for all the patients recruited in this study. Specifically, we reported the presence of 42, 28 and 14 patients with a CC, CT and TT genotype, respectively.

N° cases	SNP	Major allele frequency (%)	Minor allele frequency (%)	Homozygosity of major allele (%)	Heterzygosity (%)	Homozygosity of minor allele (%)
84	C924T	66.7	33.3	50	33.4	16.6

Table 1. C924T polymorphism of the coding sequence of the TP receptor gene in an Italian population study.

Platelet aggregation analysis

Platelet aggregation for the TBXA2R gene polymorphism C924T was analyzed in all patients, stratified on the basis of their genotype profile (CC, CT and TT), using a PFA-100 analyzer. As shown on table 2, the platelet aggregation induced by ADP was more effective on patients with at least one C allele. Furthermore, these results point out that the platelet aggregation induced by ADP was less efficient in the mutant genotype (TT), with respect to the wild type (n.r. 55-137 sec.). In addition, platelet aggregation induced by challenging with Adenosine diphosphate (ADP) at 2 and 6 μM, epinephrine at 0.6 and 10 μg/ml and collagen at 1 and 9 mg/ml final concentrations was analyzed for the TBXA2R gene polymorphism in wild type patients (CC) and in mutated patients (TT) using a multichannel aggregation analyzer (ARKRAY), according to the Born method (Fig. 4). These results confirmed the data obtained by PFA-100 highlighting a less effective platelet aggregation on patients with a TT genotype compared to those with a CC genotype.

Genotype	N° of cases	Platelet aggregation (sec.)	ANOVA (p)
CC	42	80.3 (± 2.1)	< 0.05
CT	28	102 (± 8.4)	
TT	14	242.5 (± 42.2)	

Table 2. Platelet aggregations analysis of CC, CT and TT patients for the polymorphism C924T of the TBXA2R gene was evaluated as closure time using a PFA-100 analyzer. Patients with a TT genotype showed a less efficient platelet aggregation compared to those with at least one C allele (ANOVA p< 0.05; Bonferroni test between TT and CT or CC: p< 0.05)

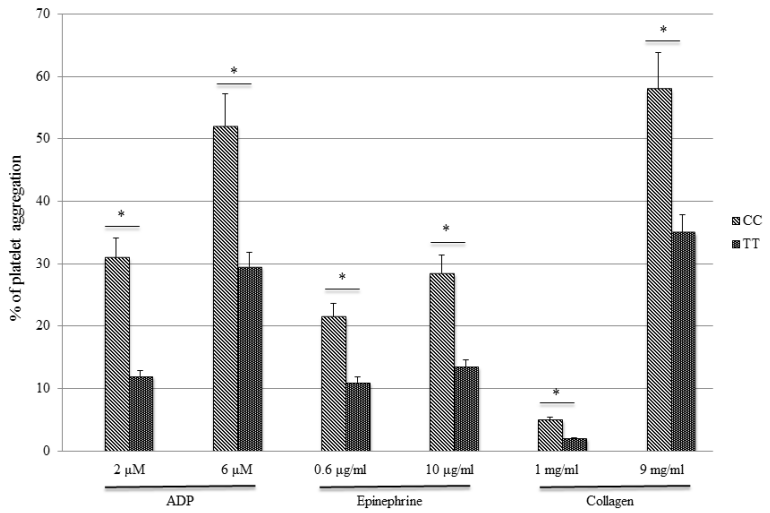


Fig. 4. Platelet aggregation analysis of CC and TT patients for the polymorphism C924T of the TBXA2R gene was evaluated using ADP at 2 and 6 µM, epinephrine at 0.6 and 10 µg/ml and collagen at 1 and 9 mg/ml final concentrations with using a multichannel aggregation analyzer (ARKRAY), according to the Born method. * p< 0.05

TBXA2R protein expression

6 and 5 patients, respectively with a CC and a TT genotype based on the C924T polymorphism, were randomly selected in order to evaluate the protein expression of the TP receptor (Fig. 5). The CC patients showed a higher level of expression of TBXA2 receptor, with two different degrees of glycosylation (55 and 64 kDa), when compared to patients with a TT genotype ($p < 0.05$). The data showed a higher stability of the wild type transcript respect to the mutant type, confirming the results obtained through the analysis of the steady state mRNA level of TBXA2R by qRT-PCR after Actinomycin D treatment on MEG-01 cell line.

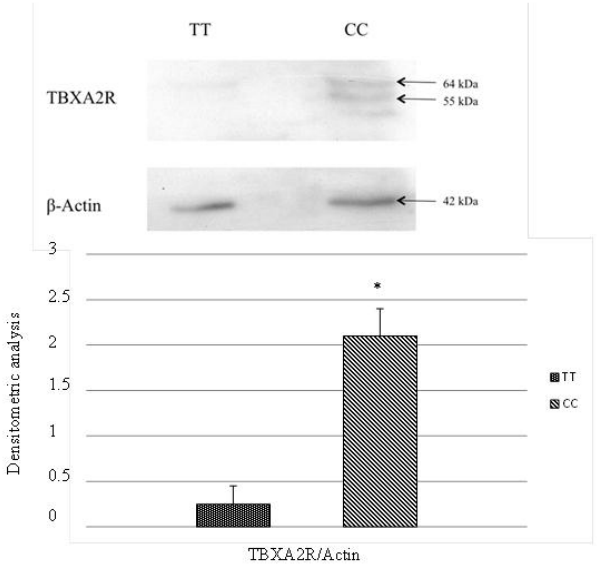


Fig. 5 The protein expression of TBXA2R on wild-type (CC) and mutant type (TT) patients was analyzed. Wild type (CC) shows a higher expression level of TBXA2 receptor, recognizing two different bands, with a different degree of glycosylation, (55 and 64 kDa), compared to the mutant type (TT) (*: $p < 0.05$). This data strongly support the evidence of a higher stability of the wild type transcript respect to the mutant type.

Discussion

Previous studies have shown that leukocytes, platelets and endothelial cells (21, 22) are involved in the biosynthesis of bioactive lipid mediators derived from arachidonic acid. For instance, stimulation of neutrophils (23, 26) results in production of LTA4 [21, 22]. On the other hand, the reduction in LTC4 biosynthesis observed in patients with unstable angina during 6-methylprednisolone (6-MP) treatment could be explained by the glucocorticoid-dependent decrease of adhesion molecule expression on blood and vascular cell surfaces [27].

Furthermore, a study by Vejar *et al.* examined the rate of thromboxane biosynthesis in the setting of acute coronary syndromes through measurements of plasma levels of thromboxane B_2, and of the urinary excretion of major enzymatic metabolites such as 11-dehydro-thromboxane B_2 and 2,3-dinor-thromboxane B_2. This study demonstrated that the administration of low-doses of aspirin are associated with statistically significant reduction in thromboxane biosynthesis by 70% [28].

Moreover, experimental and clinical studies have shown the characterization of variable patterns of COX-2 expression as a key player in atherothrombosis and led to hypothesize a role for COX-2–derived prostanoids in vascular disease progression and its thrombotic complications [29, 30]. The results of morphological, pharmacological, and genetic studies of the human carotid plaque model are consistent with the hypothesis that down-regulation of COX-2 expression in inflammatory cells may protect against atherothrombosis in high-risk aspirin-treated patients [31, 32].

In light of the above findings, in the present study we focused our attention on the $TBXA_2$ receptor as an important contributor on atherosclerosis lesion formation and development. We investigated the possibility that post-transcriptional mechanisms of regulation may be involved in the expression and stability of TBXA2R, related to the C924T genotype. We used, as bio-molecular model, a T924C heterozygosis cell clone derived from the MEG-01 cell line. The results evidenced that these cells treated with increasing doses of actinomycin D had a reduction in viability. In particular, the decrease in cell viability was more relevant for doses higher than 5 μM and for exposure times longer than 24 h, which is consistent with a role for the TBXA2R in mediating cell survival. Furthermore, the TBXA2R polymorphism expression was greater for the C allele compared to the T allele, regardless of the actinomycin D exposure times. The analysis of the steady state mRNA level of TBXA2R by qRT-PCR demonstrated that the transcript stability was affected by the C924T polymorphism: in fact,

the mRNA stability was greater for the wild type (CC) compared to the mutant type (TT), and the latter showed a decrease of its transcript levels as early as 3 hours after treatment. This evidence suggests a protective role for the TBXA2R TT genotype in the development and progression of atherosclerosis lesions.

In order to better characterize the TBXA2R, its polymorphisms and the expression levels of the two different alleles were studied using lymphomonocytes isolated from target patients. The Western blot analysis showed that the wild type (CC) had a higher expression level of the TBXA2 receptor with two different degrees of glycosylation (55 and 64 kDa), when compared to the mutant type (TT) on at least twelve different genotyped patients. This data evidenced an increased instability of the mutant (TT) type transcript in respect to the wild type, confirming the results obtained through the analysis of the steady state mRNA level of TBXA2R by qRT-PCR after actinomycin D treatment of MEG-01 cell line. Moreover, a platelet aggregation analysis on selected patients, a wild (CC) and a mutant type (TT) for the C924T polymorphism, was performed. The platelet aggregation induced by ADP, epinephrine and collagen, at different concentrations, was less effective for the mutant type (TT). This is consistent with a reduced thrombus formation and hemostasis. Thus, the instability of the TBXA2R transcript and the associated reduction of platelet aggregation [33] might be associated with a protective role for the TBXA2R TT genotype against atherothrombosis and its complications in high-risk aspirin-treated patients. These findings could represent the basis for the prediction of plaque formation and atherosclerosis by analyzing the patients' genotype for the TBXA2R C924T polymorphism. This approach could allow the identification of subjects more susceptible to atherothrombosis, in particular among high-risk aspirin-treated patients.

Compliance with Ethical Standards

Ethical Approvals: Informed consent was given by patients whose retrospective data were used for this study. In addition, according to the guidelines of the declaration of Helsinki for retrospective studies, we notified such project to the University Department where experiments were conducted and to the local Ethics Committee.

Conflicts of interest: none.

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