Biomarkers, 2011; 16(1): 58–64

informa
healthcare

ORIGINAL ARTICLE

Tissue factor as a novel marker for detection of circulating cancer cells

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Abstract

Tissue factor (TF) is a molecular marker that is up-regulated in cancer cells and aids tumoral dissemination. Our purpose was to develop a nested RT-PCR strategy against TF for detecting blood-borne tumour cells. Our method detected TF expression in a minimum of 1.5 pg total RNA from MCF7 cells. A preliminary study in blood samples from 16 advanced breast carcinoma patients showed that 80% of patients with high TF load progressed and died, while only 18% with low TF load showed the same behaviour. Kaplan-Meier analysis confirmed worse overall survival in patients with high TF load.

Key words: *Biomarker; tissue factor; RT-PCR; nested PCR; breast cancer*

Introduction

Tissue Factor (TF), also known as thromboplastin or coagulation factor III, is a 47 kDa transmembrane glycoprotein that acts as the main initiator of the coagulation cascade (Mackman *et. al.* 1989). In non-pathological conditions, TF is constitutively expressed by many cell types. However, in cells directly exposed to flowing blood this expression is abolished (Drake, Morrissey & Edgington, 1989; Fleck *et. al.* 1990). Thus, its procoagulant activity is triggered by exposure to circulating coagulation factors that penetrate surrounding tissues after disruption of the endothelium (Scarpati *et. al.* 1987).

Besides its role in blood coagulation, TF has also been reported to play an important part amidst diverse pathological scenarios, such as sepsis (Edgington *et. al.* 1992), inflammation (Cunningham *et. al.* 1999) and cancer (Semeraro & Colucci, 1997). In cancer, the role of TF in the process of metastasis has been widely documented (Versteeg *et. al.* 2004; Bromberg *et. al.* 1999).

TF levels are known to be upregulated in metastatic cells, suggesting a direct role for this molecule in the establishment of metastatic foci (Mueller *et. al.* 1992). Interestingly, TF deficient tumour cells –by means of an anti-TF antibody or expressing truncated forms of TF– become impaired in their capacity to form distant metastatic nodules (Bromberg *et. al.* 1995; Bromberg *et. al.* 1999; Milsom & Rak, 2008). Moreover, TF expression correlates with the grade of malignancy in certain types of cancer, its upregulation often being associated with advanced cancer stages (Hamada *et. al.* 1996; Milsom & Rak, 2008; Ruf, Yokota & Schaffner, 2010). It is believed that TF on the surface of cancer cells interacts with procoagulant factors in the bloodstream and creates a thrombin coat that would enable such cells to escape immune surveillance (Li *et. al.* 2006). In addition, TF is thought to aid metastatic dissemination by activating certain intracellular pathways that are independent of its habitual procoagulant functions (Bromberg *et. al.* 1995; Bromberg *et. al.* 1999; Palumbo *et. al.* 2007).

It has long been known that deep venous thrombosis and pulmonary embolism often appears as a complication of carcinomas of various stages (Carrier *et. al.* 2008) and its association with TF has been reported in the past (Sato *et. al.* 2006; Callander & Rapaport, 1993; Zacharski, Schned

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Grant Support: Quilmes National University, National Agency of Scientific and Technological Promotion (ANPCyT, Argentina) and Elea Laboratories.

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(Received 25 June 2010; revised 22 September 2010; accepted 16 October 2010)

ISSN 1354-750X print/ISSN 1366-5804 online © 2011 Informa UK, Ltd.
DOI: 10.3109/1354750X.2010.533282

& Sorenson, 1983). Currently, the release of TF-bearing microvesicles of membrane fragments by tumour cells is believed to be responsible for the appearance of thrombosis at sites distant from the primary tumour (Varki, 2007).

Circulating tumour cells from solid tumours are detectable in patient blood circulation and are considered a "real time" biopsy since they represent a surrogate source of tissue. Reverse transcriptase polymerase chain reaction (RT-PCR) is a highly sensitive molecular technique capable of detecting cancer cells gene expression in peripheral circulation, lymph nodes and bone marrow in patients with localized and metastatic disease (Pipinikas *et. al.* 2007). Early detection of minimal disseminated disease (MDD) in cancer patients, although still a matter of debate, could impact the clinical management of the disease in the future. RT-PCR of specific target sequences has been proposed by different authors as an attractive tool to reach this objective in different types of cancers (Vázquez *et. al.* 2009).

Taking together the evidence that states that TF is involved in the establishment of metastasis and in venous thrombosis, and taking into account that MDD can be detected by RT-PCR, our aim was to assess if TF mRNA was a suitable marker for detecting blood-borne cancer cells by means of an RT-PCR/nested PCR strategy. The assay was specifically engineered in such a way that it could encompass as many of the known splice variants of the TF gene as possible, whether membrane-bound or soluble (Chand, Ness & Kisiel, 2006; Bogdanov *et. al.* 2003; Mackman, 2007). Since it has previously been shown that levels of the TF sequence in malignant tissues can be considered a risk factor (Golding-lang *et. al.* 2008), we started the evaluation of TF as a putative marker of circulating cancer cells for the first time.

Methods

Tumour cells. The following cell lines were used in this work: MCF7 (human breast cancer), Skmel-28 (human melanoma), H125 (human lung carcinoma), HeLa (human cervical carcinoma), WERI-Rb-1 and Y79 (both human retinoblastomas). MCF7 cells were used to optimize the nested RT-PCR strategy employed to detect TF in cancer patient samples; non-carcinoma cell lines were used to test the specificity of the method. All cell lines but H125 were maintained in DMEM-F12 culture medium (Invitrogen, Carlsbad, CA, US) supplemented with 10% heat-inactivated foetal calf serum (FBS) and 80 mg/L Gentamicin (Invitrogen, Carlsbad, CA, US). H125 cells were maintained in RPMI medium (Invitrogen, Carlsbad, CA, US) supplemented with 10% FBS and Gentamicin as all others. Passages were performed as often as needed by trypsinization and subsequent dilution of the monolayers.

Human blood samples. Blood samples from 16 stage III breast carcinoma patients were obtained and processed for TF expression. Patients were enrolled in an immunotherapy protocol explained in detail previously (Guthmann *et. al.* 2006) approved by the National Administration for Medication, Food and Medical Technologies (ANMAT, Argentina).The research was carried out in accordance with the declaration of Helsinki (2000) of the World Medical Association. Ethical approval for this study was obtained from the Institute of Oncology Angel H. Roffo Ethics Committee. 5 ml of blood were mixed immediately with 7.5 ml of stabilization buffer (6M guanidinium thiocyanate, 37.5 mM sodium citrate, and 0.75% N-laurylsarcosine). Baseline samples were obtained after surgical treatment and afterwards sampling was performed every 3 months for over a year. Samples were stored at -70°C until RNA extraction. TF expression did not influence the therapeutic decisions or the follow-up schedule.

RNA extraction. RNA from cell lines or blood samples was obtained with Trizol LS Reagent (Invitrogen, Carlsbad, CA, US), following the manufacturer´s instructions. RNA pellets obtained from cell lines were resuspended in 30 µl DEPC-treated H_2O, while those derived from blood samples were resuspended in an equal volume of RNAsecure resuspension solution (Ambion, Austin, TX, US). RNA integrity was assessed by electrophoresis in 2% denaturing agarose gels.

Nested RT-PCR assay. High-affinity primers for the mRNA sequence of the human TF gene (F3, NM_001993) were designed using the *Primer Select* software package (DNAStar Inc, Madison, WI, US). The primers were intended to amplify 6 splice variants of the F3 gene (Chand, Ness & Kisiel, 2006) as well as a soluble form (Bogdanov *et. al.* 2003). Our assay consisted of two PCR rounds: the first one was performed on cDNA obtained after an RT step and the second one was designed as a nested PCR. The primer sequences were: first-round forward primer: 5′-GAA CCC AAA CCC GTC AAT CAA-3′, first-round reverse primer: 5′-AAA ACA TCC CGG AGG CTT AGG-3′; nested forward primer: 5′-GTA CTT GGC ACG GGT CTT CTC-3′, nested reverse primer: 5′-GTT CAT CTT CTA CGG TCA CAT TCA-3′. Both sets of primers amplified specific sequences comprised between exons II and IV of the F3 gene. First-round primers amplified a 365 bp fragment after 60 cycles of amplification with a 56.4°C annealing temperature while nested primers amplified a 182 bp one after 30 cycles at 55.2°C. As a positive control, primers for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used: forward: 5′-GGG GAG CCA AAA GGG TCA TCA TCT-3′; reverse: 5′-GAC GCC TGC TTC ACC ACC TTC TTG-3′. These primers amplified a 457 bp fragment. In order to optimize the nested RT-PCR method we subjected the assay to several test rounds which included testing primer concentration,

annealing temperature, DTT addition, $MgCl_2$ concentration and RNAse inhibitor addition. Optimized RT-PCR reactions were performed using the *Ready-to-Go RT-PCR Beads* kit (GE Healthcare, UK) and consisted of variable RNA amounts (RNA from blood samples was resuspended in a 30 µl volume, 25 µl were used as template for TF and 5 µl for GAPDH amplification), 0.5 µg $pd(N)_6$ as first-strand primer (provided with the kit), 24 pmol of each PCR primer and 2.5 mM $MgCl_2$ while nested PCR reactions were performed with *Platinum PCR Supermix* (Invitrogen, Carlsbad, CA, US) and consisted of 25 µl supermix, 12 pmol of each nested primer and 1 µl of the RT-PCR mixture as template. PCR products were analyzed by electrophoresis on 2% agarose gels which were later stained in TBE buffer supplemented with 0.5 µg/ml ethidium bromide.

Sensitivity and specificity of the Nested RT-PCR assay. The sensitivity of the assay was determined by using five-fold serial dilutions of total RNA from MCF7 cells (5 ng – 1.5 pg) and assessing the presence/absence of bands for either the RT-PCR or the nested rounds. Specificity was ascertained by using 5 ng total RNA from different human cell lines (SKmel, H125, HeLa, Y79 and WERI-Rb-1).

Cell spiking assay. 2.5 ml of blood from a TF-negative healthy donor (1 ml blood + 1.5 ml stabilization buffer) were mixed with different numbers of MCF7 cells (10^6, 10^5, 10^4, 100, or 10) resuspended in 100 µl D-MEM. For the negative control, 100 µl cell-free D-MEM was added to the blood sample. After mixing by inversion, blood samples were subjected to RNA extraction as previously described. RNA was stored at -70ºC until used for RT-PCR.

Indirect Immunoperoxidase Staining Assay. 7.5×10^5 cells (H125, Skmel or MCF7) were seeded onto a 12 well plate, each well containing a sterile round glass cover. After reaching the desired confluence (~ 70%), cells were fixed for 1 h at room temperature in a 3% paraformaldehyde / PBS solution. Subsequently, monolayers were stained with the Vectastain kit (Vector Laboratories, Burlingame, CA, US) according to the manufacturer's instructions. 100 µl/ml of a mouse anti-Human Tissue Factor IgG mAb (USBiological, Swampscott, MA, US) was used as primary antibody. Monolayers were counterstained with hematoxylin.

Statistical Analysis. Patient samples were grouped according to TF load after subjecting them to our nested RT-PCR design: samples which turned out positive for both the RT-PCR and nested PCR were considered as having a high TF load, while those which were positive only for nested PCR or those that remained negative throughout the whole assay were considered as having low/null TF load. When having more than one sample belonging to the same patient, the highest TF load score obtained was assigned to that particular patient. TF load results for each patient, either during the whole time span or the first 6 months since their enrollment, were then analyzed with MedCalc software (MedCalc software bvba, Belgium). Time to progression was represented by a Kaplan-Meier curve. Statistical data was obtained with a univariate Cox-Mantel test. Total follow-up lasted 36 months.

Results

The sensitivity of the amplification design was first assessed with serially diluted amounts of MCF7 RNA. TF mRNA was detected when using up to 40 pg of total RNA during the RT-PCR stage and this sensibility was increased to 1.5 pg with the nested PCR (Figure 1A). We also measured sensitivity by means of a cell spiking assay (Figure 1B). In this case, the TF transcript could be detected from a minimum of 1×10^5 cells during the RT-PCR stage while in the nested step 10 cells became the detection limit. No amplification was observed in the negative control. The specificity of the method was also assessed by subjecting RNA from different cell lines to the optimized assay. TF transcript was found in all carcinoma cell lines tested (MCF7, H125 and HeLa) as well as the ones corresponding to human retinoblastomas (WERI-Rb-1 and Y79). No TF expression was found in the human melanoma cell line Skmel (Figure 2).

Expression of the TF protein was determined by means of an indirect immunoperoxidase staining assay using an anti-TF antibody on three cell lines tested for TF mRNA expression (Figure 3). Results were concomitant with those obtained with the nested RT-PCR assay: cell lines MCF7 and H125 were positive for TF antigen while Skmel cells showed no TF protein expression.

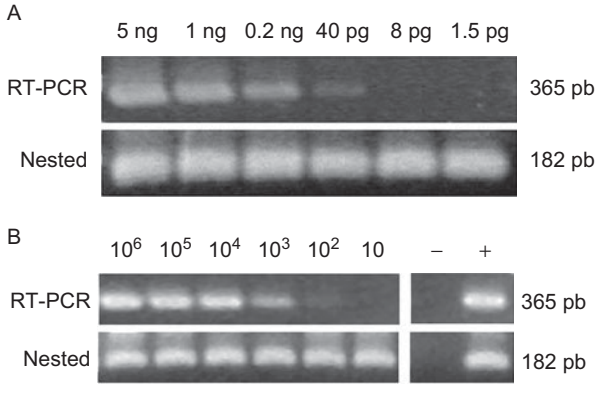

Figure 1. (A) *In vitro* sensitivity of the RT-PCR assay. Detection of human TF mRNA in serially decreasing amounts of total RNA from MCF7 mammary carcinoma cells by means of a single PCR step or nested PCR. (B) Cell spiking assay. 1 ml blood aliquots were mixed with stated numbers of MCF7 cells. -: Blood without cells, +: 5 ng RNA from *in vitro* cultured MCF7 cells. In all cases, RNA integrity was assessed by amplification with specific primers for the human GAPDH mRNA (not shown).

Afterwards we tested blood samples from 16 advanced breast cancer patients for TF expression in order to preliminarily examine its value as a marker for detecting blood-borne cancer cells. It can be interpreted that those patients whose samples resulted positive for both PCR rounds had a higher starting amount of the TF transcript than those that were positive for just the nested step. Taking this into account, we regarded those samples being positive for both the RT-PCR and the nested round as having a high TF load, while those who were only positive for the nested round and those negative for the whole assay were regarded as having low or null TF loads, respectively. 31.3 % (5/16) of the patients had a high TF load and the remaining 68.7 % (11/16) had a low/null TF load.

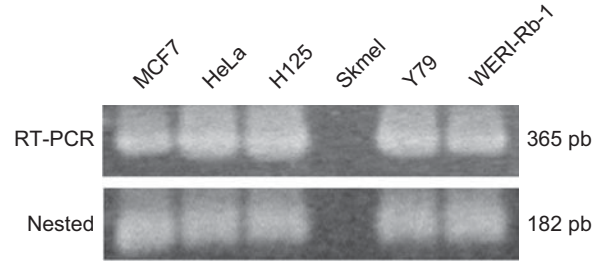

Figure 2. *In vitro* specificity of the RT-PCR assay by detection of TF mRNA in different human cell lines: HeLa, cervical carcinoma; H125, lung carcinoma; Skmel melanoma; Y79 and WERI-RB1, retinoblastomas. In all cases, RNA integrity was assessed by amplification with specific primers for the human GAPDH mRNA (not shown).

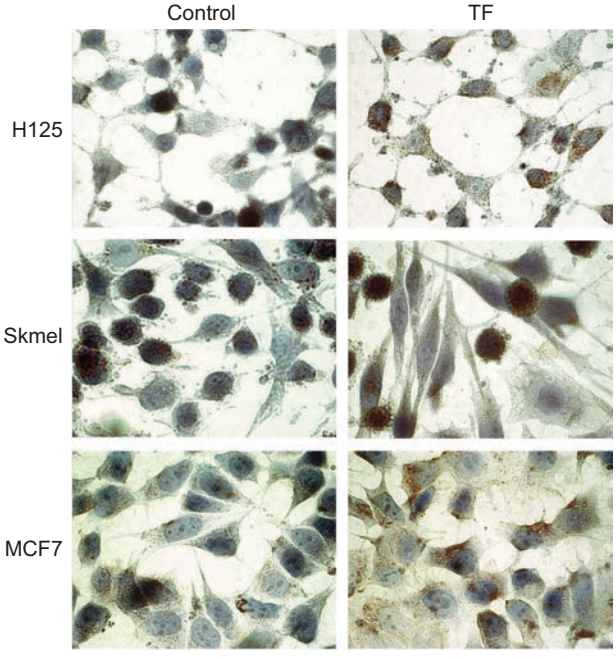

Figure 3. Indirect immunoperoxidase staining of TF antigen on H125, Skmel and MCF7 monolayers, using a mouse anti-Human IgG mAb as primary antibody (100 µg/ml).

We then correlated our PCR results with the follow-up information of each of the 16 patients obtained during a 36 month span. 4/5 (80 %) patients with high TF load showed signs of disease relapse while only one remained disease-free. On the other hand, of those patients who had low/null TF load, 5/11 (45.4%) relapsed while 6/11 (54.5%) showed no signs of disease when the follow-up schedule ended. There was a significant difference in the number of deceased patients between high and low/null TF load groups; 80% (4/5) and 18% (2/11), respectively (Table I). It is noteworthy that these results showed no correlation with the patients´ ER status (Data not shown). Figure 4) depicts time to progression by means of two Kaplan-Meier survival curves; the first one considering TF load determination throughout the complete duration of the follow-up scheme (36 months, figure 4A) and the second considering only the first 6 months since patient enrollment in the clinical trial (figure 4B). While none of the curves showed a significant difference between high and low/null TF load groups, it is interesting to note that when analyzing the first 6 months of trial the difference between groups becomes much more evident, showing certain level of discrimination that should be further evaluated. ($p = 0.564$ vs. $p = 0.053$, Cox-Mantel Test). The mean value of the time to progression was 5 months (95% Cl -1.0-36.0) for the high TF load group and 20 months (95% Cl 1.0-9.0) for the low/null TF load group.

Discussion

TF, an integral plasma membrane glycoprotein, was first described in the sixties as being one of the main molecules involved in triggering the coagulation cascade. It activates both the intrinsic and extrinsic pathways (Mackman, 2009). Given its procoagulant properties, TF is known to play a predominant role in guarding hemostasis, process by which the body responds rapidly to vascular injury to prevent blood loss (Breitenstein, Tanner & Lüscher, 2010).

For playing such an important part in maintaining vascular integrity, TF expression and function are both highly regulated. TF has been reported to be constitutively

Table 1. Patient evolution related to TF load status.

	High TF load, n (%) (n = 5)	Low/null TF load, n (%) (n = 11)
Disease-free	1 (20%)	6 (54%)
Progressed	4 (80%)	5 (45%)
Alive	1 (20%)	9 (82%)
Deceased	4 (80%)	2 (18%)*

High TF load: patients whose samples were positive for both the first PCR round and the nested PCR. Low/null TF load: patients whose samples were only positive in the nested PCR step or were negative throughout the whole assay. * $p<0.005$ vs. deceased in high TF load, Fisher exact test.

expressed by vascular smooth muscle cells, epidermal cells and cells which form the adventitial layer of blood large vessels (Drake, Morrissey & Edgington, 1989) but it is virtually absent in cells directly exposed to flowing blood; generating an "hemostatic envelope" that protects blood from clotting when it is not needed. Nevertheless, monocytes, endothelial cells and macrophages express TF at very low levels which can be augmented by stimulation with cytokines (Rivers, Hathaway & Weston 1975; Osterud & Bjørklid 1982).

Our interest in TF as a molecular marker is centred around several factors. Not only its use as a means of detecting circulating cells is novel but its direct implication in the metastatic dissemination process may render it suitable for its use as a prognostic factor, more so since its expression has been directly associated with poor outcome in several types of cancer. One of the most interesting aims of such a marker is the possibility of detecting circulating cells before the establishment of macroscopic metastatic foci, thus allowing treatment to be administered in individuals who are regarded as being clinically healthy but at high risk of recurrence.

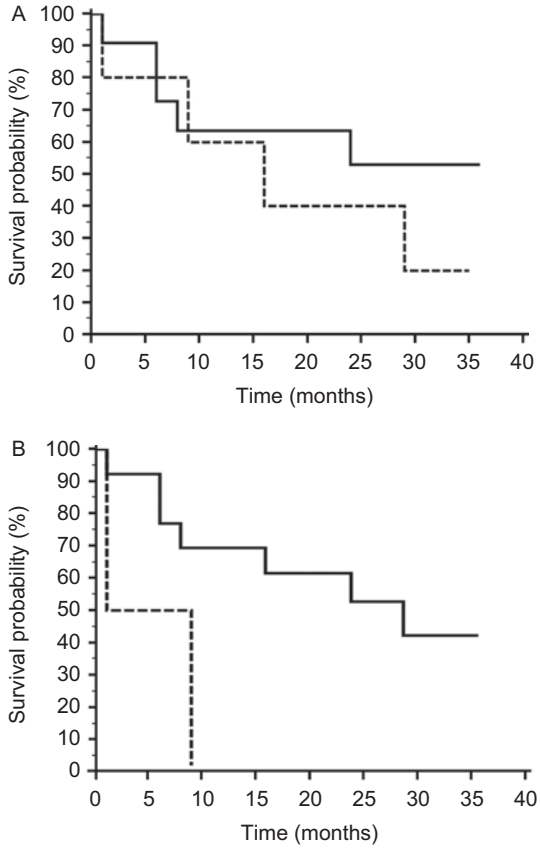

Figure 4. Kaplan-Meier progression curves. High TF load: dashed lines; low/null TF load: bold lines. The progression percentage is depicted in the Y-axis while time to progression in months is represented in the X-axis. TF load determination was done (A) throughout the complete duration of the follow-up scheme ($p = 0.5644$, Cox-Mantel test) or (B) within first 6 months since patient enrollment in the clinical trial ($p = 0.0536$, Cox-mantel test).

In the present work, we have successfully established a sensitive nested RT-PCR assay for detecting TF mRNA. In its design, we took into account the fact that many splice variants of the F3 gene have been reported (Chand, Ness & Kisiel, 2006) as well as a soluble variant of TF (Bogdanov *et. al.* 2003). Since these variants are generated by alternative splicing of exons I or V, we decided to design our primers over exons II and IV, since they seem to be common to all of them. Our optimized method detects up to 10 malignant cells per ml of whole blood or up to 1.5 pg total RNA *in vitro* and results indicate that this method can be used on frozen blood samples, which makes it suitable for cases in which samples need to be stored for long periods of time.

We could also correlate the results from the nested RT-PCR assay with protein expression using a specific monoclonal antibody against TF in some of the cell lines tested for TF expression, thus proving that the nested RT-PCR assay is indeed specific for the TF transcript.

After subjecting patient samples to our optimized method, we found that almost all patients that had a high TF load had signs of relapsed disease and all of these were deceased at the end of the follow-up period. This could suggest that high TF load and patient outcome could be indeed related. This relationship proved to be somewhat clearer when taking into account only the first 6 months since patient enrolment in a clinical trial, which states that there is a possibility that TF′s usefulness as a prognostic marker could depend on the time lapse when its expression is measured. It is important to point out that there was no difference or any imbalance in treatment between patients which then turned out to have high or low/null TF loads. Though these results were performed on a small cohort of patients, taken together with data obtained with *in* vitro assays they suggest that TF has the potential of becoming a prognostic factor. This conclusion is also supported by previous reports which state its association with poor outcome. Nevertheless, further studies are needed in order to establish if TF can undoubtedly be regarded as a prognostic marker and its scope.

In inflammation or in pathological conditions such as atherosclerosis, sepsis and cancer, TF expression within the blood compartment is no longer maintained at low levels, rapidly generating intravascular thrombosis (Wilcox *et. al.* 1989; Levi *et. al.* 2003; Levi, van der Poll & ten Cate, 2006; Mackman, 2009). In cancer, participation of TF has been widely documented with different processes that contribute to malignancy such as angiogenesis (Belting *et. al.* 2004) and metastatic spread (Mueller *et. al.* 1992; Versteeg *et. al.* 2004; Bromberg *et. al.* 1999; Sawada *et. al.* 1999). Moreover, idiopathic venous thrombosis are quite common in several types of cancer, process in

which TF is thought to be involved (Rickles & Levine, 2001; Boccaccio & Comoglio, 2005).

Since TF expression has been reported on inflammatory settings, next we want to explore if our results are associated with this kind of process or not. For that, we wish to adapt our method to a quantitative real-time PCR platform which would allow us to set a cut-off value that could discriminate between inflammatory and cancer-related TF levels. This swap in technologies for detecting TF in blood samples is currently being undertaken in our laboratory.

In conclusion, we have designed a TF detection method that is both novel and simple; its novelty relies on the choice of marker and in its robust design while its simplicity is based on the type of technology used. TF has previously been the target of certain detection strategies (Kirschmann *et. al.* 1999; Mueller *et. al.* 1992; Sawada *et. al.* 1999; Koomägi & Volm, 1998; Bromberg *et. al.* 1999; Adamson *et. al.* 1994), but it has never been used as a biomarker for a PCR-based method designed for frozen blood samples. Regarding the simplicity of our design, it is known that nested RT-PCR assays are routinely performed on many laboratories and its effectiveness as a cancer cell-detecting means has already been proved (Gabri *et. al.* 2008; Jółkowska, Derwich & Dawidowska, 2007; Gilbey *et. al.* 2004), thus making it a suitable procedure for any group that wishes to reproduce or further investigate our results.

This is the first report in which TF is used as a potential marker of cancer circulating cells and the results obtained so far. Nonetheless, we are aware that further studies are needed to clarify the variability that we may encounter in healthy people, patients with other diseases and establish their respective cut-off values.

Acknowledgements

We would like to thank Dr. Marcelo Guthmann for patient data mining. M.R. Gabri, D.E. Gomez and D.F. Alonso are members and L.L. Otero is a fellow of CONICET. This work was supported by Quilmes National University, Elea Laboratories and a grant from ANPCYT (Argentina).

Declaration of interest

The authors report no declarations of interest.

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