Interplay between cancer and thrombosis; identification of key factors

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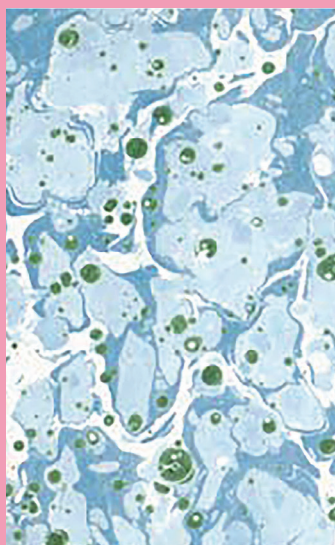

Chapter 6

Genes associated with venous thromboembolism in colorectal cancer patients

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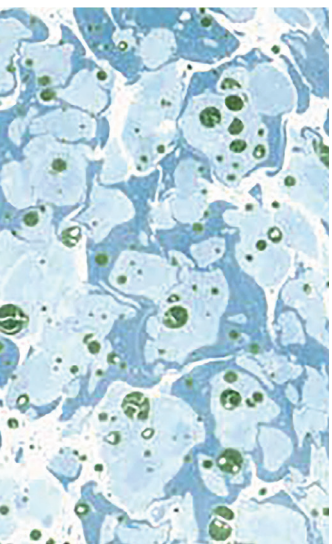

Essentials

- The underlying pathophysiological mechanisms behind cancer-associated thrombosis are unknown.
- We compared expression profiles in tumor cells from patients with and without thrombosis.
- Tumors from patients with thrombosis showed significant differential gene expression profiles.
- Patients with thrombosis had a pro-inflammatory status and increased fibrin levels in the tumor.

ABSTRACT

Background: Venous thromboembolism (VTE) is a frequent complication in patients with cancer and is associated with significant morbidity and mortality. However, the mechanisms behind cancer-associated thrombosis are still incompletely understood.

Objectives: The aim is to identify novel genes that associate with VTE in patients with colorectal cancer (CRC).

Methods: Twelve CRC patients with VTE were age- and sex-matched to twelve CRC patients without VTE. Tumor cells were isolated from surgical samples using Laser Capture Microdissection approaches and mRNA profiles were measured with next generation RNA sequencing.

Results: This approach led to the identification of new genes and pathways that might contribute to VTE in CRC patients. Ingenuity Pathway Analysis indicated significant links with inflammation, methionine degradation pathway and increased platelet function, which are all key processes in thrombus formation. Tumor samples of patients with VTE had a pro-inflammatory status and contained increased fibrin and fibrin degradation products compared to samples of those without VTE.

Conclusion: This case-control study provides proof-of-principle that tumor gene expression can discriminate between cancer patients with low and high risk of VTE. These findings may help further unravel the pathogenesis of cancer-related VTE. The identified genes could potentially be used as candidate biomarkers to select high risk colorectal cancer patients for thromboprophylaxis.

Keywords: case-control studies, colorectal cancer, homocysteine, inflammation, RNA sequencing

INTRODUCTION

Venous thromboembolism (VTE) is a frequent complication in patients with cancer and is associated with substantial morbidity and mortality. Cancer patients have a 7-fold higher risk of VTE compared to those without cancer. It has been estimated that 20% of all VTE events are related to cancer [1, 2]. Although the relation between cancer and thrombosis is well established, the underlying pathophysiological mechanisms are still incompletely understood. Many clinical and patient-related factors are known to contribute to the risk of VTE in cancer patients, such as high age, genetic disposition, immobility and prior history of VTE [3, 4]. However, the VTE risk in these patients appears to be mainly driven by cancer-related factors, such as high tumor grade, advanced disease stage, antineoplastic therapies and tumor type. Tumor types are often classified into high (pancreas, brain), moderate (colon, lung) and low (breast, prostate) VTE risk groups [5]. Furthermore, cancer treatment affects the risk of VTE after surgery and chemotherapy, by 2-fold and 6-fold, respectively [3, 6].

Biomarkers hold promise for risk stratification. Knowledge about the pathophysiological mechanism underlying the pro-thrombotic state in cancer patients could identify new candidate biomarkers. For example, high procoagulant activity of circulating tumor cells and tumor-derived extracellular vesicles (EVs) that expose tissue factor (TF) – the primary initiator of blood coagulation - has been associated with VTE, although this finding has been questioned by others [7, 8]. Other coagulation factors, such as factor VII – the protease that binds TF to start coagulation - can also be upregulated in tumors [8], potentially contributing to development of cancer-associated thrombosis. Finally, neutrophil extracellular traps (NETs) have also been proposed to contribute to cancer-associated thrombosis (CAT) [9], although, such a role for NETs in cancer patients has not yet been firmly established. Taken together, this indicates that, the biological factors that contribute to cancer-associated thrombosis remain largely unidentified.

On average, cancer patients with VTE have a 5-fold increased risk of death compared to those without VTE [4]. After cancer itself, thrombosis is the second cause of death in these patients [10]. Despite the high risk of VTE in cancer patients, routine thromboprophylaxis in outpatients is not recommended, since it increases the risk of (fatal) major bleeding leading to an unfavorable risk-benefit ratio when applied in all ambulant cancer patients. Selection of high risk patients may guide decisions about thromboprophylaxis, but current prediction scores appear to perform poorly and are therefore infrequently used. Hence, it is essential

to be able to predict which patients will develop VTE and who will benefit from prophylactic anticoagulants.

The aim of this study was to identify tumor-expressed genes that associate with VTE in cancer patients and may be used as novel biomarkers for cancer-associated thrombosis. This study focused on colorectal cancer (CRC), because the prevalence of CRC is high, while it associated with a moderate to high risk of VTE. We isolated mRNA of tumor cells with Laser-Capture Microdissection (LCM) method after which a gene expression profile was determined via Next Generation RNAsequencing. This enabled us to study gene expression exclusively in tumor cells, but not the stromal compartment. Finally, we identified biological processes associated with CAT using Ingenuity Pathway analysis.

METHODS

Patient cohort

With a cohort of unselected patients who underwent curative or palliative surgery for CRC at the Slotervaartziekenhuis (Amsterdam, The Netherlands) between January 2008 and August 2013, a total of 206 patients were identified, of whom 19 (9.2%) patients were diagnosed with objectively confirmed VTE. As we defined cancer-associated thrombosis as VTE occurring within one year before or after CRC diagnosis, and based on the availability of snap-frozen colorectal tumor specimens, we excluded 5 patients. A case-control study was performed on the remaining twelve patients with confirmed VTE and patients without VTE that were individually matched on sex, age and tumor type. Additionally, sub-group analyses were performed based on the timing of diagnosis of VTE; VTE prior to CRC diagnosis (defined as VTE max. 12 months before; 4 patient couples) and VTE around the time of CRC diagnosis (defined as VTE max. 3 months before or 3 months after cancer diagnosis; 5 patient couples) (Fig. 1). Of note, because of these criteria patient couple 1 was included in both groups. Also, based on these criteria, patient couples 9-12 were excluded from these subgroup analyses. Furthermore, in this cohort patients were included with a first VTE which was at least five years before the second (cancer-associated) VTE.

RNAsequencing

Tumor cells were isolated from tumor specimens based on morphological differences between tumor cells and stromal cells, using the Laser-Capture Microdissection (LCM) meth-

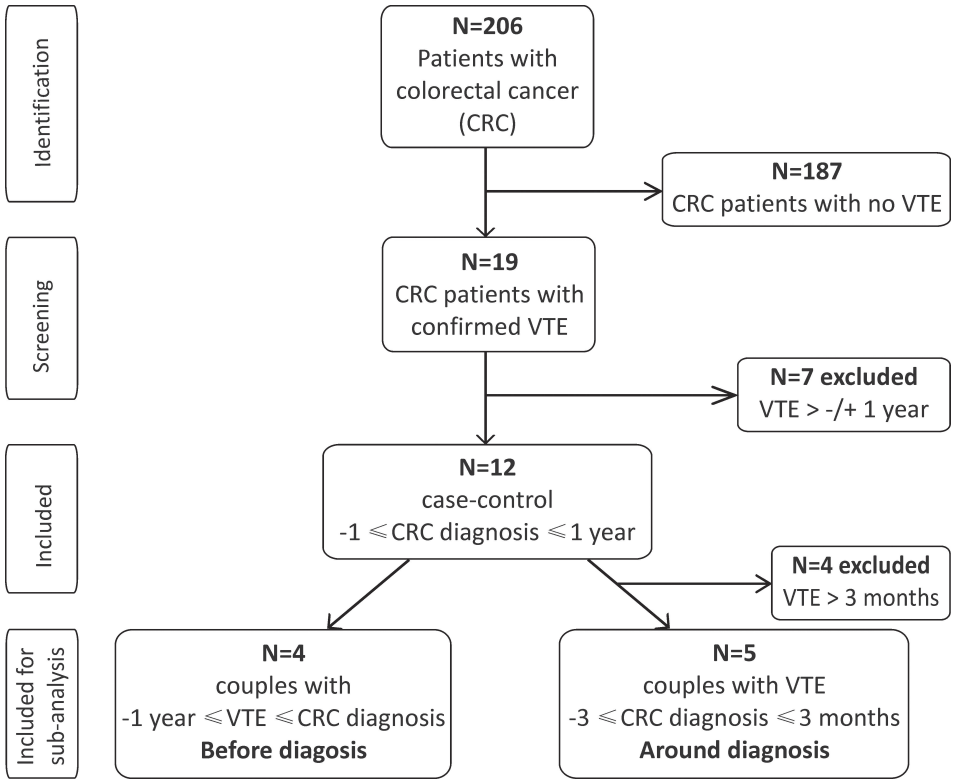

Figure 1 Design and inclusion flowchart of the study.

CRC, colorectal; VTE, venous thromboembolism.

od. Briefly, 7 µm sections were mounted on Membrane Slide NF 1.0 PEN glasses, rehydrated, hematoxylin treated to stain nuclei, washed in 1% ammonia and finally dehydrated. Sections were processed on the PALM MicroBeam (Zeiss) with a UV laser and tumor cells were collected into adhesive clear cap tubes (Zeiss). To prevent RNA degradation, tumor slices were subjected to LCM within 60 min after thawing as our pilot experiments showed that RNA is stable within a 60 min time frame-, and isolated tissue was snap-frozen. LCM dissected tissue from each patient was pooled and RNA was isolated with the NucleoSpin RNA XS kit (Magery-Nagel) according to manufacturer's instructions.

A Fragment Analyzer was used to assess the quality of the mRNA samples before RNAseq was started. The NEBNext Ultra Directional RNA library Prep Kit for Illumina was used to process the isolated RNA, after which clustering and RNA sequencing with the Illumina and

NextSeq 2500 was performed according to manufacturer's protocols at GenomeScan B.V. (Leiden, The Netherlands).

Analysis of RNA sequencing data

All RNA sequence files were processed using the BIOPET Gentrap pipeline version 0.5 developed at the LUMC (http://biopet-docs.readthedocs.io/en/latest/releasenotes/release_notes_0.5.0/). The BIOPET Gentrap pipeline consists of FASTQ preprocessing (including quality control, quality trimming and adapter clipping), read alignment, read and base quantification, and optionally transcript assembly. FastQC version 0.11.2 was used for raw read quality control. Low quality read trimming was done using sickle version 1.33 with default settings. Cutadapt version 1.9.1 with default settings was used for adapter clipping based on the detected adapter sequences by FastQC toolkit. The reads were aligned against the human reference genome GRCh38 using RNAseq aligner GSNAP version 2014-12-23 with settings "--npaths 1 --quiet-if-excessive". GENCODE genome annotation version 20 was used for raw read counting. This gene read quantification step was performed using htseq-count version 0.6.1p1 with settings of "--stranded=no". After TMM normalization, the differential gene expression analysis was performed with edgeR 3.14.0 using a model with the VTE effect and effects of matched patient-control pairs (http://bioconductor.org/packages/release/bioc/html/edgeR.html) [11]. IPA core analysis (Ingenuity Systems, Inc) was performed on the top 10 genes to identify the most significant pathways and (upstream) regulators.

Quantitative real-time PCR

Selected up- or down-regulated gene expression levels were validated by real-time PCR. In brief, RNA from snap-frozen tissues was isolated with Machery-Nagel kits. Reverse transcription was performed with Superscript II (Thermo Fisher scientific) and gene expression was performed using SYBR Select (Life Technologies) on a CFX284 Touch real-time PCR detection system (BioRad). See supplementary table 2 for primer sequences.

Western Blotting

For detection of fibrin deposition in tumors, samples were processed as described elsewhere [12]. Briefly, equal concentration of protein lysates were loaded on 4-12% Bis-Tris Plus Gels (Thermo Fischer Scientific) and separated for 18 min at 200V; blotted on 0.2 µm pore PVDF membranes and blocked for 1h in 5% milk-TBST. Fibrin was detected with mAb 59D8 antibody (a kind gift from prof. dr. Charles Esmon; Oklahoma, USA) and the primary antibody was incubated O/N at 4°C. After multiple TBST washing steps, membranes were incubated

Table 1

Characteristics of the twelve CRC patients with VTE and their individually matched patient controls on sex and age.

Couple	Sex	Tumor site	TNM stage	Age	VTE pre/ post diagnosis	days VTE diagnosis	VTE pre/ post surgery	days VTE surgery	VTE type	Type of therapy	days start chemo-therapy	History VTE	Years VTE before diagnosis	Hb levels	Leukocyte counts	Platelet counts	D-dimer
1	M	ascending colon	T3N0M0	76.35						-	-	no	-	9.7	30.1	583	-
			T3N0M0	76.41	pre	-13	pre	-104	PE	-	-	no	-	6.0	16.3	388	1032
2	M	ascending colon	T3N1M0	57.87						chemo	72	no	-	6.9	7.5	243	-
			T3N2aM0	55.76	pre	-319	pre	-349	PE	chemo	77	yes	13	5.8	6.9	291	618
3	M	rectosigmoid	T2N0M0	67.41						-	-	no	-	9.6	11.1	311	-
			T3N0M0	65.76	pre	-143	pre	-198	PE	-	-	yes	5	8.8	8.1	288	-
4	F	ascending colon	T3N0M0	83.34						-	-	no	-	9.1	6.2	184	-
			T3N0M0	83.04	pre	-263	pre	-288	DVT	-	-	no	-	6.9	4.8	319	-
5	M	rectum	T3N1M0	72.47						chemo	84	no	-	9.9	-	-	-
			T3N0M0	72.76	post	48	pre	-84	DVT	pre-surgery chemoradiation	-10	no	-	5.9	6.0	-	-
6	M	rectosigmoid	T3N2M0	66.32						chemo	62	no	-	9.4	10.5	-	-
			T1N0M0	62.36	post	45	post	4	Fatal PE	pre-surgery chemoradiation	unknown	no	-	10.2	-	223	>10000
7	M	descending colon	T2N1M0	60.89						chemo	54	no	-	9.1	7.9	253	-
			T3N0M0	58.93	post	12	post	4	PE	chemo	49	no	-	8.1	13.4	286	-
8	F	ascending colon	T3N0M0	77.54						-	-	no	-	8.3	11.5	355	-
			T3N1bM0	77.36	post	35	post	3	PE	-	-	no	-	6.8	8.0	220	-
9	M	ascending colon	T3N0M0	51.96						pre-surgery chemoradiation	unknown	no	-	5.2	-	275	-
			T3N2bM1	55.18	post	799	post	763	DVT	chemo	77	no	-	7.0	10.1	303	>10000
10	M	rectosigmoid	T3-4N0M0	78.11						-	-	no	-	7.6	-	-	-
			T3N2bM1	77.59	post	415	post	391	DVT	chemo	269	yes	45	7.9	5.4	145	6431
11	F	ascending colon	T3N0M0	85.32						-	-	yes	18	7.9	9.2	499	-
			T3N2bM1	93.19	post	208	post	209	DVT	-	-	yes	7	9.0	16.1	133	-
12	F	transvering colon	T4N0M1	68.61						chemo	196	no	-	7.1	11.1	432	-
			T3N0M0	68.37	post	320	post	305	DVT	chemo	264	yes	7	8.1	5.8	227	-

M, male; F, female; VTE, venous thromboembolism; T = tumor, N = lymph node, M = metastasis; Hb, hemoglobin is in mmol/L; Leukocyte and platelet counts are $*10^9$/L; D-dimer levels are in ng/mL; -, unknown values.

with an HRP-conjugated secondary antibody (Abcam) for 1h at RT. Antigens were visualized with Western lightning Plus ECL (Perkin-Elmer) using the ChemiDoc imaging system (BioRad).

RESULTS

Mean age of the selected cohort with twelve patient couples was 71 years and 66 % of the patients were male (Table 1). The distribution of DVT and PE was similar within this case-control study. No clear trends were observed in pre-surgery measured hemoglobin levels, leukocyte and platelet counts in patients with VTE compared to their control patients.

Next-generation RNA-sequencing analysis of tumor cells showed no significant differential gene expression in tumors of the selected twelve patients with VTE compared to CRC patients without VTE (data not shown). However, when the group was separated according to the timing of the VTE in relation to the cancer diagnosis - VTE before, and VTE around the time of CRC diagnosis (maximum -/+ 3 months) - different gene profiles were observed. Analysis of the first group revealed 20 genes that were significantly up- or down-regulated in cancer cells from patients with VTE compared to their controls (Table 2, left panel). After correction for multiple testing, four genes remained significantly differently expressed. Differential gene expression analysis of patients with VTE around CRC diagnosis (-/+3 months) showed a panel of 30 significant differently-expressed genes, which remained after adjustment for multiple testing (Table 2 right panel, and Supplementary Table 1).

Differentially expressed genes within these two selected patient groups are associated with a broad and diffuse set of biological pathways. Therefore, to narrow down relevant pathways and upstream regulators, we selected the top 10 differently expressed genes and performed core analysis using Ingenuity Pathway Analysis (IPA) Software. IPA is a web-based set of algorithms that predict upstream and downstream cellular processes, diseases and signaling pathways associated with the observed gene expression profile. The predictions are based on comparisons with previously performed gene expression analyses. A summary of the results are listed in Table 3. Multiple top canonical pathways and upstream regulators were identified, such as the coagulation pathway, methionine degradation and inflammation pathways via liver- and retinoid X receptor (LXR/RXR) pathway and interferons.

Significant upregulation of the genes ***SPINK4, SERPINA1*** and ***REG4*** in samples derived from patients with VTE pre-CRC diagnosis was confirmed using quantitative-PCR, except for patient couple 4 (Fig. 2A-C). ***SPINK4*** and ***SERPINA1*** (Fig. 2A, B) were also increased in at least 60% of the patients with a VTE around CRC diagnosis. ***REG4*** transcript levels were also elevated in the 'VTE around diagnosis' patient couples (Fig. 2C), except for couple 6. ***XKR9,*** a gene identified in the 'VTE around CRC diagnosis' group, was indeed increased in couples 1, 6 and 7 by at least 3-fold (Fig. 2D), while down regulated expression was found in patients with VTE prior to CRC diagnosis. Both ***SORBS1*** and ***NES*** were at least 2-fold downregulated in all patient couples (Fig. 2E, F). Additionally, expression of the tissue factor gene (***F3)*** was increased in most patients who developed VTE before cancer diagnosis (Fig. 3A), however, whether ***F3*** was also upregulated in the patients with VTE around CRC diagnosis remained inconclusive.

Table 2

Top 10 differently-expressed genes in tumor cells from patients with VTE before CRC diagnosis (left) and VTE around CRC diagnosis (right), compared to their controls. Genes, average log2^fold change, p-values and adjusted p-values are listed.

Before diagnosis				Around diagnosis			
Genes	*AvgLog2FC*	*p-value*	*adjusted p-value*	*Genes*	*AvgLog2FC*	*p-value*	*adjusted p-value*
REG4	7,32	7,02E-14	1,18E-09	*GBP4*	3,88	1,92E-11	3,07E-07
SPINK4	6,67	1,93E-09	1,63E-05	*XKR9*	6,21	1,35E-10	1,08E-06
SERPINA1	6,84	9,71E-08	5,45E-04	*CTSE*	7,21	3,50E-10	1,87E-06
SLITRK6	4,05	1,53E-06	6,44E-03	*AHCYL2*	2,77	6,37E-09	2,55E-05
SBSPON	4,17	2,52E-05	8,49E-02	*GRM8*	-5,05	8,63E-09	2,77E-05
DEFA5	4,32	4,02E-05	1,13E-01	*REG4*	5,50	5,56E-08	1,49E-04
KLHL32	3,04	4,87E-05	1,13E-01	*PTPRR*	4,75	2,21E-07	5,07E-04
LPL	3,83	5,37E-05	1,13E-01	*PIGR*	7,34	1,04E-06	1,94E-03
NTRK2	-4,31	8,52E-05	1,53E-01	*SORBS1*	-2,31	1,09E-06	1,94E-03
RNF217	4,89	9,59E-05	1,53E-01	*SAMD9*	2,37	1,44E-06	2,30E-03

Since inflammation was one of the top canonical pathways identified by IPA, we questioned if a pro-inflammatory status was present in these tumor specimens. Therefore we investigated the levels of CCL2, a key chemokine that regulates migration and infiltration

Table 3

Summary of IPA analysis on the list of top 10 differentially expressed genes in patients with VTE before and around CRC diagnosis. Table shows top IPA canonical pathways, diseases, molecular functions and upstream regulators.

Before diagnosis		Around diagnosis	
Top canonical pathways		***Top canonical pathways***	
	p-value		p-value
LXR/RXR activation	1,39E-03	Methionine degradation I (to Homocysteine)	9,79E-03
FXR/RXR activation	1,51E-03	Cysteine biosynthesis III	1,07E-02
Atherosclerosis signaling	1,53E-03	Superpathway of Methionine degradation	1,67E-02
Coagulation system	1,63E-02	Glutamate receptor signaling	2,64E-02
Thyroid cancer signaling	1,86E-02	Autophagy	2,86E-02
Diseases and disorders		***Diseases and disorders***	
	p-value range		p-value range
Auditory disease	1,63E-2 - 2,95E-6	Cancer	4,95E-2 - 1,64E-5
Neurological disease	4,67E-2 - 2,95E-6	Dermatological diseases and conditions	4,90E-2 - 1,64E-4
Organismal Injury and abnormalities	4,85E-2 - 2,95E-6	Organismal injury and abnormalities	1,95E-2 - 1,64E-4
Cancer	4,76E-2 - 3,94E-4	Connective tissue disorders	4,68E-4 - 4,68E-4
Developmental disorder	3,77E-2 - 4,68E-4	Developmental disorder	4,76E-2 - 4,68E-4
Molecular and cellular functions		***Molecular and cellular functions***	
	p-value range		p-value range
Cell morphology	4,04E-2 - 1,08E-5	Cell-to-cell signaling and interaction	4,85E-2 - 4,68E-4
cellular movement	4,80E-2 - 1,08E-5	Cellular function and maintenance	3,37E-2 - 4,68E-4
cell death and survival	4,67E-2 - 4,68E-4	Cellular movement	4,63E-2 - 4,68E-4
Cell-to-cell signaling and interaction	3,86E-2 - 4,68E-4	Protein synthesis	4,00E-2 - 8,96E-4
Cellular assembly and organization	4,45E-2 - 4,68E-4	Cell signaling	1,87E-3 - 1,87E-3
Top Upstream regulators		***Top Upstream regulators***	
	p-value of overlap		p-value of overlap
TCF	7,84E-04	*IFNG*	2,25E-03
SLC9A6	9,32E-04	*ADAM9*	2,33E-03
RRAD	9,32E-04	*BICC1*	2,79E-03
miR-185-3p	9,32E-04	Metribolone	4,04E-03
Oleylamide	9,32E-04	*IFNA2*	5,03E-03

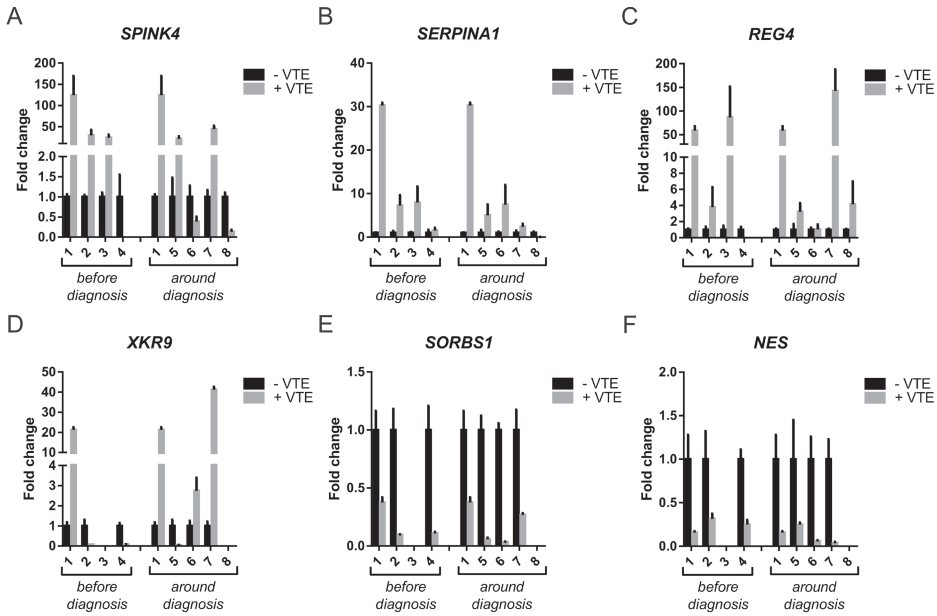

Figure 2 Validation of differentially expressed genes with a quantitative-PCR.

Fold change of mRNA expression of *SPINK4* (**A**), *SERPINA1* (**B**), *REG4* (**C**), *XKR9* (**D**), *SORBS1* (**E**), and *NES* (**F**) from patients with VTE (grey bars) normalized to their control patients (black bars). Data are shown as the mean ± SD.

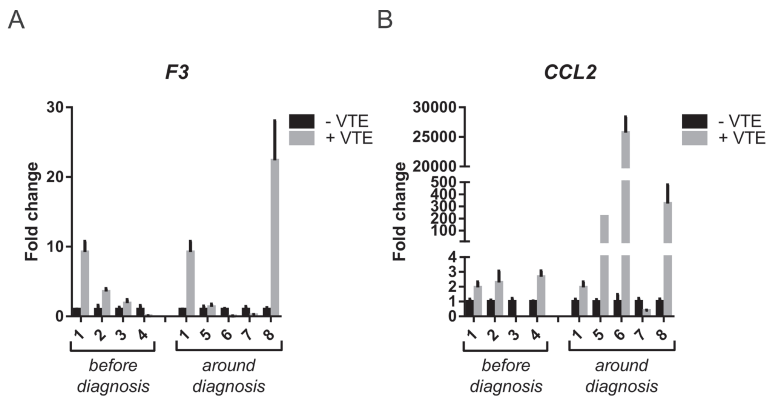

Figure 3 Assessment of tissue factor and *CCL2* expression in CRC patients with VTE.

Fold change expression of *F3* (**A**) and *CCL2* (**B**) in tumors from patients with VTE (grey bars) normalized to their control patients (black bars). Data are shown as the mean ± SD.

of monocytes [13]. Increased *CCL2* mRNA levels were observed in most patients with VTE compared to their controls, except for couples 3 and 7 (Fig. 3B).

Furthermore, we hypothesized that tumors from patients with VTE contain more fibrin deposition due to the hypercoagulant state. Therefore fibrin levels in tumor specimens were studied with an antibody that recognizes the amino-terminus of the beta-chain of fibrin [12]. All tumors from patients with VTE had higher fibrin antigen levels compared to their individual control patients, except for couple 6 (Fig. 4). This antibody also recognized fibrin degradation products in tumors from patients 3, 5 and 8 with VTE.

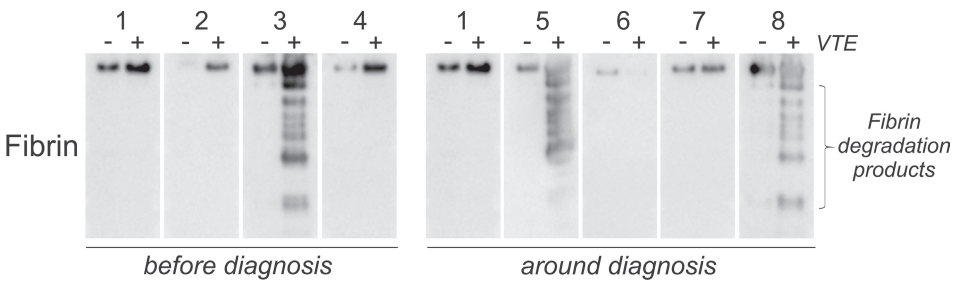

Figure 4 Expression of β-fibrin deposition in tumor specimens from CRC patients with and without VTE.

Also, fibrin degradation products were visualized. Equal protein lysates were loaded.

DISCUSSION

In this study, genes were identified that associate with development of VTE in colorectal cancer patients. RNAseq results were validated using qPCR on RNA isolated from whole tumors. IPA analysis was performed on the top 10 genes of the RNAseq analysis, showing that multiple pathways are affected in tumor cells from patients with VTE compared to controls, confirming a complex relationship. An increased pro-inflammatory state was shown in tumors from patients with VTE. Additionally, fibrin and fibrin degradation products were elevated, which suggests that a procoagulant phenotype was present in tumors of patients with VTE. Overall, these findings indicate a differential expression profile in cancer cells from patients diagnosed with VTE compared to those without VTE.

Multiple genes associated with inflammation were significantly up- and down-regulated in both VTE pre-CRC diagnosis and around CRC diagnosis (Table 2). Of note, ***REG4*** and ***SPINK4*** are tightly regulated in inflammatory bowel disease (IBD), thus co-expression of these two genes in cancer may indicate a pro-inflammatory status of the tumors. However, none of the patients in our cohort had a history of (known) IBD. Eventually, one in five IBD patients develop colitis-associated cancer over time [14]. Apart from the association of ***REG4*** with inflammation, overexpression in colorectal cancer has been linked to increased tumor progression [15, 16], suggesting that REG4 might affect both cancer and VTE.

CCL2 is a strong chemoattractant for inflammatory monocytes, which are involved in the early onset of thrombus formation [13, 17]. Indeed, elevated ***CCL2*** mRNA transcript (Fig. 3B) was detected in tumor specimens from cancer patients with thrombosis.

Our study also implicates involvement of the LXR/RXR and FXR/RXR pathway. Some studies have suggested that ligands of RXR negatively regulate VTE, as increased antithrombin production and decreased platelet activation and aggregation were observed ***in vitro*** and ***in vivo.*** [18, 19]. However, it must be mentioned that there are 2 types of platelet populations during thrombosis formation: 1) activation and aggregation of platelets which support thrombus growth; 2) loosely bound phosphatidylserine (PS)-exposing platelets with procoagulant properties [20]. Of note, ligands of LXR and FXR, such as cholesterol derivatives, positively affect the coagulant state of platelets by increased PS-exposure, EV shedding and volume size [21]. Membrane incorporated cholesterol positively regulates TF coagulant activity and hypercoagulability [22-24]. These considerations make it likely that activation of LXR/RXR and/or FXR/RXR pathways contribute to thrombosis in colorectal cancer patients. A hypercoagulant state could be further induced by apoptosis of tumor cells and increased ***XKR9*** expression, leading to elevated EV shedding and phosphatidylserine-exposure [25].

One other pathway identified by IPA was methionine degradation into homocysteine. Altered methionine metabolism by tumor cells could result in increased shedding of homocysteine into tumormilieu and/or plasma, which could result in systemic increased homocysteine levels. Multiple (epidemiological) studies have shown that increased homocysteine levels in plasma are mildly associated with VTE [26-28]. Homocysteine could elevate the risk of thrombosis by influencing TF and factor V function via inhibition of activated protein C (reviewed by Undas [27]). However, in mouse models displaying elevated homocysteine levels, no pro-thrombotic phenotype was observed [29]. This suggests that dysfunction of

methionine metabolism might be a contributor to VTE, and elevated homocysteine plasma levels, as a caudal consequence, could serve as a potential biomarker for patients at risk.

One remarkable finding was that tumors from CRC patients with a VTE diagnosis before cancer showed a different gene profile than those with a VTE diagnosis around the time of cancer diagnosis. Two possible explanations for these disparate results can be put forward. Firsts of all, patients with a VTE diagnosis around the time of cancer diagnosis have undergone cancer treatment. Surgery increases the risk of VTE by 2-fold and chemotherapy even by a 6-fold, thus in these patients chemotherapy and/or surgery could have contributed to the VTE risk, and thus this group may be biologically different from the group with a VTE diagnosis before cancer diagnosis. Secondly, during analysis of RNAseq data not all RNA reads could be aligned to the human RNA library. This might have resulted in an underestimation of significantly differentially expressed genes in patients with VTE compared to controls. Indeed, qPCR analysis (Fig. 2) demonstrated that selected genes that were identified in our RNAseq approach were similarly up- or downregulated in both groups.

Our study has a number of other limitations. We realize that the number of patients analyzed in our study was relatively small. Nevertheless, we were able to show significant expression profiles in tumors from patients with VTE compared to non-VTE controls, providing proof-of-concept that certain tumor-expressed genes associate and maybe even dictate a pro-thrombotic state in cancer patients. Increased patient numbers would perhaps give more detailed insights into the pathways involved. However, in general, it may reveal similar contributors to increased VTE risk such as inflammation, cellular metabolism and modulators of the coagulation system. Furthermore, an external validation is needed to validate these results in another CRC cohort. Additionally, we were able to detect some variation in biological processes involved in cancer patients with VTE-events at different time-points, suggesting that a more pro-inflammatory status was present in patients with VTE before diagnosis. Patients who developed VTE around CRC diagnosis might have deregulated cellular metabolism and increased apoptosis. Unfortunately, no plasma samples of patients were available, so potential plasma biomarkers could not be evaluated in this cohort.

Our cohort contains five patients with a prior history of VTE. The timing of a first VTE was at least five years before the second VTE, which makes it unlikely that the first VTE is cancer-related. However, VTE is a multifactorial disease, and even when a predisposing (genetic) factor is present, cancer is likely to be a contributing factor in causing the second VTE, especially as cancer is the largest risk factor for VTE [30]. Unfortunately, we could not

experimentally address whether our results would have been different if the patients with a prior history of VTE were to be excluded, as this would have undermined the power of our analysis.

Although literature suggests increased hemoglobin levels, leukocyte and/or platelet counts as a predictive biomarker(s) of VTE in cancer patients [31], associations between these parameters and VTE were rather weak or absent in our cohort, but this may be the result of the relative small cohort size. Therefore, we postulate that differential gene expression profiles show better associations with VTE in colorectal cancer patients than blood cell counts or Hb levels, even in a small cohort.

This study focused on CRC, and therefore our results cannot be extrapolated to other types of cancer. Thus, it would be interesting to investigate if similar, or alternatively, unique tumor genes and pathways are associated with VTE in other cancer types.

In conclusion, tumor specimens of colorectal cancer patients with VTE show a different gene profile, presence of fibrin deposition and increased inflammation compared to that of patients without VTE. Distinct pathways might be detected and discriminated into biological pathways affecting cancer-associated thrombosis or cancer related treatment. The newly identified genes from patients with VTE around cancer diagnosis could potentially be used as candidate biomarkers in order to predict thrombosis in colorectal cancer patients who might benefit from prophylactic anticoagulants.

Addendum

Conceived and designed the project: B. Ünlü, N. van Es, H.M. Otten, S. Middeldorp, P.J.K. Kuppen, S.C. Cannegieter and H.H. Versteeg. Provided study material and/or patients: J. Westerga and H.M. Otten. Performed the experiments: B. Ünlü. Bioinformatics and statistical analyses: W. Arindrarto, S.M. Kiełbasa and H. Mei. Analyzed the data: B. Ünlü, H.H. Versteeg. Wrote the paper: B. Ünlü, H.H. Versteeg. Reviewed and approved the paper: all authors.

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Disclosure of conflict of interest

Authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

List of all differently expressed genes after RNAseq analysis in tumor cells from patients with VTE before CRC diagnosis (left) and VTE around CRC diagnosis (right).

Before diagnosis				Around diagnosis			
Genes	*AvgLog2FC*	*p-value*	*adjusted p-value*	*Genes*	*AvgLog2FC*	*p-value*	*adjusted p-value*
REG4	7,32	7,02E-14	1,18E-09	*GBP4*	3,88	1,92E-11	3,07E-07
SPINK4	6,67	1,93E-09	1,63E-05	*XKR9*	6,21	1,35E-10	1,08E-06
SERPINA1	6,84	9,71E-08	5,45E-04	*CTSE*	7,21	3,50E-10	1,87E-06
SLITRK6	4,05	1,53E-06	6,44E-03	*AHCYL2*	2,77	6,37E-09	2,55E-05
SBSPON	4,17	2,52E-05	8,49E-02	*GRM8*	-5,05	8,63E-09	2,77E-05
DEFA5	4,32	4,02E-05	1,13E-01	*REG4*	5,50	5,56E-08	1,49E-04
KLHL32	3,04	4,87E-05	1,13E-01	*PTPRR*	4,75	2,21E-07	5,07E-04
LPL	3,83	5,37E-05	1,13E-01	*PIGR*	7,34	1,04E-06	1,94E-03
NTRK2	-4,31	8,52E-05	1,53E-01	*SORBS1*	-2,31	1,09E-06	1,94E-03
RNF217	4,89	9,59E-05	1,53E-01	*SAMD9*	2,37	1,44E-06	2,30E-03
LINC00261	6,30	9,99E-05	1,53E-01	*REEP1*	-4,46	1,63E-06	2,37E-03
B3GNT6	6,02	1,24E-04	1,73E-01	*NOTUM*	-5,95	1,81E-06	2,41E-03
AKR1B10	4,66	2,17E-04	2,74E-01	*FRMD3*	3,95	2,30E-06	2,78E-03
HSPA8P1	2,13	2,51E-04	2,74E-01	*MYOM3*	-3,49	2,43E-06	2,78E-03
THRB	-3,32	2,61E-04	2,74E-01	*C2orf72*	3,80	3,19E-06	3,41E-03
PDIA2	-3,25	2,73E-04	2,74E-01	*HIF3A*	-4,75	4,33E-06	4,34E-03
SPDEF	4,20	2,76E-04	2,74E-01	*B3GALT5*	3,37	4,68E-06	4,40E-03
IGHG4	3,37	4,31E-04	4,03E-01	*RP11-401P9.6*	-4,06	4,94E-06	4,40E-03
RAP1GAP	5,73	4,58E-04	4,06E-01	*CD274*	4,72	5,66E-06	4,77E-03
COL4A1	7,50	6,39E-04	5,38E-01	*TNFSF13B*	5,24	7,28E-06	5,83E-03
				PTP4A3	-2,51	7,91E-06	6,04E-03
				CD74	2,08	1,02E-05	7,15E-03
				ST6GALNAC1	3,61	1,03E-05	7,15E-03
				LAP3	2,13	1,20E-05	8,02E-03
				PTK7	-2,21	1,76E-05	1,13E-02
				MYEF2	-4,25	2,63E-05	1,62E-02
				UGT2A3	-5,51	2,78E-05	1,65E-02
				GALNT7	1,76	3,20E-05	1,83E-02
				SYT7	-1,88	4,89E-05	2,55E-02
				PGM1	3,85	4,92E-05	2,55E-02

Supplementary Table 2

Primer sequences used for the qPCR.

Gene	Primer sequence		
SPINK4	Forward 5'-	CAGTGGGTAATCGCCCTGG	-3'
	Reverse 5'-	CACAGATGGGCATTCTTGAGAAA	-3'
SERPINA1	Forward 5'-	ATGCTGCCCAGAAGACAGATA	-3'
	Reverse 5'-	CTGAAGGCGAACTCAGCCA	-3'
REG4	Forward 5'-	CTGCTCCTATTGCTGAGCTG	-3'
	Reverse 5'-	GGACTTGTGGTAAAACCATCCAG	-3'
XKR9	Forward 5'-	ACTTGTGGCTCAGTGTTTTAGTT	-3'
	Reverse 5'-	AGCTGCATGGTAACCCCTTTT	-3'
SORBS1	Forward 5'-	ATTCCCAAGCCTTTCCATCAG	-3'
	Reverse 5'-	TTTTGCTGTTCTCGATTGTGTTG	-3'
NES	Forward 5'-	CTGCTACCCTTGAGACACCTG	-3'
	Reverse 5'-	GGGCTCTGATCTCTGCATCTAC	-3'
F3	Forward 5'-	CTG CTC GGC TGG GTC TTC	-3'
	Reverse 5'-	AATTATATGCTGCCACAGTATTTGTAGTG	-3'
CCL2	Forward 5'-	AAGATCTCAGTGCAGAGGCTCG	-3'
	Reverse 5'-	TTGCTTGTCCAGGTGGTCCAT	-3'
GAPDH	Forward 5'-	TTCCAGGAGCGAGATCCCT	-3'
	Reverse 5'-	CACCCATGACGAACATGGG	-3'