Interplay between cancer and thrombosis; identification of key factors

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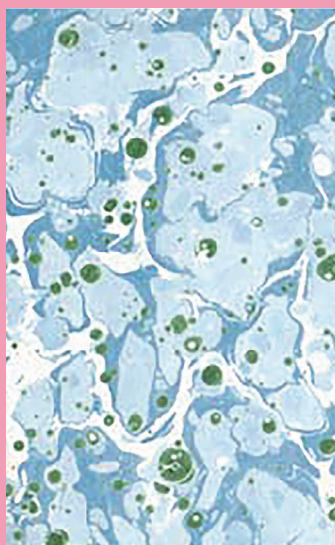

Chapter 7

Cancer-associated thrombosis: the search for the Holy Grail continues

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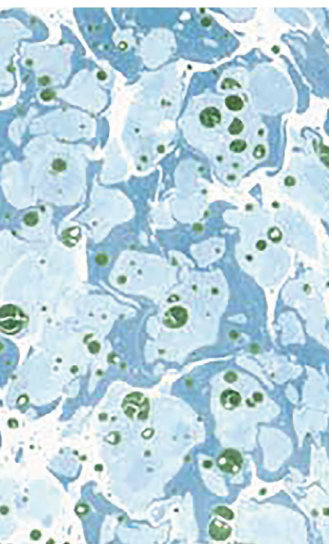

Essentials:

- The mechanisms behind cancer-associated thrombosis are poorly understood.
- The link between mutations and risk of cancer-associated thrombosis is discussed.
- Genetic profiling of tumors from patients may elucidate the underlying mechanisms.
- An unbiased molecular profiling could form a diagnostic tool to predict thrombosis in cancer.

SUMMARY

Cancer patients have an increased risk of developing venous thromboembolism (VTE), a condition that is associated with increased morbidity and mortality. Although risk assessment tools have been developed, it is still very challenging to predict which cancer patients will suffer from VTE. The scope of this review is to summarize and discuss studies focusing on the link between genetic alterations and risk of cancer-associated thrombosis (CAT). Thus far, classical risk factors that contribute to VTE have been tried as risk factors of CAT, with low success. In support, hypercoagulant plasma profiles in patients with CAT differ from those with only VTE, indicating other risk factors that contribute to VTE in cancer. As germline mutations do not significantly contribute to elevated risk of VTE, somatic mutations in tumors may significantly associate with and contribute to CAT. As it is very time-consuming to investigate each and every mutation, an unbiased approach is warranted. In this light we discuss our own recent unbiased proof-of-principle study using RNA sequencing in isolated colorectal cancer cells. Our work has uncovered candidate genes that associate with VTE in colorectal cancer, and these gene profiles associated with VTE more significantly than classical parameters such as platelet counts, D-dimer and P-selectin levels. Genes associated with VTE could be linked to pathways being involved in coagulation, inflammation and methionine degradation. We conclude that tumor cell-specific gene expression profiles and/or mutational status have superior potential as predictors of VTE in cancer patients.

Keywords: cancer, germline mutation, risk factors, RNA sequence analysis, venous thromboembolism

INTRODUCTION

The relationship between cancer and venous thromboembolism (VTE) is well established, however the underlying pathogenic mechanism has remained elusive. Among all patients with VTE, approximately one in five is diagnosed with cancer, whereas cancer patients have a 4-7-fold increased risk for a VTE-event [1-3]. Furthermore, cancer-associated thrombosis (CAT) contributes to high morbidity and mortality, with VTE being the second cause of death – after cancer itself [4, 5]. Besides the 'classical' patient-related factors like age, ethnicity and prior history of VTE, several other risk factors intrinsic to cancer that may contribute to CAT have been addressed, such as higher tumor grade, metastatic disease and cancer type. Cancer types may be classified into those that confer a high risk (pancreas, brain), moderate risk (lung, colon) and low risk (prostate, breast) of VTE [6, 7]. In addition, cancer treatment such as surgery and chemotherapy further increases the risk of VTE [8, 9].

In order to select those patients that are at (high) risk for VTE and those who might benefit from thromboprophylaxis, development of an accurate prediction models is key. These models will undoubtedly become more accurate as we learn more on the mechanisms underlying CAT. Although extensive research has been performed on finding biomarkers that predict VTE in cancer patients, the focus in most studies was on coagulation factors – either in terms of expression or genetic variants - and mediators. In this review we will discuss some of these risk factors, focusing mainly on potential tumor-derived biomarkers. Furthermore, we will present future directions that may be taken to increase the accuracy of CAT prediction models.

Risk assessment tools

Over the years several risk assessment tools have been developed to estimate the risk of CAT [6, 10-12] but unfortunately, the accuracy of such tools is very low [9, 13, 14]. The main limitations of these risk assessment tools are; i) while performing better in large cohort studies these tools are unable to predict CAT at the individual level, ii) these models are not developed for specific cancer types, iii) they underperform when used to predict risk of recurrence VTE and iv) they poorly predict increased risk of mortality. Inclusion of variables classically associated with VTE, such as platelet counts, D-Dimer and P-selectin levels, moderately improves power of such models. At the same time, it should be noted that these plasma-derived biomarkers are sensitive to circumstances like inflammation, surgery and chemotherapy, and therefore introduce a wide variability in their plasma concentrations.

The most recently developed risk score, TiC-Onco - that also includes genetic risk factors - showed a positive predictive value of up to 37%, which is only an incremental increase over the predictive values obtained after using the Khorana score that correctly predicted VTE in only 22% of the CAT patients [15].

The main reason why progress in understanding and predicting CAT is slow is the fact that many investigators extrapolate classical VTE risk factors to CAT patients, with addition of a few extra risk factors related to cancer. However, a recent publication indicates that cancer patients with VTE have different plasma profiles compared to patients with VTE only [16]. In this study the authors measured concentrations of 31 plasma proteins using multiplexed targeted proteomics. Here, the authors were able to identify and cluster 17 out of 25 cancer patients with VTE compared to healthy controls and patients with VTE only, based on their plasma protein levels. This research indicates that a 'unique fingerprint' protein profile in CAT patients, and a combination of coagulation factors that differs from those in patients with VTE only, should be considered. Unfortunately, the authors do not explain what this unique barcode in their plasma is. Yet, while these findings need to be validated in other cohorts this approach holds promise for the future.

Tissue Factor

A protein that is considered the center of cancer-associated thrombosis is Tissue Factor (TF) as it plays a role in both tumor progression and VTE. Since the association and putative role of TF in CAT is extensively investigated and reviewed [17-21] we will only briefly summarize the most important findings. TF is the activator of the extrinsic coagulation pathway, ultimately resulting in fibrin degradation and platelet activation. TF overexpression has been associated with reduced survival, increased angiogenesis, migration and invasive capacity of tumor cells in a number of cancer types (previously reviewed in [19, 20]). At present only a hand full of studies have investigated the clinical association between tumor-expressed TF and the incidence of VTE. In pancreatic cancer the risk of VTE was increased 4-fold in patients with high tumor TF expression when compared to those with low TF levels [22]. Furthermore, in a relatively small cohort, consisting of 32 ovarian cancer patients, TF expression showed a correlation with the incidence of thrombosis and D-dimer levels [23]. However, not all studies confirm a link between TF and VTE. In a prospective study on non-small cell lung carcinoma (n=39), TF expression did not associate with increased risk of VTE [24]. Similarly, in a study by Thaler et al. TF expression in brain tumors did not associate with increased VTE events [25]. Thus, high TF expression in tumors does

not lead to VTE in cancer patients per se, while associations between TF and VTE risks may very well be cancer type specific.

Stubborn as scientists may be – including ourselves -, the search for a 'black-and-white' association between TF and VTE in cancer patients continued. The majority of research attention then focused on associations between VTE and TF-positive extracellular vesicles (TF^+ EVs). Tumor cells may shed EVs into the bloodstream as a consequence of cellular activation or cell death. As EV's typically contain similar membrane-bound proteins as their mother cell, EVs can possess procoagulant activity that may contribute to VTE. Preclinical mouse models have demonstrated that TF^+ EVs are being shed from pancreatic cancer cells into the bloodstream, mediating platelet activation and thrombus formation [26-28]. Unfortunately, a relationship between circulating TF^+ EVs and VTE in a clinical setting was only established in pancreas cancer patients, while no correlation was found in other moderate-to-high risk groups such as brain, colorectal or lung cancer patients [29, 30]. Although we would have wished to consider TF the center of cancer-associated thrombosis, no evidence has been found to consider TF (EVs) as the one and only risk factor or biomarker.

Host-specific genetics

Mutations in coagulation related genes are known contributors of VTE in non-cancer patients. Therefore, initial studies investigating CAT have focused on these 'classical' targets. Factor V Leiden (FVL) – a genetic variant that is resistant to inactivation by activated protein C - confers an increased risk of VTE with an odds ratio of 3.49 in the healthy population [31]. While some studies suggest a 2-5-fold increased risk of VTE in cancer patients with FVL [32-34], other cohort studies were unable to confirm this association [35-37]. Similarly, polymorphisms in other coagulation-related genes, such as FII G20210A, FIII -603A/G, FIII +5466A>G, FXIII Val34Leu and methylenetetrahydrofolate reductase (MTHFR) C667T, showed no effect on VTE incidence in patients with and without cancer [34-36, 38, 39]. Altogether, these studies suggest that host-specific mutations and SNPs in coagulation factors are not main contributors of VTE in cancer patients, and therefore should not be considered as potential biomarkers.

In recent years studies have also addressed involvement of unsuspected gene variants as contributors to VTE in cancer patients. One example is a study in which colorectal cancer (CRC) patients with a β3 integrin rs3809865 A/A genotype were shown to have an increased risk of VTE compared to CRC patients with an A/T or T/T genotype [40]. Although the causality between this gene variant and CAT remains unknown, the authors speculate that this

variant might lead to an increased expression of β3 integrin, as this genotype is less susceptible to microRNA-mediated downregulation. To our knowledge, rs3809865 A/A–dependent β3 integrin expression on endothelial cells and platelets has not been investigated. Moreover, the risk of VTE in non-cancer patients with this genotype is unknown. Thus, while it is tempting to speculate on a link between β3 integrin rs3809865 A/A and an increased risk of VTE in patients with cancer, this hypothesis cannot be validated.

Others have reported synergistic effects of germline polymorphisms and chemotherapy - an anticancer strategy that increases the risk of VTE 6-fold - on the incidence of VTE in cancer patients [8]. Specifically, patients with a polymorphism in the promoter region of vascular endothelial growth factor (VEGFA), at location -1154, appear to have a 4-fold reduced risk of VTE (OR=0.26) while treated with standard chemotherapies, like fluorouracil, irinotecan or platinum-based drugs [41]. Gastrointestinal cancer patients carrying the tumor necrosis factor alpha (TNFα) -857 C/T polymorphism or a five-loci CTGGG haplotype (-863C/-857T/-376G/-308G/-238G) are at increased risk of VTE during fluorouracil-based chemotherapy [42].

Tumor-specific genetics

Tumor cells contain an abundance of mutations and show different gene expression profiles when compared to their untransformed counterparts. It is now believed that both somatic mutations and tumor cell-specific gene profiles might contribute to increased risk of CAT.

A number of studies have shown that mutational status associates with TF expression. For instance, in colorectal cancer TF expression is upregulated via MAPK and PI3K signaling pathways due to mutations in K-ras and loss of the tumor suppressor p53 [19, 20]. In glioblastoma, TF expression is regulated in an EGFR-dependent manner together with loss of PTEN [21]. In support, the link between elevated TF levels and mutations in K-ras, PTEN and p53 were confirmed in tumor specimens derived from patients with non-small cell lung cancer [22, 23]. Although TF expression does not necessarily associate with a high risk of VTE, as discussed above, it may very well be that K-ras, p53, EGFR and PTEN mutations have an impact on VTE (summarized in Table 1).

In a multicenter retrospective study cohort (activating) mutations in K-ras – specifically in codons 12 and 13 –associated with a 2-fold increased risk of VTE in metastatic colorectal cancer patients when compared to those patients bearing a wild-type K-ras in colon tumors (OR=2.21). Interestingly, when VTE was separated into patients with DVT or PE

the odds ratio changed to 2.62 and 1.36, respectively. Although, investigation of the seven most common K-ras mutation types did not reveal a specific variant that associates with VTE, suggesting that hyperactivation of K-ras ***in general*** contributes to VTE in metastatic colorectal cancer patients [43]. A retrospective case-control study in lung cancer confirmed the association between K-ras mutation and increased VTE risk (OR=2.67) [44]. Mechanistic studies have given more insight in the consequences of K-ras activation on tumor progression. K-ras promotes several signaling pathways, resulting in increased angiogenesis, inflammation and invasion [45, 46]. Moreover, elevated levels of inflammatory mediators, e.g. IL-6 and IL-8, may be found in tumor cells harboring a K-ras mutation [45]. Interestingly, increased IL-6 and IL-8 levels in plasma are associated with increased risk of VTE in non-cancer patients [47]. It should, however, be noted that no correlations were found between interleukins and VTE in the Vienna Cancer and Thrombosis Study (CATS) cohort [48], except for patients with pancreatic cancer. This might be attributed to the relatively low incidence of VTE in the cohort (7.2%) and that plasma was collected prior to cancer-related therapy, ruling out contributions of surgery and/or chemotherapy. Overall, this suggests that mutational status of K-ras might serve as a potential biomarker, and might serve as an upstream regulator of CAT.

Unfortunately, associations between EGFR and VTE in cancer are less obvious. Although tumor specimens of high-grade astrocytoma (a specific type of brain cancer) showed a strong correlation between TF and EGFR expression coinciding with an increase in intravascular thrombosis in the tumor, it was not examined if these patients indeed had (a)symptomatic VTE [49]. In contrast with these data, a retrospective study showed a decreased hazard risk of VTE in EGFR-mutation bearing lung adenocarcinoma patients [50]. Yet, in another retrospective case-control study, no association of VTE events in EGFR mutated patients was found when compared to those without [44]. This latter group included all types of non-small cell lung carcinoma, with lung carcinoma consisting in 72% and 57% of case and control patients, respectively. The majority of VTE events in lung cancer is associated with non-small cell lung carcinoma [51].

Another mutation found in 5% of the tumors from non-small cell lung carcinoma patients is chromosomal rearrangement of anaplastic lymphoma kinase (ALK). The first study on this mutation and its link with CAT showed an increased risk of VTE [52]. In a cohort of Canadian lung adenocarcinoma patients VTE was diagnosed in over 40% of patients with ALK rearrangements, and in an Israeli validation cohort 28% of the patients with ALK rearrangements had VTE. In this latter cohort patients were not screened for asymptomatic

VTE diagnosis, which could explain the lower incidence rate. In contrast, in a retrospective study that consisted of a similar group size a trend of decreased VTE risk in patients with ALK rearrangement in lung adenocarcinoma was determined [50]. Thus, ALK mutational status as a marker or even a driver for increased VTE risk in non-small cell lung carcinoma remains controversial.

In brain cancer, aggressive glioblastoma frequently harbor the wild-type variant of isocitrate dehydrogenase 1 or 2 (IDH1/2) [53], while somatic point mutations in IDH1/2 are associated with less aggressive behavior and less necrosis [54, 55]. A recent study has investigated whether patients with wild-type IDH1/2 glioblastoma are more likely to develop VTE. Interestingly, patients harboring IDH1/2 mutations did not develop VTE neither in a discovery nor in a validation cohort, both consisting of approximately 150 patients [56]. Furthermore, only 2% of the tumors with IDH1/2 mutation showed intratumoral microthrombi versus 86% in wild-type IDH1/2 tumors. This association could be linked to reduced TF expression in the tumors and circulating procoagulant active TF^+ EVs. Therefore, IDH1/2 mutation might be an interesting biomarker to predict which cancer patients have a decreased risk of VTE.

Unbiased screen for risk factors in CAT

It is a time-consuming effort to identify all mutations in tumors and to link them individually to risks of VTE in cancer. Therefore, we have previously proposed to screen – in an unbiased manner – tumor gene expression profiles and/or mutations that associate with VTE in cancer patients. In a proof-of-principle study we showed that it is feasible to link tumor-specific gene expression profiles with VTE in colorectal cancer patients [57]. In this study RNA from isolated tumor cells was subjected to next generation RNAsequencing, making it possible to compare expression profiles in tumor cells from colorectal cancer patients with VTE compared to colorectal cancer patients without VTE. Tumors from CAT patients had different expression profiles that involved pathways related to coagulation, inflammation, homocysteine production and liver- and retinoid X receptor (LXR/RXR) function (Table 2). In addition, tumor specimens from CAT patients displayed a pro-inflammatory state and elevated fibrin deposition levels. Stratification of patients for timing of VTE (i.e. VTE before CRC diagnosis or VTE around the time of CRC diagnosis), suggested that time of a VTE event influenced the set of observed gene expression profiles. Particularly, gene expression profiles suggested a pro-inflammatory status in patients with VTE prior to CRC diagnosis, and altered cellular metabolism in patients included in the group that experienced VTE around the time of CRC diagnosis. This may suggest that altered expression

profiles within the tumor are affected by cancer treatment like surgery or chemotherapy. Hence, we assume that treatment-related CAT and CAT in the absence of such treatment have different etiologies, and this warrants further investigation.

This study opens up new possibilities in improving our understanding of the pathophysiological mechanism of CAT, to better treat CAT, and to improve CAT prediction models. It would be of interest to further investigate whether single or co-expression of the top 3 genes as identified in the patient group experiencing VTE before CRC diagnosis (***REG4***, ***SPINK4*** and ***SERPINA1***) could serve as a strong predictor of VTE in CRC patients. Additionally, these 3 genes encode secretable proteins and therefore future work is required to study if plasma levels could also serve as prognostic biomarkers [58-60]. Furthermore, it would be interesting to investigate if there is a relationship and/or synergism with mutational status of K-ras, as this is already associated with CAT in colon cancer [43]. Finally, future work may demonstrate that there is a link between the expression profiles in CAT and different subtypes of colon cancer [61], as Magnus et al. previously reported glioblastoma subtype-specific phenotypes and altered coagulation-related genes. Such identification may allow for personalized treatment of CRC patients to prevent CAT.

Significant upregulation of ***REG4*** was detected both in patients with VTE before, as well as around CRC diagnosis. Overexpression of Reg4 is associated with tumor progression, metastasis and reduced survival [62-64]. As mentioned before, risk of CAT increases dramatically in patients experiencing metastasis compared to non-metastatic cancer patients [65-67]. Tumor cells must gain cancer stem cell (CSC) properties and should undergo epithelial-to-mesenchymal transition (EMT), for successful metastasis [68, 69]. In the bloodstream procoagulant functions rescue the circulating tumor cell (CTC) from immune attack and shear stress, which additionally supports metastasis [70-72]. Of note, REG4 is associated with cancer stemness and metastasis [73, 74], suggesting that Reg4-dependent metastasis may be another mechanism leading to CAT. Unfortunately, thus far, no (genetic) reports have been published on the mechanism linking metastasis to increased risk of VTE. We believe that rather than the metastatic lesion itself, CTCs contribute to VTE, as they possess i) procoagulant activity, ii) may consist of large clumps of multiple cells and iii) are found in thrombi [75, 76]. Although, studies on this particular topic are rather inconclusive, Mego et al. recently reported in a US-based retrospective study that 9% of (metastatic) breast cancer patients experienced CAT with CTCs detectable, whereas patients without detectable CTCs had no VTE [77]. Discrimination of CTCs into epithelial or mesenchymal-like CTCs in a Slovakian cohort with 116 early breast cancer patients showed no differences, with

only 1 patient with mesenchymal-like CTCs eventually developing VTE [78]. Therefore, future research directions may include genetic profiling, using RNAseq, of CTCs, primary and metastatic tumors in patients with and without CAT.

CONCLUSION

Despite over 150 years of effort to elucidate mechanisms behind CAT, or to accurately predict which cancer patients have an increased risk of CAT, research has made only incremental steps forward. With the most recently developed risk assessment tools only 37% cases of CAT can be predicted, which is – in our opinion – not accurate enough. Therefore, scientists should change their view on the mechanisms behind VTE in cancer patients. Classical risk factors of VTE cannot be extrapolated to cancer patients, nor do (mutations in) coagulation-related genes significantly contribute to CAT. So far, germline variants have only been shown to affect VTE risk during chemotherapy. Thus, we believe that understanding the mechanism behind CAT comes from genetic profiling of tumors. At present, only mutations in K-ras in colon and lung cancer show an association with increased risk of VTE, while IDH1/2 mutations are associated with a decrease in VTE risk in glioblastoma patients. As it is time consuming to investigate the role of every gene in CAT one by one searching for genes that associate with CAT an unbiased manner may be more appropriate. This should ultimately lead to the discovery of novel biomarkers that potentially serve as a diagnostic tool. Furthermore, it will also give more insight in the upstream biological processes that provoke a hypercoagulant state, leading to VTE. We furthermore recommend assessing genetic profiles in each cancer (sub)type separately, since different genetic events that associate with CAT may be dependent on processes that are cancer type-specific.

Author contributions

BU wrote the manuscript, HHV supervised and edited the manuscript.

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Conflict of interest statement

The authors have no relevant conflicts to declare in relation to this paper.

Table 1

Clinical studies on mutational status and cancer-associated thrombosis

Cancer	cohort type	cohort size	tumor type	Gene of interest	Outcome	Remarks	Ref
Colorectal	Retrospective	172	metastatic colorectal cancer	K-ras	OR=2.21	Bevacizumab independent; Multicenter	Ades et al. 2015
Lung	Retrospective case-control	159	non-small cell lung carcinoma	K-ras	OR=2.67		Corrales-Rodriguez et al. 2014
				EGFR	OR=0.99		
	Retrospective	293	lung adenocarcinoma	EGFR	HR=0.46	Tyrosine kinase inhibitor (TKI) treatment reduces VTE risk	Davidsson et al. 2017
				ALK	HR=0.61 (ns)		
	Discovery	55	lung adenocarcinoma	ALK	41.8% VTE	All patients had ALK rearrangement; included patients with VTE history and thromboprofylaxis	Zer et al. 2017
	Validation	43	non-small cell lung carcinoma	ALK	27.9% VTE	All patients had ALK rearrangement; included patients with VTE history and thromboprofylaxis	
Brain	Discovery	169	glioma	IDH1/2	0% VTE	wild-type: 25.5% VTE; microthrombi in 85.5% WT vs 1.9% in mutant	Unruh et al. 2016
	Validation	148	glioma	IDH1/2	0% VTE	wild-type: 29.5% VTE; microthrombi in 90.4% WT vs 5.9% in mutant	

Table 2

Expression profile and associated canonical pathways in CRC patients with VTE before or around diagnosis. Table adjusted from Ünlü et al. [57].

−1 year ≤ CRC diagnosis			−3 ≤ CRC diagnosis ≤ +3 months		
Top canonical pathways					
		p-value			p-value
LXR/RXR activation		1,39E-03	Methionine degradation I		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
Gene expression profile					
Genes	*AvgLog2FC*	Adjusted p-value	Genes	*AvgLog2FC*	Adjusted p-value
REG4	7,3	1,18E-09	*GBP4*	3,9	3,07E-07
SPINK4	6,7	1,63E-05	*XKR9*	6,2	1,08E-06
SERPINA1	6,8	5,45E-04	*CTSE*	7,2	1,87E-06
SLITRK6	4,0	6,44E-03	*AHCYL2*	2,8	2,55E-05
SBSPON	4,2	8,49E-02	*GRM8*	-5,1	2,77E-05
DEFA	4,3	1,13E-01	*REG4*	5,5	1,49E-04

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