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Increased In Vivo Thrombin Generation in Patients with Localized Non-Small Cell Lung Cancer Unfit for Surgery

Johanne Andersen Hojbjerg, MD, PhD[1,2], Kristian Kirkelund Bentsen, MD[3,4,5], Pernille Just Vinholt, MD, PhD[5,6], Olfred Hansen, MD, PhD[3,4,5], Stefan Starup Jeppesen, MD, PhD[3,4,5], and Anne-Mette Hvas, MD, PhD[1,7]

Abstract

Patients with lung cancer face a substantially increased risk of thromboembolic disease. Patients with localized non-small cell lung cancer (NSCLC) who are unfit for surgery due to age or comorbidity have additional thrombotic risk factors. Thus, we aimed to investigate markers of primary and secondary hemostasis, since this could assist in treatment decisions. We included 105 patients with localized NSCLC. Ex vivo thrombin generation was determined by calibrated automated thrombogram and in vivo thrombin generation was determined by measurement of thrombin–antithrombin complex (TAT) levels and prothrombin fragment F1 + 2 concentrations (F1 + 2). Platelet aggregation was investigated by impedance aggregometry. Healthy controls were used for comparison. TAT and F1 + 2 concentrations were significantly higher in NSCLC patients than in healthy controls ($P < .001$). The levels of ex vivo thrombin generation and platelet aggregation were not increased in the NSCLC patients. Patients with localized NSCLC considered unfit for surgery had significantly increased in vivo thrombin generation. This finding should be further investigated as it could be relevant for the choice of thromboprophylaxis in these patients.

Keywords

lung neoplasms, hemostasis, platelet aggregation, thrombin generation

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Introduction

Lung cancer is one of the most thrombogenic cancers, with thromboembolism occurring in up to 14% of patients.[1] The increased risk of thromboembolism in cancer patients[2] has been attributed to several factors including immobilization, central venous catheters, and endothelial dysfunction and damage[3] due to tumor growth[4] or surgery.[5] The cancer cells also directly activate the coagulation system through signals that stimulate leukocytes and platelets among others.[6,7] Furthermore, treatment-related risk factors also contribute to the increased thromboembolic risk in patients with cancer, and it is well-known that both chemotherapy, antiangiogenic agents, and immunotherapy increase the risk of thromboembolism.[8]

Both primary and secondary hemostasis is involved in cancer-associated thrombosis (CAT). Platelets are a well-established risk marker of CAT[9] and are incorporated in the

[1] Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark
[2] Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
[3] Department of Oncology, Odense University Hospital, Odense, Denmark
[4] Academy of Geriatric Cancer Research (AgeCare), Odense University Hospital, Odense, Denmark
[5] Institute of Clinical Research, University of Southern Denmark, Odense, Denmark
[6] Department of Clinical Biochemistry, Odense University Hospital, Odense, Denmark
[7] Faculty of Health, Aarhus University, Aarhus, Denmark

Corresponding Author:
Johanne Andersen Hojbjerg, Department of Clinical Biochemistry, Aarhus University Hospital, Palle Juul-Jensens Boulevard 99, DK-8200 Aarhus N, Denmark.
Email: johanand@rm.dk

Khorana score used for the prediction of venous thromboembolism in cancer patients.[10] Thrombin generation is a global marker of coagulation activity and has also been studied in relation to the risk of CAT. However, the determination of real-time thrombin formation in the patient is complicated, due to the rapid inhibition of thrombin in vivo. Hence, by-products of thrombin generation, prothrombin fragment (F1 + F2) and thrombin-antithrombin complex (TAT), can be used to estimate in vivo thrombin generation.[11,12] Therefore, they represent markers of in vivo generation of thrombin[13] and have furthermore been demonstrated to predict the risk of CAT[14,15]

Stereotactic body radiation therapy (SBRT) is high-dose radiotherapy delivered in a few fractions to the tumor. SBRT is offered to inoperable patients with localized non-small cell lung cancer (NSCLC) and secures the same local control of the tumor as surgery.[16,17] As a result, SBRT is an alternative treatment offered to medically inoperable cancer patients. Since approximately 50% of lung cancers are diagnosed in patients aged 70 years or older, patients are often inoperable due to comorbidity, and are consequently treated with SBRT.[17] Age is known to be an independent risk factor for thromboembolism,[18] which further increases the risk of thromboembolic events in these patients. As a result, NSCLC patients unfit for surgery have several thromboembolic risk factors beyond cancer, such as high age and substantial comorbidity. Thus, the aim of this study was to investigate markers of primary and secondary hemostasis, since this could assist in treatment decisions.

Methods

Study Population

The study population was enrolled in this prospective single-institutional study from October 2018 to April 2021 at the Department of Oncology, Odense University Hospital, Denmark. A total of 105 consecutive patients with newly diagnosed localized NSCLC (T1-T3N0M0) referred to SBRT were included in the study. The patients met the following inclusion criteria: (1) age >18 years, (2) planned SBRT as standard treatment, which was considered best practice. Exclusion criteria were inability to provide informed consent, venous thrombosis within the last 3 months or a concurrent active cancer within the previous year except for lung cancer. The study was conducted in accordance with the declaration of Helsinki and was approved by the regional ethical committee (project ID no. S-20180109). Oral and written informed consent was obtained from all participants.

All patients were assessed by the Charlson comorbidity index (scores 0-1, 2-3, and $\geq$ 4 were considered normal, medium, and high, respectively).[19]

Blood samples were collected within 2 weeks prior to initiation of SBRT.

Blood Sampling

Venous blood samples from both healthy controls and patients were collected by antecubital vein puncture with a minimum of stasis. The samples were kept at room temperature for a maximum of 2 h before processing.

Blood samples for measuring thrombin generation, TAT levels, and F1 + 2 concentrations were collected in 3.2% sodium citrate tubes (Vacuette®, Greiner Bio-One International GmbH, Kremsmünster, Austria). Within 30 min from the collection, blood was centrifuged at 3000 relative centrifugal force for 25 min. Plasma was subsequently frozen at −80 °C until analysis.

Platelet aggregation was determined in whole blood collected in hirudin tubes. Blood for fibrin d-dimer, fibrinogen, international normalized ratio (INR), and activated partial thromboplastin time (aPTT) was drawn into citrate tubes, whereas hemoglobin, leukocytes, platelets, and immature platelet count (IPC), were collected in K2-EDTA tubes. Blood for C-reactive protein (CRP), estimated glomerular filtration rate (eGFR), alanine transaminase (ALAT), and bilirubin was collected in lithium heparin tubes

Laboratory Analysis

Thrombin generation was measured in platelet-poor plasma after the addition of tissue factor (1 pM) and phospholipids (4 μM) using a calibrated automated thrombogram (Thrombinoscope BV, Maastricht, the Netherlands) as previously described.[20] The following parameters were reported: Lag time (min) as a measure of the time to clot initiation, time to peak (min) as a measure of the time to reach the maximum peak height, peak height (nanomolar thrombin) as a measure of the highest thrombin concentrations, and endogenous thrombin potential (ETP) (area under the thrombogram, nanomolar × min) representing the total amount of thrombin being generated during the analysis.

TAT and F1 + 2 concentrations were analyzed in platelet-poor plasma as described by Lundbech et al[21] using commercial enzyme-linked immunosorbent assays (Enzygnost® TAT Micro, Siemens Healthcare GmbH, Erlangen, Germany) and (Enzygnost® F1 + 2 Mono, Siemens Healthcare GmbH). Samples were analyzed in duplicates and results were reported as the mean value. Results were accepted if the coefficient of variation (CV) between duplicates was <10%, or else the analysis was repeated. The F1 + 2 analysis had a measurement range from 20 to 1200 pmol/L. The measurement range of TAT was 2-60 μg/L, and results <2 μg/L were registered as 1 μg/L and results >60 μg/L were registered as 65 μg/L in the statistical analysis. These arbitrary values were used since they are identical to the values underlying the reference intervals published by Lundbech et al.[21]

Platelet aggregation was analyzed in unprocessed whole blood using multiple electrode aggregometry (Multiplate 5.0 Analyzer, Roche Diagnostics, Switzerland). Samples rested for 30 min before being analyzed within 2 h from vein puncture. Platelet aggregation was induced by three different agonists; ADP 6.4 μM, (ADPtest), arachidonic acid 0.5 mM (ASPItest), and thrombin receptor activator peptide-6 32 μM

(TRAPtest). Results were reported as the area under the curve (AUC) (aggregation units [AU] × min).

Hematology parameters were measured on Sysmex XN-9000 (Sysmex, Kobe, Japan). INR, aPTT (Actin FS), fibrinogen activity, and fibrin d-dimer were evaluated on CS5100 (Siemens Healthineers, Erlangen, Germany) with reagents from the manufacturer. CRP, eGFR, and ALAT were measured on Cobas 8000 (Roche Diagnostics, Basel, Schweiz) with reagents from the manufacturer.

Healthy Controls

Data from healthy controls regarding thrombin generation, TAT, and F1 + 2 concentrations were generated in a previous study by Lundbech et al.[21] This cohort consisted of 57 (46%) females and 67 (54%) males with a mean age of 42 years (range 21-66).

Likewise, data on platelet aggregation in healthy controls were obtained from a cohort collected by Ostrowski et al[22] with a median age of 49 years and 73% women.

Results from healthy controls were all generated in the same laboratory using identical equipment and methods as for the study population.

Statistical Analysis

Data distribution was evaluated by Q-Q plots to determine if they followed the Gaussian distribution. Normally distributed data are presented by mean ± standard deviation (SD) whereas data that was not normally distributed are presented as median with interquartile ranges. Differences between healthy controls and patients with lung cancer were calculated using an unpaired t-test if data were normally distributed and by Mann–Whitney U test if data did not follow the Gaussian distribution. Statistics were performed using GraphPad® Software Version 8.0.1; San Diego, USA.

The sample size was fixed because the patients are part of a larger study. With 105 patients included and 124 healthy controls, we would have a power of 98% to detect a difference of 15% in ETP between the groups (mean ETP = 1293 nm × min, 2alpha = 0.05). The power calculation was performed using STATA/MP 17.0 for Windows (64-bit × 86-64); College Station, Texas, USA

Patients receiving anticoagulant treatment were subsequently excluded from the statistical analyses of thrombin generation, TAT, and F1 + 2 concentrations. Likewise, patients receiving antiplatelet therapy were excluded from the platelet aggregation analysis.

Results

A total of 105 patients with localized NSCLC and planned for SBRT were enrolled in the study. The median age was 74 years with an even distribution of sex. The majority of patients were comorbid with 61% scoring ≥ 2 using the Charlson Comorbidity Index. A total of 61 patients received antithrombotic treatment at the time of enrollment; 39 patients received antiplatelet therapy of which 6 patients received double antiplatelet therapy and 23 patients were treated with anticoagulant therapy (direct oral anticoagulants [DOACs; n = 14], vitamin K-antagonists [VKA; n = 6], or low molecular weight heparin [LMWH; n = 3]). Two patients received both anticoagulant therapy and antiplatelet therapy. Patient characteristics are presented in Table 1.

Patients with localized NSCLC not receiving anticoagulants (n = 83) had thrombin generation in the same range as healthy controls (n = 124) expressed by no difference in lag time (P = .81), peak (P = .10), and time-to-peak (P = .81). ETP was

Table 1. Demographic and Clinical Characteristics Including Routine Biochemistry in 105 Patients with Localized Non-Small Cell Lung Cancer.

Variables	Results
Sex, female n(%), [105]	50 (48%)
Age (years), median (range)	74 (55-93)
Smoking n(%)[105]	
Present	47 (45%)
Former[a]	55 (52%)
Never	3 (3%)
Histology n(%)[105]	
Adenocarcinoma	63 (60%)
Non-adenocarcinoma	42 (40%)
Lung cancer stage n(%)[105]	
1A	67 (64%)
1B	26 (25%)
2A	7 (6%)
2B	5 (5%)
Khorana score n(%)[105]	
1	59 (56%)
2	36 (34%)
3 +	10 (10%)
Charlson Comorbidity Index n(%)[105]	
0-1	41 (39%)
2-3	50 (48%)
≥4	16 (13%)
Routine biochemistry, (reference interval)	Median (25%;75% percentiles)
Fibrin D-dimer, mg/l FEU, (<0.8), [102]	0.69 (0.37;1.12)
Fibrinogen, µmol/l, (5.2-12.6), [103]	12.2 (10.9;13.8)
International normalized ratio (INR), <1.2, [103]	1.0 (0.9;1.1)
Activated partial thromboplastin time (APTT), s, (22-28), [103]	25 (23;27)
Hemoglobin, mmol/l, (7.3-10.5), [105]	8.6 (8.0;9.1)
Leukocytes, 10^9/l, (3.5-8.8), [105]	8.0 (6.9;9.5)
Platelet count, 10^9/l, (145-400), [105]	287 (221;359)
Immature platelet count, 10^9/l, (0.01-0.06), [104]	0.03 (0.02;0.05)
C-reactive protein, mg/l, (<6), [104]	2.9 (1.4;8.0)
Estimated glomerular filtration rate, mL/min, (>59), [98]	79 (61;88)
Alanine transaminase, U/l, (10-70), [97]	18 (14;25)
Bilirubin, µmol/l, (5-25), [97]	6 (5;8)

Due to a few missing data, [n] is indicated for each variable in the table.
aFormer smoker is defined as an adult who has smoked at least 100 cigarettes in his or her lifetime but who had quit smoking at the time of interview.

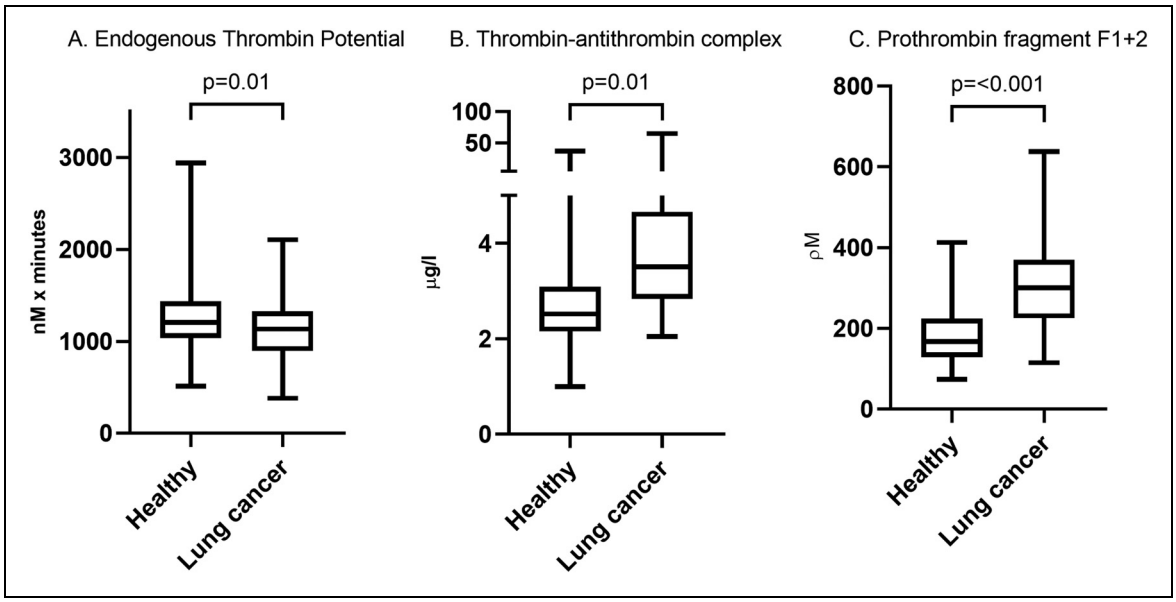

Figure 1. Endogenous thrombin potential (A), thrombin-antithrombin complex levels (B), and prothrombin fragment F1 + 2 concentrations (C) in healthy controls and in patients with localized lung cancer unfit for surgery presented as median and ranges. Patients receiving anticoagulants were excluded.

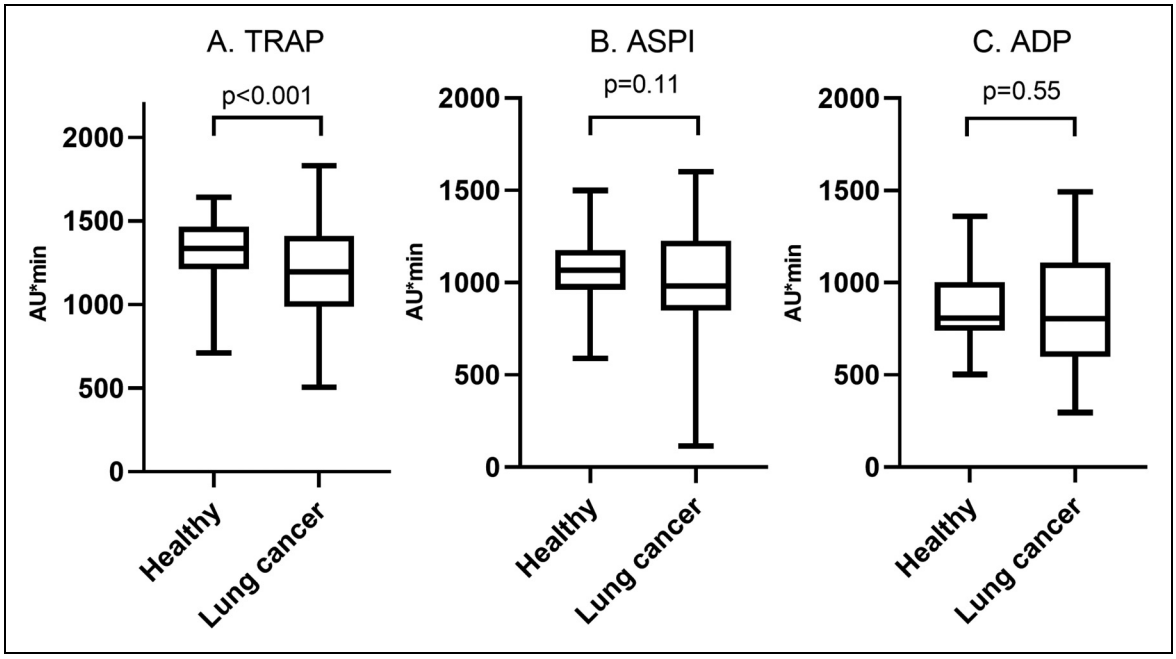

Figure 2. Platelet aggregation presented as area under the curve (AUC) in healthy controls and in patients with localized lung cancer unfit for surgery, activated with three different agonists presented as median and ranges. Patients receiving antiplatelet therapy were excluded.

significantly lower in lung cancer patients than in healthy controls ($P = .01$), Figure 1A. Patients with localized NSCLC (n = 74) showed significantly higher TAT complex levels than healthy controls (n = 122) ($P < .001$), Figure 1B. Also, F1 + 2 concentrations were significantly higher in the patients with localized NSCLC (n = 66) than in healthy controls (n = 124) ($P < .001$), Figure 1C.

For patients not receiving antiplatelet therapy, all median platelet aggregation values expressed by AUC were within the reference interval (n = 66). Patients with localized NSCLC showed lower platelet aggregation than healthy controls (n = 80) for TRAP ($P < .001$) and no significant difference between the groups for ASPI ($P = .11$) or ADP ($P = .55$), Figure 2.

The medians of all routine biochemical parameters including INR, aPTT, platelet count, and fibrinogen were within the respective reference intervals as presented in Table 1.

Discussion

In this study, we demonstrated that in vivo thrombin generation, reflected by TAT and F1 + 2 concentrations, was significantly increased in patients with localized NSCLC compared to healthy controls. Thrombin plays a pivotal role in the coagulation process,[11] and the analysis of ex vivo thrombin generation determines the capacity of the coagulation system. In contrast, both TAT and F1 + 2 concentrations are markers of in vivo thrombin generation since they are generated in the conversion from prothrombin to thrombin.[23]

Here, we report increased in vivo thrombin generation reflected by increased TAT complex levels and F1 + 2 concentrations indicating that these co-morbid NSCLC patients generate more thrombin in vivo than healthy controls. On the contrary, ex vivo thrombin generation was not elevated. The lack of increased ex vivo thrombin generation in combination with elevated TAT and F1 + 2 concentrations is in accordance with a previous study comparing early-stage operable lung cancer patients with healthy controls.[21,24] This pattern is hypothesized to be due to an exhaustion phenomenon caused by an increased in vivo production of thrombin resulting in a decreased ability to generate thrombin ex vivo. This phenomenon has also been described in patients with arterial thrombosis by Hansen et al in which thrombin generation in patients with ST-segment elevation myocardial infarction was evaluated.[25]

Platelets also play an essential role in cancer-related thrombosis.[26,27] However, we do not find that the patients with localized NSCLC in this study have increased platelet aggregation. This is in contrast to our expectations based on a previous study by Hvas et al, showing increased platelet aggregation in patients with operable lung cancer compared to healthy controls.[28] The stage of cancer in the study by Hvas et al is like the one in the present study, but Charlson Comorbidity Index was not calculated by Hvas et al, hence a strict comparison is therefore not possible.

The present study is limited by the uncertainty of consumption of over-the-counter drugs and natural therapeutics, for example, fish oil, since these might be unreported by the patients. The finding of slightly lower platelet aggregation in comparison to healthy controls could be due to interaction with over-the-counter drugs as platelet aggregation is reduced by analgesics in the form of non-steroidal anti-inflammatory drugs and acetylsalicylic acid.[29] In addition, natural therapeutics such as fish oil among others can also inhibit platelet aggregation.[30]

Furthermore, 21% of the included patients were treated with anticoagulants and because of this were excluded from the statistical analysis to avoid bias. This impairs over results, however, substantiates the hypothesis of this comorbide population being hypercoagulable and underlines the need for larger studies to overcome this impediment caused by antithrombotic medication.

Conclusion

We have demonstrated increased in vivo thrombin generation in patients with localized NSCLC unfit for surgery with considerable comorbidities. The potential clinical implications of this finding must be further investigated as enhanced in vivo thrombin generation could be a significant contributor to the increased thrombosis risk in this high-risk population.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD

Johanne Andersen Hojbjerg https://orcid.org/0000-0002-5604-3451

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