Clonal Hematopoiesis in patients with cancer and its impact on risk of cancer-associated thrombosis

Project proposal

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1. Study Synopsis

Title	Clonal Hematopoiesis (CH) in patients with cancer and its impact on risk of cancer-associated thrombosis
Précis	In this project, I will establish an easy and cost-effective approach to screen for clonal hematopoiesis of indeterminate potential (CHIP)-like mutations and assess the prevalence of CH in patients with cancer to evaluate a possible association with cancer-associated VTE.
Objectives	The **primary objectives** are (1) to establish a custom Amplicon-based Next Generation Sequencing Assay to rapidly and cost-effectively screen for CHIP-like mutations in patient cohorts and (2) to assess the prevalence of CHIP-like mutations in a cohort of patients with cancer. **Secondary objectives** are to evaluate a possible association between CH and (1) cancer-associated VTE and ATE, (2) VTE and ATE case fatality rate, and (3) all-cause mortality in patients with cancer.
Methods	Custom Amplicon-based Next Generation Sequencing Assay for CHIP-like mutations in the genes DNMT3A, TET2, ASXL1, JAK2, PPM1D, TP53 and CHEK2
Study population	Vienna Cancer and Thrombosis Study (CATS, EK: 126/2003): Patients with newly diagnosed or recurrent cancer Prospective observational cohort study Designed to identify risk factors for cancer-associated VTE
Expected time-frame	18 months

3. Introduction

Venous thromboembolism (VTE; including deep vein thrombosis (DVT) and pulmonary embolism (PE)) is a common complication in patients with cancer. About 20% of all VTE events occur in patients with cancer and the VTE risk is about 9-fold higher in this patient population.(1, 2) Thus, VTE is a leading cause of morbidity, mortality, and associated with a poor prognosis in this patient population.(3-5) Lately the evidence that patients with cancer are at an increased risk for arterial thrombotic events (ATE) is increasing as well.(1) Although various risk factors for VTE in patients with cancer are known, the exact underlying pathophysiology could not be shown so far.(3) With our current knowledge we still cannot explain all thrombotic events in cancer patients and with our tools for individual risk assessment in the clinical setting we still miss over 40% of thrombotic events.(6)

In the last years, clonal hematopoiesis (CH), defined as clonal expansion of a subset of hematopoietic stem cells (HSCs) due to an acquired somatic mutation providing them with a survival benefit, is gaining increasing interest in research. It became evident that clonal hematopoiesis is highly prevalent in the general population.(7-11) As a consequence the term clonal hematopoiesis of indeterminate potential (CHIP) was proposed. Currently, it is defined as the presence of clonal hematopoiesis due to a somatic mutation in a known CHIP-like gene with a variant allelic frequency of at least 2% or other non-disease-defining clonal cytogenetic alterations.(12) The majority of mutations occur in the genes DNMT3A, TET2, ASXL1 and JAK2.(7-11) Before diagnosing an individual with CHIP, one has to rule out the presence of dysplasia and blasts in the bone marrow as well as paroxysmal nocturnal hemoglobinuria, monoclonal gammopathy of undetermined significance, and monoclonal B-cell lymphocytosis as possible differential diagnoses.(12)

CH was assessed either by whole-exome sequencing or whole-genome-sequencing.(7, 11) Those approaches are expensive and time-consuming because of the huge amount of data that has to be analyzed. Thus, an easy and cost-effective approach to screen for mutations in the most common underlying genes would be helpful. In the future, this approach could further be useful to rapidly screen other patient populations for CH for research purposes.

The incidence of CHIP increases with age and is estimated to be about 5% in 60-year-olds but rises to nearly 20% in those older than 90 years.(7, 9) Not surprisingly the presence of CHIP increases the risk of being diagnosed with hematological cancer quite substantially, namely about 10-fold.(7, 9) Nevertheless, as the progression rate is estimated to be 0.5-1% per year, most individuals with CHIP will never be diagnosed with a hematological malignancy.(12) However, CHIP is associated with a high all-cause mortality that cannot solemnly be explained by the progression to cancer. Recently an association with coronary heart disease (CHD), ischemic stroke, and chronic cardiovascular disease was shown.(7, 8) Individuals with CHIP

are two times more likely to suffer from CHD, making it a risk factor as potent as smoking, hypertension, hyperlipidemia and diabetes.(7, 8)

The proposed mechanism responsible for this increased risk of atherothrombosis was closer investigated for some common CHIP-like mutations and is thought to act mainly through increased inflammatory responses of monocytic, granulocytic, and lymphocytic cells.(8, 13-16) Further, the activation of platelets and endothelial cells leading to increased p-selectin expression was proposed as a possible underlying pathophysiological pathway.(13, 15) As platelet and endothelial cell activation, innate immune cells, and inflammation play an important role in venous thrombus formation as well (17, 18), an additional association with venous thrombosis was suggested. The fact that certain single nucleotide polymorphisms in inflammatory genes appear to increase the risk of VTE development further supports the hypothesis that CHIP leading to an elevated inflammatory environment may raise the risk of VTE development.(19) In addition, age is a known risk factor for VTE development and the incidence of CHIP is increasing with age as well.(7, 20) Indeed healthy individuals with JAK2 driven CHIP were found to have a 2.8-fold higher risk for VTE development.(21) However, such a possible association was not evaluated in other CHIP-like mutations.

Clonal hematopoiesis was found to be highly prevalent in patients with solid tumors and the most frequently mutated CHIP-like genes (CHEK2, TP53, PPM1D) differed from those seen in the non-cancer population.(22-25) In addition, CHIP has been associated with poor survival in diverse cancer patient cohorts, indicating its potential usefulness as a prognostic marker.(22, 24)

This leads to the question if CHIP increases the already high risk of VTE development in cancer patients, and if so, what pathomechanism underlies this possible observation.

We hypothesize that through activation of inflammatory pathways in leukocytes, endothelial cell activation, and increased monocyte-platelet interaction, CH may lead to an increased VTE risk.

3. Aims

Thus, one aim is to assess the prevalence of CH in patients with cancer in a fast and cost-effective manner.
Therefore, I want to establish an Amplicon-based Next Generation Sequencing (NGS) Analysis of the most common CHIP-like mutations in patients with cancer. This allows for an easy and cost-effective screening for these mutations in various cohorts.
First, custom primers (Sigma-Aldrich, St. Louis, MO) will be designed to flank the exonic regions with known hotspot mutations involved in CH (18, 21) of the genes DNMT3A, TET2, ASXL1, JAK2, PPM1D, TP53 and CHEK2. In total, 55 primer pairs (55 Forward and 55 Reverse Primer) will be designed. In the next step, a Multiplex PCR (Qiagen Multiplex PCR Kit; Qiagen, Hilden, Germany) will be performed to amplify the desired exon loci simultaneously. To evaluate if the primers are designed correctly to enable the amplification, an agarose gel electrophoresis will be performed subsequently. Furthermore, cycling conditions will be optimized by checking the results of the different conditions again with gel electrophoresis and further on by using an Agilent 2100 Bioanalyzer Instrument (Agilent, CA, USA) as it has a higher sensitivity to assess the PCR products. This is important to minimize the likelihood of increasing the false positive rate of mutations detected in the sequencing run at the end.
After passing the initial evaluation, the PCR product will be enzymatically cleaned up using Exonuclease 1 and Shrimp Alkaline Phosphatase (New England Biolabs, Ipswich, MA) to remove the remaining primers and dNTPs. Then an index PCR will be performed (KapaHifi HotStart Polymerase, KAPA Biosystems, Cape Town, ZA) to add Nextera XT Index primers (Illumina, San Diego, CA), so that every sample is labeled individually allowing for pooling of samples. After pooling the samples, I will perform a second clean-up to discard the remaining primers and dNTPs using a SPRI approach. With this approach, it is possible to select specific sizes of fragments for the library that will be sequenced. As our amplicons are in the range of 200-600 base pairs (bp), a left-sided selection with AMPure XP (Beckman Coultier, Indianapolis, IN) to discard any fragments smaller than 100bp will be performed.
Lastly, sequencing of the pooled samples will be performed on an Illumina MiSeq (Illumina, San Diego, CA) to get our desired read depth.
The MiSeq produces FASTQ and BAM files, which are computational de-multiplexed by the MiSeq Local Run Manager, which results in fully annotated variants tables as an output. Results will be filtered to detect variants with a total depth of more than 200 reads and a VAF higher than 0.05 (VAF >5%). Variants with a VAF of around 0.00 (0%), 0.50 (50%), and 1.00 (100%) will be filtered as well, as they mostly characterize germline variants. Furthermore

synonymous single nucleotide variants will be filtered. All other variants will be considered as “likely somatic” and thus further evaluated and investigated.

Then patients in whom mutations were detected will undergo an additional systematic evaluation of patient records. As a bone marrow histology is not available in these patients, a complete exclusion of dysplasia is not possible and thus blood counts at study inclusion will be assessed to differentiate CH from possible differential diagnoses that present with blood count abnormalities (e.g. myeloproliferative neoplasms (MPN), myelodysplastic syndrome (MDS), or clonal cytopenia of undetermined significance (CCUS) (26)). If a blood count abnormality is discovered, the possibility of an underlying hematologic disease will be discussed internally. The diagnosis of a myeloid malignancy will be an additional exclusion criterion.

Moreover, I want to evaluate a potential association between CH and overall survival, cancer-associated VTE, and ATE.

To achieve this goal we will use samples from our cohort of patients with cancer from the Vienna Cancer and Thrombosis Study (CATS, EK: 126/2003). CATS is a prospective observational cohort study that included patients with newly diagnosed or recurrent cancer. It was designed to identify risk factors for cancer-associated VTE and thus followed patients for 2 years for the primary outcome VTE.

An individual who met any of the following criteria was excluded from participation in this study:

- incapacity or refusal of informed consent
- <18 years of age
- overt bacterial or viral infection, venous or arterial thromboembolism within the last 3 months
- continuous anticoagulation with vitamin K antagonists or low-molecular-weight heparin (LMWH)
- surgery or radiotherapy within the past 2 weeks and chemotherapy within the past 3 months

This study is conducted in full conformity with the International Conference of Harmonization (ICH) guidelines on Good Clinical Practice (GCP) and the Declaration of Helsinki by the World Medical Association (WMA).

4. Rationale

The conceptual base of our study is provided by 4 points: **1.** CH is common in the general population. It has been associated with various diseases, as it seems to influence the inflammatory responses of leukocytes. Thus, it would be useful to have a method to rapidly and cost-effectively screen for CH in various study cohorts. **2.** The prevalence of CH is very high in patients with cancer. Furthermore, it has been found to be associated with poor overall survival in this patient population, thus it would be of interest to identify the pathomechanism responsible for this observation. **3.** The burden and incidence of ATE and VTE are high in patients with cancer. The exact pathophysiologic mechanism underlying this observation could not be defined so far. **4.** Due to the proposed pathomechanism involving increased inflammation monocytes, and platelets that lead to increased ATE risk in patients with CH, an additional association with VTE seems likely.

2.1 Objectives

Primary objective:

- To establish a custom Amplicon-based NGS Analysis for assessing the most common CHIP-like mutations
- To quantify the frequency of CH in our cohort of patients with cancer

Secondary objective:

- To identify a possible association between CH and VTE, ATE, VTE and ATE case-fatality rate in a cancer cohort
- To identify the impact of CH on all-cause mortality in patients with cancer

Strategies to achieve our objectives:

- Analysis of a predefined cohort of patients with cancer by quantifying CHIP-like mutations using Targeted Next Generation Sequencing (NGS) of genomic DNA derived from peripheral whole blood samples
- Data and statistical analysis to assess the influence of CH on clinical outcomes and overall survival

5. Expected impact of this project

One main expected impact of this project is to establish a new approach for CH assessment. A time- and cost-effective assay to screen for CHIP-like mutations in other research cohorts would be useful in the future. This would enable an easy screening and evaluation if there is an association for example between CH and VTE in other high thrombotic risk populations. Furthermore, as CH seems to have an impact on the inflammatory environment by increasing the inflammatory responses of leukocytes, it is an area of huge interest to evaluate its influence on various other diseases. This ongoing and future research could profit from such an approach significantly.
In addition, this project should answer the question if CH is associated with cancer-associated thrombosis. Further, it should give more insight into why patients with CH have a poor prognosis. This would be of interest as it could lead to an improved risk assessment in patients with cancer and further on lead to preventive strategies. It could also help in answering the very difficult question of how one should deal with cancer patients with CH.

6. Work and time plan

Tasks	Months 0-3	Months 3-6	Months 6-9	Months 9-12	Months 12-15	Months 15-18
Establishing Method ○ Designing custom primers for exons of interest ○ Evaluating correct binding of custom primers ○ Evaluating correct amplification of exon loci of interest ○ Evaluating optimal cycling conditions of both Multiplex and Index PCR ○ Performing Spike-In NGS test run						
Performing Analysis ○ Conducting NGS analysis of 1,000 patient samples						
NGS Data Analysis ○ Filtering of variants detected ○ Variant calling						
Interpreting Results ○ Interpreting pathogenicity of Variants ○ Comparing results with literature						
Final Report and Manuscript Writing ○ Statistical analysis ○ Summarizing data and final analysis of results ○ Critical discussion of results ○ Writing of manuscript						

7. Financial Aspects

Laboratory costs

Product	Estimated costs	Company
Qiagen Multiplex PCR Kit	2,500€	Qiagen
2x Kapa HiFi HotStart ReadyMix PCR Kit	700€	Kapa Biosystems
XT Nextera Index primer kit v2 sets (A, B, C, D)	4,500€	Illumina
Exonuclease	250€	New England Biolabs
Shrimp Alkaline Phosphatase	220€	New England Biolabs
Custom Primers	1,000€	Sigma Aldrich
Tris Buffer Solution	100€	VWR
6x Illumina Flow Cell	6,500€	Illumina
AMPure XP	400€	Beckman Coultier

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