Platelet RNA as Pan-Tumor Biomarker for Cancer Detection
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ABSTRACT

Blood-based liquid biopsies are considered a screening approach for early cancer detection. Sequencing technologies enable in-depth analyses of nucleic acids, including mutant cell-free (cf) DNA in the plasma. However, in the blood of patients with early-stage cancer the detection level of mutant cfDNA is relatively low, and complicated by the natural presence of non-cancer cfDNA mutants attributed to aging-related processes. Consequently, analysis of methylated cfDNA patterns and alternative approaches such as tumor-educated platelets are gaining traction for the detection of early-stage tumors. Here, we dissect the use of platelet RNA as a potential biomarker for the development of early-stage, pan-cancer blood tests.

Nucleic acids-based detection of cancer with liquid biopsies has gained significant attention in recent years. Various blood-based biosources are being evaluated, including plasma, serum, extracellular vesicles, and circulating tumor cells (1). Blood platelets are a relatively new biosource for the detection of cancer, even though their role in cancer biology was already established more than a century ago (2, 3). Platelets are widely known for their function in blood clotting. Moreover, their involvement in inflammation, cancer progression, and metastasis has been extensively studied. Platelets are present in the bloodstream in large numbers and can be easily isolated. They lack a nucleus but do contain pre-mRNA transcripts that can be spliced into mature mRNA and translated into thousands of different proteins once activated by external signals (2, 3). Platelets are known to play an intricate role in cancer signaling, in particular in angiogenesis and immune evasion, providing platelets with an abundance of tumor-associated “educational” signals and potentially resulting in an altered pattern of spliced RNA biomarkers of value for the detection of cancer. It was demonstrated that RNA-seq of platelets via the thromboSeq assay resulted in different spliced RNA profiles when comparing patients with cancer and healthy controls. The thromboSeq method allows for the detection of brain tumors as well, which are notoriously hard to detect by blood-based liquid biopsies. In the past years, thromboSeq has been evaluated, optimized, and standardized, enabling the development of a pan-cancer detection and tumor organ-of-origin (TOO) localization tests on the basis of altered spliced RNA profiles in platelets (4). Here, we discuss the potential of tumor-educated platelets (TEP) as a pan-cancer biomarker trove.

An emerging strategy to prevent cancer-related death is to focus on earlier detection of primary and recurrent tumors (5). Blood-based liquid biopsies are therefore considered to be a holy grail for earlier detection of cancer. It has been known for decades, if not longer, that blood is a rich source of tumor-associated biomarkers. Moreover, analysis of blood enables screening for multiple tumor types in parallel, from a single or a few tubes of blood, as was shown for spliced RNA patterns of TEPs and mutated or methylated cell-free (cf) DNA patterns of plasma.

Recently, it was revealed that mutant cfDNA analysis for the detection of early-stage cancer may be compromised by the presence of mutant cfDNA in individuals without cancer (1, 5). One could consider this a setback for the blood-based earlier detection of cancer via mutational analyses. The cfDNA mutants measured in blood were initially regarded to be directly and only derived from cancer cells, and hence preferred over any alternative measurement of indirect so-called surrogate markers. However, aging-related (noncancer) mutant cfDNA may affect the specificity of these presumed direct cancer biomarkers. The detection of tumor recurrence through mutant cfDNA may be less affected by the presence of age-related cancer cfDNA molecules, in particular if specific and unique mutations from the initial tumor tissue biopsy can be tracked via cfDNA. Moving to methylated cfDNA patterns may overcome some of the limitations of mutant cfDNA for early-stage cancer detection. It should be noted that patterns of cfDNA methylation, like platelet RNA, can be derived from many different origins, including stromal and immune cells, and not only from cancer cells per se. It could be argued that especially for early cancer detection even more attention should be given to the measurement of microenvironmental factors. In particular early-stage cancer may well be measured via mutation-independent aberrations in the initial tumor, its microenvironment, and the systemic response toward the originating and progressing oncogenic events (5).

As circulating platelets interact with various cells and molecules in the blood stream and their transcriptome is altered in response to extracellular queues, they can be considered as early responders to local and systemic perturbations in the human body (2). Platelets also infiltrate in tumor tissue (6), suggesting that platelets continuously colonize and evacuate from the tumor microenvironment. Platelets originate from megakaryocytes in the bone marrow and lung. They are the second most-abundant cell type in peripheral blood, after red blood cells. Although the size of the platelet population can increase or decrease during infection, cancer, or bone marrow disease, there is general consensus that healthy individuals have 200–500 million platelets per mL of whole blood. For blood-based cancer diagnostics and prognostics in multiple tumor types and stages, reports dating back to decades describe the potential diagnostic use of platelet counts, platelet size, and platelet protein markers such as platelet factor 4, thrombopsondin, thrombopoietin, and P-selectin. In addition to internal alterations of the platelet RNA content, complete platelet
subpopulations may be shifted as well in patients with cancer. For instance, in patients with mammary lung metastasis, as opposed to patients with primary lung cancer, a distinct population of larger CD44+ platelets was recently identified, at least partly explaining differences in platelet content observed in patients with different types of cancer. Platelets circulate for approximately 7–10 days in the bloodstream prior to degradation in the spleen. During that time, the combination of specific splice events in response to external signals and the capacity of platelets to directly ingest (spliced) circulating RNA can provide platelets with a highly versatile RNA repertoire. We previously observed that TEPs from patients with glioblastoma seques- ter tumor-derived EGFRVIII-mutant RNA molecules, and uptake of clinically relevant biomarkers in TEPs from patients with non–small cell lung cancer (NSCLC) or prostate cancer was con-

sidered. However, the measurement of such directly ingested tumor-derived TEP markers is only feasible with very sensitive and targeted methods, and not so much via shallow sequencing methods such as thromboSeq. The detection sensitivity of tumor-derived transcripts may also depend on the expression of the correlating wild-type transcripts in platelets, and hence targeted TEP approach does not seem very promising for the detection of early-stage cancer, also due to the relatively low shedding of tumor-derived nucleic acids and the potential noise caused by the presence of naturally occurring age-related–mutant nucleic acids.

After introduction of high-throughput characterization methods, such as RNA-sequencing techniques, a much deeper insight on the entire platelet RNA repertoire has been achieved, including the discovery of small and longer regulatory RNAs. So far, several RNA families have been identified in platelets, including precursor and mature miRNAs, mRNA fragments, ribosomal RNAs, small nuclear and small nucleolar RNAs, antisense RNAs, and transfer RNAs (2, 3). Advances in molecular analysis techniques allow for the high-throughput profiling of platelet RNAs from relatively small blood volumes, matching the equivalent of a single drop of blood (which equals ~50 mL of whole blood). Quantification of the RNA content in platelets indicated that a single platelet can contain an estimated approximately 2 fg of RNA. It has been described that younger, reticulated platelets have a 20–40-fold enriched total RNA concentration, as measured by RNA-binding thiazole orange staining. This suggests that younger platelets contain more (unspliced) RNAs that might be subjected to decay or released while platelets circulate or become activated (2). The presence of a functional spliceosome, required for splicing of premature mRNA into mature mRNAs molecules for protein translation, and previously thought to be present in only nucleated cells, was somewhat unexpected. The enriched levels of RNA in younger platelets and the presence of a splicing machinery suggests that this mechanism might serve as a gatekeeper for pre-mRNA processing and protein translation. Measuring the spliced mRNAs in conjunction with other RNA families may further increase the potential of TEPs for earlier cancer detection (3).

In 2010, Calverley and colleagues (7) used microarray analysis to profile the platelet mRNA of seven healthy individuals and 5 patients with metastatic NSCLC (reviewed in refs. 2, 3). The platelet samples of the patients with lung cancer were collected prior to any treatment, and 200 altered RNAs were discovered between the healthy individuals and the patients with NSCLC, of which, 197 transcripts (99%) were decreased in platelets of patients with NSCLC. This study also identified the presence of alternative mRNA splicing mechanisms in platelets of patients with NSCLC as compared with healthy individuals, and proposed that platelet RNA might have a predictive role for the detection of metastatic lung cancer. The thromboSeq platform was developed as a shallow sequencing platform, and based on RNA-sequencing for the identification of spliced RNA profiles from minute amounts of platelet RNA (100–500 pg). The thromboSeq assay is combined with machine learning classification algorithms to profile and classify shallow RNA profiles of hundreds of platelet samples as surrogate markers for the detection of cancer. In an initial study, patients with localized and metastasized cancer were discriminated from healthy individuals with 84%–96% accuracy, and TEP RNA profiles pinpointed the TOO of the primary tumor with 71% accuracy. In addition, spliced RNA surrogate signatures associated with the molecular subtype of the tumor tissue were identified, although here the sample numbers were relatively low and the data may therefore be subject to some degree of overfitting. This proof-of-concept study in late-stage cancers was followed by a study that included age-matched series and patients with inflammatory conditions, enabling the diagnosis of late-stage NSCLC with an AUC of 0.94 in an n = 518 late-stage NSCLC-independent validation cohort, and with an AUC of 0.89 in an n = 106 locally advanced stage I–II NSCLC-independent validation cohort (reviewed in refs. 2, 3). Since the Calverley, Nilsson and Best and colleagues’ studies (2, 3, 7, 8), other groups have demonstrated the use of TEPs. Recently, Xing and colleagues (9) confirmed that RNA-sequencing of platelets can identify TEP biomarkers for the detection of lung cancer, and ITGA2 was identified and validated by RT-PCR to distinguish patients with stage I–II NSCLC (n = 243), healthy controls (n = 150), and patients with benign lung nodules (n = 141), with an AUC of 0.92. Liu and colleagues (10) demonstrated that a three- marker TEP signature (MAX, MUTCN, and HLA-B) enabled detection of early-stage NSCLC with AUCs up to 0.82, and Yang and colleagues (11) used a similar approach and identified through RNA-sequencing TIMP1 as a TEP biomarker for the detection of early- and late-stage colorectal cancer (n = 286) with an AUC of 0.96. Moreover, the TEP TIMP1 assay also enabled a similar discrimination efficiency between ulcerative colitis (n = 22) and Crohn disease (n = 23) and stage I–IV colorectal cancer (n = 286). Recently, Yao and colleagues (6) employed the publicly available thromboSeq files and validated TPM3 by qRT-PCR as platelet RNA marker for the detection of breast cancer, with an AUC of 0.971 for healthy controls (n = 109) versus stage I–IV breast cancer (n = 504) and an AUC of 0.840 for nonmetastatic (n = 42) versus metastatic (n = 49) breast cancer. Furthermore, analysis by Takagi and colleagues (12) of patients with smoldering multiple myeloma revealed differential TEP RNA profiles between patients with myeloma and healthy controls, potentially further broadening the multi-cancer applicability of TEPs to hematologic malignancies. Several of these studies included confirmation that the thromboSeq protocol for platelet isolation results in relatively pure platelets, with only a minimal contamination by leucocytes. Further studies to determine the value of TEPs for the detection of early-stage cancer are ongoing, including in patients with idiopathic venous thromboembolism at risk for having (occult) cancer, in which TEPs, cfDNA, and serum protein analyses are aimed to be compared and combined (NCT02739867).

The use of TEPs also has limitations that require attention and which are listed here. Currently, cfDNA is often isolated from Streck or other preserving blood collection tubes to protect the cfDNA in the blood after withdrawal and to prevent nucleated cells from dying and releasing their nonnuclear DNA in the tube. However, such tubes are not per se suited for platelet isolation, and for thromboSeq it is recommended to use standard EDTA-coated tubes instead. In addition, whereas urine is explored as an alternative source of tumor-derived cfDNA, this fluid clearly is not an optimal source for platelets, limiting the thromboSeq measurements to blood. More research also...
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needs to focus on the effects of drugs on the spliced platelet RNA profiles, including the use of aspirin and other drugs that may directly affect platelet activity. Other factors that may influence the spliced platelet RNA profiles may be due to effects of other diseases such as infection and inflammation. Although the ultimate goal is to screen asymptomatic individuals for the presence of cancer, the assessment of noncancerous diseases requires careful assessment, also in the context of comorbidities that may potentially overshadow the early detection of cancer. Another application of platelet RNA in addition to early detection could be the monitoring of tumor progression as a potential alternative to imaging techniques or the possibility of molecular risk stratification. Longitudinal studies are currently performed to determine the stability of the TEP signatures per patient during treatment, and possibly allowing platelet-based monitoring of treatment responses. Last but not the least, the biological functions of the altered platelet transcripts require attention and may help to further define the most robust TEP signature. It was recently shown that tropomyosin 3 (TPM3) mRNA is increased in platelets of patients with breast cancer (6). TPM3 is a member of the tropomyosin family of actin-binding proteins, associated with multiple cancers including breast cancer, and capable of inducing cancer cell invasion. TPM3 delivered via platelet microparticles to breast cancer cells induced their migration and invasion, which could be blocked by inhibition of TPM3 via siRNAs (6).

More research is warranted to understand how platelet “education” is caused and what the functional implications are. Moreover, it is not unlikely that a further understanding of the biological function of altered platelet transcripts may provide new therapeutic opportunities. Apart from the detection of cancer, a blood test requires to provide a lead for clinical follow-up diagnostics, that is, to identify where the tumor can be located. Early reports have already shown that both spliced RNA patterns of TEPs and mutated or methylated cfDNA patterns of plasma can contain information on the presence of a tumor and its TOO. It is not unlikely that within the coming years a first-generation multi-cancer cfDNA-based blood test for early-stage cancer detection will become available. For screening, a test specificity of 99% or more is needed, which will come at the cost of sensitivity. Previous studies suggest that cfDNA and TEPs have the potential to reach 99% specificity (1–5), and large-scale validations in early-stage pan-cancer settings are ongoing. A step to further improve such first-generation blood tests could be by the combination of different biomarker types in a multi-analyte blood test, for example, by combining methylated cfDNA and platelet-derived features. A key challenge will be to subject the various blood analytes to concerted big data analyses to identify a reproducible combination of features representing cancerous “needles in the haystack.”

Disclosure of Potential Conflicts of Interest

T. Wurdinger has ownership interest (including patents) in inventorships and shares. No potential conflicts of interest were disclosed by the other authors.

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