

Tumor-Educated Platelets as a Noninvasive Biomarker Source for Cancer Detection and Progression Monitoring

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Abstract

Liquid biopsies represent a potential revolution in cancer diagnostics as a noninvasive method for detecting and monitoring diseases, complementary to or even replacing current tissue biopsy approaches. Several blood-based biosources and biomolecules, such as cell-free DNA and RNA, proteins, circulating tumor cells, and extracellular vesicles, have been explored for molecular test development. We recently discovered the potential of tumor-educated blood platelets (TEP) as a noninvasive biomarker source for RNA biomarker panels. TEPs are involved in the progression and spread of several solid tumors, and spliced TEP RNA surrogate signatures can provide specific information on the presence, location, and molecular characteristics of cancers. So far,

TEP samples from patients with different tumor types, including lung, brain, and breast cancers, have been tested, and it has been shown that TEPs from patients with cancer are distinct from those with inflammatory and other noncancerous diseases. It remains to be investigated how platelets are "educated," which mechanisms cause intraplatelet RNA splicing, and whether the relative contribution of specific platelet subpopulations changes in patients with cancer. Ultimately, TEP RNA may complement currently used biosources and biomolecules employed for liquid biopsy diagnosis, potentially enhancing the detection of cancer in an early stage and facilitating noninvasive disease monitoring. *Cancer Res*; 78(13): 3407–12. ©2018 AACR.

Introduction

Liquid biopsies are considered to represent a promising tool for the early detection of cancer and subsequent noninvasive tumor profiling and monitoring. Currently, cancer is diagnosed by clinical presentation, radiology, and biochemical tests, while for definitive diagnostics, pathologic analysis of tumor tissue is generally required. Due to the increasing number of cancer screening tests, for example, by mammography and low-dose computed tomography lung imaging, tumors can be detected at an earlier stage while patients are still asymptomatic. In addition, clinical oncology practice relies on (repeated) removal of tumor tissue through biopsies for analysis of tumor-linked genetic alterations and other cancer biomarkers. Although tumor tissue biopsy is the current gold standard for cancer diagnosis and represents an essential tool in cancer management, it has become clear in recent years that the information acquired from a single biopsy provides a spatially and temporally limited snapshot of a tumor and often fails to reflect the heterogeneity of the disease. Moreover, the

fact that tumor biopsies are invasive poses a limitation for repeated sampling (1).

It has been known for decades that blood is a rich source of tumor-associated biomarkers, which can be isolated from several biosources (1, 2). These include the mononuclear cell fraction, encompassing leucocytes, circulating tumor cells (CTC), circulating endothelial cells (CEC), and plasma and serum, which contain extracellular vesicles (EV), cell-free DNA (cfDNA) and RNA (cfRNA), plasma proteins and metabolites, and tumor-educated platelets (TEP; ref. 2). These biomolecules and biosources are being regarded as part of the protumorigenic and systemic activity of the primary tumor (e.g., CTCs, CECs, EVs, cfRNA, and TEPs) or are considered to derive from merely passive release during tumor cell apoptosis and necrosis (e.g., cfDNA). It has been shown that each biosource and biomolecule in blood has its potential for blood-based cancer diagnostics, companion diagnostics, prognostics, and therapy monitoring. For example, analysis of cfDNA can be used for detection of point mutations or structural variants but also of copy-number aberrations, differential cfDNA length, and methylation status. Furthermore, EVs have been shown to harbor proteins and RNA biomarker molecules of variable length, but also surface membrane proteins that are correlated to organ tropism for cancer metastasis (3). TEPs are considered as local and systemic responders to the presence of cancer (4), thereby sequestering EV-derived RNAs (5–7) and proteins (8), as well as altering their spliced RNA profile (9–12). Interestingly, a combined readout of multiple biosources and biomolecules, here plasma-derived cfDNA and proteins, might leverage the information included in each individual source (13), resulting in the detection of 62% of stage I–III cancers with >99% test specificity, and providing an innovative approach for detection of cancer in an early stage. In all, liquid biopsies may

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doi: 10.1158/0008-5472.CAN-18-0887

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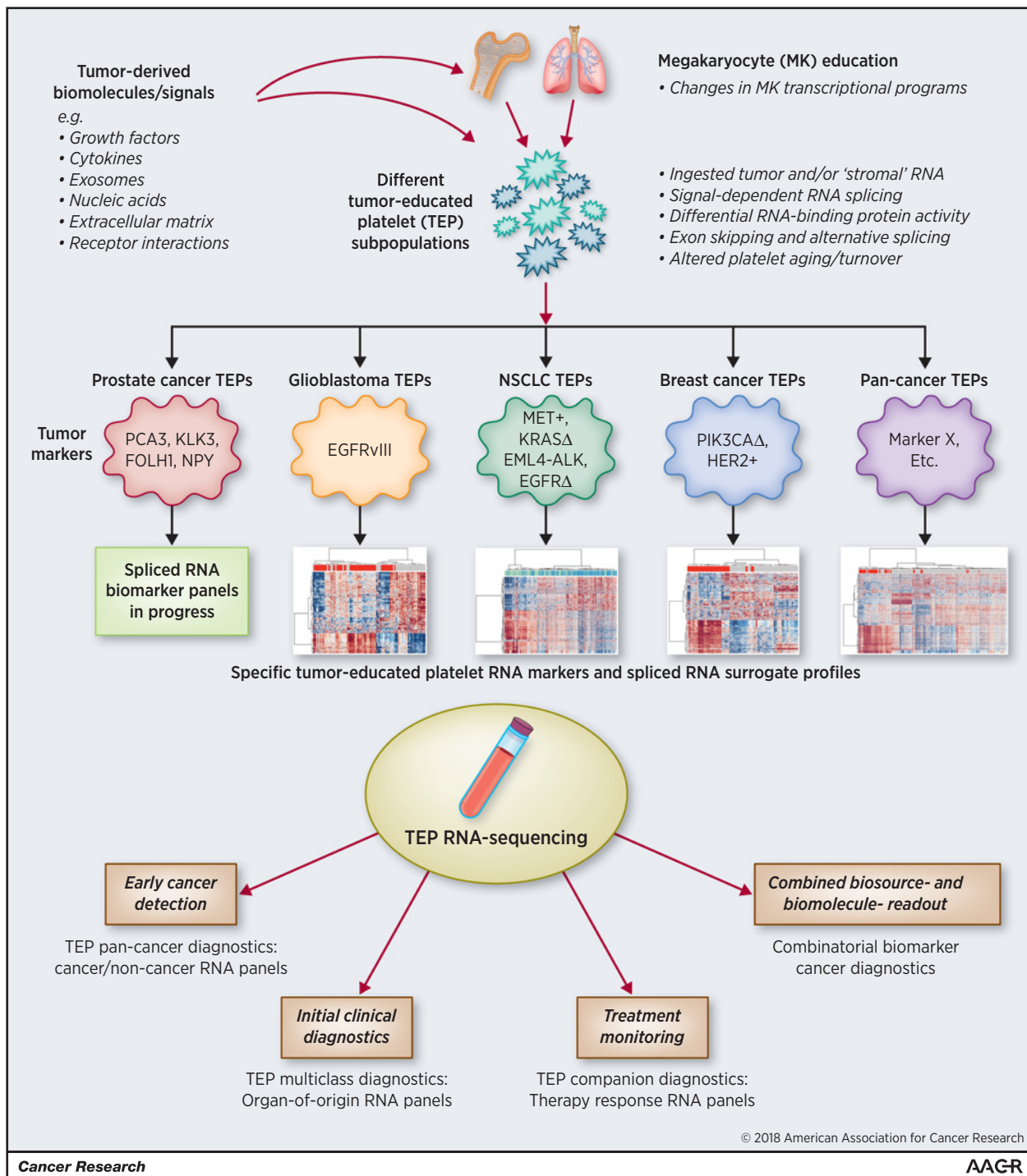
enable (i) early detection of cancer (screening), (ii) prognostication for the individual patient by providing information about stage and spread of the disease, (iii) identification of new targets for personalized treatment, (iv) pretreatment therapy response prediction, (v) early therapy response monitoring and "real-time" assessment of treatment effectiveness, and (vi) early detection of recurrence of the disease.

The Contribution of TEPs to the Spread of Cancer

In the past decade, appreciation has been growing for the contribution of platelets to immune and inflammatory activities in health and disease, including the progression of cancer (4). Platelets can be regarded as "scanning soldiers" of the immune system, thereby sensing the presence of bacteria entering the bloodstream, cross-communicating with lymphocytes, and partially regulating immune cell extravasation (14). During the presence of cancer, platelets have an effect on the tumor-resident cancer cells as well as the cancer cells that have entered the bloodstream. First, platelets create an environment supportive of neovascularization by supplying the tumor with multiple proangiogenic factors such as VEGF, PDGF, and bFGF and by stimulating expression of these factors (15). Next, platelets reduce local tumor cell apoptosis and anoikis (16) and are able to induce the epithelial–mesenchymal switch in tumor cells by direct physical interaction and release of TGF β molecules (17). Subsequently, the ability of platelets to protect tumor cells in the circulation from the normal immune response is likely to significantly contribute to the metastatic process (4). Then, platelets form a cell–fibrin–platelet aggregate surrounding CTCs or arrested tumor cells, providing mechanical protection, and transfer MHC class I proteins to the CTCs, resulting in additional protection against natural killer cells. Recent lab-on-a-chip microfluidic device analysis revealed the presence of a potential CTC subpopulation with enhanced platelet coverage and possibly platelet-mediated protection (18). Upon activation, platelets can release several growth and proangiogenic factors (8), and once these factors are released at a metastatic niche, a tumor-stimulating growth microenvironment is created (4). Thus, platelets are a fundamental component of the tumor microenvironment and are considered an important aspect of cancer biology as they contribute to tumor initiation, tumor progression, and therapy response. Interest has been renewed in cancer prevention mediated by daily use of aspirin, a controversial topic spanning several decades. Aspirin may both inhibit tumor-intrinsic cyclooxygenase activity, which might control intracellular signaling pathways and the local inflammatory response, and platelet-resident cyclooxygenase activity, which can influence processes such as platelet activation and aggregation (19). Platelet aggregation inhibitors, such as ticagrelor, clopidogrel, and prasugrel used in cardiology, provoked interest and exerted an inhibitory effect on metastatic spread in intravenous and intrasplenic melanoma and breast cancer mouse models (20). Also, these drugs target receptors essential for thrombus formation (e.g., adenosine diphosphate receptor) that were found to influence mechanisms leading to cancer progression as well, mainly by regulating tumor cell/platelet interaction and angiogenesis. Hence, platelets mediate crucial steps in tumor progression and the systemic spread of cancer.

TEPs Enable for Blood-Based Cancer Diagnostics and Treatment Monitoring

Studies have shown that platelet count and platelet size can already provide clinically relevant information about the presence of cancer (21). High platelet count is associated with increased mortality in a variety of cancers such as malignant mesothelioma, gynecological malignancies, and lung, renal, gastric, colorectal, and breast cancers. It has indeed been shown that tumor-derived platelet factor 4 (PF4, CXCL4) promotes bone marrow megakaryocyte-mediated platelet production in patients with non-small cell lung cancer (NSCLC; ref. 22). Also, elevated pretreatment platelet-to-lymphocyte ratios have been reported to correlate with a reduced response rate to nivolumab anti-PDL1 immunotherapy (23), indicating that circulating platelets may enhance a protumorigenic effect in the presence of an antitumor immune response (24). Interestingly, it has also been shown that platelets can infiltrate in tumor tissue (22), suggesting that platelets may continuously colonize and evacuate from the tumor microenvironment. We initially focused on the noninvasive diagnostics of brain cancer and observed that TEPs from patients with glioblastoma sequester tumor-derived EGFRvIII mutant RNA molecules (5). Next, uptake of clinically relevant biomarkers in TEPs from patients with NSCLC or prostate cancer was confirmed (6, 7). Then, microarray analysis revealed that a panel of RNAs was altered in the TEPs from patients with glioblastoma as compared with those from healthy controls (5). Similar observations were made in patients with metastatic NSCLC (11). Subsequently, the thromboSeq platform was developed, an RNA-sequencing-based methodology that enables identification of spliced RNA profiles from minute amounts of platelet RNA (100–500 picogram, the equivalent of platelets in a single drop of blood) combined with sophisticated machine learning-based classification algorithms, to profile and classify the RNA profiles of hundreds of platelet samples (Fig. 1). This enabled discrimination of patients with localized and metastasized cancer from healthy individuals with 84% to 96% accuracy (9). Also, we were able to pinpoint the organ-of-origin of the primary tumor with 71% accuracy and, if both the first and second best suspected organ as indicated by the classification algorithm were selected, even up to 89% accuracy. Moreover, spliced RNA surrogate signatures were identified, associated with the tumor tissue molecular subtype, such as *EGFR* and *KRAS* mutations and *HER2* and *MET* amplifications with 85% to 95% accuracy, though the number of samples analyzed here remained relatively low (9). By gene ontology analysis, an enrichment of RNAs implicated in platelet activity and platelet vesicles and a decrease in RNAs implicated in RNA maintenance and splicing was observed (9). This proof-of-concept study was followed by a follow-up study that included additional analyses of age-matched cohorts and patients with inflammatory conditions (10). In this follow-up study, time between blood and platelet isolation was also standardized as this potentially influences platelet RNA profiles. In addition, a notorious challenge in biomarker gene or RNA panel discovery studies was addressed: selection of a robust biomarker set. To overcome this challenge, an algorithm iteratively optimizing the biomarker spliced RNA panel during the training process, termed swarm-intelligence, was implemented (10). This approach enabled the diagnosis of late-stage NSCLC with an accuracy of 89% in an >500-sample late-stage NSCLC-independent validation cohort, and with an accuracy of 81% in an >100 locally advanced-stage I–III NSCLC-

**Figure 1.**

TEPs for blood-based cancer diagnostics. **A**, Altered TEP RNA profiles may be caused by multiple processes, including tumor-derived biomolecules and signals, alterations in the megakaryocytes transcriptional programs, and shifts in platelet subpopulations. These queues may induce ingestion of tumor- or stromal-derived RNAs, differences in RNA splicing, altered RNA-binding protein activity, alternative splicing mechanisms, and platelet aging. This has resulted in the detection of specific TEP RNA markers [e.g., PCA3, KLK3, FOLH1, and NPY in prostate cancer (7), EGFRvIII in glioblastoma (5), and EML4-ALK in NSCLC (6)] and spliced RNA surrogate profiles [e.g., diagnostic panels for the detection of glioblastoma (5, 9), NSCLC (9, 10), breast cancer (9), and a pan-cancer cohort (9), or KRAS, EGFR, PIK3CA, and HER2 mutants in NSCLC or breast cancer (9)]. **B**, The altered TEP RNA repertoire may allow for blood-based pan-cancer, organ-of-origin, and companion diagnostics, possibly combined with readout of other biosources/biomolecules of the same blood sample.

independent validation cohort (10). Recent analysis in patients with multiple myeloma identified differential TEP RNA profiles between healthy individuals and patients with smoldering multiple myeloma (12), indicating that platelet education also occurs in hematologic malignancies.

Apart from detection of cancer, TEPs have been investigated for the monitoring of cancer progression. As TEPs sequester tumor-derived RNA molecules, including the EML4-ALK fusion transcripts, it has been shown that effective anti-EML4-ALK crizotinib therapy in a patient with NSCLC lowered the number of EML4-ALK transcripts in circulating TEPs (6). Due to the lifespan of ~7 to 10 days of a regular platelet, tumor-derived transcript can accumulate in the TEPs and be protected for circulating plasma-derived RNases, and thus it can be expected that TEP RNA analysis will reveal an up-to-date, enhanced, and dynamic reflection of the tumor's activity.

Biological Mechanisms Possibly Responsible for Education of Blood Platelets

The exact queues and subsequent specific effects causing alterations in the TEP RNA repertoire remain unknown. The current hypothesis to explain this observation is based on the potential effect of external queues derived from the tumor and its micro-environment causing intraplatelet premature mRNA (pre-mRNA) splicing. In all, platelets in patients with cancer have phenotypically a proactive state, as indicated by a reduced threshold to present surface-membrane activation markers such as p-selectin, and the enhanced incidence of thrombo-embolism in these patient populations. Though platelets are anucleated cell fragments and lack a nucleus, they are packed by the megakaryocyte—their precursor cell present in both the bone marrow and, as recently identified, the lungs—with a pool of premature RNAs (pre-mRNAs), a functional spliceosome, and a protein translation machinery (25). Aside presence of (pre-)mRNAs, platelets contain small RNAs including microRNAs (miRNA), long noncoding RNAs, circular RNAs (circRNA), and mitochondrial DNA (26). Younger platelets have a higher RNA content, as opposed to "older" platelets (27), indicating that the RNA supplied by the megakaryocyte is required by the platelets for activities while circulating in the bloodstream or homing/infiltrating to areas of primary tumor burden and/or metastatic sites. Indeed, platelet activation induced via several stimuli for example released by bacteria, such as LPS and staphylococcal-derived alpha-toxin, can induce subsequent pre-mRNA splicing and protein translation (28). Similarly, release of biomolecules by cancer cells and the tumor microenvironment (stromal and immune cells) may induce splicing events, though the exact factors and accompanying splice alterations remain unknown. It has been suggested that the splicing events can only partially be explained by alternative splicing events and that the splicing events may be partially dependent on the activity of RNA-binding proteins (RBP; ref. 10). *In vitro* analyses revealed that RBP Clk1/SRSF1 enables for splicing of tissue factor mRNA (25). Similarly, we hypothesized that enriched binding sites for RBPs in the 5'- and 3' untranslated region sequences, partially responsible for splicing induction and correlating with enriched RNA levels in TEPs, may result in differential RBP panels and that more RBP binding sites enable more splicing (10). Additional regulation of pre-mRNAs may be performed by microRNAs and other small RNAs (29), including

perhaps circularization and decircularization of circRNAs (26). Also, the effect of tumor-derived queues on the bone marrow and lung-resident megakaryocytes and their RNA production remains unknown, but it is expected that stressed megakaryocytes alter their RNA repertoire (30).

Besides intraplatelet RNA splicing, platelets are able to continuously physiologically exchange nucleic acids and proteins with other platelets, immune cells, endothelial cells, but also tumor cells, for example, by vesicle-mediated transport mechanisms. It was shown that tumor-associated biomolecules are transferred to platelets, leading to their "education" (5, 6, 9). Alternatively, platelet-derived microparticles shuttle to tumor cells, thereby regulating both anti- and protumorigenic gene expression programs in a miRNA-dependent manner (31). The capability to sequester proteins and nucleic acids, possibly also including cfDNA, seems to be inherent to platelets during their entire lifespan (5), although the exact mechanism of EV sequestration and internalization and whether the platelets have a selection mechanism for specific vesicle sizes and types remains unknown. Also, whether a subpopulation of platelets more sensitive for biomolecule sequestration exists, for example, young, reticulated platelets, in which platelet compartment the sequestered material is stored, and whether platelets actively translate sequestered nucleic acids remains unknown. Thus far, we expect, as indicated by sequestration of only low copy numbers of tumor-specific mutant RNAs, that the relative level of sequestered RNAs as opposed to the megakaryocyte-inherited RNAs remains low. Thus, the combination of specific splice events in response to external signals and the capacity of platelets to directly ingest (spliced) circulating mRNA is providing platelets with a highly dynamic mRNA content. It has indeed been observed that in the TEPs of patients with cancer an enrichment of spliced RNAs associated with cytoskeleton activation, platelet activation, and ATP activity is present (11, 12).

In addition to internal alterations of the TEP RNA content, platelet subpopulations in patients with cancer might be shifted as well. Platelets circulate for approximately 7 to 10 days in the bloodstream, after which they are degraded in the spleen. They are continuously shed by the bone marrow and lung-resident megakaryocytes, and circulate their first days as young, reticulated, RNA-rich platelets (27). Toward the end of their lifespan, they will become desialylated urging the liver via the Ashwell-Morell receptor to produce thrombopoietin to stimulate subsequent production of new platelets. This process mediates the continuous cycle of platelet generation and breakdown, resulting in multiple platelet subpopulations such as reticulated platelets, procoagulant or 'coated' platelets, and 'old' platelets, in blood (32). As each subpopulation has its particular content, effect, and expected remaining life span, this process is of particular interest for the research of platelets for transfusion medicine, and perhaps also cancer. It has been shown that NSCLC can enhance platelet production by release of PF4 (22), after which platelets are recruited to the lung and tumor parenchyma. Thrombocytosis was associated with increased plasma levels of thrombopoietin and IL6 for patients with ovarian cancer (21). Recent analysis of the effect of platelets in patients with systemic septic shock has uncovered the potential of platelets to extravasate in lung and brain tissue, release proinflammatory mediators in these tissues, and subsequently leave these organs with empty granula (33). We have shown that a TEP-RNA panel containing 698 RNAs associated with the presence of NSCLC shows large overlap (77%) with

a 1,820-RNA gene panel associated with p-selectin, a marker for reticulated platelets (10). Together, we measured enriched total RNA recovery from platelets collected from patients with NSCLC as compared with noncancer controls (10). Recently, Clancy and colleagues profiled the RNA content of small and large platelets, and observed in large platelets an enrichment for gene ontologies associated with platelets/vesicles and hemostasis (34). This observation supports the gene ontologies associated with previous TEP analyses, indicating similar biological processes in the enriched RNAs in patients with cancer (9, 10). In all, it can be hypothesized that in patients with cancer, younger platelets become increasingly represented in blood, possibly resulting in enhanced thrombotic potential. Perhaps, reticulated platelets are able to more efficiently sequester tumor-derived nucleic acid and protein biomarkers, or provide improved shielding capacity for blood-born CTCs (18). It has been proposed that the older, smaller platelets may have sequestered a rich repertoire of vascular cell-derived RNAs during their lifespan (34), indicating that platelet subpopulations correlate with enrichment or depletion of specific transcripts. Further identification of the efficiency of the educational process in cancer for each specific platelet subfraction is of particular interest. Also, shifts in platelet subpopulations during cancer progression and the course of therapy may be of interest for platelet-based liquid biopsies. Additionally, the contribution and role of the "coated" platelets in patients with cancer might be of value. Further, platelet RNA heterogeneity may be uncovered on a single platelet level by, for example, single platelet RNA-sequencing, though technical hurdles such as highly specific, low-activation platelet sorting, and ultra-low RNA input RNA-sequencing remain to be overcome. The identification of specific membrane markers for each of the currently identified and potentially unidentified platelet subpopulations may be crucial in this process.

Concluding Remarks

Liquid biopsies are considered to be a holy grail for earlier, noninvasive detection of cancer. Analysis of blood may enable future screening of multiple tumor types at once with high patient convenience and as opposed to the currently used organ-by-organ tests. Though early detection of cancer may be complemented by lead-time bias, indicating that the cancer is detected earlier, however, not resulting in improved survival rate and detection of potential nonlethal lesions, tumors may be more easily treated at an early, non-advanced stage. To overcome this hurdle, it may be anticipated that early-stage, localized, and slow-growing tumors, such as prostate cancers, are first subject to a "watchful waiting" strategy with frequent liquid biopsy monitoring, meanwhile adequately managing the anxiety that will be elicited in such a situation. Alternatively, faster growing tumors may be more appropriately treated by a rapid therapeutic intervention. For the early detection of cancer, a blood-based test ideally has an extremely high specificity. This is especially of relevance in a general screening population with a low pretest probability of cancer, and the risk of a large number of false-positive test results. The high test specificity may be accepted at cost of sensitivity,

especially once these blood-based screening tests are performed annually. Alternatively, in high-risk patient populations, such as women with a BRCA mutation with a life-time risk for the development of breast cancer of 60% to 80%, a blood-based screening test with high sensitivity and moderate specificity may be considered, thereby ensuring that each early-stage cancer is detected at the cost of test specificity. Development of blood-based tests for the screening of cancer requires inclusion of individuals with inflammatory and other noncancerous disease as controls and need to take into account the accumulation of somatic alterations in both solid tissues and the hematopoietic system as a function of age (35). Apart from the detection of cancer, the blood test requires to provide a lead for clinical follow-up diagnostics. It has been shown that both TEPs and cfDNA can provide information on the organ of origin (9, 13, 36), though the accuracy has to be further investigated and independently validated in blood of patients with or suspected of (early-stage) cancer. In addition, the blood-based test ideally provides a guide on the most effective therapy, from surgery to immunotherapy, according to a "detect and select" concept. It can be expected that the coming years the first commercial blood-based tests for early-stage cancer detection will become available (35). To progress toward national blood-based screening programs for early cancer detection, future clinical validation studies need to be performed in (i) large-sample sized cohorts (>10,000 individuals tested, as recently performed for nasopharyngeal cancer; ref. 37); (ii) well-characterized cancer-confirmed and cancer-suspected patients and noncancer controls matched for potential confounding variables, such as age, gender, and smoking status; (iii) standardized blood processing and storage conditions; and (iv) the combined readout of multiple biosources and biomolecules in parallel. The wealth of data that can be obtained in this way may be subjected to novel bioinformatics algorithms and machine learning software, possibly resulting in previously unidentified patterns in blood indicating the presence and location of a primary tumor. In short, further evaluation and ultimately large-scale validation of the available liquid biopsy biosources and biomolecules, ideally in a combined manner including the TEP RNA repertoire, is thus advised.

Disclosure of Potential Conflicts of Interest

T. Wurdinger reports receiving a commercial research grant from Bristol-Myers Squibb, other commercial research support from Illumina, and has ownership interest (including patents) in GRAIL. No potential conflict of interest were disclosed by the other authors.

Acknowledgments

This work was supported by the European Research Council #336540 and PoC #713727 (T. Wurdinger), the Dutch Cancer Society (T. Wurdinger), Stichting STOPHersentumoren.nl (M.G. Best, P. Wesseling, and T. Wurdinger), and Horizon 2020 Marie-Curie European Liquid Biopsy Academy #765492 (T. Wurdinger). We apologize for all of the excellent reports that we could not cite because of space restrictions.

Received March 23, 2018; revised April 17, 2018; accepted May 2, 2018; published first June 19, 2018.

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