LIQUID BIOPSY AS A TOOL FOR PRECISION ONCOLOGY: NEW CHALLENGES TO ASSESS CLINICAL RESPONSE

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EDITORIAL
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Editorial: Liquid Biopsy as a Tool for Precision Oncology: New Challenges to Assess Clinical Response

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Editorial on the Research Topic

Liquid Biopsy as a Tool for Precision Oncology: New Challenges to Assess Clinical Response

The Research Topic "Liquid Biopsy as a Tool for Precision Oncology: New Challenges to Assess Clinical Response" includes eight papers with more than 50 authors contributing as experts in the field. The Research Topic has the aim to reflect the state of the art on this emerging technology, which is revolutionizing the clinical approach in oncology.

Despite the numerous progresses in early diagnosis and in target therapies, cancer remains the second leading cause of death worldwide. Tumor molecular characterization plays a key role in choosing the right treatment among the armamentarium available to the oncologist. Unfortunately, with the traditional tissue biopsy in some cases analysis of tumor is not feasible due to insufficient or poor quality material (Siravegna et al., 2019). Liquid biopsy has started to be considered a new standard of care for oncological patients mainly after the FDA approval on 2016 of the first blood-based test, to detect EGFR mutations for selecting patients who may benefit from the target therapy (Torres et al, 2020). Indeed, liquid biopsy has several advantages, including its minimal invasiveness and highly repeatability over the time, which potentially guarantees a dynamic picture of the tumor and the chance to monitor pharmacological responses. This last characteristic makes liquid biopsy particularly attractive within the oncological context. Indeed, to date, diagnosis and clinical monitoring have been the two major applications of liquid biopsy, and are well described in our Research Topic.

Kamatham et al., who used circulating tumor DNA (ctDNA) to identify, microsatellite instability status in a pancreatic cancer patient. This led to switch the therapy from chemo to immuno-therapy, with an excellent response. A different case report is presented by Nagaya et al., which uses circulating tumor cell (CTC) analysis to select the right treatment in castration-resistant prostate cancer (CRPC) patients. In this article, the authors evaluated AR-V7 expression in CTC in serial blood tests; based on the results, abiraterone was selected as re-challenge in the setting of post-chemo androgen-targeted-therapy.

Another example of a clinical application of liquid biopsy is presented by Dalle Fratte et al., present a case report of a patient with KIT/PDGFRA wild-type gastrointestinal stromal tumor (GIST). The patient was resistant to the standard treatment imatinib, even after increasing the dose. By using an

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NGS based approach, the authors analyzed circulating free DNA (cfDNA) to investigate somatic changes responsible for imatinib resistance, and identified a sharp increase in the allele frequency of a TP53 mutation, responsible for a protein loss of function, never described before. The same TP53 mutation was retrospectively identified in the primary tumor and in the metastatic hepatic lesion, suggesting a rapid clonal selection of the mutation during tumor progression. Besides these case reports, that highlight the potential of liquid biopsy in different cancer types with no previous clinical indications, two contributions describe the application of liquid biopsy in a high number of patients. Buderath et al. evaluated levels of soluble PD-L1 and PD-L2 in sera of 83 primary epithelial ovarian cancer (EOC) patients and related the results with the presence of CTCs, clinical characteristics and with PFS and OS. The results showed that sPD-L1 and sPD-L2 could be used as complementary biomarkers reflecting clinical status, treatment response and disease outcome of EOC patients. In particular, sPD-L1 may facilitate the identification of high-risk patients with unfavorable disease outcomes despite platinum-sensitivity arguing for additional therapeutic approaches. Pazdirek et al. investigated the potential of ctDNA as blood-based biomarker in patients with locally advanced rectal cancer (LARC) undergoing neoadjuvant chemoradiotherapy (NCRT) prior to surgery. The study included 36 LARC patients undergoing NCRT followed by surgical treatment. Somatic mutations were characterized in tissue biopsies and ctDNA from plasma samples prior to therapy and at the end of the first week of treatment (plasma only). The analysis revealed a ctDNA reduction within the 1st week of therapy, not associated with the clinical response determined. The rapid ctDNA disappearance, unrelated with the therapy outcome, suggests the need for further studies to better understand its clinical significance in LARC patients within the neoadjuvant setting.

Finally, three reviews illustrate the state of the art about the scientific progress in liquid biopsy in different cancer types, highlighting novel aspects. Cavallari et al. provide a critical summary on the potential applications and its pitfalls of circulating molecules in Malignant Pleural Mesothelioma (MPM), an aggressive tumor linked to asbestos exposure. This is often diagnosed at an advanced stage with consequent poor prognosis. MPM diagnosis relies on pleural biopsies, therefore, the development of circulating biomarkers for early diagnosis is of great importance. In particular, the identification of robust non-invasive tests for the screening and risk assessment of asbestos-exposed subjects is still an important unmet clinical need. In this context, the authors reviewed the recent literature with the aim to identify novel blood-based circulating biomarkers for the early diagnosis and prognostic stratification of MPM patients. Despite some other interesting potential biomarkers, including miRNAs, CTCs, and ctDNA, validation in large prospective studies is still awaited.

Chennakrishnaiah et al. focus their attention on the so-called leukobiopsy. Recently, it has been reported that white blood cells (WBCs) may represent a sort of reservoir of circulating oncogenic DNA. Indeed, circulating WBCs, especially neutrophils, may contain high levels of oncogenic DNA, exceeding the amount of normally recovered from soluble fractions of plasma, circulating extracellular vesicles, platelets, and others. In some settings, the WBC content of cancer specific DNA (csDNA) may represent an important source of csDNA contained in serum or in plasma. Another thrilling topic is presented by Tieng et al., who describes the importance of CTC single-cell transcriptomics analyses in colorectal cancer (CRC). Single-cell RNA sequencing (scRNA-seq) is emerging as a breakthrough technology which provides the potential to dissect the different cellular populations of cancers and possibly the intra tumor heterogeneity (ITH). An important point raised by the authors is the role of CTC in CRC metastasis and their characterization through scRNA-seq.

In conclusion, the "Liquid Biopsy as a Tool for Precision Oncology: New Challenges to Assess Clinical Response" Research Topic highlights the importance of circulating molecules as a new tool to achieve personalized therapy. In the last decade, many progresses have been done, but at the same time, many clinical aspects remain to be elucidated. In this context, the papers enclosed in this topic offer the chance to generate a collaborative discussion, contributing to the future direction of liquid biopsy.

AUTHOR CONTRIBUTIONS

GR, MR, AG, RS, and SA wrote the editorial and revised the final drafts. All authors contributed to the article and approved the submitted version.

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REVIEW
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Liquid Biopsy in Malignant Pleural Mesothelioma: State of the Art, Pitfalls, and Perspectives

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Malignant pleural mesothelioma (MPM) is an aggressive tumor linked to asbestos exposure. Although the risk factors for MPM are well-known, the majority of MPM patients are diagnosed at an advanced stage and have a very poor prognosis. Circulating biomarkers for early diagnosis remain to be identified, and the current standard for MPM diagnosis relies on pleural biopsies. Robust non-invasive tests for the screening of asbestos-exposed subjects are therefore an important unmet clinical need. This review provides a critical summary of recent liquid biopsy-based studies aimed at discovering novel blood-based circulating biomarkers for the early diagnosis and prognostic stratification of MPM patients.

Keywords: microRNA, prognostic stratification, early diagnosis, asbestos exposure, liquid biopsy, mesothelioma

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INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare cancer with increasing incidence and dismal prognosis due to its aggressiveness and lack of effective treatments (1–3).

Asbestos exposure is considered the main causative factor for MPM, with a decades-long latency between start of exposure and clinical diagnosis (4). Prolonged exposure to inhaled asbestos fibers trigger an increase in reactive oxygen species (ROS) and inflammatory cytokines in the pleural microenvironment, both of which are key drivers of MPM carcinogenesis (5, 6). However, despite the high ROS burden, MPM is characterized by a low mutation load (7), with tumor suppressors (CDKN2A, BAP1, NF2, LATS2) the most frequently mutated genes involved in MPM pathogenesis (8).

The current standard for the diagnosis and genetic profiling of most tumors involves the use of tissue biopsies (9). However, given its invasive nature, tissue biopsy is burdened with considerable patient morbidity and costs for the health care systems (9, 10). The histopathological diagnosis of pleural biopsies is difficult and may require FISH (fluorescent *in situ* hybridization) of the CDKN2A locus and immunohistochemistry for p16 and BAP1 (11) when invasion is not clearly demonstrated based on the histology, and to confirm the diagnosis of mesothelioma in pleural effusions.

The onset of MPM is insidious and most patients have advanced disease at presentation. Current imaging methods are inadequate for screening and for differential diagnosis of pleural plaques vs. malignant mesothelioma (12). The availability of a robust non-invasive test for the screening of asbestos-exposed subjects is therefore an important unmet clinical need.

"Liquid biopsy" of biological fluids (e.g., plasma and serum, urine, saliva, cerebrospinal fluid, pleural fluid, ascites, stool) is emerging as a powerful tool for non-invasive diagnosis, screening, prognosis, and stratification of cancer patients. This approach is based on the fact that tumor cells

release molecules (proteins, DNA, RNA), circulating tumor cells (CTC), and extra cellular vesicles (EV) that can be used as biomarkers (13) (**Figure 1**). Liquid biopsies may be repeated frequently to provide a more detailed picture of the natural history of the disease with a longitudinal assessment of tumor burden and clues about clonal evolution and emergence of drug-resistant clones leading to clinical relapse (9, 14).

The following sections provide a critical overview of recent studies describing novel circulating biomarkers for the early diagnosis and prognostic stratification of MPM patients.

CIRCULATING TUMOR PROTEINS

Mesothelin

Mesothelin (MSLN) is a cell-surface glycoprotein expressed by mesothelial cells. It is synthesized as a 70-kDa precursor which is cleaved by Furin protease to produce the mature form of Mesothelin and Megakariocyte Potentiating Factor (MPF) (15). Mesothelin is overexpressed in ovarian cancer, pancreatic cancer (15) and MPM, especially in the epithelioid and biphasic subtypes (16).

A soluble form of Mesothelin, named Soluble Mesothelin-related peptide (SMRP), is shed by the tumor cells into the circulation (17–19). Although SMRP is not specific for MPM (17, 20, 21), its role as early biomarker for the screening of asbestos-exposed subjects has been extensively studied (**Figure 1**, **Table 1**).

Almost all of these studies used the U. S. Food and Drug Administration (FDA)-approved Mesomark ELISA assay to detect all soluble forms of the protein. Mesomark is a reliable assay that is not affected by the presence of other molecules (e.g., hemoglobin, triglycerides, bilirubin) (20).

The first study investigating SMRP in the context of MPM showed increased SMRP levels in serum samples from 37 out of 44 MPM patients compared to 40 healthy asbestos-exposed subjects (22). The test also distinguished MPM patients from 18 patients affected by non-neoplastic asbestos-related disease and 122 patients with inflammatory lung diseases or other cancers. Unfortunately, SMRP levels showed low accuracy in identifying patients with sarcomatoid MPM and low tumor size ($<$1 cm). In this study, the authors did not take into account confounding factors such as age, renal dysfunction, and body mass index (BMI) that may "per se" increase SMRP levels (37–40). Other studies confirmed these results (23, 24, 26–28, 41, 42), but were characterized by high heterogeneity regarding the selection of the control study population and cut-off values (18). A meta-analysis of data from 16 different studies indicated low accuracy for early diagnosis because of low sensitivity. Indeed, low SMRP levels did not exclude the presence of malignancy, especially in early-stage disease (18).

Although SMRP cannot be considered an early diagnostic biomarker for surveillance programs, it seemed to be effective in predicting response to chemotherapy and patient survival. This is an important issue, as quantitative radiological measures are difficult for this cancer. In three prospective studies enrolling, respectively, 96, 107, and 100 MPM patients, high baseline SMRP levels significantly correlated with shorter survival (24, 25, 43). In 2010, Creaney et al. performed a prospective evaluation of serum SMRP levels over time in patients undergoing chemotherapy ($N = 55$). These authors found a strong correlation between radiological responses (measured by CT scans according to modified RECIST criteria) and SMRP variations. Specifically, an increase $>$25% was associated with progressive disease (PD), a decrease $>$25% with partial response (PR), and no changes with stable disease (SD). Log-rank analysis showed that a decrease in SMRP was strongly associated with longer survival (44). The results were confirmed in another study, although the authors measured SMRP in the plasma and set the cut-off at 10% variation (45). These studies have some limitations such as the small sample size and the heterogeneity of the treatments received; nevertheless, the usefulness of SMRP as an indicator of tumor response deserves further investigation.

Anti-Mesothelin antibodies (e.g., immunotoxin SS1P) are being tested for MPM and other cancers. In these patients MPF (Megakariocyte Potentiating Factor) may be used as a biomarker to evaluate response to therapy as it does not bind the therapeutic antibodies (46). Serum MPF was analyzed in patients enrolled in two clinical trials evaluating SS1P efficacy: a phase I trial tested first line treatment with SS1P in combination with Cisplatin/Pemetrexed (47) and a pilot study investigated SS1P in association with cyclophosphamide and pentostatin in previously treated patients (48); results showed that all patients who experienced PR showed a strong decrease in MPF expression, suggesting that serum MPF might predict clinical outcome (49). However, these studies were carried out on a low number of patients.

Osteopontin

Osteopontin is an integrin-binding protein, implicated in cell-matrix interactions. It is overexpressed in several types of cancers (50), including MPM (29). Pass et al. analyzed serum samples from 69 asbestos-exposed subjects, 45 smoking subjects and 75 MPM patients. The duration of asbestos exposure independently correlated with Osteopontin serum levels (**Figure 1**). Furthermore, serum Osteopontin was higher in MPM patients than in asbestos-exposed controls (133 ± 10 vs. 30 ± 3 ng/ml). Receiver Operating Characteristic (ROC) analysis demonstrated that the most accurate cut-off value for stage I disease was 62.4 ng/ml, with 84.6% sensitivity and 88.4% specificity. Unfortunately, in another study serum Osteopontin failed to distinguish MPM patients from patients with pleural metastasis of different carcinomas or from subjects with non-tumoral asbestos-related diseases (25). Another study suggested that plasma Osteopontin is a more stable and reliable marker than serum Osteopontin; nevertheless, conclusive data about its diagnostic accuracy are still lacking (30, 31, 51). Combined assessment of SMRP and Osteopontin, was not more informative than SMRP alone (30, 31, 45, 52) (**Table 1**).

Fibulin-3

Research for circulating biomarkers of MPM also included Fibulin-3 as a single biomarker or in combination with Mesothelin. Fibulin-3 is a secreted glycoprotein implicated in cell proliferation and migration (34). In the first report, plasma

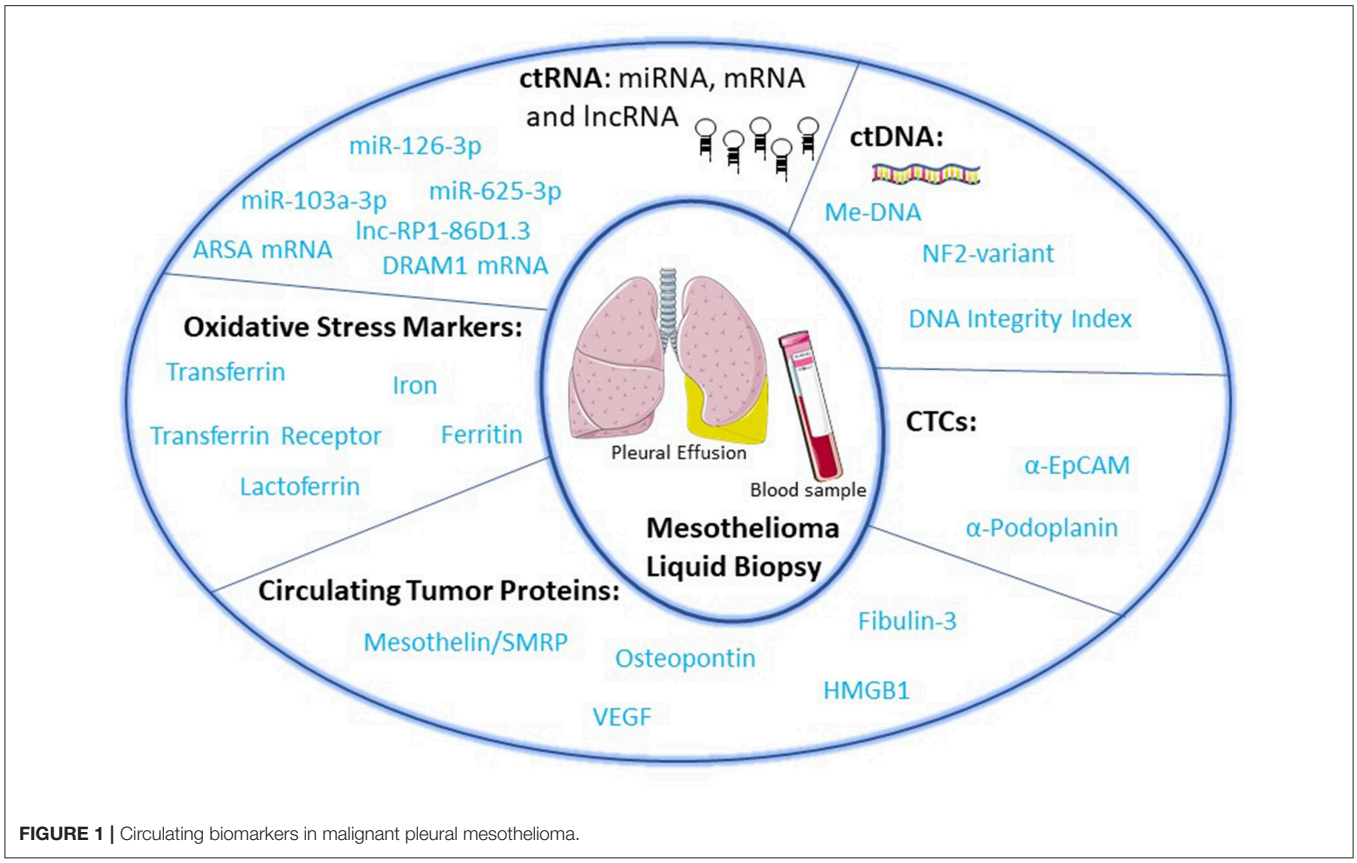

FIGURE 1 | Circulating biomarkers in malignant pleural mesothelioma.

levels of Fibulin-3 were measured in a total of 92 MPM patients, 136 healthy asbestos-exposed controls and 93 patients affected by other cancers. The subjects enrolled in the study belonged to two different cohorts. Fibulin-3 was found to be higher in MPM patients compared to control groups; this alteration was not related to duration of asbestos exposure, age, sex, histologic subtype or tumor stage (32). ROC analysis showed an AUC of 0.99 and the best cut-off value was 52.8 ng/ml for all MPM patients and 46 ng/ml for stage I/II disease. Based on these results the authors concluded that Fibulin-3 was the best biomarker analyzed so far. However, in an analysis of a validation cohort comprising 48 MPM patients and 96 asbestos-exposed controls, the accuracy of Fibulin-3 did not differ from that reported for Mesothelin (AUC 0.87) (32). This discrepancy between training and validation sets may be due to differences in the cohorts analyzed (36, 53). Other studies indicated that Fibulin-3 was not useful for discriminating MPM patients from patients affected by other diseases (33), and did not perform as well as Mesothelin (54) (**Table 1**).

Inflammatory and Angiogenic Factors

Chronic inflammation is considered a key determinant of MPM carcinogenesis (55). Inhaled asbestos fibers accumulate in the pleura and activate an inflammatory response. As macrophages cannot eliminate these fibers, inflammatory cytokines and growth factors are continuously produced, promoting malignant transformation. Tumor-associated macrophages (TAM) also produce, and induce production of cytokines and growth factors that enhance tumor growth and invasiveness (56).

The tight link between inflammation and cancer aggressiveness is supported by several studies demonstrating that the neutrophil-to-lymphocyte ratio (NLR), an indicator of systemic inflammation, is an independent predictor of poor prognosis in several cancers, including MPM (57–59). Based on this knowledge, the evaluation of inflammation markers was proposed for the diagnostic/prognostic stratification of MPM patients.

High Mobility Group B 1 (HMGB1) belongs to the family of damage-associated molecular pattern proteins (DAMPs) and is considered a key mediator of asbestos-induced inflammation (60, 61). In physiological conditions, HMGB1 is localized in the nucleus, where it functions as a chromatin-binding protein and is released by cells undergoing necrosis. In pathological conditions, myeloid cells and cancer cells can actively secrete a hyper-acetylated form of HMGB1. In the extracellular space, HMGB1 activates innate and adaptive immunity and acts as a pro-oncogenic factor binding to Toll like Receptors (TLRs) and RAGE (receptor of advanced glycation end products) (62). Jube et al. demonstrated that HMGB1 and its receptors are highly expressed in MPM tissues and cell lines. Exposure of normal mesothelial cells to asbestos induces necrosis, resulting in release of HMGB1 (**Figure 1**). Transformed MPM cells actively secrete acetylated HMGB1, which promotes cell proliferation and invasiveness in an autocrine manner (61). Consistent with this

TABLE 1 | Circulating protein biomarkers evaluated for early diagnosis in MPM.

Protein biomarker	Study design	Method	Sample	Study results	References
SMRP	AEXP = 40, MPM = 44, ARD = 38, ILD = 92, OC = 38	ELISA	Serum	MPM vs. AEXP cut-off 0.22 nmol/L Sensitivity: 84%, Specificity: 83% MPM vs. PD cut-off 0.22 nmol/L Sensitivity: 84%, Specificity: 100% MPM vs. OC cut-off 0.22 nmol/L Sensitivity: 84%, Specificity: 95%	(22)
	MPM = 60, Mets = 30, ARD = 23	ELISA	Serum	MPM vs. ARD AUC = 0.87 cut-off 0.93 nmol/L Sensitivity: 80% Specificity: 82.6% MPM vs. Mets AUC = 0.693 cut-off 1.85 nmol/L Sensitivity: 58.3% Specificity: 73.3%	(23)
	HC = 54, AEXP = 203, ARD = 130, MPM = 107	ELISA	Serum	MPM vs. All AUC = 0.77 cut-off 1 nmol/L Sensitivity: 68.2% Specificity: 80.5%	(24)
	AEXP = 112, Mets = 43, ARD = 33, MPM = 96	ELISA	Serum	MPM vs. AEXP AUC = 0.866 MPM vs. ARD AUC = 0.719	(25)
	AEXP/ARD = 66, MPM = 90	ELISA	Serum	MPM vs. AEXP/ARD AUC = 0.81 cut-off 1.9 nmol/L Sensitivity: 60% Specificity: 89.2%	(26)
	HC = 48, AEXP = 177, ARD = 101, MPM = 36	ELISA	Serum	MPM vs. All AUC = 0.75 cut-off 0.55 nmol/L Sensitivity: 72% Specificity: 72%	(27)
	HC = 120, AEXP = 123, ARD = 279, MPM = 24	ELISA	Serum	MPM vs. All AUC 0.74 cut-off 1.63 nmol/L Sensitivity: 58% Specificity: 83%	(28)
Osteopontin	AEXP/ARD = 69, FS = 45, MPM = 75	ELISA	Serum	MPM vs. AEXP/ARD AUC = 0.888 cut-off 48.3 ng/ml Sensitivity: 77.6% Specificity: 85.5% Stage I MPM vs. AEXP/ARD AUC = 0.906 cut-off 62.4 ng/ml Sensitivity: 84.6% Specificity: 88.4%	(29)
	AEXP = 112, Mets = 43, ARD = 33, MPM = 96	ELISA	Serum, Plasma	MPM vs. AEXP AUC 0.724 MPM vs. Mets AUC = 0.689 MPM vs. ARD AUC = 0.677	(25)
	AEXP = 93, ARD = 111, MPM = 31	ELISA	Plasma	MPM vs. AEXP/ARD AUC = 0.785	(30)

(Continued)

TABLE 1 | Continued

Protein biomarker	Study design	Method	Sample	Study results	References
	ARD = 89, MPM = 66	ELISA	Plasma	MPM vs. ARD AUC = 0.763	(31)
	HC = 120, AEXP = 123, ARD = 279, MPM = 24	ELISA	Serum	MPM vs. All AUC 0.86 cut-off 17.273 nmol/L Sensitivity: 75% Specificity: 86%	(28)
Fibulin-3	AEXP = 136, OC = 93, MPM = 92	ELISA	Plasma	MPM vs. All AUC = 0.99	(32)
	Validation study: AEXP = 96, MPM = 48	ELISA	Plasma	MPM vs. AEXP AUC = 0.87	
	Non-MPM = 56, MPM = 84	ELISA	Plasma	MPM vs. Non-MPM AUC = 0.632	(33)
	ARD = 49, BE = 35, OC = 36, MPM 82	ELISA	Plasma	MPM vs. All AUC = 0.671 Cut-off 52 ng/ml Sensitivity: 22% Specificity: 95%	(34)
Acetylated HMGB1	HC = 20, AEXP = 20, BE = 13, OC = 25, MPM = 22	Mass Spectrometry	Serum	MPM vs. AEXP AUC = 1 Cut-off 2 ng/ml Sensitivity: 100% Specificity: 100% MPM vs. BE/OC AUC = 0.837 Cut-off 9.70 ng/ml Sensitivity: 81.82% Specificity: 89.47%	(35)

AEXP, healthy asbestos-exposed individuals; MPM, Malignant Mesothelioma Patients; ILD, Inflammatory Lung Disease patients; OC, patients affected by other cancers; Mets, pleural metastasis of carcinomas; HC, non-exposed healthy controls; ARD, benign asbestos-related disease patients; FS, former smoker individuals; BE, patients with benign effusion.

notion, Napolitano et al. showed that asbestos-exposed subjects ($N = 42$) had higher serum levels of total HMGB1 compared to non-exposed control ($N = 20$). In healthy exposed individuals ($N = 20$) the majority of serum HMGB1 was in the non-acetylated form (90%), while in MPM patients ($N = 22$) the acetylated form was prevalent (67%). ROC analysis showed that serum levels of acetylated HMGB1 discriminated healthy exposed controls from MPM patients with high accuracy (cut-off = 2 ng/ml; AUC =1; 100% specificity, 100% sensitivity). Importantly, tumor stage did not influence acetylated HMGB1 levels (35) (**Table 1**). Although these results were obtained with a small number of subjects, they provide groundwork for future investigations on larger cohorts aimed at validating acetylated HMGB1 as an early diagnostic marker.

The angiogenic factor VEGF, a key stimulator of tumor neoangiogenesis, is overexpressed in MPM tissues (63–65). VEGF levels are also increased in pleural effusions (PE) of MPM patients compared to patients affected by non-malignant pleural diseases or lung cancer (66). Yasumitsu et al. showed that serum VEGF was higher in MPM patients ($N = 51$) compared to control patients with non-tumoral asbestos-related diseases ($N = 29$). Setting a cut-off at 460 pg/ml, these authors showed a strong correlation between high serum VEGF and shorter patient survival (67). A predictive/prognostic role of VEGF in MPM has also been described. Baseline serum levels of VEGF-A and VEGF receptor 2 (VEGFR-2) correlated with radiological response in patients treated with the multitarget tyrosine kinase inhibitor Sunitinib Malate (68). In patients with high baseline serum levels of VEGF, its decrease after 8 weeks of thalidomide treatment correlated with longer patient survival (69). Although these results are promising, they should be considered cautiously. Serum VEGF may not really reflect its circulating levels because it may be released by platelets during *in vitro* blood clotting (70). Considering that platelet count is an independent prognostic factor for MPM patient survival (71), VEGF should probably be evaluated in plasma instead of serum samples.

Markers of Oxidative Stress

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are key mediators of asbestos toxicity (72). ROS and RNS are generated by asbestos through two main mechanisms. First, the different forms of asbestos fibers contain iron, which increases the generation of hydroxyl radicals through the reactions of Fenton and Haber-Weiss (73, 74). Consistent with this notion, X-ray imaging and spectroscopy studies showed that asbestos fibers in tissues contain iron in the form of ferritin and haematite (75). Second, inhaled asbestos fibers are internalized by alveolar epithelial cells (AEC) and alveolar macrophages (AM); the activation and attempted phagocytosis by AM and neutrophils lead to activation of vacuolar NADPH oxidase and myeloperoxidase, which generate ROS and hypochlorite radicals in the microenvironment. Undigested asbestos fibers are coated

with a mucopolysaccharide, generating the pathognomonic asbestos bodies, and with iron protein complexes, resulting in the ferruginous bodies, which further enhance ROS production and local inflammation.

Bronchoalveolar lavage fluid (BALF) of asbestos-exposed patients ($N = 14$) exhibited an increase in several markers of inflammation and altered iron and ROS homeostasis (i.e., iron, transferrin, transferrin receptors, lactoferrin, and ferritin) compared to unexposed controls ($N = 10$) and asbestos-exposed subjects ($N = 14$) (76) (**Figure 1**). It will be interesting to test ROS-related markers in the peripheral blood of MPM patients and asbestos-exposed individuals.

CIRCULATING microRNAs (miRNAs)

miRNAs are small non-coding RNAs that regulate the expression of a vast number of mRNAs (77). Tumor cells exhibit distinctive miRNA signatures (78, 79) and can release miRNAs as a result of cell death and active secretion (80). Such cell-free circulating miRNAs (cfmiRNA) are relatively stable, since they are incorporated into membrane-bound vesicles or bound to ribonucleoprotein complexes (81).

Studies of cfmiRNAs are curbed by major problems in data normalization, given the difficulties in identifying "bona fide" housekeeping miRNA in biological fluids that can be used for normalization. Biases linked to the choice of an appropriate reference can be circumvented by using the miRNA ratio approach (82, 83), which is based on the calculation of ratios between upregulated and downregulated miRNAs in the same patient.

Several studies investigated the cfmiRNA profile in mesothelioma patients with the aim of identifying markers for early diagnosis and prognostic stratification (84). This section focuses mainly on miR-126-3p, miR-103a-3p, and miR-625, 3 miRNAs that appear to be consistently altered in MPM patients (**Table 2**).

Santarelli et al. showed that miR-126-3p is strongly downregulated in serum samples from MPM patients ($N = 44$) compared to samples from healthy volunteers ($N = 50$) or asbestos-exposed subjects ($N = 196$) (85). ROC curve analysis indicated that this miRNA distinguished MPM patients from asbestos-exposed individuals with 73% sensitivity and 74% specificity (85). The combined upregulation of soluble SMRP and downregulation of miR-126-3p was associated with a high risk of mesothelioma development. However, these data were normalized using the small nucleolar RNA RNU6 (U6), which is known to be present at low and variable levels in blood (83) and may be altered in chronic inflammation (95), which is very common in asbestos-exposed individuals.

Tomasetti et al. (86) confirmed that miR-126-3p discriminates MPM patients ($N = 45$) from healthy controls ($N = 56$) (sensitivity 80%, specificity 60%) and that its levels are lower in MPM patients with poor prognosis compared to those with better clinical outcome and to patients with non-small cell lung cancer ($N = 20$). In this study, the samples were normalized to spiked-in cel-miR-39, endogenous U6 or both (86).

Interestingly, the diagnostic performance of miR-126-3p was significantly improved when combined with Mesothelin and methylation of the thrombomodulin promoter (AUC 0.857, 95% CI 0.767–0.927) (87) (see also section on DNA methylation).

In apparent contrast with these studies, Mozzoni et al. (88) did not confirm the ability of miR-126-3p to discriminate MPM patients ($N = 32$) from asbestos-exposed controls ($N = 14$), and did not observe a correlation between the levels of miR-126-3p in the plasma and in the MPM tissues ($N = 24$). However, miR-126-3p was able to distinguish MPM patients ($N = 32$) and asbestos-exposed patients ($N = 14$) from control subjects with non-cancerous pulmonary diseases ($N = 15$). It must be noted that in this study, the authors used different normalizer RNAs for plasma (miR-146) and tissue (U6, RNU44, RNU48). More recently, Weber et al. (89) analyzed the levels of miR-126-3p, miR-132-3p, and miR-103a-3p in plasma samples obtained a median of 8.9 months prior to the diagnosis of MPM ($N = 17$), and compared them to asbestos-exposed controls ($N = 34$). This study indicated 0% sensitivity of these miRNAs considering a specificity of 98%. Based on these findings, the authors concluded that these miRNAs are unsuitable as biomarkers for early detection of MPM in asbestos-exposed individuals. However, it must be noted that, to permit a comparison with previous studies, the authors normalized miR-126-3p against U6, miR-132-3p against miR-146b-5p, and miR-103a-3p against miR-125a (89). As the authors suggest, it would be desirable to employ a common normalizer for all these miRNAs.

Kirschner et al. demonstrated higher levels of miR-625-3p in the serum of MPM patients ($N = 30$) compared to asbestos-exposed subjects ($N = 10$) (accuracy 79.3%, sensitivity 70% and specificity 90%) (90). However, these data were normalized against miR-16, which is known to be highly dependent on the haemolysis of the sample (90) and was also reported to be altered in MPM (88, 96).

Weber et al. took a different approach and analyzed the cell fraction obtained by centrifugation of whole blood; in this fraction miR-103a-3p was downregulated in MPM patients ($N = 23$) compared to asbestos-exposed ($N = 17$) and healthy control subjects ($N = 25$). miR-103a-3p discriminated MPM patients from asbestos-exposed subjects with a 83% sensitivity and 71% specificity, and from healthy controls with 78% sensitivity and 76% specificity (91). In a subsequent study the authors confirmed this finding and provided evidence that the association of reduced levels of miR-103a-3p in blood cells with elevated Mesothelin in plasma improved the discrimination of MPM patients ($N = 43$) from asbestos-exposed ($N = 52$) individuals (92). However, these findings were not confirmed in a follow-up study of prediagnostic MPM samples (89). In all these studies data were normalized to miR-125a measured in the cell fraction of whole blood.

Cavalleri et al. analyzed the levels of miR-103a-3p along with miR-30e-3p in extracellular vesicles and showed that the

TABLE 2 | Circulating miRNAs in MPM.

miRNAs	miRNA expression in MPM	Study design	Samples	Reference gene, method of analysis	Study results	References
miR-126-3p	Reduced	MPM = 10, NMT = 5 (frozen biopsy); MPM = 27, adjNCT = 27 (FFPE); MPM = 44, HC = 50, AEXP = 196;	Frozen Biopsy, FFPE, tissue, Serum	Ref gene: RNU6 Method: TaqMan MicroRNA Assay	MPM vs. AEXP sensitivity: 73%, specificity: 74% AEXP vs. HC sensitivity: 60%, specificity: 74%	(85)
miR-126-3p	Reduced	MPM = 45, HC = 56, NSCLC = 20;	Serum	Ref gene: RNU6, cel-miR-39 Method: TaqMan microRNA Assay	MPM vs. HC sensitivity: 80%, specificity: 60%	(86)
miR-126-3p	Reduced	MPM = 45, AEXP = 99, HC = 44 (discovery group); MPM = 18, AEXP = 50, HC = 20, LC = 42 (validation group);	Serum	Ref gene: RNU6, cel-miR-39, Method: TaqMan MicroRNA Assay Circulating methylated TM DNA assay and ELISA	MPM vs. HC miR-126-3p sensitivity: 75%, low specificity: 54% miR-126 + SMRP + Met-TM: AUC = 0.857	(87)
miR-16 miR-17 miR-126 miR-486	Reduced	MPM = 32, AEXP = 14, NCP = 15; 24 MPM (FFPE)	Plasma, FFPE Tissue	Ref gene: miRNA-146 for plasma RNU6B, RNU44, RNU48 for tissue Method: TaqMan microRNA Assay	MPM ± AEXP vs. NCP miR-16: AUC = 0.89, cut-off 77.5, sensitivity: 86.7%, specificity: 82.2% miR-17: AUC = 0.88, cut-off 5.9, sensitivity: 80%, specificity: 84.4% miR-126: AUC = 0.95, cut-off 5.4, sensitivity: 80%, specificity: 97.8% miR-486 AUC = 0.88, cut-off 9.2, sensitivity: 80%, specificity: 89.1%	(88)
miR-126-3p miR-132-3p miR-103a-3p	Reduced	MPM = 17, AEXP = 34;	Plasma, Blood Cells	Ref gene: RNU6 for miR-126-3p, miR-146b-5p for miR-132-3p, miR-125a for miR-103a-3p Method: TaqMan microRNA Assay; miR-103a in whole blood cell fraction	MPM vs. AEXP miR-126-3p: AUC = 0.614, sensitivity: 0%, specificity: 98% miR-132-3p: AUC = 0.542, sensitivity: 0%, specificity: 98% miR-103a-3p: AUC = 0.603, sensitivity: 0%, specificity: 98% miR-126-3p + miR-132-3p + miR-103a-3p: AUC = 0.605, sensitivity: 0%, specificity: 98%	(89)
miR-625-3p	Increased	MPM = 5, HC = 3 (plasma); MPM = 15, CS = 14 (plasma); (test cohort) MPM = 30, AEXP = 10 (serum); (validation cohort) MPM = 18, CS = 7 (FFPE);	Plasma, Serum, FFPE Tissue	Ref gene: miR-16 (plasma), RNU6B (FFPE) Method: Human miRNA, Microarray Agilent; TaqMan miRNA Assay, OpenArray Analysis	MPM vs. AEXP test cohort AUC = 0.824 sensitivity: 73.33% specificity: 78.57% MPM vs. AEXP validation cohort AUC = 0.793 sensitivity: 70% specificity: 90%	(90)
miR-103a-3p	Reduced	MPM = 23, AEXP = 17, HC = 25;	Blood cells	Ref gene: miR-125a Method: miRNA Microarray, TaqMan miRNA Assay	MPM vs. AEXP AUC = 0.757, cut-off 0.621 sensitivity: 83% specificity: 71% MPM vs. HC AUC = 0.871, cut-off 0.621 sensitivity: 78% specificity: 76%	(91)

(Continued)

TABLE 2 | Continued

miRNAs	miRNA expression in MPM	Study design	Samples	Reference gene, method of analysis	Study results	References
miR-103a-3p	Reduced	MPM = 43, AEXP = 52;	Blood cells	Ref gene: miR-125a. Method: TaqMan miRNA Assay	MPM vs. AEXP miR-103a-3p: AUC = 0.76, cut-off 749.61 sensitivity: 86% specificity: 63% miR-103a-3p + Mesothelin: AUC = 0.90, sensitivity: 86% specificity: 85%	(92)
miR-103a-3p, miR-30e-3p	Reduced	MPM = 23, AEXP = 19;	Extra-Cellular Vescicles (EV)	Ref gene: RNU48, average of miR-99a, miR-638, miR-720, miR-1274a. Method: OpenArray qRT-PCR, Custom TaqMan^{TM} Low Density Array	MPM vs. AEXP miR-103a-3p + miR-30e-3p: AUC = 0.942 sensitivity: 95.5% specificity: 80%	(93)
miR-2053	Increased	MPM = 100, AEXP = 20, HC = 20;	Serum	Ref gene: RNU6B (serum), ACTB (RNAs) Method: miScript SYBR Green PCR, miScript Primer Assay	MPM vs. HC miR-2053: AUC = 0.91, cut-off 1.25 sensitivity: 85 % specificity: 97.5 % miR-2053 + lnc-RP1-86D1.3 + ARSA + DRAM1: AUC = 0.94, sensitivity: 100 %, specificity: 85%	(94)

MPM, malignant pleural mesothelioma; NSCLC, non-small cell lung cancer; LC, lung cancer; CS, control subjects (patients with coronary artery disease or healthy subjects); AEXP, asbestos exposed patients; HC, healthy volunteers; NCP, controls with noncancerous pulmonary diseases; SMRP, soluble mesothelin-related proteins; Met, TM-methylated thrombomodulin promoter; adjNCT, adjacent non-cancerous tissue; NMT, non-malignant tissue.

combination of these two markers discriminated MPM patients from asbestos-exposed subjects with a 95.5% sensitivity and 80% specificity. These findings were confirmed by normalizing the data to RNU48, miR-99a, miR-638, miR-720, and miR-1274a (93).

A recent study by Matboli et al. (94) detected increased levels of the long non-coding-RNA RP1-86D1.3 and miR-2053 and downregulation of the mRNAs coding for DRAM1 (damage-regulated autophagy modulator) and ARSA (arylsulfatase A) in MPM patients ($N = 100$) compared to asbestos-exposed subjects ($N = 20$) and healthy controls ($N = 20$). Data were normalized to RNU6B for miR-2053 and to beta actin for the other RNAs. The function of long non-coding-RP1-86D1.3 is obscure at present, although its expression is frequently altered in lung, breast, colon, and gastric cancer (97, 98). DRAM1 is a p53 responsive gene that encodes a lysosomal membrane protein involved in autophagy (99). ARSA is a lysosomal enzyme that is necessary for the correct function of autophagosomes (100). These mRNAs could be considered as biomarkers of asbestos exposure rather than disease. Moreover, the authors suggest that the upregulation of miR-2053 is a good prognostic marker of MPM, which will be validated in a large sample cohort (94).

CIRCULATING TUMOR DNA (ctDNA)

ctDNA comprises the fraction of circulating cell-free DNA (cfDNA) that is released by the tumor through apoptosis, necrosis, or active tumor secretion (13). cfDNA is typically found as double-stranded fragments measuring 180-200 base pairs in length, corresponding to nucleosome-associated DNA (101, 102). Cancer patients commonly exhibit a higher concentration of cfDNA (103) that contains the mutations found in the tumor.

The detection of ctDNA variants in MPM holds promise as a potential biomarker for the diagnosis and stratification of MPM patients.

Sriram et al. showed that the DNA integrity index (i.e., the ratio between ALU fragments of 247 and 115 bp) in pleural fluid was higher in MPM patients ($N = 16$) than in benign pleural effusions ($N = 23$) (median: 1.2 vs. 0.8 with $p < 0.001$) (104). ROC analysis of this cohort revealed that serum Mesothelin had the highest predictive value (AUC: 0.94) followed by pleural fluid Mesothelin (AUC: 0.89) and DNA integrity index in pleural effusion (AUC: 0.82). Using a cut-off of 1.06 for the DNA integrity index, a cut-off of 12.91 nM for pleural fluid Mesothelin and a cut-off of 1.34 nM for serum Mesothelin, the authors obtained a specificity of

90% and a sensitivity of 75% to distinguish malignant pleural mesothelioma from benign pleural effusion. Further studies are needed to test whether the DNA integrity index in pleural fluid may provide additional information about the progression of disease.

A comprehensive genomic analysis conducted by Bueno et al. on a large cohort of MPM tissue samples revealed mutations in the BAP1, NF2, TP53, SETd2, DDX3X, ULK2, RYR2, CFAP45, SETDB1, and DDX51 genes (105). Using an integrated analysis, these authors identified alterations in the Hippo, mTOR, histone methylation, RNA helicase and p53 signaling pathways.

However, to date none of these mutations have been systematically investigated using a liquid biopsy approach. In a recent study, Hylebos et al. (106) performed whole exome sequencing (WES) of tumor and germline DNAs of ten MPM patients and confirmed the mutation described by Bueno et al. Selected tumor-specific variants of ctDNA were detected in serum samples using ddPCR (Droplet Digital PCR), but the mutation in NF2 was clearly and reproducible detectable in only one patient (fraction of mutated DNA = 0.8%).

DNA METHYLATION

DNA methylation is an epigenetic modification that usually occurs at regions of DNA rich in CpG dinucleotides, which are located mainly in 5' regulatory regions of genes. The DNA methylation pattern may be modified following environment exposure, therapy, aging and disease. Studies have demonstrated that promoter methylation, and alterations of gene expression are a common occurrence in mesothelioma and that the DNA methylation profile in tissue samples was able to distinguish normal pleura from mesothelioma (107). Detection of changes in the methylation profile of ctDNA might thus be a tool for early diagnosis and prognostic stratification of MPM patients (108).

Santarelli et al. (87) evaluated alterations in the methylation of the thrombomodulin (TM) gene in serum in association with serum levels of SMRP and miR-126 in MPM patients ($N = 45$), asbestos-exposed healthy subjects ($N = 99$), and healthy donors ($N = 44$). The model based on the combination of these 3 parameters improved the differential diagnosis of MPM, with an AUC of 0.857. A significant risk of disease (odds ratio >4) was found in the presence of high levels of SMRP in association with altered levels of either miR-126 or TM promoter methylation, and when both miR-126 and TM promoter methylation were altered even at low SMRP concentration. It will be interesting to test the validity of these findings in large prospective longitudinal cohorts (87, 109).

In a very recent study, Guarrera et al. (110) investigated peripheral blood DNA methylation as a biomarker of MPM in a large cohort of patients and controls. Results showed a distinct methylation signature in MPM patients compared to controls, with more than 800 differentially methylated (DM) CpG sites and significant enrichment for genes controlling innate immunity and neutrophil degranulation. The authors identified seven top DM-CpGs, three of which were hypomethylated (FOXK1, MYB, and TAF4) and four hypermethylated (CXCR6/FYCO1, TAP1, MORC2, and LIME1). ROC analysis showed a diagnostic value of the methylation levels of the seven top DM-CpGs in association with age, sex and asbestos exposure levels (AUC: 0.89). Univariate regression analysis showed no clear evidence for differences in the seven DM-CpGs among the different histotypes of mesothelioma. Overall, the results obtained in these studies are very promising but need to be validated in a longitudinal study.

CIRCULATING TUMOR CELLS (CTCs)

CTCs are intact tumor cells derived from primary or metastatic tumor sites. The number of CTCs present in the blood is very low at early stages and increases in advanced stages of cancer (111).

To date, CELLSEARCH® is the only FDA-approved test for capturing and counting CTCs. This method consists of magnetic particles coated with antibodies targeting the Epithelial Cell Adhesion Molecule (EpCAM), an antigen present on most epithelial tumor cells (112).

For MPM, which originates from the mesothelium, the CELLSEARCH technique has demonstrated a very low diagnostic sensitivity (113–115).

More recently, Chikaishi et al. developed a "CTC-chip" that was able to capture the Ep-Cam negative CTCs by targeting podoplanin (116), a mucine-type transmembrane glycoprotein whose expression is increased in malignant cells of mesothelial origin (117). Yoneda et al. (118) evaluated CTCs in a small cohort of 16 MPM patients using the CellSearch and CTC-chip techniques. The CTC-chip performed better than CellSearch, and demonstrated a significant diagnostic value in discriminating between early and advanced disease (AUC = 0.851).

CONCLUSIONS AND PERSPECTIVES

At present, there is no reliable marker for the longitudinal monitoring and risk assessment of asbestos-exposed individuals. Although liquid biopsy is still far to replace tissue biopsy for MPM diagnosis, it holds great promise for non-invasive tracking of the follow-up of asbestos-exposed individuals. Plasma and serum samples represent minimally invasive, low risk, and easily obtained biological fluids and many studies have indicated potentially interesting biomarkers, including Mesothelin (early diagnosis and prognostic stratification of MPM), Osteopontin (early diagnosis), Fibulin-3 (early diagnosis), HMGB1 (early diagnosis), VEGF (early diagnosis and prognostic stratification) and miRNAs (early diagnosis). More recent studies have also suggested that markers of oxidative stress, CTCs and ctDNA might also be useful for the screening/early diagnosis of

MPM. Furthermore, a study by Zucali et al. demonstrated that TS (Thymidylate Synthase) is overexpressed in MPM tissues and is a strong predictor of responsiveness of MPM patients to Pemetrexed/Carboplatin (119). It is thus possible that detection of TS in circulating MPM cells or as circulating cell-free RNA might prove to be an interesting predictive biomarker (120, 121).

However, most of these markers were studied in restricted patients' cohorts, and the conclusive identification of robust circulating biomarkers for early diagnosis and prognostic stratification of MPM patients awaits validation in large prospective studies. Furthermore, most of these studies were highly heterogeneous in terms of preanalytical and analytical protocols employed. Therefore, future efforts should be focused on reaching a consensus on the standardization and normalization of the different assays to achieve robust and reproducible results. Multivariate analyses of multiple biomarkers may also improve the diagnostic power of these assays.

AUTHOR CONTRIBUTIONS

IC prepared the paragraphs on ctDNA, DNA methylation, CTC, and revised the final version of the paper. LU prepared the paragraph on Circulating Tumor Proteins and revised the final version of the paper. ES prepared the paragraph on Circulating miRNAs and revised the final version of the paper. GP prepared the Introduction and revised the final version of the paper. VC prepared the paragraphs on Oxidative Stress Markers, on Perspectives, and revised the final version of the paper.

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Soluble Programmed Death Receptor Ligands sPD-L1 and sPD-L2 as Liquid Biopsy Markers for Prognosis and Platinum Response in Epithelial Ovarian Cancer

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Introduction: Response to platinum-based therapy is a major prognostic factor in epithelial ovarian cancer (EOC) and reliable prognostic biomarkers are urgently needed to identify patients at high risk. Since ligands of the Programmed Death Receptor-1 (PD-L1 and PD-L2) play a crucial role within the tumor microenvironment for tumorigenesis, we investigated levels of sPD-L1 and sPD-L2 in liquid biopsies of serum samples, and correlated the results with the clinical status, presence of circulating tumor cells (CTCs) and disease outcome in primary EOC patients.

Methods: sPD-L1 and sPD-L2 were determined by ELISA in patients ($N = 83$) and healthy females ($N = 29$). Gene expression analysis of EpCAM, MUC-1, CA-125, and ERCC1 was performed by RT-PCR after CTCs enrichment.

Results: sPD-L1 was significantly ($p = 0.0001$) increased and sPD-L2 decreased ($p = 0.003$) in EOC patients compared to controls. While enhanced sPD-L1 was associated with residual tumor burden ($p = 0.022$), reduced sPD-L2 levels were related to platinum-resistance ($p < 0.01$) and the presence of ERCC1+ CTCs ($p < 0.0001$). High sPD-L1 levels were associated with a reduced 5 year overall survival (OS, $p = 0.003$) and progression-free survival (PFS, $p = 0.019$). Strikingly, sPD-L1 levels >6.4 pg/ml were indicative of a reduced OS ($p = 0.035$) and PFS ($p = 0.083$) in platinum-sensitive patients, while OS and PFS in platinum-resistant patients did not differ when patients were stratified to this cut-off.

Conclusions: Our study highlights sPD-L1 and sPD-L2 as complementary biomarkers reflecting clinical status, treatment response and disease outcome of EOC patients. Especially, sPD-L1 may facilitate the identification of high-risk patients with unfavorable disease outcomes despite platinum-sensitivity arguing for additional therapeutic approaches. As sPD-L1 and sPD-L2 are easily accessible via liquid biopsy, the inclusion of sPD-L1 and sPD-L2 in addition to CTC investigation as markers for risk assessment during patient therapy planning and follow-up appears to be a valuable approach.

Keywords: epithelial ovarian cancer (EOC), soluble PD-L1 (sPD-L1), soluble PD-L2 (sPD-L2), liquid biopsy, biomarkers, platinum therapy, residual tumor burden, circulating tumor cells

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INTRODUCTION

Epithelial ovarian cancer (EOC) is the most lethal of gynecological malignancies worldwide (1). In Germany, EOC is responsible for 3.2% of all malignant neoplasms and 5.3% of all cancer deaths (2). Due to the lack of early symptoms, most patients are diagnosed at an advanced stage. Standard therapy consists of radical cytoreductive surgery and a platinum-based chemotherapy. In recent years, the anti-VEGF antibody Bevacizumab has become part of the standard adjuvant treatment for advanced EOC. Despite a high initial response rate, the majority of patients eventually relapse (3), leading to a relative 5-year survival of 41% (2). The platinum-free interval to relapse has been identified as a predictive factor for the response to subsequent platinum-based therapy (4, 5). Patients relapsing within 6 months after initial platinum-based chemotherapy are generally considered platinum-resistant with subsequent platinum-based therapy not being an option. Thus, treatment options for this group of patients are limited, making these women the most challenging to treat. As there is currently no clinically established method of predicting response to first-line platinum-based treatment, reliable predictive biomarkers are urgently needed to estimate the risk of relapse and to improve treatment management. In this regard, it has already been reported that the characterization of disseminated tumor cells (DTCs) in the bone marrow (BM), and circulating tumor cells (CTCs) in blood has identified stem cell-like DTCs and CTCs, tumor cells in epithelial-mesenchymal transition (EMT) as well as resistant cells, and all associated with poor outcomes and clinical platinum resistance [reviewed in Giannopoulou et al. (6)]. Interestingly, we were able to show that immunotherapy based on the intraperitoneal trifunctional bispecific antibody Catumaxomab was successful in the elimination of DTCs and CTCs in patients with advanced EOC (7). Thus, the tumor microenvironment might play a crucial role in tumor control and tumorigenesis of these patients.

The immune-regulatory protein PD-1, expressed by different immune cells, and its ligands PD-L1 and PD-L2, expressed by tumor cells and a variety of immune cells, have gained attention for treatment options in EOC (8). Hitherto, anti-PD-1 and anti-PD-L1 antibodies have already been successfully used in early clinical studies (9). PD-L1 was shown to be primarily expressed by tumor cells and correlated with worst prognosis (10). However, subsequent studies showed contradictory results with PD-L1 being expressed primarily by macrophages resulting in a longer OS (11). Recently, the soluble forms of PD-1 and PD-L1 (sPD-1 and sPD-L1) in serum samples were considered to be effective for the prediction of prognosis and treatment response (12, 13). sPD-L1 plasma levels were shown to be significantly increased in EOC patients compared to women with benign tumors and healthy controls (14). However, studies elucidating the role of sPD-L1 and sPD-L2 in EOC are rare.

Although the cellular expression of the immune checkpoint molecule PD-1 and its ligands PD-L1 and PD-L2 in the tumor microenvironment play crucial roles in tumor control and tumorigenesis, the routine clinical analysis is problematic due to the need to obtain representative biopsies of the entire tumor. Thus, we hypothesized that assessing the soluble forms of PD-L1 and PD-L2 in peripheral blood of EOC patients might represent a feasible approach in the search for "liquid biomarkers" to target patients accordingly.

To introduce sPD-L1 and sPD-L2 as biomarkers for disease status and outcome, we evaluated levels of sPD-L1 and sPD-L2 in sera of 83 primary EOC patients retrospectively and related these results with the presence of CTCs, clinical characteristics including FIGO-stage, tumor grade, lymph node infiltration, presence of metastases, residual tumor burden and platinum resistance as well as PFS and OS.

MATERIALS AND METHODS

Patient Characteristics

A total number of 83 patients diagnosed with histologically confirmed EOC between 2007 and 2014 at the Department of Gynecology and Obstetrics, University Hospital Essen, were analyzed. Histological subtype was serous except for 3 cases with poorly differentiated tumors which could not be assigned to a specific subtype by the pathologist. Written informed consent was obtained from all participants and the study was approved by the local ethics committee (Essen 05-2870 and 17-7859) and performed according to the Declaration of Helsinki. Tumors were classified according to the WHO classification of tumors of the female genital tract. Grading was conducted using the grading system proposed by Silverberg and tumor staging was classified according to the Fédération Internationale de Gynécology et d'Obstétrique (FIGO). The whole study population underwent primary radical surgery. Total abdominal hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy, and peritoneal stripping were performed. The most important aim of surgery was to achieve macroscopic complete tumor resection. Radical pelvic and para-aortic lymphadenectomy were only performed if macroscopic complete tumor resection was achieved intraperitoneally following guideline recommendation during the reported time period. All patients received at least six cycles of carboplatinum AUC 5 and paclitaxel 175 mg/m^2. Tumors were clinically defined as platinum-resistant if they recurred within 6 months after the completion of platinum-based chemotherapy. Any macroscopic residual disease at the end of primary surgery was defined as residual tumor. Inclusion criteria were: histologically confirmed EOC, primary radical surgery, platinum-based chemotherapy, availability of serum-samples, and follow-up information. All patients from the selected time period who met these criteria were included. Chemotherapy was administered postoperatively in all patients during this period. Clinical characteristics of the patients and association to sPD-L1 and sPD-L2 levels are documented in **Table 1**.

Patients had a median age of 68 (range: 32–98) years, whereas the control cohort consisted of 29 healthy women with a median age of 55 ranging from 35 to 70 years. Overall survival (OS) and progression-free survival (PFS) ranged from 1 to 118 months with a median of 30 months and 1 to 111 with a median of 18 months, respectively.

TABLE 1 | Patients' characteristics and association to sPD-L1 and sPD-L2 serum-levels.

		n	sPD-L1†	*p*	sPD-L2†	*p*
FIGO stage	FIGO II	6	3.8; 0.0–9.2	n.s.	1,870; 906–5,925	n.s.
	FIGO III	55	5.7; 0.0–32.9		1,968; 260–6,300	
	FIGO IV	22	8.4; 1.2–24.0		1,773; 712–6,300	
Grading	G1-G2	33	6.8; 0.0–32.9	n.s.	1,710; 675–5,925	n.s.
	G3	50	6.0; 0.0–24.0		1,906; 260–6,300	
Histo-pathological type	Serous	80	6.0; 0.0–32.9	n.s.	1,906; 260–6,300	n.s.
	Non-specified	3	10.6; 9.4–10.6		1,250; 778–1,435	
Progression	No	28	4.3. 0.0–19.7	0.03	1,906; 850–5,925	n.s.
	Yes	55	7.0; 0.0–32.9		1,831; 260–6,300	
Survival	No	40	7.3; 1.1–32.9	0.003	1,712: 260–6,300	n.s.
	Yes	43	4.2; 0.0–23.6		1,919; 850–5,925	
Platinum resistance	No	55	5.9; 0.0–32.9	n.s.	1,919; 686–6,300	0.0096
	Yes	13	4.5; 0.0–13.1		1,338; 352–3,061	
	Unknown	15	7.4; 1.2–21.9		1,919; 260–6,300	
Residual tumor	No	40	5.5; 0.0–21.9	0.022	1,907; 352–6,300	n.s.
	Yes	43	7.5; 0.0–32.9		1,831; 260–6,300	
CTC before therapy	No	60	5.9; 0.0–32.9	n.s.	2,100; 686–6,300	<0.0001
	Yes	22	7.2; 0.0–23.2		1,324; 260–3,019	
	Unknown	1	10.5; 10.5–10.5		778; 778–788	
CTC after therapy	No	21	3.3; 0.0–23.9	n.s.	2,354; 850–6,300	n.s.
	Yes	10	3.7; 0.0–11.9		1,656; 675–3,019	
	Unknown	52	7.4; 0.0–32.9		1,853; 260–6,300	

† *Given as median; minimum—maximum in pg/ml; n.s., not significant; platinum resistance was defined as recurrence <6 months after completion of adjuvant platinum therapy; residual tumor was defined as any macroscopic disease at the end of primary surgery.*

Sampling of Serum

Serum samples of healthy women or of EOC patients at the time of first diagnosis were collected and centrifuged for 10 min at 2,500×g. Subsequently, the upper phase was stored at −20°C until usage.

Assessment of Soluble PD-L1 and PD-L2

Serum concentrations of sPD-L1 and sPD-L2 were measured by commercial ELISA kits (R&D Systems, GmbH, Wiesbaden-Nordenstadt, Germany) using the manufacturer's protocols with minor modifications. In brief, microtiter plates with high binding surface (Costar Corning, Bodenheim, Germany) were coated with anti-human PD-L1 or PD-L2 antibody at 4°C overnight in a final concentration of 4 and 2 µg/ml, respectively. For the detection of bound sPD-L1 or sPD-L2, biotin-coupled polyclonal goat anti-human PD-L1 and PD-2, respectively, was used diluted to 50 ng/ml or 1 µg/ml in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA, AppliChem GmbH, Darmstadt, Germany). Bound detection antibodies were recognized by streptavidin conjugated with horseradish peroxidase being diluted 1:200 in PBS containing 1% BSA; 3,3,5,5-tetramethybenzidine substrate reagent set (Becton Dickinson, Franklin Lakes, USA) was used for visualizing immune complexes. Substrate reaction was terminated using 2N H2SO4 and optical density was measured at 450 nm (Biotek Instruments, Winooski, VT).

All serum samples were tested undiluted. Recombinant PD-L1 and PD-L2 protein fused with Fc portion of human IgG were used as standard reagents. PD-L1 or PD-L2 standard was serially diluted from 0 to 1,250 pg/ml or 0 to 6,000 pg/ml. Quantification of sPD-L1 and sPD-L2 serum levels were performed by four-parameter curve fitting. For sPD-L1 and sPD-L2, the intra-assay coefficients of variations were 6.6 and 5.2%, respectively, whereas the inter-assay coefficients of variations were 15.0% for sPD-L1 and 9.1% for sPD-L2.

Selection and Detection of CTCs

Ethylenediaminetetraacetic acid blood samples were collected and processed within 4 h for the enrichment of CTCs. In briefly, CTCs were immunomagnetically selected using the AdnaTest *OvarianCancerSelect* (QIAGEN, Hilden, Germany). After RNA isolation, gene expression analysis was done by

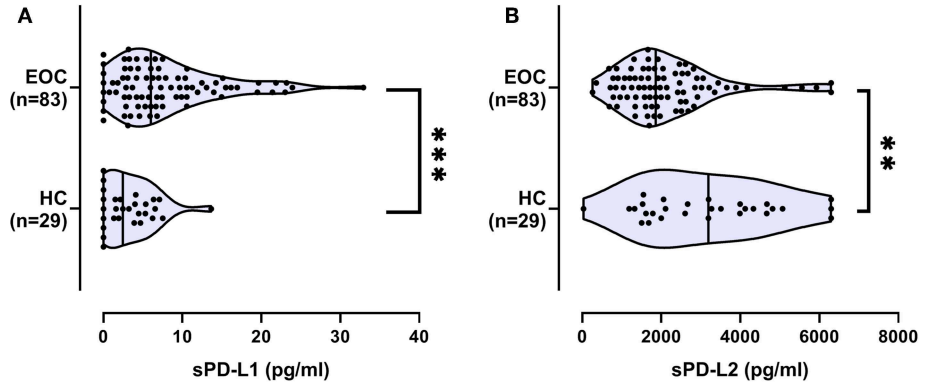

FIGURE 1 | Serum levels of sPD-L1 **(A)** and sPD-L2 **(B)** in healthy controls (HC) and ovarian cancer patients (EOC). Straight line within the violin plot indicates the median. $^{**}p < 0.01$, $^{***}p < 0.001$.

reverse-transcription (RT) and multiplex RT-PCR, detecting EpCAM, MUC-1, and CA-125 (AdnaTest *OvarianCancerDetect*). ERCC1-transcripts were studied by a singleplex RT-PCR (n = 57/83 patients). β-actin served as an internal control. Assays have been described in detail elsewhere (15, 16).

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics Version 24. Continuous and categorical variables were compared using the Mann-Whitney *U*, Kruskal–Wallis test, or Fisher's exact test, as appropriate. Receiver operating curve (ROC) analysis was performed to obtain cut-off values for categorization of continuous patient characteristics into dichotomous variables representing the optimal separation of survival curve by using the BIAS 11.01 software program (http://www.bias-online.de/). Probabilities of OS and PFS were analyzed using the Kaplan-Meier method in combination with the log-rank test implemented in the R package survminer (version 0.4.0; https://CRAN.R-project.or/package=survminer). Starting points were time point of diagnosis (blood collection) and endpoints were death from EOC, progress or relapse of EOC disease (therapy requirement). Differences with a p-value < 0.05 were considered statistically significant.

RESULTS

Increased sPD-L1 but Decreased sPD-L2 Serum Levels in EOC Patients

Serum levels of sPD-L1 and sPD-L2 are given as median (range) pg/ml. sPD-L1 levels were significantly ($p = 0.0001$) higher in 83 EOC patients [6.0 (0–32.9)] when compared to 29 healthy females [2.5 (0–13.7); **Figure 1A**]. At variance to sPD-L1, the sPD-L2 serum levels of EOC patients were significantly lower 1,862 (260–6,300) ($p = 0.003$) than levels observed in healthy controls [3,193 (34–6,300); **Figure 1B**]. No correlation was observed between sPD-L1 levels or sPD-L2 and age in EOC patients or controls (**Supplementary Figures 1A,B**).

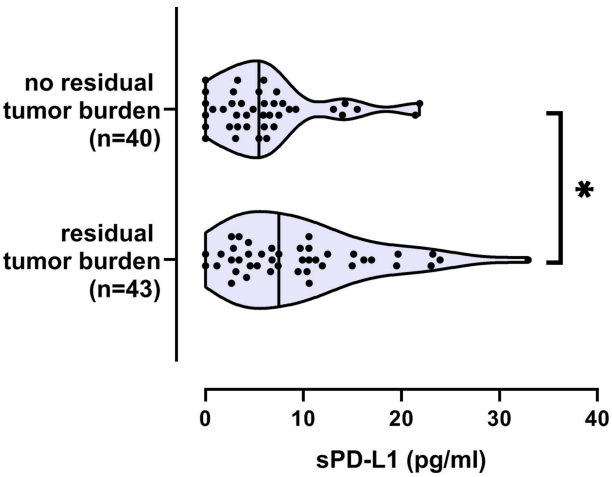

FIGURE 2 | Increased sPD-L1 serum levels in EOC patients with residual tumor burden. Straight line within the violin plot indicates the median. $^{*}p < 0.05$.

Association of sPD-L1 and sPD-L2 Serum Levels With Clinical Characteristics

Concerning clinical characteristics, sPD-L1 levels and sPD-L2 did not show any association to FIGO-stage, tumor grade, lymph node infiltration, or presence of metastases (**Table 1**). However, increased sPD-L1 levels were significantly associated with residual tumor burden ($p = 0.022$; **Table 1**; **Figure 2**) and reduced sPD-L2 levels were significantly ($p = 0.0096$) associated with platinum-resistance (**Table 1**; **Figure 3**).

Association of Decreased sPD-L2 Serum Levels With the Presence of CTCs

The presence of CTCs before therapy was associated with lower sPD-L2 levels [1,324 (260–3,019), $N = 22$], whereas the absence of CTCs was accompanied by increased levels of sPD-L2 [2,100 (686–6,300); $p < 0.0001$; **Figure 4A**]. With regard to CTC subtypes, ERCC1+ CTCs were significantly associated with lower

levels of sPD-L2 ($p < 0.0001$; **Figure 4B**). No association between the presence of CTCs and sPD-L1 was observed.

Association of High sPD-L1 Levels With Reduced Overall and Progression-Free Survival

As shown in **Table 1**, samples from patients who were alive at the time point of analysis displayed significantly ($p = 0.003$) lower sPD-L1 levels [4.2 (0–23.6); $N = 43$] than samples from patients who did not survive [7.3 (1.1–32.9); $N = 40$]. Similarly, patients without disease progression exhibited lower sPD-L1 levels [4.3 (0.0–19.7); $N = 28$] than patients with progression [7.0 (0.0–32.9); $N = 55$; $p = 0.03$]. With regard to the predictive value for a 5 year PFS and OS, an optimal cut-off value of 6.4 pg/ml was calculated with a sensitivity of 58.0 or 69.25% and specificity of 76.0 or 70.4% by ROC analysis. Using this cut-off, a similar Odds-Ratio (OR) for sPD-L1 (5.37; 95% CI: 2.14–13.42) was obtained as for the presence/absence of CTC before therapy (OR: 4.61; 95% CI: 1.63–13.01, $p = 0.003$) and platinum-resistance/sensitivity (OR: 5.48; 95% CI: 1.58–19.04; $p = 0.007$) with respect to 5-year OS (**Table 2A**). Nevertheless, for 5 years PFS, the OR of sPD-L1 was 4-fold lower compared to the one of Platinum-resistance/sensitivity (**Table 2B**). No significant association of 5-year PFS or OS with sPD-L2 was observed.

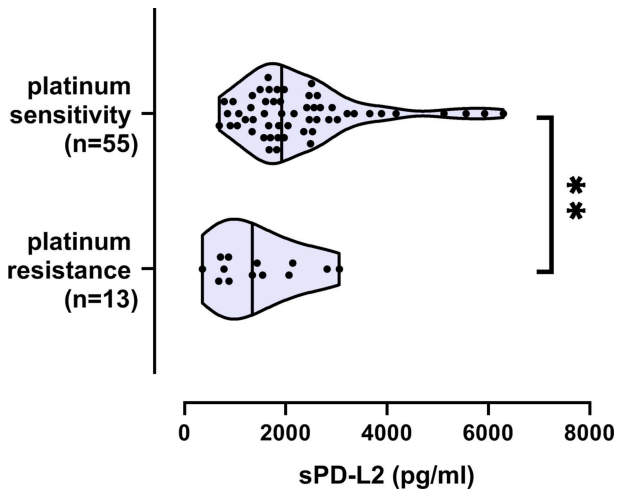

FIGURE 3 | Decreased sPD-L2 serum levels in EOC patients with platinum resistance. Straight line within the violin plot indicates the median. Platinum resistance/sensitivity was available for 68 EOC patients. $^{**}p < 0.01$.

Kaplan-Meier analysis combined with Log-rank testing revealed that patients with sPD-L1 of >6.4 pg/ml experienced a reduced OS (median OS: 30 months) compared to patients with sPD-L1 levels of <6.4 pg/ml [median OS: undefined; Hazard Ratio (HR): 2.67; 95% CI: 1.35–5.28] within an observation time of 60 months ($p = 0.0031$; **Figure 5A**). Considering 5-year PFS, patients with sPD-L1 <6.4 pg/ml showed a 2-fold prolonged PFS (median: 29 months) compared to patients with sPD-L1 >6.4 pg/ml (median: 14 months; $p = 0.019$, HR: 1.84; 95% CI: 1.09–3.10; **Figure 5B**). For CTC-negative patients, a prolonged 5-year OS (median: undefined, $p = 0.007$) and PFS (28 months; $p = 0.053$) were observed compared to CTC-positive patients (median: 26 and 15 months, respectively). As expected, patients with platinum-sensitive tumors ($N = 55$) displayed a prolonged 5-year OS (median OS: undefined, HR: 3.86; 95% CI: 1.69–8.78; $p = 0.001$) and PFS (median PFS: 32 months, HR: 20.2; 95% CI: 8.5–48.4; $p < 0.0001$) compared to patients with platinum-resistant disease (median OS: 21 months, $N = 13$; median PFS: 8 months, $N = 13$).

High sPD-L1 Levels as a Prognostic Marker for Disease Progression and Outcome in Platinum-Sensitive EOC Patients

As platinum-resistance is indicative for early disease progress and reduced OS, a stratification of patients with sPD-L1 levels <6.4 and >6.4 pg/ml was performed in platinum-sensitive and in platinum-resistant patients, respectively. In the group of platinum-sensitive patients, patients with sPD-L1 levels >6.4 pg/ml showed a significantly ($p = 0.035$) reduced probability of 5-year OS (HZ: 3.96; 95% CI: 1.27–12.30)

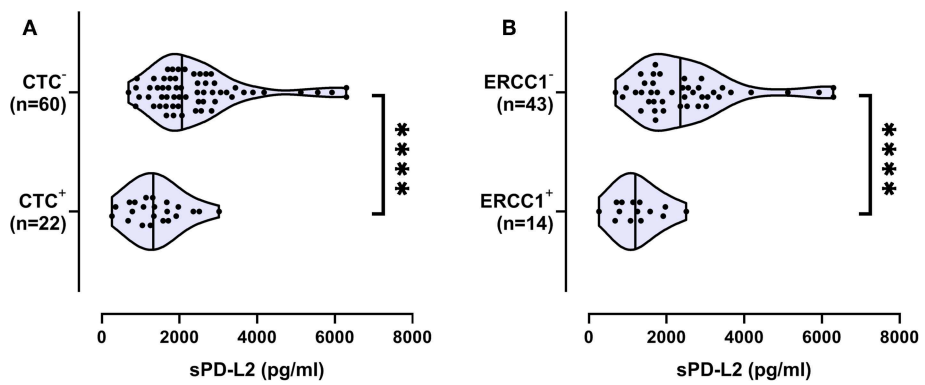

FIGURE 4 | Association of decreased sPD-L2 serum levels (pg/ml) with the presence of circulating tumor cells (CTC) and the ERCC1+CTC subpopulation. Data about the presence of CTC **(A)** or ERCC1+CTC **(B)** was available for 82 and for 57 EOC patients, respectively. Straight line within the violin plot indicates the median. $^{****}p < 0.0001$.

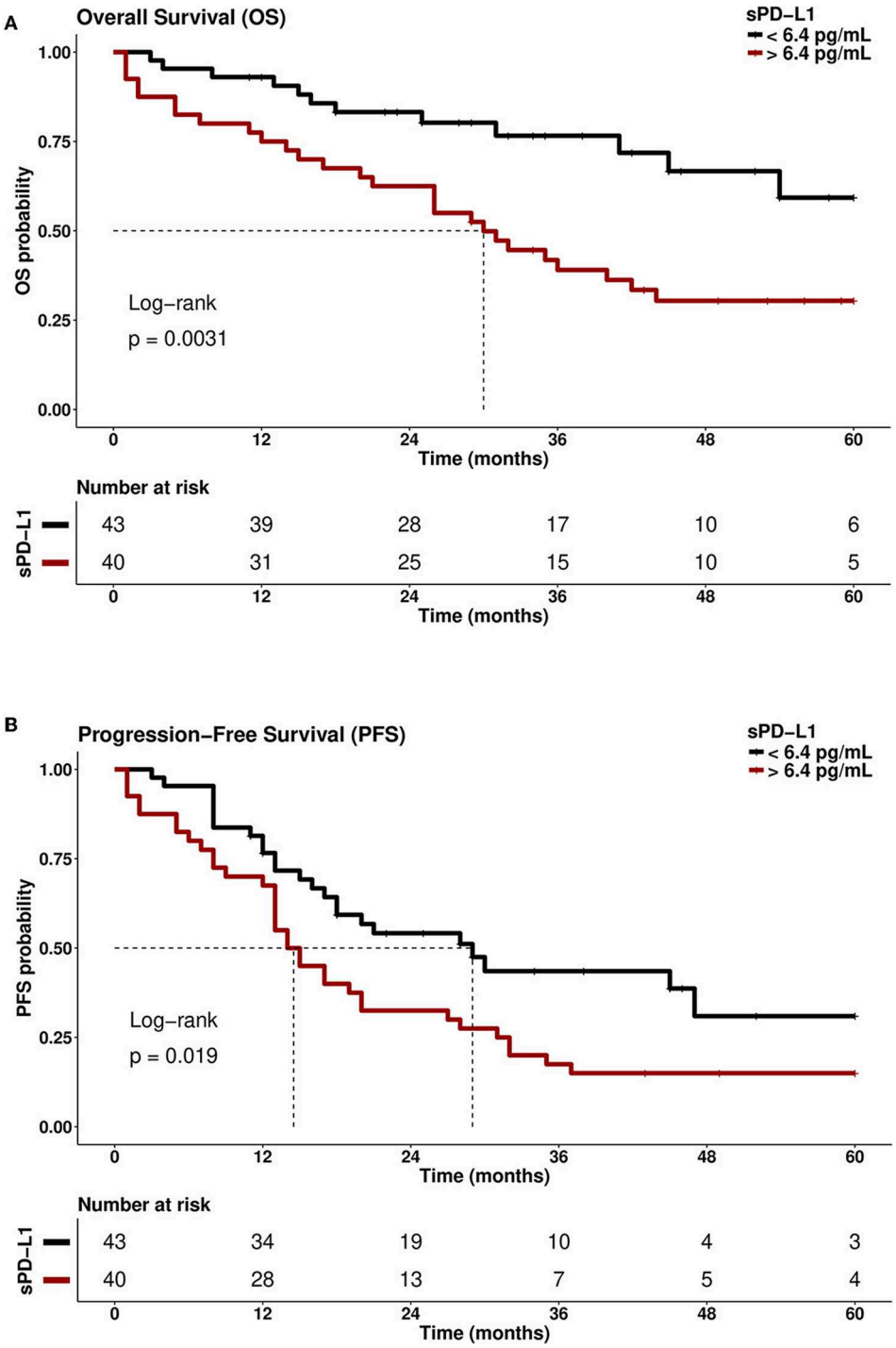

FIGURE 5 | Kaplan-Meier curve of survival probability with respect to sPD-L1 serum levels (pg/ml). Patients with high sPD-L1 serum levels (>6.4 pg/mL) had a reduced **(A)** overall survival (OS; $p = 0.0031$) and **(B)** progression-free survival (PFS; $p = 0.019$) compared with patients who had low sPD-L1 levels (<6.4 pg/ml). Time was calculated from blood sampling to event (death/progression) or last follows up. Dotted line indicates the median survival time of EOC patients in the respective group.

compared to patients below this cut-off (**Figure 6A**). A similar trend was observed for 5-year PFS ($p = 0.083$; **Figure 6B**): Platinum-sensitive patients having sPD-L1 levels >6.4 pg/ml presented a shortened PFS (median: 27 months) compared to patients with levels <6.4 pg/ml (median: 47 months HZ: 1.85; 95%CI: 0.91–3.79). Contrary to these findings, the 5-year OS

TABLE 2 | Association of sPD-L1, CTCs, and platinum-resistance/sensitivity status of EOC Patients with 5-year overall survival (OS) **(A)** and progression-free survival (PFS) **(B)**.

Parameter		No	Yes	$p^{\dagger}$	OR (95% CI)
(A)					
5-year OS					
sPD-L1	>6.4 pg/mL	12	13	0.0003	5.37 (2.14–13.42)
	<6.4 pg/mL	27	31		
CTC[b]	Positive	16	6	0.003	4.61 (1.63–13.01)
	Negative[b]	22	38		
Platinum[‡]	Resistant	4	9	0.007	5.48 (1.58–19.04)
	Sensitive	38	17		
(B)					
5-year PFS					
sPD-L1	>6.4 pg/mL	34	6	0.0004	4.49 (1.61–12.47)
	<6.4 pg/mL	24	19		
CTC[b]	Positive	19	3	0.046	3.67 (1.02–13.14)
	Negative[b]	38	22		
Platinum[‡]	Resistant	13	0	0.003	21.00 (2.76–159.94)
	Sensitive	31	24		

[†] p-values were calculated by Fisher's exact test, OR, Odds ratio; CI, confidence interval.
[‡] Platinum resistance was defined as recurrence <6 months after completion of adjuvant platinum therapy.
[b] The presence of CTC in the blood was unknown for one patient.

and PFS were very similar for patients >6.4 and <6.4 pg/ml in the group of platinum-resistant patients (data not shown). Furthermore, the CTC status did not identify patients with high risk of early progression and reduced OS in the group of platinum-sensitive patients (data not shown).

DISCUSSION

In recent years, many inhibitory molecules and cells have been identified, which facilitate the escape of tumor cells from immune surveillance by creating an immunosuppressive microenvironment either locally at the site of the tumor or systemically. Among the different immune escape mechanisms, the interaction of PD-1 expressed on immune cells with its ligands PD-L1 or PD-L2, expressed on tumor cells or antigen presenting cells such as macrophages and dendritic cells, represents an important immune evasion pathway in EOC. In our study we focused on the diagnostic and prognostic potential of PD-L1 and PD-L2 serum levels in a cohort of 83 primary EOC patients. We were able to demonstrate different clinicopathologic significances for sPD-L1 and sPD-L2 levels: (i) sPD-L1 level was increased and sPD-L2 was decreased in EOC patients compared to healthy controls. (ii) High sPD-L1 levels were related to residual tumor burden, reduced PFS and OS, whereas low levels of sPD-L2 were associated with platinum-resistance and the presence of CTCs, especially ERCC1+ CTCs. (iii) In platinum-sensitive patients, sPD-L1 levels above 6.4 pg/ml were significantly associated with a reduced probability of 5 years OS and mildly related to a reduced 5 years PFS, while OS and PFS in platinum-resistant patients did not differ when patients were stratified according to this cut-off.

In line with our results, a recent study demonstrated enhanced sPD-L1 levels in 174 EOC patients compared to healthy women and patients with benign ovarian tumors (14). Likewise, a consistent negative effect of high sPD-L1 levels on OS has also been described in a recent meta-analysis summarizing 1,040 patients with different solid tumors including lung, gastrointestinal and renal cancer (17). While cell surface-expressed PD-L1 on macrophages has been shown to be associated with favorable prognosis in ovarian cancer (11), soluble forms of PD-L1 are thus likely to play a significant role for immune escape-mechanisms in different tumor entities. This hypothesis is further supported by a recent study demonstrating that the secretion of sPD-L1 as extracellular vesicle correlates with tumor size and inhibits the proliferation, cytokine production, and cytotoxicity of CD8 T cells in malignant melanoma (18).

Little is known about the functional consequence of cell surfaced-expressed PD-L2 or sPD-L2 in oncologic diseases. A recent meta-analysis on the correlation between PD-L2 expression and clinical outcomes in solid tumors supports the notion that high PD-L2 expression favors tumor metastasis and unfavorable prognosis in solid cancer patients after surgery, especially in hepatocellular carcinoma (19). To the best of our knowledge, PD-L2 expression at tumor sites in EOC patients has not been investigated. Only one study was able to analyze the PD-L2 expression on HLA-DR-positive cells found in ascitic fluids from EOC patients but no relationship with the clinical outcome was observed (20). In our study, EOC patients showed significantly lower sPD-L2 compared to healthy controls and within patients, those with platinum-resistant tumors showed substantially lower sPD-L2 levels than those with platinum-sensitive disease. These results suggest an important role of sPD-L2 in the host immune response to the tumor and further point to the significance of immune processes for the response to platinum therapy and outcome in EOC. Notably, low sPD-L2 levels were significantly related to the presence of ERCC1+CTCs, a CTC-subgroup associated with platinum resistance and worse outcome in EOC (16, 21). Hypothesizing a causal relationship between low sPD-L2 levels and the presence of ERCC1+CTC, one might argue that sPD-L2 mediated anti-tumor activity might play an important role in the prevention of tumor spread to the bone marrow and the elimination of CTCs.

In a previous study, we showed that immunotherapy, applying the intraperitoneal trifunctional bispecific antibody Catumaxomab, was successful in the elimination of CTCs as well as DTCs in the BM in patients with advanced ovarian cancer (7), hinting at the inverse relationship between anti-tumor immunoactivity and tumor cell spread to the BM. Other immunotherapeutic approaches have been reported to show activity in EOC such as the cytotoxic T-lymphocyte associated protein 4 (CTLA-4) antibody Ipilimumab (22). Regarding the PD-1/PD-L1/2 axis, the PD-1 antibody Nivolumab (23) as well as the anti PD-L1

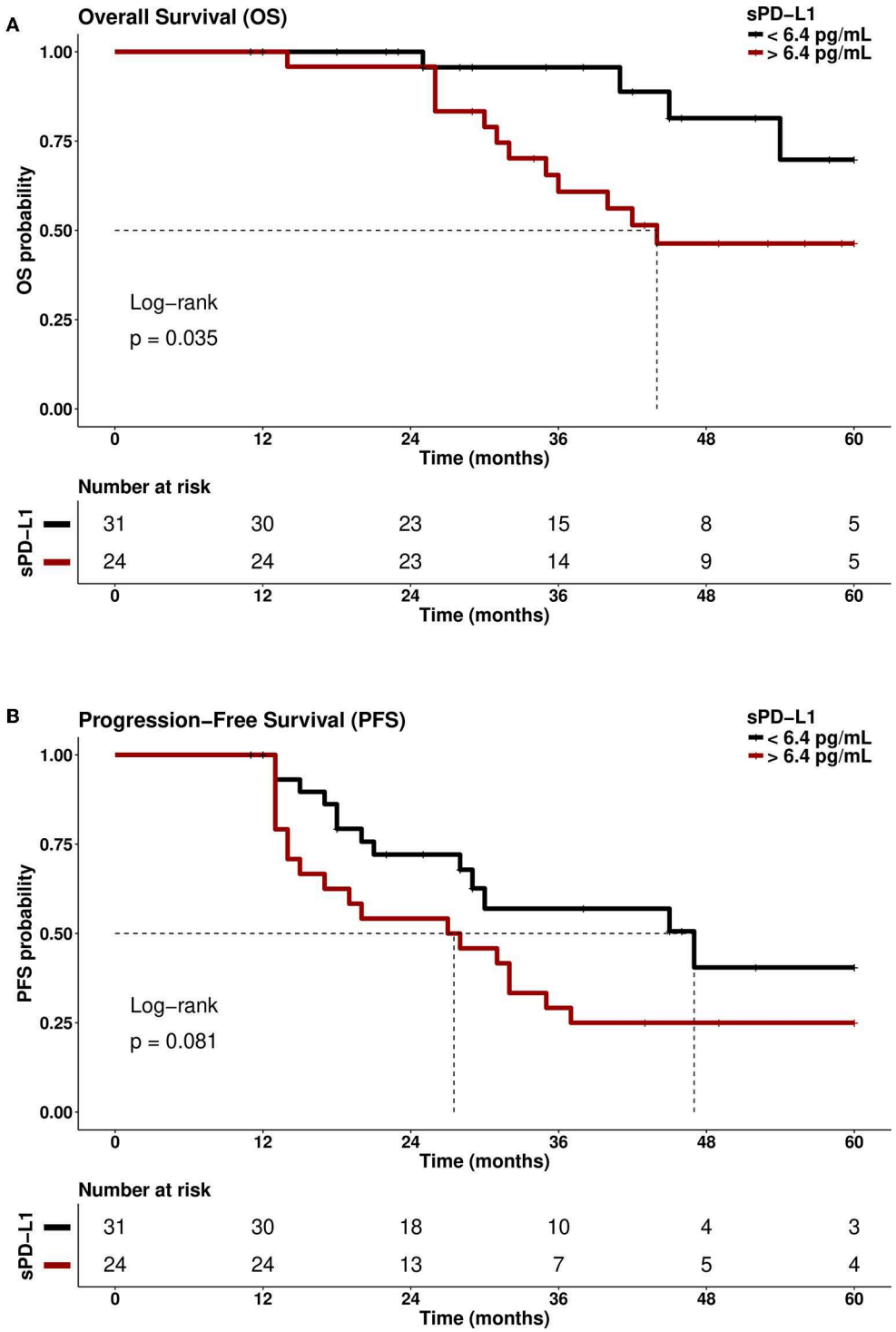

FIGURE 6 | Association of high sPD-L1 levels with reduced overall survival (OS) and progression-free survival (PFS) in platinum-sensitive patients. Platinum-sensitive EOC patients with high sPD-L1 serum levels (>6.4 pg/mL) had a reduced **(A)** overall survival (OS; $p = 0.035$) and **(B)** progression-free survival (PFS; $p = 0.081$) compared with platinum-sensitive patients with low sPD-L1 levels (<6.4 pg/ml). Time was calculated from blood sampling to event (death/progression) or last follows up. Dotted line indicates the median survival time of EOC patients.

antibody Atezolizumab (9) have been successfully used in EOC patients. However, little is known about the effect of these therapies on the presence of CTCs. It will be interesting to determine if the activation of different anti-tumor immune-pathways can help to eliminate CTCs as shown before for Catumaxomab.

Forthcoming, research will have to elucidate the complex mechanisms involving the cellular-expressed and soluble forms of PD-L1 and PD-L2 in EOC. However, in our study sPD-L1 helped to differentiate EOC patients from healthy controls and predict prognosis. Although our study has several limitations, mostly attributed to the small sample size and its retrospective study design, the clinical relevance of our findings is especially of interest for patients with a rather good prognosis, as defined by sensitivity to platinum therapy. In these patient cohort sPD-L1 levels helped to identify high risk patients for unfavorable disease outcome despite platinum-sensitivity, which may argue for anti-PD-1 and anti-PD-L1 antibodies treatment as additional therapeutic approaches being already successfully used in early stage clinical studies (9). Nevertheless, the value of sPD-L1 as a biomarker for the administration of anti-PD-L1/PD-1 therapy needs to be evaluated. As sPD-L2 is related with platinum sensitivity and the occurrence of CTC, both markers are promising candidates in the context of the development of new, reliable prognostic biomarkers easily accessible via liquid biopsy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University Hospital Essen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PB: study design, data acquisition, providing of blood and tissue samples, statistical analysis, and manuscript writing. ES: study design, serum analyses, statistical analysis, and manuscript editing. CJ: serum analyses, statistical analysis, and manuscript editing. PH: manuscript editing. RK: providing of blood and tissue samples and manuscript editing. SK-B: study design, data acquisition, providing of blood and tissue samples, and manuscript editing. VR: study design, data acquisition, serum analyses, statistical analysis, and manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.01015/full#supplementary-material

Supplementary Figure 1 | No correlation between age and sPD-L1 **(A)** and sPD-L2 **(B)** serum levels in healthy controls (HC) and patients with epithelial ovarian cancer (EOC). A filled triangle indicates correlation points of HC and open circle indicate correlation points EOC patients.

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REVIEW
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Leukobiopsy – A Possible New Liquid Biopsy Platform for Detecting Oncogenic Mutations

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Detection of unique oncogenic alterations encoded by the sequence or biochemical modification in cancer-associated transforming macromolecules has revolutionized diagnosis, classification and management of human cancers. While these signatures were traditionally regarded as largely intracellular and confined to the tumor mass, oncogenic mutations and actionable cancer-related molecular alterations can also be accessed remotely through their recovery from biofluids of either rare circulating tumor cells (CTCs), or of more abundant non-cellular carriers, such as extracellular vesicles (EVs), protein complexes, or cell-free tumor DNA (ctDNA). Tumor-related macromolecules may also accumulate in circulating platelets. Collectively, these approaches are known as liquid biopsy and hold promise as non-invasive, real-time opportunities to access to the evolving molecular landscape of human malignancies. More recently, a possibility of recovering cancer-specific DNA sequences from circulating leukocytes has also been postulated using experimental models. While it is often assumed that these and other liquid biopsy approaches rely on material passively shed from the tumor mass or its debris, recent evidence suggests that several regulated processes contribute to the abundance, nature, half-life, and turnover of different circulating cancer-related molecular signals. Moreover, many of these signals possess biological activity and may elicit local and systemic regulatory responses. Thus, a better understanding of the biology of liquid biopsy platforms and analytes may enable achieving improved performance of this promising and emerging diagnostic strategy in cancer.

Keywords: oncogenes, neutrophils, extracellular vesicles, exosomes, thrombosis, liquid biopsy, cancer, diagnosis

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INTRODUCTION

In this overview we wish to articulate and contextualize two frequently overlooked considerations. First, the liquid biopsy paradigm sweeping through the diagnostic space in cancer might have an important functional component as the respective analytes, be it soluble nucleic acids or circulating cells are under influence of regulatory processes that control their abundance. They also may possess poorly understood biological activities of their own. Second, the list of biological carriers that can be remotely intercepted and used as liquid biopsy diagnosis platforms continues to expand, one novel possible addition being leukocyte cargo of cancer-related nucleic acids, that can be retrieved in the process that can be termed "leukobiopsy". While we discuss the necessary context of these

considerations this article is not intended as a systematic overview of liquid biopsy as such, a topic that has been amply covered by leaders in the field as, at least partially, reflected in citations used in the remainder of the text.

LIQUID BIOPSY PLATFORMS IN CANCER

It could be argued that progress achieved in the management of human cancers during the past several decades is largely attributable to a better understanding of the biological, cellular and molecular underpinnings of different malignant states (Vogelstein and Kinzler, 2004). While functional significance of the hallmarks of cancer (Hanahan and Weinberg, 2011) has illuminated the salient commonalities of neoplastic processes, it is the understanding of tumor diversity, existence of disease subtypes, and aspects of their molecular uniqueness, that has guided successes in targeted and biological therapy efforts (Gotwals et al., 2017). Indeed, it can be argued that differences between disease entities are often more actionable than similarities.

The centrepiece in this paradigm and the source of hope for developing more effective, personalized anticancer treatment strategies, is the notion of striking at crucial oncogenic drivers, either genetic or epigenetic, that are implicated in cancer causation (Vogelstein and Kinzler, 2004), but which may be highly context-specific (Ben-David et al., 2019). While compiling the related molecular information is often illuminating, and has led to the discovery of new therapeutic targets as well as the remarkable molecular diversity of major human cancers (Curtis et al., 2012; Reifenberger et al., 2017), it does not necessarily, by itself, lead to successful therapies for several reasons. First, driver genes often unleash a chain reaction of stromal and host responses, such as angiogenesis (Rak et al., 1995), inflammation (Sparmann and Bar-Sagi, 2004), immunosuppression (Spranger and Gajewski, 2018), coagulopathy (Yu et al., 2005), or other complex microenvironmental changes (Finak et al., 2008), which assume a pathogenetic role of their own (Magnus et al., 2014) and may not be readily reversible even upon suppression of the oncogenic signal. Second, the genetic evolution of cancer cell clones generates perpetual drift in their oncogenic repertoires, resulting in heterogeneity and co-existence of several disease-causing mechanisms often obscured by dominant cell populations (Gerlinger et al., 2012; Ben-David et al., 2019). Third, processes of invasion and metastasis result in the formation of a multifocal malignant disease where different tumors progress independently in the same individual (Fidler, 2003; Gerlinger et al., 2012). Fourth, anticancer therapies often result in a profound re-alignment of the molecular repertoire of cancer cell populations, due to mutations, selection, or the entry of minor clones into the disease process (Johnson et al., 2014; Wang et al., 2016; Garnier et al., 2018). This happens at the time when the recurrent disease no longer responds to prior therapy, while new vulnerabilities are often still unknown (Wang et al., 2016).

These biological considerations complicate molecular diagnosis of cancers, which is traditionally predicated on the analysis of surgical or biopsy tissue specimens. Such one-time snapshots of limited spectrum of tumor microregions is simply insufficient to accurately reflect the spatial and temporal complexity and cellular heterogeneity of the progressive and evolving disease (Siravegna et al., 2017). While serial biopsy programs may alleviate these challenges, at least to some extent, the invasive nature of these procedures, risk of complications, tissue sampling errors, ethical considerations and inaccessibility of anatomically difficult sites or disseminated tumor foci may severely curtail the expected gains (Siravegna et al., 2017).

Many (if not all) human cancers, even if anatomically localized, exert a level of systemic impact through the release of tumor cells and their products into biofluids, such as blood, cerebrospinal fluid, urine, ascites, pleural effusion, or glandular secretions. Collection and analysis of these remote signals, long known as liquid biopsy (**Figure 1**), offers a remote, continuous and non-invasive access to salient characteristics of all tumor cell subpopulations (and stroma) with adequate representation in the appropriate biofluid (Pantel and Alix-Panabieres, 2019). While this is an area of intense interest, and one extensively reviewed in recent literature (Crowley et al., 2013; Siravegna et al., 2017; Wan et al., 2017; Heitzer et al., 2019; Pantel and Alix-Panabieres, 2019), it deserves a few general comments and context.

First, the nature of tumor-related material that is recovered from biofluids, such as plasma, ranges from simple molecular biomarkers such as PSA, CA125, or CEA to more comprehensive representations of cancer complexity and driving mechanisms (bona fide liquid biopsy), such as circulating tumor cells (CTCs) (Pantel and Alix-Panabieres, 2019), circulating cell-free DNA (cfDNA) (Schwarzenbach et al., 2011), tumor-educated platelets (TEPs) carrying tumor-related biomolecules (Cervi et al., 2008), especially RNA (Nilsson et al., 2011; In 't Veld and Wurdinger, 2019), and an array of extracellular particles (EPs) and vesicles (EVs) containing molecular and mutational fingerprints (proteins, RNA and DNA) of their donor cancer cells (Al-Nedawi et al., 2008; Skog et al., 2008; Balaj et al., 2011; Kahlert et al., 2014; Lazaro-Ibanez et al., 2014; Lee et al., 2014; Thakur et al., 2014; Siravegna et al., 2017; Miller et al., 2019). Each of these liquid biopsy platforms is based on a different, defined or presumed biological process resulting in the shedding of cancer-related material into the circulation. For example, while CTCs are generally believed to represent a part of (though not tantamount to) the metastatic intravasation process, ctDNA is often viewed as a result of cellular breakdown and release of debris from the tumor mass or from other poorly defined release mechanisms (Siravegna et al., 2017).

By their very nature, different liquid biopsy platforms pose different analytical challenges. For example, the relative abundance of CTCs is thought to be low (1–10 CTCs/ml of plasma), which underrepresents the cellular heterogeneity of the corresponding cancer, while ctDNA is often present at low levels, but more importantly, in a largely fragmented molecular form. Extraction of RNA signals from circulating ribonucleoprotein

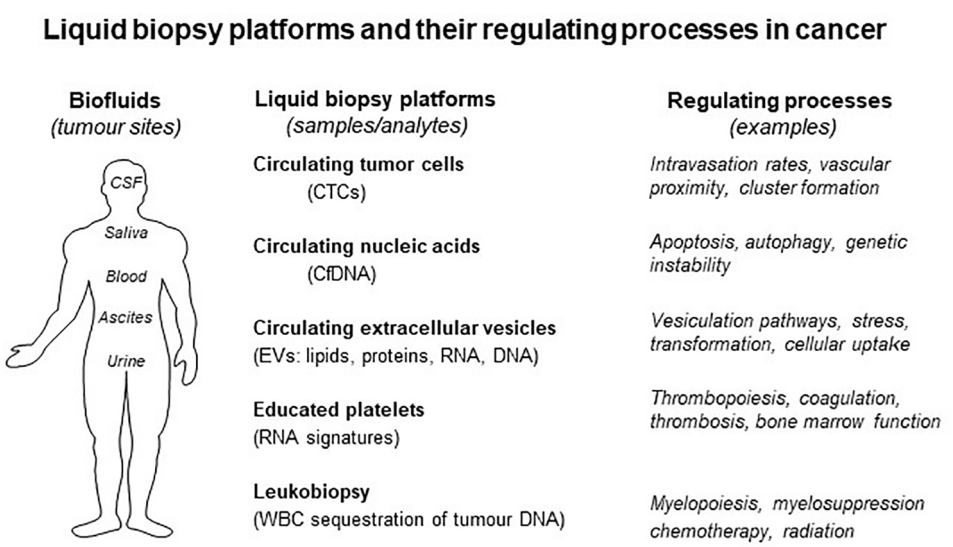

FIGURE 1 | Liquid biopsy platforms and processes that regulate them in cancer. Several processes affect the state of biofluids (right) and their content of liquid biopsy analytes. Each form of liquid biopsy (CTCs, cfDNA, EVs, platelets, and leukocytes) may be affected by unique regulatory influences that may change the content of biological information (see text for details).

complexes, platelets or EVs may be technically complex and burdened with variability (Das et al., 2019; Jeppesen et al., 2019). While some of these challenges are often perceived as "technical" in nature, they may result from our limited understanding of the biological processes leading to the presence of specific liquid biopsy forms and analytes in biofluids, as well as mechanisms regulating the fate, half-life, content and diagnostic meaning of the related molecular signals.

EXTRACELLULAR VESICLES AS CARRIERS OF DIAGNOSTIC INFORMATION

EV/EPs exemplify the link between cancer biology and its extracellular and systemic representation (Rak, 2013). Cells are programmed to export complex multi-molecular packets either as membrane-less EPs, such as exomeres (Zhang et al., 2018), or as a wide spectrum of EVs (Zijlstra and Di Vizio, 2018), of which exosomes originate intracellularly, within the late endosome, while ectosomes (microvesicles) and many other EVs result from outward budding of the plasma membrane (Van Niel et al., 2018).

Apart from their unique biogenesis, exosomes possess several distinctive properties. They tend to be under 150 nm in size, float at low density of sucrose (1.11–1.19) and carry a repertoire of surface markers enriched in tertraspanins (CD9, CD63, CD81, and CD82), components of endosomal sorting complex required for transport (ESCRT; TSG101) and related proteins (ALIX, Syntenin 1). While this places ESCRT at the centre of EV biogenesis, exosomes may also be generated in an ESCRT-independent manner linked to production of ceramide within the vesicle membrane by neutral sphingomyelinases (SMPD3) (Van Niel et al., 2018; Xu et al., 2018). Therefore, inhibitors of SMPD3 may reduce cellular exosome production (Trajkovic et al., 2008), as could targeting elements of vesicular transport system (Rab27a/b, SYT7) or tetraspanins (Chairoungdua et al., 2010; Luga et al., 2012; Sung et al., 2015).

Budding from the plasma membrane gives rise to a large spectrum of EVs ranging from 40nm–100nm (ARMMs), 150–1,000 nm (microvesicles), over 1um (migrasomes), and 1um–10 um (large oncosomes) with different properties, biogenetic origins, cargo packaging mechanisms, molecular make ups and biological activities, as reviewed extensively in the recent literature (Muralidharan-Chari et al., 2009; Thery et al., 2009; Abels and Breakefield, 2016; Kowal et al., 2016; French et al., 2017; Xu et al., 2018; Jeppesen et al., 2019). Moreover, molecular predictions based on the composition of the EV proteome suggest the existence of even greater diversity (dozens or more) of distinct EV subtypes that are only beginning to emerge. Efforts are underway to map EV landscapes (Choi et al., 2017) in various settings using single EV analysis afforded by nano-flow cytometry (Nolan, 2015; Choi et al., 2018) and microfluidics (Fraser et al., 2019).

EVs represent a conserved regulatory mechanism spanning the evolutionary spectrum from prokaryotic (Ibanez de Aldecoa et al., 2017) to mammalian cells (Van Niel et al., 2018) and endowed with two fundamentally important functions. First, EVs enable a rapid active expulsion of large amounts of molecular material including effector and signalling proteins,

as well as lipids, RNA, and DNA from their parental cells. This may enable cellular adaptation to differentiation programs (Johnstone, 2006), noxious signalling events (Chairoungdua et al., 2010), as well as molecular (Takahashi et al., 2017) and therapeutic stresses (Montermini et al., 2015; Garnier et al., 2018). Second, EVs are capable of delivering their molecular content to other cells thereby mediating intercellular communication, integration and molecular flux (Mulcahy et al., 2014). In cancer, EVs mediate transmission of mutant oncogenes between cells resulting in biological responses resembling malignant transformation (Al-Nedawi et al., 2008; Lee et al., 2016; Choi et al., 2017). Both of these properties (expulsion and uptake) are relevant for the emerging diagnostic use of EVs in cancer and other diseases.

From the liquid biopsy perspective EVs offer unprecedented advantages, but also pose some challenges. For example, in cancer, tumor-derived EVs carry a wealth of clinically important information as to driver mutations (Al-Nedawi et al., 2008; Skog et al., 2008; Choi et al., 2017), drug resistance markers (Bebawy et al., 2009; Boelens et al., 2014), determinants of immunoregulation (e.g. PD-L1) (Ricklefs et al., 2018) and other salient features of tumor and stroma. With up to 10^{12} EVs per ml of plasma, EVs exceed numbers of CTCs (<10 per ml) by several orders of magnitude, which also translates into favorable complexity profiles, which likely approximates the representation of the true heterogeneity of parental cancer cell populations. Unlike tumor-educated platelets that may undergo activation and sequestration, EVs circulate in biofluids in relatively stable biological form. Unlike ctDNA, EVs co-express informative and diverse biological signals, such as nucleic acid sequences and protein lineage markers, which makes them amenable to multiplexing and tracing cellular sources of cargo (Choi et al., 2017). This may enhance the specificity of detection and offer several other advantages (Shao et al., 2015; Zachariah et al., 2018). Moreover, EVs protect their molecular cargo from degradation and enable recovery of meaningful signals even from archival samples (Skog et al., 2008).

On the other hand, the abundance of cancer-related EVs in blood is estimated to be low, in single digit percentages (Abels and Breakefield, 2016), which poses sensitivity and background challenges. Their heterogeneous content of informative signals (Choi et al., 2019) may further impact detectability, all of which is compounded by a poor understanding of cargo packaging mechanisms, their regulation, and of the processes governing EV half-lives and fate upon entry into biofluids (Peinado et al., 2012; Hoshino et al., 2015; Chennakrishnaiah et al., 2018; Thery et al., 2018).

LEUKOBIOPSY

While bolus injection of EVs into the circulation leads to their rapid elimination within minutes to hours, due to the uptake in major organs (liver, spleen) (Wang et al., 2012; Thery et al., 2018), less is known about the natural turnover of EVs released into the circulation spontaneously. Notably, CD47 expression ("don't eat me signal") prolongs the half life of exosomes in the circulation suggesting that their uptake by phagocytes controls their fate (Kamerkar et al., 2017). Such uptake can also be demonstrated directly by EV-mediated transfer of cancer-related signalling receptors (e.g. MET)(Peinado et al., 2012), or RNA (Ridder et al., 2015) from cancer cells to myeloid cells *in vivo*.

Since the half-life of circulating blood cells is much longer than that of EVs and varies between 8 h for neutrophils (Summers et al., 2010), 3 days for monocytes (Patel et al., 2017) to 120 days for red blood cells (Franco, 2012), their possible retention of EV related material could prolong the availability of such molecules in the circulation. For this reason we assessed the distribution in blood of oncogenic DNA associated with the EV fraction of the cellular secretome in the case of cancer cells driven by either mutant HRAS (Lee et al., 2014) or amplified HER2 oncogenes (Chennakrishnaiah et al., 2018). The respective cancer cell lines (RAS3 and BT474) were inoculated into immune deficient mice and once tumors were established and reached readily palpable sizes blood was collected from individual mice and carefully fractionated by centrifugation into cellular compartments, such as red blood cells (RBCs), white blood cells (WBCs, buffy coats) and platelets (PLTs), while platelet poor plasma (PPP) was further separated by ultracentrifugation into the EV pellet and EV-free plasma supernatant (SUP). The DNA was extracted from these respective blood compartments and human oncogene (HRAS or HER2) copy number per ml of mouse blood was assessed using sequence specific droplet digital PCR (ddPCR) protocol (Chennakrishnaiah et al., 2018). Surprisingly, while EV and SUP fractions predictably contained oncogenic DNA, as did PLTs, the content of cancer-related genomic sequences were the highest in the WBC fraction, while RBC were virtually devoid of this signal. The contribution of CTC contamination was ruled out through the use of fluorescently labelled cancer cells. Furthermore, the circulating levels of cancer-specific DNA (csDNA) contained in the WBC fraction were higher than those recovered from the liver, spleen or bone marrow, organs where the uptake of EVs is thought to take place, suggesting that it is the circulating fraction of WBCs that sequesters this oncogenic material (Chennakrishnaiah et al., 2018).

Of note is the fact that in small blood samples (up to 100 ul) collected serially from mice harboring RAS3 tumors, the amount of HRAS DNA at the baseline was far more robust in WBCs than the signal recovered from the corresponding cell-free serum. Moreover, a surgical removal of the primary tumor led to extinction of the WBC-associated HRAS DNA signal within 2–3 days (estimated half life of these cells), while the signal in serum remained low and changed minimally over time. This suggests that, at least in some settings, the WBC content of csDNA may serve as a far more robust source of biologically meaningful representation of the driver mutation than csDNA contained in cell-free serum or in plasma (Chennakrishnaiah et al., 2018).

To understand which WBC population may have taken a role of the apparent reservoir of csDNA, buffy coat cellular isolates

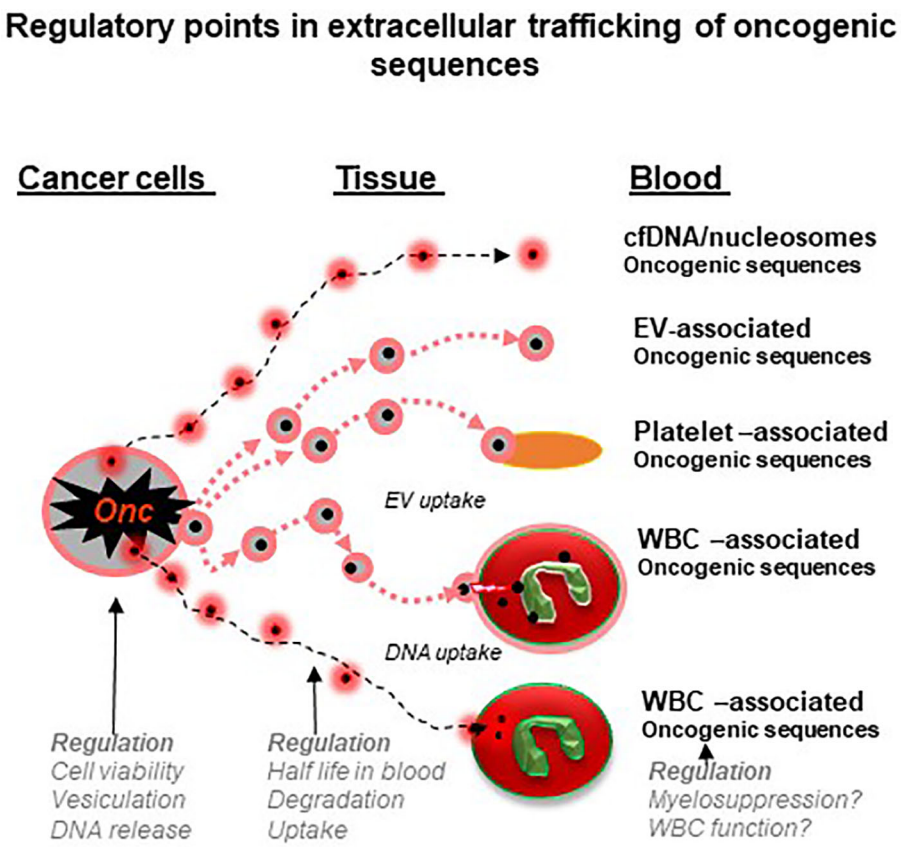

FIGURE 2 | Regulatory points in extracellular trafficking of oncogenic sequences in cancer (a hypothesis). Genomic DNA and mutant nucleic acids exit cancer cells either through secretory mechanisms, vesiculation or cell death. This material circulates in blood and biofluids as either particles (e.g. nucleosomes) or extracellular vesicles (EVs) and both may be ingested by platelets or white blood cells (WBCs). These cells may therefore act as reservoirs and regulatory mechanisms to control the levels of cancer-related nucleic acids. In addition, processes that influence the state and function of blood cells may influence their storage capacity for cancer-related material and possibly the circulating levels of liquid biopsy analytes (see text for details).

from blood of RAS3 tumor-bearing mice were sorted into neutrophils, monocytes and lymphocytes using appropriate markers. Interestingly, the greatest amount of human HRAS DNA was found in neutrophils, and the least in lymphocytes, as measured both per blood volume and per cell. This is understandable as neutrophils (and monocytes) possess a professional phagocytic activity and could play a role in clearing circulating particulate matter containing tumor DNA. Moreover, systemic depletion of neutrophils using anti-Ly6G antibody resulted in a shift of circulating csDNA to the EV and SUP (ctDNA) fractions of blood suggesting that, indeed, these cells control the distribution of tumor-related material in the circulation, possibly by ingesting EV-associated or free extracellular DNA (Chennakrishnaiah et al., 2018).

Interestingly, EVs or particles appear to be required for the uptake of extracellular DNA by leukocytes. This is because incubation of RAS3 cell-derived EVs or nucleosomes with myeloid HL-60 cells in culture resulted in the internalization and retention of tumor DNA in these cells for several days, while soluble DNA purified from EVs failed to penetrate into recipient cells (Chennakrishnaiah et al., 2018). Thus, interactions of EVs with circulating professional phagocytes may lead to the accumulation of EV-related DNA in these cells, which therefore may serve as a unique reservoir of cancer specific biomarkers. This experimental approach termed *leukobiopsy* still remains to be tested in diverse experimental models and in clinical settings to assess its possible diagnostic utility.

There are also several expected limitations that may be associated with this particular prospective diagnostic approach. First, the content of germline nuclear DNA may present dilution challenges with respect to detection of small amounts of cancer-related sequences, especially those without unique mutations. Second, the numbers of leukocytes and their phagocytic activities may vary as a function of chemotherapy, infection, inflammation

or myelosuppression (in a similar manner as experimental treatment with anti-Ly6G antibody), all of which could affect the ability of these cells to accumulate and retain cancer-related material. Indeed, different forms of biological regulation are likely a relevant consideration for all liquid biopsy platforms.

BIOLOGICAL REGULATORS OF LIQUID BIOPSY SIGNALS

While liquid biopsy is often regarded as a passive manifestation in biofluids of the remotely located tumor mass, several poorly understood processes are likely to control the levels of circulating analytes and their carriers, be it CTCs, EVs or ctDNA. The aforementioned example of experimental leukobiopsy exemplifies at least two levels of such a regulation. First, the uptake of EVs and nucleosomes by leukocytes reduces the levels of cell-free mutant DNA in plasma and shifts it to another blood compartment (WBCs) that is not routinely analysed in this setting. Second, as mentioned, neutrophil depletion dramatically increases the EV and ctDNA content of the mutant HRAS signal in blood (Chennakrishnaiah et al., 2018). Since numbers, compositions and states of myeloid cells in blood are regulated by several cancer associated processes, such as inflammation, secondary infections, immunomodulation, or myelosuppression, including the effects of cytotoxic therapy and radiation, it could be argued that the performance of ctDNA or EV-based liquid biopsy assays may be affected by these conditions.

Similar considerations may apply to tumor-educated platelets as a reservoir of cancer-related macromolecules (e.g. RNA)(In 't Veld and Wurdinger, 2019). For example, cancer-associated thrombosis (CAT) is a condition that affects variable numbers of patients with the severity that largely depends on cancer site (Wun and White, 2009), type (Hisada and Mackman, 2017), and molecular subtype (Magnus et al., 2013; Unruh et al., 2016). However, the overall prevalence of CAT is very high as autopsy data estimate it to occur in approximately 50% of cases (Timp et al., 2013). CAT involves either an increase (Haemmerle et al., 2018), or consumptive reduction of circulating platelets (Riedl et al., 2017). The latter is often the case in glioblastoma due to the expression of platelet activating surface glycoprotein, podoplanin, on the surface of cancer cells (Riedl et al., 2017). Interestingly, platelets ingest glioblastoma EVs containing oncogenic transcript for EGFRvIII and carry this signature into the circulation (Nilsson et al., 2011). However, certain subtypes of glioblastoma are spared form CAT, due to protective effects associated with the expression of specific transforming mutations, such as those of isocitrate dehydrogenase (IDH1), a phenotype that correlates with reduced expression of tissue factor, podoplanin and other genes (Unruh et al., 2016; Tawil et al., 2019) and with normal platelet counts (Unruh et al., 2016). It is presently unknown, but remains of great interest whether these events affect the levels of blood-borne cancer biomarkers associated with platelets, EVs, or cell-free nucleic acids.

Chemotherapy and radiation could potentially exert complex influences on liquid biopsy analytes. It is possible that in some instances the amounts of molecularly informative cfDNA or DNA associated with EVs could be increased post-treatment due to cell death processes occurring at the tumor site (Swystun et al., 2011). On the other hand, chemotherapy and radiation often trigger myelosuppression and reduced WBC and platelet counts in the circulation. It is possible, but remains to be thoroughly investigated, that these events may shift the content of circulating cancer DNA from leukocytes or plasma and affect assay sensitivity (Ritch and Wyatt, 2018). Nonetheless, the relationship between the effects of therapeutic interventions and the performance of liquid biopsy platforms requires further study and possibly preanalytical adjustments (e.g. timing of sample collection).

Not all cancer cells with comparable biology release oncogenic proteins and nucleic acids into the circulation. For example, in leukemic cells driven by oncogenic PML-RARa this fusion product triggers profound changes in the molecular repertoire of EVs released into the culture media, but such EVs do not contain measurable amounts of the PML-RARA oncoprotein and neither do they transfer this signal to recipient endothelial cells (Fang et al., 2016). Giant cell tumors of the bone (GCT) release certain amounts of oncogenic DNA with mutant oncohistone sequences ($H3.3^{G34W}$), but the levels of this material differ between cell lines and, at least in some cases, sequence specific amplification of DNA contained in EVs is dramatically less efficient than in the case of equivalent amounts of cellular DNA suggesting EV-related biochemical changes or fragmentation (Aprikian – unpublished). Certain cancer cells harboring oncogenic RAS exhibit high level of genetic instability and produce ample cytoplasmic DNA (Dou et al., 2015), which is associated with emission of genomic DNA into the EV fraction of conditioned media (Lee et al., 2014) and into blood of tumor bearing mice (Chennakrishnaiah et al., 2018). This process is likely driven by compromised integrity of the nuclear envelope, instability of the cellular genome, onset of autophagy and other processes (Dou et al., 2015) (Tsering – unpublished). Their regulation might therefore change the emission and detection of extracellular DNA. Tumors driven by other oncogenes may produce lower amounts of EV-associated DNA and the levels of this signal in blood of tumor-bearing mice and in cancer patients may also exhibit considerable variability, impacting the sensitivity of detection and the robustness of the respective liquid biopsy tests.

It is also of note that the choice of biofluids may affect the performance of liquid biopsy assays. For example, while in glioblastoma the detection of tumor-specific mutations is possible in both peripheral blood and cerebrospinal fluid (Al-Nedawi et al., 2008; Skog et al., 2008; Graner et al., 2009; Figueroa et al., 2017), the latter represents the liquid space more proximal to cancer cells, rendering signal detection more robust (Zachariah et al., 2018; Seoane et al., 2019). Thus, a better understanding of biological processes and regulatory mechanisms that control the release of liquid biopsy analytes

may hold the key to a more rational use of biofluid sources, molecular signal recovery methods and detection techniques for specific cancers and medical purposes.

BIOLOGICAL EFFECTS OF LIQUID BIOPSY ANALYTES

It is increasingly clear that liquid biopsy analytes possess important biological activities, which may add meaning and complexity to their detection. In this regard the emerging biological effects of traditional cancer biomarkers, such as PSA (Niu et al., 2008), or CEA (Bramswig et al., 2013) have attracted recent attention as regulators of cellular growth and angiogenesis. While the contribution of CTCs to metastasis is implicit, their interactions with plasma, platelets and other cells in the circulation may result in additional biological perturbations of significance, for instance in the context of systemic CAT and thrombotic events in cancer patients (Beinse et al., 2017). Similarly, extracellular DNA and chromatin released from cancer cells may induce thrombosis (Swystun et al., 2011) and interfere with the function of leukocytes acting as damage recognition molecular patterns (Swystun and Liaw, 2016).

In some of these instances the oncogenic activity of liquid biopsy-associated macromolecules and their carriers (EVs/EPs) may play a role in biological events. For example, oncogenic EGFR released by cancer cells as cargo of EVs detectable in blood (Montermini et al., 2015) may be taken up by endothelial cells and reprogram their biological responses, including activation of the VEGF pathway and angiogenesis (Al-Nedawi et al., 2009). Detection of RAS mutations in circulating blood of cancer patients remains among the most attractive examples of liquid biopsy developments reported to date (Diehl et al., 2008; Bardelli and Pantel, 2017) with several recent promising follow up studies (Cohen et al., 2018). In many of these instances mutant sequences are found in association with circulating EVs (Thakur et al., 2014). However, in the aforementioned study it was found that while oncogenic RAS drove a release of genomic DNA, as well as mutant RNA and altered protein repertoire of cancer cell-related EVs, these EVs were not only informative as to the existence of oncogenic mutation but also highly bioactive (Lee et al., 2014; Lee et al., 2016). Indeed, the uptake of EVs from RAS-driven cells, but not EVs from their isogenic, non-transformed counterparts by cultured leukocytes, resulted in a dramatic change in phenotype, marked by an increase in procoagulant activity associated with tissue factor and elevated release of interleukin 8 (Chennakrishnaiah et al., 2018). These examples merely signal a much broader question of biological activities associated with liquid biopsy analytes and their carriers including extracellular DNA, RNA and proteins (Siravegna et al., 2017), a question that still requires more extensive studies.

CONCLUSIONS AND FUTURE PROSPECTS

It could be argued that a biomarker of a pathological process, such as cancer, would ideally be the molecular driver, or an indispensable and unique attribute of it. Oncogenic mutations would meet these criteria if not for dynamic evolution, molecular complexity and cellular heterogeneity of cancer cell populations, which often also depend on exogenous triggers to manifest their disease-causing potential (Ghajar et al., 2013; Magnus et al., 2014; Martincorena et al., 2015). Still, detection of unique molecular changes occurring in the cancer cell genome and epigenome in real time could carry enormous value in developing more adaptive, personalized and ultimately more effective care for cancer patients.

Cancer cells exteriorize these unique signatures though a multitude of regulated processes ranging from the shedding of CTCs, apoptotic bodies, vesiculation and secretory mechanisms, resulting in the influx of analytes, such as cfDNA and other into remote biofluid compartments (Siravegna et al., 2017). In this article, we argued that the release and biological turnover of this material are not necessarily "unspecific", steady or passive, but instead multiple regulatory steps may perturb the levels, timing and tumor representation in different liquid biopsy settings (**Figure 2**). These regulatory influences may affect their choice and performance of liquid biopsies and require optimization and/or use of multiple approaches simultaneously (e.g. CTCs and EVs). In this regard, we propose that the sequestration of mutant macromolecules by circulating phagocytes may offer a hitherto unappreciated diagnostic opportunity (leukobiopsy), which while presently experimental, is worthy of further exploration.

Future efforts will be required to determine whether experimental promise of leukobiopsy will be borne out in the clinical reality and whether there may be advantages (beyond technical) to apply specific liquid biopsy platforms to specific different cancer contexts.

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SC, JR, SA, and TT wrote the manuscript and contributed ideas.

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Conflict of Interest: SC and JR filed a patent application for diagnostic use of leukocytes as carriers of cancer-causing mutations.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Clonal Selection of a Novel Deleterious TP53 Somatic Mutation Discovered in ctDNA of a KIT/PDGFRA Wild-Type Gastrointestinal Stromal Tumor Resistant to Imatinib

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The standard of care for the first-line treatment of advanced gastrointestinal stromal tumor (GIST) is represented by imatinib, which is given daily at a standard dosage until tumor progression. Resistance to imatinib commonly occurs through the clonal selection of genetic mutations in the tumor DNA, and an increase in imatinib dosage was demonstrated to be efficacious to overcome imatinib resistance. Wild-type GISTs, which do not display KIT or platelet-derived growth factor receptor alpha (PDGFRA) mutations, are usually primarily insensitive to imatinib and tend to rapidly relapse in course of treatment. Here we report the case of a 53-year-old male patient with gastric GIST who primarily did not respond to imatinib and that, despite the administration of an increased imatinib dose, led to patient death. By using a deep next-generation sequencing barcode-aware approach, we analyzed a panel of actionable cancer-related genes in the patient cfDNA to investigate somatic changes responsible for imatinib resistance. We identified, in two serial circulating tumor DNA (ctDNA) samples, a sharp increase in the allele frequency of a never described TP53 mutation (c.560-7_560-2delCTCTTAinsT) located in a splice acceptor site and responsible for a protein loss of function. The same TP53 mutation was retrospectively identified in the primary tumor by digital droplet PCR at a subclonal frequency (0.1%). The mutation was detected at a very high allelic frequency (99%) in the metastatic hepatic lesion, suggesting a rapid clonal selection of the mutation during tumor progression. Imatinib plasma concentration at steady state was above the threshold of 760 ng/ml reported in the literature for the minimum efficacious concentration. The *de novo* TP53 (c.560-7_560-2delCTCTTAinsT) mutation was *in silico* predicted to be associated with an aberrant RNA splicing and with an aggressive phenotype which might have contributed to a rapid disease spread despite the administration of an

increased imatinib dosage. This result underlies the need of a better investigation upon the role of TP53 in the pathogenesis of GISTs and sustains the use of next-generation sequencing (NGS) in cfDNA for the identification of novel genetic markers in wild-type GISTs.

Keywords: circulating tumor DNA, *TP53*, gastrointestinal stromal tumor, imatinib, liquid biopsy

BACKGROUND

Gastrointestinal stromal tumors (GISTs) are the most common soft tissue tumors arising in the gastrointestinal tract. Common sites of GIST in the gastrointestinal tract include stomach (50%), small intestine (25%), rectum (5%), esophagus (< 5%), while extra-intestinal localizations are rare (< 5%). (DeMatteo et al., 2000; Casali et al., 2018) The diagnosis of GIST commonly relies on immunohistochemical (IHC) analysis of tumor tissue and is based on the assessment of KIT and DOG1 positivity. Based on histopathological features including mitotic index, tumor size, and primary site, risk-stratification schemes have been formulated. (Fletcher et al., 2002; Miettinen and Lasota, 2006).

By a molecular point of view, GISTs are characterized by gain-of-function mutations in *KIT* (70%–75% of cases) or *PDGFRA* (platelet-derived growth factor receptor, alpha polypeptide) genes (5%–10% of cases). (Corless et al., 2004) The mutational status of *KIT/PDGFRA* represents a significant predictive factor for response to the targeted drug imatinib mesylate. In particular, patients displaying *KIT* exon 11 mutations are usually sensitive toward imatinib, whereas patients bearing *KIT* exon 9 mutations are less sensitive and benefit from a higher drug's starting dosage. (Debiec-Rychter et al., 2006) On the other hand, *KIT* exon 13 and 17 mutations are usually insensitive to imatinib and commonly arise later during treatment, leading to secondary acquired resistance. (Lasota et al., 2008) Concerning the less common *PDGFRA* mutations, the p.D842V substitution is associated to primary insensitivity to imatinib, thus suggesting an alternative drug, i.e., sunitinib. (Heinrich et al., 2003) The subpopulation of GIST patients who do not show *KIT* or *PDGFRA* mutations has been historically classified as "wild-type" and only in the last years the contribution of other genes, such as *BRAF*, *SDH*, and *NF1*, has emerged to play a role in the pathogenesis of GIST (Boikos et al., 2016).

Only a narrow panel of mutations is known to be directly associated with the primary or secondary resistance to imatinib. Indeed, so far, imatinib resistance is mainly attributed to mutations located in *KIT*, *PDGFRA*, *BRAF*, and *SDH* genes, and thus other "noncanonical" genes remain less investigated. The inclusion of other oncogenes into the panels which are routinely screened for imatinib treatment monitoring could lead to the identification of novel biomarkers for the early diagnosis of treatment failure. Moreover, the use of wider panels of genes could be of particular interest in *wild-type* GIST, for which the dynamic tracking of driver known mutations is not feasible.

The strategy of tumor mutations dynamic monitoring for early detection of imatinib resistant clones is of special worth, as alternative therapeutic approaches, after imatinib failure, are available in the clinical care, such as sunitinib and regorafenib. A suitable method for the dynamic monitoring of tumor behavior is offered by the circulating tumor DNA (ctDNA) analysis. In the framework of liquid biopsies, ctDNA represents a specific biomarker which displays the same molecular characteristics (i.e., mutations) of the tissue of origin thus representing a real-time source of tumor-derived DNA. In the clinical setting, ctDNA analysis has shown to be of prognostic significance in predicting the targeted therapy's response, as well as in the early identification of disease relapse and/or progression (Tie et al., 2016; Coombes et al., 2019; Siravegna et al., 2019).

With the aim of developing an innovative approach for the dynamic monitoring of imatinib in GIST patients, we set up a joint research project for the ctDNA analysis and for the monitoring of imatinib plasma C_{min} in blood samples collected during patients' follow-up. The ctDNA analysis, by means of targeted deep sequencing, is focused on detecting tumor-related mutations which could be informative about disease status and treatment response.

CASE PRESENTATION

A 53-year-old male patient was diagnosed with gastric GIST in May 2015 at Azienda Ospedaliera ULSS 9 of Monselice (PD) for which he underwent a total gastrectomy with no evidence of residual disease. The tumor tissue examination revealed the characteristic spindle cell morphology of GIST and displayed a low mitotic index (< 1/50 HPF). Immunohistochemical stain revealed positivity for Ki67 and CD117 (c-KIT) antigens, confirming the diagnosis of GIST, whereas stains for smooth muscle alpha-actin, desmin, CD34, and S-100 were negative. A *post hoc* molecular analysis did not highlight any somatic mutation in *KIT* or *PDGFRA*, allowing to define it as a wild-type tumor. The patient was classified as a low risk of recurrence, and the wait-and-see approach was preferred to adjuvant treatment with imatinib. In November 2015, magnetic resonance showed the presence of six hepatic nodules with maximum diameter of 2.5 cm consistent with metastatic GIST lesions, so imatinib first-line therapy was started at the standard dosage of 400 mg/day. In March 2016, the patient accessed medical care in our hospital in which a magnetic resonance showed hepatic disease progression. The GIST derivation of hepatic lesions was confirmed through a tissue biopsy staining positively for c-KIT antigen (**Figure 1A**); therefore, imatinib dosage was increased to 800 mg/day. In October 2017, PET imaging revealed further hepatic disease progression in addition

to bone and intra-abdominal metastatic spread, so the patient was switched to sunitinib. During the overall course of therapy, the patient displayed a primary resistance against imatinib since he never experienced a clinical benefit from treatment. The patient died because of disease progression in March 2018.

METHODS

(For a detailed description of *Methods*, see **Supplementary Methods**).

Biological Sample Collection and Ethics Approval

The patient provided a signed informed consent at the time of enrollment. Blood samples were collected in January 2017 (sample IM_21.1) and in July 2017 (sample IM_21.2). A diagnostic residue of the formalin-fixed paraffin-embedded (FFPE) bioptic tissue derived from the hepatic lesion was provided by the Pathological Anatomy Division of IRCCS CRO, whereas primary FFPE surgical tissue was kindly provided by Azienda Ospedaliera ULSS 9 of Monselice (PD).

DNA Extraction and Quality Control (QC)

Cell-free DNA (cfDNA) was extracted from 4 ml of plasma using the QIAamp MinElute ccfDNA Kit (Qiagen) and quantified with Quantus Fluorometer (Promega). Fragment size distribution was assessed by High Sensitivity TapeStation (Applied Biosystems). Germline DNA was extracted from 200 µl of buffy-coat using the automated BioRobot EZ1 (Qiagen). DNA from FFPE tissue (both primary tumor and hepatic metastasis) was extracted using the GeneRead DNA FFPE Kit (Qiagen) according to the manufacturer's instructions. The median tumor cell content was 80%, as established by a trained expert pathologist (**Figure 1B**).

Library Preparation, Sequencing, and Data Analysis

Genomic libraries were prepared using QiaSeq Human Actionable Solid Tumor Panel DNA (Qiagen). Regions covered by the panel are listed in **Supplementary Table S1**. Pooled libraries were paired-end (151 × 2) sequenced in an Illumina platform (MiSeq). Bioinformatic analysis was performed on a workstation with a 30-core Intel Core i7 and 64 GB of memory running Centos 7.5. Raw reads after trimming for quality were aligned against the reference genome hg19 (UCSC) using bwa aligner. (Li and Durbin, 2009) Variants were called using smCounter v 2 with default parameters. (Xu et al., 2019) Identified variants were manually verified using Integrative Genomics Viewer (IGV, https://www.broadinstitute.org/igv).

ddPCR Assay

cfDNA, DNA from primary tumor, and DNA from metastatic tissue were interrogated for the presence of *TP53* indel (c.560-7_560-2delCTCTTAinsT) by a ddPCR custom assay developed from BioRad (Bio-Rad Laboratories, Hercules, CA, USA). ddPCR was performed using primers and specific TaqMan probes targeting the wild-type *TP53* sequence and the aberrant *TP53* sequence bearing the indel c.560-7_560-2delCTCTTAinsT. As reference mutated control, a synthetic oligonucleotide bearing the *TP53* indel c.560-7_560-2delCTCTTAinsT was used (Sigma Aldrich, St. Louis, MO, USA). As a reference wild-type control, a germline DNA in which the presence of the *TP53* indel (c.560-7_560-2delCTCTTAinsT) was excluded through next-generation sequencing (NGS) was used. Droplet generation was performed using QX200™ Droplet Generator™ (Bio-Rad Laboratories, Hercules, CA, USA), and fluorescence emitted from droplets was measured using QX200™ Droplet Reader (Bio-Rad Laboratories, Hercules, CA, USA). Sample analysis was performed using QuantaSoft v1.7.4.0917 software (Bio-Rad Laboratories, Hercules, CA, USA). Details concerning ddPCR conditions and data analysis are reported in **Supplementary Methods**.

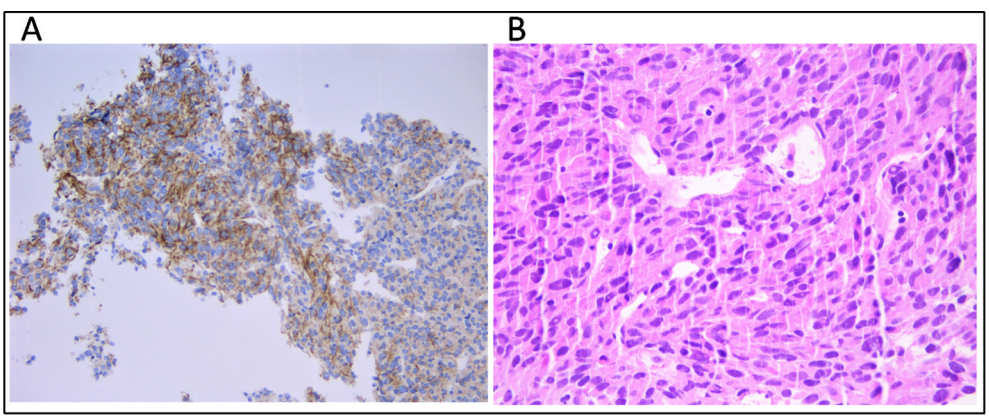

FIGURE 1 | (A) Immunhistochemical staining for CD117 (c-KIT) and **(B)** tumor composition of spindle cells and eosinophilic cytoplasm (hematoxylin and eosin) on the metastatic hepatic tissue.

Computational Prediction of Splicing Alteration

Six freely available *in silico* tools were used to predict the impact of the splice-site mutation in *TP53* gene on pre-mRNA splicing. Tools used are SpliceView (http://bioinfo.itb.cnr.it/~webgene/wwwspliceview.html), GENSCAN (http://hollywood.mit.edu/GENSCAN.html), NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/), MaxEntScan (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), and Human Splicing Finder (HSF, http://www.umd.be/HSF/HSF.shtml) (Shapiro and Senapathy, 1987; Brunak et al., 1991; Hebsgaard et al., 1996; Burge and Karlin, 1997; Reese et al., 1997; Yeo and Burge, 2004; Houdayer et al., 2008; Desmet et al., 2009). To facilitate the output interpretation, we compared the score of the mutant with the score of the reference sequence.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Quantification of Imatinib Plasma Concentrations

The quantification of imatinib was obtained using an LC-MS/MS apparatus consisting of a Prominence UFLC XR (Shimadzu) coupled with an API 4000 QTrap mass spectrometer (SCIEX). Details concerning sample processing and experimental conditions are reported in **Supplementary Methods**.

RESULTS

Next-Generation Sequencing of DNA Derived From Plasma (cfDNA), Primary Tumor Tissue, and Metastatic Tissue

After variants calling by smCounter v2, genetic variants were filtered *per* the following criteria: passing filter (PASS), quality score ≥100, frequency of mutated allele ≥0.5%, and total number of reads mapping the chromosomal location (reads depth) ≥2,500 X. All the genetic variants were compared to those obtained by matched germline DNA sequencing to exclude from the analysis nonsomatic variants.

In the two serial cfDNA samples, one somatic indel affecting the exon 6 flanking site of *TP53* gene indel (c.560-7_560-2delCTCTTAinsT) at nucleotide position c.560-2_c.560-7 was identified at a minor allele frequency (MAF) of 2.7% in the first plasma sample (IM_21.1) and of 9.7% in the second plasma sample (IM_21.2). No other somatic mutation was detected in cfDNA. The same *TP53* indel was detected through NGS in the DNA from the metastatic tissue with a MAF of 99%. The mutation identified is an intronic indel that affects the canonical AG/GT splice site motif by the deletion of the nucleotide in position –2 upstream exon 6.

The identified variant was automatically annotated against the human *TP53* genomic sequence NC_000017.10 (chr 17:7,571,720-7,590,868) corresponding to isoform NM_00546.5. The latest release of the International Agency for Research on Cancer (IARC) *TP53* Mutation Database (Database R19, released on August 2018) (http://p53.iarc.fr/TP53GeneVariations.aspx) was used to check the *de novo TP53* variant, confirming our novel finding. (Bouaoun et al., 2016) The characteristics of *TP53* mutation are listed in **Table 1**.

DNA derived from the primary tumor tissue at surgery resulted wild-type for the regions analyzed, as no somatic mutation was detected.

ddPCR Analysis on Plasma, Primary Tumor Tissue, and Metastatic Tissue-Derived DNA

The custom ddPCR assay was harnessed to validate the presence of *TP53* indel (c.560-7_560-2delCTCTTAinsT) in cfDNA and metastatic tissue. Primary tumor tissue was interrogated as well to perform a cross-platform comparison and increase the sensitivity in mutation detection.

Since cfDNA IM_21.1 was completely depleted to perform NGS, only cfDNA IM_21.2 was analyzed through ddPCR. In cfDNA IM_21.2, the presence of the *TP53* indels was confirmed by ddPCR, which revealed 16 mutated copies/μl, corresponding to 277 mutated copies/ml of plasma. So, in cfDNA, the MAF estimated by ddPCR was 17%, superior to that reported by NGS (9.7%). The MAF revealed in the metastatic tissue-derived DNA by ddPCR was comparable to that obtained by NGS analysis, confirming a complete selection of the mutated clone in the metastatic lesion (MAF ~100%). Notably, the use of ddPCR allowed the identification of *TP53* indel also in the DNA derived from the primary tumor tissue with a mutated allele frequency of 0.1% that was not detectable by means of NGS (**Figure 2**).

Computational Prediction of *TP53*c.560-7_560-2delCTCTTAinsT

Tools used to predict the effect of the *TP53* indel at mRNA and the respective scores generated are listed in **Table 2**. All but one tool agree in identifying the canonical splice site in the wild-type *TP53* sequence, and all of them predicted the splice site destruction in the mutated sequence. Consequently, the effect

TABLE 1 | *TP53* somatic mutation identified by next-generation sequencing (NGS). Genomic coordinates of the mutation and the read depth in that chromosomal location are reported.

Sample ID	Genomic coordinates	Read depth	UMT	VMF	Mutation frequency (%)	cDNA change	Type of mutation
IM_21.1	17:7,578,291	6,672	1,023	28	2.7	c.560-7_560-2delCTCTTAinsT	Indel
IM_21.2	17:7,578,291	5,868	876	85	9.7	c.560-7_560-2delCTCTTAinsT	Indel

The total number of reads bearing the same Unique Molecular Target (UMT) and those reporting the mutation [Variant Mutational Fraction (VMF)] was used to calculate the mutation frequency in each sample.

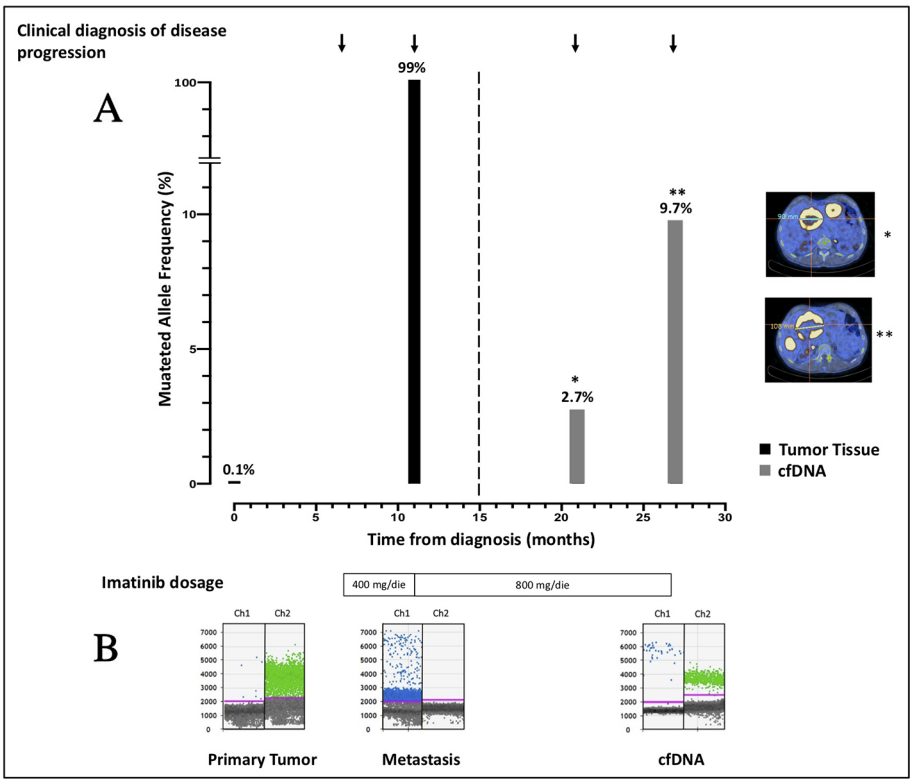

FIGURE 2 | (A) Course of disease from the time of diagnosis, treatment administered, PET/CT images, and allele frequency of *TP53* indel are shown. The mutated allele frequency in tumor tissue (black columns) is reported for the primary tumor [minor allele frequency (MAF) 0.1%, ddPCR] and for the metastatic lesion (MAF 99%, ddPCR) at the time of surgery and biopsic sampling, respectively. The ctDNA fraction (gray columns) is reported for the sample IM_21.1 [MAF 2.7%, next-generation sequencing (NGS)] and sample IM_21.2 (MAF 9.7%, NGS). PET/CT scans reporting the diameter of target lesions and performed in concomitance to blood sampling are shown as well. On the bottom of the plot, the imatinib dosage administered is indicated. **(B)** ddPCR plots reporting the signal generated from the wild-type (green dots) and the mutated (blue dots) sequence are shown. In chronological order are reported the primary tumor DNA, the metastatic DNA, and the IM_21.1 cfDNA.

TABLE 2 | Computational prediction of the effect of the mutation on the *TP53* splice site by the use of six different bioinformatic tools.

Tool	Output	Reference score	Mutated score	Predicted effect
SpliceView	Score (0–100)	83	Not detected	Deleterious
GENSCAN	Probability score (0–1)	0.120	Not detected	Deleterious
NetGene2	Confidence score	0.00	Not detected	Not evaluable
NNSplice 0.9	Score (0–1)	0.94	Not detected	Deleterious
Human Splicing Finder (HSF)	Score (0–100)	80.49	Not detected	Deleterious
MaxEntScan	Maximum entropy score	1.08	–2.91	Deleterious

The wild-type DNA sequence was compared with the mutated one, and the effect was predicted by comparing the two generated scores.

of the mutation was supposed to be deleterious by five out of six predictive tools, not being evaluable by means of NetGene2, which did not detect the wild-type splice site. The activation of an alternative splice site was predicted by HSF that identified a likely new splicing acceptor site located 30 nucleotides downstream from the canonical site. The new splice site was scored 50.40 by HSF (**Table 2**), and it is weaker than the canonical ones, which was scored 80.49. The activation of the new cryptic splice site would lead to an in-frame deletion of 10 amino acids from the position 187 to 196 in the mature protein. The description of the *TP53* indel and its predicted effect on mRNA strand are depicted in **Figure 3**.

Molecular biology analyses to confirm the mutation's impact on TP53 mRNA splicing were attempted but not feasible due to the very poor quality of FFPE RNA (data not shown).

Imatinib Plasma Levels

The sample IM_21.1 for C_{min} analysis was collected 17.5 h after the previous imatinib; drug plasma concentration was determined as 987 ng/ml. The sample IM_21.2 was collected a week after the last intake of the drug. Therefore, this last sample was not suitable

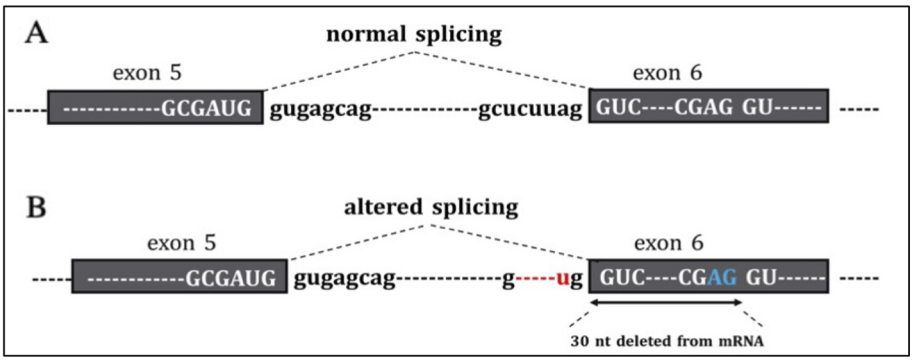

FIGURE 3 | In the figure are reported **(A)** the normal sequence and exon splicing of TP53 pre-mRNA (exons 5–6) and **(B)** aberrant splicing caused by the c.560-7_560-2delCTCTTAinsT (red) likely to generate an in-frame deletion of 30 nucleotides from mRNA due to the activation of a cryptic splice site (blu).

for C_{min} determination given that the possible plasma imatinib concentration was not associated with steady state kinetics.

DISCUSSION

The use of imatinib has favorably rewritten the natural history of GIST, improving the patients' outcome in terms of survival. However, primary and secondary resistance is the main weakness of imatinib and represents the leading cause of progression.

The primary resistance is related to tumor's molecular features at baseline and is assessed on tumor at the time of diagnosis, as recommended by clinical guidelines. (Casali et al., 2018) Wild-type GISTs, characterized by the absence of *KIT*/*PDGFRA* activating mutations, are a heterogeneous class of tumors showing multiple genetic and morphological features. Therapeutic strategies for the treatment of these tumor subtypes are not defined yet.

Secondary imatinib resistance is commonly observed after 2 years of treatment in half of primarily responder patients as a consequence of the selective pressure exerted by the drug. In *KIT*/*PDGFRA*-mutated GISTs, the development of acquired resistance is commonly restricted to secondary mutations in these genes, enabling a more handling monitoring of disease progression by the detection of target mutations. In wild-type GISTs, which do not display a shared evolutionary path, the identification of genetic markers to assess tumor's evolution is urgently needed (Wei et al., 2018).

The possibility of interrogating ctDNA as a surrogate of tumor tissue by massive parallel sequencing has enabled the real-time detection of emergent resistance clones in several kinds of malignancies in a less invasive manner (Russo et al., 2016).

In this study, using a targeted NGS panel of hotspot regions of 23 cancer-related genes, we assessed the molecular evolution of a wild-type GIST by analyzing two serial cfDNA samples collected 6 months apart, the primary tumor tissue and the hepatic metastasis tissue. We found that a novel *TP53* indel (c.560-7_560-2delCTCTTAinsT) was detected in cfDNA samples with an allele frequency of 2.7% (IM_21.1) and 9.7% (IM_21.2). The primary and relapsed tumors did not show *KIT*/*PDGFRA* mutations, but they harbored the same *TP53* indel with an allele frequency of 0.1% and ~100%, respectively.

The functional impact of *TP53* indel (c.560-7_560-2delCTCTTAinsT) was postulated on the basis of its localization in a highly conserved region. We hypothesized a misrecognition of the canonical splice site from the splicing machinery, which would result in the translation of a truncated or nonfunctional protein. Our hypothesis was sustained by five different *in vitro* algorithms that predicted the canonical splice site destruction and the likely activation of noncanonical ones, located 30 base pairs downstream of the canonical one in the intron 5/exon 6 boundary. At the protein level, the TP53 region excised from canonical mRNA transcription, both in the case of a complete or a partial loss of exon 6, is of pivotal relevance for the interaction with other proteins involved in the cell cycle regulation, such as AXIN1, HIPK1, and ZNF385A and is located in the DNA-binding domain. (Das et al., 2007; Sun et al., 2009) The production of an aberrant *TP53* transcript leads to the transduction of a truncated and nonfunctional TP53 protein but could also drive the reduction of the TP53 expression as a consequence of a nonsense-mediated mRNA decay.

TP53 is a tumor suppressor gene well known to play a pivotal role in the DNA repair process and in the apoptosis initiation. Its inactivation is a frequent event in cancer and is commonly associated with a worst prognosis (Basu and Murphy, 2016).

Although only few studies have investigated the role of *TP53* mutations in GIST, a consensus upon their association with imatinib resistance has been achieved. The first evidence demonstrating the correlation between *TP53* mutations and imatinib insensitivity was described by Wendel et al. (2006) in BCR-ABL-positive leukemic cells. They observed that the mechanism of imatinib resistance was independent of the chemical inhibition of BCR-ABL kinase by imatinib, suggesting

a downstream involvement of *TP53* mutations in leading the drug's resistance. Further studies confirmed the loss of TP53 in chronic myeloid leukemia as a molecular feature associated with imatinib resistance (Al-achkar et al., 2012).

Recently, in a study aimed at identifying genes involved in imatinib resistance in GIST-T1 cells through a CRISP-Cas9 knockout genome-wide screening, Cao et al. (2018) identified *TP53* as one of the main genes associated to imatinib resistance. These evidences suggest that genomic alterations in genes related to the apoptosis pathway might represent an escape route exploited by tumor cells to evade imatinib therapy.

In *KIT*/*PDGFRA* mutant GISTs, there is no doubt upon the origin of the oncogenic signaling, and the development of imatinib resistance is mainly restricted to the acquisition of secondary *KIT*/*PDGFRA* mutant clones bearing novel mutations. In these groups of GISTs, the overall *TP53* mutation rate was reported as low, emphasizing the oncogenic reliance on kinase-mediated signaling. However, a straightforward association between presence of *TP53* aberrations and GIST malignancy has been observed, with a significant increase of *TP53* aberrations in high-risk rather than in low-risk tumors. (Merten et al., 2016; Ihle et al., 2018; Heinrich et al., 2019) On the other hand, *TP53* has emerged as one of the main mutated genes in wild-type GISTs, supporting its likely role in the pathogenesis of these tumor subtypes (Pantaleo et al., 2017).

In this clinical case study, the rapid metastatic evolution is consistent with the *TP53* mutant clonal selection from the primary to the relapsed tumor. The homozygous presence of *TP53* indel (c.560-7_560-2delCTCTTAinsT) in fundamentally all hepatic relapsed cells suggests once again an indisputable association between *TP53* deleterious mutations in GIST and the establishment of an aggressive phenotype insensitive to imatinib.

Moreover, the observation of no clinically actionable mutations, which might represent a molecular target for currently available therapeutic options, corroborates the lack of sensitivity toward imatinib reported here and implies the impossibility of prescribing further targeted drugs. Indeed, the administration of targeted therapies is limited to the presence of specific overexpressed or mutated molecular targets in tumor cells, thus making the management of wild-type tumors a challenging task.

In this case, the clinical tumor progression was well recapitulated by the longitudinal sequencing of ctDNA, which revealed the presence of *TP53* c.560-7_560-2delCTCTTAinsT at increasing allele frequency over 6 months. This finding is significant since it sustains the feasibility of relying on information obtained by liquid rather than tissue biopsies for the assessment of genetic features in metastatic GISTs. A good concordance between mutated cfDNA and tumor tissue in GIST patients was reported by previous studies, which observed a higher detection rate of ctDNA in patients with active disease and high tumor burden, rather than in patients with complete response or localized disease. (Maier et al., 2013; Xu et al., 2018) In this frame, the allele frequency of mutated cfDNA was shown to increase or decrease according to disease progression or tumor shrinkage, respectively, allowing the dynamic monitoring of tumor changes in advance GIST.

In sample IM_21.1, C_{min} resulted equal to 987 ng/ml (higher than the recommended threshold of 760 ng/ml); therefore, we reasonably consider adequate the imatinib level in patient's plasma and assume that his lack of response to therapy was not due to a concentration issue but more probably to the biological aggressiveness of the disease (Bouchet et al., 2016).

In summary, our work sustains the applicability of NGS of cfDNA for the monitoring of GIST patients on treatment with imatinib, and for the characterization of the mutational pattern of GISTs, especially in those classified as wild-type, for whom the identification of genetic markers is even more urgent due to the lack of targetable mutations. The screening of a panel of actionable genes offers the possibility of identifying new tumor markers, which may be relevant for the surveillance of tumor's evolution and for the development of new drugs. In the era of precision oncology, the baseline profiling of tumor is an imperative need for choosing the best therapeutic option and for avoiding the prolonged administration of ineffective drugs. Moreover, this procedure should be accompanied by the longitudinal follow-up of tumor genetics for the early identification of tumor changes and the emergence of resistance subclones. In this field, the use of liquid biopsy coupled with NGS represents a valuable tool to explore in parallel a wide range of genomic regions and to broader horizons upon tumor's evolutionary process. In the case here reported, the identification of a novel and deleterious *TP53* indel (c.560-7_560-2delCTCTTAinsT), compatible with an aggressive and drug resistance phenotype, remarks the need for further investigations upon the role of *TP53* in wild-type GISTs as well as on its involvement in the development of acquired resistance toward tyrosine kinase inhibitors. The clinical management of wild-type GIST remains a subject of open debate, and effective therapeutic strategies are still lacking. Even though this class of tumors usually displays an indolent course, the development of unpredicted outcomes such as the evolution into a more aggressive form must be considered. An accurate noninvasive molecular monitoring by the use of the liquid biopsy is of primary relevance to identify effective therapeutic strategies and to personalize the therapeutic strategies.

DATA AVAILABILITY STATEMENT

The data cannot be made publicly available because the consent was not acquired from the patient to publish his genetic profile. Reasonable requests for data should be directed to: ececchin@cro.it.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Indipendente-Centro di Riferimento Oncologico di Aviano. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the

individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

CDF, EC, EDM, MG, and GT were involved in designing the study, critically revising the results, and preparing the manuscript, CDF, RBR, LR, MP, EM, and SG were involved in the molecular, bioinformatic and biochemical analyses. MG, AB, RBS and AF collected samples and clinical data, EB provided the PET/CT scans and VC did the histopathological analysis. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00036/full#supplementary-material

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CASE REPORT
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Circulating Tumor DNA-Based Detection of Microsatellite Instability and Response to Immunotherapy in Pancreatic Cancer

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Pancreatic cancer is an aggressive malignancy with poor survival. Research has indicated the association of few genetic aberrations with pancreatic cancer. The data regarding the prevalence of microsatellite instability in pancreatic cancer is diverse and controversial. However, it could be an actionable target in pancreatic cancer especially due to availability of immune checkpoint inhibitors which has demonstrated promising results in different types of cancers. We present a case of pancreatic cancer whose microsatellite instability status was identified on liquid biopsy (circulating tumor DNA testing). Our patient showed a dramatic ongoing durable response to immunotherapy. We were able to do serial monitoring with liquid biopsy that showed clinical utility and validity.

Keywords: circulating tumor DNA, microsatellite instability, pancreatic cancer, immunotherapy, pembrolizumab

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BACKGROUND

Pancreatic cancer is a challenging disease with unfavorable outcomes. Pancreatic ductal adenocarcinoma (PDAC) constitutes around 90% of all malignant pancreatic cancers (Hackeng et al., 2016). As opposed to other common cancers, there has been a rise in incidence and mortality rates of PDAC (Ryerson et al., 2016). According to the American Cancer Society, it is the fourth leading cause of cancer-related mortality in both males and females with an estimated number of new cases and deaths in the United States in 2019 as 56,770 and 45,750, respectively (Siegel et al., 2019). PDAC is associated with poor prognosis, having a 5-year survival rate of 8%, owing to its presentation as an advanced disease, being resistant to different drug regimens and a distinct tumor microenvironment with condensed desmoplasia (Mahadevan and Von Hoff, 2007; Yachida and Iacobuzio-Donahue, 2009; Erkan et al., 2010; Erkan et al., 2012; Liu et al., 2017). Chemotherapy, both in adjuvant and neoadjuvant settings, has remained the treatment of choice for most patients with PDAC. However, it has not been helpful in a significant improvement of survival of these patients. This leaves surgery as the only curative option but fewer than 20% of patients present with a resectable disease at diagnosis (DeWitt et al., 2004). The unavailability of a useful diagnostic and prognostic biomarker has always been a concern for PDAC (Herreros-Villanueva and Bujanda, 2016).

PDAC could be associated with several targets including *KRAS*, *TP53*, *TGF-β*, *WNT*, *NOTCH*, *SMAD4*, *CDKN2A*, *ARID1A*, *MLL3*, and *TGFBR2* (Bailey et al., 2016; Chou et al., 2018). Important are aberrations in BRCA and DNA repair for whom PARP-inhibitors are now an option. Small proportion (1%–2%) of PDAC is also associated with microsatellite instability (MSI) (Barrett et al., 2017; Humphris et al., 2017; Hu et al., 2018). MSI results from mismatch repair deficiency (dMMR) and consists of repetitive 1–6 base pairs of DNA (Umar and Kunkel, 1996; Humphris et al., 2017). dMMR or loss of functional ability of any of the mismatch repair proteins (MLH1, MSH2, MSH6, and PMS2) hinders the effective DNA replication process. Research has indicated that cancers with dMMR and MSI-H respond very well to immune checkpoint inhibitors. This implies that targeting the immune checkpoints including programmed cell death-1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4) encourages T cells to fight cancer cells. This has been well-elaborated for melanoma and lung cancer, but data is lacking for PDAC.

Herein, we report a case of PDAC whose MSI-H status was identified on liquid biopsy which is also known as circulating tumor DNA (ctDNA) testing. Our patient showed a dramatic response to immunotherapy which was again assessed on ctDNA assays.

CASE PRESENTATION

Patient is an 81-year old female who had unrelenting pain in her stomach in January 2018. She had an ultrasound done by her gastroenterologist that showed a mass in the pancreatic head versus body. A follow-up CT scan confirmed the ultrasound findings and also showed one possible enlarged lymph node. This was followed up by MRI at Mayo Clinic, Florida in March 2018, which showed locally advanced pancreatic mass centered at the junction of the body and tail measuring 5.1 × 6.4 × 5.2 cm. The results also showed encasement of the first jejunal arterial and venous branches with adjacent mass effect causing narrowing of the superior mesenteric vein confluence. Enlarged right common iliac artery chain lymph node measuring 1.4 × 1.1 cm and indeterminate lesions in the posterior aspect of the vertebral body at T9 were noted.

A baseline ctDNA test was obtained, which is at present part of our standard of care at Mayo Clinic, Florida for patients with GI malignancies. Testing is performed through commercially available platforms. In this particular patient, this was done through Guardant360 that showed *SMAD4 R361H*, *TP53R213L*, *KRAS G12D*, *RET A640A*, *KIT K412K*, *NTRK3 R630G*, *ARID1A G1711fs* and the highest variant allele fraction was noted to be 2.2% as shown in **Table 1**. She was started on neoadjuvant chemotherapy with gemcitabine and paclitaxel since surgery was not an option, but there was not much response in her repeat scans in May 2018, though there was a decrease in the highest variant allele fraction to 1.2%. She was then switched to 5-fluorouracil with nanoliposomal irinotecan (5-FU was held due to *DPD* heterozygosity on pharmacogenomics testing). Repeat CT scan in July 2018 showed decrease in the size of a mass in the pancreas which measures 3.4 × 3.1 cm compared with 6.5 × 5.1 cm.

TABLE 1 | Serial circulating tumor DNA evaluation in our patient with MSI-H/dMMR pancreatic ductal adenocarcinoma and excellent response to pembrolizumab. As noted, the circulating tumor DNA in the *SMAD4 R361H* mutation has gone down from 2.2% to 0%.

Serial ctDNA(liquid biopsy)testing results						
	March 2018	**May 2018**	**July 2018**	**Dec 2018**	**Jan 2019**	**Feb 2019**
Highest Variant Allele Fraction						
	2.2%	**1.2%**	**0.8%**	**0.7%**	**0.5%**	**0.4%**
Clonal Mutations						
SMAD4 R361H	2.2%	0.9%	0.6%	0.7%	0%	0%
TP53 R213L	1.9%	0.8%	0.5%	0.6%	0%	0%
KRAS G12D	1.9%	1.2%	0.4%	0.4%	0%	0%
RET A640A	1.6%	0.5%	0.5%	0.5%	0%	0%
KIT K412K	1.5%	0.4%*	0.3%*	0.6%	0%	0%
NTRK3 R630G	1.4%	0.6%	0.4%	0.4%	0%	0%
ARID1A G1711fs	1.1%	0.8%	0.3%*	0.3%*	0%	0%
Subclonal Mutations						
MTOR I486V	1.0%	0.1%	0.3%	0.4%*	0%	0%
BRCA2 A1572T	0.9%	1.2%*	0.8%*	0.5%*	0.5%	0.4%
HNF1A A209T	0.9%	0.4%	0.4%	0.3%	0%	0%
ARID1A G1847G	0.9%	0%	0%	0%	0%	0%
DDR2 K699	0.7%	0%	0.2%	0%	0%	0%
MPL S505N	0.5%	0%	0.2%	0%	0%	0%
PIK3CA W11R	0.4%	0%	0%	0.3%	0%	0%
MET N1081S	0.2%	0%	0%	0.2%	0%	0%
ATM G2675	0.2%	0%	0%	0%	0%	0%
EGFR E543G	0.1%	0%	0%	0%	0%	0%
MSI status	ND	ND	ND	**high**	ND	ND
ǂ ***Concordance with tissue biopsy*** –				**clinical concordance: yes clonal concordance: yes MSI-high detection: yes**		
Treatment	**Neoadjuvant chemotherapy (gemcitabine and paclitaxel/ 5-fluorouracil with nanoliposomal irinotecan)**			**Immunotherapy (Pembrolizumab)**		
Tumor markers						
CEA (ng/ml)	15	11.4	16.2	22.1	8.7	3.3
CA-19-9 (Units/ml)	5	4	6	6	7	6

*50% of the highest variant allele fraction value has been used to differentiate clonal from sub clonal mutations. *indicates change from clonal to Subclonal and vice-versa; ND, Not Detected.*

ǂBiomarker profiling on tissue sample detected SMAD4 R361H, KRAS G12D, ARID1A G1711fs mutations and MSI-high status. Therefore, ctDNA (liquid biopsy testing) is concordant with tissue biopsy testing in this patient.

Moreover, highest variant allele fraction dropped down to 0.8% on ctDNA testing in July 2018. Chemoradiation with capecitabine was added to her treatment plan in August 2018.

In December 2018, CT scan of the chest showed a new right lower lung lobe nodule suspicious for metastatic disease along with a persistent mass in the body of the pancreas. In the repeat ctDNA testing in December 2018, she was noted to be MSI-High/mismatch repair deficient (dMMR). Furthermore, mismatch repair immunohistochemistry on the tissue sample showed loss of

MLH1 and PSM2 proteins. Therefore, she was considered a great candidate for immunotherapy pembrolizumab on-label and was started on it. Dramatic improvement was noticed within 4 weeks of treatment with pembrolizumab and repeat ctDNA testing in January 2019 showed loss of all the above noted mutations. Furthermore, the highest variant allele fraction dropped down to 0.5% and patient continues to be on this therapy as depicted in **Figure 1**.

DISCUSSION

The management of PDAC is a complex task. To begin with, tissue biopsy has been the diagnostic test of choice historically which is a cumbersome technique. Moreover, it is troublesome for the patients to have repeated serial tissue biopsies in order to monitor the response to therapy. In the past decade, liquid biopsy has emerged as a noninvasive and patient-convenient technique that has demonstrated the clinical utility and validity for various cancers. However, it has not been widely incorporated into clinical practice for the management of all types of cancers especially PDAC because of sparsity of evidence. Our case showed the promising role of liquid biopsy not only in detection of the underlying genetic aberration but also in determination of the treatment response in PDAC.

The available data on the prevalence of dMMR and MSI-H in PDAC is limited and heterogeneous. Previous studies have demonstrated the existence of MSI-H in PDAC, however, the findings were controversial. Yamamoto et al. investigated 103 PDAC patients and the reported incidence of MSI-H in their cohort was 16%. The researchers also reported the finding of MSI-H in 100% of their patients with hereditary PDAC (n = 3) (Yamamoto et al., 2001). Goggins et al. studied 82 xenografts of pancreatic carcinoma and reported that 3.7% of their specimens had MSI-H (Goggins et al., 1998). Ouyang et al. investigated 60 pancreatic cancer patients and found MSI-H in 9 (15%) patients of their cohort (Ouyang et al., 1998). Venkatasubbarao et al. reported the presence of MSI-H in 4 (29%) out of their 14 surgically resected samples of pancreatic adenocarcinoma (Venkatasubbarao et al., 1998). Recently, Hu et al. investigated 833 PDAC patients and found dMMR in 7 (0.8%) patients of their cohort (Hu et al., 2018). The summary of studies looking at pancreatic carcinoma and MSI-H/dMMR expression are demonstrated in **Table 2**. The disagreement in prevalence of MSI-H in PDAC might be due to confounding factors including differences in sample sizes, test techniques and diversity of tumor histology (Macherla et al., 2018).

Our patient demonstrated a phenomenal response to immunotherapy. The tumor mutation burden pre- and post-treatment was well-picked on ctDNA assays. Recent research has highlighted the clinical utility and validity of ctDNA testing in

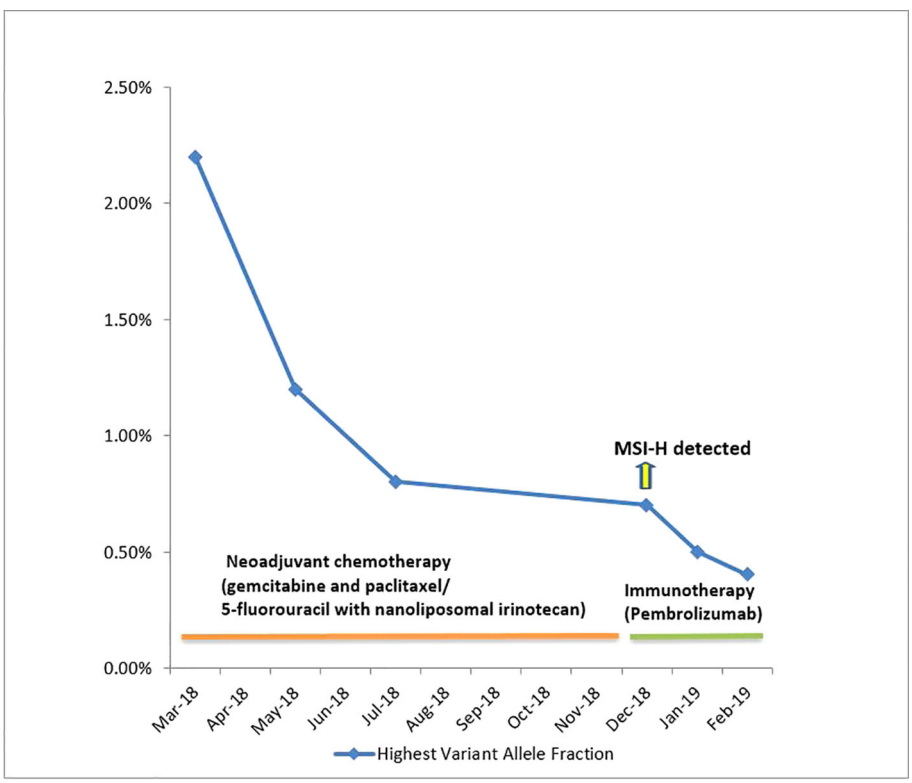

FIGURE 1 | Serial liquid biopsies and changes in Highest Variant Allele Fractions of ctDNA over the course of therapy. MSI-H detected in ctDNA testing done in December 2018. MSI-H was not detected in ctDNA reports of January 2019 and February 2019.

TABLE 2 | Summary of studies looking at pancreatic carcinoma and MSI-H/dMMR expression.

Study	Year	Study group	MSI/MMR status	Data source
Singhi et al. (2019)	2019	3,594 PDAC samples	MSI-H was detected in 0.5% of samples	International cohort
Hu et al. (2018)	2018	833 pancreatic adenocarcinoma patients	7 (0.8%) patients had dMMR, all 7 dMMR patients had lynch syndrome	Multiple hospitals, USA
Lupinacci et al. (2018)	2018	445 pancreatic ductal adenocarcinoma tissue samples	1.6% samples were dMMR	Multiple centers
Humphris et al. (2017)	2017	385 pancreatic cancer tissue samples	1% specimens were dMMR	Multiple centers of Australia, Germany, UK and USA
Connor et al. (2017)	2017	160 pancreatic adenocarcinoma cases from 154 patients (148 primary, 12 metastases)	4 cases were dMMR (3 had germline and 1 had somatic mutations in MMR genes)	International Cancer Genome Consortium data portal
Grant et al. (2015)	2015	290 pancreatic ductal adenocarcinoma patients	4 (1.38%) patients were dMMR	Ontario Pancreas Cancer Study (Ontario population-based registry)
Riazy et al. (2015)	2015	265 pancreatic ductal adenocarcinoma cases	41 (15%) were dMMR	Vancouver Coastal Health Region
Mitsuhashi et al. (2015)	2015	282 pancreatic ductal adenocarcinoma patients	None of the patients had MSI-H	3 hospitals database, Japan
Liu et al. (2014)	2014	36 acinar cell carcinoma of pancreas cases	5 (14%) patients were dMMR	Single academic center, USA
Ottenhof et al. (2012)	2012	78 pancreatic adenocarcinoma patients	13% tumors were dMMR	3 cancer treatment centers, Netherlands
Maple et al. (2005))	2005	35 pancreatic cancer patients	3 (8.6%) were dMMR who also had lynch syndrome	Single academic center, USA
Nakata et al. (2003)	2003	55 pancreatic carcinoma patients	4 (7.3%) tumors had abnormal hMS2-negative staining	Single university medical center, Japan
Yamamoto et al. (2001)	2001	103 pancreatic ductal adenocarcinoma (100 sporadic, 3 hereditary) patients	13% patients had MSI-H among sporadic PDAC group; 100% of hereditary PDAC patients had MSI-H	Single university medical center, Japan
Goggins et al. (1998)	1998	82 xenografted pancreatic carcinomas	3 (3.7%) specimens had MSI-H	Single academic center, USA
Ouyang et al. (1998)	1998	60 pancreatic cancer patients	9 (15%) patients had MSI-H	Multiple hospitals, Japan
Venkatasubbarao et al. (1998)	1998	14 surgically resected pancreatic adenocarcinoma tissue samples	4 (29%) had MSI-H	Single university medical center, USA

the management of various gastrointestinal cancers including PDAC (Shahjehan et al., 2019). Our findings also suggest that serial monitoring of tumor mutation burden can serve as a potential prognostic biomarker in pancreatic cancer. Previously, this was not feasible before the implication of ctDNA testing. The advent of immune checkpoint inhibitors has revolutionized the management of various MSI-H/dMMR tumors especially non-small cell lung carcinoma and melanoma. In May 2017, the United States Food and Drug Administration (FDA) approved pembrolizumab, a PD-1 inhibitor, for the treatment of MSI-H/dMMR solid tumors regardless of site of origin or histology that didn't respond or have metastasized after the introduction of first line agents (Lemery et al., 2017). The role of immunotherapy in PDAC has been studied in various clinical trials, however, no objective/complete response was achieved (Brahmer et al., 2012; Patnaik et al., 2015). The potential factors responsible for resistance of PDAC to immunotherapy could be low immunogenicity, decreased tumor mutation burden and inherent quality of being unlikely to be detected by the immune system (Evans et al., 2016).

There are several ongoing clinical trials that are investigating the role of various anti-PD-1/PD-L1 agents including pembrolizumab, nivolumab, etc. as monotherapy as well as combination regimens in PDAC. A recent phase I study investigated the role of a combination regimen consisting of a tumor-associated macrophage-targeting agent cabiralizumab and the anti-PD-1 nivolumab in metastatic PDAC. The combination regimen demonstrated a confirmed objective response in 4 of 31 (13%) patients. The researchers also revealed that all of these 4 patients with confirmed objective response had MSI-H and didn't show response to anti-PD-1 or PD-L1 monotherapy (NCT02526017) (Wainberg ZA et al., 2017).

Le et al. investigated the efficacy of PD-1 blockade in the management of 12 types of solid tumors including pancreatic cancer in a multicenter phase 2 study. They enrolled all dMMR cancer patients (n = 86) who had at least one prior therapy and developed a progressive disease. The estimated objective and complete response rates were 53% and 21%, respectively. Of note, the subset analysis for pancreatic cancer (n = 8) demonstrated an objective response rate of 62%. The researchers indicated that the complete and partial responses were attained in 2 (25%) and 3 (37%) pancreatic cancer patients respectively. For the colorectal cancer (n = 40), their results showed the objective and complete response rates of 52% and 12%, respectively (NCT01876511). The study is still ongoing and manifests the value of immune checkpoint inhibitors in pancreatic cancer (Le et al., 2017).

CONCLUSION AND FUTURE DIRECTIONS

Pancreatic cancer is a lethal cancer with poor outcomes in spite of the recent breakthroughs in combination chemotherapy regimens. Immune checkpoint inhibitors have exhibited strong responses in several MSI-H solid tumors but there is lack of

evidence regarding pancreatic cancer. Liquid biopsy could be incorporated in the management of these patients to record serial assessments of tumor mutation burden and to detect microsatellite instability, where obtaining tissue is often very difficult.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

Written informed consent was obtained from the patient for the publication of this case report.

AUTHOR CONTRIBUTIONS

SK and FS drafted the initial draft manuscript with guidance from PK. Author PK revised the manuscript and further edited by all the authors. All authors approved the final draft for publication.

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MINI REVIEW
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Single Cell Transcriptome in Colorectal Cancer—Current Updates on Its Application in Metastasis, Chemoresistance and the Roles of Circulating Tumor Cells

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Colorectal cancer (CRC) is among the most common cancer worldwide, a challenge for research, and a model for studying the molecular mechanisms involved in its development. Previously, bulk transcriptomics analyses were utilized to classify CRC based on its distinct molecular and clinicopathological features for prognosis and diagnosis of patients. The introduction of single-cell transcriptomics completely turned the table by enabling the examination of the expression levels of individual cancer cell within a single tumor. In this review, we highlighted the importance of these single-cell transcriptomics analyses as well as suggesting circulating tumor cells (CTCs) as the main focus of single-cell RNA sequencing. Characterization of these cells might reveal the intratumoral heterogeneity present in CRC while providing critical insights into cancer metastasis. To summarize, we believed the analysis of gene expression patterns of CTC from CRC at single-cell resolution holds the potential to provide key information for identification of prognostic and diagnostic markers as well as the development of precise and personalized cancer treatment.

Keywords: single-cell RNA sequencing, colorectal cancer, metastasis, chemoresistance, tumor heterogeneity, circulating tumor cells

INTRODUCTION

Colorectal cancer (CRC) is among major cancer worldwide in terms of incidence and mortality, with increasing trend, particularly in developing countries (Granados-Romero et al., 2017). According to global cancer statistics 2018, CRC was the third most commonly diagnosed cancer (10.2% of total cases) and ranked second for cancer-related deaths (9.2% of the total cancer deaths)

Abbreviations: CRC, Colorectal cancer; CTC, Circulating tumor cell; DNA, Deoxyribonucleic acid; ITH, Intratumoral heterogeneity; mCRC, Metastatic colorectal cancer; RNA, Ribonucleic acid; scDNA-seq, Single cell DNA sequencing; scRNA-seq, Single cell RNA sequencing; scTrio-seq, Single-cell triple omics sequencing.

(Bray et al., 2018). Approximately 30 to 50% of newly diagnosed patients will progress into metastatic CRC (mCRC) with 5-year survival rate of 50 to 60% (Arvelo, 2015; Engstrand et al., 2018). Despite the advancement in cancer detection tools and treatment options, metastasis remains a hindrance for effective treatment (Chakraborty and Rahman, 2012). Over the years, several mechanisms have been proposed to explain the metastatic progression in CRC, mainly *via* disturbance of cellular processes, epigenetic modifications, and genomic alterations (Herceg and Hainaut, 2007; Wong et al., 2007; Kanwal and Gupta, 2012; Arvelo, 2015). Aside from conventional treatment, in particular, chemotherapy (5-Fluorouracil, Oxaliplatin, Irinotecan and Capecitabine), many new targeted agents are also available for metastatic CRC (mCRC), including vascular endothelial growth factor (VEGF)-targeted therapy (Bevacizumab) and anti-epidermal growth factor receptor (EGFR)-targeted therapy (Cetuximab and Panitumumab) (Riihimäki et al., 2016; Burz et al., 2018). Nevertheless, metastasis remains a challenge in treating CRC, and among the main reasons are mostly attributable to intratumoral heterogeneity (ITH) and the presence of circulating tumor cells (CTCs) (Worthley and Leggett, 2010; Séronie-Vivien, 2014).

Intratumoral heterogeneity (ITH) refers to the differences in genetic and molecular characteristics between cancer cells within a single tumor or due to the various degrees of cellular differentiation (Punt et al., 2017), whereas precision treatment, often called personalized treatment, exploits patient's as well as cancer-specific molecular and pathologic signatures to target cancerous cells (Xue and Wilcox, 2016). However, in an actual scenario, not only did these precision therapies remain unresponsive to a significant amount of patients, but also promote acquired drug resistance if inhibitors were added to maximize cancer cell death at initial stage, resulting in the rapid outgrowth of resistant clones and reoccurrence of CRC (Molinari et al., 2011). One plausible explanation to this matter was that current precision medicine was tailored based on transcriptome analyses, which utilized bulk tumor data but lacked the ability to capture ITH (Valdes-Mora et al., 2018). The presence of ITH, in turn, obscured precision cancer treatment (Hutchinson, 2014; Seoane and De Mattos-Arruda, 2014). Hence, studying the cancerous cells in single-cell resolution, at molecular level, in order to understand ITH, is necessary for precision therapy and the prediction of therapeutic efficacy (Punt et al., 2017). With the recent development of high-throughput single-cell RNA sequencing (scRNA-seq), scientists now have the power to dissect the diverse cellular populations of cancers (Bagnoli et al., 2019). In the future, it is possible that the scRNA-seq technique is applied to guide the selection of targeted combination therapies and assist in determining the enrolment criteria for clinical trials.

SINGLE CELL TRANSCRIPTOME ANALYSIS IN CRC

At present, transcriptome analyses have been intensively applied to understand the heterogeneity of tumors *via* examining the gene expression level (mRNA) present in bulk tumor cell populations (Marisa et al., 2013; Sadanandam et al., 2013; Sadanandam et al., 2014). The two most recent advances in molecular pathological classification systems for CRC are The Cancer Genome Atlas (TCGA) (The Cancer Genome Atlas Network, 2012) and Consensus Molecular Subtypes (Guinney et al., 2015). The classification systems are capable of classifying tumors into subgroups with distinct molecular and establishing signatures/clinical features to predict treatment response and patient outcomes (Budinska et al., 2013; Roepman et al., 2014). Nonetheless, the overall progress is still largely hindered because of the limitation of these bulk profiling technologies in capturing ITH (Seoane and De Mattos-Arruda, 2014). Therefore, there has been rising attention in the study of single-cell transcriptomics which is capable of examining the expression levels of individual cells within a given population.

Single-cell sequencing is a powerful technology for investigating ITH by identifying genomic alterations and distinct transcriptomic states in single tumor cells (Patel et al., 2014). To date there are only a few published studies on single-cell transcriptomes of CRC. One of the first studies was published in 2017 by Li and his colleagues, which includes 11 primary CRCs (375 cells) and matched normal mucosa (215 cells) (Li et al., 2017). Single-cell RNA sequencing was performed on 969 resected primary tumor cells from 11 CRC patients, and 622 single cells from the nearby normal mucosa of seven of the patients. The authors developed a novel clustering method, named reference component analysis (RCA) and obtained seven distinct cell clusters, which were annotated as epithelial cells, fibroblasts, endothelial cells, B cells, T cells, mast cells and myeloid cells. Interestingly, although the differentially expressed genes identified by scRNA-seq and bulk analyses were significantly concordant, the majority of differentially expressed genes from scRNA-seq were undetected in bulk analysis. Epithelial–mesenchymal transition (EMT)-related genes were upregulated only in the cancer-associated fibroblast subpopulation of CRC samples. Their results indicated that *via* projecting bulk-tumor transcriptomes onto single-cell transcriptomes, existing CRC classification system could be further refined.

In another attempt to reveal CRC tumor heterogeneity, Ono and her coworkers combined single-cell DNA and RNA sequencing technologies with a mouse CRC model, ideal for time-series analysis (Ono et al., 2019). Single-cell exome and transcriptome sequencing of 200 cells were performed to identify ITH from one single cell. The authors demonstrated that mouse cancer cells, after undergoing alteration in transcriptional and genetic ITH, can adapt to the drastic environmental changes of allograft into a mouse. During this process, new subpopulations of cells, showing mesenchymal–epithelial transformation (MET), were generated. In addition, human CRC data from TCGA revealed a remarkable trend of metastasis in a fraction of human patients whose expression patterns were similar to those of the mouse-cell subpopulations. In a nutshell, their study revealed an evolutionary pattern of single-cell RNA and DNA changes in cancer progression and a superior CRC

classification based on its ITH. **Table 1** summarizes the recent findings from single-cell transcriptome studies in CRC.

THE NECESSITY OF SINGLE-CELL TRANSCRIPTOME ANALYSIS IN METASTATIC CRC

Majority of CRC-related deaths were related to metastatic progression (Riihimäki et al., 2016). The high metastatic rate of CRC (approximately 30 to 50%) exacerbated the situation (Engstrand et al., 2018). In this context, there is an uprising interest in the discovery of new target agents for cytotoxic drugs. However, there are limited approved targeted therapy for treating mCRC, and some of these examples are EGFR monoclonal antibodies (mAbs), VEGF mAbs, anti-VEGF receptor-2 mAbs, recombinant fusion protein (Zivaflibercept) and oral multikinase inhibitor (Regorafenib). The former is available for RAS wild-type patients, while the remaining are effective for those with RAS mutation (Martini et al., 2017). Unfortunately, all these treatments are unresponsive towards CRC with alterations in genes such as *BRAF* and *PIK3CA*, posing a greater risk to patients with these subtypes (Sartore-Bianchi et al., 2009; De Roock et al., 2010; Tamborero et al., 2018). Thus, new targets for drug development and techniques in identifying the complex molecular heterogeneity of mCRC, in particular, are urgently needed (Lim et al., 2019).

Single-cell transcriptomics is most likely to contribute more specific diagnostic and prognostic markers, and actionable therapeutic targets for personalized cancer medicine than bulk transcriptomics (Zhang et al., 2014). However, it was limited in terms of characterization of multiple layers of molecular features in each genetic lineage. Therefore in the year 2018, a powerful scTrio-seq (single-cell triple omics sequencing) technique was established, which was capable of examining mutations, transcriptome, and methylome simultaneously from a single cell. CRC tumors and metastases from 10 individual patients (stage III or IV) were subjected to scTrio-seq, and their analysis provided insights into tumor evolution linked DNA methylation to genetic lineages and confirmed that DNA methylation levels were consistent within lineages but can differ substantially among clones (Bian et al., 2018). To summarize, all recently conducted research converged to the necessity to tailor individualized cancer treatment based on the analysis of gene expression patterns at single-cell resolution, which hold the potential to shed light on a key mechanism behind the development of metastasis.

POSSIBLE SOLUTION TO CRC CHEMORESISTANCE

Existing advances in cancer treatment fall short of offering an adequate solution to chemoresistance, especially among patients at the advanced stages of CRC (Hammond et al., 2016). The formation of chemoresistant cells is frequently attributed to the presence of rare drug-resistant clones in the tumor before or after treatment (Shi et al., 2018). In general, these chemoresistant

TABLE 1 | Summary of recent findings of single-cell RNA-seq and bulk analysis of CTCs in CRC.

Transcriptome	Type	Sample	Finding	Citation
Single cell analysis	Primary CRC	Primary CRC cells from CRC patients	Development of reference component analysis (RCA) which obtained seven distinct cell clusters (epithelial cells, fibroblasts, endothelial cells, B cells, T cells, mast cells and myeloid cells).	(Li et al., 2017)
		Organoids from primary CRC human tumors (stage 1)	Detection of four newly emerged chemoresistant cell subtypes (c29, c30, c31, and c32) after Oxaliplatin treatment with different drug responses.	(Chen et al., 2018)
		2824 primary CRC cells from a CRC patient	Detection of five distinct cell clusters from a CRC patient with clear sign of heterogeneity, where each cluster consisted of specific cell markers with different functions.	(Dai et al., 2019)
	Metastatic CRC	Metastatic single cells from CRC patients (stage III or IV)	Confirmation of the feasibility of genetic lineages reconstruction together with their epigenomic and transcriptomic dynamics.	(Bian et al., 2018)
		CRC mouse model and metastatic mouse cancer cells	Validation of the dynamics of ITH in mouse CRC models and relate to CRC in humans *via* comparison with TCGA data.	(Ono et al., 2019)
		Samples of liver metastasis cancer tissue and adjacent tissue from CRC patients	Identification of a total of 12 clusters corresponding to 6 cell types, including cancer cells, T cells, myeloid cells, endothelial cells, fibroblasts and B cells from patient sample of CRC liver metastasis.	(Zhang et al., 2019)
	CRC cell line	Secondary cell line (HCT116 cells)	Identification of multiple adaptive resistance mechanisms to regorafenib in CRC *via* single cell RNA sequencing.	(Sathe et al., 2019)
Bulk analysis	Metastatic CRC	EpCAM-based immunoisolation of CTC from 6 mCRC patients	Identification of 410 genes related to cell movement and adhesion, cell death and proliferation, and cell signaling and interaction *via* cDNA microarray.	(Barbazán et al., 2012)
		CTC lines from the blood of 3 advanced mCRC patients	Validation of genetically and phenotypically heterogeneity in CTC lines. Identification of gene subset commonly enriched in cultured CTC and CTCs from colon and other cancers. CTC lines expressed high levels of drug metabolism genes and were resistant to conventional therapies.	(Grillet et al., 2017)

subtypes of CRC attain the ability to disrupt drug transport, dysregulate cellular processes, alter drug sensitivity (via genetic or epigenetic modifications) and targets of therapy, that subsequently limit the efficacy of existing anti-cancer therapies (Holohan et al., 2013; Panczyk, 2014; Hu et al., 2016; Zhang and Wang, 2017; Hon et al., 2018; Abu et al., 2019). Since there are hints that metastasis and chemoresistance can be interconnected (Zheng, 2017; Durinikova et al., 2018), the previous can be prevented if chemoresistant subtypes are identified early for optimal or more aggressive treatment. Unfortunately, the mechanisms responsible for chemotherapy resistance by CRC have not been clearly identified. Moreover, current chemotherapy does not possess the strength to fully eradicate solid tumors, resulting in secondary tumor and relapse. Owing to this, numerous efforts have been made to dissect the chemoresistant cancer cells based on the genes expressions, epigenetics, pathways signatures and therapeutic responses (Datta et al., 2016; Baharudin et al., 2017; Abu et al., 2019; Li et al., 2019).

Although bulk transcriptomics is adequate to study the average gene expression signatures related to chemoresistance, they generally involve bulk tissue with assumption that all the cells obtained are of homogeneous material, thereby ignoring the stochasticity of gene expression (Raj and van Oudenaarden, 2008; Stegle et al., 2015). Single-cell transcriptomics analyses, on the other hand, are capable of studying the transcriptomes of individual cells and are more preferable for cell-specific precision therapies. For instance, a recent research by Chen et al. (2018) confirmed the capability of scRNA-seq in characterizing four different types of cellular subtypes from organoids, including drug-induced group (c29, c30, c31, and c32), drug insensitive group (c2, c5, c6, c7, c13, c22, c21, c24, c27, and c28), drug-sensitive group (c1, c3, c4, c8, c9, c11, c15, c19, c25, and c26), and drug ultrasensitive group (c10, c12, c14, c16, c17, c18, c20, c23), after treatment of Oxaliplatin. The first group displayed chemoresistance properties and appeared only after treatment. Studying of these subtypes would enable further detailed categorization based on the differential responses, genes and pathways involved, leading towards better therapeutic selection for CRC patients who might or already displayed chemoresistance before or after chemotherapy. Hence, scRNA-seq is foreseen to be applied to guide the selection of anticancer therapies and even in the prevention of chemoresistance in the future.

CTC CHARACTERISTICS AS A SNAPSHOT OF TUMOR HETEROGENEITY

Circulating tumor cells (CTCs) are rare metastatic cells shed from the primary tumor into the circulatory system, forming secondary tumor at distant tissues (Ferreira et al., 2016) (**Figure 1**). According to the 'seed and soil' hypothesis by Stephen Paget in 1889, a seed (in this case, CTC) have the ability to form metastasis only in a location suited for this process (organs like liver, lungs), whereas the mechanistic theory based on the direction of blood flow from tumor proposed by James Ewing in 1920 assumes that potential of metastasis is dependent on drainage anatomy from the primary tumor. Both these two complementary hypotheses point out the

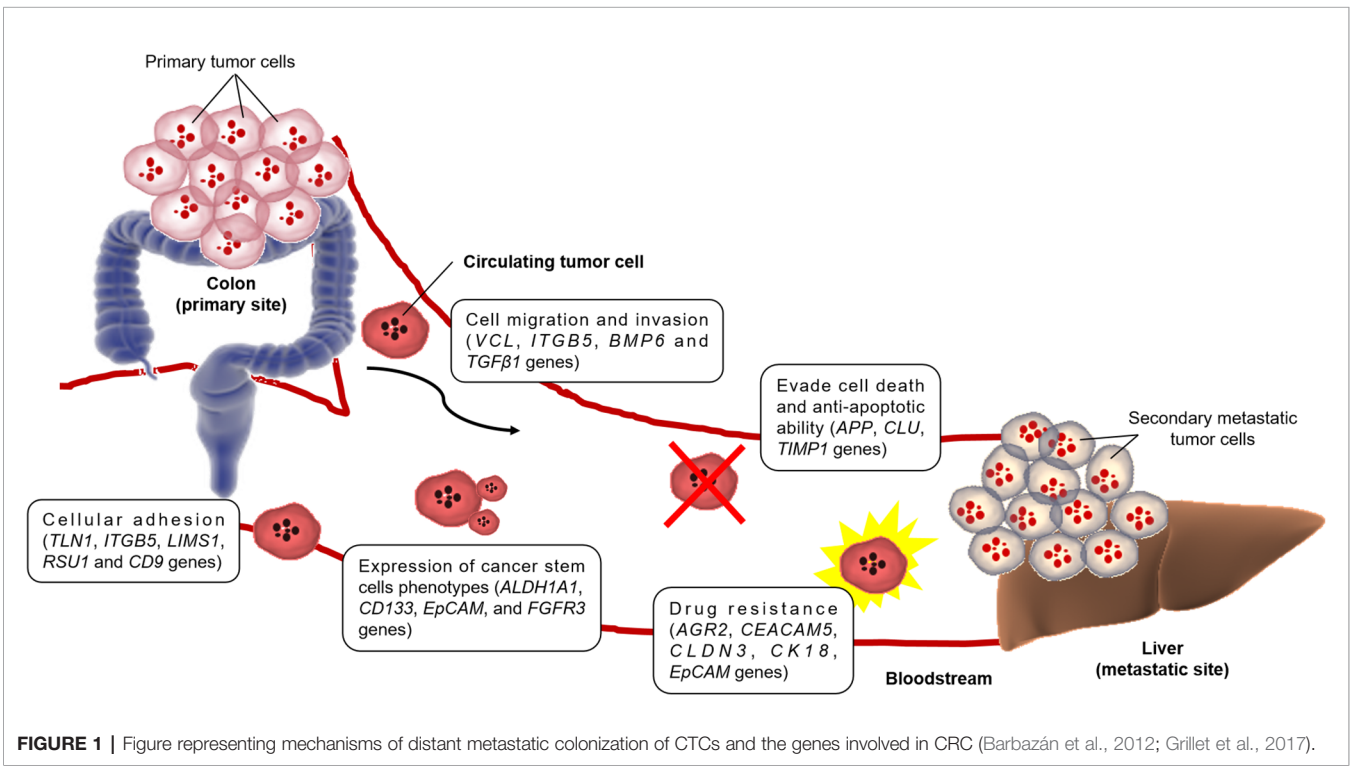

FIGURE 1 | Figure representing mechanisms of distant metastatic colonization of CTCs and the genes involved in CRC (Barbazán et al., 2012; Grillet et al., 2017).

potential of CTCs in causing metastasis during the progression of CRC. Since then, there have been several examples of validated clinical applications for CTC detection, proving its existence in CRC (Bork et al., 2015) and mCRC (Cohen et al., 2008), and also as a prognostic and predictive marker in CRC patients (Huang et al., 2014). As such, single-cell analyses of these CTCs are believed to provide critical insights into CRC cancer metastasis (Pantel and Alix-Panabières, 2012; Pantel and Speicher, 2016). However, characterization of CTCs from CRC, at the single-cell level, are as yet unknown. At present, there are only a few published papers on the bulk RNA sequencing analysis in CTCs.

As presumptive founders in the metastasis formation, CTC is becoming a field of interest, and the understanding of their biology may open new perspectives in oncology (Lim et al., 2019). In 2012, a group of Spain researchers performed molecular characterization of CTCs in human mCRC. Their objective was to investigate the biology of CTCs and improving their clinical utility in the CRC patients' management (Barbazán et al., 2012). For this, EpCAM-based immunoisolation of CTC from six mCRC patients was combined with whole-transcriptome microarrays, revealing 410 genes related to cell movement and adhesion, cell death and proliferation, and cell signaling and interaction. All these genes characterized the CTC populations. Their study suggested CTCs as the diagnostic and prognostic biomarkers, which represented an innovative and promising approach in the clinical management of CRC patients in the foreseeable future.

Although CTCs have attracted a broad interest as potential markers of tumor progression and treatment response, the lack of functional characterization of these cells has become a bottleneck in taking these observations to the clinic. In addition, *in vitro* CTC models are lacking. Following this, Grillet and her coworkers generated several CTC lines from the blood of three advanced mCRC patients (Grillet et al., 2017). Characterization of these cells verified the presence of multipotent cells responsible for genetic and phenotypic heterogeneity, endowing them with strong metastatic potential. In addition, six genes (*AGR2*, *CEACAM5*, *CLDN3*, *KRT18*, *EpCAM* and *FGFR3*) were detected as differentially expressed in the generated CTC cell lines, which was similar to primary CRC cells grown under similar conditions (Smirnov et al., 2005; Mostert et al., 2015; Onstenk et al., 2015). The CTC lines also displayed enhanced drug/xenobiotics metabolizing activity, in particular *via* cytochrome P450 pathway, suggesting resistance to conventional therapies. To sum up, their study was the first experimental demonstration that CTCs isolated from mCRC patient could be used to determine drug sensitivity which aided in the formulation of personalized cancer medicine, even though the heterogeneity of CTCs was not investigated in single-cell resolution at molecular level.

CHALLENGES AND FUTURE DIRECTIONS

Colon is a relatively large organ, categorized into four parts (the ascending colon, the transverse colon, the descending colon and the sigmoid colon) and is composed of multiple different cells (epithelial cells, stroma, muscle cells, fat, etc.). In order to obtain a single-cell suspension, the bulk tissue has to undergo mechanical or enzymatic dissociation. Isolating the single-cell suspension containing 'healthy' and contamination-free viable cells are indeed a challenge for scRNA-seq studies. Moreover, there are several technical limitations in scRNA-seq like the number of cells that can be studied at a time and the depth of sequencing required. The higher the number of cells, the higher the sequencing depth needed, hence increasing the overall cost of scRNA-seq experiment. If the number of cells must be limited to a certain amount, then the overall representative of the single-cell transcriptome will be questioned. Therefore, CTCs are believed to be the versatile components which warrant a spotlight in CRC research in order to identify biomarkers that will benefit the metastatic or chemoresistant CRC patients, while providing representative data. Also, scRNA-seq requires the cells to be intact after sorting or enrichment, prior to library preparation to prevent RNA degradation. The ability of CTCs to survive from harsh environments in the bloodstream makes them the 'tough' cells and the best candidate for scRNA-seq in studying CRC (Steinert et al., 2014).

At present, various technologies are developed for CTCs isolation, however, they are mainly used for research purposes rather than clinical applications (Bankó et al., 2019). Among the obstacles are the presence of ITH (Levitin et al., 2018; Lim et al., 2019), limited knowledge on CTCs mechanism of action in cancer progression (shedding from the primary/metastasis tumor, survival in bloodstream, avoidance of apoptosis, colonization potentials and settlement in distant organs), the rarity of CTCs (0 to 10 CTCs/ml whole blood in 30% to 50% mCRC patients) (Zieglschmid et al., 2005), various sizes of CTCs and the lack of clinical validation (Millner et al., 2013; Rejniak, 2016; Kowalik et al., 2017; Bankó et al., 2019). In addition, there are only a few studies relating to CTCs pharmacogenomics and underlying survival mechanisms (Wang et al., 2018) as well as the cell-cell interactions in CRC microenvironment (Krog and Henry, 2018), and majority of them are based on experimental and theoretical extrapolations (Burz et al., 2018). For instance, Yu et al. (2014) suggested that pharmacogenomic profiling of invasive CTCs could predict chemotherapy response and resistance, whereas Steinert et al. (2014) identified upregulation of *CD47* in concordance with the mark down-regulation of calreticulin, which were believed to mediate immune escape and survival mechanisms of CTCs in CRC. In 2016, Ning et al. (2018) proposed CTCs as a clinically useful prognostic marker in mCRC patients as they displayed Akt-2 expression that mediated epithelial mesenchymal transition. Although CTCs demonstrated potential as a predictive marker (Yap et al., 2014) and matched most of the cancer hallmarks described by Hanahan and Weinberg (Hanahan and Weinberg, 2011; Hanahan and Weinberg, 2017; Fouad and Aanei, 2017), current understanding about their pharmacological and clinical knowledge is still limited. Therefore, we anticipate that, in the near future, with the advancement of single cell technologies, CTCs could be proven useful in CRC management and a routine screening for cancer patients.

Another bottleneck in scRNAseq is the bioinformatics data analysis. Various algorithms have been developed to infer cell types by clustering scRNAseq profiles, however, a robust algorithm is yet to be developed due to high noise levels, technical variability and batch effects (Gao, 2018; Chen et al., 2019; Choi and Kim, 2019). To date, gene expression in such tumors has been profiled using bulk transcriptome methods, providing a single transcriptome measure that represents many cell types (Barbazán et al., 2012; Grillet et al., 2017). By employing single-cell transcriptomic technology, it is now possible to deconstruct a tumor into its component cell-type parts and therefore gain a better understanding of the underlying biology. In conclusion, the analysis of gene expression patterns of CTCs from CRC at single-cell resolution holds the potential to provide key information for identification of prognostic and diagnostic markers as well as the development of precise and personalized cancer treatment.

AUTHOR CONTRIBUTIONS

N-SM is the principal investigator that conceptualize the idea of this manuscript. FT, RB and N-SM wrote the manuscript, while R-IM, NA and L-HL provide critical insights.

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CASE REPORT
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Abiraterone Rechallenge Based on Sequential Testing of Androgen Receptor Splice Variant 7 Expression in Circulating Tumor Cells: A Case Report

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Serial analysis of circulating tumor cells (CTCs) such as androgen receptor splice variant 7 is useful in selecting treatments for castration-resistant prostate cancer (CRPC). We report a case who had been positive for androgen receptor splice variant 7 in CTCs before docetaxel, and was subsequently treated with abiraterone rechallenge because of the negative conversion of androgen receptor splice variant 7 following docetaxel. Although, the rechallenge of anti-androgen agent based on CTCs analysis is expected to be an effective approach, it is yet to be reported. Thus, we chose the candidate for abiraterone rechallenge based on serial CTCs analyses by the AdnaTest. As a result, the patient responded to abiraterone that he once had developed resistance to. Our findings reinforce the utility of AR-V7 as a biomarker in the setting of post-chemo androgen-targeted-therapy rechallenge.

Keywords: abitaterone, androgen receptor splice variant 7, castration-resistant prostate cancer, circulating tumor cells, rechallenge

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BACKGROUND

Most metastatic prostate cancers are treated with androgen deprivation therapy (ADT) at the outset. Nevertheless, most of them progress to acquire resistance to the primary ADT, which is a state called castration-resistant prostate cancer (CRPC). Novel anti-androgen agents (enzalutamide, apalutamide, and darolutamide), CYP17A1 inhibitor that inhibit the production of testosterone (abiraterone), two taxane-based chemotherapies (docetaxel and cabazitaxel) and radium-223 have been approved by the US Food and Drug Administration for CRPC treatments (1–3).

Despite a variety of treatment options available for CRPC, there is no predictive biomarker used for treatment selection. Instead, clinicians decide on a course of treatment based on the several prospective randomized controlled phase 3 trials. For instance, most prostate cancer experts have consensus that asymptomatic men with metastatic CRPC should receive abiraterone or enzalutamide as the first-line treatment (4).

However, from the perspective of personalized medicine, we should choose treatments based on the genomic profiles of CRPC. Since genomic analysis of biopsy samples from metastatic lesions is not practical, liquid biopsy such as circulating tumor cells (CTCs) and circulating tumor DNA are drawing attention in recent years. In terms of CTCs, Antonarakis's study propounded testing

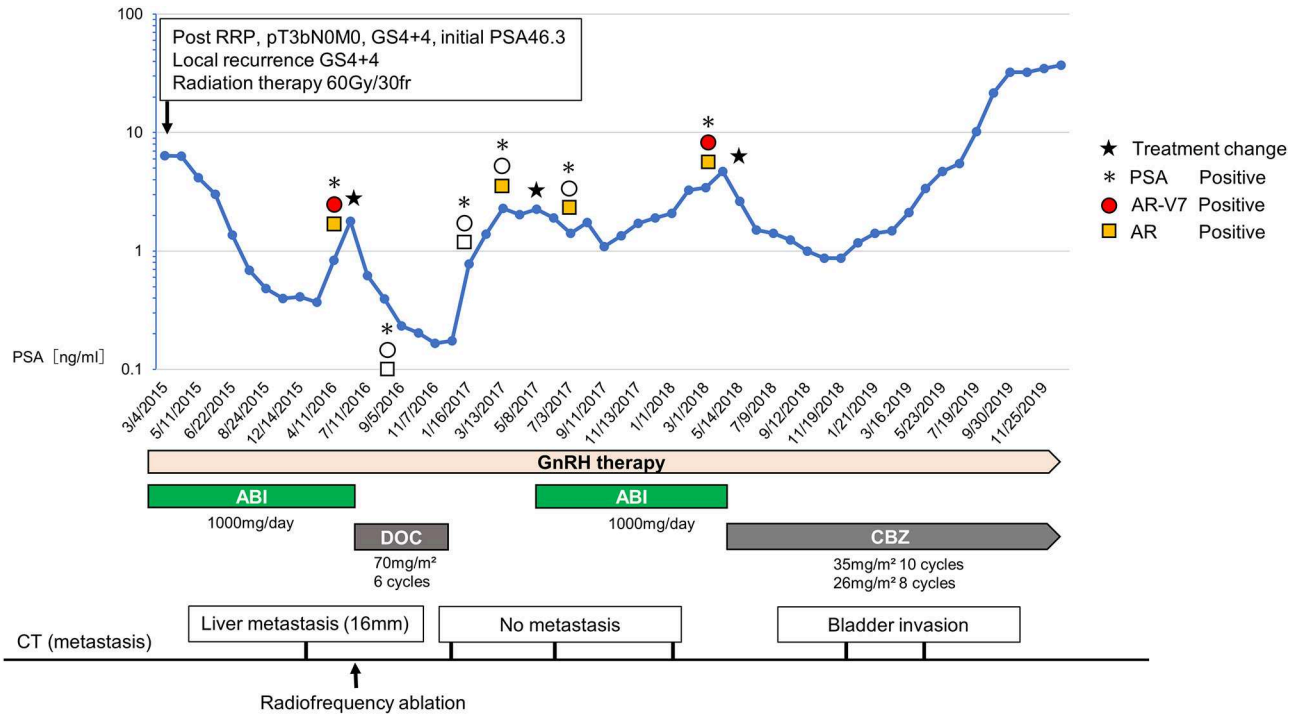

FIGURE 1 | Changes of the serum PSA, treatment course, metastasis lesion, and the status of CTCs. The upper graph shows the changes of serum PSA and CTC status including AR-V7 expression. The treatment course is showed in the middle and the status of metastasis lesion is shown in the bottom. During the first-round abiraterone administration, when the serum PSA level rose twice, we analyzed AR-V7 expression in CTCs. Due to positive AR-V7 expression, we chose docetaxel as the second-line CRPC treatment. Because of rising PSA level during a drug holiday after docetaxel, we rechallenged abiraterone based on the negative conversion of AR-V7 expression, which resulted in PSA decline. When PSA rose to 4.00 ng/dl 10 months after abiraterone rechallenge, the expression of AR-V7 was converted to positive. Subsequently, cabazitaxel was started.

androgen receptor splice variant 7 (AR-V7) expression in CTCs (5, 6). In their study, AR-V7-negative cohort showed a better prostate-specific antigen (PSA) response to novel anti-androgen agents than AR-V7-positive cohort. Furthermore, serial testing of AR-V7 in CTCs can be useful in selecting treatments for CRPC. However, this CTC analysis is yet to be acknowledged as the standard companion diagnosis. To further verify the utility of CTC analysis, prospective studies are currently underway (6).

To this end, we performed a feasible bedside CTC testing for CRPC patients. Herein, we report a case of a CRPC patient who was successfully treated based on sequential CTC analysis (**Figure 1**).

This study was approved by the institutional review board of Juntendo hospital (admission number: 14-052), and all experiments were carried out in accordance with approved guidelines. Written informed consent was obtained from the patient for the publication of any potentially identifiable images or data included in this article.

CASE PRESENTATION

In November 2007, a 62-year-old man was diagnosed as localized prostate cancer with no metastasis (cT3bN0M0, Gleason score 4+4, initial PSA 46.3 ng/dl) and underwent radical prostatectomy. In April 2008, he had a PSA recurrence (2.4 ng/dl) and the combination androgen blockade therapy (LH-RH agonist + bicalutamide 80 mg/day) was initiated. In March 2015, he presented a local recurrence, and the treatment was switched to abiraterone (1,000 mg/day) combined with LH-RH antagonist and prednisolone. In addition, radiation therapy (60 Gy/30 fr) was done against the local recurrence, which resulted in marked tumor disappearance. His disease was well-managed with abiraterone for 13 months. In June 2016, he showed PSA progression and a liver metastasis occurred. At this point, AR-V7 was positive by the AdnaTest (QIAGEN, Germany). Therefore, we selected docetaxel of every 4 week (70 mg/m^2) and a total of six cycles was given. Radiofrequency ablation (RFA) was performed for a liver metastasis since it was a singular and small lesion (<30 mm), which is the indication for RFA. After the administration of docetaxel, PSA declined to 0.166 ng/dl. Furthermore, CTC analysis confirmed the negative conversion of AR-V7 in CTCs. Due to the favorable response to docetaxel, docetaxel treatment was suspended and only LH-RH antagonist was continued for the following 5 months as a drug holiday. Subsequently, when PSA rose to the pre-docetaxel level (2.29 ng/dl), we tested AR-V7 expression again. Resultantly, because AR-V7 still remained negative, we opted for abiraterone rechallenge based on the following discussion

with patient. The attending physician explained to the patient that enzalutamide may cause adverse events, such as fatigue and anorexia. Because the patient had no adverse events during the administration of abiraterone, he wanted to resume abiraterone. Also, because he had experienced symptoms of alopecia and numbness that adversely affected his job at the time of docetaxel administration, he did not want to be treated with chemotherapy while at work.

As a result, 6 months after the abiraterone rechallenge, PSA value became lower (1.08 ng/dl) than the level before abiraterone rechallenge. In line with this, no other metastases were found. PSA elevation was not observed for 8 months. Afterward, when PSA rose to 4.00 ng/dl 10 months after abiraterone rechallenge, we analyzed AR-V7 expression in CTC again. CTC analysis showed that the expression of AR-V7 was converted to positive. Based on the CTC analysis, subsequent cabazitaxel was started. Although, PSA was well-controlled for 9 months after cabazitaxel administration, PSA rose continually after 10 cycles of cabazitaxel, and computed tomography image showed the emerge of the bladder invasion.

DISCUSSION AND CONCLUSIONS

Here, we reported the case of a CRPC patient with liver metastases, who was treated with abiraterone rechallenge based on AR-V7 status in CTCs.

Nakazawa et al. (7) reported the dynamic transition of AR-V7 status in CTCs and its utility for treatment selections. Their study showed the results of detailed CTC profiles and treatment courses in 14 patients. In this study, there was one patient showing continuous AR-V7 positivity, who was treated with androgen-targeted therapy rechallenge after docetaxel. However, he did not benefit from this rechallenge. Also, there was another patient who benefited from the first time abiraterone after docetaxel, when CTCs' AR-V7 status changed from positive to negative following docetaxel. However, unlike our case, there was no patient who benefited from the androgen-targeted-therapy rechallenge following chemotherapies in Nakazawa's study.

The clinical outcome of treatment with abiraterone or enzalutamide for patients who progressed on abiraterone has been shown in COU-AA-302 trial. Most of these patients received chemotherapy before subsequent abiraterone or enzalutamide. PSA response (defined as a PSA reduction of at least 50% from baseline) was observed in 24 of 55 patients who received subsequent abiraterone and in 22 of 33 patients who received subsequent enzalutamide (8). Besides, the results of phase III clinical trial, CARD trial, showed that cabazitaxel significantly improved clinical outcomes compared with androgen-targeted therapy (abiraterone or enzalutamide) in patients with metastatic CRPC previously treated with docetaxel (9). Although, the benefit of post-chemotherapy abiraterone or enzalutamide rechallenge was limited to some patient, these results still suggested that some cancer regained abiraterone or enzalutamide sensitivity after chemotherapy. However, we need to accurately identify the candidate patient who potentially benefit from androgen-targeted-therapy rechallenge. Therefore, our case provides clinical and biological rationale for AR-V7-based patients selection for the androgen-targeted-therapy rechallenge following chemotherapies.

In our report, changes in AR-V7 expression appeared to be reflective of the treatment effect. For example, the negative conversion of AR-V7 occurred after docetaxel and RFA for liver metastasis, both of which could have eliminated AR-V7-positive CTCs. Then, the withdrawal of docetaxel possibly stimulated the expansion of AR-V7-negative cells expressing full-length AR, which might have resulted in regained susceptibility to abiraterone rechallenge. Although, PSA elevation was observed after the abiraterone rechallenge, this treatment was able to suppress the increase in PSA for the following 8 months and delay the initiation of subsequent cabazitaxel treatment. Furthermore, abiraterone was effective only when AR-V7 was negative. These findings reinforce the utility of AR-V7 as a biomarker in the setting of post-chemo androgen-targeted-therapy rechallenge.

On the other hand, we have a variety of issues we need to address in CTC research. Firstly, whether CTCs represent the entire tumor characteristics is questionable. Secondly, AR-V7 is not the only mechanism driving CRPC progression. The states of other mechanisms responsible for treatment resistance in CRPC such as dysregulated PI3K–AKT signaling, WNT signaling pathway and DNA repair defects need to be elucidated (10). Thirdly, not only the wild-type full-length AR and AR splice variants mediate AR-signaling in CRPC, but also gene amplification and gain-of-function mutations are reportedly implicated in the sustained AR signaling in CRPC (11). Further investigations and technical developments are required to clarify the underlying heterogeneity of CTCs and CRPC biology in CTCs.

To the best of our knowledge, this is the first reported case of regaining susceptibility to the post-chemo anti-androgen agent with the concurrent negative conversion of AR-V7. Our result suggests that the rechallenge of post-chemo AR-targeted therapy based on AR-V7 testing can be a good strategy in treating CRPC.

ETHICS STATEMENT

Written informed consent was obtained from the patient for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors have significantly contributed to the study and are in agreement with the content of the manuscript. Each author's contribution is as follows: NN and MK performed the experiments. NN, MK, MN, and SH designed the study. NN and MK wrote the manuscript, and MN and SH revised it.

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ORIGINAL RESEARCH
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Monitoring of Early Changes of Circulating Tumor DNA in the Plasma of Rectal Cancer Patients Receiving Neoadjuvant Concomitant Chemoradiotherapy: Evaluation for Prognosis and Prediction of Therapeutic Response

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Introduction: Patients with locally advanced rectal cancer (LARC) are undergoing neoadjuvant chemoradiotherapy (NCRT) prior to surgery. Although in some patients the NCRT is known to prevent local recurrence, it is also accompanied by side effects. Accordingly, there is an unmet need to identify predictive markers allowing to identify non-responders to avoid its adverse effects. We monitored circulating tumor DNA (ctDNA) as a potential liquid biopsy-based biomarker. We have investigated ctDNA changes plasma during the early days of NCRT and its relationship to the overall therapy outcome.

Methods and Patients: The studied cohort included 36 LARC patients (stage II or III) undergoing NCRT with subsequent surgical treatment. We have detected somatic mutations in tissue biopsies taken during endoscopic examination prior to the therapy. CtDNA was extracted from patient plasma samples prior to therapy and at the end of the first week. In order to optimize the analytical costs of liquid-biopsy testing, we have utilized a two-level approach in which first a low-cost detection method of denaturing capillary electrophoresis was used followed by examination of initially negative samples by a high-sensitivity BEAMING assay. The ctDNA was related to clinical parameters including tumor regression grade (TRG) and TNM tumor staging.

Results: We have detected a somatic mutation in 33 out of 36 patients (91.7%). Seven patients (7/33, 21.2%) had ctDNA present prior to therapy. The ctDNA positivity before treatment reduced post-operative disease-free survival and overall survival by an average of 1.47 and 1.41 years, respectively ($p = 0.015$, and $p = 0.010$). In all patients, ctDNA was strongly reduced or completely eliminated from plasma by the end of the first week of NCRT, with no correlation to any of the parameters analyzed.

Conclusions: The baseline ctDNA presence represented a statistically significant negative prognostic biomarker for the overall patient survival. As ctDNA was reduced indiscriminately from circulation of all patients, dynamics during the first week of NCRT is not suited for predicting the outcome of LARC. However, the general effect of rapid ctDNA disappearance apparently occurring during the initial days of NCRT is noteworthy and should further be studied.

Keywords: rectal cancer, neoadjuvant chemoradiotherapy, circulating tumor ctDNA, prediction, prognosis, response, biomarker

INTRODUCTION

Current treatment of rectal cancer is based on a multimodal approach involving surgery, radiation, and systemic therapies (1). The actual therapeutic decision process is based on a precise disease staging with early tumors being preferentially considered for direct surgical treatment. Patients with locally advanced rectal cancer (LARC), that is, stages II and III, should undergo radiation in combination with systemic chemotherapy prior to surgery. This neoadjuvant chemoradiotherapy (NCRT) typically includes 5-fluoropyrimidine administered within either a "long regimen" involving a total dosage 50.4 Gy (45 Gy split into 25 fractions received within 5 weeks and initial boost 5.4 Gy) or a "short regimen" of 25 Gy in five fractions during the first week. Although records show that NCRT in general does not improve the overall survival (OS), it reduces the local recurrence of RC to under 10% of all cases (2). Patients showing appropriate response to NCRT have a significantly better prognosis in the long-term perspective (3, 4).

The clinical and laboratory evaluation following NCRT includes endoscopic examination, MRI, and histopathology (5). The objective response to NCRT may include (i) a complete elimination of the tumor, (ii) partial regression of the tumor size, (iii) elimination or reduction of the number of tumor-positive lymph nodes in the mesorectum, or (iv) any combination thereof. Although a complete clinical response is found in 15–30% of all cases and partial response is reached in 20–5% of all patients, half of the tumors will remain principally unaffected following NCRT (3, 4). At the same time, however, it is known that NCRT induces a variety of adverse side effects, most importantly development of fibrosis due to the post-radiative pelvic damage leading to impairment of the anal sphincter, leading to incontinence and erectile dysfunction. It is also associated with post-operative complications and worsening of post-operative functional results (6, 7). In order to avoid and/or minimize the adverse effects of NCRT, it is highly desirable to modify or even eliminate preoperative NCRT in potential non-responders.

Identification of patients non-responding to NCRT has recently become a focus of sustained clinical research. A wide spectrum of approaches has been investigated including application of various predictive markers. The traditional clinical parameters such as tumor size and distance from anal margin (evaluated by endoscopy and MRI) were complemented by laboratory biomarkers comprising, for example, hemoglobin, carcinoembryonic antigen (CEA) serum levels, or tumor-infiltrating lymphocytes (8). Furthermore, use of a contrast MRI with diffusion-weighted imaging (DWI), which accounts for perfusion and cellular density and thus reflects tumor biology, has also been utilized. However, to this point, study results have been generally contradictory (9–15).

More recently, a role of molecular genetic markers of NCRT response prediction was investigated. Among others, variants of the *KRAS* gene (MIM# 190070), which is mutated in 30–60% sporadic colorectal cancer (CRC), are the most frequently studied genetic "biomarkers" (16). Activating pathogenic variants (henceforward termed in legacy nomenclature "mutations") in *KRAS* are associated with poor response to biological therapy as monitored by monoclonal anti-epidermal growth factor receptor (anti-EGFR) antibodies (17). Several studies have assessed *KRAS* as a predictive marker for the therapeutic effect of NCRT, indicating better response of the wild type over its mutated alleles (18, 19). This concept, however, has not been confirmed by others (20, 21). In addition, because mutations in the *TP53* gene (MIM# 191170) have also been frequently observed in CRC, it was suggested that it could serve as a potential predictor of resistance to NCRT. Several groups implied that *TP53* gene wild-type constitution and a lower expression of the p53 protein product are both associated with proper therapeutic response (22–24). Nonetheless, these studies have not been universally accepted, and further studies are needed (25, 26).

Detection of DNA circulating in the blood of patients [cell-free DNA (cfDNA)] has recently gained considerable interest as a potentially new class of molecular markers in the area of cancer diagnostics and management (27). CfDNA consists of short DNA fragments released from decomposing tumor cells mainly through necrotic and apoptotic processes as well as active endosomal release. Its levels are significantly elevated in organisms undergoing cellular decomposition induced by immune response such as owing to infection or inflammation as well as cancer. The typical levels of cfDNA range approximately from 10 to 100 ng/ml of plasma (∼3,000–30,000 DNA copies/ml) in healthy individuals up to mg/ml levels in cancer patients (28).

Abbreviations: CEA, carcinoembryonic antigen; cfDNA, cell-free DNA; CRC, colorectal cancer; CT, computed tomography; ctDNA, circulating tumor DNA; DCE, denaturing capillary electrophoresis; DWI, diffusion-weighted imaging; LARC, locally advanced rectal cancer; MRI, magnetic resonance imaging; NCRT, neoadjuvant chemoradiotherapy; RC, rectal cancer; TNM staging, TNM Classification of Malignant Tumors; TRG, Dworak histopathological tumor regression grade.

The part originating from cancerous cells is referred to as a circulating tumor DNA (ctDNA). CtDNA, which represents only a very small fraction of the overall cfDNA, typically contains somatic mutations, which are only present in tumor and not in the "healthy" cells, hence, presents an alternative source for predictive cancer-specific mutation detection. This fact has been intensely investigated giving rise to a whole new area of ctDNA "liquid biopsy-based" diagnostic strategies. Aside from being used as an alternative source of material for tumor diagnostics, the relative changes in ctDNA levels are known to correlate with the overall tumor burden in a given patient, thus indirectly reflecting the overall size and number of cancerous lesions present in the body (29). Therefore, ctDNA evaluation has a great potential as a marker for monitoring the disease and course of the treatment (30). It has long been recognized that in a positive response to chemotherapy, the ctDNA is reduced or eliminated completely from the peripheral circulation (31–33).

According to recent reports, the natural kinetics of cfDNA levels in blood is a bimodal process in which initially high levels are rapidly decreasing within minutes as the cfDNA fragments are being distributed across highly vascularized organs/tissues and in other biological fluids followed by a period of slower elimination processes (34). The elimination process is through DNA degradation by ribonucleases present in the blood with a typical half-life of 1.5–3 h. The equilibrium between release and elimination supports the use of cfDNA as a marker of concurrent processes taking place in the body. It is universally accepted that tumors responding to radiotherapy exhibit cell damage leading to necrosis by which DNA is rapidly released into the bloodstream. Hence, upon administration of the radiotherapy, ctDNA levels should rise momentarily before being removed from the circulation by natural homeostatic processes.

In this study, we investigated tumor-derived DNA in plasma (ctDNA) in a pilot cohort of patients with LARC undergoing NCRT. The aim of the present work was to observe changes in ctDNA levels in rectal cancer patients during the initial days of the NCRT treatment and to correlate these to the overall clinical outcome of RC therapy.

PATIENTS AND METHODS

Patient Cohort and Neoadjuvant Chemoradiotherapy Therapy

Our prospective study included 36 patients with LARC who had been recruited between 2013 and 2017. The group included 27 men and 9 women with an average age of 64.1 years, capable of undergoing repeated blood sampling during therapy. Patient characteristics are listed in **Table 1**. The study protocol was approved by the ethics committee of Motol University Hospital, and patients confirmed their study participation by signing an informed consent form. Upon initial recommendation of the committee, only patients with good performance status and compliance were included in the study.

Initial endoscopic biopsy was performed, and the tumor was histologically verified. Staging of the disease was determined based on the CT and MRI (**Table 1**), and the respective treatment was protocol based on these examinations. Tumor tissues (typically a total of three samples acquired by biopsy forceps) collected during the initial endoscopy prior to oncological treatment were immediately post-operatively frozen at −30°C and sent to the collaborating laboratory for genetic testing. Subsequently, tumor tissues were examined for the presence of the most common mutations previously observed in CRC (comprising *KRAS /MIM# 190070/*, *TP53 /MIM# 191170/*, *APC /MIM# 611731/*, *PIK3CA /MIM#* 171834/, *BRAF /MIM# 164757/*, and *CTNNB1 /MIM# 166806/*). Plasma was obtained by centrifugation from blood samples taken prior to and during NCRT.

All patients underwent NCRT consisting of 50.4 Gy of radiation and concomitant administration of Xeloda™ (*capecitabine*) at a dose of 825 mg/m^2. Irradiation was carried out by 25 fractions with initial boost of 5.4 Gy. At the end of the first week of NCRT, another blood sample was taken for ctDNA examination. A control MRI of the pelvis was performed 6 weeks after termination of NCRT. At 8–10 weeks after the end of NCRT, all patients with LARC underwent surgery. Biopsy samples were evaluated in detail by an expert histopathologist using standard TNM staging. In addition, the "Dworak histopathological tumor regression grade" (TRG) was also determined. Patients were followed up for at least 3 years after surgery. Standard tumor marker examination, colonoscopy, and computed tomography (CT) imaging were performed at regular intervals.

Tissue and Circulating Tumor DNA Mutation Testing

DNA extraction from tumor tissue bioptates and plasma was performed using standard spin-column procedures. A GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich, St. Louis, Missouri, USA) was used for extraction from the tissue samples. Extraction of ctDNA from blood plasma samples was performed using NucleoSpin Plasma XS kit (Macherey-Nagel, Düren, Germany), the volume of plasma processed was 600 μl, yielding typically between 5 and 50 ng of cfDNA per sample determined by Qubit 2.0 Fluorometer (Life Technologies, Camarillo, CA).

Similarly to works of others (31–33), the mutation analysis of tissue samples was focused on a panel of selected oncogenes, with the highest proportion of somatic mutations in rectal cancer according to the international "COSMIC database" (https://cancer.sanger.ac.uk/cosmic). This panel included the hotspots in *KRAS*, *BRAF*, *PIK3CA*, and *CTNNB1*, as well as selected areas of tumor suppressor genes *APC* (mutation cluster region) and *TP53* (exons 5–8). Mutation analyses were performed using the denaturing capillary electrophoresis (DCE) method using the previously described experimental parameters (35–38). Somatic mutations detected in the tissue samples were subsequently examined in samples of cfDNA obtained from plasma. Genetic testing was conducted using two separate methods as discussed further. A subgroup of ctDNA samples assigned as negative by DCE method (39) was subsequently retested using a high-resolution "BEAMING assay" (40) directed at the detection of *KRAS*-specific ctDNA and provided by an external contracted

TABLE 1 | Patient characteristics.

				TNM staging I			TNM staging II						
Patient	**Gender**	**Age**	**Stage**	**T**	**N**	**M**	**T**	**N**	**M**	**L**	**TRG**	**Mutation**	**Baseline ctDNA**
1	M	68	2	3	0	0	3	0	0	3	2	*TP53*	0
2	M	63	2	3	0	0	0	0	0	1	4	*KRAS*	0
3	M	73	3	3	1	0	3	2a	0	2	2	*KRAS+APC*	0
4	M	74	2	3	0	0	3	2a	0	2	1	*KRAS*	0
5	F	73	3	3	1	0	2	0	0	2	2	*PIK3CA*	0
6	M	64	3	4	1	0	3	1c	0	3	1	*APC*	0
7	M	62	3	3	1	0	3	2b	0	3	2	*TP53+APC*	0
8	F	36	3	3	1	0	2	0	0	2	2	*APC*	0
9	F	61	3	3	1	0	0	0	0	2	4	*TP53*	0
10	M	70	3	3	1	0	2	0	0	2	2	*KRAS+PIK3CA*	0
11	M	64	2	3	0	0	2	0	0	2	3	0	0
12	M	79	2	3	0	0	3	1a	0	2	2	*TP53*	0
13	F	59	2	3	0	0	3	0	0	2	1	*KRAS+TP53*	0
14	M	61	3	3	1	0	3	2a	0	2	1	*KRAS+TP53*	x
15	M	62	2	3	0	0	3	0	0	3	2	*TP53/PIK3CA*	0
16	M	53	3	3	1	0	3	1a	0	2	2	*APC*	0
17	M	79	2	3	0	0	2	0	0	1	2	*KRAS*	0
18	M	64	2	3	1	0	3	1a	0	2	2	*TP53*	0
19	M	72	3	2	1	0	2	2b	0	3	2	*BRAF/TP53*	0
20	F	30	3	1	1	0	1	1b	0	2	1	*KRAS/TP53*	x
21	F	64	3	2	1	0	1	0	0	2	2	*KRAS/APC*	0
22	M	60	3	3	1	0	3	0	0	2	2	0	0
23	F	74	3	3	1	0	3	2b	0	2	1	*KRAS*	0
24	M	63	3	3	1	0	1	0	0	2	2	*TP53*	0
25	M	74	3	3	1	0	1	0	0	1	2	*KRAS*	x
26	F	66	2	4	0	0	2	0	0	3	1	*KRAS*	x
27	M	52	3	3	1	0	2	0	0	2	1	*KRAS/APC*	x
28	M	64	2	3	0	0	2	1c	0	2	3	*KRAS*	x
29	M	83	3	3	1	0	3	2a	0	1	2	*KRAS*	x
30	M	74	3	3	2	0	3	0	0	2	1	*KRAS*	0
31	M	62	3	3	2	0	3	2	0	2	1	*TP53*	0
32	F	63	3	3	1	0	0	0	0	3	4	*APC*	0
33	M	58	3	3	1	0	2	0	0	3	2	*KRAS*	0
34	M	58	3	3	0	0	3	0	0	3	2	*KRAS*	0
35	M	63	3	3	1	0	2	1	0	1	1	*KRAS*	0
36	M	62	3	3	1	0	3	0	0	3	2	0	0

ctDNA, circulating tumor DNA; Stage, clinical stage of the disease; TNM staging I, before treatment; TNM staging II, after surgery according to histopathology report; L, tumor location (1, upper rectum; 2, middle rectum; 3, lower rectum); TRG, tumor regression grade according to Dworak (0, no response; 1, minimal response; 2, moderate response; 3, near complete response; 4, complete response).

laboratory (Department of Pathology, Jessenius Medical Faculty of Comenius University in Martin, Slovakia). The details of the multilevel ctDNA testing approach are illustrated in **Figure 1** and further detailed in the *Discussion* section.

Statistical Methods

All statistical analyses were performed using R language for statistical computing and graphics (41). Associations between survival period and other predictors such as the presence of ctDNA prior and during NCRT were analyzed using the Cox proportional hazard model and *t*-tests and plotted using boxplots and Kaplan–Meier curves, respectively. Outputs with *p*-values below 0.05 were assumed to be statistically significant.

RESULTS

The objective response to NCRT is included in patient characteristics listed in **Table 1**. The best response characterized by Dworak TRG score of 3 or 4 was observed in five patients (5/36, 13.9%), whereas 11 patients (11/36, 30.5%) showed none or very limited tumor regression (TRG score 0 or 1). Both results were in a range of typically observed response frequencies as

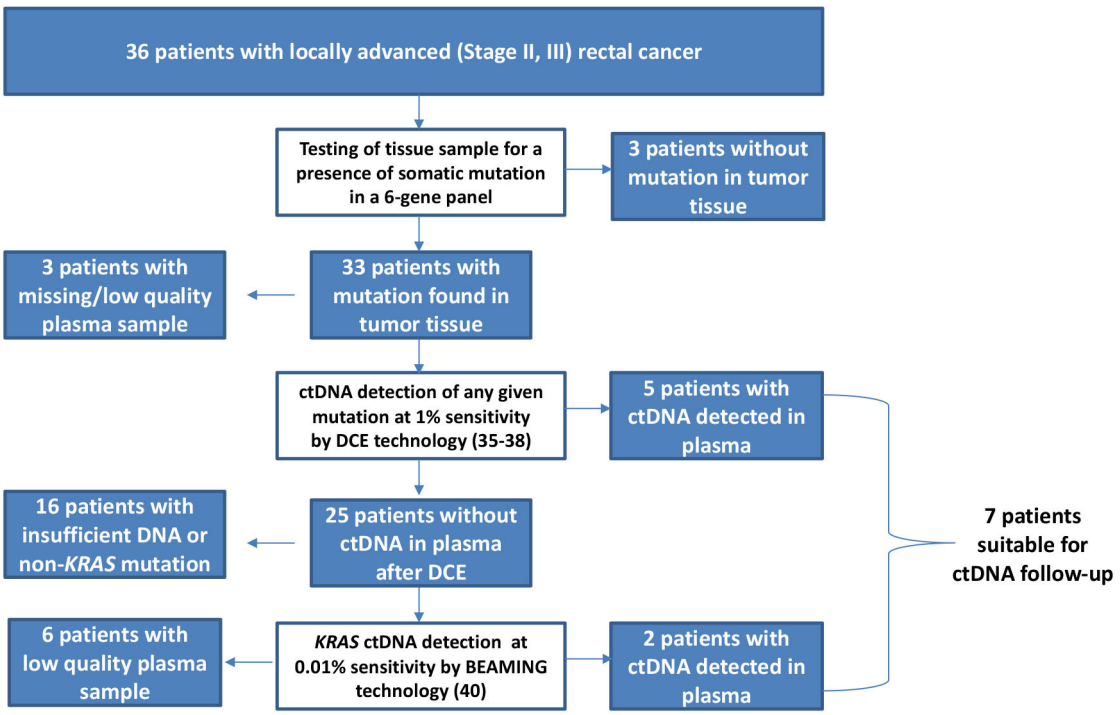

FIGURE 1 | A multilevel cascade testing algorithm for evaluation of circulating tumor DNA (ctDNA) involving standard [denaturing capillary electrophoresis (DCE)] and high-sensitivity (BEAMING*) approaches. *The BEAMING technology was performed using an experimental setting on a set of archived ctDNA samples. This research setting is different from that of protocols applied in regular testing under the IVD-CE certification.

described by others (3, 4). When evaluating the effect of NCRT on the disease stage, some patients exhibited a shift toward the lower TNM (such as that illustrated in **Figures 2**, **3**) mainly owing to the reduction of tumor mass and nodal status.

As the tumor tissue biopsies were evaluated prior to therapy, somatic mutations were detected in samples from 33 out of 36 patients (91.7%) using the six-gene panel. As expected, the mutation testing has revealed the presence of combinations of multiple mutations, mainly with concurrent presence of *KRAS* or *TP53* with another mutation type (shown in **Table 1**). There was no relation between the presence of a specific mutation (or a mutation combination) in the tumor tissue and the ultimate outcome of NCRT evaluated by either TRG or TNM staging.

With the combination of the low-resolution and high-resolution methods, ctDNA was detected in plasma samples of seven patients prior to NCRT (7/33, 21.2%), and it showed a prognostic role. Whereas, the overall probability of a 3-year survival in all patients was 86.7%, the value was 91.2% in ctDNA-negative subgroup and 71.4% in ctDNA-positive subgroup. Hence, as shown using boxplots in **Figure 4**, comparing both groups of patients with positive and negative ctDNA and not considering the time-event dimension and proved by *t*-tests, the ctDNA-positive status prior to NCRT was significantly associated with an shorter disease-free survival (DFS) and a shorter OS by an average of 1.47 and 1.41 years, respectively [$t_{(DFS)} = 2.95$, $df_{(DFS)} = 9.88$, $p_{(DFS)} = 0.015$ (approx.), and $t_{(OS)} = 3.15$, $df_{(OS)} = 10.31$, $p_{(OS)} = 0.010$ (approx.)]. The effect is further documented within, assuming the time-event associations by Kaplan–Meier analysis for DFS and OS (**Figure 5**).

The early dynamics of ctDNA revealed an interesting phenomenon as shown in **Figure 6**. Surprisingly, during the first week of NCRT, ctDNA has been indiscriminately eliminated or significantly reduced from circulation in all patients. Accordingly, there was no association between the change in ctDNA levels (before and during NCRT) and TRG or TNM staging.

DISCUSSION

In this pilot study, we aimed to provide additional evidence of the effect of NCRT in LARC using various predictive biomarkers. There were several reports in the literature investigating the association between the presence of cfDNA as well as ctDNA and the prediction of an NCRT therapeutic response in rectal tumors. Zitt et al. evaluated cfDNA levels in LARC before and after NCRT and following surgical interventions (42). Studied cases were divided into NCRT non-responders and responders. The median level of pretreatment cfDNA was 4.2 ng/ml, after termination of CRT 1 ng/ml, and after surgery 4.1 ng/ml. The authors found that pretreatment levels of cfDNA of non-responders and responders do not significantly differ. At the end of treatment, cfDNA levels were higher in the non-responder cohort. In almost all patients, cfDNA levels substantially decreased toward the end of the NCRT

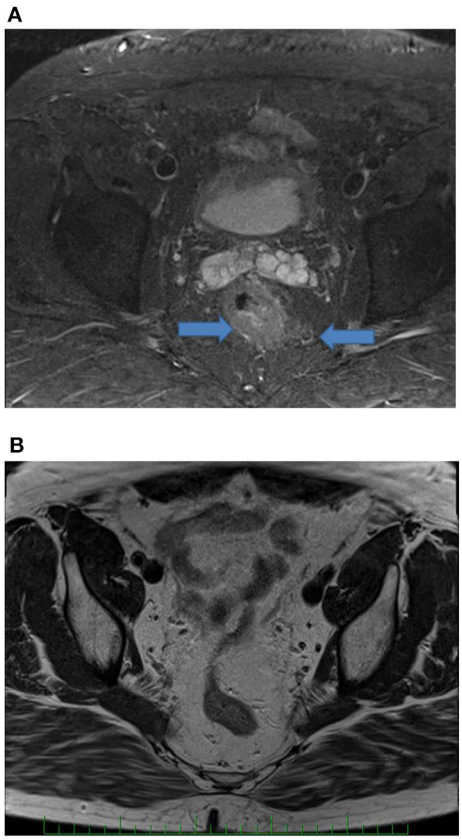

FIGURE 2 | MRI of the rectal tumor (initially T3, N1) before **(A)** and after **(B)** treatment. Arrows show tumor and enlarged lymph node. After treatment, no tumor and lymph nodes are presented (complete clinical tumor response).

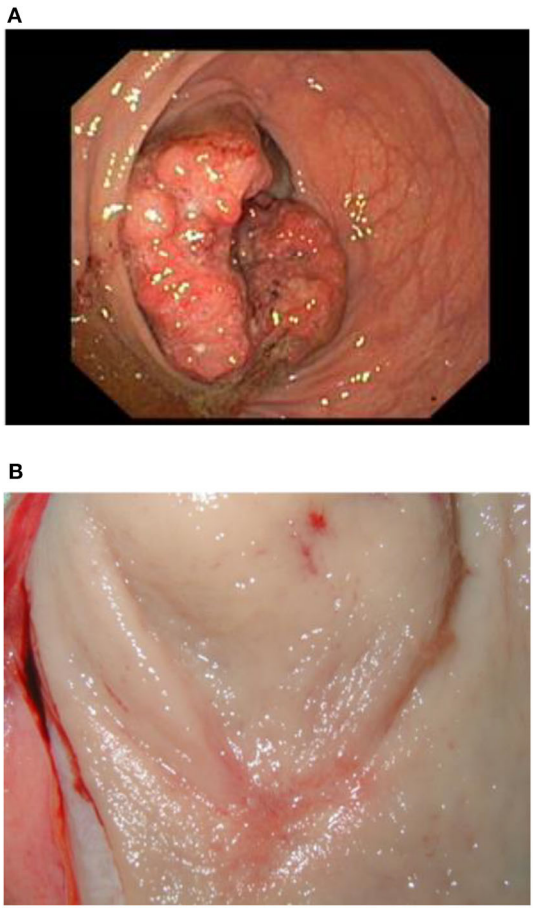

FIGURE 3 | Illustration of a complete therapy response in a patient. Pretreatment endoscopic examination of the tumor **(A)** and surgical specimen **(B)**.

regimen. However, the major limitation of this study is the overall small number of analyzed cases ($n = 26$).

In 2011, Agostini et al. published a set of 67 cases with LARC (43). These clinical investigators measured cfDNA levels before, during, and after NCRT. They determined the total cfDNA concentration and the proportion of long and short DNA fragments, thus establishing a "DNA integrity index." Like Zitt et al., also, these authors did not observe a significant correlation between the pretreatment cfDNA levels and the response to NCRT. However, they provided evidence that NCRT responders had a significantly lower DNA integrity index than non-responders following NCRT.

Sun et al. have verified that cfDNA levels in CRC patients are significantly higher than in healthy subjects (44). Furthermore, they determined the plasma concentration of two DNA fragments (100 and 400 bp) before and after NCRT, and they found that the 400-bp fragment concentration was significantly lower in the responder group after termination of NCRT, thus demonstrating a higher level of fragmentation.

Carpinetti et al. performed whole-genome sequencing of tumor DNA and thus determined specific DNA fragments for each of four patients (19). The authors searched for ctDNA fragments in patients' plasma and demonstrated that in patients with good treatment response, ctDNA levels decreased during NCRT. When ctDNA increased again, it was associated with cancer progression and preceded the rise of CEA and the manifestation of recurrence detected by various imaging approaches. However, this study suffers from a rather small number of analyzed cases. Similarly, Li et al. detected ctDNA levels before and during NCRT. In this study, the prediction of treatment response based on ctDNA positivity before NCRT was 70% (45). On the contrary, Yang et al., in a larger group of patients, did not confirm the association between the pretreatment level of ctDNA and the response to NCRT in patients with RC (46).

In our study, we have hypothesized that early changes in plasma ctDNA reflect the immediate effect of NCRT and thus will be of utility in predicting its therapeutic efficiency. Hence, we expected that upon evaluation of ctDNA plasma dynamics, we will be able to differentiate NCRT non-responders, sparing them from adverse effects of continued/aggressive cancer treatment schemes. Accordingly, we have determined the ctDNA levels prior to NCRT and immediately at the end of the first week of NCRT. In addition, we aimed to find potential correlations with mutations in a panel of six commonly examined genes.

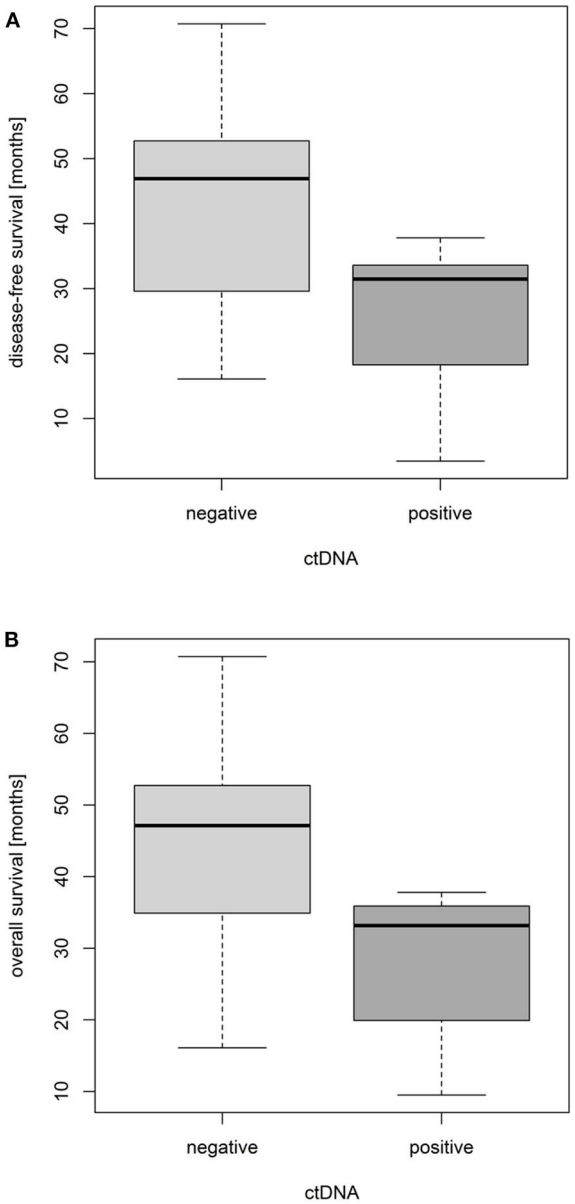

FIGURE 4 | Impact of pretreatment circulating tumor DNA (ctDNA) positivity on disease-free survival **(A)** and overall survival **(B)** of locally advanced rectal cancer (LARC) patients.

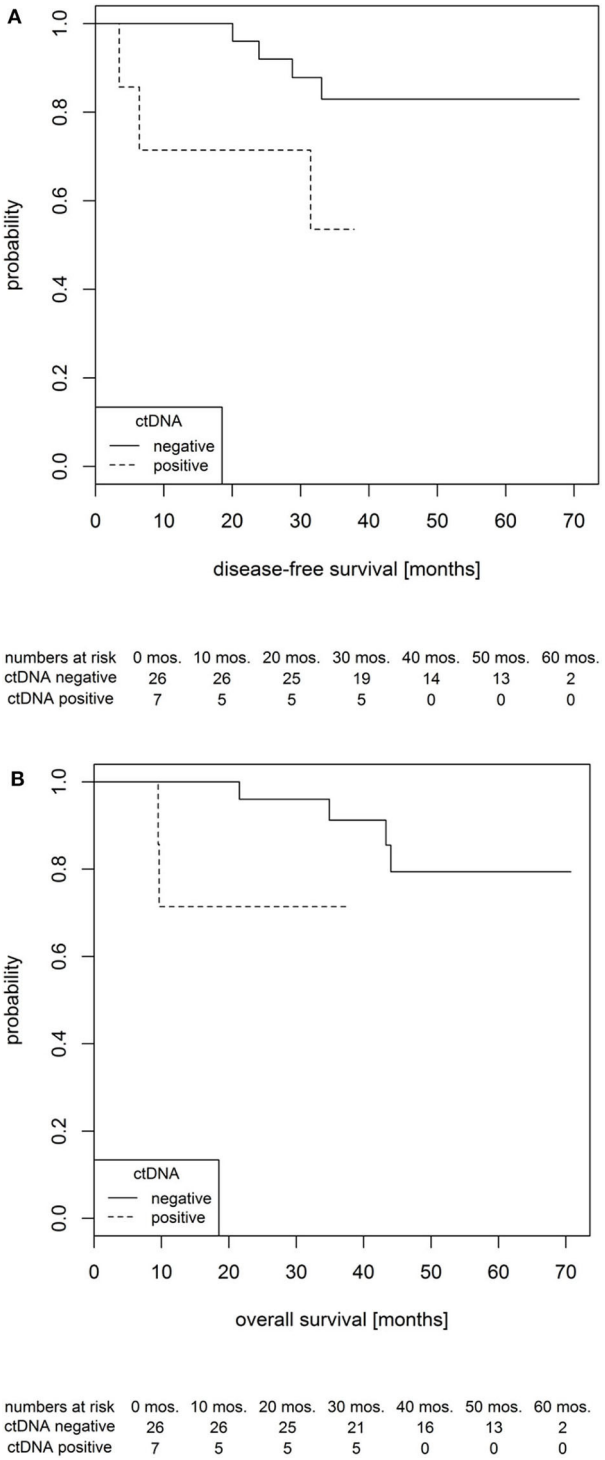

FIGURE 5 | Probability of disease-free survival **(A)** and overall survival **(B)** in locally advanced rectal cancer (LARC) patients according to circulating tumor DNA (ctDNA) status prior to the therapy onset.

A large variation in baseline ctDNA positivity is apparent from previous reports ranging from 15 to 77% in various groups of LARC patients (47). In order to reduce high cost associated with liquid biopsy testing to enable for future cost-effective routine diagnostic approach, we have in this work employed a "cascade" approach (**Figure 1**). In this regard, we have always started evaluation with a relatively very simple and fast singleplex PCR method that required only a relatively small amount of samples (600 μl of plasma). This "Level 1" method, the DCE, was capable of revealing a ctDNA presence at >1% of minor allele fraction (MAF) (39, 47) in just 2 h. In case of a negative result, we performed in "Level 2" a high-resolution BEAMING assay. BEAMING (which stands for beads, emulsion, amplification, and magnetics) utilizes a digital droplet PCR

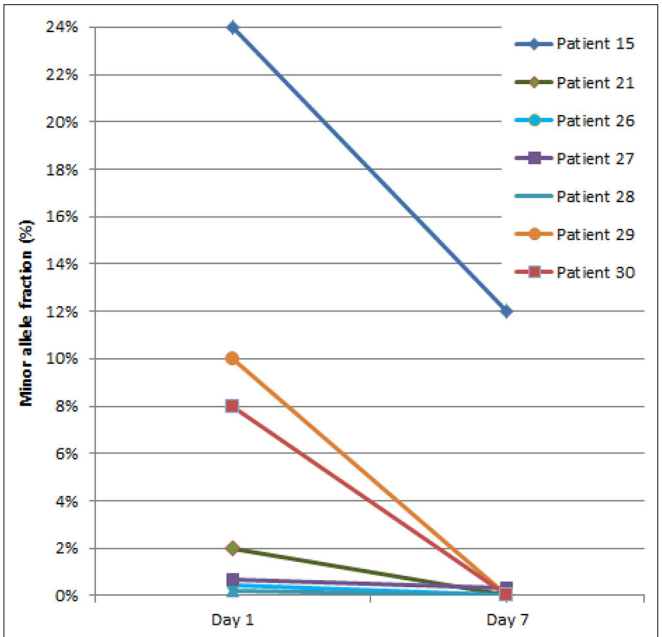

FIGURE 6 | Dynamics of circulating tumor DNA (ctDNA) during the first week of neoadjuvant chemoradiotherapy (NCRT). The ctDNA quantity is presented as a percentage of DNA fragments bearing tumor-specific mutation detected in plasma [denoted as minor allele fraction (MAF)].

resulting in amplified product of individual ctDNA fragments being bound to individual magnetic beads and subsequently detected by flow cytometry. The approach, which is performed using a dedicated instrumentation, exhibits sensitivities down to 0.01% (MAF) on residual amounts of archived ctDNA that were left after DCE Level 1 testing. In this study, only patients exhibiting tumors with *KRAS* mutation could be subjected to Level 2 testing, owing to the specificity of the BEAMING technology. Level 1 (DCE) testing revealed ctDNA positivity in five patients (5/30, 16.7%), and Level 2 (BEAMING) revealed ctDNA positivity in two more patients (2/4, 50%). The overall yield from this multilevel testing approach was 23.3% (7/30), mainly due to the limitation of BEAMING testing directed at *KRAS* ctDNA mutations only. Indeed, other high-sensitivity alternative techniques would improve this; nonetheless, the obtained frequency is comparable with previously observed results for stages II and III in rectal cancer (29).

Contrary to our expectations, we have not observed any predictive correlation between the baseline ctDNA levels and the actual outcome of NCRT in terms of TRG or TNM staging. Yet when the prognostic effect was evaluated, patients showing baseline ctDNA positivity have exhibited a shorter progression-free survival and OS (**Figure 6**). This is in agreement with previous work by Tie et al., who have reported ctDNA as a negative prognostic factor for the overall patient survival (48). For most ctDNA-positive patients, imaging has, indeed, subsequently revealed a presence of previously unrecognized micrometastatic sites. The ctDNA positivity should therefore be considered to guide therapy-related decisions following surgical treatment as similarly applied in breast (49) or colorectal cancer (50, 51).

Intriguingly, in all patients, we have observed a strong reduction or complete elimination of ctDNA at the end of the first NCRT week. Counterintuitively, ctDNA levels were reduced regardless of the eventual clinical outcome. Apparently, this unequivocal rapid ctDNA clearance following the therapy dose suggests presence of a more general phenomenon not related to the actual patient characteristics or specific tumor biology. The ctDNA removal from plasma is primarily a result of enzymatic digestion (34, 52). It has been reported recently that some DNA exonucleases active in DNA repair are released as a result of radiation damage (53). It can only be speculated that such a radiation-induced activity of exonucleases could result in a temporal effect of ctDNA clearance following the administration of NCRT. In order to elucidate the aforementioned phenomena, ctDNA monitoring should be performed at even shorter time intervals. Although most papers describing the use of ctDNA in palliative chemotherapy (54, 55) and, more recently, immunotherapy (56) apply monitoring with initial sampling at day 7 or later, it may well be necessary to perform examination at even shorter intervals of days or hours from the therapy start, respectively. Recently, a similar approach directed at the evaluation of ctDNA in urine has recently been recently applied for monitoring of daily dynamics of tumor response to targeted anticancer therapy in non-small-cell lung cancer (NSCLC) (57). When performed during the initial phase of the NCRT, possibly within hours from receiving the first radiation fraction together with chemotherapy and continuing for the next several days, such approach could substantiate ctDNA dynamics underlying eventual transient changes in tumor morphology and its damage, including subsequent ctDNA uptake resulting from the administered multimodal therapy. Thus, understanding of the detailed timing of ctDNA release and clearance may be essential for the long-awaited applicability of the ctDNA-based therapy outcome prediction for NCRT treatment of LARC patients (58) and beyond.

CONCLUSIONS

In the present work, we have demonstrated the utility of monitoring of early changes in ctDNA levels in patients with LARC undergoing NCRT prior to surgical treatment. By applying a multilevel ctDNA detection approach, we were able to monitor ctDNA dynamics in seven patients receiving NCRT. We have evaluated the previously reported preoperative presence of ctDNA as a negative prognostic factor, which may be useful in direction of patients for adjuvant therapy following surgery.

We have observed a clear reduction of ctDNA levels in all patients during the initial week of NCRT, but without any direct association to the objective clinical response evaluated by TRG or TNM. As a consequence, we could not predict the response to preoperative NCRT in LARC on the basis of the ctDNA levels. Although such observations might exclude the use of early ctDNA changes as a predictive biomarker of NCRT outcome, our findings may open new research avenues on the mechanisms of

ctDNA release and clearance upon cellular damage due to the combined effects of chemoradiation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee Motol University Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

FP: designed study, organized and followed up the patient cohort, collected samples, and prepared and drafted manuscript. MMi: designed study, and prepared and drafted manuscript. TH and BB: processed and analyzed samples. MMa: prepared a revised manuscript. LS: performed a statistical analysis. LB: managed molecular testing of patients and revised manuscript. JH: designed study and overseeing clinical part of project. All authors contributed to the article and approved the submitted version.

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