bio-techne® White Paper

Harnessing the Power of Exosomes to Improve Sensitivity for Cancer Mutation Detection

Introduction

Liquid biopsies are non-invasive mechanisms to allow for the characterization of disease from a freely accessible biofluid, such as blood or urine. With respect to solid tumors, liquid biopsies are not necessarily a replacement for conventional tissue-based profiling, but rather serve as a complementary tool for the continuous molecular profiling of cancer and the optimization of cancer treatment. One significant advantage of liquid biopsies is their ability to be collected often without inconveniencing or harming the patient; samples can be collected from the point of diagnosis and throughout the course of disease to assess overall prognosis, response to therapy, and likelihood of relapse or progression. In addition, they are less costly to perform than a tissue biopsy.[1] While liquid biopsies are not yet widely considered the gold standard for molecular profiling over conventional tissue biopsies, they are making inroads into mainstream diagnostics via a series of landmark FDA approvals. Therefore, it is essential for liquid biopsybased tests to be clinically validated, and their specificity and sensitivity determined and compared with the gold standard to support the clinical utility of these tests moving forward.

Despite considerable advances in molecular profiling, there remains a high unmet medical need for early cancer detection. Liquid biopsies are a rich source of biomarkers, such as circulating tumor cells (CTC), cell free DNA (cfDNA) and extracellular vesicles containing exosomal RNA (exoRNA).[2] Cancer patients' biofluids contain circulating tumor DNA (ctDNA), which is shed from tumor cells undergoing necrosis, apoptosis or other cell cycle regulatory mechanisms. By interrogating these analytes, many clinically actionable biomarkers such as B-Raf proto-oncogene serine/threonine kinase (BRAF), epidermal growth factor receptor (*EGFR*) and Kirsten rat sarcoma virus (KRAS) can be detected without the need for surgical tissue resection.[3]

These markers may indicate the emergence of a therapy-sensitive clone; resistance to existing treatment; or inform overall disease prognosis. The analysis of cfDNA presents new opportunities to analyze low-expression biomarkers due to the high signal-to-noise ratio; to detect cancer early; to distinguish cancer from benign conditions; and to identify fast- and slow-growing cancers.[4]

ExoRNA contains a mixture of coding and non-coding RNA that regulates intracellular processes. It has also proven to be a useful source of disease biomarkers and novel targets for cancer therapies. ExoRNA can also be used to detect tumor-derived mutations from biofluids.[5, 6, 10, 13, 19] Tumor-derived mutations can be found on both the exoRNA and cfDNA in biofluids, so combining the exoRNA and cfDNA can achieve more copies and higher sensitivity for the specific mutation. The combined exoRNA and cfDNA is hereafter referred to as exoNA (exogenous nucleic acid). Exosomes affect the initiation and progression of cancers and act as a communicator among cells through the transport of RNAs, proteins and other molecules. Several studies have shown that the exoRNA changes during tumor invasion, metastasis and progression, and exoRNA is important in the occurrence and development of drug resistance. Unlike cfDNA, exoRNA is derived from living cells and is released as an active process from living tumor cells. In contrast, cfDNA is primarily released by cells that die through apoptosis or necrosis. Active release of this exoRNA during the early stage of cancer development can potentially enable earlier detection.[6] Preliminary research suggests that screening patients for exoRNA and cfDNA is superior to either cfDNA or exoRNA alone. This white paper summarizes the evidence from four peer-reviewed articles supporting the combination of exoRNA and cfDNA in cancer diagnosis and treatment.

Key Findings

- Blood-based liquid biopsies are making inroads in cancer management and are increasingly used in conjunction with tissue biopsies. Plasma cfDNA, ctDNA and exoRNA can be used to molecularly profile cancer patients and guide downstream treatment decisions.
- Krug and colleagues (2018) demonstrated that data from both exoRNA and ctDNA can increase the level of sensitivity of mutation detection in NSCLC patients, particularly in more localized disease (M0/M1a), where cfDNA alone is less abundant.
- Möhrmann and colleagues (2018) showed that common mutations – BRAF, KRAS and *EGFR* – can be detected in plasma exoRNA and cfDNA with a high degree of sensitivity; plasma exoRNA is an independent, prognostic biomarker of patient survival.
- Castellanos-Rizaldos and colleagues (2018) have developed a method for single-step preparation for plasma exoRNA/DNA and cfDNA. Followed by qPCR, it can determine the *EGFR* T790M mutation status in the plasma of NSCLC patients. In addition, combining exoRNA/DNA and cfDNA in a single PCR diagnostic test provides higher sensitivity and specificity than cfDNA alone, and may result in more patients being eligible for the optimal treatment.
- Castellanos-Rizaldos and colleagues (2019) have developed a qPCRbased test, ExoDx *EGFR* assay, that can detect *EGFR* mutations (e.g., *EGFR* L858R, T790M and exon 19 indels) in plasma exoRNA/DNA and cfDNA. The qPCR test provides higher sensitivity and specificity than cfDNA alone, and can detect patients in early-stage disease.
- Overall, data from four peer-reviewed articles indicate that plasma exoRNA/DNA combined with plasma cfDNA can be tested in a single qPCR test or through next generation sequencing panels – and this can increase the potential to detect patients with cancer, particularly those with early-stage disease. The ExoDx *EGFR* assay offers high sensitivity and specificity for *EGFR* mutations; and a low risk of false positives compared to cfDNA testing alone.
- The analysis of plasma exoRNA in combination with plasma cfDNA has potentially important implications in the molecular diagnosis of various conditions beyond cancer, including immunoinflammatory, musculoskeletal and neurodegenerative diseases.

Advances in Blood-Based Liquid Biopsies Testing

Analysis of cfDNA can be assessed using a wide range of analytical platforms, including BEAMing (beads, emulsion, amplification, magnetics) PCR; digital polymerase chain reaction (dPCR); qPCR; and next generation sequencing (NGS). BEAMing and dPCR are generally more sensitive for individual mutation targets than NGS and have been shown to detect somatic alterations from minute cfDNA samples with mutant allele fractions (MAF) of less than 0.1%, dependent on the specific sequence and assay optimization.[7,8] Despite this high level of sensitivity, cfDNA is susceptible to clonal hematopoiesis, and cancer patients are heterogenous in cfDNA expression.[9] Moreover, for early stages of cancer detection, the number of copies of the mutated cfDNA can be very low, and even the most sensitive assay cannot detect the mutation if there is no detectable copy in the sample. By contrast, qPCR, dPCR or NGS of exoNA offers greater sensitivity because of the increased copy numbers of available mutations when combining the mutations on exoRNA and cfDNA. This can enable the detection of early- or localized-disease stages where the use of cfDNA is limited.[10, 11]

Case Study: Combining Plasma exoRNA Plus cfDNA Increases Mutation Detection in Early-Stage Cancer Patients

Krug and colleagues (2018) investigated whether combining plasma exoRNA and cfDNA could improve *EGFR* mutation detection in NSCLC patients. The study analyzed 84 tumor samples from NSCLC patients enrolled in the TIGER-X Phase I/II trial, and discovered that the combination of exoRNA and cfDNA not only increased the level of sensitivity for *EGFR* mutation detection, but also enabled the identification of patients with intrathoracic disease (M0/M1a), which is known to release fewer copies of tumor mutations via cfDNA (Table 1).

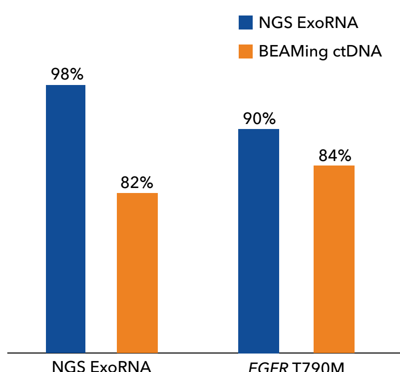
Table 1: Combining exoRNA and ctDNA increased sensitivity for detecting *EGFR* Exon 19 deletion and T790M mutations in the plasma of patients with intrathoracic metastatic disease (M0/M1a)

Genetic mutations tested (n=21)	Sensitivity ctDNA	Sensitivity exoNA	P value
EGFR Exon 19 deletion	26% (5/19)	74% (14/19)	0.0003
EGFR T790M	19% (3/16)	31% (5/16)	0.5

Krug *et al.* 2018[10]

Furthermore, this combined approach demonstrated that analysis of plasma exoRNA and ctDNA helps to boost assay sensitivity in technologies which otherwise may not be as analytically sensitive. In this study, exoRNA and ctDNA analyzed by NGS was determined to be more sensitive for both *EGFR* Exon 19 deletion and *EGFR* T790M mutations against ctDNA alone analyzed by the highly sensitive BEAMing approach (Figure 1).

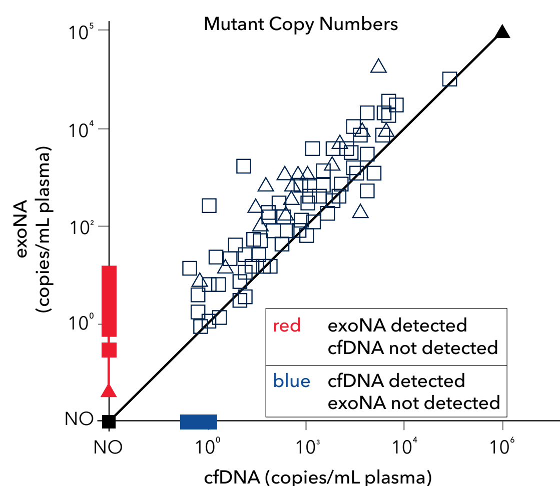
Figure 1: Sensitivity of exoRNA ctDNA testing of *EGFR* and *EGFR* T790M mutations



Krug *et al.* 2018[10]

Lastly, by analyzing both exoRNA and cfDNA, the number of mutant copies of target mutations is greatly increased, enhancing the assay's ability to detect rare variants. In this particular study, the number of copies of *EGFR* activating mutations was increased nearly 10-fold over what was detected in cfDNA alone

Figure 2. Mutant Copy Numbers Detected by exoRNA+cfDNA vs cfDNA Alone



Krug *et al.* 2018[10]

Case Study: Combination of exoRNA/DNA and cfDNA Results in Higher Sensitivity and Specificity Than Using cfDNA Alone for Activating *EGFR* Mutations

The PCR amplicon used for the exoNA T790M assay was unusually large – larger than the activating *EGFR* mutations used to generate the results in Table 1. We postulated that we could increase the sensitivity further by making the footprint of the *EGFR* T790M assay smaller. To achieve this, Castellanos-Rizaldos and colleagues (2018) developed a qPCR assay with a smaller amplicon size. This very sensitive allelespecific quantitative PCR (qPCR) was then used to determine the *EGFR* T790M mutation status in the NSCLC patients (n=110) on exoNA material that was isolated through a single step process.13 The qPCR assay achieved high sensitivity (92%) and specificity (89%) compared to tissue biopsy and also demonstrated high sensitivity in samples derived from intrathoracic disease (M0/M1a) patients.

Liquid biopsies can provide a comprehensive view of the disease, whereas tissue-based profiling is highly dependent on the tumor sample excised. Thus, if cells expressing a particular target of interest are not present, they will not be detected via a tissue test. Conversely, the liquid biopsy provides a holistic picture of the disease and can help overcome the challenge of tumor heterogeneity.

Combining exoRNA/DNA and cfDNA in a single PCR diagnostic test provides higher sensitivity and specificity than cfDNA alone, helping avoid unnecessary tissue biopsies.

Case Study: A Single qPCR-based Test can be Used to Interrogate Mutations within *EGFR* Using exoRNA/DNA and cfDNA Derived from Patient Plasma with NCSLC

A later study by Castellanos-Rizaldos and colleagues (2019) showed that the *EGFR* T790M assay could be successfully multiplexed with the activating *EGFR* mutations in an assay that looks for 29 mutations in the *EGFR* gene (e.g., T790M, L858R and exon 19 indels) in plasma exoRNA/DNA and cfDNA from NSCLC patients (Table 2). The ExoDx *EGFR* assay provides higher sensitivity and specificity than using cfDNA alone, and demonstrated high performance among patient samples.

Table 2: ExoDx *EGFR* assay sensitivity and specificity in NSCLC plasma samples

Genetic Mutations Tested	Assay Sensitivity	Assay Specificity
All patients (n=110)		
***EGFR* L858R**	90%	100%
***EGFR* T790M**	83%	100%
***EGFR* exon 19 indels**	73%	96%
Subcohort of patients with extrathoracic disease M1b/MX (n=33)		
***EGFR* L858R**	92%	100%
***EGFR* T790M**	95%	100%
***EGFR* exon 19 indels**	86%	94%

Castellanos-Rizaldos. 2019[9]

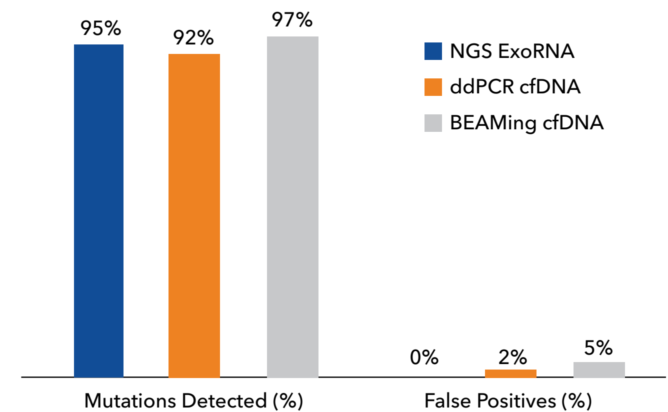
Combining exoRNA/DNA and cfDNA in a single PCR diagnostic test provides higher sensitivity and specificity than cfDNA alone. This enables the detection of biomarkers at exceedingly low levels – even in patients with local/regional disease – and can overcome the challenge of tumor heterogeneity, which may increase the rate of false positives for tissue-based assays.

Case Study: Testing for Plasma exoRNA as Sensitive as cfDNA in Detecting Mutations in Advanced Cancer Patients

In 2018, Möhrmann and colleagues published a study analyzing archival tumor tissue from 43 patients with progressing advanced cancer. Of these, 41 expressed BRAF, KRAS or *EGFR* mutations detected by NGS plasma exoRNA, ddPCR and BEAMing PCR of plasma cfDNA (Figure 3). Importantly, NGS of exoRNA did not detect any mutations not present in the tumor such as false positives (Figure 3).

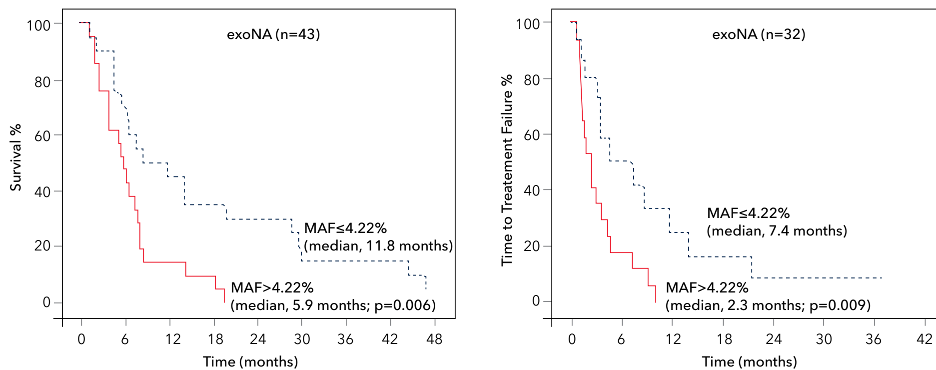
Based on clinical outcomes data, patients with high exoRNA MAF had shorter median survival and time to treatment failure compared to those with low MAF (Figure 4). Low expression of exoRNA was associated with partial response and stable disease ≥6 months (P = 0.006).

Figure 3: Percentage mutations detected in tumor tissue samples by NGS exoRNA, ddPCR and BEAMing cfDNA analysis



Möhrmann *et al.* 2018[5,7]

Figure 4: Correlation of exoRNA MAF versus clinical outcomes



A. Overall survival (OS) per mutation allelic frequency (MAF) of KRAS, BRAF or *EGFR* mutations in plasma. Twenty-one patients with a low MAF (≤median; blue dashed line) in plasma exoRNA had a significantly longer median OS duration than 22 patients with a high MAF (>median; red line; 11.8 vs. 5.9 months; P=0.006).

B. Time to treatment failure (TTF) per pre-treatment mutation allelic frequency (MAF) of KRAS, BRAF or *EGFR* mutations in plasma. Fifteen patients with a low MAF (≤median; blue dashed line) in plasma exoNA had a significantly longer median TTF than 17 patients with a high MAF (>median; red line; 7.4 vs. 2.3 months; P=0.009)

Möhrmann *et al.* 2018[7]

These data suggest that clinical testing using NGS analysis of plasma exoRNA for BRAF, KRAS and *EGFR* mutations using exoNA correlate well to treatment response, and low exoNA MAF may be an independent prognostic factor of patient survival.

Future Applications of exoRNA and exoNA

Liquid biopsies have shown great promise in the clinical management of cancer patients. However, the analysis of plasma ctDNA to assess mutation status alone may be limited by stability and amount of tumor DNA available, especially in early-stage disease. Conversely, exosomes are packages of RNA and DNA that can be isolated from biofluids and tissues to provide additional information on altered cellular or tissue states.[14]

Preliminary data suggest that analyzing plasma exoRNA in combination with plasma cfDNA to assess mutation status is superior to either cfDNA or exoRNA alone across multiple tumor types. This may also facilitate earlier detection of cancer and be used to monitor the development of treatment resistance mutation in the longitudinal setting.

The analysis of exoRNA in combination with cfDNA has important implications beyond cancer and has a potential role to play in the molecular diagnosis of various conditions, including immunoinflammatory, musculoskeletal and neurodegenerative diseases.[4, 15]

The development of CLIA-validated qPCR tests from BioTechne provides the biopharmaceutical industry with a cost-effective, sensitive and specific method of identifying genetic mutations. This holds true in a wide array of samples for a broad range of diseases in both the preclinical and clinical setting.

For instance, the ExoDX™ Prostate Test is a clinically validated, noninvasive, urine-based liquid biopsy test that predicts the presence of high-grade (Gleason score ≥7) prostate cancer for men 50 years of age and older with a prostate-specific antigen (PSA) 2 - 10 ng/mL presenting for a biopsy.[16] The test can help urologists identify patients who have a low versus high risk of clinically significant disease, independent of PSA testing and other standard-of-care factors.[17]

In the clinical setting, the diagnostic test ExoTRU™ (Exosome Transplant Rejection Urine) is a non-invasive, multigene urine-based exoRNA assay designed to support the management and care of kidney transplant patients. Bio-Techne developed the ExoTRU™ test in collaboration with Azzi Laboratory at the Transplantation Research Center at Brigham and Women's Hospital, Harvard Medical School, and it is capable of discriminating between types of kidney rejection. This provides critical information to assist clinicians' decision-making and to optimize patient care.[18]

Abbreviations

BEAMing PCR	Beads, emulsion, amplification, magnetics polymerase chain reaction
BRAF	Raf proto-oncogene serine/threonine kinase
CLIA	Clinical laboratory improvement amendments
CRC	Colorectal cancer
ctDNA	Cell-free deoxyribonucleic acid
ddPCR	Droplet digital polymerase chain reaction
EGFR	Epidermal growth factor
exoRNA	Exosomal ribonucleic acid
KRAS	V-Ki-Ras2 Kirsten rat sarcoma virus
MAF	Mutant allele fraction
NSCLC	Non-small cell lung cancer
NGS	Next-generation sequencing
PSA	Prostate-specific antigen
qPCR	Quantitative polymerase chain reaction

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