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Minimal Residual Disease in Solid Tumors: Shifting the Focus from Cell-free DNA to Cell-Free RNA

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Abstract

In the fight against cancer, actionable information is one of our most important weapons. On an individual basis, to predict a patient's prognosis, their response to prior therapies and regular surveillance empowers them, together with their doctor, to optimize treatment and forge a secure path forward. This is especially crucial today where new therapies are needed and many types of cancer can take a long and unpredictable course. Recently, extensive interest has grown for a measure known as minimal residual disease (MRD) status that is emerging as a key surrogate biomarker for monitoring disease recurrence. Residual cancerous cells following treatment, known as MRD, can linger in tiny numbers that evade detection by traditional tests and slowly regrow and are responsible for the relapses seen in many types of blood cancer. It's only until recently that technological advances have made MRD tests a reality for solid tumors. Identifying the presence or absence of residual tumor cells (i.e., MRD-positive or -negative) with blood-based testing can provide a gauge of the extent of a person's response to treatment as well as the risk of relapse more accurate and earlier than traditional means. Ultimately, MRD information obtained via circulating cell-free DNA and/or RNA can guide better treatment decisions and expedite the development of new medicines.

Keywords: Minimal Residual Disease; Solid Tumor; Cell-free RNA; Biomarker

Current Mrd Status

Cancer is a worldwide epidemic with 18 million people currently affected in the US alone. Each year in the United States, more than 1.9 million people are diagnosed with cancer, and nearly 610,000 die from it [1]. The annual global tally of new cases is expected to grow, fueled largely by aging populations and risk factors like smoking, poor diet, sedentary lifestyles and excessive body weight. Improved outcomes in cancer management are becoming a reality with advancements in both personalized genomic profiling and targeted therapy. Still, there is a growing unmet medical demand to detect the small number of cancer cells (by measuring tumor-specific genetic alterations) remaining after curative treatment and post-adjuvant therapy-called minimal residual disease (MRD)-to predict risk of relapse, monitor and respond earlier in cases of recurrence. Unfortunately, even with today's gold standard approaches, primarily imaging techniques, can't accurately measure MRD; instead, highly sensitive molecular tests targeting specific mutations are used to detect MRD, and numerous studies have demonstrated that these genetic tests can provide vital insight into patients' treatment trajectories, accelerating their use in the clinic.

The current diagnostic workup is when a patient is suspected of having cancer, they must first undergo diagnostic procedures to image and molecularly characterize the tumor, followed by neoadjuvant therapy to reduce tumor size prior to a curative treatment such as tumor resection, radiotherapy and/or chemotherapy. Subsequently, longitudinal MRD measurements are taken after curative treatments to better assess a patient's response, prognosis and recurrence risk [2,3]. Overall, MRD testing, initially for hematologic malignancies, could also improve outcomes of solid tumor therapy by enabling clinicians to deliver faster and earlier personalized care via a simple blood draw.

For solid tumors, the existing imaging surveillance and tissue biopsy tests have several drawbacks. First, imaging is a low-sensitivity and low-resolution technique, only visible tumors can be detected, MRD information can't be captured; Second, these procedures are invasive, time-consuming and expensive. Most significantly, repetitive imaging and tumor sampling are not practical. In contrast, liquid biopsy offers a golden opportunity for MRD detection. High-sensitivity, real-time and longitudinal liquid biopsy can capture MRD information that would have been invisible by imaging, it can provide actionable and dynamic genetic profile much earlier. It also reduces the test turnaround time, cost and complications associated with invasive tumor biopsy. One key form of liquid biopsy is circulating cell-free DNA (cfDNA) in blood where tumor-specific genetic biomarkers reside. Circulating cfDNA testing will become a major driver of the MRD market moving forward [4-6]. More importantly, improved targeted treatments are increasingly successful for solid tumors, leading to a larger number of surviving patients who need to be monitored long-term for relapse via MRD testing [7]. As a result, a rising demand for cfDNA-based MRD testing is warranted.

It is imperative to monitor MRD in cancers with well-established genetic biomarkers, for example, KRAS for colorectal, lung and pancreatic cancer, EGFR in metastatic lung cancer, BRAF and NRAS for metastatic melanoma, and BCR-ABL1 in chronic myeloid leukemia (CML) (8-10). However, each patient's disease has a unique mutational profile, the MRD testing approach can vary accordingly. The current standard of care is to comprehensively analyze the patient's tumor first by next-generation sequencing (NGS) to establish the baseline or reference mutational profile. Tumor-informed biomarkers are then selected from the NGS data to become the targets of personalized cfDNA-based MRD tests. The wealth of data recently generated highlighted that MRD–negative status can be achieved in a large proportion of patients. There is an evolving consensus that achieving MRD–negative status should become the ultimate goal of therapeutic intervention. Future efforts should now be directed at determining how MRD status can be used to guide and personalize further therapy including type of consolidation and maintenance therapy.

Current Blood-Based Mrd Technologies

As the use of MRD evolves, it's time to standardize testing. While conversations are still going, many doctors and regulatory authorities have noted that MRD tests should be developed to be highly sensitive, broadly applicable, accurate, reliable, fast, and affordable before they can become widely adopted. MRD testing must be optimized and validated (both analytically and clinically) for each specific cancer. Technologies such as quantitative real-time PCR (qPCR), digital PCR (dPCR), multiparameter flow cytometry (MFC) or NGS have been employed to evaluate their utility in MRD assessment (Table 1) [11].

Table 1: Current blood-based MRD technologies

	qPCR/dPCR	MFC	NGS Sequencing
Need for Baseline Sample	Yes	No	Yes
Sample Requirements	Cell-free portion of blood (Plasma)	Require > 5 million cells	Cell-free portion of blood (Plasma)
Sample Processing	Cell-free DNA or RNA extraction	Needs assessment within 24-48 hours (requires a fresh sample)	Cell-free DNA or RNA extraction
Sensitivity	0.01 - 0.001%	≥ 1 in 100,000 cells	0.5% for cfDNA MAF; 0.0001% for cfRNA transcript expression
Turnaround and Complexity	Requires development of cancer type-specific biomarkers; may take 3-5 days	Labor intensive with long hand-on time; may take 1-2 days	Labor intensive; requires robust bioinformatics support; may take 2-3 weeks
Clonal Consideration	Detects major clones	Considers all clones with similar phenotype	Covers major and minor clones
Cost per Sample	Low	low	High

The prototype of MRD assay is the quantification of BCR-ABL1 fusion transcripts by RT-qPCR to monitor the treatment response with TKI in CML. Detection of low levels of BCR-ABL1 fusion transcripts to monitor MRD has been performed quickly, efficiently, and at a reasonable cost. The National Comprehensive Cancer Network (NCCN) have recommended monitoring BCR-ABL1 mRNA levels by RT-qPCR for molecular response in CML [12]. The International Randomized Study of Interferon versus STI571 (IRIS) also proposed that log reduction of BCR-ABL1^{IS} (IS ratio) during therapy, compared with baseline IS ratio at diagnosis (BCR-ABL1^{IS}, 100%), should be evaluated to monitor MRD. Clinical data have demonstrated that the RT-qPCR method is sufficiently sensitive to detect MRD and recurrence. The main advantages of this assay lie in the promptness with which results are obtained and its ease of use.

This technology is capable of identifying chimeric fusion, gene rearrangements, genetic alterations, and differentially expressed genes. Being the gold standard platform in many laboratories, RT-qPCR-based MRD test thus could be advantageous for broad and quick implementation.

Digital PCR (dPCR) is the latest generation of PCR technology. A single qPCR reaction is partitioned into hundreds to millions of droplets or wells and each containing a single or few copies of the target template. The partitioned reaction undergoes thermocycling with each of these partitions constituting an individual qPCR reaction during the cycling process. Fluorescent signal is measured after amplification for each partition individually, resulting in millions of data points. Therefore, the number of positive molecules is determined by counting the number of successfully amplified fluorescent partitions. In addition, through absolute counting, the technique obviates the requirement for copy number standards as is required for qPCR. This technique has demonstrated promising results in the monitoring of MRD in hematological malignancies using both RNA-based and DNA-based methods. The sensitivity of dPCR is comparable to qPCR and has demonstrated special promise for detecting single nucleotide variations (SNVs) due to the greater capability to differentiate the mutant versus normal allele in the absence of competing normal allele in each partition. [13,14].

Multiparameter flow cytometry (MFC) is a cell-based immunophenotyping assay. This technique is based on antigen expression patterns that characterize the diverse lineages of normal hematopoietic cells, for example, Acute myeloid leukemia (AML) blasts have aberrant antigen expression patterns that are not detectable on the surface of bone marrow cells from healthy donors. These distinct immunophenotypic patterns are present in 80% to 100% of AML patients. The prognostic significance of MRD detection using MFC has been examined in numerous studies performed in adults and children (13). Overall, these studies demonstrated that MFC-detected MRD is prognostically significant in virtually all AML patients over a wide array of patient populations and treatment strategies. However, there have also been several inconsistencies due to variation in methodology, thresholds of positivity, patient population and treatment strategies. Nevertheless, guidelines regarding the advantages and limitations of MRD measurement by MFC can be established [15].

NGS is the latest generation of sequencing technology upgraded from Sanger sequencing. The use of single target assays for molecular MRD detection remains dominant in the field. However, given well-known patterns of clonal evolution evident in cancers and the need for highly personalized assays in patients with no recurrent or common lesions, there is a need for economical alternatives that enable more patients to be tested for MRD in a manner that is robust to clonal evolution (molecular MRD) and/or phenotypic switching (flow cytometry MRD). Going forward, NGS represents a potentially powerful alternative. Owing to the heterogeneity of the mutation repertoire in AML and the lack of hot spots in important but frequently mutated genes, it seems unfeasible to develop standardized NGS assays on a per-patient basis. In addition, when tracking with a multigene panel, it has been observed that some cancer patients will relapse with different mutant clones with distinct mutation landscapes, and in these cases, the marker being monitored is not informative to predict relapse. With NGS platform, there is no need for patient-specific assays as practically all mutations are detected. One caveat of NGS is that has limited its use for MRD assessment in the past few years, is the sequencing error rate and its impact on the sensitivity of the technique compared to the previously discussed methods. The recent introduction of unique molecular index and error-corrected read technologies and has helped overcome this limitation and greatly improving its sensitivity. However, even the advantages of error correction are likely to be compromised in certain gene regions by mappability limitations and errors arising from potential factors including gene paralogs. Further improvements in read length and read mapping algorithms coupled with statistically principled variant calling techniques are likely to extend the sensitivity, specificity, and thus overall utility of NGS in MRD monitoring. Overall, ultra-deep NGS-based methods provide the ability to detect new emerging therapy-related mutations that would otherwise be missed, opening the possibility to measure MRD in large patient cohorts [16,17]. While none of these tests fully satisfy all of the ideal characteristics currently, qPCR/dPCR and NGS fulfill most of them and can be translated into an advanced platform that can be uniformly applied. Inclusion of both methodologies should be done in prospective trials to collect data that would allow the better understanding of the advantages and disadvantages of the individual approaches as well as the limit of detection required in various clinical settings.

In the era of personalized and precision therapies achieving very high conventional complete remission rates, the MRD status is beginning to play an important role in predicting molecular response, recurrence and clinical outcome. MRD may serve as a biomarker to inform therapy, assess relapse risk and as a surrogate for overall survival. Specifically, achieving MRD-negative status may become a goal of future studies using induction, transplant, consolidation, and/or maintenance therapies. With available technologies and ease of MRD measurement, additional large prospective studies should be performed to define the clinical significance of MRD and its impact on patient outcome. In our quest toward personalizing therapy for patients, it may now be possible to both assess and monitor MRD using standardized assays and decide both intensity and length of therapy for individual patients to improve patient outcomes [18].

Circulating Cell-Free Rna Biomarkers for Mrd in Solid Tumors

Several limitations of cfDNA-based liquid biopsy have been addressed over the last few years. Complications include low concentration, highly fragmented, short half-life as well as wild-type DNA contamination from leukocyte lysis [19]. Furthermore, most cfDNA alterations are not tissue-specific, making it difficult to predict the tumor tissue of origin in positively identified patients with cancer. RNA serves not only as translators of genetic information, but also subjects of gene expression regulation [20,21]. Compared with cfDNA molecules, cfRNA biomarkers possess higher sensitivity and specificity, and have the advantage of providing dynamic and deeper insights into tempo-spatial distribution and regulatory processes including tumor clonal evolution, changes in tumor microenvironment, immune responses, and blood vessel epithelium function. Besides, cfRNA existed as multiple copies with various spliced variants, providing a much higher chance to be detected than cfDNA (Table 2). PCR also enables traces of RNA sequences to be amplified and thus captured specifically with high sensitivity. Moreover, cfRNA transcripts usually have stable secondary and tertiary structures, and complexed with proteins or lipids, thereby protecting them from degradation in circulation. Although naked, full-length cfRNA transcripts are unstable in circulation, shorter and complexed forms of cfRNA fragments are readily detectable and quantifiable at low abundance [22]. Cell-free RNA also presents an opportunity to detect cancer in patients with low tumor shedding rates, as overexpression of tumor-specific transcripts could

lead to amplification of tumor-derived signals in the blood. Plasma cfRNA may be released into the blood through mechanisms other than cell death, such as exosome-mediated signaling by living cells [23]. Consequently, tumor-derived cfDNA and cfRNA may originate from distinct cell populations, potentially expanding the opportunities for MRD detection through the combined screening of multiple analytes beyond cfDNA.

Table 2: Comparison of cfDNA- and cfRNA-derived molecular diagnostic biomarkers

	cfDNA	cfRNA
Biomarker	SNV, indels, CNV, limited fusions and rearrangements, TMB, MSI, methylation	Gene fusions, translocations, gene expression signatures
Composition	98% non-coding; only 2% gene-coding	rRNA, tRNA, mRNA, microRNA, non-coding RNA
Forms in circulation	Heterogeneous species in size and form (50 bp – 20 kb in nucleoprotein complexes)	Heterogeneous species in size and form (50 nt – 2,000 nt in highly complexed forms)
Gene Copy Number	2 copies per cell	Hundreds or thousands copies per cell
Tumor Information	Tumor-specific genetic alterations	A full spectrum of physiological states reflecting tumor evolution, tumor microenvironment and immune response
Clinical Applications	Cancer early detection, treatment selection and monitoring, prognosis, MRD for recurrence	Cancer early detection, treatment selection and monitoring, prognosis, MRD for recurrence
Assay Platform	NGS, dPCR, qPCR, mass spectrometry	RNA-Seq, dPCR, qPCR, microarray

Studies have demonstrated the requirement of ultra-sensitive MRD testing, i.e., capable of detecting 0.01% mutant allele frequency (MAF), due to the low abundance of fragmented cfDNA [9]. Recently, NGS technology capable of the quantified measurements of RNA expression levels at whole transcriptome level has been established. Increasing depth of RNA sequencing (RNA-Seq) and quantified as TPM (transcripts per million) or FPKM (fragments per kilobase of transcript per million reads mapped) enables the detection of novel and rare transcripts, and subtle variations in expression with greater accuracy [24,25]. Large scale expression profiling by RNA-Seq provides dynamic and gene regulatory information, and thus can act as accurate and direct markers of tumor physiological state which truly open the door for MRD application in solid tumor [26].

Studies of circulating mRNA have more clinical applications but are limited to the detection of known oncogenic mRNA markers, often with prior knowledge of mutations harbored in matched tumor tissue. Such approaches are invaluable for treatment guidance and monitoring but do not address the potential application of cfRNA for MRD. In a study aimed to establish the roadmap of plasma cell-free oncogenic transcripts and to identify cell-free mRNA biomarkers specific to patients with lung cancer, we have performed targeted expression profiling based on multiplex RT-qPCR followed by quantitative analysis of cfRNA abundance by delta Ct, the difference of Ct values between reference gene (18S rRNA) and target gene. A panel of 750 cancer-associated genes were profiled and categorized into 8 major cancer signaling pathways: immune response, transcription factors, DNA repair, oncogenesis, tumor metastasis, TP53 signaling, MAP kinases, and cell surface markers. In addition to providing gene-level information, the panel condenses genes measuring similar biology into signatures that provide robust characterization of a given pathway, thus enabling greater insights to be gained from fewer samples. The most abundant circulating cell-free transcripts among these 8 clusters from the lung cancer cohort was demonstrated in Table 3.

Table 3: Highly expressed transcripts identified in plasma of the lung cancer patients by cfRNA profiling of 750 genes associated with 8 major cancer pathways

Immune Response	Transcription Factors	DNA Repair	Oncogenesis	Tumor Metastasis	TP53 Signaling	MAP Kinases	Cell Surface Markers
CCL5	ETS1	APEX1	BCL2L1	FXYD5	FOS	DUSP1	PGK1
NFATC3		DCLRE1A	CCND3		GADD45B	FOS	UBC
PF4		MAPK14	CDK4		GSK3B	GRB2	PPIA
		POLH	RHOA		HDAC1	MAP2K3	CD63
		POLR2A			HIF1A	MAP3K3	CD74
		PSMB9			HIPK2	MAPK1	CD79A
		RPA2			PSMB4	MAPK6	HLA-A
		TP53			PSMD4	MAPKAPK3	HLA-DRA
		XRCC1			SIRT2	PTK2B	MS4A1
					SIRT6	RAF1	MYH9
					THBS1		PECAM1
					UBB		S100A8
					UBC		

In parallel, transcriptome-wide characterization of tissue mRNA in lung cancer was also conducted using RNA-Seq technology. Of 17,780 detected and annotated genes, 5,185 (29%) displayed at least 1.2-fold higher expression over non-cancer samples. Within those low-variation genes, we identified lung cancer-specific transcripts that are recurrently detected in plasma and tissue. These transcripts met our set criteria: [1] they were not detected in non-cancer or other cancer plasma, [2] they were upregulated in the cancer group compared to the non-cancer group, and [3] they were detected in more than one cancer sample in our cohort. Lung cancer-specific cfRNA levels in plasma correlate with RNA expression in tissue, suggesting that these genes with relatively high expression in tumor tissue could enhance cancer detection in patients with circulating cfRNA (Figure 1). Overall, our data indicated that there is a detectable population of tumor-derived cfRNA circulating in cancer plasma, and cfRNA provides a unique opportunity to detect cancer, predict the tumor recurrence, and determine the cancer MRD.

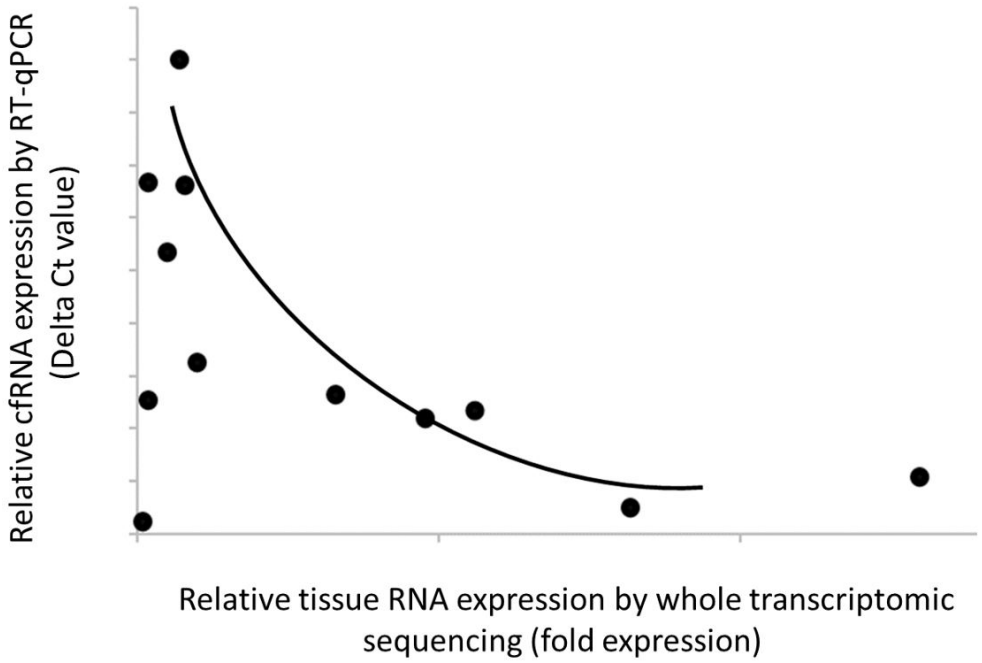

Figure 1: Correlation of expression of 12 lung cancer-specific mRNA biomarkers between tissue and plasma samples. Relative cfRNA levels were expressed as delta Ct values, whereas tissue RNA expression was normalized as fold expression. The data showed a good positive correlation between cfRNA and tissue RNA expression (i.e., an inverse relationship between delta Ct and fold expression).

Future Perspectives of Cell-Free Rna-Based Mrd Testing

The existence of cfRNA in plasma has been well-established for more than 20 years. However, several factors may have hindered its widespread adoption for cancer diagnostics. For example, circulating RNA is assumed to be low abundant, unstable, and highly fragmented. This perception arises from the relative instability of RNA compared to DNA and the high concentration of RNases present in circulation. The preanalytical approach to cfRNA extraction should be agnostic to the origin of the extracellular material and seeks to isolate all available RNA from the cell-free fraction. This precludes speculation about the potential function of these transcripts in circulation but allows a comprehensive characterization of cfRNA and provide a preanalytical workflow for future studies seeking to quantify circulating RNA in a robust and reproducible manner.

Circulating cfRNA biomarkers address two major MRD challenges: highly expressed RNA biomarkers for ultra-sensitive cancer detection while avoiding a large number of false positives from low-frequency "driver" somatic mutations derived from healthy cells. By focusing on cancer type-specific panels that are free of background, non-specific signals from healthy cells and other cancer types, we can ensure high cancer specificity of the identified cfRNA biomarkers. The 2-tier approach using both RT-qPCR and RNA-Seq on cfRNA and tissue RNA respectively, effectively reduces the likelihood of identifying false positive cfRNA biomarkers in plasma e.g., due to technical variations or batch effects. Circulating cfRNA biomarkers exhibit several distinct characteristics that support their validity as cancer-specific biomarkers for MRD: [1] tumor-specific cfRNA species were highly enriched, [2] their expression was correlated with expression in tumor tissue, and [3] they can be detected by existing highly sensitive technologies.

Our results suggest that tumor-derived signals are amplified in cfRNA due to increased expression of these markers in tumor tissue. The ability to measure MRD through the analysis of cfRNA could lead to the amplification of cancer-specific signal by orders of magnitude compared to mutation fraction alone. This increase in signal-to-noise may enable detection for patients with cancer of low mutation fraction that might otherwise be missed by cfDNA-based detection approaches. We now have a comprehensive picture of the types of plasma oncogenic cfRNA and have identified a class of cancer-specific cfRNA biomarkers in low-noise regions of the cell-free transcriptome. Ultimately, we hope cfRNA can enable the MRD measurement of a variety of cancers besides hematological malignancies to reduce cancer mortality. The MRD testing strategy can predict risk of recurrence more precisely, making it possible to reduce unnecessary therapy to improve quality of life for cancer patients. In the future, cfRNA-based MRD testing of patients with localized solid tumor may help clinicians better tailor adjuvant strategies to their patients' needs: reducing treatment for those likely to be cured and starting treatment earlier for those at higher risk.

Although MRD testing can provide incredible insight, several barriers have slowed full clinical adoption. MRD tests typically must assess one or a few biomarkers. For some cancers with well-defined biomarkers, this is relatively easy and cheap. For others, it is costly because NGS must be utilized establish personalized biomarkers. This challenge is especially relevant when considering MRD testing in solid tumor cancers. Furthermore, the approach to selecting biomarkers and measuring MRD is not standardized: different labs employ various NGS platforms, qPCR, RT-qPCR, dPCR and a host of other tests, leaving questions on how to standardize result interpretation and readout.

Ultra-sensitive methods for MRD detection have proven advantageous in numerous clinical studies. Efforts continue toward standardization and will help expand what can be gleaned, leading to more widespread adoption of the available techniques. Simultaneously, as biomarker research uncovers more cfRNA common to whole cancer types, these may be converted into inexpensive, commercial tests. As MRD testing strategies become better established and researchers uncover new uses, expanding applications in the clinic will match pace. However, clinical studies must continue to define standardized diagnostic metrics such as MRD presence or quantity to understand how these translate into prognosis and risk. As medical advances continue to drive growth, the momentum will promote increased awareness and education amongst the clinical community and patients about MRD testing, further unifying the field and leading to better patient outcomes.

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