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Integrating contextual miRNA and protein signatures for diagnostic and treatment decisions in cancer

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Abstract

The promise of personalized medicine is highly dependent on the identification of biomarkers that inform diagnostic decisions and treatment options, as well as on the accurate, rapid and cost-effective detection and interpretation of these biomarkers. miRNAs, which are short noncoding regulatory RNAs, are rapidly emerging as a novel class of biomarkers with a unique set of biological and chemical properties that makes them very appealing candidates for theranostic applications in cancer. Since the utility of some protein-encoding gene biomarkers is already exploited in routine clinical practice, it will be important to identify areas in which miRNAs provide complementary or superior information to these existing (and other translational) biomarkers to enhance the diagnostic, prognostic and predictive power of molecular characterization of tumors. In this article, the challenges and opportunities for integration of miRNA-based assays in the clinical toolkit to improve care and management of patients afflicted with solid tumors will be discussed.

Keywords

biomarkers; breast cancer; colorectal cancer; expression profiling; immunohistochemistry; *in situ* hybridization; lung cancer; miRNA; multiplexing; proteins; quantitative reverse-transcriptase polymerase chain reaction

Cancer causes approximately 23% of all deaths in the USA [1]. Standard clinical parameters such as tumor size, grade, lymph node involvement and tumor-node-metastasis (TNM) staging [201] correlate with outcome and serve to stratify patients with respect to (neo)adjuvant chemotherapy and/or radiotherapy regimens. Incorporation of molecular markers in clinical practice has defined tumor subtypes that are more likely to respond to targeted therapy, as is the case for the response of breast cancers overexpressing estrogen receptor (ER) to hormone therapy (e.g., tamoxifen and aromatase inhibitors). However, stage-matched tumors grouped by histological or molecular subtypes respond differently to the same treatment regimen. This indicates that additional key genetic and epigenetic alterations exist, with important etiological contributions. A more detailed understanding of the molecular mechanisms and regulatory pathways at work in cancer cells and the tumor microenvironment (TME) could dramatically improve the design of novel anti-tumor drugs

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and inform the selection of optimal thera peutic strategies. Thus, there is an urgent need to develop and implement diagnostic, prognostic and therapeutic biomarkers to characterize the biology of each tumor and to assist clinicians in making important decisions with regard to individual patient care and treatment [2,3].

In the last 5–10 years, high-throughput expression analysis platforms have been widely used to study tumors at a molecular level in order to further refine patient subgroups. While the general strategy and approach of these technologies are applicable and have been applied to the mole cular characterization of hematologic (leukemias and lymphomas) and solid (carcinomas, sarcomas and melanomas) tumors, this article will focus on recent biomarker discoveries in breast, colorectal and lung cancer as examples of prevalent and representative solid tumors of epithelial origin (carcinomas). I will place a particular emphasis on recent clinical findings on miRNAs and will discuss how inherently distinct biological properties of miRNA and protein-encoding genes are important determinants of the clinical utility and performance of miRNAs as novel biomarkers.

miRNAs as emerging clinical biomarkers

MicroRNAS are evolutionary conserved short noncoding RNA genes that were discovered just 10 years ago [4–6]. While their discovery occurred in studies of the nematode *Caenorhabditis elegans* [7–9], we now know that miRNAs are an important and pervasive regulatory layer of gene expression that acts at the post-transcriptional level in all animals, including humans [10,11] miRNAs have been implicated as pivotal players in prevalent human diseases, including cancer and neurodegenerative conditions [12–16]. The mature and biologically active miRNA is released from a much longer RNA molecule after sequential enzymatic cleavage. A primary 5′-capped and 3′-polyadenylated transcript is cleaved in the nucleus by the RNAse type III enzyme Drosha and associated proteins of the microprocessor, and yields a 70-nucleotide precursor RNA hairpin, which is exported via the Expo5 pathway into the cytoplasm [17]. Once in the cytoplasm, the approximately 21–23 nucleotide-long mature miRNA is released after cleavage by another RNAse type III, Dicer, and loaded an Argonaute-containing multiprotein miRNA-induced silencing complex [17]. By binding to partially complementary sites in the 3′-untranslated region of mRNAs, the miRNA brings the miRNA-induced silencing complex in close proximity to target mRNA, which triggers increased mRNA degradation and/or inhibition of protein synthesis [18].

The human genome is estimated to contain more than 1000 miRNA genes and a third of protein-encoding genes are predicted to be under miRNA-mediated regulation [19–23]. This suggests that miRNAs constitute a novel and global mechanism to modulate gene expression, comparable to the genome-wide epigenetic and transcriptional changes associated with cancer. Indeed, the recent explosion in miRNA research has brought conceptual and technological advances and innovations that are expanding our understanding in the fields of cancer biology and molecular pathology [12–14,24]. Unique chemical and biological characteristics make miRNAs very attractive candidates for theranostic applications in cancer as the expression and function of miRNAs are tightly associated. A single miRNA can regulate and modulate levels of hundreds of targets, some of which are components of the same signaling pathway and/or biological process [21,22]. Accordingly, functional manipulation of a single miRNA could concertedly affect multiple target mRNAs (one drug, multiple hits), unlike siRNA-, antibody-or small molecule inhibitor-based therapies (one drug, one hit). Synthetic compounds exist to eliminate or decrease miRNA activity (e.g., antagomirs, tiny antisense locked nucleic acids [LNAs] and oligonucleotides) and to replenish or increase miRNA activity (e.g., siRNA-like compounds). Proof-of-concept experiments have demonstrated effective and safe *in vivo*

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delivery of these compounds in rodents and nonhuman primates [25–30]. Therefore, the utility of key miRNAs as biomarkers for interrogating tumor biology could also be exploited to discover pharmacological miRNA targets that affect tumor biology.

Assay platforms & approaches for biomarker detection

Molecular characterization of tumors based on the expression of genes and gene products can be divided into two broad and distinct categories: RNA-based and tissue slide-based assays. RNA-based assays follow similar procedures for miRNA and mRNA detection. Typically, total RNA is extracted from whole tissue biopsies (fresh, frozen or fixed) to determine mRNA and miRNA levels. High-throughput platforms, such as microarray chips and deep sequencing have enabled transcriptome- and miRNome-wide tumor tissue characterizations. Tissue slide-based morphology-driven assays use archived formalin-fixed paraffin-embedded (FFPE) specimens to determine gene copy number by fluorescence *in situ* hybridization, mRNA or miRNA levels by *in situ* hybridization (ISH) and protein levels by immunohistochemistry (IHC). IHC is the most common assay for expression analysis of protein-encoding genes in routine clinical practice, as is ISH for expression of miRNAs in translational research.

The use of miRNA biomarkers have a distinct set of advantages over protein-encoding genes:

- miRNAs are more stable and are better preserved than mRNAs in biological specimens, including archived FFPE tissue blocks and blood samples. Thus, poor RNA quality and RNA degradation may compromise the information content and accuracy of mRNA signatures much more than that of miRNA signatures;
- The human miRNome is relatively simple, with approximately 1000 genes, enabling detection of all miRNAs in a single-tube reaction and quick generation of miRNA signatures, which are less bioinformatically demanding than mRNA expression signatures;
- RNA-based and tissue slide-based assays measure the same bioanalyte for miRNA genes - namely, the RNA levels of mature miRNA - whereas RNA-based assays measure mRNA levels (intermediary carrier of genetic information) and tissue slide-based assays measure protein levels (gene product) for protein-encoding genes;
- More straightforward interpretations of miRNA function can be drawn based on expression changes of protein function, as mature miRNA levels generally correlate with miRNA activity, whereas post-transcriptional regulation and post-translational modifications render a more complex correlation between protein activity and expression levels of mRNAs or proteins.

RNA-based assays

Expression profiling in whole tumor tissue samples has provided sets of differentially expressed mRNAs and miRNAs (expression signatures), with potential links to the etiology of the disease. However, this type of assay does not distinguish between altered RNA expression within the cancer cell compartment or other elements of the TME, including reactive stroma and infiltrating immune cells. This level of cellular complexity is probably captured in expression signatures obtained from whole-tissue RNA analysis, and thus it is not straightforward to interpret what these signatures measure or indicate biologically. Changes in RNA levels between normal and tumor tissues and among tumors from different patients can, in some cases, simply reflect a different ratio of cell type representation and tissue heterogeneity, whereas, in other instances, it can reflect specific molecular aberrations

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in the cancer cell or other cellular compartments of the TME that affect cancer cell behavior and aggressiveness. For protein-encoding genes, gene ontology analysis provides annotated information on expression and function that facilitates ascribing changes in mRNA levels to a specific cell type [202]. For miRNA genes, there is only a fragmentary knowledge of cell type and tissue-specific expression and function in these different cellular contexts under physiological and pathological conditions. Upregulation of a miRNA within cancer cells or immune cells may have both etiological relevance and clinical significance, but obviously indicates different biological processes and has different diagnostic and therapeutic implications.

While one should be mindful of these caveats and limitations, high-throughput discovery tools have identified expression signatures with potential clinical utility for the diagnosis and prognosis of cancer that can be measured in single-tube highly sensitive multiplex quantitative reverse-transcriptase PCR assays. Some of these refined mRNA signatures have already received or are seeking US FDA approval for clinical use (see later). A miRNA signature for the diagnosis of pancreatic adenocarcinoma (miRInform® Pancreas) is currently offered by Asuragen, Inc. (TX, USA) as laboratory-developed test in a Clinical Laboratory Improvement Amendments-certified environment [31].

Tissue slide-based assays

Tissue slide-based morphology-driven assays are the workhorse of clinical pathology. These include morphological and histological characterization of tumor lesions with specialized stains and dyes (e.g., hematoxylin and eosin), as well as molecular assays such as IHC (e.g., ER, MSH2 and EGF receptor [EGFR]) and fluorescence ISH (e.g, human EGF receptor 2 [HER2]). Routine clinical IHC assays detect one or two markers by chromogenic staining [32]. The use of fluorescence-based staining can increase the multiplexing capability of this assay to three or more independent markers [33,34]. To meet this increasing demand for digital pathology, several companies already offer multispectral whole slide scanning instruments and companion software packages for computer-assisted image analysis that enable fast and quantitative scoring of markers in specific cellular compartments [33–36].

Protein molecules can accumulate in cells at several orders of magnitude (10^2–10^4) higher than their corresponding mRNAs [37], whereas the maximum number of mature miRNA molecules equals the number of primary miRNA transcripts. Thus, sensitivity has been a challenge for detection of miRNAs by ISH on FFPE clinical specimens. The introduction of LNAs, high-affinity bycyclic RNA analogs [38], for the synthesis of chimeric LNA/DNA probes has overcome the technical limitations of achieving specific and avid hybridization to the short RNA sequence of mature miRNAs. This has allowed several groups to characterize miRNA expression at single-cell resolution in different types of cancer [39–53]. The ISH assay is an important validation tool to correctly interpret the biological basis and clinical relevance of altered expression of cancer-associated miRNAs, as determined by RNA-based assays on whole tissue samples [52,53]. A highly sensitive fluorescence-based method for the codetection of multiple RNA and protein markers on the same tissue section has recently been reported [52]. Sequential rounds of tyramide signal amplification reactions [54] with in-house-synthesized fluorochromes enables the detection of additional RNA markers, such as abundant noncoding RNAs (e.g., U6 snRNA and 18S rRNA) that can be used to assess RNA quality and integrity, and protein biomarkers that can be used: to highlight a specific cellular compartment (e.g., CK19 for epithelial/cancer cells, CD45 for leukocytes) and to assess the cell source of miRNA expression; to characterize signaling pathways and cellular processes (e.g., Ki-67 for proliferation, p53 for compromised DNA damage and apoptotic responses); and to interrogate miRNA-target interaction networks (e.g., PTEN and PDCD4 for miR-21) [52]. This ISH/IHC assay was deployed to characterize the spatial expression of cancer-associated miRNAs, including miR-21 and −155, two of the most frequently

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upregulated miRNAs in cancer, which are thought to be highly oncogenic [12–14,24]. In a panel of breast, colorectal, lung, pancreatic and prostate carcinomas, the authors identified a subpopulation of immune cells to be the predominant source of miR-155 expression in these prevalent solid tumors, whereas miR-21 expression was upregulated within cancer cells and reactive fibroblasts to different extents depending on the organ site [52]. By contrast, miR-34a expression was detected at lower levels in cancer whereas the surrounding stromal cells retained high levels of miR-34a in breast and lung cancer specimens, suggesting dynamic and independent regulation of this miRNA in different cellular compartments [52]. In an effort to bring this type of miRNA-based assay closer to the clinic, the authors have already performed a fully automated protocol for the described IHC/ISH assay in FDA-approved automated staining stations in a laboratory environment that is compliant with gold-standard practice of current clinical IHC assays [52,55].

Molecular assays in breast cancer

Breast cancer (BC) is the most prevalent type of cancer and is the second most common cause of cancer-related death of women in the USA [56]. BC is not a single disease, but rather a complex and heterogeneous group of diseases with different molecular alterations [57,58]. Current management of BC is mainly based on the activation status of ER, progesterone receptor (PR) and HER2. ER/PR/HER2 classification is used for diagnosis, prognosis and treatment selection, as targeted drugs have been developed to block these signaling pathways [3]. Global mRNA expression studies have identified four major biological subtypes of BC, which differ with respect to their biology, prognosis and susceptibility to specific treatments [59,60]. These molecular intrinsic subtypes closely correspond to ER/PR/HER2 classification [61]: the immense majority of luminal A cases are ER^+ and/or PR^+(ER/PR^+)$HER2^-$; luminal B cases are ER/PR^+HER2^- (with a higher proliferation rate) or ER/PR^+HER2^+; HER2-overexpressing cases are ER/PR^-HER2^+; and basal cases are $ER^-PR^-HER2^-$. These gene-expression studies also indicate the involvement of distinct subpopulations of epithelial cells among tumor subtypes; cells with myoepithelial/basal characteristics contribute almost exclusively to the aggressive basal subtype. This underscores the importance of ER and HER2 signaling in tumor initiation and evolution.

Given the technical and financial difficulties of conducting global mRNA expression analysis in all clinical samples, multiprotein marker panels and refined mRNA signatures have been identified for diagnosis and subtyping, prognosis and treatment selection [62,63]. An eight-marker IHC assay has been proposed as a refinement of ER/PR/HER2 classification and as a better surrogate marker of intrinsic subtypes [61,64,65]. In addition to ER, PR and HER2, this eight-marker IHC assay also includes CK5, CK14, EGFR, Ki-67 and p53. CK5 and CK14 are cytokeratins expressed by basal/myoepithelial cells and are used to identify $ER^-PR^-HER2^-$ breast cancer cases with a basal phenotype [61,64]. Human EGFR is overexpressed in a large number of $ER^-PR^-HER2^-$ cases with a basal phenotype and is a potential druggable target. The Ki-67 proliferation index is used for grading and prognostics in BC and other cancer types. High proliferation index and elevated *p53* expression (indicative of a mutant and dysfunctional *p53*) can be used to distinguish luminal A from luminal B tumors, which have a slightly less favorable outcome owing to reduced response to hormone therapy and chemotherapy [65]. Refined mRNA signatures such as the 21-gene Oncotype DX® (Genomic Health Inc., CA, USA) [66], 70-gene MammaPrint® [67] and 50-gene PAM50 [68] can predict the risk of recurrence and thus inform the course and intensity of treatment. For ER^+ cases with a low risk of recurrence as assessed by these tests, adjuvant chemotherapy is not recommended since the adverse side effects and morbidity would outweigh any potential clinical benefit. Oncotype Dx and MammaPrint are the only RNA-based assays so far that have received approval or clearance by the FDA for

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use in the clinic [62]. Remarkably, only one gene is shared in common between the Oncotype Dx and MammaPrint mRNA signatures, suggesting that these genes, besides the obvious exception of *ER* and *HER2*, probably serve more as readout of biological processes rather than the identification of key driver alterations in the cancer cells. For example, *CD68*, one of the genes in the Oncotype Dx assay, is a cell type-specific marker of monocytes and macrophages; mRNA levels of CD68 probably reflect the composition of immune cell infiltrates and by inference the overall nature of immune programs elicited against (tumoricidal) or recruited by (tumorigenic) the cancer cells. Indeed, three-marker IHC-based immune cell signature that measures the ratio of $CD4^+$ T cells, $CD8^+$ T cells and $CD68^+$ monocytes/macrophages was recently shown to be a prognostic indicator of recurrence-free and overall survival in BC [69]. The authors suggest that this deleterious effect is a result of decreased treatment response to chemotherapy in patients with a high number of infiltrating $CD68^+$ cells [69]. Similarly, RNA-based assays using microdissected stromal tissue as the input source have also revealed the influence of noncancer cell compartments in the course of the disease [70–75]. Stromal signatures can stratify patients based on outcome independent of standard clinical prognostic factors and global mRNA expression signatures such as MammaPrint [75]. These stromal signatures reflect a combination of biological processes (angiogenic, hypoxic and immune responses) in the different constituent cellular compartments such as reactive fibroblasts, vasculature and leukocytes.

Global miRNA expression studies have revealed consistent changes between normal and tumor tissues. miR-125b and miR-145 are frequently detected at lower levels, and miR-21, miR-155 and miR-210 at higher levels in tumor tissues [40,76–79]. Unsupervised hierarchical clustering of cases based on global miRNA signatures differs from that of global mRNA signatures and well-established IHC surrogate markers, suggesting that miRNAs could provide additional diagnostic information [80,81]. Other miRNAs have been shown to closely correlate with ER, PR and HER2 status, and these miRNAs could be useful to detect alterations in these signaling pathways and inform treatment response [82–86]. Yet, there is little overlap of differentially expressed miRNAs among studies, and additional investigations will be needed to select the most informative and relevant miRNAs [78,87]. Other clinical applications have been proposed for single miRNAs and refined miRNA signatures in specific intrinsic subtypes or subgroups of patients (Tables 1 & 2). miR-210 is a robust and independently validated prognostic biomarker in BC. Higher miR-210 levels have been associated with poor outcome in ER^+ cases with negative lymph node involvement [80,88,89] and in ER^- cases with negative lymph node involvement [88,90,91]. It is noteworthy that miR-210-based quantitative reverse-transcriptase PCR assay performed similarly to the 21-mRNA Oncotype Dx assay to predict outcome in ER^+ cases that received adjuvant tamoxifen treatment for 5 years [80]. miR-210 expression is induced by hypoxic conditions and has been shown to mediate anti-apoptotic, migratory and proinvasive programs in ER^+ and ER^- BC cell lines [80,92]. Thus, miR-210 expression could serve as a hypoxic sensor and indicator of an aggressive phenotype in clinical samples.

The overwhelming majority of miRNA expression studies utilize RNA-based rather than tissue slide-based assays. Tissue slide-based assays provide an independent platform and a different vantage point to validate and refine expression profiling results. An ISH-based study found a subset of BC-associated miRNAs whose expression was altered within distinct subpopulations of mammary epithelial cells [40]. Expression of let-7a and miR-141 was detected at varying levels, predominantly within luminal epithelial cells in normal tissue [40]. Conversely, miR-145 and miR-205 expression was restricted to myoepithelial cells in normal epithelial structures, whereas their expression was reduced or completely eliminated in matching tumor specimens [40]. The authors proposed miR-145 as a novel biomarker for early detection of malignancy and disease progression from noninvasive to preinvasive

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lesions based on the early manifestation of altered miR-145 expression in carcinoma *in situ* lesions adjacent to invasive carcinoma and thus presumed preinvasive [40]. The authors also observed a positive correlation between miR-205 expression and favorable clinical outcome in $ER^{-}PR^{-}HER2^{-}$ cases and proposed miR-205 as a prognostic indicator for the aggressive $ER^{-}PR^{-}HER2^{-}$ (basal) subtype [40].

Molecular assays in colorectal cancer

Colorectal cancer (CRC) is the third leading cause of cancer-related death in both men and women in the USA [56]. CRC can be divided into two molecular subtypes: microsatellite stable (MSS) and microsatellite instable (MSI). The MSI phenotype is observed in approximately 15% of cases, is associated with a better prognosis and appears to have a different chemoresponse to therapeutic agents such as 5-fluorouracil (more resistant) and irinotecan (more sensitive) [93,94]. MSI was first described in connection to Lynch syndrome or hereditary nonpolyposis CRC [95]. The MSI phenotype can be determined by PCR-based genomic DNA analysis of monoand di-nucleotide repeats of representative microsatellite sequences or by IHC-based assay of DNA mismatch repair proteins (MHL1, MSH2, MSH6 and PMS2) [93,94]. The latter is a preferred screening assay in routine clinical practice; absence of one or more of these proteins is indicative of the MSI pheno type. This may be followed by microsatellite analysis to confirm diagnosis and/or DNA sequencing to determine hereditary or sporadic origin of the tumor [94].

Surgical resection of the primary tumor is a successful curative treatment for approximately 80% of stage I and II CRC cases. Prognostic mRNA signatures have been identified to stratify cases into low risk and high-recurrence groups to minimize patient over-treatment with adjuvant chemotherapy [96]. As noted earlier for BC, there is little overlap in the gene lists that compose these expression signatures [96]. A recent study with large sample sizes for both training and validating sets of patients developed an 18 mRNA prognostic signature (ColoPrint®; Agendia, CA, USA) for stage II and III cases that has better predictive value than mutation status of *BRAF*, *KRAS* and/or *PI3KCA* genes or standard clinical parameters [97]. Unsupervised hierarchical clustering analysis uncovered three main molecular subtypes in the training set. Overall, 86% of the MSI cases were included in the low-risk recurrence subtype in the training set, and 90% of the MSI cases in the validation set [97]. This indicates that ColoPrint captures the already known good prognosis of MSI cases. Importantly, ColoPrint also serves to stratify MSS cases with a more mixed prognosis *a priori* [97]. The current standard of care for stage III cases, which are more often MSS tumors, is adjuvant chemotherapy. Results from these RNA-based assays may prompt a revision of this practice, at least for a subgroup of stage III cases with a low-risk recurrence signature.

Global miRNA expression studies have revealed consistent changes between normal and tumor tissues. miR-125a, miR-143 and miR-145 are frequently detected at lower levels and miR-31, miR-106a and miR-203 at higher levels in tumor tissues [77,98,99]. An eight-miRNA signature was developed to classify CRC tissues as the MSS or MSI subtype [100]. Two of these miRNAs, miR-25 and miR-92, are consistently detected at higher levels in MSS tissue samples and detection of these miRNAs in CRC could complement current diagnostic assays [100]. Other studies have investigated the prognostic value of single miRNAs or refined miRNA signatures(Tables 1 & 2). Independent reports have associated high miR-21 expression with shorter recurrence-free and overall survival [51,101–103]. One of these studies determined miR-21 expression by ISH analysis in a cohort of 197 stage II CRC cases [51]. Upregulation of miR-21 expression was predominantly detected in the stromal cell compartment, mainly in tumor-associated fibroblasts [51]. Interestingly, higher

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expression of miR-21 in reactive fibroblasts was associated with a poor outcome in colon cancer (n = 130), but not in rectal cancer (n = 67) specimens [51].

Epidermal growth factor receptor is overexpressed in the majority of metastatic CRC (mCRC) cases [104]. Treatment of mCRC with monoclonal blocking anti-EGFR antibodies (i.e., cetuximab or panitumumab) alone, or in combination with, chemotherapy has been shown to increase progression-free and overall survival in several randomized clinical trials [104–106]. While EGFR over-expression correlates with treatment response to cetuximab [104], wild-type status of the *KRAS* gene is currently a more accurate predictor of treatment response to cetuximab- and panitumumab-containing therapies [105,106]. Since patients whose tumors harbor mutated *KRAS* gene as a group are unlikely to benefit from anti-EGFR therapies, these may exclude individual patients with mutated *KRAS* tumors for whom this treatment could be efficacious and include some patients with wild-type *KRAS* for whom this treatment would not be efficacious. RNA-based studies in CRC cell lines have reported mRNA and miRNA signatures that could improve prediction of treatment response over *KRAS* mutation status alone and thus inform patient eligibility to anti-EGFR-based treatments [107,108]. Using cetuximab-sensitive and -resistant CRC cell lines to profile miRNA expression and mining miRNA expression data from previous studies in clinical samples, a three-miRNA signature was proposed to predict treatment response to cetuximab [108]. Two of these miRNAs, let-7b and let-7e, are negative regulators of *KRAS* expression. The importance of let-7's role is reinforced by molecular epidemiologic studies that indicate a poor outcome of cetuximab-treated mCRC patients whose tumors harbor a single-nucleotide polymorphism variant in the 3′-untranslated region of *KRAS* mRNA that disrupts let-7 binding [109,110].

Molecular assays in lung cancer

Lung cancer (LC) is the leading cause of cancer-related death for men and women in the USA [56]. LC can be divided into two major histological subtypes: small-cell LC and non-small-cell LC (NSCLC). Small cell LC has an endocrine cell of origin and an overall even worse prognosis than NSCLC, partly owing to its late presentation [111]. NSCLC represent approximately 80% of all LC cases and can be divided into three histological groups: large cell carcinoma, squamous cell carcinoma (SCC) and adenocarcinoma (AdCa) [111]. AdCa and SCC are the two most common subtypes and have traditionally received the same treatment regimens with equally disappointing results.

To gain further insight into the biological differences between these histological subtypes, global mRNA profiling experiments have been conducted with normal adjacent and tumor tissues. Initial studies identified a close correlation between histological subtypes and expression signatures, with the exception of AdCa cases, which were separated into three to four major groups with different outcomes [112–114]. Two-marker (p63 and TFF-1) and six-marker (TRIM29, CEACAM5, SLC7A5, MUC1, CK5 and CK6) IHC assays can also accurately distinguish AdCa and SCC subtypes [115]. Consensus expression signatures from multiple studies that compared normal tissues with AdCa tissues [112,113,116–118] and normal tissues with SCC tissues [112–114] contain some, but not all, of the genes that these IHC assays are based on. Numerous studies have described prognostic signatures for early-stage NSCLC cases; some studies combined expression data of AdCA and SCC cases while other studies focused on a specific histological subtype [119,120]. As noted for BC and CRC, the overlap of genes in these signatures was low [119]. Gene ontology analysis indicates that these signatures capture different alterations in both cancer cells and other cellular elements of the TME. For example, a 72-gene expression prognostic NSCLC classifier was noticed to be highly enriched for immune cell-expressed genes, suggesting the influence of differential immune cell responses in different tumors [121].

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Global miRNA expression studies have revealed consistent changes between normal and tumor tissues. Let-7a, miR-34a, −126 and −145 are frequently detected at lower levels, and miR-21, −155 and −221 at higher levels in tumor tissues [77,122–124]. A 34-miRNA signature was developed to classify NSCLC tissues in the AdCa and SCC subtypes [125]. Surprisingly, miR-205 was not part of this diagnostic signature. Several independent studies reported that high expression levels of miR-205 are associated with SCC cases and can be used to accurately separate AdCa and SCC histologies, even in samples with little tissue material [126–130]. However, these studies did not test whether differential expression levels of miR-205 could have a prognostic value to stratify SCC cases into low- and high-risk groups. Other studies have investigated the prognostic value of single miRNAs or refined miRNA signatures (Tables 1 & 2). In one of the first miRNA studies in LC, low expression of let-7a and high expression of miR-155 correlated with poor outcome in mixed staged AdCa cases [131]. High levels of miR-155 expression were also correlated with poor prognosis in 41 stage I AdCa cases [131]. These findings on miR-155 expression have been difficult to validate in independent studies [47,132,133].

The advent of systemic targeted therapies against EGFR with cetuximab or with tyrosine kinase inhibitors (i.e., gefitinib and erlotinib) and against processes with anti-VEGF-blocking antibody (i.e., bevacizumab) has exposed the need for molecular characterization to preselect patients who will benefit from these treatments [134]. Unlike in mCRC, cetuximab does not appear to be efficacious in advanced NSCLC patients and the *KRAS* mutation status is not predictive of treatment response [134,135]. Gefitinib and erlotinib are only efficacious in tumors with a mutated *EGFR* gene. These tumors mainly present with an AdCa histology and are more frequent among nonsmoker female patients [134]. Approximately 15% of AdCa cases harbor a mutated *EGFR* gene and are eligible for gefitinib or erlotinib treatment; this excludes 85% of AdCa and virtually all SCC cases. Similarly, bevacizumab treatment is contraindicated due to an increased risk of severe bleeding events in SCC cases [134]. Thus, the great majority of cases currently lack a targeted treatment. mRNA and miRNA expression analysis could provide a means to identify subgroups that may benefit from other therapies. let-7a-mediated regulation of *KRAS* expression was first described in LC [136]. A SNP variant in the 3′-untranslated region of *KRAS* mRNA that disrupts let-7 binding is associated with an increased risk of developing NSCLC in moderate smokers [137]. An independent study in AdCa cases did not find an association of this SNP variant with mutation status of *KRAS* gene or overall survival [138]. However, expression levels of let-7 family members are much lower in SCC than in AdCa cases, and expression levels of let-7 family members only have prognostic value in SCC cases [125]. SCC cases have virtually no incidence of mutated *KRAS* gene, whereas approximately 30% of AdCa tumors harbor activating *KRAS* mutation. The authors speculate that compromised let-7-mediated regulation in SCC cases increases levels of wild-type *KRAS* expression and consequently tumor aggressiveness and resistance to treatment, whereas activating *KRAS* mutations in AdCa cases renders these tumors less susceptible to let-7-mediated regulation [125]. Other miRNAs, including miR-7 and miR-128b, have been shown to regulate *EGFR* expression in NSCLC cell lines [139–141]. Loss of miR-128b expression by chromosomal deletion (loss of heterogeneity) correlated with a better clinical response and improved overall survival in a group of 58 NSCLC patients treated with gefitinib-containing therapy [139]. The authors suggest that attenuation of miR-128b-mediated regulation of *EGFR* expression was responsible for this effect, as tumor expressing higher levels of EGFR may be more responsive to gefitinib [139]. Thus, miR-128b and other miRNAs that modulate *EGFR* expression could be used to inform treatment response to anti-EGFR therapies.

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Expert commentary

Discrepancies between studies and independent validation of most expression signatures remain a tall challenge. Heterogeneity of patient cohorts (subtypes, stages and age), heterogeneity of tissue specimens and RNA quality, different experimental designs, different detection platforms, different statistical analysis and data mining algorithms are all contributing and confounding factors to the generation of different expression signatures for the `same' clinical questions [142,143]. Nonetheless, gene-expression signatures defining intrinsic BC subtypes have been reproduced using different microarray platforms [144] and salient miRNA examples have been independently validated (Table 2). This suggests that information about robust and frequent biological processes can be obtained with these high-throughput discovery platforms, but as such further validation and understanding of what these signatures reflect is required.

Cellular complexity of tumor tissues is often overlooked in the design, analysis and interpretation of RNA-based assays. Each tumor lesion has a different ratio of cancer cells to reactive stroma and infiltrating immune cells; a biomarker can be expressed at different levels in different cell types. Therefore, the most appropriate molecular assay and biomarkers should be deployed to extract the contextual information embedded within different cell types to indicate aberrant and dysfunctional pathways in cancer cells or other cellular compartments of the TME and to avoid confounding effects of tissue heterogeneity (see later). This will be an especially important consideration to validate reported miRNA signatures in large cohorts of patients with similar clinical characteristics and to identify the cellular compartment of altered miRNA expression. A miRNA biomarker may only be informative if its overall levels can be specifically assessed in a particular cellular compartment or if a differential expression pattern between cancer cell compartment versus another compartment can be measured.

miR-21, miR-34a and miR-210 are among the few salient examples of miRNA biomarkers that are ready to be tested in a clinical setting. A critical head-to-head comparison of the performance of these miRNA biomarkers with existing and translational protein-encoding genes will determine whether miRNAs provide superior or complementary information to increase the molecular characterization of tumors and enhance the diagnostic power of current clinical assays. Pioneered global miRNA profiling indicated that miRNA signatures outperformed mRNA signatures to group together tumors derived from the GI tract from other carcinomas and to classify poorly differentiated tumors into the appropriate organ site [145]. Other studies have also shown the utility of miRNA signatures to identify the organ site of carcinomas of unknown primary origin [146,147]. This information can be useful to improve clinical outcome by matching treatment of the carcinomas of an unknown primary tumor with that recommended for the organ site. A miR-210-based assay yields comparable information to the 21-mRNA Oncotype Dx assay to predict response to tamoxifen in BC patients [80]. It is likely that both assays reflect the coordinated effects of different biological processes, but a single miRNA assay will be more amenable to clinical implementation and readout interpretation.

While this is beyond the scope of this article, miRNA-based assays in blood samples is a rapidly expanding field with the potential to improve disease management [148–151]. A blood test is a noninvasive procedure in which samples from the same patient can be collected repeatedly in a longitudinal manner. miRNA-based blood assays could be useful for early disease detection and for monitoring disease progression, especially after treatment. However, the exact mechanism and cell source(s) for altered miRNA levels in blood are not currently known. Do they mainly represent the content of lysed cancer cells or other cellular components of the TME? Do they mainly reflect an anti-tumor immune response or

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protumoral inflammation? Do cancer cells communicate systemically by secreting specific miRNAs in exosomes, lipoprotein complexes or other organized macrostructures?

Five-year view

As the clinical utility of more miRNA- and protein-encoding biomarkers is validated by independent prospective and longitudinal studies, it will be important to translate and integrate these findings into routine clinical practice. Innovative technological and conceptual approaches that combine the unique capabilities of RNA-based (multiple marker detection) and tissue slide-based (cell type-specific information) assays will enhance the diagnostic power and accuracy of molecular analysis. Enrichment of specific cell types by laser capture microdissection or similar techniques prior to RNA quantification is a feasible option to provide RNA-based assays with contextual information [152,153]. However, this type of assay will be technically demanding and will require further processing steps. This contextual RNA-based assay will be amenable to implementation in a research setting, but it will probably be difficult to incorporate into routine clinical practice unless further technological advances are introduced, such as procedural automatization. Instead, I propose a transformative technological platform that shares the same technical principles and instruments as current tissue slide-based clinical assays and is designed to be seamlessly adaptable with minimal time loss or procedural modifications in high-volume clinical pathology laboratories. In order to enable codetection of an unprecedented large number of protein, DNA and RNA biomarkers on a single tissue slide, this impending platform will couple tissue compartmentalization for parallel marker detection with sequential rounds of permanent stains for cumulative marker detection. In 2010, Kim *et al.* engineered a four-chambered microfluidic device that was pressed down on top of BC tissue slides to physically separate independent compartments and dispense different solutions and antibodies to each compartment [154]. Kim *et al.* demonstrated codetection of ER, PR, HER2 and Ki-67 on contiguous but insulated (there is no antibody cross-contamination) compartments on a single BC tissue section using chromogenic staining [154]. Also in 2010, Sempere *et al.* demonstrated cumulative codetection of up to five independent RNA and protein markers on the same tissue section using multicolor fluorescent staining [52]. Figure 1 illustrates the concept of this tissue slide-based high-density marker detection assay and how it could be deployed to maximize the informational content of a single tissue section using translational and clinically established biomarkers in BC, CRC and LC. Briefly, the whole tissue section is physically compartmentalized in large insulated fields. Each field contains a similar representation of tumor tissue from the core to the periphery of the tumor mass and adjacent normal tissue (Figure 1). Marker expression in cancer, stromal and immune cells in these different tissue regions can then be analyzed side by side. The use of cell type-specific protein markers to highlight and enumerate cancer cells ($CK19^{+}$), reactive fibroblasts ($vimentin^{+}$), and immune cells ($CD45^{+}$) will enable the generation of contextual signatures in which the expression changes of each biomarker will be determined in the appropriate cellular compartment(s). In the next 5 years, as the necessary technologies continue to evolve, it will be feasible to acquire contextual expression signatures of 60 or more miRNA and/or protein biomarkers (within the range of current gene-expression signatures) on a single tissue slide in a fully automated manner from multiplex marker detection to computer-assisted image analysis. This exquisitely detailed molecular characterization of tumors will be conducive to personalized cancer treatment.

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Key issues

- miRNAs are short noncoding regulatory RNAs. Chemical and biological features make miRNAs attractive candidates as novel clinical biomarkers.
- Expression profiling experiments have identified diagnostic and prognostic mRNA and miRNA signatures that refine the classification and treatment of tumors based on current pathological and clinical criteria.
- Discrepancies among different studies of individual genes that compose these signatures present a challenge for the selection and clinical implementation of the most robust biomarkers.
- Specific miRNAs have demonstrated clinical utility to improve current diagnostic and prognostic assays. Single miRNAs provide comparable information to multigene mRNA signatures.
- Head-to-head comparisons of performance for miRNA and protein-encoding gene biomarkers will determine redundancy or synergy of combining miRNAs with other translational and clinically established biomarkers.
- Tissue heterogeneity and cell type complexity are confounding and contributing factors to accurate interpretation of expression signatures.
- Molecular assays that extract contextual information from tissue samples should improve the diagnostic power and accuracy of expression analysis.
- RNA-based and tissue slide-based assays can be developed to extract this contextual information. However, tissue slide-based assays will be easier to incorporate in high-volume clinical pathology laboratories.

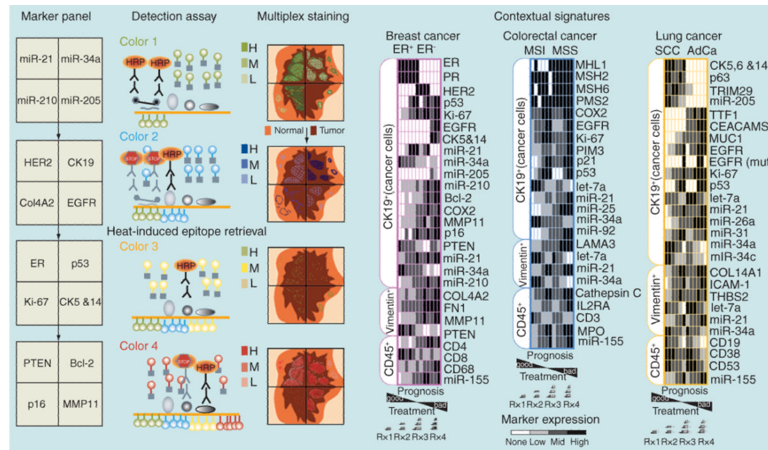

Figure 1. Contextual miRNA and protein signatures for cancer diagnostics and treatment

On the left is a flow chart of a conceptual high-density marker detection platform. Physical compartmentalization yields four contiguous but insulated compartments for the parallel detection of independent biomarkers from the tumor core to adjacent normal tissue. Specific locked nucleid acid-modified DNA probes are dispensed in each compartment (marker panel) and tissue slides are subjected to *in situ* hybridization assay. miRNA signal is revealed by tyramide signal amplification (TSA) reactions with green fluorochrome-tyramine substrate (color 1; detection assay). After hydrogen peroxide incubation to inactivate HRP from the preceding TSA reaction, protein expression is revealed by a new round of TSA reactions with a different fluorochrome–tyramine substrate (color 2). After heat-induced epitope retrieval, expression of additional proteins is revealed by sequential TSA reactions with other fluorochrome–tyramine substrates (colors 3 and 4). In this example, the colorized tissue cartoon provides a virtual rendition of the expected staining pattern for each marker on an $ER^{+}PR^{+}HER2^{-}$ breast cancer specimen (multiplex staining). On the right are tentative and speculative multimarker panels based on biomarkers already incorporated in routine clinical practice and promising translational biomarkers. Computer-assisted image analysis will be used to quantitate the expression levels of each marker. This information will be used to generate contextual signatures that reflect molecular changes within the cancer cells ($CK19^{+}$) or other cellular compartments of the tumor microenvironment, such as reactive fibroblast ($vimentin^{+}$) and immune cells ($CD45^{+}$), to inform treatment selection and intensity in prevalent solid tumors. AdCa: Adenocarcinoma; EGFR: EGF receptor; ER: Estrogen receptor; H: High; HER2: Human EGF receptor 2; HRP: Horseradish peroxidase; L: Low; M: Medium; MMP: Matrix metalloproteinase; MSI: Microsatellite instable: MSS: Microsatellite stable; PR: Progesterone receptor; SCC: Squamous cell carcinoma.

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Table 1

Diagnostic and prognostic applications of miRNA signatures in solid tumors.

Signature	Clinical application(s)	Number in study (n)	Ref.
Breast cancer			
miR-7,-128a,-210,-516-3p	Disease progression (distant metastasis) in $ER^{+}LN^{-}$ cases	147	[88]
miR-30a-3p, -30c, -182	Response to adjuvant tamoxifen in advanced ER^{+} cases	246	[155]
miR-128a, -135a, -767-3p, -769-3p	Recurrence-free survival in ER^{+}cases	207	[90]
miR-27b, -30c, -144, -150, -210, -342	Recurrence-free survival in ER^{-}cases	207	[90]
miR-21,-181a	Recurrence-free and overall survival in all-comers	291	[156]
miR-21,-210,-221,-222	Recurrence-free and overall survival in $ER^{-}PR^{-}HER2^{-}$ cases	49	[91]
Colorectal cancer			
miR-142-3p,-144,-151,-212	Subtype classification: MSS vs MSI-high	59	[50]
miR-17, -20, -25, -32, -92, -93, -106a, -125a, -155, -191,-192,-203,-215,-223	Subtype classification: MSS vs MSI-high	39	[100]
miR-320, -498	Recurrence-free survival in stage II MSS cases	37	[50]
Lung cancer			
let-7a, -7b, -7c, -7d, -7e, -7f, -7g, -7i; miR-16, -17, -19b, -20a, -26a, -26b, -29a, -29b, -29c, -30b, -30d, -98, -103, -106a, -106b, -107, -146b-5p, -181a, -191, -195, -453, -491-5p, -498, -509-3p, -654-5p, -663	Subtype classification: AdCa vs SCC in male smokers	205	[125]
let-7a; miR-221, 137, -182*, -372	Recurrence-free and overall survival in NSCLC cases	112	[157]
let-7e; miR-17-5p, -20a, -20b, -21, -93, -106a, -106b, -126, -146b, -155, -182, -183-191, -200a, -200c, -203, -210, -224	Overall survival in SCC cases	54	[158]
let-7e; miR-34a, -34c-5p, -25, -191	Overall survival in male smoker SCC cases	107	[125]

AdCa: Adenocarcinoma; ER: Estrogen receptor; HER2: Human EGF receptor 2; LN: Lymph node; MSI: Microsatellite instable; MSS: Microsatellite stable; NSCLC: Non-small-cell lung cancer; PR: Progesterone receptor; SCC: Squamous cell carcinoma.

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Table 2

Diagnostic and prognostic applications of single miRNAs in solid tumors.

miRNA	Clinical application(s)	Number in study (n)	Ref.
Breast cancer			
miR-21	Response to neoadjuvant trastuzumab treatment	32	[159]
	Recurrence-free survival in stage I/II all-comers	270	[160]
	Overall survival in all-comers	113	[161]
miR-205	Disease-recurrence and overall survival in $ER^-PR^-HER2^-$ cases	20	[40]
miR-210	Disease progression (distant metastasis) in ER-LN- cases	114	[88]
	Disease progression (distant metastasis) in $ER^-PR^-HER2^-LN^-$ cases	69	[88]
	Recurrence-free and overall survival in all-comers	219,79	[80,89]
	Response to tamoxifen in ER^+ cases	89	[80]
Colorectal cancer			
miR-21	Response to neoadjuvant chemoradiotherapy in advanced rectal cancer	40	[162]
	Recurrence-free survival in stage II colon cases	130	[51]
	Recurrence-free survival in all-comers	44	[101]
	Recurrence-free and overall in all-comers	156	[103]
	Overall survival in all-comers	84 (training) +113 (validation)	[102]
miR-106a	Recurrence-free and overall survival in all-comers	110	[163]
miR-125b	Overall survival in all-comers	89	[164]
miR-145	Response to neoadjuvant chemoradiotherapy in advanced rectal cancer	40	[162]
miR-155	Recurrence-free and overall survival in all-comers	156	[103]
Lung cancer			
let-7a	Overall survival in NSCLC cases	143	[165]
	Overall survival in AdCa cases	52	[131]
miR-21	Overall survival in NSCLC cases	48	[166]
	Overall survival in SCC cases	30	[167]
miR-34a	Recurrence-free and overall survival in NSCLC cases	70	[168]
miR-155	Overall survival in AdCa cases	55	[131]
miR-205	Subtype classification: AdCa vs SCC	122, 102,31	[126,127,130]

AdCa: Adenocarcinoma; ER: Estrogen receptor; HER2: Human EGF receptor 2; LN: Lymph node; NSCLC: Non-small-cell lung cancer; PR: Progesterone receptor; SCC: Squamous cell carcinoma.