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The next thing in prognostic molecular markers: microRNA signatures of cancer

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Cancer is known to be a consequence of the accumulation of mutations and epigenetic alterations of oncogenes and tumor suppressor genes, which cooperate to cause a normal cell to transform into a cancer cell. Thus, the clinical behavior of cancer cells has been logically predicted to reflect the consequences of these mutated and altered genes. This hypothesis has led to the hope that measurement of gene activity or expression can be used as biomarkers to accurately determine which tumors will recur and which are likely to respond to specific treatments. Consequently, for over three decades, there has been an ongoing search to identify mutated or otherwise altered genes that can precisely predict the behavior of tumors with regards to risk for recurrence and response to chemotherapy.

In this issue of *Gut*, Li et al. report on the discovery of a microRNA gene expression signature that shows considerable promise for determining the prognosis of individuals with gastric cancer. Their identification of this panel of prognostic microRNAs for gastric cancer is one of several recent studies that have found this class of small noncoding RNAs to be useful for predicting the behavior of a variety of different cancers, including acute myeloid leukemia, chronic lymphocytic leukemia, colon cancer, pancreatic cancer, and non-small cell lung cancer [1]. These reports highlight that this relatively newly identified class of RNA molecules is showing substantial potential to be used as diagnostic and prognostic biomarkers for cancer. Beyond the promise that microRNAs hold as candidate biomarkers for the early detection and management of cancer, there is also considerable excitement for the use of miRNAs as a novel class of therapeutic targets and as an entirely new class of therapeutic agents for the treatment of cancers.

RNA and microRNA

In order to appreciate where our understanding of microRNAs is with regards to cell physiology and disease, it is important to place them in the context of our current understanding of RNA and DNA in the molecular biology of the cell. The role of RNA as a messenger for translating DNA sequence code into proteins has been understood for decades and is a canonical function of messenger RNA. Additional functions of RNA that have been discovered include the identification of ribozymes and RNA species with structural roles. Over the last decade, we have come to appreciate that in addition to messenger RNAs, ribozymes, and structural RNAs, there is a class of small RNA molecules, called microRNAs, that represent an important layer of the gene regulation network, which traditionally has been known to include transcription factors, proteins that regulate mRNA

stability, proteins that regulate the translation of mRNA into protein, and factors that control protein degradation. Remarkably, microRNAs may regulate the expression levels of nearly all genes in most multi-cellular organisms, and appear to provide a means for modulating the mRNA and proteins levels in cells [2]. The profound significance of microRNAs and the process of gene expression regulation by small RNAs (also known as ‘RNA interference’) in biology was recognized in 2006 by the award of the Nobel Prize in Physiology or Medicine to Andrew Z. Fire and Craig C. Mello, who carried out some of the seminal studies that defined the effect of miRNAs and other small RNAs on gene regulation [3].

What are microRNAs?

So, what exactly are these remarkable molecules that appear to have a fundamental role in the biology of the cell? MicroRNAs are a class of non-protein encoding RNA molecules that are approximately 22 nucleotides in length and that function in most cases to repress the activity of specific mRNA molecules, either by promoting their degradation or by preventing their translation into protein. Currently, there are at least 700 microRNAs known to be encoded by the human genome. MicroRNAs function by direct binding to target mRNAs via sequence-specific complementarity, and a single microRNA can control the activity of multiple mRNA targets. MicroRNAs have been estimated to play a role in regulating anywhere from 30–100% of proteins through modulating the expression levels of the genes encoding these proteins. [1]. MicroRNAs are evolutionarily conserved and can be located in the introns or exons of genes, or in the sequence between genes (intergenic sequence). The microRNAs that are embedded in the introns and exons of genes are most often in the sense orientation of the host gene and are usually transcribed when the rest of the gene is transcribed. MicroRNAs located in intergenic regions are regulated as independent transcriptional units. The miRNAs undergo a relatively complicated biogenesis that starts with being synthesized as a long primary transcript, typically by RNA polymerase II. The primary transcript (pri-miRNA) can be up to several kilobases in length and undergoes processing in the nucleus by a “microprocessor” protein complex composed of the proteins DGCR8 and Drosha, generating an approximately 70 nucleotide long precursor that has a characteristic “hairpin” double-stranded RNA secondary structure due to self-complementarity within the molecule (pre-miRNA) [4]. The pre-miRNA is then exported out of the nucleus and into the cytoplasm by the protein exportin 5. In the cytoplasm, pre-miRNA molecules are bound to the RNase Dicer, which cleaves them to produce a 22 base-pair, double-stranded RNA molecule. One strand (the active, or ‘guide’ strand) is then loaded into the RNA-induced silencing complex (RISC), while the inactive, ‘passenger’ strand is removed and degraded. RISC is composed of the transactivation-responsive RNA binding protein (TRBP) and Argonaute 2 (Argo2). The guide strand can then recognize complementary sequence of messenger RNA via its association with RISC. Target recognition is directed by sequence complementarity between nucleotide positions 2–8 in the miRNA (the “seed” region) and sequences located in the 3’ untranslated region of target mRNAs. The binding of the miRNA to the messenger RNA guides the miRNA-RISC complex to repress gene expression by inducing mRNA degradation and/or inhibiting the translation of the mRNA. Although it is known that a single species of miRNA can affect the expression levels of many genes, it is not yet clear how the specificity of this function of the miRNAs is regulated. Nonetheless, it is clear that during the biogenesis process there are a host of mechanisms that govern the transcription and post-transcriptional regulation of the miRNAs. The discovery of these mechanisms has helped in our understanding of how miRNAs can be deregulated in a variety of disease states, including cancer, although there is still a great deal of work that needs to be done to obtain a more precise understanding of how miRNAs regulate the expression levels of the genes that they affect.

What do microRNAs do, and what role do they play in cancer?

In light of the broad ranging effects of microRNAs on proteins in cells, it is not unexpected that microRNAs (miRNAs) have been found to be involved with a myriad of biological processes, including but not limited to proliferation, apoptosis, metabolism, differentiation, epithelial-mesenchymal transition, etc., and they have been implicated in many benign and malignant diseases [1, 5, 6, 7, 8, 9]. With regards to cancer, it is notable that approximately half of the microRNAs discovered to date are located near fragile sites, in loci associated with loss of heterozygosity, in loci associated with DNA amplification, and in common breakpoints [10]. This finding raises the possibility that the targets of these DNA alterations may actually be the microRNAs and not the conventional genes located at these loci. In light of this interesting association of cancer associated genomic alterations and miRNAs, the development of high throughput technologies for assessing microRNAs has led to the use of these techniques to study the global expression patterns of microRNAs in cancer, which has been called the microRNAome [11, 12].

Although dozens to hundreds of microRNAs have been shown to be deregulated in cancers, the set of microRNAs that actually play a pathogenic role in cancer has not yet been fully defined. It is suspected that a subset of microRNAs will be shown to be functionally important in primary cancers because of the following pieces of evidence: 1) There are tumor-specific microRNA signatures which accurately distinguish different sub-types of cancers, suggesting that specific microRNAs provide a clonal growth advantage in different tumor types, likely depending on the context of the other gene mutations present in the cancer [13, 14]; 2) manipulation of microRNAs in cancer cell lines can directly regulate fundamental behaviors of cancer cells, such as proliferation, apoptosis, etc. [15]; and 3) many of the microRNAs deregulated in cancers have been shown to have oncogenes, tumor suppressor genes, and signaling pathway components as direct targets [3, 16]. Thus, microRNAs are another class of molecules whose deregulation ultimately contributes to cancer formation, and they likely cooperate with the other classic oncogenes and tumor suppressor genes in the cancer cells to drive the behavior of the tumors.

What do we know about the role of miRNAs in gastric cancer?

Over the last several years the expression of microRNAs has been assessed in a variety of cancers, including gastric cancer. In essentially all cancers studied to date, at least a subset of microRNAs are over- or under-expressed. Some of these appear to be commonly deregulated in a broad range of cancer types, such as miR-21 and let-7a, while others appear to be more specific for distinct tumor types [4, 17]. With regard to gastric cancer, investigators have identified a number of microRNAs whose expression is deregulated when compared to normal stomach, including miR-21, miR-34b, miR-34c, miR-27a, miR-106a, miR-128a, miR-128b, miR-129, miR-148, miR-433, and miR-9 [18, 19, 20, 21]. In a comprehensive screen of microRNAs, Guo and colleagues identified miR-20b, miR-20a, miR-17, miR-106a, miR-18a, miR-21, miR-106b, miR-18b, miR-421, miR-340, miR-19a and miR-658 as being over- or under-expressed in a set of three moderately differentiated intestinal type gastric cancers [22]. Studies in cell lines has provided evidence that at least some of these microRNAs affect the expression of signaling pathway proteins, like Grb2 (an adapter protein that transmits activation states from ligand-bound receptors), PTEN, Rb, etc. [21, 22]. In their study published in *Gut*, Li and colleagues have now logically extended our understanding of microRNAs in gastric cancer by correlating microRNA deregulation with the clinical behavior of gastric cancer. They have identified and validated a microRNA expression signature that can distinguish patients with a poor prognosis from those with a good prognosis. These findings are significant because they address a key clinical problem at this time, namely the suboptimal state of the current parameters used for determining the

prognosis of patients with stomach cancer [23]. Indeed, the modest performance of pathological features for determining the prognosis of gastric cancer has led to an intense effort to identify specific genes and global gene expression patterns that can better predict the behavior of the tumors [24].

Current state of molecular prognostic markers and gastric cancer

Given the heterogeneous behavior of gastric cancer and the modest predictive value of pathologic staging for determining the prognosis of these patients, the correlation between the clinical behavior of stomach cancer and the presence of specific oncogenes or mutated tumor suppressor genes has been assessed extensively. Furthermore, the expression patterns of specific genes and gene clusters has also been subject to intense investigation to determine if they can serve as molecular markers for the clinical behavior of stomach cancer [24]. However, to date, the vast majority of these studies have only been conducted using small, retrospectively collected tissue sets and have used non-validated assays that are of questionable reproducibility [23, 25, 26]. The consequence is that despite the effort that has gone into identifying biomarkers for gastric cancer, there are essentially no biomarkers that have been proven to be useful clinically at this time. The most promising biomarkers that have been discovered to date include thymidylate synthase (TS) and excision repair cross complementing (ERCC) 1 gene expression for predicting response to 5-fluorouracil and cisplatin, respectively; HER2 expression in differentiated gastric cancer; *TP53* mutation; and the high expression of claudin-4, vascular endothelial growth factor, interleukin-8, cyclin E, urokinase-plasminogen activator, and xanthine oxidoreductase [26, 27, 28, 29, 30, 31, 32]. However, none of these biomarkers are currently recommended for clinical use in managing patients with gastric cancer [33].

miRNAs and prognosis in gastric cancer

In light of the dearth of clinically useful biomarkers for gastric cancer, the studies by Li et al. address a critical need in gastrointestinal oncology. Furthermore, their studies highlight the potential of microRNAs to be used as cancer biomarkers. microRNAs are particularly attractive in this role because their expression appears to be stable, they can be detected with robust clinical assays, and they can be detected in tissues that have been formalin-fixed using standard clinical protocols [4]. The prognostic value of microRNAs has already been assessed in esophageal squamous cell cancer (ESCC), colon cancer, pancreatic cancer and liver cancer [17]. MicroRNAs found to associate with a poor prognosis include miR-103, miR-107, (ESCC); miR-21 (colon cancer); miR-106a (pancreatic ductal adenocarcinoma); miR-125b, miR-100, miR-150, miR-99a, miR-31, miR-220, miR-26b, and miR-29a (hepatocellular carcinoma). Li et al. now demonstrate that a panel of seven microRNAs that include miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p, and miR-126 are robust stage-independent markers for the prognosis of gastric cancer. A strength of their study is the study design which included not only a test set of cancers after a study was performed to identify the most promising microRNAs (training set), but an independent validation set of cases. The confirmation of the signature in the validation set makes it likely that an independent assessment of this miRNA panel in other patient sample sets will further validate their findings and ultimately lead to biomarkers that can be translated into the clinic. An important extension of these results will be to determine whether these microRNAs, or others, can be used to predict the response to specific treatments for gastric cancer.

What remains to be determined about microRNAs and gastric cancer?

We have only a nascent understanding of the role of microRNAs in cancer in general, and particularly in gastric cancer. Given the rapid pace of improvements in microRNA expression profiling technologies and given that new microRNA continue to be discovered,

it is likely that future studies will identify other expression signatures that may be even more informative for predicting the behavior of gastric cancer. Furthermore, the vast majority of studies to date have been done with gastric cancer from geographic areas with high rates of gastric cancer (e.g. China, Korea, and Japan). Similar studies from low frequency areas (e.g. western Europe, United States, etc.) are needed to determine whether the same patterns of microRNAs are observed and whether they have the same correlations with clinical behaviors of the tumors. The study by Li et al. suggests that microRNAs may ultimately be more successful at prediction than the other potential biomarkers discovered to date for gastric cancer, and that this newly discovered class of molecules may prove to be the clinically useful molecular markers that were predicted when oncogenes and tumor suppressor genes were first discovered decades ago.

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