

Molecular Pathways: microRNAs, Cancer Cells, and Microenvironment

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Abstract

One of the most unexpected discoveries in molecular oncology over the last decade is the interplay between abnormalities in protein-coding genes and short noncoding microRNAs (miRNA) that are causally involved in cancer initiation, progression, and dissemination. This phenomenon was initially defined in malignant cells; however, in recent years, more data have accumulated describing the active participation of miRNAs produced by microenvironment cells. As hormones, miRNAs can be released by a donor cell in various forms of vesicles or as "free" molecules secreted by active mechanisms. These miRNAs spread as signaling molecules that are uptaken either as exosomes or as "free" RNAs, by cells located in other parts of the organism. Here, we discuss the communication between cancer cells and the microenvironment through miRNAs. We further expand this in a more translational context and present miRNAs as predictors of treatment response, as crucial agents in targeted therapeutics, and as significant molecules to target. *Clin Cancer Res*; 20(24); 6247–53. ©2014 AACR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Editor's Disclosures

The following editor(s) reported relevant financial relationships: P.S. Steeg reports receiving commercial research grants from Geron, GlaxoSmithKline, and Sanofi.

CME Staff Planners' Disclosures

The members of the planning committee have no real or apparent conflicts of interest to disclose.

Learning Objectives

Upon completion of this activity, the participant should have a better understanding of what the short noncoding microRNAs are and how they work as hormones, their contribution to the interplay between the cancer cells and the tumor microenvironment, and how clinicians can use this knowledge to develop better predictors for resistance to therapy and new therapeutic strategies for cancer patients, including those with advanced metastatic disease.

Acknowledgment of Financial or Other Support

This activity does not receive commercial support.

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doi: 10.1158/1078-0432.CCR-13-2500

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Background

The relationship between cancer cells and their surrounding microenvironment is recognized as fundamental for tumor development, progression, invasion, and metastasis, which generally results in patient death (1). Several studies have demonstrated that the role of the microenvironment, composed of stromal stem/progenitor cells, cancer-associated fibroblasts (CAF), immune inflammatory cells, endothelial cells, and pericytes, is that of a "game changer," modifying the progression of a tumor at its site or keeping it in a dormant stage (1–4). Within the past few years, a plethora of studies have demonstrated that the communication between various types of tumor microenvironment cells and cancer cells is regulated by a peculiar category of short transcripts that do not codify for proteins but certainly regulate protein expression (5). These molecules, called microRNAs (miRNA), are small 19- to 25-

nucleotide noncoding RNAs (ncRNA) that regulate gene expression by hybridizing to complementary target messenger RNAs (mRNA), resulting in either the translation inhibition or mRNA degradation (6). miRNAs are phylogenetically conserved and are involved in most biologic processes, including cell-cycle control, apoptosis, vascular development, cell differentiation, immune control, and metabolism (7–10). Besides acting as oncogenes or tumor suppressors in signaling pathways involved in cancer initiation, progression, and development of metastatic patterns (11), miRNAs appear to be involved in a large spectrum of disorders, including cardiovascular, immune, and neurologic diseases (12).

"The RNA world" hypothesis describes the primordial origin of "living" organisms billions of years ago as containing only RNA as genetic material. The first signaling molecules throughout the genome were most likely short, stable RNA sequences, quite similar to circulating miRNAs (13). Although the secretory mechanisms involving miRNAs remain yet unclear, suggested mechanisms include passive leakage from cells with short half-lives, such as platelets, or cells that undergo apoptosis or necrosis (14), active secretion via cell-derived membrane, including exosomes, microvesicles, and apoptotic bodies (nanovesicles; ref. 15), and active secretion of miRNAs in complexes with lipoproteins (e.g., high-density lipoprotein) or with proteins (e.g., Ago2; ref. 16). Consequently, miRNAs shuttle between various types of cells using short distance cell-to-cell movements or long distance tissue-to-tissue movements (Fig. 1).

Fundamentals of communication by miRNA

Functional effects of miRNAs transferred between various types of cells present at the tumor site. Until recently, the effects of extracellular miRNAs on receptor cells (defined as the cells that absorb external miRNAs) had not been experimentally proven. New evidence has shown that miRNAs move from one type of cell to another and that they produce functional effects that generally inhibit tumor development. For example, normal epithelial prostate PNT-2 cells release the tumor suppressor miR-143 that has been shown to induce growth inhibition *in vitro* and *in vivo* exclusively in prostate cancer cells (17). Intercellular transfer of miR-142 and miR-223 from immune cells to malignant cells (hepatocellular carcinoma cells) inhibits proliferation of malignant cells and also causes a reduction in endogenous levels of STMN1 (stathmin-1), involved in the regulation of the microtubule filament system, specifically by destabilizing microtubules (18).

On the other hand, the malignant compartment of the tumor can also influence the microenvironment by coordinated miRNA release. Exosomes derived from hypoxic leukemic K562 cells have been found to carry and release miR-210, among other angiogenic miRNAs, increasing tube formation by human umbilical vein endothelial cells (19). The direction of miRNA signaling is critical in order to determine the subsequent effect. For example, when miR-223 circulates from tumor-associated

macrophages (TAM) to breast cancer cells, it reduces the expression of myocyte-specific enhancer factor 2C (Mef2C) leading to nuclear accumulation of CTNNB1 (β -catenin) and an increase in cell migration (20). From the opposite direction, miR-210, the most overexpressed miRNA, under hypoxic conditions (21), is released from metastatic breast cancer cells to endothelial cells, promoting angiogenesis and metastasis through a neutral sphingomyelinase 2-dependent exosomal transfer (22).

Clustered miRNAs acting synergistically on targets in both malignant and stromal cells. miRNA clusters are made up of miRNAs that are located in very close genomic loci separated by less than a few hundred nucleotides and are under the same transcriptional control. For example, the miR-15a/miR-16-1 cluster, located on chromosome 13q, has been found to be deleted in the most common type of leukemia in the Western world, chronic lymphocytic leukemia (CLL; ref. 23). These two clustered miRNAs share a similar 5' or seed region that is important for pairing with target mRNA, including mRNA of several important cancer-related genes, such as BCL2, MCL1, MSH2, c-JUN, WT1, and TP53 (24, 25). It has been reported that both miR-16-1 and miR-15a are present in microvesicles and can be secreted from leukemic cells (26). Furthermore, although they are downregulated in malignant cells due to their location in a deleted genomic region, the levels of these miRNAs in plasma are high and do not differ significantly from the levels in normal individuals, supporting the concept that both miRNAs are secreted not only by malignant cells but also by microenvironment cells (26). In concordance with this, miR-15a and miR-16-1 are also important in prostate cancer stroma in which they were found to be downregulated in CAFs surrounding the prostate tumor. Both genes promoted tumor growth and progression through the reduced posttranscriptional repression of FGF-2 and its receptor FGFR1, which act on both stromal and tumor cells to enhance cancer cell survival, proliferation, and migration (27). In this case, both miRNAs have concordant effects when coexpressed either in malignant cells or stromal cells.

miRNA regulation of multiple tumor components. Maintenance of epithelial tissues requires the stroma (3, 28, 29). Both stroma and epithelium change and adjust as the tumor undergoes transformation toward being more invasive and therefore metastatic. The primary tumor microenvironment is characteristically different from a metastatic microenvironment (3, 28–30) as we will present in the following examples. Natural killer (NK) cells are potent malignant cells killers, and it has been shown that TGF β treatment abrogates their killer effect. A recent study revealed that TGF β induced the expression of miR-183, which represses DAP12 transcription/translation in tumor-associated NK cells, a protein important for signaling pathways involving several cytotoxicity receptors (31). An additional example involves alternatively associated macrophages (AAM) that can be identified by the presence of the MRC1/CD206 mannose receptor. In MRC1-positive AAMs (as well as TAMs), the involvement

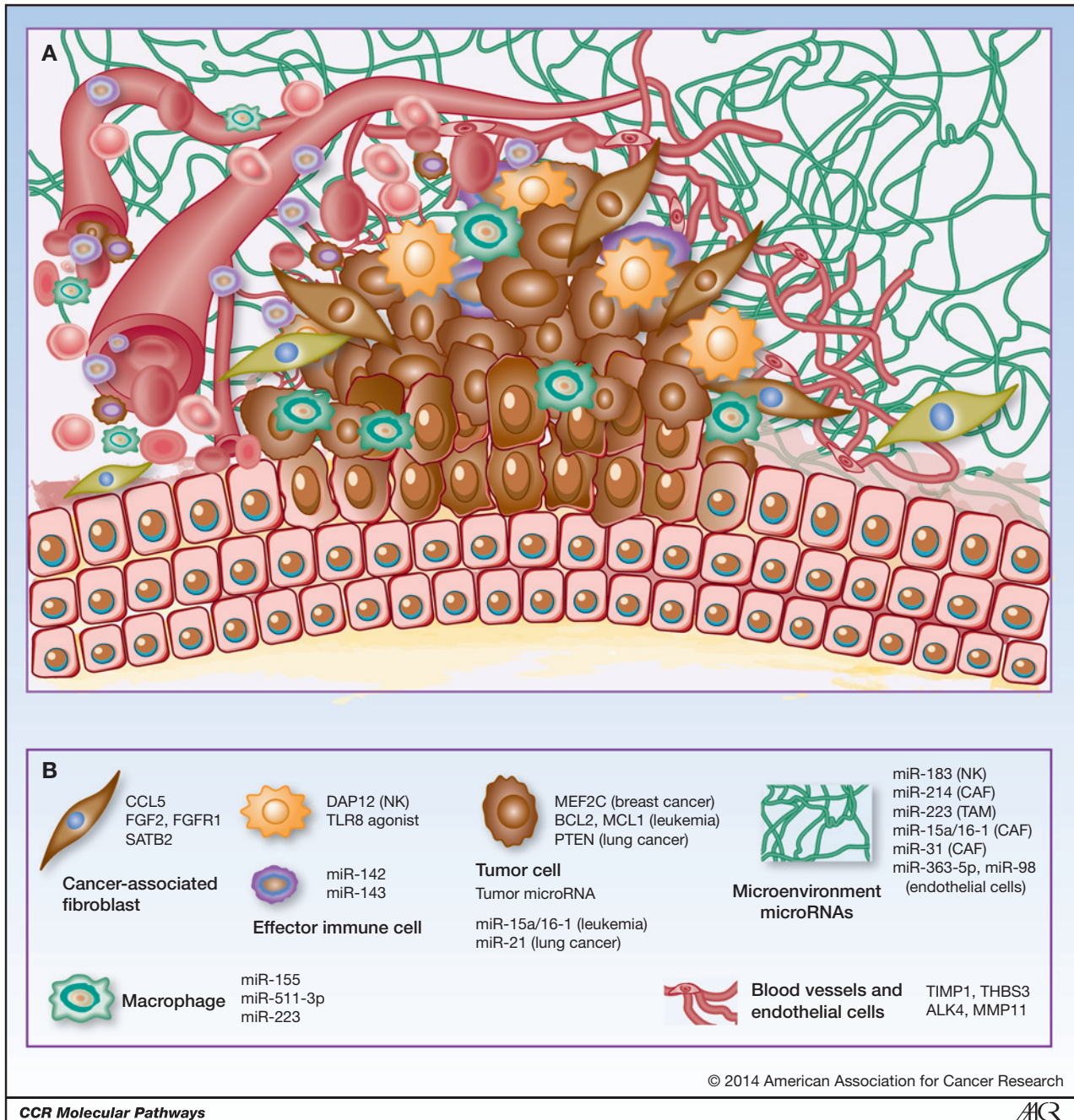


Figure 1. miRNAs as signaling molecules between malignant cells and microenvironment cells. A, the secreted miRNAs by the malignant or microenvironment component of the tumor contribute to tumor development and migration; consequently the patient develops metastases and the overall survival is reduced. Because of the therapeutic intervention on malignant cells (blocking of oncogenic miRNAs) and microenvironment cells (stimulation of secretion of suppressor miRNAs), the tumor can be reduced, but rarely disappears without surgical intervention where possible. B, examples of miRNA interaction with target coding genes and the specific types of cells where this occurs. Each color represents an interactor pair, miRNA–mRNA.

of miR-155 and miR-511-3p in protumoral activity has been identified (32).

Fibroblasts are known to represent major constituents of the extracellular matrix (ECM) and are involved in several cellular mechanisms, including wound repair (29). At a tumor site, fibroblasts acquire, through miRNA and other

signaling molecules, a distinct phenotype and become CAFs. Low expression of miR-31 and miR-214 and high expression of miR-155 have been found to be involved in reprogramming quiescent fibroblasts to CAFs in ovarian cancers. At least one of these miRNAs, namely miR-214, was found to directly target the CCL5 (C–C motif ligand 5)

chemokine important for CAF function (33). Furthermore, miR-31 was reported to be the most downregulated miRNA in CAFs isolated from endometrial cancer when compared with normal endometrial fibroblasts. miR-31 directly targets the homeobox gene SATB2, which is responsible for chromatin remodeling and regulation of gene expression, and is significantly elevated in CAFs. Overexpression of miR-31 significantly impaired the ability of CAFs to stimulate tumor cell migration and invasion, without affecting tumor cell proliferation. Increased levels of SATB2 homeobox in CAFs revealed a reciprocal finding to miR-31: SATB2 increased tumor cell migration and invasion, whereas knockdown of endogenous SATB2 in CAFs reversed this phenotype (34). Likewise, miR-21 is known to be an activator in the transition of fibroblasts into CAFs and is associated with stroma that is secreted by fibroblasts involved in the migration and invasion capacity of esophageal squamous cell carcinoma (35).

miRNAs as angiogenic signals for vascular cells. Cancer metastasis is a long and inefficient process (4); however, when it becomes efficient (i.e., the cancer cells prevail against the microenvironment), metastasis greatly limits the effects of various cancer therapies and reduces overall survival. Several miRNAs have been identified as involved in the angiogenic process that ensures the survival of cancer cells. An example is miR-130, known to be involved in angiogenic mechanisms in colorectal cancer. Under hypoxic conditions, miR-130 modulates HIF1 α , an inducer of VEGF (36). miR-200, previously described as a regulator of angiogenesis, is downregulated by miR-130b in endometrial cell lines through DICER1 reduction (37). miR-363-5p regulates endothelial cell properties and their communication with hematopoietic precursor cells (38). At the post-transcriptional level, tissue inhibitors of metalloproteinase-1 (TIMP-1) and thrombospondin-3 (THBS3) are regulated by miR-363-5p. Furthermore, miR-363-5p inhibition using anti-miRs has been shown to affect endothelial cell angiogenic properties (such as the response to stimulation by angiogenic factors) and the interaction between endothelial cells and hematopoietic precursors. miR-98 inhibits angiogenesis by modulating endothelial cell activities, including cell spreading, cell invasion, and tubule formation (39). This occurs by targeting activin receptor-like kinase-4 (ALK4) and matrix metalloproteinase-11 (MMP-11). Likewise, rescue experiments reversed the antiproliferative, anti-invasive, and antiangiogenic effects of miR-98 (Fig. 1; ref. 39).

Clinical-Translational Advances

miRNAs as therapeutic response predictors

As hormones, miRNAs are released by a donor cell and spread signals that affect cells in other parts of the organism (20). The development of sensitive detection technologies (such as quantitative real-time PCR, microarray, and deep sequencing), has produced strong evidence that serum/plasma, urine, and saliva, as well as seminal, amniotic, and pleural effusions from patients with cancer have a distinct

miRNA expression profile. In addition to their diagnostic or prognostic value, circulating miRNAs can serve as predictors of chemotherapeutic response (40). The main advantage of using miRNA signatures (combinations of multiple miRNA expressions) in body fluids versus tumoral cells as predictors of response or prognosis results from their direct transmission between cancer cells and associated stromal cells. For example, serum/plasma levels of miR-21 successfully predicted chemotherapy response in multiple cancer types, including cancers of the prostate (41), lung (42), esophagus (43), and pancreas (44). Very likely the extracellular levels of this miRNA reflect the levels from the full tumor, including malignant and adjacent stromal cells, as miR-21 is overexpressed in every type of tumor studied to date (45). miR-155 was identified in circulating microvesicles from both subjects with a premalignant condition called monoclonal B-cell lymphocytosis, as well as from patients with CLL, the consequent malignant stage (26). It was further found that patients who failed to achieve a complete response (CR) to initial therapy had significantly higher miR-155 expression levels than did patients who obtained a CR.

Targeting miRNAs from microvesicles and exosomes

miRNA therapeutics represents the full arsenal of strategies used to restore or to block the functions of suppressor miRNAs or oncogenic miRNAs, respectively (46). These strategies take advantage of the ability of miRNAs to target genes implicated in the same pathway and/or in interacting pathways; therefore, by targeting multiple miRNAs, miRNAs could control the targets in a more powerful fashion than using siRNAs, small molecules, or ribozymes that interact, by design, with only one specific target. A peculiarity of this approach is that miRNA activity can be dependent on the cellular environment, and the same miRNA can have different sets of coding and noncoding gene targets in different cell types. For example, miR-21 expression promotes growth, metastasis, and chemo- or radioresistance by targeting PTEN in non-small cell lung cancer cells (47), whereas the same miRNA binds to Toll-like receptors (TLR) as an agonist to induce a prometastatic inflammatory response in immune cells associated with lung tumors (48). Therefore, the modulation of a specific miRNA may have concordant effects by complementary mechanisms in distinct cell types.

As miRNAs shuttle between various types of cells composing a tumor, one way to "perturb" this mode of transport is to stop extracellular miRNAs in exosomes. It has been shown that the small molecule GW4869, an inhibitor of neutral sphingomyelinase that is also known to inhibit miRNA and exosome secretion, can be effectively used to interrupt miRNA-mediated aberrant cross-talk between cancer cells and surrounding immune cells within the tumor microenvironment (48, 49). miR-21 and miR-29a can be released by cancer cells within exosomes and are engulfed by macrophages in the tumor microenvironment expressing TLRs. These miRNAs bind to and activate TLR8, specific for single-stranded RNA, leading to increased

secretion of IL6 and TNF α by immune cells and increased cancer cell proliferation and metastatic potential (48). It has also been shown in mice that extracellular let-7 (a highly abundant regulator of gene expression in the central nervous system) can activate *TLR7* and induce neurodegeneration through neuronal *TLR7* (50). Intriguingly, let-7b levels are higher in the cerebrospinal fluid of patients with Alzheimer disease, indicating that miRNA-mediated activation of TLRs may have implications beyond cancer.

The use of molecules that block the functions of specific miRNAs (such as LNA anti-miR-21 and LNA anti-miR-29a) in tumor cells could reduce miRNA levels in exosomes released by cancer cells and effectively decrease miRNA-mediated TLR activation (48). Likewise, it can be hypothesized that miR-21 or miR-29a could be mutated in such a way that they retain the ability to bind to TLRs but fail to activate them, thereby counteracting the cross-talk between cancer-released miRNAs and TLRs. Moreover, genetically engineered TLR decoy molecules could be designed to bind and sequester miRNAs released by cancer cells in the tumor microenvironment without triggering TLR-activated signaling transduction pathways.

An additional strategy targeting miRNA transport involves the use of antibodies that recognize tumor-specific antigens expressed by cancer-released exosomes; some of the antigens most likely have reduced antigenic properties and permit the production of cancer-released exosomes without any obvious stimulation of the immune system. Finally, we can envision a therapeutic strategy in which cells are stimulated to secrete oncogenic miRNA-loaded nanovesicles and the patient with cancer is subsequently treated with dialysis, as a way to "wash out" oncogenes from cancer cells.

First clinical trials with miRNAs

miRNAs represent promising therapeutic agents, and several pharmaceutical companies already have miRNA therapeutics in their developmental pipeline (48, 51). For instance, Regulus Therapeutics is actively exploring the value of anti-miRs in the treatment of diseases such as fibrosis, hepatitis C virus (HCV) infection, atherosclerosis, and cancer. MIRagen Therapeutics is using chemically modified structures of miRNA (including miR-15/195, miR-29, and others) in work that has reached preclinical development in pathologies such as metabolic and cardiovascular diseases. MRX34, a liposome-formulated mimic of the tumor suppressor miR-34, developed by Mirna Therapeutics, produced complete tumor regression in orthotopic mouse models of liver cancer, with no observed immunostimulatory activity or toxicity to normal tissues. These results have prompted a phase I clinical trial, which is currently recruiting patients with advanced or metastatic liver cancer.

Along this line of strategy, Santaris Pharma A/S has developed Miravirsin, an anti-miR-122 agent for treatment of HCV infection, a risk factor for the development of the hepatocellular carcinoma. miR-122 is an abundant liver-specific miRNA crucial for efficient replication of the virus,

and its downregulation in hepatocellular carcinomas is associated with a poor prognosis (52, 53). When miR-122 is inhibited, the replication of the RNA is halted in cultured cells, supporting the development of anti-miR-122 compounds for the treatment of HCV. A phase I clinical trial demonstrated that antagomiR-122 has dose-dependent pharmacology and is well tolerated. When investigated in a phase II clinical trial, Miravirsin was found to be well tolerated in patients with HCV, with mild side effects, including light coryza, diarrhea, and headache. Importantly, the administration of Miravirsin in patients with chronic HCV-1 displayed extended dose-dependent diminutions in HCV RNA levels without any manifestation of viral resistance (52).

Conclusions

The discovery that miRNAs can circulate between various types of cells and provoke biologic effects, either supportive or inhibitory for tumor growth or dissemination, has revolutionized the way we think about tumor initiation and metastatic pyramidal cascades. Important players in this process include various other extracellular components such as cytokines (IL1, TNF α or IL6), chemokines (CXCL1, CXCL2, or CXCL12), the NF- κ B activation pathway, VEGF, platelet-derived growth factors (PDGF), and components of the ECM (MMP, collagen type 1, or fibronectin). The principles presented in this review apply to any type of human disease or biologic process in which miRNAs are involved. For example, it has recently been shown that extracellular let-7 miRNA activates nociceptor neurons to elicit pain via direct binding to *TLR7* and its coupling with TRPA1 ion channel (54). Given our current knowledge, we have confidence that miRNA-mediated intercellular communication will be identified with increasing frequency as a fundamental biologic process that can be manipulated by drugs for the treatment of cancer as well as many other diseases. Ultimately, as the ncRNA world is quickly populated by longer transcripts (55), development of successful therapeutic strategies will require scientists to identify and comprehend the functions of these ncRNAs in both malignant and microenvironment cells.

Authors' Contributions

Conception and design: I. Berindan-Neagoe, G.A. Calin

Development of methodology: I. Berindan-Neagoe

Writing, review, and/or revision of the manuscript: I. Berindan-Neagoe, G.A. Calin

Study supervision: I. Berindan-Neagoe

Acknowledgments

The authors apologize to colleagues whose important work was not cited due to space limitations. The authors thank Julianna Bronk for the critical reading of this article and editing.

Grant Support

G.A. Calin is the Alan M. Gewirtz Leukemia & Lymphoma Society Scholar. Work in Dr. Calin's laboratory is supported in part by the NIH/NCI grants 1UH2TR00943 and 1R01CA182905; Developmental Research Awards in Prostate Cancer, Multiple Myeloma, Leukemia (P50CA100632), and Head and Neck (P50CA097007) SPORes; a Sister Institution Network Fund (SINF) University of Texas MD Anderson Cancer Center DKFZ grant in chronic lymphocytic leukemia (CLL); a SINF grant in colon cancer; a Kidney Cancer Pilot Project; the Duncan Family Institutional Seed Funds; the Blanton-Davis

Ovarian Cancer—2013 Sprint for Life Research Award; the Laura and John Arnold Foundation; the RKG Foundation and the Estate of C.G. Johnson Jr; and the CLL Global Research Foundation. I. Berindan-Neagoe is a Fulbright Scholar. Work in Dr. Berindan-Neagoe's laboratory was financed by POSCCE 709/2010 grant, with the title "Clinical and economical impact of proteome

and transcriptome molecular profiling in neoadjuvant therapy of triple negative breast cancer (BREASTIMPACT)."

Received April 8, 2014; revised June 27, 2014; accepted July 18, 2014; published online December 15, 2014.

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