Published OnlineFirst December 7, 2012; DOI: 10.1158/0008-5472.CAN-12-3229

Review

Cancer Research

TLRs as miRNA Receptors

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Abstract

MicroRNAs (miRNA) are small noncoding RNAs with gene regulatory functions. Their expression is frequently dysregulated in almost all human tumors and they can be found circulating within exosomes secreted by cancer cells. In addition to being promising cancer biomarkers with diagnostic, prognostic, and theranostic implications, circulating miRNAs have also important biologic functions: they can be engulfed by immune cells surrounding cancer cells within the tumor microenvironment and bind to toll-like receptors (*TLR7* in mice and TLR8 in human) expressed by the immune cells. As a result, the binding miRNAs function as agonists of these single-stranded RNA-binding TLRs, leading to NF-κB signaling activation and secretion of interleukin (IL)-6 and TNF-α, which promote cancer cell growth and metastasization. This novel miRNA mechanism of action suggests that these small noncoding RNAs can act as hormones (we call these miRNAs hormone miRNAs or H-miRNAs). The discovery that miRNAs released by cancer cells can bind to a receptor in a surrounding immune cell is completely novel. Other receptors (in addition to *TLR7* and TLR8) are likely to be found, but this is the first identified miRNA receptor and it is relevant to cancer. This review discusses the meaning of this discovery and comments on the exciting future implications of these findings in the context of tumor microenvironment biology as well as of other human diseases. *Cancer Res; 72(24); 6333–7. ©2012 AACR.*

Introduction

miRNAs are small noncoding RNAs, 19 to 24 nucleotides (nt) in length, with gene regulatory functions (1), whose expression is frequently dysregulated in tumors (both solid and hematologic malignancies) with respect to the normal tissue counterpart (2). miRNAs are involved in human carcinogenesis by directly silencing tumor-suppressor genes or releasing oncogenes from their inhibitory posttranscriptional regulation (these properties are excellently reviewed in refs. 2 and 3). Several studies have shown that both genetic and epigenetic factors are responsible for miRNA aberrant levels in tumor cells. For instance, it has been shown that miRNAs can be transactivated or silenced by specific transcription factors (4–7), chromosomal deletions or amplifications (5, 8), as well as point mutations (9, 10), and epigenetic factors (such as promoter methylation status and histone modifications) also affect miRNA expression levels (11–13). Overall, it can be concluded that miRNAs essentially undergo the same regulatory mechanisms of any other protein coding gene (PCG). Specific signatures of dysregulated miRNAs harbor diagnostic, prognostic, and theranostic implications (10, 14). Interestingly, several groups have shown that miRNAs can be detected in blood, saliva, urine, and pleural effusions, and different levels of specific circulating miRNAs differentiate patients with cancer from healthy donors (15, 16). It has been shown that miRNAs can be released by cancer cells in 2 forms: within microvesicles called exosomes (15, 17) or bound to the Argonaute2 protein, a key component of the miRNA-mediated silencing machinery (18). The outbreaking discovery that miRNAs can be detected in blood (and other human body fluids) and can identify patients with cancer from healthy individuals generated a lot of enthusiasm in the scientific community because it revealed that miRNAs can be considered as novel cancer biomarkers. Although this is certainly true and justifies the efforts of translational researchers to investigate sensitivity and specificity of circulating miRNAs in the diagnosis (and prognosis) of cancer, there is certainly another dimension to this discovery: the biologic significance of miRNA secretion by cancer cells. In their seminal discovery, Valadi and colleagues showed that miRNAs released by mast cells within exosomes can actually be taken up by another mast cell and target mRNAs in the recipient cell, showing that exosome-released miRNAs represent a novel mechanism of cross-talk and genetic exchange between cells (19). A role for miRNAs as molecules involved in intercellular communication is particularly fascinating, especially in light of the elegant demonstration that RNA represents the most evolutionarily ancient form of nucleic acid (20), and therefore, also likely the primordial cell–cell cross-talk mediator.

miRNAs as receptor ligands

The identification of circulating miRNAs released by cells within exosomes and the demonstration of their ability to be

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doi: 10.1158/0008-5472.CAN-12-3229

Published OnlineFirst December 7, 2012; DOI: 10.1158/0008-5472.CAN-12-3229

transferred from 1 cell to another provocatively suggests that miRNAs might act as hormones do. On the basis of this hypothesis, hormonal miRNAs (H-miRNA) should also have a receptor to which they bind. Interestingly, Eiring and colleagues have recently shown that, in addition to their role as gene-expression regulators, miRNAs can also directly interact with proteins (21), supporting the possibility that H-miRNAs might be able to bind to a proteic receptor. They showed that miR-328 can directly bind to the poly(rC)-binding protein hnRNP E2, which normally interacts with the 5′-UTR of CCAAT/enhancer binding protein (C/EBP) alpha (CEBPA) mRNAs, with an inhibitory effect. By binding to hnRNP E2, miR-328 prevents this protein from binding to CEBPA mRNA and increases CEBPA expression, leading to differentiation of chronic myelogenous leukemia blasts (21). Other miRNA–protein interactions have been observed (such as protein–miRNA interaction during miRNA biogenesis or protein stabilizing the miRNA), but none has shown the activation of an intracellular signal transduction as a consequence of this interaction, and as expected by an H-miRNA. Among the several possible candidates as H-miRNA receptors, the toll-like receptor (TLR) family is particularly interesting. TLRs are a family of receptors through which the mammalian innate immune system recognizes the presence of invading pathogens (22, 23). It has been shown that members of the TLR family (i.e., murine *TLR7* and human TLR8) on dendritic cells and B lymphocytes can recognize and bind viral single-stranded RNA (ssRNA) sequences, leading to cell activation and production of cytokines (24, 25). Both murine *TLR7* and human TLR8 bind to and are activated by 20-nt long ssRNAs, which represent physiologic ligands for these 2 receptors (25), located in intracellular endosomes. Circulating mature miRNAs are 19 to 24 nt in length, and could represent endogenous agonists of ssRNA-binding TLRs involved in intercellular communication within a tumor, which releases miRNAs in exosomes. To show this, we first investigated which miRNAs are secreted by cancer cells in exosomes. The models we used were non–small cell lung cancer (NSCLC) cells A549 and SK-MES. We showed that among the most represented miRNAs in the exosomes released by these 2 cell lines in their supernatant were miR-16, miR-21, and miR-29a (26). Interestingly, while miR-16 was also present in the exosomes from HEK-293 cells, high levels of miR-21 and miR-29a were only present in the exosomes from NSCLC cell lines, suggesting a lung cancer-specific profile of exosome-secreted miRNAs. Because both ssRNA-binding TLRs (TLR7 and TLR8) are located in intracellular endosomes, we investigated whether exosome-secreted miRNAs were able to reach the endosome compartment in a recipient cell. By conducting colabeling experiments, we showed that this was the case, and we also observed that miR-21 and miR-29a (but not miR-16) were able to bind to TLR8 (in human), by carrying out coimmunoprecipitation assays (26). Functionally, we showed that by binding to TLR8 (in human) and its functional equivalent *TLR7* (in mouse) in immune cells, miR-21 and miR-29a (but not miR-16) activated a TLR-mediated NF-κB signaling that led to increased secretion of proinflammatory cytokines interleukin (IL)-6 and TNF-α (26). As a result, cancer cells increased their proliferation rate and metastatic potential. Interestingly, this effect was highly (but not completely) reduced in *TLR7*$^{-/-}$ mice, suggesting the existence of TLR-independent signals promoting cancer growth and dissemination in the cancer-immune cell–exosome–mediated cross-talk within the tumor microenvironment. Previously, Kim and colleagues showed that tumor secretion of the extracellular matrix proteoglycan versican is able to induce a proinflammatory response by activating TLR2:TLR6 complexes in myeloid cells leading to increased metastatic potential (27). Therefore, it is clear that cancer cells release signals to the surrounding cells of the tumor microenvironment that ultimately promote tumor growth and dissemination, and these signals involve different subgroups of TLRs. The H-miRNA–mediated signaling involves murine *TLR7* and human TLR8, but not human TLR7. We observed that the proinflammatory and prometastatic response triggered by H-miRNAs (miR-21 and miR-29a) in human cancer is mediated by TLR8, not by human TLR7 (26). This is an intriguing finding, as there is no conclusive evidence of a specific function for the 2 ssRNA-binding TLRs in human (TLR7 and TLR8), and a better understanding of their selective role is the necessary rationale to properly develop selective and specific TLR7- (or TLR8-) binding anticancer drugs. In an attempt to understand why miR-21 and miR-29a bind to and activate murine *TLR7* and human TLR8, whereas miR-16 does not, we analyzed the nucleotide sequence of these 3 mature miRNAs. We observed that while all 3 miRNAs begin with the same 4-nt motif at their 5′-end (UAGC), the 3′-end (nt 18–21) of miR-21 and miR-29a (but not the 3′-end of miR-16) harbors a GU-motif that is predominant in the ssRNA-binding TLR activator RNA33. By carrying out single-nucleotide mutagenesis, we were able to show that nt. 20 was very important in modulating TLR-mediated activation of NF-κB for both miR-21 and miR-29a, suggesting indeed a sequence-driven and nucleotide-specific mechanism of activation of TLRs by H-miRNAs (26). Despite these preliminary findings, it is still controversial which factors affect the ability of miRNAs to become part of the exosome cargo (compared with which ones remain in the cell) and which factors determine their ability to bind and activate TLRs. Interestingly, the implications of H-miRNA binding to TLRs are not disease specific and go beyond cancer. Lehmann and colleagues showed that let-7b, a key regulator of gene expression in the central nervous system, is expressed at higher concentration in the cerebrospinal fluid of patients with Alzheimer's disease, and extracellular introduction of let-7b into the cerebrospinal fluid of wild-type mice by intrathecal injection resulted in neurodegeneration. Mice lacking *TLR7* were resistant to this neurodegenerative effect, but this susceptibility to let-7b was restored in neurons transfected with *TLR7* by intrauterine electroporation of *TLR7*$^{-/-}$ fetuses (28). These findings suggest that in addition to miR-21 and miR-29a, let-7b can bind to *TLR7* and indicate that the H-miRNA–mediated activation of murine *TLR7* is also relevant in neurodegenerative diseases, suggesting a broader mechanism involved in several different inflammatory diseases and providing additional evidence, at a molecular level, of a connection between cancer and

Published OnlineFirst December 7, 2012; DOI: 10.1158/0008-5472.CAN-12-3229

inflammation. Interestingly, we also showed that GW4869, an inhibitor of miRNA and exosome secretion, is able to significantly reduce the metastatic potential of lung cancer cells, whereas the injection of cancer-released exosomes was able to rescue the effect of GW4869 toward an increased metastasis formation (26). These findings indicate that the inhibition of cancer-released exosomes by drugs like GW4869 represents a promising novel anticancer strategy, impairing the perverse paracrine cross-talk of cancer cells to surrounding immune cells mediated by exosome-secreted H-miRNAs in the tumor microenvironment.

Future Implications

The discovery that miRNAs released by cancer cells can bind to a receptor in a surrounding immune cell is completely novel. Other receptors (in addition to *TLR7* and TLR8) are likely to be found, but this is the first identified miRNA receptor and it is relevant to cancer. Intriguingly, there is a large group of orphan receptors, whose ligand has not been identified yet. An appealing hypothesis is that at least some of these receptors might actually bind to miRNAs (or other nucleic acids) released by cells. According to this point of view, they are orphan simply because researchers never looked in the right direction, assuming that ligands might be only proteins or steroids. Should this theory be embraced by scientists, it will be extremely interesting to identify whether nucleic acids are indeed ligands of some of these receptors, because this discovery could open new and yet-unconceivable molecular mechanisms involved in cancer and several other human diseases. The finding that cell-released H-miRNAs bind to TLRs leads to another question: is this pathway relevant only for pathology or is it a physiologic pathway (H-miRNAs then representing the normal, endogenous ligands of ssRNA-binding TLRs) normally used in intercellular communication, which is altered in human diseases and contributes to the onset and maintenance of a pathologic status? While these intriguing questions suggest that the identification of TLR-binding miRNAs might be a scratch in the surface of a broader molecular world still in need to be discovered, the discovery of H-miRNAs, of their ability to bind and activate human TLR8, and of their importance in cancer growth and spreading already harbors profound translational implications. Indeed, several strategies can be envisioned to interrupt the protumoral aberrant cross-talk initiated by cancer cells and occurring between them and the surrounding immune cells, mediated by exosome-released H-miRNAs in the tumor microenvironment. As summarized in Fig. 1, this signaling can be blocked

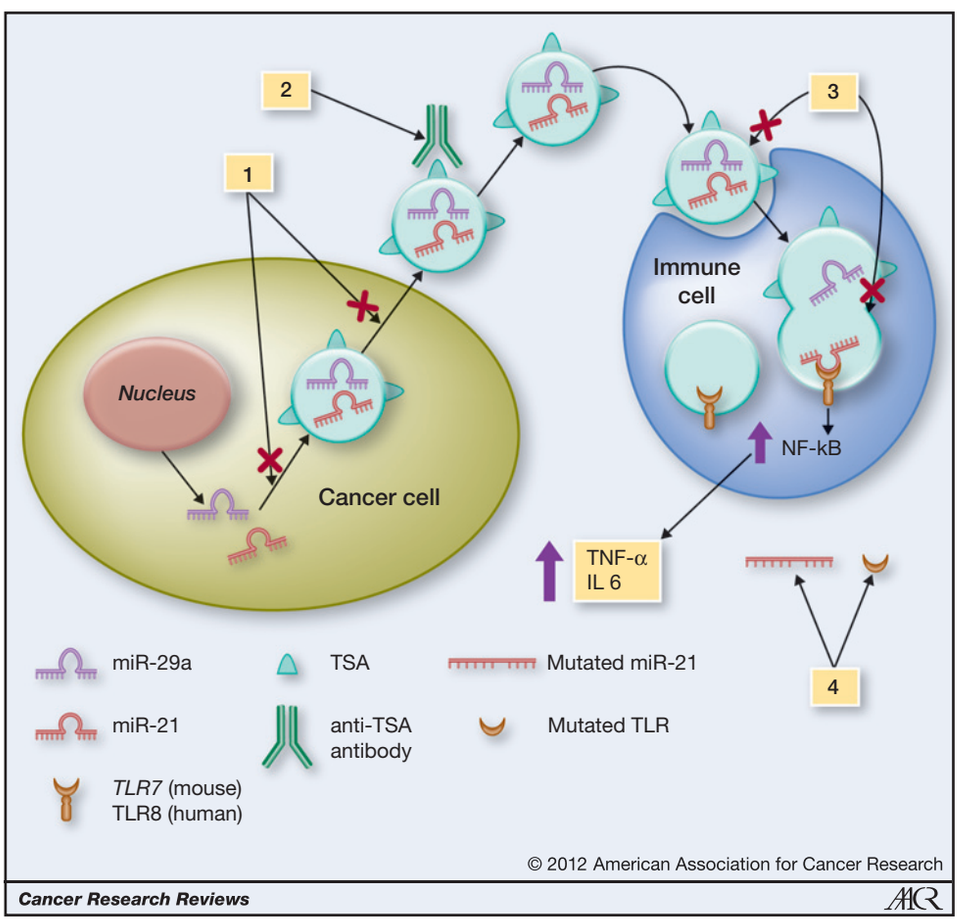

Figure 1. miRNAs released by cancer cells within exosomes can bind to TLRs in surrounding immune cells, leading to NFκB pathway activation and increased secretion of IL-6 and TNF-α that ultimately promotes cancer cell proliferation and metastatic potential. This aberrant loop can be impaired by several different strategies. 1, inhibition of miRNA and exosome secretion by cancer cells (this mechanism is used, for instance, by GW4869); 2, antibodies directed against tumor-specific antigens (TSA) on the surface of cancer cell-released exosomes could label these exosomes and help clearing them out of the tumor microenvironment/general circulation; 3, inhibition of exosome internalization and exosome–endosome fusion in the immune cells; 4, generation of miRNA mutants and/or TLR mutants that compete for the wild-type molecules and prevent the miRNA–TLR binding that leads to the activation of the downstream protumoral signaling.

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at several levels. First, it is possible to inhibit exosomes and H-miRNA secretion by cancer cells. This goal has already been shown to be successful by using inhibitors of neutral sphingomyelinase, such as GW4869. It has also been shown that silencing miRNAs in cancer cells (for instance, with anti-miRNA oligonucleotides) also reduces the content of miRNAs in the exosomes derived by those cells (26). Another strategy consists of preventing cancer-released exosomes from reaching the TLR-expressing target cell. This aim could be achieved by labeling exosomes with antibodies against tumor-specific antigens at the surface of cancer-released exosomes, and clearing these microvesicles from circulation. Interestingly, this approach has been already proposed by using the adaptive dialysis-like affinity platform technology (Aethlon ADAPT) system, an affinity plasmapheresis platform allowing extracorporeal capture and selective retention of target particles less than 200 nm from the entire circulatory system (29). Because exosomes are 30 to 100 nm membrane vesicles, this system might be effectively used to clear cancer patients of the protumoral signals released by cancer cells in exosomes. Such an approach is being considered in HER2 (epidermal growth factor receptor type 2)-positive breast cancer-released exosomes (29). The ultimate mechanism responsible for exosome-contained H-miRNAs to reach immune cells endosomes is still poorly understood. However, another possible approach is to be able to interfere with the exosome internalization and the exosome–endosome fusion in the immune cells, preventing H-miRNAs from reaching ssRNA-binding TLRs. Finally, a mechanism to prevent H-miRNAs from binding to TLRs can be envisioned. This can occur in 2 ways: by generating modified (mutated?) H-miRNAs with a different affinity to TLRs and able to compete for the binding to the TLR, or by generating peptides that harbor the H-miRNA binding region of the TLR and could bind and trap H-miRNAs, preventing their binding to a full-length and functionally active ssRNA-binding TLR. Because it can be theorized that other receptors are able to bind H-miRNAs and the activation of these receptors might lead to an antitumoral effect instead (depending on the nature of the binding H-miRNA and/or of the receptor), it can also be hypothesized that artificial exosomes containing these tumor suppressor H-miRNAs can be used alone or in combination with existing drugs as a novel anticancer treatment. In summary, the scenario opened by the discovery of H-miRNAs and their ability to bind and activate specific receptors is extremely wide and the translational implications of this discovery are broad, spanning from cancer to neurodegenerative diseases and reasonably extending to autoimmune and other inflammatory diseases. While the introduction of this new chapter of medicine has been written, certainly several more pages are still white and will hopefully be filled by new exciting discoveries leading to a better comprehension and more efficient treatment of cancer and other human diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

Received August 14, 2012; revised October 2, 2012; accepted October 10, 2012; published OnlineFirst December 7, 2012.

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Published OnlineFirst December 7, 2012; DOI: 10.1158/0008-5472.CAN-12-3229

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Cancer Res 2012;72:6333-6337. Published OnlineFirst December 7, 2012.