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Review

Circulating free xeno-microRNAs – The new kids on the block☆

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ABSTRACT

The role of circulating free microRNAs (cfmiRNAs) as promising tools for cancer screening, prognosis and monitoring of anticancer therapies has been widely studied in the past decades. cfmiRNAs have all the characteristics of the perfect biomarkers owing high stability under storage and handling conditions and being detectable not only in plasma, but in almost all body fluids. Moreover, their levels in plasma are likely to resemble ones in the primary tumor. Recently, viral and plant miRNAs have been found in plasma of healthy individuals through deep sequencing technique, and subsequently the same ones were deregulated in patients. Growing body of literature is recently focusing on understanding the potential cross-kingdom regulation of human mRNAs by miRNAs most likely absorbed with food ingestion. In this article we will review the literature concerning the xenomiRs detected in plasma and their role in influencing cancer onset and progression. XenomiRs could potentially be used not only as early screening tool, but also for patients' prognosis.

1. Introduction

microRNAs (miRNAs) are a class of evolutionary conserved, endogenous small non-coding RNA of 19–25 nucleotides long, produced from hairpin-shape precursors. miRNAs are localized in intronic or exonic regions of protein-coding genes or in non-coding genomic regions, and they account roughly for 1% of the whole genome. More than one third of human miRNAs are organized in clusters, which are likely to be a single transcriptional unit, suggesting their coordinated regulation (Yu et al., 2006). miRNAs are initially transcribed in the nucleus by RNA Polymerase II or III as long primary transcripts, which are cleaved in the nucleus by a complex involving Drosha and DGCR8 microprocessor complex subunit, forming a precursor miRNA (pre-miRNA). The pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5–Ran-GTP, where the RNase Dicer, in complex with the double-stranded RNA-binding protein TRBP, cleaves the pre-miRNA hairpin to its mature length (Kim, 2005). The mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it induces post-transcriptional gene silencing through

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inhibition of translation or messenger RNA (mRNA) cleavage. microRNAs bind their targets through sequence complementarity: incomplete complementarity leads to translational inhibition or deadenilation of the target mRNA, while perfect binding triggers for mRNA degradation (Lewis et al., 2003). Growing body of evidence demonstrated that miRNAs not only mediate translational repression, but also activate gene expression through the binding to promoter sequences (Place et al., 2008). Moreover, a single mRNA can contain binding sites for multiple different miRNA sequences, while one microRNA can regulate multiple target mRNAs, making this process both tightly regulated and difficult to decipher.

2. miRNA and cancer

Due to the several different mechanisms through which miRNAs could regulate gene expression, they have been involved in several ways to cancer onset, progression and dissemination. More than 50% of human miRNAs are localized in fragile chromosomal regions that exhibit DNA amplifications, deletions or translocations during tumor progression (Calin et al., 2004). Furthermore, expression of miRNAs in cancer has been demonstrated to be influenced also by promoter methylation or transcriptional deregulation (Scott et al., 2006).

Deregulation of a single miRNA can modify the expression of several different protein coding genes, thus influencing downstream cellular processes such as proliferation, differentiation, migration, apoptosis, and acting both as oncogene or oncosuppressor (Di Leva et al., 2014).

What makes miRNAs promising biomarkers for early cancer detection, it's their tight tissue- and time-specific expression, and their alterations (mainly in expression and rarely in sequence) consequence of tumor progression. Moreover, miRNAs have not only the potential to be tumor-specific, but are also detectable in body fluids.

The presence of cell-free nucleic acids in plasma was established many decades ago (Kamm and Smith, 1972). A growing interest has been shown in cell-free miRNAs (cfmiRNAs) in connection with cancer (Schwarzenbach et al., 2011). cfmiRNAs exhibit remarkable stability in various types of body fluids, including whole blood, serum, plasma, urine and saliva (Weber et al., 2010), even though their levels are shown to be different depending on the body fluid and influenced by many factors, i.e. inflammation. cfmiRNAs, are resistant to plasma RNase activity as well as to severe physicochemical conditions, such as freeze-thawing and extreme pH (Chen et al., 2008); moreover, they are functionally active in deregulating targets in downstream cells (Kosaka et al., 2010). The high stability of circulating miRNAs could be related to different mechanisms: cfmiRNAs could be encapsulated into vesicular bodies such as exosomes (10–100 nm) and microvesicles (0.1–1 μm), or are present in complexes with RNA-binding proteins, such as nucleophosmin 1 (Wang et al., 2010), HDL (Vickers et al., 2011), and AGO-2 (Arroyo et al., 2011). Nonetheless, the mechanism of cfmiRNAs secretion is still under investigation. Three possible pathways have been proposed, that include passive release from apoptotic or necrotic cells, active secretion through microvesicles or via microvesicle-free RNA-binding protein-dependent pathway (Zhang et al., 2013). The first possible pathway of miRNA entry in body fluids it's through the passive release as a product of cellular activities or cell death. Active release is likely to happen through exosomes, which evolve from endosomes as microvesicles that result from inward budding of the plasma membrane into the cytoplasm, and then are secreted in a process dependent from cytoskeleton activation (Ramachandran and Palanisamy, 2012). As opposed to exosomes, shedding vesicles are formed from outward budding of the plasma membrane, and their release into the extracellular cavity occurs in a calcium-dependent manner with cytoskeleton reorganization.

The recent advances in next generation sequencing and genome-wide analysis allowed the characterization of cfmiRNAs present in the circulation and highlighted the presence of exogenous miRNAs, together with endogenous ones. In the present review we are focusing on a special subset of cfmiRNAs that are less reported and investigated due to the perception that because they are not codified by the human genome they don't have to be present in the human body. These are the xeno-miRNAs, miRNAs codified by non-human genomes and that are present in the circulation – the cfxeno-miRNAs. Several reports described the presence of miRNAs from different species in circulation of healthy individuals (Philip et al., 2015; Semenov et al., 2012; Witwer, 2012), and thus their potential role in targeting human mRNA (Zhang et al., 2012a). Supposing that nutrient-derived microRNAs are present in the circulation and might affect gene expression in endogenous human tissues, support the evidence that consumption of certain type of food is relevant in the pathogenesis of some diseases.

3. Cross-kingdom regulation of miRNA

Since the role of miRNAs as biomarkers has been established, greatly outnumbered investigations tried to analyze their contribution not only in cancer, but also in other conditions like metabolic disorders or infectious diseases (Weiland et al., 2012). A growing interest about the direct contribution and the presence in the circulation of exogenous miRNAs, or xenomiR, leaded to different hypotheses on how diet and diseases are strictly related and influence each other.

In mammals, the potential role of miRNAs in inter-individual communication has been suggested after the demonstration of their presence in both cow and human breast milk (Chen et al., 2010). The mechanism of the gastrointestinal absorption of active miRNAs seems to involve transcytosis in the gut of miRNAs robustness protected in vesicles or in protein complexes, but additional mechanisms for unprotected miRNAs or for selective uptake of specific ones seems to be more than possible. Protected miRNAs can persist several hours in the circulation, long enough to be uptake by cells throughout the body, possibly via receptor mediated endocytosis. Moreover, exogenous miRNAs and in particular plant ones, have a stability that make them bioavailable even after an extensive pretreatment, such as processing, cooking and storage (Philip et al., 2015). The uptake of microRNAs by immune cells through their direct binding to TLR8 has been demonstrated to contribute to inflammatory

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response (Fabbri et al., 2012), and possibly exogenous miRNAs can use the same pathway of other still uncharacterized receptors.

The evidence of the active role of miRNAs in inter-individual communication was observed in maternal-to-newborn stimulation of immunity, where exosomal miR-155 has been proposed to have a crucial role in Thymic T-cell maturation during breastfeeding (Melnik et al., 2014). Also miR-29 present in cow milk has been shown to be absorbed in the human gut, being stable in circulation for at least 9 h, altering osteoblast and osteoclast differentiation (Baier et al., 2014). Together with milk-derived ones, absorption of nutrition-derived miRNAs from different species has been envisaged, even if their role has not been completely understood.

The first and maybe the most debated paper showing that plant miRNAs are not only present in sera and tissue of various animals, but they can also regulate human mRNAs has been published in 2012 (Zhang et al., 2012a). The authors found rice miR-168a in sera of Chinese subjects, demonstrating how it regulates low-density lipoprotein receptor adapter protein 1 (LDLRAP1), a gene involved in cholesterol metabolism, influencing LDL removal from plasma, at least in a mouse model. These data were not reproduced by a subsequent paper in which cfxeno-miRNA levels were measured in non-human primates before and after ingestion of a miRNA-rich soy and fruit mixture (Witwer et al., 2013) and a consistent response to dietary intake was not observed. Even if the findings from Zhang et al. where debated and the results were suggested to be due to errors in sequencing and to the high homology of miR-168a with human one (Zhang et al., 2012b), this finding provides the first evidence that food-derived exogenous plant miRNAs enter into the circulation and organs of mammals. Clinical observations partially support the hypothesis that rice consumption directly correlate with high serum LDL levels and thus atherosclerotic risk (Chen and Cheng, 2006), and this is a clear starting point to analyze the potential of xenomiRs as plasma biomarkers. It's still unclear how efficiently miRNAs are absorbed in the gastrointestinal tract or if there is a selection for assisted transport of some miRNAs rather than others. A recently published bioinformatics analysis tried to solve this issue, analyzing the probability of xeno-miRNA to be transported and to regulate human mRNAs, based on their homology with human ones (Shu et al., 2015), confirming the importance of diet for cross-kingdom regulation. Interestingly, miRNAs from plants are 2′-O-methylated and this modification protects their 3′ ends from truncation and uridylation (Zhao et al., 2012). Methylation of plant miRNAs influences negatively the qRT-PCR, and is considered a reason for they under-representation in screening experiments (Yu et al., 2005), but could also be a way for selective absorption in the gut.

In Figure 1 is depicted the potential route of viral and plant miRNAs. Their presence and circulation and uptake by cells have the ultimate role to deregulate human mRNAs, which have a high level of conservation in the seed sequences. Consequently, they have been associated with cancer. The most important xenomiRs detected in human and with a demonstrated role in cancer are summarized in Table 1. Together with plant miRNAs, another example of the relevant role of xenomiRs in cross-species communication comes from virus–host interactions. Viruses are able to sequester host cell machinery to produce not only their own proteins, but also miRNAs (Pfeffer et al., 2004), which are targeting pathways

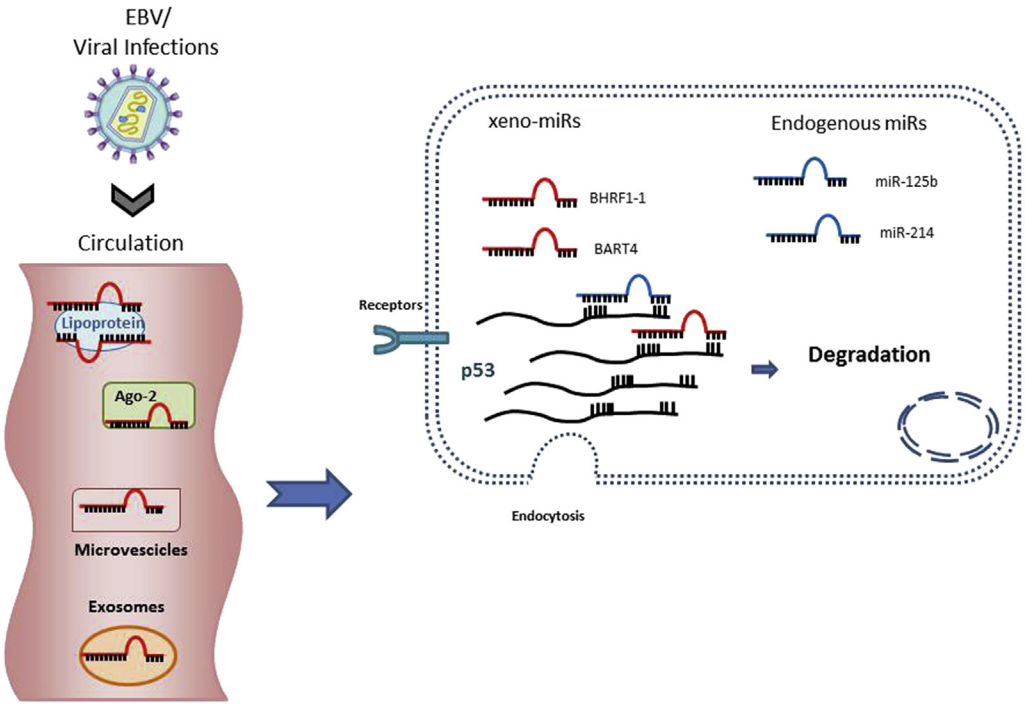

Figure 1 – Route of viral and plant microRNAs from the circulation to the deregulation of cellular targets.

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Table 1 – Circulating-free xeno-microRNAs in human plasma.

Xeno-miRNAs	Putative target	Disease	Reference
Rice miR-168a	LDLRAP1	Atherosclerosis	(Zhang et al., 2012a, 2012b)
KSHV-miR-K12-11	C/EBPβ	Lymphoma	(Boss et al., 2011)
KSHV-miR-12-7	MICB	Glioblastoma	(Herman et al., 2015)
miR-K-12-10b, miR-K-12-12*	Agonist TLR8	Sepsis	(Tudor et al., 2014)
Cow miR-29	HDAC4, TGFbeta3	Osteoclast differentiation	(Baier et al., 2014)
BHRF1-1 miRNA	p53	CLL	(Ferrajoli et al., 2015)
miR-BART16-1	LMP1	HLH	(Zhou et al., 2015)
miR-BART3, miR-BART7	Unknown	NPC	(Zhang et al., 2015)

crucial for cell survival or for evasion from immune recognition (Nachmani et al., 2009). Moreover, some viral miRNAs exhibit homology to human oncogenic miRNAs, as Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded miR-K12-11, which is an ortholog of miR-155 (Gottwein et al., 2007). miR-K12-11 can mimic the documented lymphoproliferative activity of miR-155 during hematopoiesis *in vivo*, promoting lymphomagenesis and thus contributing to the induction of KSHV-positive B-cell tumors in infected patients (Boss et al., 2011). During active KSHV infection, 12 pre-miRNAs and 18 mature miRNAs are present in the hosting environment. KSHV miRNAs has been also identified as potential biomarker for other types of cancers: glioblastoma patients have significantly higher plasma levels of KSHV-miR-12-7 compared to healthy individuals. This miRNA has been shown to target MICB, a stress-induced non-classical major histocompatibility class I molecule, reducing the cytotoxic death of the infected cells mediated by this molecule through the interaction with the natural killer and $CD8^+$ T cells (Herman et al., 2015).

Other than their potential role in etiology of lymphomas, individuals positive for KSHV infection seem to have a different response to septic shock, a deadly disease characterized by multiple organ dysfunction syndrome (Tudor et al., 2014). In particular, miR-K12-10 and miR-K12-12* during the viral lytic phase antagonize TLR8 and stimulate IL6 and IL10 secretion, thus relying on negative outcome in case of sepsis.

Viruses might utilize the molecular mimicry of miRNA genes to regulate host cellular environment and influence disease progression and outcome. It has been demonstrated also for Epstein–Barr Virus (EBV), where previous infection negatively influences Chronic Lymphocytic Leukemia (CLL) progression, at least partially by down regulating p53 levels in pre malignant B cells (Ferrajoli et al., 2015). The Figure 1 pictures the example of EBV to summarize how xenomiRs, present in circulation, can easily reach cellular targets and contribute to their regulation, due to seed sequence conservation. EBV miRNAs have been associated not only to CLL outcome, but as for KSHV, its infection has been correlated to other type of malignancies. In hemophagocytic lymphohistiocytosis (HLH), a complication of EBV infection, plasma levels miR-BART16-1 seems to be a good candidate for monitoring disease progression, since they were observed to continue decreasing during the whole chemotherapy (Zhou et al., 2015).

On the other hand, the association between EBV and nasopharyngeal carcinoma (NPC) is quite established (Sam et al., 1993), while the mechanism that regulates its contribution to the development of this disease is not still understood. Different studies focused on the potential use of EBV microRNAs as detection marker and for diagnosis confirmation, but circulating plasma EBV miRNAs could also predict therapy response or tumor stage. For example, miR-BART3, miR-BART7 and miR-BART13 are microRNAs actively secreted from NPC cells, and even if their biological functions are still not clear, they may be good biomarkers for NPC (Zhang et al., 2015). Moreover, the plasma levels of miR-BART7 and miR-BART13 decrease significantly after radiotherapy treatment of NPC, suggesting that they could be used as marker also for response to therapy.

4. Conclusions

Growing evidences demonstrate that circulating cfmiRNAs could be a potential valuable marker for early detection diagnosis and prognosis in cancer (Chen et al., 2014; Schwarzenbach et al., 2014). cfmiRNAs have all the characteristics of the perfect cancer biomarker, such as stability to different storage conditions and freeze-thaw cycles, presence not only in tumors, but also in almost all body fluids with a discrete correlation with the ones altered in the primary tumor. Despite this, their use in clinic is still limited, mostly for the limited sample size and the lacking of an independent validation cohort.

Together with deregulation of endogenous miRNAs, and they tumor-associated deregulation, the interest toward xeno-miRNAs as biomarkers, not only in cancer but also in other deadly diseases, has continuously grown over the last years. The potential cross-interaction between plant or viral miRNAs and human mRNAs has been envisaged by different authors. This regulation is based on the high level of conservation in the seed sequences and in the target sites across species (Berezikov et al., 2005). While for viral miRNAs the correlation between infection and regulation of endogenous targets has been widely proved (Carl et al., 2013); regarding plant miRNAs several concerns were expressed about absorption and minimal plasma concentration needed to be effective (Liang et al., 2015). Nonetheless, they have been detected in human plasma of healthy individuals, suggesting that despite potential preferential absorption of degradation, they have a role in human microenvironment (Liang et al., 2015; Zhang et al., 2012a). These evidences not only confirm the well-known relationship between diet and cancer, but also suggest

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that xenomiRs could be useful biomarker for diagnosis and early detection.

On the other hand, the studies regarding the role of viral miRNAs and their role in cancer onset are more advanced. EBV microRNAs are known to target b-cells and be involved in lymph-proliferative diseases, while with the same machinery endogenous miRNAs (i.e. the oncogenic miR-155) are deregulated by viral latent or lytic proteins (Skalsky et al., 2012). KSHV, another γ-herpesvirus, is known to regulate, as for EBV, viral reactivation, apoptosis and viral immune evasion in infectious context (Ramalingam et al., 2012), while its strict correlation with cancer came out with the similarities between KSHV-miR-K12-11 and, again, miR-155, making it a candidate that contribute to lymphomagenesis (Boss et al., 2011).

But even if there are several example of how viral miRNAs are important for infection and replication in different contexts such as HIV (Barichievy et al., 2015) or HBV (Sarkar and Chakravarty, 2015), their role in deregulating human mRNAs in different contexts has not been clearly understood.

Further studies are needed to understand the mechanism of xenomiRs in an endogenous context, and common guidelines as regards sample collection, treatment and sequencing are necessary to obtain consistent results. Despite some technical issues, xenomiRs are involved in carcinogenesis and probably also in tumor spreading, making then suitable not only for early detection, but also useful for targeted-gene delivery to the tumor cells.

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