MINIREVIEW

BJC

British Journal of Cancer (2015) 112, 777–782 | doi: 10.1038/bjc.2015.6

Keywords: microRNA; miR; radiation; radiotherapy; cancer; radioresistance; biomarkers; therapeutics

MicroRNA in radiotherapy: miRage or miRador?

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At least half of all cancer patients will receive radiation therapy. Tumour radioresistance, or the failure to control certain tumours with this treatment, can result in locoregional recurrence; thus there is great interest in understanding the underlying biology and developing strategies to overcome this problem. The expanding investigation of microRNA in cancer suggests that these regulatory factors can influence the DNA damage response, the microenvironment and survival pathways, among other processes, and thereby may affect tumour radioresistance. As microRNA are readily detectable in tumours and biofluids, they hold promise as predictive biomarkers for therapy response and prognosis. This review highlights the current insights on the major ways that microRNA may contribute to tumour radiation response and whether their levels reflect treatment success. We conclude by applying the potential framework of future roles of miR in personalised radiotherapy using prostate cancer clinical management as an example.

INTRODUCTION

Cancer radiotherapy and tumour radioresistance. The treatment of solid tumours using radiotherapy (RT) is a cornerstone of cancer treatment. This treatment modality is predicated on the idea that cancerous lesions can be destroyed by targeted irradiation, or ionising radiation (IR) exposure, while the surrounding, normal tissue can withstand and recover from IR exposure. Central to this is the 'therapeutic ratio', wherein an optimal dose of radiation will provide maximal tumour kill while minimising the risk of significant late toxicity to the patient. However, biological heterogeneity complicates this issue, as certain patient tumours may be inherently more insensitive to a given dose of IR. Failure to control a tumour with a seemingly curative dose would suggest that the tumour is 'radioresistant' (i.e., resists radiation treatment), whereas a 'radiosensitive' tumour would be controlled. Radioresistance may arise from microenvironmental hypoxia, abnormal intrinsic DNA damage response (DDR) activity, deregulated survival pathway engagement (e.g., ERK or AKT) through constitutive activation of growth factor receptors, or mutations of oncogenes (e.g., KRas) or tumour suppressors (e.g., PTEN) (comprehensively reviewed by Begg *et al* (2011)). In addition, certain patients have a greater predilection to develop late radiation toxicity. Predictive strategies to determine the radiosensitivity of patient tumours and normal tissue *a priori* are required to facilitate the future delivery of personalised cancer RT.

Endogenous microRNA (miR) are short non-coding ribonucleic acid molecules whose functions are only recently being appreciated for the important role that they have in radiation response processes through regulation of gene expression. MiRs are emerging as a promising class of biologics pursued for their biomarker and future therapeutic potential in RT. At present, the uses of miR for these purposes are still in their infancy and remain to be rigorously validated in clinical studies. To speculate about the potential clinical implications of miR to RT, we discuss these hypothetical uses in the management of prostate cancer (PCa).

MiR biogenesis. The biological processing and negative transcript regulatory function of miR have been well characterised and reviewed (Ameres and Zamore, 2013). Initially, miR originates in the nucleus as a primary or pri-miR that is transcribed directly from a promoter or from gene introns. The polyadenylated and capped pri-miR is processed by the RNAse III enzyme Drosha into pre-miR, exported to the cytosol and further processed by the

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Received 20 September 2014; revised 17 December 2014; accepted 19 December 2014; published online 22 January 2015

RNAse III enzyme Dicer into mature miR. The 19–23-nucleotide RNA duplex is then bound by the RNA-induced silencing complex (RISC) and dissociates into a single-stranded species upon interaction with RISC. The nucleotide sequence of the single-stranded mature miR forms the basis for the recognition of target mRNA transcripts. The RISC subunit Argonaute catalyses the cleavage of mRNA molecules that are perfectly complimentary to the RISC:miR complex, leading to transcript degradation. If the complimentarity of the miR to an mRNA transcript is imperfect, translation is repressed through a variety of mechanisms. As there are about 2000 unique human miR (miRBase.org), with up to hundreds of predicted targets per miR, their role in cellular regulation is widespread.

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MiR involvement in the tumour radioresponse. MiR levels are associated with cancer radioresistance, and the topic has been recently reviewed (Metheetrairut and Slack, 2013). Knockdown experiments of the miR biogenesis machinery are used to evaluate a potential role of miR in the cellular response to radiation (or the 'radioresponse'). For example, Francia *et al* (2012) discovered that Drosha and Dicer knockdown in cells reduced DDR foci in response to IR, although cell survival beyond impaired cell cycle checkpoints and proliferation was not assessed. Likewise, two other groups have demonstrated that Dicer levels affect the DDR and cell radiosensitivity (Kraemer *et al*, 2011; Surova *et al*, 2012). We are now beginning to understand how different miR species downstream of their biogenesis machinery contribute to the observed radiobiological effects. Accordingly, the following sections briefly outline how miR dysregulation *in vitro* and *in vivo* contributes to several radiobiological mechanisms. Notwithstanding, endogenous miR dysregulation may be reflective of underlying genetic alterations rather than the driving force behind observed effects. Also, clinical validation for the effects of this dysregulation on RT is currently lacking and will need to be an important focus for translational research. In addition, and even more fundamentally, it should be noted that the causal link between the often quantified outcomes *in vitro* and *in vivo* (e.g., apoptosis, proliferation), and human tumour radioresponse has yet to be shown; these outcomes may not truly reflect patient tumour radiosensitivity.

DNA damage response (DDR). IR induces double-stranded DNA (dsDNA) breaks in genomic DNA. These are sensed and transduced by factors (e.g., ataxia telangiectasia mutated (ATM), histone H2AX phosphorylation) that recruit DNA repair machinery effector protein complexes (e.g., DNA-dependent protein kinases (DNA-PKcs), BRCA1). Failure to restore genomic integrity before mitosis can lead to cell death or malignant transformation.

Several well-studied miRs repress DDR pathway components in cells, which impairs DNA damage sensing or repair and results in increased radiosensitivity. For example, miR-421 and miR-24 downregulate ATM and H2AX, respectively, resulting in increased IR-induced genomic instability and cell death *in vitro* (Lal *et al*, 2009; Hu *et al*, 2010). MiR that target transcripts of fast error-prone (*DNA-PKc*) or slow error-free (*BRCA1*) dsDNA break repair genes also radiosensitise cancer cells *in vitro* and cancer xenografts (Yan *et al*, 2010; Moskwa *et al*, 2011). In sum, the underexpression of these miR in cancer cells may enable them to resist radiation damage during RT.

Hypoxic tumour microenvironment. There are several avenues by which the heterogenous hypoxic intratumoural landscapes can protect cancer cells from irradiation. First, the paucity of oxygen impairs the chemical fixation of DNA lesions, leading to the creation of fewer lethal dsDNA breaks. Second, the hypoxic environment can influence radiosensitivity through activation of the hypoxia-inducible factor-1 (HIF-1) pathway and transcription of HIF-1-responsive genes (Meijer *et al*, 2012). Several hypoxia-induced miR, notably miR-210, can stabilise the HIF-1 complex and enhance radioresistance *in vitro* (Grosso *et al*, 2013). Third, it is now recognised that hypoxia can downregulate Dicer *in vitro* and *in vivo*, shaping global miR expression to maintain the induction of hypoxia-responsive genes (Ho *et al*, 2012). Hence, the complex regulatory interface between the hypoxic tumour microenvironment and miR expression may be another source of radioresistance during RT.

Cancer stem cells. Cancer stem cells (CSCs), which reportedly reside in hypoxic niches and possess self-renewal and differentiation potential, may have intrinsic resistance to radiation and chemotherapy (Pajonk *et al*, 2010). Emerging evidence suggests that miR, such as miR-34a and miR-145, are involved in regulating CSCs. For example, miR-34a negatively regulates human primary tumour-derived CD44+ prostate CSCs, impairing prostate tumour development and metastasis *in vivo* (Liu *et al*, 2011). More specifically, miR-145 targets transcription factors (OCT4, SOX2 and KLF4) that are central to maintaining cell stemness (Xu *et al*, 2009), and is implicated in regulating CSCs in a range of different tumour types, including PCa (Huang *et al*, 2012). Thus, the involvement of miR in regulating CSC self-renewal capacity is a potential mechanism influencing tumour radioresistance.

Survival pathway alterations. Irradiation-induced DNA damage triggers p53 transcription factor activity that induces the expression of miR-34a (among others) to support cell cycle arrest, senescence or apoptosis (He *et al*, 2007). However, such miR are often underexpressed in primary human tumours, such as let-7c in PCa (Nadiminty *et al*, 2012). Several of these also participate in p53 feedback regulatory loops by targeting regulators of p53 or p53 transcripts directly, adding another regulatory layer to the radiation response (Feng *et al*, 2011).

MiR-dependent alterations in key survival signalling pathways are also common ways cancer cells circumvent irradiation-induced growth arrest and death. For example, miR-21 and miR-95 promote phosphatidylinositol 3 kinase-AKT-pathway-mediated survival by suppressing its direct and indirect negative regulators PTEN and SGPP1, respectively (Meng *et al*, 2007; Huang *et al*, 2013). Conversely, miR-9 and let-7 target the NF-κB1 transcript, abrogating the pro-survival effects of NF-κB signalling (Arora *et al*, 2011). The entanglement of miR in these already well-established tumour survival pathways highlights the opportunity to develop therapeutic strategies aimed at these pathways.

MiR involvement in the normal tissue radioresponse. In contrast to the role of miRs in cancer cells exposed to irradiation, there are few reports detailing how miRs contribute to normal tissue radiation responses. MiRs may be involved in the development of radiation-induced late-onset tissue fibrosis, which can have serious ramifications on cancer patients' quality of life (Weigel *et al*, 2014). For example, miR-34a expression was associated with the pathology of irradiation-induced fibrosis in a murine model, and found to target an anti-fibrotic protein transcript (Simone *et al*, 2014). The most headway has been made characterising the miR landscape of irradiated endothelial cells, where some species show substantial dysregulation following IR exposure, and exert influence over several cellular processes, particularly inflammatory ones (Palayoor *et al*, 2014). Indeed, the responses of the normal tissue endothelial cell compartment affect the development of acute and late normal tissue toxicities (Stewart *et al*, 2013; Korpela *et al*, 2014). In summary, miRs that contribute and correlate with normal tissue radiation toxicity development

remain to be better characterised in preclinical models and validated in human subjects.

MiRs as predictive biomarkers of tumour radioresponse. Despite our molecular knowledge of tumour radioresponse, personalised clinical indicators of tumour radioresistance are poorly defined. Positron emission tomography molecular imaging with [^{18}F]-fluorodeoxyglucose or fluoromisonidazole tracers to non-invasively visualise tumour metabolism or hypoxia is a promising approach being evaluated for adaptive RT planning (Bussink *et al*, 2011). In addition, genetic approaches are being investigated as biomarkers to predict RT treatment outcome. Copy-number alterations of *PTEN* and *c-MYC*, or haploinsufficiency of *NKX3.1* are prognostic for relapse after PCa RT (Bristow *et al*, 2014). Recently, a combined tumour hypoxia, genomic instability and genomic subtype signature has demonstrated prognostic significance in men with PCa treated with external beam RT (Lalonde *et al*, 2014).

The rapidly expanding field of miRs as biomarkers may provide a versatile method of tumour radioresponse prediction and monitoring. In fact, Schwarzenbach *et al* (2014) review how and why hundreds of circulating miRs have been proposed as biomarkers for human cancer diagnosis and prognostication. The practical utility of miR arises mainly from their relative stability in tissues and their release in stable form into a range of biofluids (e.g., blood, urine). Although individual miR species are subject to decay by newly emerging mechanisms and the purpose of their release remains unknown, they resist decay in biofluids due to their association with Argonaute proteins or encapsulation by vesicles. Even after routine sample tissue processing (e.g., formalin-fixed paraffin-embedded sections) or years of sample storage at $-20\,^{\circ}$C, miR levels remain readily detectable (Hall *et al*, 2012). A signature of miR species is amenable to rapid quantification by reverse transcriptase-PCR, whereas a global profile can be quantified by microarray or next-generation sequencing platforms. Although these techniques have high sensitivity and specificity, consensus has not been reached regarding best normalisation protocols or controls to address heterogeneity, let alone biofluid choice or standardised sample-processing methods.

Despite the immense potential clinical utility of miRs, very few studies have investigated this avenue specifically in the context of patients receiving RT. The few available clinical studies offer correlative data and are often statistically underpowered and confounded by other treatments (e.g., concurrent chemotherapy). For example, among a hundred inoperable non-small cell lung cancer (NSCLC) samples from an ongoing trial (NCT01190527), miR-885 and miR-7 were identified as potential serum biomarkers of better overall survival following chemoRT (Bi *et al*, 2013). In forty advanced rectal cancers, low intratumoural miR-145 expression post chemoRT correlated with poor neoadjuvant chemoRT response (Drebber *et al*, 2011). In a study not confounded by chemotherapy treatment, Wang *et al* reported on 15 postoperative RT-resistant and 15 postoperative RT-sensitive patient NSCLC samples. The RT-resistant cases were associated with low intratumoural miR-126 and let-7a levels (among 10 other dysregulated miRs), as well as higher recurrence rates and poorer overall survival when compared with the RT-sensitive cases. Correspondingly, miR-126 overexpression in a lung cancer cell line sensitised them to irradiation-induced apoptosis (Wang *et al*, 2011). Ke *et al* found that out of 18 human cervical carcinoma biopsies, the seven that were resistant to RT had high miR-181a expression levels. Likewise, miR-181 overexpression in cell lines and tumour xenografts conferred resistance to IR treatment (Ke *et al*, 2013).

Indeed, these preliminary studies demonstrate that evaluation of miRs as potential predictors of tumour response to RT are underway, but more non-confounded studies are needed. Statistically rigorous independent validation studies are also critical to confirm the prognostic or predictive value of these emerging findings. Future implementation of miR biomarkers might combine additional genomic and non-invasive imaging approaches (i.e., radiogenomics) to optimise personalised clinical management decisions (Aerts *et al*, 2014).

MiR-based treatments to increase tumour radiosensitivity. Inoperable melanomas or sarcomas and locally advanced carcinomas are relatively radioresistant when compared with other solid tumours, and are associated with poor patient outcomes. Against the backdrop of chemotherapy or small-molecule tumour radiosensitizers that can exacerbate the side effects of RT in patients, miR-based biologics may be a novel way to combat this resistant phenotype.

Numerous studies have identified endogenous miR species which when expressed, radiosensitise cancer cells *in vitro* (see the section 'MiR involvement in the tumour radioresponse'). Some reports further demonstrate radiosensitisation *in vivo* using cell line xenografts that overexpress particular miR. More clinically relevant investigations would test the delivery of synthetic miR made of oligonucleotides (called miR mimics or morpholinos) as tumour suppressor replacement therapies. For example, one group treated lung cancer xenografts using liposomal nanoparticles loaded with miR-200c mimics, and showed that this sensitised tumours to irradiation by regulating the cell oxidative stress response (Cortez *et al*, 2014).

Conversely, endogenous miR species that cause radioresistance when expressed could be silenced using synthetic antisense oligonucleotides that bind them (called antagomiR or antimiR). The design of delivery systems that ensure the stability of synthetic miR in the circulation and provide tumour-specific targeting and delivery is integral to translating miR therapy to the clinic (Babar *et al*, 2012; Cortez *et al*, 2014). Frank Slack's group has recently demonstrated the specific targeting of miR-155 in a mouse model of lymphoma using a novel construct that is selective for the acidic tumour microenvironment, highlighting the progress made in this field (Cheng *et al*, 2015). The feasibility of an antimiR treatment has recently been demonstrated in phase II clinical trials for chronic hepatitis C viral infection in humans (Janssen *et al*, 2013).

Future clinical implementation of miR for personalised RT. It is becoming clear that miRs are involved in the radiation response, and early data suggest that they may serve as promising predictive and prognostic biomarkers. Thus, miRs may serve as valuable clinical tools in the future to aid clinicians in RT management decisions; here we use localised PCa management to highlight general concepts of potential clinical integration (Figure 1).

PCa is the most common cancer diagnosed in men, with the majority of men presenting with localised, low-grade PCa. This group consists of excellent candidates for active surveillance, which carefully follows men to pick up early signs of disease progression prior to proceeding with curative treatment (Klotz *et al*, 2014). The lack of robust biomarkers that identify the presence of, or the potential to develop, higher-risk disease remains a barrier to widespread adoption of active surveillance (Vesprini *et al*, 2013). The identification of a miR signature obtained from biofluids that independently predicts the presence of high-grade PCa would therefore enable proper stratification of patients who require upfront treatment. Indeed, one group recently demonstrated that a three-member miR signature present in pre-radical prostatectomy sera showed an AUC of 0.94 in predicting poorer pathological disease compared with the biopsy result, suggesting promise in this approach (Wang *et al*, 2014).

For men with localised PCa who are not candidates for, or who are adverse to active surveillance, the standard treatment options are radical prostatectomy or RT. Although the RT dose is standardised among patients, isolated local recurrences can occur;

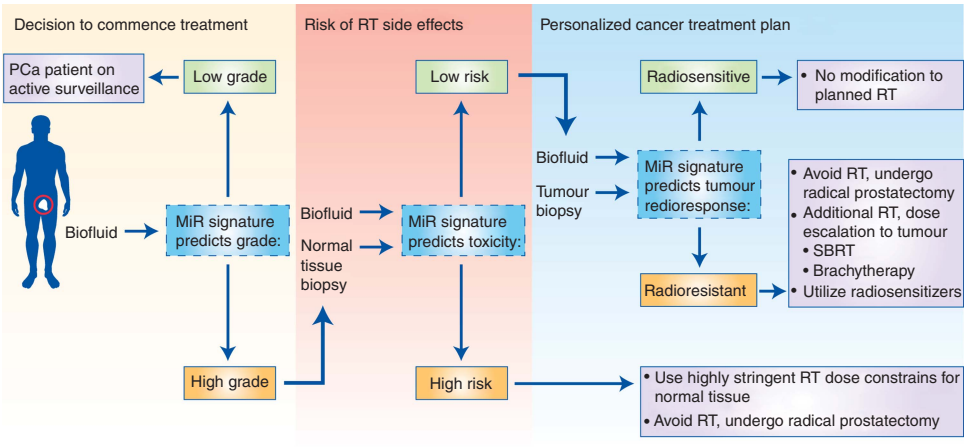

Figure 1. The potential utility for miR-predictive signatures in personalising the management of cancer and radiotherapy treatment. Using prostate cancer as an example, validated miR signatures could assist in determining which patients undergoing active surveillance should proceed to definitive treatment. In addition, they could aid in identifying those patients who potentially require additional radiotherapy dose escalation due to a cancer radioresistant signature, or are at increased risk of developing significant normal tissue radiotoxicity.

even in the modern era of dose escalation, post-treatment biopsies show a 15–20% residual disease rate (Zelefsky *et al*, 2008). This indicates that the current dose is not optimal for all patients, and implies the failure of RT in eliminating all PCa clonogens in those patients. A miR signature predictive of RT treatment response (i.e., radiosensitivity in the tumour phenotype) *a priori* would be invaluable in personalising the RT treatment approach. For example, a PCa patient with a radioresistant tumour signature could be treated with a radical prostatectomy, or treated with more ablative doses of radiation using brachytherapy or stereotactic body RT in order to overcome the radioresistance seen with standard RT doses. Biological modifiers of radioresponse could be employed to biologically dose-escalate the tumour (e.g., androgen deprivation therapy, poly (ADP-ribose) polymerase inhibitors to suppress DNA repair, chemotherapy or synthetic miR/antagomirs).

The consequence of escalating RT dose to treat PCa is the increased risk to develop both acute and late toxicity in adjacent normal organs. To potentially predict which patients are at increased risk for normal tissue toxicity, researchers have investigated the use of the patient's normal cells in functional *in vitro* assays and performed genomic analyses for single-nucleotide polymorphisms. However, to date, none of these biomarkers have demonstrated highly significant results that would be clinically useful (Barnett *et al*, 2009). This may be partly due to a lack of a distinct subpopulation of patients at risk of developing significant radiotoxicity, and also the need to account for radiation dose-volume metrics (Bentzen *et al*, 2010). The expression of serum miRs are rapidly altered in response to IR, and can serve as biomarkers for radiation exposure (Jacob *et al*, 2013) and potentially for radiotoxicity. Indeed, it was recently reported that miR-210 expression is increased in patients with radiation enteropathy, and repressed by anti-fibrotic therapy (Hamama *et al*, 2014). However, investigation into miRs as predictors for normal tissue radiotoxicity is still in its infancy; it remains to be determined whether a miR signature can be discovered and validated for prediction of acute or late radiation toxicity in normal tissue. Such a finding could revolutionise the development of personalised RT (e.g., utilising very stringent dose constraints for surrounding normal tissue or avoidance of RT if feasible for patients predicted to be at higher risk for developing radiotoxicity).

The future challenges to adopting miR-based diagnostic, predictive and therapeutic applications for clinical use involve further technical and scientific developments. First, standardised protocols for sample processing, data normalisation and clinical result interpretation require further investigation. Similarly, the optimal biofluid and the choice of an acceptably sensitive and specific detection method remain unidentified. Second, there are great variations in the global expression patterns of miR among different human cancer types, and imperfect overlap of identified signatures even among studies of the same tumour types (Calin and Croce, 2006). Third, genetic and microenvironmental tumour heterogeneity may confound miR expression pattern identification. Proposed miR signatures require rigorous biological and statistical validation in addition to clear definitions of applicable target populations. Future clinical research designs should consider prospectively incorporating pre-treatment tumour biopsies and biofluid collection during and after the RT course to track miR biomarkers in a temporal manner. Molecular elucidation of the radiobiological mechanisms and targets of miR are also critical for the future implementation of synthetic miR or antagomir therapy. Addressing these development and knowledge gap areas will facilitate the development of predictive and prognostic miR biomarkers that will lead us towards personalised RT.

CONCLUSION

The study of miRs in regulating essential cellular processes is deepening our understanding of the intricacies of tumour radioresistance, and may lead to novel therapeutic strategies employing miR mimics or antagomirs. The expanding interest in miRs as disease biomarkers extends to the realm of personalised RT, where the investigation of miRs in tumours or more readily obtainable biofluid samples is just starting to unfold. We envision that in the clinical setting of low-risk PCa diagnosis and treatment, miR signatures may have the potential to find occult diseases not suitable for surveillance, and identifying patients that are more or less likely to respond to RT, thereby facilitating personalised treatment choices.

ACKNOWLEDGEMENTS

This work was supported by research grants from Prostate Cancer Canada and the Movember Foundation (Grant No. D2013-24, and RS2014-03: 2014 Movember Rising Star in Prostate Cancer Research), the Canada Foundation for Innovation-MEDI ORF, and the Motorcycle Ride for Dad (Huronia branch). EK was supported by the Canada Graduate Scholarships Master's Award

(CIHR CGS-M), the Scace Graduate Fellowship in Prostate Cancer Research and the Paul Starita Graduate Student Fellowship (in molecular prostate cancer research).

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