Functional microRNA binding site variants
Ye Yuan and Joanne B. Weidhaas
Department of Radiation Oncology, UCLA, Los Angeles, CA, USA

Keywords
biomarkers; functional variants; KRAS-variant; microRNA binding sites; microRNAs; polymorphisms

Correspondence
J. B. Weidhaas, Department of Radiation Oncology, UCLA, Los Angeles, CA, USA
Tel: +1 310 825 4966
E-mail: jweidhaas@mednet.ucla.edu
(Received 28 September 2018, revised 15 November 2018, accepted 26 November 2018, available online 26 December 2018)
doi:10.1002/1878-0261.12421

1. Introduction
Single nucleotide polymorphisms (SNPs) are the most common source of variation within human genomes, and there are currently over 80 million mapped SNPs (1000 Genomes Project Consortium, 2015). In some cases, SNPs in coding regions of oncogenes or tumor suppressor genes can lead to gain-of-function or loss-of-function mutations resulting in malignant transformation. Although this association of functional SNPs in gene coding regions with cancer is well known, individuals who harbor these specific mutations represent an extremely small proportion of cancer patients.

Single nucleotide polymorphisms are found throughout the genome, and studies have predicted that the majority of disease-associated SNPs reside in noncoding regions (Tak and Farnham, 2015; Yao et al., 2014). Genomewide association studies, or GWAS, which were the first approach to try to identify germ-line disease-associated SNPs, appear to have difficulty capturing clinically relevant noncoding region SNPs, perhaps in part due to the complexity of accurate annotation (Nishizaki and Boyle, 2017), or limitations in SNP inclusion due to platform restraints. However, subsequent direct experimental testing of noncoding region SNPs has shown they can have significant functional effects on gene expression by disrupting transcriptional regulatory sites (Kasowski et al., 2010; Maurano et al., 2012) or altering the binding of other recently discovered regulatory factors such as microRNAs (miRNAs) (Saunders et al., 2007). miRNAs are short 18- to 24-nucleotide RNA molecules that play an important role in regulating many biologic pathways including pathways involved in cancer progression (Caldas and Brenton, 2005; Calin and Croce, 2006; Ceppi and Peter, 2014; Kent and Mendell, 2006; Kong et al., 2012; Lujambio and Lowe, 2012). They exert their regulatory control by binding via complete or partial complementarity with sequences in the 3' UTR of a target mRNA. This subsequently results in

Abbreviations
3'-UTR, 3'-untranslated region; 5-FU, 5-fluorouracil; ADT, androgen deprivation therapy; BER, base excision repair; CSS, cancer-specific survival; DSB, double-strand break repair; ER, estrogen receptor; GWAS, genomewide association studies; HR, homologous recombination; miRNAs, microRNAs; miR-SNP, microRNA-associated single nucleotide polymorphism; MTX, methotrexate; NHEJ, nonhomologous end joining; SNP, single nucleotide polymorphism.
silencing of gene expression through either sequestration or degradation of the target mRNA. Slight changes in the miRNA binding sequence in the 3' UTR can change miRNA and mRNA binding leading to alterations of these key regulatory interactions (Saunders et al., 2007) so that even single nucleotide changes introduced by germline polymorphisms within miRNA binding sites (miR-SNPs) can have profound downstream effects. The aims of this review were to (a) define the biological effects of miR-SNPs, (b) distinguish between prognostic and predictive miR-SNP-based biomarkers, and (c) provide clinically promising examples of each.

1.1. Alterations of cancer pathways by miR-SNPs

Hanahan and Weinberg proposed that cancer develops and progresses through aberrations in key biological pathways and there is strong evidence that miRNAs are key players in maintaining these hallmark pathways (Hanahan and Weinberg, 2011). Aberrations in miRNA expression or alterations in their binding can lead to tumorigenesis and cancer progression via one of these canonical pathways. For instance, the miR-15 and miR-16-1 family of miRNAs downregulate the expression of the anti-apoptotic protein BCL2 and loss of these two miRNAs leads to the development of B-cell chronic lymphocytic leukemia (Fabbri et al., 2009). Several SNPs have been identified in the non-coding regions of BCL2 including rs1564483 (G>A) a functional variant in the BCL2 3' UTR that is associated with decreased risk for non-small-cell lung cancer (NSCLC) (Xu et al., 2013) while yet another variant rs2279115 (C>A) appears to increase risk of esophageal SCC (Pan et al., 2015).

Similarly, unfettered activation of proliferative pathways is central to malignant transformation. For example, mutated KRAS leads to constitutive activation of proproliferative signaling pathways downstream of EGFR and is implicated in a significant proportion of colorectal cancers (Normanno et al., 2009). However, even in KRAS wild-type patients, a germline variant in the let-7 miRNA binding site of the KRAS 3' UTR (rs61764370, KRAS-variant) has been shown to increase risk for certain types of cancers and to predict treatment outcomes.

Functional variants have also been uncovered in genes involved in cell cycle progression and DNA repair pathways. XRCC1 is a DNA repair pathway gene involved in single-strand repair that harbors a miR-SNP. Bioinformatic screens uncovered a functional variant, rs1799782 (C>T), within the 3' UTR of XRCC1, a key gene in the DNA single-strand break repair pathway, and gene reporter assays confirmed that this variant strengthens the binding of miR-138. This resulted in higher XRCC1 expression in the presence of miR-138 (Nicoloso et al., 2010). These results highlight the fact that nucleotide changes introduced by miR-SNPs can also create new binding sites for miRNAs leading to previously unforeseen regulatory interactions.

Germline mutations in coding regions of the BRCA1 gene greatly increase the risk for hereditary breast and ovarian cancers due to defective double-stranded DNA damage repair pathways. A functional variant, rs799917 (C>T) in an intron of the BRCA1 coding sequence, was found to be associated with increased breast cancer risk (Nicoloso et al., 2010). Interestingly, the authors found that rs799917 resided in a binding site for miR-638 within the BRCA1 coding sequence and that the minor allele (T) diminished miR-638's ability to repress BRCA1 gene expression. These seemingly contradictory findings highlight the sometimes heterogeneous effects of miRNAs. In fact, miRNA binding can lead to transcriptional activation in cell type- and cell cycle-dependent contexts (Shohba Vasudevan et al., 2008; Shohba Vasudevan et al., 2007).

Given the involvement of miR-SNPs in disrupting the regulation of hallmark tumorigenic pathways, it is not surprising that there is increasing research on how miR-SNPs relate to cancer risk and prognosis. Many studies have established a link between functional miR-SNPs and increased risk for a variety of cancer types, and this has been recently reviewed in depth (Cipollini et al., 2014; Moszyńska et al., 2017). For the purposes of this review, we will focus specifically on functional miR-SNPs that may develop into biomarkers that can help clinicians select the optimal treatment for cancer patients. Thus, we must distinguish between biomarkers that are prognostic versus those that are predictive. Prognostic biomarkers are genetic or genomic variations that are associated with certain clinical outcomes regardless of the selected treatment regimen. Predictive biomarkers, on the other hand, can potentially identify what subset of patients may have better outcomes from one type of treatment versus another. With this distinction in mind, we will discuss some promising miR-SNP-based biomarkers under development.

1.2. miR-SNPs as prognostic biomarkers

Much work has been done to find miR-SNPs that are prognostic in cancer patients. In particular, we will focus on specific variants that are prognostic for outcomes after treatment with chemotherapy, radiation, or targeted agents.
Several studies have investigated the association between miR-SNPs and survival after chemotherapy. Wynendaele and colleagues found a variant, rs4245739 (A>C), in the 3′ UTR of MDM4 that led to the creation of a binding site for miR-191 and resulted in transcriptional repression of MDM4 with the AC and CC alleles. MDM4 is an oncprotein that represses the activity of p53, and the authors found that in patients with ovarian cancer the A-allele MDM4 was associated with better median overall survival versus the miR-191-associated C-allele, especially for women with ER-negative tumors. Furthermore, patients with the MDM4 A-allele were at increased risk for relapse following chemotherapy (Wynendaele et al., 2010). Another case-control study in ovarian cancer patients found 24 miR-SNPs associated with ovarian cancer survival and 17 miR-SNPs that were prognostic of treatment outcome. Of these, the rs1425486 (G>A) variant in the 3′ UTR of PDGFC was the most prognostic and disrupted a binding site for miR-425 (Liang et al., 2010).

DNA repair pathways are important for cell survival in response to therapeutic doses of ionizing radiation, and DNA repair genes are often dysregulated in cancer cells. A screen of miR-SNPs within 20 genes involved in DNA repair pathways including base excision repair (BER), nucleotide excision repair, nonhomologous end joining (NHEJ), homologous recombination (HR), and double-strand break repair (DSB) revealed 7 miR-SNPs in LIG3, ATM, BRCA1, PARP1, NBS1, and RAD51 of which the RAD51-associated variant rs7180135 (A>G) was prognostic for 5-year cancer-specific survival (CSS) following radiation in patients with muscle-invasive bladder cancer (Teo et al., 2012). Bioinformatic analyses of the rs7180135 site revealed a potential binding site for miR-197 that is weakened by the G-allele.

Finally, there are also miR-SNPs that are prognostic for treatment outcomes following targeted therapies. A case-control study of prostate cancer patients receiving androgen deprivation therapy (ADT) uncovered a germline variant signature consisting of three (rs6728684/KIF3C, rs3737336/CDON, rs1045747/IFI30), four (rs6728684/KIF3C, rs1071738/PALLD, rs998754/GABRA1, rs4351800/SYT9), and one (rs4351800/SYT9) miR-SNPs that were significantly correlated for disease progression, prostate cancer-specific mortality, and all-cause mortality, respectively. Interestingly, the multivariant signatures showed significant gene-dosage effect with worsening prognosis in patients with increasing numbers of variants (Bao et al., 2011).

### 1.3. miR-SNPs as predictive biomarkers

Predictive biomarkers can be used to classify patients based on their expected response to one treatment versus another. The presence of somatic BRAF V600 mutations, for instance, is a clinically significant predictive biomarker for response to small-molecule inhibitors of BRAF in patients with metastatic melanoma (Chapman et al., 2011; Hauschild et al., 2012). In contrast to many prognostic biomarkers, predictive biomarkers can have direct clinical utility and can be used to select between treatment regimens. However, discovery of such predictive biomarkers requires careful study design to develop and ultimately validate a potential signature. Since the functional consequences of miR-SNPs have only recently been appreciated, there are comparatively few germline miR-SNPs that have been demonstrated to be predictive biomarkers at this time.

A few miR-SNPs have shown potential as predictive markers in preclinical studies. Rs34764978 is a variant in the 3′ UTR of DHFR, a critical gene in purine biosynthesis that is targeted by the chemotherapy agent methotrexate (MTX). This variant also appears to disrupt the binding site for miR-24 resulting in higher expression DHFR in variant-harboring cells (Mishra et al., 2007). The authors found that DHFR levels were higher in cells with the rs34764978 variant in the presence of miR-24 and were more resistant to treatment with MTX. Additional follow-up studies, including well-controlled clinical studies, will be needed to determine the predictive power of the rs34764978 variant in patients who receive MTX versus those who do not. In another study, Pardini and colleagues looked at miR-SNPs in genes of the BER pathway to determine whether any would be prognostic for colorectal patients treated with 5-fluorouracil chemotherapy (5-FU). They hypothesized that since BER is the predominant mechanism for repairing 5-FU-induced DNA lesions, alterations of BER pathway genes by miR-SNPs would be important. One variant, rs2233921 (G>T), was indeed predictive for patients who were homozygous for the T-allele and received 5-FU showing the best survival (Pardini et al., 2013).

Currently, the KRAS-variant is a miR-SNP with the best clinical evidence as a predictive biomarker. Recently, the impact of the KRAS-variant on treatment outcomes was analyzed in a secondary analysis of a large multi-institutional randomized trial (Weidhaas et al., 2017). This trial, NRG Oncology RTOG 0522, randomized 891 patients with locally advanced oropharyngeal HNSCC between the standard of care...
of cisplatin-based chemoradiation or cisplatin-based chemoradiation with the anti-EGFR monoclonal antibody, cetuximab. Of the 70 patients found to have the KRAS-variant, the addition of cetuximab to cisplatin and radiation significantly increased both progression-free survival (PFS) and overall survival (OS). Further validation of the KRAS-variant’s efficacy as a predictive biomarker in a dedicated clinical trial of this regimen that randomizes patients into treatment groups based on KRAS-variant status is currently planned.

2. Discussion

miRNAs are noncoding RNAs that post-transcriptionally regulate much of the coding genome. Disruptions in miRNA::mRNA regulatory interactions are known to lead to tumorigenesis and cancer progression. Germline variants in conserved miRNA binding sites, known as miR-SNPs, have recently been shown to play an important role in pathogenic alterations of miRNA regulatory networks including those that modulate hallmark tumorigenic pathways.

A more complete molecular understanding of how miR-SNPs alter miRNA regulatory networks is still needed. In particular, variations in miRNA binding sites can lead to a range of effects on miRNA::mRNA interactions, from complete disruption of binding to the creation of a new miRNA binding site. While in silico prediction software can be useful in screening for miR-SNPs and effected miRNAs, gene reporter assays remain the gold standard for experimentally verifying functional variants. Interpreting the effects of miR-SNPs on biological pathways can sometimes be complicated by the fact that miRNAs can have cell type-dependent and cell cycle-dependent effects on target mRNAs. Furthermore, because miR-SNPs are germline variants present in both normal host and malignant cells it is important to consider the effects of these polymorphisms on both tumor cells and peritumoral normal cells.

While many questions still remain regarding the downstream biological effects of miR-SNPs, there is much work being done to see whether these germline polymorphisms can be used to risk-stratify cancer patients. Especially with improvements in the efficiency and cost of DNA sequencing technology, screening for miR-SNPs can potentially be easily integrated into the clinical workflow with potentially far-reaching clinical application. Many miR-SNPs have already been shown to be useful in a variety of cancer types as prognostic biomarkers. However, there are still relatively few predictive miR-SNP-based biomarkers that can help clinicians and patients personalize treatment decisions. Development of such predictive miR-SNP biomarkers will require careful patient selection and validation with clinical trials that randomize patients into treatment groups based on their biomarker status.

Conflicts of interest and disclosure

Weidhaas is an inventor on a patent filed by Yale University regarding the KRAS-variant and the founder of a company that has licensed the patent from Yale University. Yuan has no conflicts.

References

1000 Genomes Project Consortium (2015) A global reference for human genetic variation. Nature 526, 68–74.
Bao B-Y, Pao J-B, Huang C-N, Pu Y-S, Chang T-Y, Lan Y-H, Lu TL, Lee HZ, Jiang SH, Chen LM et al. (2011) Polymorphisms inside MicroRNAs and MicroRNA target sites predict clinical outcomes in prostate cancer patients receiving androgen-deprivation therapy. Clin Cancer Res 17, 928–936.
Caldas C and Brenton JD (2005) Sizing up miRNAs as cancer genes. Nat Med 11, 712–714.
Calin GA and Croce CM (2006) MicroRNA signatures in cancer genes. Cancer 6, 857–866.
Ceppi P and Peter ME (2014) MicroRNAs regulate both epithelial-to-mesenchymal transition and cancer stem cells. Oncogene 33, 269–278.
Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M et al. (2011) Improved survival with Vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med 364, 2507–2516.
Cipollini M, Landi S and Gemignani F (2014) MicroRNAs bind site polymorphisms as biomarkers in cancer research and management. Pharmgenomics Pers Med 7, 173–191.
Fabbri M, Valeri N and Calin GA (2009) MicroRNAs and genomic variations: from Proteus tricks to Prometheus gift. Carcinogenesis 30, 912–917.
Hanahan D and Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144, 646–674.
Hauschild A, Grob JJ, Demidov LV, Jovary T, Gutzmer R, Millward M, Rutkowski P, Blank CU, Miller WH Jr, Kaemppgen E et al. (2012) Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. Lancet 380, 358–365.
Kasowski M, Grubert F, Heffelfinger C, Hariharan M, Asabere A, Waszak SM, Habegger L, Rozowsky J, Shi M, Urban AE et al. (2010) (suppl.) Variation in transcription factor binding among humans. Science 328, 232–235.
Kent OA and Mendell JT (2006) A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. *Oncogene* 25, 6188–6196.

Kong YW, Ferland-mccollough D, Jackson TJ and Bushell M (2012) microRNAs in cancer management. *Lancet Oncol* 13, e249–e258.

Liang D, Meyer L, Chang DW, Lin J, Pu X, Ye Y, Ye Y, Gu J, Wu X and Lu K (2010) Genetic variants in microRNA biosynthesis pathways and binding sites modify ovarian cancer risk, survival, and treatment response. *Cancer Res* 70, 9765–9776.

Lujambio A and Lowe SW (2012) The microcosmos of cancer. *Nature* 482, 347–355.

Maurano MT, Humbert R, Rynees E, Thurman RE, Haugen E, Wang H, Reynolds AP, Sandstrom R, Qu H, Brody J et al. (2012) Systematic localization of common disease-associate variation in regulatory DNA. *Science (80-)* 337, 1190–1195.

Mishra PJ, Humeniuk R, Mishra PJ, Longo-Sorbello GSA, Banerjee D and Bertino JR (2007) A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. *Proc Natl Acad Sci USA* 104, 13513–13518.

Moszyńska A, Gebert M, Collawn JF and Bartoszewski R (2017) SNPs in microRNA target sites and their potential role in human disease. *Open Biol* 7, 1–13.

Nicoloso MS, Sun H, Spizzo R, Kim H, Wickramasinghe P, Shimizu M, Wojcik SE, Ferdin J, Kunej T, Xiao L et al. (2010) Single-nucleotide polymorphisms inside microRNA target sites influence tumor susceptibility. *Cancer Res* 70, 2789–2798.

Nishizaki SS and Boyle AP (2017) Mining the unknown: assigning function to noncoding single nucleotide polymorphisms. *Trends Genet* 33, 34–45.

Normanno N, Tejpar S, Morgillo F, De Luca A, Van Cutsem E and Ciardiello F (2009) Implications for KRAS status and EGFR-targeted therapies in metastatic CRC. *Nat Rev Clin Oncol* 6, 519–527.

Pan W, Yang J, Wei J, Chen H, Ge Y, Zhang J, Wang Z, Zhou C, Yuan Q, Zhou L et al. (2015) Functional BCL-2 regulatory genetic variants contribute to susceptibility of esophageal squamous cell carcinoma. *Sci Rep* 5, 1–8.

Pardini B, Rosa F, Barone E, Di Gaetano C, Slysikova J, Novotny J, Levy M, Garritano S, Vodickova L, Buchler T et al. (2013) Variation within 3′-UTRs of base excision repair genes and response to therapy in colorectal cancer patients: a potential modulation of microRNAs binding. *Clin Cancer Res* 19, 6044–6056.

Saunders MA, Liang H and Li W-H (2007) Human polymorphism at microRNAs and microRNA target sites. *Proc Natl Acad Sci USA* 104, 3300–3305.

Tak YG and Farnham PJ (2015) Making sense of GWAS: using epigenomics and genome engineering to understand the functional relevance of SNPs in non-coding regions of the human genome. *Epigenetics and Chromatin* 8, 1–18.

Teo MTW, Landi D, Taylor CF, Elliott F, Vaslin L, Cox DG, Hall J, Landi S, Bishop DT and Kiltie AE (2012) The role of microrna-binding site polymorphisms in DNA repair genes as risk factors for bladder cancer and breast cancer and their impact on radiotherapy outcomes. *Carcinogenesis* 33, 581–586.

Vasudevan S, Tong Y and Steitz JA (2007) Switching from repression to activation: MicroRNAs can up-regulate translation. *Science* 318, 1931–1934.

Vasudevan S, Tong Y and Steitz JA (2008) Cell cycle control of microRNA-mediated translation regulation. *Cell Cycle* 4101, 1545–1549.

Weidhaas JB, Harris J, Schau D, Chen AM, Chin R, Axelrod R, El-Naggar AK, Singh AK, Galloway TJ, Raben D et al. (2017) The KRAS-Variant and Cetuximab response in head and neck squamous cell cancer A secondary analysis of a randomized clinical trial. *JAMA Oncol* 3, 1–9.

Wyndendaue J, Böhne A, Leucci E, Nielsen SJ, Lambertz I, Hammer S, Sbrzesny N, Kubitz D, Wolf A, Gradhand E et al. (2010) An illegitimate microRNA target site within the 3′ UTR of MDM4 affects ovarian cancer progression and chemosensitivity. *Cancer Res* 70, 9641–9649.

Xu P, Liu L, Wang J, Zhang K, Hong X, Deng Q, Xiang J, Zhang X, He M, Wu T et al. (2013) Genetic variation in BCL2 3′-UTR was associated with lung cancer risk and prognosis in male Chinese population. *PLoS ONE* 8, 1–8.

Yao L, Tak YG, Berman BP and Farnham PJ (2014) Functional annotation of colon cancer risk SNPs. *Nat Commun* 5, 1–13.