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MicroRNA binding site polymorphisms as biomarkers of cancer risk

Cory Pelletier¹ and Joanne B Weidhaas^{†,1}

¹Yale University School of Medicine, 15 York St, New Haven, CT 06510 USA

Abstract

MicroRNAs (miRNAs) are well established as global gene regulators and thus, slight alterations in miRNA levels as well as their ability to regulate their targets may cause important cellular changes leading to cancer risk. 3' untranslated region (UTR) miRNA binding site single nucleotide polymorphisms (SNPs) have added another layer of possible genetic variation involved in the complex process of oncogenesis. Identifying these key genetically inherited effectors of miRNA functioning has improved our understanding of the complexity of disease. Interest in the field has grown rapidly in only the last 5 years, with several studies reporting on the role of 3' UTR binding site SNPs as genetic markers of increased cancer susceptibility, as well as biomarkers of cancer type, outcome and response to therapy. Currently, there are numerous known miRNA binding site SNPs associated with multiple cancer subtypes.

Keywords

3' UTR; cancer; miRNA; miRNA binding site; SNP

MicroRNAs (miRNAs) are a new and large class of small, noncoding RNAs that regulate gene expression by sequence-specific binding to target mRNAs (Figure 1) [1]. These miRNAs work to inhibit the mRNA translation into protein or lead to degradation of the mRNAs (Figure 2) [2]. miRNAs were originally discovered in 1993 in the nematode *Caenorhabditis elegans*. The discovery of the second miRNA, *let-7*, and its conservation across organisms generated intense interest in the identification of miRNAs. It was first estimated that there may be 1000 miRNA genes in the human genome [3]. However, a recent study estimates that the human genome contains over 3000 miRNAs [4], of which over 700 have been identified experimentally [5,201]. miRNA genes represent approximately 2–3% of the human genome [6], and each of them have hundreds of different conserved and non-conserved targets. It has been estimated that approximately 30% of genes are regulated by at least one miRNA [7].

Every cellular process is likely regulated by miRNAs, and an aberrant miRNA expression signature is a trademark of several diseases, including cancer, where they function as a novel class of oncogenes or tumor suppressors (Figure 3) [8]. Because miRNAs are global gene regulators, even small irregularities in miRNA levels or their target control leads to important cellular changes. The ability of miRNAs to bind to mRNA is critical for regulating mRNA level and protein expression. This binding can be affected by single

nucleotide polymorphisms (SNPs) that can reside in miRNA target sites and either eliminate an existing binding site or create an erroneous binding site (Figure 4) [1]. The role of miRNA target site SNPs in cancer is just beginning to be defined.

miRNA methods of action

How miRNAs target their mRNA transcripts remains elusive. The 5' end of the mature miRNA plays an important role in target recognition, often referred to as the seed region, and is crucial for both the stability of the mature miRNA and its incorporation into the miRISC complex [9–13]. The mature miRNA associates with target sites primarily in the 3' untranslated region (UTR) of mRNAs, directed by RNA–RNA interactions. If complementarity between the miRNA and the target mRNA is perfect, cleavage follows and the mRNA levels are decreased. If the interaction is imperfect, translational repression occurs [14,15]. The mechanism of translational repression is unclear [16,17].

The identification of miRNA targets is essential to the understanding of their regulatory function. There are a number of algorithms to help identify putative miRNA binding sites. MiRanda [18], DIANA-microT [19], RNAhybrid [20], MicroInspector [21], TargetScan [22] and TargetScanS [23] are based mainly on three characteristic properties: the complementarity of the 5' seed of the miRNA to the 3' UTR of the target mRNA, the negative folding free energy of the RNA–RNA duplex, and the conservation from species to species of the miRNA binding site and the resulting duplex [24]. PicTar [25] scans the 3' UTR for seed matches to miRNAs and filters results based on thermodynamic stability [24]. Probability of Interaction by Target Accessibility (PITA) computes the difference between the free energy gained from the formation of the microRNA–target duplex and the energetic cost of unpairing the target to make it accessible to the miRNA [26]. Similarly, MicroTar incorporates site accessibility with free energy measurements of unbound mRNA and miRNA–mRNA duplexes, without consideration for conservation of functional targets [27]. RNA22 identifies miRNA binding sites [28] and the resulting miRNA–mRNA duplex without regard for cross-species conservation[24].

Recently, two independent studies evaluating protein expression alterations resulting from miRNA activity tested target prediction sites. The first study evaluated five commonly used algorithms and found TargetScan and PicTar performed the best based on protein downregulation of predicted miRNA targets [29]. Finding similar results, Selbach *et al.*, concluded that PicTar, DIANA-MicroT, and TargetScanS supplied the most accurate predictions based on proteomic data [30]. Overall, these *in silico* approaches provide important tools for miRNA target detection and insight into the role of miRNAs in gene regulation. However, there are exceptions to these generalized rules on which the algorithms are based and therefore sometimes bioinformatics approaches lack accuracy. Combining computational approaches with experimental analysis is the next step in gaining a comprehensive understanding of miRNAs, their functions, and their targets.

Role for miRNAs in cancer

Cancer is the second leading cause of death, accounting for nearly a quarter of deaths in the USA, exceeded only by heart disease [31]. Worldwide, cancer is the third leading cause of mortality after cardiovascular and infectious diseases. Although major advances have been made in the understanding of cancer biology and pathogenesis as well as in the development of new targeted therapies, the progress in developing improved early diagnosis and screening tests has been inadequate. As a result, most cancers are diagnosed in advanced stages when treatment is less effective, leading to poor outcomes. Intense research today is focused on seeking specific molecular changes that are able to identify patients at high risk,

patients with early cancer or patients with precursor lesions [32]. These efforts are especially important as we are entering an era of personalized cancer medicine, where the goal is for treatment to be tailored to the unique genetic make-up of the individual and their tumor.

High-throughput expression profiling has been used for genome-wide assessment of mature miRNAs. Microarrays have revealed that miRNAs often exhibit tissue-specific expression signatures and these signatures might be implicated in tissue differentiation [33]. Recent studies have shown that miRNAs are also atypically expressed in virtually all cancers, where they are known to function as oncogenes or tumor-suppressor genes (Figure 3) [34]. Not surprisingly, expression-profiling analysis has revealed that there is differential miRNA expression across different types of cancers as well [35]. Furthermore, miRNA mis-expression can control initiating events resulting in a cancerous phenotype [36].

Examples of miRNA binding site SNPs & cancer risk

Since the discovery of the global role miRNAs play in biological processes, it is likely that mutations affecting miRNA function play a pathogenic role in human diseases. The ability of miRNAs to locate and bind mRNA is critical for regulating mRNA level and protein expression. SNPs are the most common germline variants that influence cancer susceptibility [37]. They can reside in miRNA target sites, altering the binding capacity of miRNAs by eliminating an existing binding site, creating an erroneous binding site [1] or affecting binding affinity. Mutations in miRNA binding sites can result in deleterious effects on gene-expression control [38].

There is ever-increasing evidence of SNPs in miRNA binding sites of protein-coding genes correlating to altered functions of proteins and resulting in increased susceptibility to a wide-range of cancers. miRNA binding site SNPs play a role as novel biomarkers of cancer risk and clinical cancer diagnosis, as well as outcome. This review will focus on known miRNA binding site SNPs and their correlations to multiple cancer subtypes (Table 1).

miRNA binding site SNPs & papillary thyroid cancer

Papillary thyroid cancer (PTC) is the most common malignancy in thyroid tissue, accounting for approximately 80% of all thyroid cancers [39]. It is also the most common thyroid carcinoma in the USA, characterized by changes in the RET/PTC–RAS–BRAF pathway [40,41]. Although classic Mendelian inheritance patterns have not been observed in PTC, He *et al.* showed that molecular regulation of PTC at the genetic level results from both protein coding genes and regulatory elements like miRNAs [39]. They demonstrated that five miRNAs (miR-146, miR-221, miR-222, miR-155 and miR-181a) were upregulated in PTC compared with normal tissue.

MicroRNA-221 and miR-222 are most consistently upregulated in PTC [42] and *KIT* is one of the genes that is targeted by these miRNAs [43]. *KIT* is a tyrosine kinase receptor involved in cell differentiation and growth [42]. It has been known for many years that one of the signatures of PTC is reduced *KIT* levels; however, the mechanism of downregulation was not clear [44]. Recently, He *et al.* showed that a number of miRNAs are overexpressed in PTC tumors compared with unaffected thyroid tissue from nine tumor samples paired with unaffected thyroid tissue. A subset of five miRNAs, including the three most upregulated ones (miR-221, miR-222 and miR-146), were found to be sufficient to predict cancer status in an additional 12 samples. Additionally, miR-221 was upregulated in unaffected thyroid tissue adjacent to PTC tumors from all papillary thyroid patients, potentially an early event in carcinogenesis. In tumors with the highest upregulation of miR-221, miR-222 and miR-146 (11–19-fold increase) there was a dramatic loss of *KIT* transcript and protein [39]. The group then sequenced the miR-221, miR-222 and miR-146

binding domains in 48 patient and three cell-lines. They found a 3' UTR SNP G3169A (rs17084733) in one of the recognition sites of miR-221 and miR-222, and five patients sequenced were heterozygous for this SNP. Additionally, a synonymous G2607C SNP (rs3733542) in exon 18 was located within miR-146a and miR-146b binding sites. Heterozygosity was found in the same five patients observed with SNP G3169A, suggesting linkage disequilibrium. The tumors of all five double heterozygous patients displayed downregulation of the *KIT* transcript. Based on *in silico* analysis, these miRNA-binding site SNPs are predicted to modify miRNA binding to *KIT* mRNA, thereby increasing the efficiency of miRNA-mediated translational inhibition of *KIT* [39].

The proposed miRNA binding site SNP-induced misregulation of *KIT* presented here will be important to verify *in vitro*, exemplified by the fact that little is currently known about genetic changes involved in PTC tumorigenesis.

miRNA binding site SNPs & breast & ovarian cancer risk

Breast cancer is the second leading cause of cancer-related deaths in women today, accounting for 26% of new cancer diagnoses in 2008 [45]. Additionally, ovarian cancer is the second most common gynecologic malignancy and the most common cause of death from gynecologic malignancies [45]. Carriers of germline *BRCA1* or *BRCA2* gene mutations are at a substantially increased risk for developing breast and ovarian cancer; the estimated lifetime risk for developing breast cancer is approximately 80%, and approximately 50% for ovarian cancer [46,47]. However, there is considerable variation in the phenotype of mutation carriers, as well as variation in penetrance in *BRCA1* and *BRCA2* mutation carriers [48]. This implies the existence of other modifying factors, genetic and/or environmental, as players in the development of breast and ovarian malignancies. Modification of cancer risk by other genes is still being studied, and additionally, the putative role that miRNAs play in affecting breast and ovarian cancer risk is just beginning to be defined.

Several miRNAs have been linked with breast cancer. For example, in 2005 Iorio *et al.* used genome-wide miRNA expression profiling in a large set of normal and tumor breast tissues to demonstrate the existence of a breast cancer-specific miRNA signature [49]. The overall miRNA expression could clearly separate normal from breast cancer tissue. They found the most significantly aberrantly expressed miRNAs to be miR-125b, miR-145, miR-21 and miR-155. Both miR-21 and miR-155 were upregulated, suggesting that they may act as oncogenes, while the remaining deregulated miRNAs were downregulated, possibly acting as tumor-suppressor genes [49].

Differential expression of genes encoding some miRNAs are associated with pathologic features of breast cancer [50]. Downregulation of miR-30 is found in estrogen/progesterone receptor-negative cancers [49]. Additionally, Iorio *et al.* reported that miR-203 and miR-213 correlate with tumor stage. They found that increased expression of the genes encoding these miRNAs is found in higher stage breast cancers [49]. MiR-206 has been shown to be upregulated in estrogen receptor (*ER*-) negative breast cancer [49]. MiR-206 was further evaluated in *ER*- negative breast cancer to see if it regulates the expression of *ER*-protein [51]. MiR-206 decreases endogenous *ER*- mRNA and protein levels in human MCF-7 breast cancer cells by acting through two specific miR-206 target sites within the 3' UTR of the human *ER*- transcript. Adams *et al.* show with *in vitro* luciferase that the C/T SNP (rs9341070) within the first miR-206 binding site is functional; the variant allele T allows increased binding of miR-206 and downregulation of *ER*- [51]. These findings provide the first evidence of a specific miRNA involved in the regulation of the human *ER*- in breast cancer cell lines.

Brendle *et al.* found 3 UTR polymorphisms in predicted miRNA binding sites in integrin genes to be risk factors for breast cancer. The study evaluated associations between genotypes and breast cancer risk, clinical tumor characteristics and survival probability by tumor subtype. Integrins control the cell attachment to the extracellular matrix and play an important role in mediating cell proliferation, migration and survival [52]. A number of important cancer-associated integrins can be regulated by miRNAs, and therefore may contribute to altered expression of integrin genes [53]. In Brendle's case-control study, an association was observed between the A allele of rs743554 in the *ITGB4* gene and estrogen receptor-negative tumors (odds ratio [OR]: 2.09; 95% CI: 1.19–3.67). The same SNP was associated with survival; the variant allele (A) carriers had a worse survival compared with the wild-type genotype carriers (hazard ratio [HR]: 2.11; 95% CI: 1.21–3.68). The poor survival was significantly associated with aggressive tumor characteristics, such as high grade, lymph node metastasis and high stage. However, none of the SNPs evaluated in the study were significantly associated with breast cancer risk [52]. Furthermore, the variant allele (A) may cause a loss of the binding site for miR-34a, supported by evidence for an overexpression of miR-34a in breast cancer compared with normal breast tissue [49] and *in silico* approaches. By causing a loss of the miR-34a binding site in the *ITGB4* gene, the SNP rs743554 may enhance the ability of *ITGB4* to promote tumor cell growth, survival and invasion and thus partly explain the observed poor survival of the carriers of the variant allele.

Recently, 11 miRNA target site SNPs located in the 3 UTRs of genes involved in cancer and breast cancer were analyzed for their impact on breast cancer risk, using a large familial study population [54]. A significant association was revealed for the variant allele (T) at rs2747648, affecting a putative miRNA target site, miR-453, in the estrogen receptor (*ESR1*). This SNP was associated with familial breast cancer risk, especially in premenopausal and high-risk women (C vs T: OR: 0.6; 95% CI: 0.41–0.89; $p = 0.010$ and OR: 0.42; 95% CI: 0.25–0.71; $p = 0.0009$, respectively). Because clinical studies have shown that reduction of *ESR1* reduces breast cancer risk, current therapies for breast cancer block *ESR1*. Bioinformatics tools were employed to show that SNP rs2747648 in *ESR1* affects the binding capacity of miR-453, which is stronger when the C allele is present. By contrast, the T allele is predicted to attenuate the binding of miR-453, which may lead to higher *ESR1* protein levels and an increased breast cancer risk. Therefore, the protective effect observed for the C allele in premenopausal women makes sense.

MicroRNA-125b has been shown to negatively regulate *BMPR1B* and allelic variation at SNP rs1434536 exists within this 3 UTR miRNA-binding site of *BMPR1B* [55]. The SNP's C to T change is predicted to alter a 7-mer seed site for miR-125b to a 6-mer seed site, a change expected to reduce miR-125b's binding affinity to the site. Moreover, miR-125b and *BMPR1B* are both differentially expressed in normal compared with breast cancer in general, and in estrogen receptor-positive versus estrogen receptor-negative tumors in particular [49,56,57]. Additionally, this study found that allelic variation of rs1434536 likely disrupts miR-125b's regulation of *BMPR1B* and that the SNP is in linkage disequilibrium with two breast cancer-associated SNPs (rs1970801 and rs11097457), suggesting that rs1434536 has a pathologic role in breast cancer [55].

Single nucleotide polymorphisms in miRNA binding sites located in selected genes in *BRCA1* and *BRCA2* mutation carriers affected mutant *BRCA* penetrance [48]. This study genotyped SNPs in miRNA binding sites within the 3 UTR of gene transcripts of patients who had one of the predominant mutations in Jewish Israeli women in either *BRCA1* (185delAG, 5383InsC, Tyr978X) or *BRCA2* (6174delT, 8765delAG). Four polymorphic SNPs predicted to be involved in miRNA regulatory action showed association with various health status categories and disease morbidity. Two of these polymorphisms are located in

predicted miRNA binding sites: rs11169571 (miR-320) and rs3842753 (miR-491). Rs11169571 is located in the 3' UTR of the *ATF1* gene and among *BRCA2* mutation carriers, CT heterozygotes were associated with an increased risk of developing breast/ovarian cancer compared with having a TT genotype, for the ancestral T allele (relative risk [RR]: 2.05; 95% CI: 1.24–3.39, $p = 0.005$). Rs3842753 is located in the 3' UTR of the *INS* gene and among *BRCA1* carriers, AA homozygotes develop ovarian cancer at an older age compared with either AC or CC carriers. AC heterozygotes had an increased chance of developing ovarian cancer as compared with AA homozygotes (RR: 4.78; 95% CI: 1.36–16.86; $p = 0.015$) and CC homozygotes had a higher risk of developing ovarian cancer as compared with AA homozygotes (RR: 3.35; 95% CI: 0.99–11.29; $p = 0.051$) [48]. It would be important to verify these results *in vitro*, to gain insight into the exact mechanistic biological effect of these SNPs on miRNA binding or on gene regulation and cancer predisposition.

A common SNP (rs16917496) was first predicted to be in the miR-502 binding site in the 3' UTR of the *SET8* gene by Yu *et al.* in 2007 in a large-scale search for interesting miRNA target SNPs using an *in silico* screen [38]. Song *et al.* further discovered this miR-502 binding SNP to be associated with early age of breast cancer onset [58]. *SET8* methylates *TP53* and regulates genome stability. Additionally, *TP53* mutations are associated with Li-Fraumeni syndrome and contribute to an early age of onset of several cancers, including breast cancer [59,60]. The group found that the *SET8* C allele interacts synergistically with the *TP53* G allele in a dose-dependent manner to lower the age of onset of breast cancer [58]. Individuals with the *SET8* CC and *TP53* GG genotypes developed cancer at a median age of 47.7 years compared with 54.6 years for individuals with the *SET8* TT and *TP53* CC genotypes. Finally, in breast cancer tissue samples tested, the *SET8* CC genotype was associated with reduced *SET8*, but not miR-502, transcript levels [58]. No *in vitro* luciferase assays were done in this study.

The *BRCA1* 3' UTR was evaluated around the same time for miRNA binding site SNPs, specifically the derived (and less frequent) alleles at rs12516 and rs8176318 showed positive association in Thai women with familial breast and ovarian cancer [61]. The study evaluated 46 patients and 103 unaffected Thais. They found that homozygosity for the derived alleles, A, at both SNP sites are found in cancer patients at triple the frequency seen in unaffected Thais, yielding a significant cancer association ($p = 0.007$). Functional analysis showed reduced activity of *BRCA1* function with the derived alleles at both sites when present on the same chromosome, that is, in *cis*. Additionally, when the derived alleles were analyzed in separate plasmids, both showed reduced luciferase activity. However, a greater reduction was seen with the derived allele at rs8176318, suggesting altered miRNA activity at this site by way of altered gene expression. It is discussed that rs8176318 may be a miRNA binding site; miRNA binding is predicted to differ slightly based on the allele present (G allele: miR-525–5p and miR-520a–5p, T allele: miR-518d–5p, miR-523, miR-525–5p and miR-520a–5p). This study found that the 3' UTR SNPs were not associated with known *BRCA1* mutations. However, no analysis of the association of these *BRCA1* 3' UTR SNPs in the different subtypes of breast cancer was done.

A recent study by Nicoloso *et al.* looked at SNPs known to be associated with breast cancer risk, *in silico* and *in vitro*, for their ability to modify miRNA binding sites and miRNA gene regulation [62]. They identified two transcribed exonic missense mutations associated with breast cancer risk, rs1982073-*TGFB1* and rs1799782-*XRCC1*, as target SNPs whose alleles could alter gene expression by differential interaction with miR-187 and miR-138, respectively. It is interesting to note that, according to the *in silico* predictions for rs1799782, the [T] variant was the active allele, increasing the binding energy of miR-138 compared with the ancestral [C] allele. When miR-138 was assayed with pGL3-rs1799782-

XRCC1 constructs, they observed a significant increase in luciferase activity with the [T] allele compared with the [C] allele (34% increase; $p = 0.0003$) and compared with the scrambled miRNA used as a negative control. This stabilizing role of miR-138 displaying stronger binding with rs1799782 variant [T] allele suggests that miRNAs can cause translational activation [63,64]. Testing the effects of target SNPs rs1982073 and rs1799782 on endogenous protein levels in cancer cell lines carrying different SNP genotypes by transfecting with the interacting miRNAs verified the results. The group further demonstrated that rs799917-*BRCA1* exonic SNP (variant T allele) and rs334348-*TGFBRI* 3' UTR SNP (AG risk genotype) are both implicated in breast cancer risk. Luciferase activity of target SNPs, allelic variants and protein levels in cancer cell lines with different genotypes showed differential regulation of target genes following overexpression of the two interacting miRNAs (miR-638 and miR-628-5p, respectively).

Although studies on SNPs within miRNA binding sites of breast and ovarian cancer associated genes are at an early stage and the results from reported studies need replication and many still need functional validation, the above results are encouraging in the search for identifying women at high risk for developing these malignancies.

miRNA binding site SNPs & lung cancer

In 2009, 219,440 new cases of lung cancer were expected and 159,390 individuals were expected to succumb to their disease. Worldwide, lung cancer accounts for the greatest number of cancer-related deaths in both men and women. Non-small-cell lung cancer (NSCLC) is the most common cause of lung cancer-related deaths [50]. Lung cancer has traditionally been causally associated with smoking. The increased lung cancer incidence among smokers is proportional to the length and intensity of smoking history [65]. On average, a lifetime smoker has a 20-fold increase in the risk of developing lung cancer compared with a lifetime nonsmoker [66].

Despite many recent advances in lung cancer research, there are few known genetic markers to identify patients at increased risk. Altered expression of the *let-7* family of miRNAs is implicated in many human cancers. The *let-7* family of miRNAs are global gene regulators important in controlling lung cancer oncogene expression through base pairing interactions with sequences in their 3' UTRs [67]. The human *RAS* genes contain *let-7* complementary sites in their 3' UTR, and *let-7* has been shown to repress *RAS* expression [67]. This family of miRNAs is found at low levels in NSCLC and their lower levels are biomarkers for poor outcome [67–70]. Mechanistically, this leads to enhanced expression of the oncogene *KRAS* [67]. *KRAS* is a member of the *RAS* oncogenes and is activated by somatic mutation in many human cancer types [71]. The activation of the oncogene *KRAS* has been well documented in carcinomas of the lung, colon and pancreas [72]. Amplification of *KRAS* has been reported to promote growth of head and neck squamous cell carcinoma (HNSCC) as well [73].

Rs61764370 is a functional SNP located in the 3' UTR of the *KRAS* gene that leads to disruption of *let-7* binding and is referred to as the *KRAS*-variant. The *KRAS*-variant is found in approximately 6% of world populations and in 12–15% of European Caucasian populations. These figures are based on the genotyping of over 2500 people representing 46 world populations [74]. This variant, in the sixth *let-7* complementary site in the *KRAS* 3' UTR (LCS6), results in upregulation of the *KRAS* gene and is associated with low levels of *let-7* in tumors. The *KRAS*-variant is a genetic marker of increased susceptibility to NSCLC, as shown by Chin *et al.* in two USA case-control studies, where the prevalence of the variant in NSCLC patients was 18–20% and in the noncancerous USA control populations was 12–14% [74]. The *KRAS*-variant was specifically associated with an

increase in lung cancer risk among smokers with less than a 41 pack-year smoking history, suggesting that this variant is a genetic marker of susceptibility to the carcinogenic effects of tobacco smoke.

The same *KRAS*-variant was recently evaluated for its association with *KRAS* mutations (codon 12, previously described [75]) as well as patient survival [76]. Overall, the group found no significant association of the *KRAS*-variant with *KRAS* mutations. Owing to the modifying effects of smoking reported by Chin *et al.* [74], Nelson *et al.* further examined their data stratified by 40 pack years smoked, resulting in no evidence for association between *KRAS* mutation and *KRAS* variant. Lastly, Nelson *et al.* evaluated survival time as a function of genotype and found no association between the *KRAS*-variant and survival among all histologies or by histologic subgroups [76].

Given that the *KRAS* variant has been implicated in a number of cancers [74,77,78], it displays promise for a wide-range clinical utility.

miRNA binding site SNPs & oral cancer outcome

Head and neck/oral cancer (HNOC) is a devastating disease that is undertreated and understudied [79]. Oral cavity and pharynx cancers are in the ninth leading site of new cancer cases in men predicted by the American Cancer Society for 2009 [202]. Over 90% of HNOC are oral squamous cell carcinoma (HNSCC) [79]. According to the American Cancer Society cancer statistics [45,80–83], new cases for HNOC increased approximately 25% during the past 5 years and deaths associated with HNOC increased by 5% over the past 5 years as well. Improvement in diagnosis, treatment and patient survival require a better understanding of the disease progression to allow for early detection and the configuration of targeted therapies. It has been known for some time that HNOC is associated with heavy smoking, alcohol abuse and human papillomavirus [84,85]. Interestingly, in recent years there has been an increased incidence of HNOC in nonsmokers and nondrinking patients. This suggests that environmental and genetic factors also contribute to the disease [86].

Only a small number of investigations into miRNAs and oral cancers have been published. Tran *et al.* completed the largest genome-wide study of mature miRNAs in cancers of the head and neck in 2007 [87]. They investigated 261 mature miRNA genes in nine head and neck cancer cell lines. The cell lines were from carcinomas of hypopharynx, larynx, tongue and tonsil. The authors found 33 miRNAs to be highly expressed and 22 showed low levels of expression in all cell lines tested. *Let-7a* and miR-21 are among the miRNAs overexpressed in oral cancers. This study, in combination with two other studies of miRNA expression profiles in HNSCC have highlighted the importance of miRNA expression alterations in HNSCC tumorigenesis [88,89].

The variant allele in the *KRAS* 3' UTR, discovered by Chin *et al.* was examined for its association with HNSCC [77]. Christensen *et al.* examined the prevalence of the *KRAS*-variant in a population-based case–control study of HNSCC to determine if the variant allele was associated with disease occurrence and patient survival. The group did not find an association of the *KRAS*-variant with overall risk of developing HNSCC however, cases with the variant genotype had significantly reduced survival (HR: 1.6; 95% CI: 1.0–2.5). The risk was greatest in cases of oral cavity carcinoma (HR: 2.7; 95% CI: 1.4–5.3). It would be useful to investigate the mechanism by which the variant genotype alters the phenotype of these tumors leading to poor outcome.

miRNA binding site SNPs & colorectal cancer

Colorectal cancer (CRC) is both a leading site for new cancer cases and a leading cause of death from cancer in the USA [90]. CRCs share many environmental risk factors and some are found in individuals with specific genetic syndromes [90]. The survival and prognosis of patients depends on the stage of the tumor at the time of detection. Unfortunately, over half of all tumors at diagnosis have regional or distant spread of the disease [91]. Increased screening to encourage early detection of CRC has been a mainstay in clinical prevention and diagnosis of CRC [92].

Studies have recently identified specific miRNAs in colonic tissue and serum that might be used to screen for the presence of CRCs and also to help predict disease recurrence. Both overexpression and silencing of specific miRNAs have been described in the tumorigenesis of CRCs [93]. Overexpressed miRNAs, such as miR-20, miR-21, miR-17-5p, miR-15b, miR-181b, miR-191 and miR-200c, have been implicated in CRC tissues [94–96]. These tumor promotor miRNAs function by targeting and inhibiting different tumor-suppressor genes [97]. Lower levels of mature miRNAs, such as miR-34a, miR-126, miR-143, miR-145 and miR-342, are also found in CRCs, suggesting that they act as tumor-suppressor miRNAs [98–101]. Through miRNA expression profiling researchers have also demonstrated that, in addition to the distinction of tumors from normal tissue, miRNA expression can characterize tumor type, stage and other clinically relevant variables [35,95,102].

The 2008 Landi *et al.* paper was the first study to report a positive association between miRNA binding site SNPs and cancer risk [103]. The group selected 104 candidate genes for CRC and identified putative miRNA binding sites within the 3 UTRs of these candidate genes by using specialized algorithms, such as microInspector [21]. A total of 46 of the genes did not harbor any SNPs in their 3 UTRs, and 21 genes did not have SNPs in miRNA binding sites. A total of 57 SNPs were identified in miRNA binding sites. Landi *et al.* then evaluated these SNPs for their ability to impact the binding of the miRNA with its target, by assessing the Gibbs free energy between the two alleles of each SNP. They found eight known SNPs (in the 3 UTRs of *CD86*, *NOD2*, *IL16*, *IL12B*, *ALOX15*, *PLA2G2A*, *PTGER4* and *INSR*) that were further investigated by a case–control association study. The study was carried out in a series of cases and controls from the Czech Republic. They found association between CRC risk and variant alleles at rs17281995 of the *CD86* gene (OR: 2.74; 95% CI: 1.24–6.04, for the variant homozygotes) and rs1051690 of the *INSR* gene (OR: 1.94; 95% CI: 1.03–3.66, for the variant homozygotes). Five miRNAs were predicted to bind to rs17281995: miR-337, miR-582, miR-200a*, miR-184 and miR-212. When the C allele is present, miR-337, miR-582 and miR-200a* are predicted to bind less tightly to the 3 UTR SNP. By contrast, miR-184 and miR-212 increase their binding affinity with the C allele present. Two miRNAs were predicted to bind to rs1051690: miR-618 and miR-612. This study provides evidence that SNPs in miRNA binding sites may be important in cancer risk and supports future work to validate the results in other populations. Additionally, it would be interesting to understand the function of these variants *in vitro*.

Recently, another paper was published to evaluate the *let-7* complimentary site (LCS6) T more than G, the *KRAS*-variant, in the *KRAS* 3 UTR in patients with metastatic CRC treated with anti-EGF receptor therapy [78]. In this study, patients were analyzed for associations between the *KRAS*-variant and overall survival (OS) and progression-free survival (PFS). *KRAS*-variant G-allele carriers (variant allele) showed worse OS ($p = 0.001$) and PFS ($p = 0.004$) than T/T genotype carriers. Survival analyses also focused on 55 unresponsive patients whose primary tumors were positive for a *KRAS* mutation, and found that the *KRAS*-variant G-allele carriers showed worse OS and PFS times [78]. The role of

the *KRAS*-variant in treatment response in patients receiving EGF receptor inhibitors supports the function of this variant.

miRNA binding site SNPs & hepatocellular cancer

Hepatocellular carcinoma (HCC) is one of the leading malignancies worldwide, and in China it is ranked second among all malignancies [104,105]. Many of the risk factors of HCC (>85%) are preventable: hepatitis B and C, Type II diabetes, obesity, aflatoxin B1 and alcohol abuse [106,107]. Owing to the high fatality rate, the incidence and mortality rates are comparable [108]. Intense research in recent years has led to an increased understanding of the molecular biology of carcinogenesis and tumor progression of HCC. However, the molecular and cellular mechanisms of HCC pathogenesis are still poorly understood [109]. There is evidence to suggest a relationship between differential miRNA expression and HCC [110,111]. As proven in a number of cancers, variants in miRNA binding sites can alter miRNA gene regulation and consequently, contribute to cancer susceptibility. Identification of these susceptibility alleles related to HCC are important to the identification of high-risk cohorts, early diagnostic tools and therapeutic strategies that can improve treatment and outcome for this deadly disease.

Gao *et al.* studied an insertion/deletion (Indel) polymorphism (rs3783553) located in the 3' UTR of *IL-1* (*IL1A*) for a case-control study in a Chinese population [112]. *IL-1* is a pleiotropic cytokine that affects immune and inflammatory responses, regulates other homeostatic functions of the body and has important influence on the pathogenesis of disease [113]. Rs3783553 (variant homozygote, insertion/insertion) was found to statistically associate with risk of HCC (OR: 0.62; 95% CI: 0.49–0.78; $p < 0.0001$) in Chinese populations and higher *IL1A* expression was observed in patients harboring the variant allele. Furthermore, this 3' UTR polymorphism significantly affects the binding of miR-122 and miR-378 and influences the regulation of *IL1A* expression of those miRNAs *in vitro*.

More recently, Chen *et al.* conducted a case-control study on the Indel polymorphism (rs16405) in the 3' UTR of γ -Transducin repeat-containing protein (*TrCP*) gene to investigate if a particular allele or genotype of this polymorphism would modify the occurrence of HCC in the Chinese population and the potential pathogenic mechanism of HCC mediated by the Indel [109]. *TrCP* plays a key role in the S and G2 DNA-damage response checkpoint, the main function of which is to mediate cell cycle arrest to allow time to repair DNA lesions [114]. Alterations in cell cycle regulators can be a contributing factor in the uncontrolled proliferation that is common in cancer cells [109]. *TrCP* acts as an oncogene and overexpression of *TrCP* has been reported in multiple cancers [115–117]. Chen and colleagues found the nine base pair Indel, rs16405, to be associated with decreased HCC risk with the genotypes deletion/deletion and insertion/deletion (OR: 0.44; 95% CI: 0.24–0.83; $p = 0.004$ and OR = 0.56; 95% CI: 0.31–1.00; $p = 0.034$, respectively). Furthermore, *in vivo* experiments showed that mRNA levels of *TrCP* from HCC tumor tissue correlated with rs16405 genotypes; HCC tumor tissue with genotype insertion/insertion had the highest level of *TrCP*, 3.99- and 7.04-fold higher than genotypes insertion/deletion and deletion/deletion, respectively. Based on an *in silico* approach, it is predicted that the [deletion] allele would allow miR-920 to bind tightly to *TrCP* mRNA transcripts, leading to negative regulation of *TrCP* expression. Alternatively, the binding of miR-920 with mRNA transcripts containing the [insertion] allele would be disrupted, allowing upregulated *TrCP* expression. However, functional experiments have yet to be done to confirm this mechanism.

miRNA binding site SNPs & their future use: risk assessment, diagnosis, prognosis & treatment

The 3' UTR of mRNAs have, until recently, been generally neglected as potential sources of sequence variations with a possibly pathogenic effect in cancer development or as biomarkers of tumor response to treatment. The study of miRNA binding site SNPs has brought great promise for diagnosis, determining prognosis and ultimately directing therapy for cancer. Some of the candidate SNPs in miRNA binding sites discovered so far have uncovered the possible powerful roles of this form of novel gene disruption in all aspects of cancer.

Polymorphisms that are located in miRNA binding sites likely have a functional role in the regulation of gene expression and possibly further alter cellular homeostasis, therefore contributing to an individual's susceptibility to cancer, as well as their cancer's biology and response to therapy. Although current technology has made it difficult to determine biologically relevant miRNA targets, there is substantial evidence that alterations at miRNA target sequences contribute to cancer, as reviewed here. In certain cancers, once genetic risk is identified, there are specific management strategies that can be employed for early detection, risk reduction and possible prevention of cancers. Additionally, as detailed here, the same functional variants that predict cancer risk may well predict cancer outcome and response to therapy, allowing additional steps towards personalized medicine.

A better understanding of the functional role of polymorphisms in miRNA binding sites will be a key step to allow us to fully exploit these new data to change the way human cancers are categorized, diagnosed and treated in our quest for personalized cancer treatments. This review highlights some of the earliest findings in this area of study, and based on their promise supports the need to aggressively study the 3' UTR in all cancer-associated genes for additional important variants that may alter miRNA function and give further insight into cancer pathogenesis.

Expert commentary

The importance of 3' UTR miRNA binding site SNPs and their link to cancer is just beginning to be defined. Large amounts of data on various cancers have been compiled in a very short period of time. The evolution of this field may soon revolutionize how clinicians predict, classify and treat cancers.

The studies reviewed here support the possibility of developing new cancer risk predictive systems based on inherited polymorphisms that may also then be used to predict patient outcome. Currently, one of the limitations restraining the field of cancer is identification of those at greatest risk and further risk stratification to optimize treatment. Defining distinct miRNA binding site SNPs and how they predict the biology of different cancers is a significant step towards being able to accomplish these important goals. We believe these studies will help clinicians identify people at an increased risk of cancer, and achieve earlier cancer diagnosis, including type, severity and how they are likely to respond to therapy.

As the field continues to grow, it is likely that many more miRNA binding site SNPs will be discovered with links to cancer, and there may well be an interaction between such SNPs. Moving forward, it is important to pursue studies consisting of larger patient populations, with accompanying high accuracy and excellent quality of clinical data. Additionally, by improved understanding of the function of these polymorphisms we will be best poised to fully utilize the information to better shape cancer care.

Five-year view

The emergence of 3' UTR miRNA binding site polymorphisms as key disrupters of gene function leading to cancer predisposition is likely to have a large effect on future clinical treatment approaches. However, only a small number of studies have been completed examining 3' UTR SNPs located in miRNA binding sites and their correlation to cancer. In the next 5 years we hope to create a thorough library of functional 3' UTR miRNA binding site alterations impacting various cancer predispositions. Furthermore, to understand how these miRNA binding site SNPs also influence cancer type, severity, outcome and response to therapy will be important. It is probable that technology will advance to a level that will make it easier to determine biologically relevant miRNA targets for pursuit. It will be necessary to get a solid foundation in the understanding of distinct miRNA binding site SNPs and how they affect protein regulation in different cancers before we begin to utilize these new data to shape not only our overall understanding of oncogenesis, but also the way human cancers are categorized, diagnosed and treated for our ultimate goal of personalized cancer treatments in the future.

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Key issues

- MicroRNAs are a new and large class of small, noncoding RNAs that regulate gene expression by sequence-specific binding to target mRNAs.
- Recent studies show that miRNAs are aberrantly expressed in virtually all cancers studied so far.
- Given that miRNAs are global gene regulators, even small irregularities in miRNA levels or their control leads to important cellular changes.
- MicroRNA binding can be affected by single nucleotide polymorphisms (SNPs) that can reside in miRNA target sites and either eliminate an existing binding site or create an erroneous binding site.
- Several 3' untranslated region (UTR) miRNA-binding site SNPs have been correlated to multiple cancers.
- In some cases the SNPs are genetic markers of an increased cancer risk and in some cases they also act as biomarkers of cancer outcome and response to therapy.
- Moving forward, it is important to aggressively study the 3' UTR in cancer-associated genes for additional important variants that may alter miRNA function and give further insight into cancer pathogenesis.

Figure 1. miRNA biogenesis

miRNAs are transcribed by RNA polymerase II or III as long pri-miRNAs that are 5' 7-methyl-guanosine-capped and polyadenylated. Pri-miRNAs are subsequently processed by the RNase III endonuclease Drosha in conjunction with the dsRNA binding protein DGCR8/Pasha, to form pre-miRNAs. Pre-miRNAs are exported from the nucleus to the cytoplasm in a Ran-GTP-dependent manner by exportin-5 and are subject to an additional processing step by another RNase III enzyme, Dicer, releasing a dsRNA duplex, which is then incorporated into the miRISC complex. At this point the mature miRNA is capable of regulating its target genes.

pre-miRNA: Precursor miRNA; pri-miRNA: Primary miRNA

Figure 2. miRNA regulation of gene expression

miRNAs regulate gene expression through 3' untranslated region complementary binding to target mRNA. miRNAs downregulate mRNA protein translation or degrade the mRNAs.

Figure 3. MiRNA involvement in cancer pathogenesis

miRNAs are regulators of gene expression. Here we show miRNA role in cancer by regulation of tumor-suppressor genes and oncogenes. **(A)** Overexpression of miRNAs can decrease expression of tumor-suppressor genes. **(B)** Underexpression of miRNAs can increase expression of oncogenes.

Figure 4. Mutations in miRNA target sites: flawed target recognition

Mutations in the 3' untranslated region (UTR) of mRNAs can lead to the obliteration or generation of a target recognition site for a specific miRNA. 3' UTR mutations can also lead to altered binding efficiency, creating looser binding or lower recognition frequency.

Table 1

Summary of cancer risk polymorphisms thought to cause microRNA misregulation contributing to cancer predisposition.

Tumor	Gene (chromosome location) [†]	SNP (location)	microRNA	Evidence	Ref.
PTC	<i>KIT</i> (4q12)	rs17084733 (3 UTR)	miR-221, miR-222	Bioinformatics predictions	[39]
		rs3733542 (exonic)	miR-146a, miR-146b		
Breast	<i>ER-</i> (6q25.1)	rs9341070 (3 UTR)	miR-206	Bioinformatics predictions and experimental with luciferase assays	[51]
Breast	<i>ITGB4</i> (17q25.1)	rs743554 (3 UTR)	miR-34a	Bioinformatics predictions	[52]
Breast	<i>ESR1</i> (6q25.1)	rs2747648 (3 UTR)	miR-453	Bioinformatics predictions	[54]
Breast	<i>BMPR1B</i> (4q22.3)	rs1434536 (3 UTR)	miR-125b	Bioinformatics predictions and experimental with luciferase assays	[55]
Breast/ovarian	<i>ATF1</i> (12q13.12)	rs11169571 (3 UTR)	miR-320	Bioinformatics predictions	[48]
Ovarian	<i>INS</i> (11p15.5)	rs3842753 (3 UTR)	miR-491		
Breast	<i>SET8</i> (12q24.31)	rs16917496 (3 UTR)	miR-502	Bioinformatics predictions [38] No luciferase assay (real-time PCR)	[58]
Breast/ovarian	<i>BRCA1</i> (17q21.31)	rs8176318 (3 UTR)	Allele G: miR-525-5p miR-520a-5p Allele T: miR-525-5p miR-520a-5p miR-518d-5p miR-523	Bioinformatics predictions and experimental with luciferase (lacking data to show specificity with predicted miRNAs, but does show decreased luciferase activity with variant allele compared with ancestral allele)	[61]
Breast	<i>TGFB1</i> (19q13.2)	rs1982073 (exonic)	miR-187	Bioinformatics predictions experimental with luciferase	[62]
	<i>XRCC1</i> (19q13.31)	rs1799782 (exonic)	miR-138		
	<i>BRCA1</i> (17q21.31)	rs799917 (exonic)	miR-638		
	<i>TGFBR1</i> (9q22.33)	rs334348 (3 UTR)	miR-628-5p		
Lung	<i>KRAS</i> (12p12.1)	rs61764370 [‡] (3 UTR)	Let-7	Bioinformatics predictions and experimental with luciferase	[74]
CRC	<i>CD86</i> (3q13.33)	rs17281995 (3 UTR)	miR-337 miR-582 miR-200a*, miR-184 miR-212	Bioinformatics predictions	[103]
	<i>INSR</i> (19p13.2)	rs1051690 (3 UTR)	miR-618 miR-612		
HCC	<i>IL1A</i> (2q13)	rs3783553 (3 UTR)	miR-122 miR-378	Bioinformatics predictions and experimental with luciferase	[112]
HCC	<i>bTrCP</i> (10q24.32)	rs16405 (3 UTR)	miR-920	Bioinformatics predictions	[109]

[†]Chromosome locations based on dbSNP build 130.

[‡]This variant has now been implicated in CRC and head and neck/oral cancer [76,77].

CRC: Colorectal cancer; HCC: Hepatocellular carcinoma; PTC: Papillary thyroid cancer; SNP: Single nucleotide polymorphism; UTR: Untranslated region.