Chapter 10
MicroRNAs in Bladder Cancer

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Abstract Bladder cancer is the fourth most common solid malignancy in men and fifth most common overall with an estimated 70,000 new cases of urothelial carcinoma (UC) and over 14,000 deaths from the disease expected in 2010 in the United States. Although the majority of patients with invasive bladder cancer present without radiographic or clinical evidence of disease beyond the bladder, up to 56% of patients die from the result of occult metastasis not detected by current staging modalities. The potential of microRNAs (miRNAs) as novel tumor markers has been the focus of recent scrutiny because of their tissue specificity, stability, and association with clinical-pathological parameters. Prognostic tools based on conventional clinical and pathologic staging can quantify the risk of death from UC, but their accuracy is imperfect due to the heterogeneous biologic behavior of tumors. Use of biomarkers specific to the tumor and/or patient can provide prognostic utility over that available from routine clinical features. Data have emerged documenting altered systemic miRNAs expression across a spectrum of cancers including urothelial carcinoma of the bladder. Examples include *miR-21* (up-regulated), *miR-200* family (associated with epithelial-mesenchymal transition and *Zeb1/2*), and *miR-145* (apoptosis). Assessing the expression of all known and predicted non-coding RNAs species and contrasting the miRNAs in the circulation of patients with superficial or invasive disease has great potential in determining whether we can identify systemic miRNAs as screening tools for bladder cancer.

10.1 Introduction

Bladder cancer affected nearly 71,000 people in the United States in 2010 and was the fourth most common cancer diagnosis in men. Over fourteen thousand people succumbed to the disease during the same time frame (Jemal et al. 2010). For

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this chapter, bladder cancer is strictly limited to cancer of urothelial origin (also known as transitional cell carcinoma). From a clinical perspective, bladder cancer is traditionally subdivided into either superficial (Ta or T1) or invasive (T2, T3, and T4) subtypes. The two types are quite different in their overall characteristics, with superficial disease having long-recurrence free episodes, while invasive disease often requires multimodal therapies combining surgery as well as chemotherapeutic approaches.

MicroRNA (miRNAs) expression and research in bladder cancer has increased over the last several years, but remains inconclusive with so many new miRNAs being discovered on a monthly basis (Schaefer et al. 2010). This chapter will review the major findings over the last several years related to bladder cancer, as well as discuss new proposed outcomes and mechanisms.

10.2 Historical

Since the discovery by Lee and colleagues (1993) in 1993 of small non-protein encoding RNAs of approximately 22 nucleotides in length, miRNAs have been identified in multiple organisms and tissue types (Chen et al. 2004; Hanke et al. 2010; Lagos-Quintana et al. 2002; Lagos-Quintana et al. 2003). MiRNAs are thought to induce their effect on gene expression based on the amount the miRNA complements the mRNA. If it has a high amount of complement, then the miRNA leads to mRNA degradation; if the complementary sequence is low, however, the miRNA can repress translation of the mRNA (Hutvagner and Zamore 2002).

The first identified miRNA was in *C. elegans* and was labeled *lin-4*, though at the time all that was known was that this particular small RNA was complementary in sequence to *lin-14*. The binding of *lin-4* to the 3′UTR of *lin-14* resulted in decreased mRNA expression suggesting a suppressive role for this small RNA nucleotide (Lee et al. 1993). Identification of miRNAs in humans occurred over seven years later (Pasquinelli et al. 2000). Further, the expression of miRNAs is variable across organ systems, with the highest expression in kidney, lung, and brain, while the lowest expression in heart, thymus, and bone marrow. This finding is a common theme throughout the expression of mRNA with the interaction of miRNAs in varying tissues (Grosshans and Filipowicz 2008; Vasudevan et al. 2007).

10.2.1 Embryonic MiRNA Associations with Bladder Development

In order to shed light on the developmental characteristics of miRNAs in the bladder, Liu and colleagues (2009) evaluated mouse embryo at three time points correlating to the day prior to smooth muscle formation, at smooth muscle formation, and after formation with each time point separated by approximately 24–48 h. Tissue was extracted at each time point from the urothelium and then the smooth muscle with subsequent miRNA characterization via microarray technology and RT-PCR

confirmation. Recalling that epithelial cells of the bladder is derived from endoderm and that mesenchymal cells associated with smooth muscle is derived from the urogenital sinus and allantoids (Staack et al. 2005), significant differences are found during each developmental time stage. Of note, the mesenchymal cells of the bladder differentiated into smooth muscle based on signaling from the urothelium that is postulated to occur secondary to a miRNA process (Liu et al. 2009).

Specific miRNA have been identified for varying time points of the developmental process in the bladder (Liu et al. 2009). Of the 187 miRNAs discovered, however, only 16 are known miRNA including *miR-137*, *miR-467*, and *miR-503*. As might be expected, with differentiation some miRNAs increased in expression amount, while others decreased. For example, *miR-137* increased with more differentiated embryonic stages whereas, *miR-503* had a stepwise decrease. Likewise, the expression amounts changed within the same stage of development, but in different tissues. For example, *miR-137* increased in expression when moving from the outermost layer of mesenchymally derived tissue and going toward the epithelial derived tissue (Liu et al. 2009). This finding is characterized due to the extensive amount careful laser dissection performed by the authors, and this finding demonstrates the importance that may be missed by characterization of whole tumors rather than laser dissected ones and the differences that may lie within superficial and invasive cancers.

10.2.2 MiR-200 Family and Epithelial-mesenchymal Transition (EMT)

Numerous papers over the past several years have specifically targeted the five members of the *miR-200* family (*200a*, *200b*, *200c*, *141*, and *429*) and their effects on the EMT. From an embryologic standpoint, this family of miRNAs is more pronounced within differentiated epithelial tissues as opposed to mesenchymal ones (Darnell et al. 2006), and can be organized into one of two clusters based on their base sequences (Park et al. 2008). Further, *miR-200* family members demonstrate an inhibitory role on the mesenchymal associated factors of *Zeb1* and *Zeb2* (Gregory et al. 2008; Park et al. 2008). Specifically, *Zeb1* and *Zeb2* are repressed and EMT prevented when *miR-200* family expression is enforced. Conversely, the loss of *miR-200* expression can lead to elevated *Zeb1* and *Zeb2* causing silencing of *CDH1* and, consequently, increased EMT (Gregory et al. 2008).

Recently, our group published findings in UC cell lines related to the *miR-200* family and its modulatory effects on EMT (Adam et al. 2009). Utilizing nine UC cell lines classified as either epithelial or mesenchymal based on *CDH1* expression, miRNA array screening with RT-PCR validation confirmed expression patterns. Baseline expression of *miR-200c* is elevated in cell lines associated with the epithelial phenotype as compared to those of mesenchymal phenotype and this expression correlates with the expression of *CDH1*. UMUC3, a UC cell line with low baseline expression of *miR-200c*, was transfected with a stable lentivirus construct for *miR-200c* and yielded an approximate 150-fold increased expression of *miR-200c* as compared to the empty vector. Of important significance, the analysis of EMT markers (*CDH1*, *Zeb1*, and *Zeb2*) demonstrates a dramatic increase in *CDH1* with

converse decrease in *Zeb1* and *Zeb2* expression for the stable *miR-200c* construct. Confocal microscopy also demonstrates the nuclear down-regulation of *Zeb1* and *Zeb2*. Essentially, an epithelial phenotype can be achieved in a mesenchymal cell line by over-expression of *miR-200c*. Therefore, *miR-200c* is inversely correlated with *Zeb1* and *Zeb2* expression. Further characterization of the 3′UTR of *Zeb1* and *Zeb2* yields five potential binding sites for *miR-200* family members and suggests a molecular mechanism for inhibition of this mesenchymal gene expression. Overall, *miR-200* family members in UC cell lines are associated with epithelial phenotype and are inversely correlative with markers of mesenchymal phenotype.

The exact mechanisms that lead to EMT with respect to loss of *miR-200* expression are just now being characterized. *Zeb1* interacts directly as a repressor of transcription against *miR-200c* and *miR-141* via binding to the E-box promoter regions (Bracken et al. 2008). Further, both the *miR-200* family and *miR-205* are associated with increased CpG islands which suggest a mechanism of inhibition via DNA hypermethylation (Wiklund et al. 2010). Analysis of normal, non-invasive and invasive bladder tumors find confirmation of DNA hypermethylation with silencing of the *miR-200* family and *miR-205* in invasive tumors (Wiklund et al. 2010).

Although it is important to clarify the differences between miRNA expressions in invasive versus non-invasive tumors, one of the more difficult tumors clinically is the T1 UC, where a significant percentage will undergo progression to muscle invasive disease. In this regard, Wiklund and colleagues (2010) evaluated 100 T1 tumors and found loss of *miR-200c* expression was highly correlative to disease progression. This latter finding has important clinical biomarker potential if confirmed as T1 tumors have a varied clinical response and identification of poor risk patients would be extremely useful.

10.2.3 Other Identified MiRNAs

As has previously been discussed, the variability in miRNA expression can be quite high, and this carries over into the cancer phenotype (Lu et al. 2005). As compared to normal tissues, most cancerous tissues have decreased expression of miRNAs (Calin et al. 2002; Lu et al. 2005). However, some combination cancer regimens yield up-regulation of miRNAs. *MiR-127* is one such miRNA where combination treatment with histone deacetylase (HDAC) inhibition and inhibitors of DNA methylation in the T24 UC cell line yields up-regulation. Further, *miR-127* is embedded within a *CpG* island and is not expressed within the T24 invasive mesenchymal phenotype (Saito et al. 2006). Additionally, matched normal and cancerous bladder tissue demonstrate similar findings with *miR-127* over-expression in normal tissues, but non-existent in bladder tumors (Saito et al. 2006).

Utilizing computational analysis, Saito and colleagues identified *BCL6* as a possible target for *miR-127* as over-expression of *miR-127* resulted in decreased protein expression of *BCL6*. Further, when utilizing vectors with mutant and wild-type (wt) *BCL6*, *miR-127* led to decreased translational expression of *BCL6* (Saito et al. 2006). Importantly, this suggests that HDAC inhibition in conjunction with DNA

methylation inhibitors can induce expression of miRNAs associated with tumor suppressive actions in cancer cells and suggests further therapeutic targets.

Other tissue derived comparative assessments have also found unique miRNAs and proposals for biomarker evaluations. Utilizing miRNA array expression analysis, 290 unique miRNAs were evaluated in normal and cancerous bladder tissues (Dyrskjot et al. 2009) of which several demonstrated differential expression between normal and cancerous tissue as well as between stages of disease. Of note, *miR-21* was up-regulated in bladder cancer tissue, whereas *miR-143* and *miR-145* were down-regulated. When comparing invasive bladder cancer to superficial disease, *miR-200c* was down-regulated in the invasive subtypes. We have also noted similar findings in our comparison of invasive and superficial bladder tumors for the *miR-200* family (Williams et al. 2010, manuscript in preparation). Further, four miRNAs were identified as being associated with disease progression defined as worsened stage: *miR-133b*, *miR-518c*, *miR-129*, and *miR-29c* (Dyrskjot et al. 2009). These particular markers were then cross-validated resulting in a 63% sensitivity and 66% specificity for disease progression. With in situ hybridization, normal urothelium had elevated *miR-145* and *miR-129*; however, *miR-21* was only detected in carcinoma tissue. Functionally, when the T24 UC cell line was transfected with pre-*miR-129*, the result was a significant anti-proliferative effect that promoted cell death. Knockdown of *miR-129* did not have the counter effect secondary to very low basal levels found within the T24 cell line. Transfection of *miR-21* into the same T24 cell line did not result in phenotypic change. In summary, identification of key miRNAs involved in disease progression have been made, but exact molecular mechanistic identification of why a given tumor progresses as compared to another remains elusive.

The number of tissues evaluated does not limit the number of expression changes found as demonstrated by Gottardo and colleagues (2007). Utilizing an array platform for 245 miRNAs, 27-bladder primary tumor specimens were assessed along with two normal mucosa. Ten miRNAs (*miR-223*, *miR-26b*, *miR-221*, *miR-103-1*, *miR-185*, *miR-23b*, *miR-203*, *miR-17-5p*, *miR-23a*, *miR-205*) were up-regulated in the cancerous tissue as compared to normal bladder mucosa. Twenty of the 25 patients had evaluable stage information, and four of these tumors were Ta. Only *miR-26b* demonstrated a trend for decreasing expression with increasing stage, however, none of the miRNAs were significant based on Stage alone. Speculation was made that due to the known deletion of Chr2q35 in progression from Ta to T1 (Richter et al. 1999), that miRNA regulation of *miR-26b* may also be adversely affected.

A unique concept to evaluating the effects of miRNA expression within invasive and non-invasive cell lines is the comparative ratio. Neely and colleagues (2010) performed expression profiling for 343 miRNAs in 14 UC cell lines and demonstrated 9 differentially expressed miRNAs for invasive and non-invasive phenotypes, including *miR-21*, *miR-205*, and two members of the *miR-200* family (*miR-200a* and *miR-200c*). *MiR-21*, targets caspases and thus prevents apoptosis (Chan et al. 2005), and *miR-205* were targeted based on prior published data demonstrating their association with cancer phenotypes. As might be expected, invasive UC cell

Table 10.1 MicroRNA ratios associated with prognosis in bladder cancer

MicroRNA Ratio	Prognostic application	References
miR-21:miR-205	Increased with increasing stage	Chan et al. (2005)
miR-126:miR-152	Increased with cancer as compared to normal tissues	Hanke et al. (2010)
miR-182:miR-152	Increased with cancer as compared to normal tissues	Hanke et al. (2010)

lines found *miR-21* elevation and decreased expression of *miR-205,* with the converse true for non-invasive UC lines. Upon initial assessment of a small subset of bladder tumors, however, significance was lost due to lack of discriminatory power. Therefore, a ratio approach was applied to this data with invasive lines having a 10-fold higher *miR-21:miR-205* ratio as compared to non-invasive ones. This finding remained significant in a larger cohort of bladder tumors with receiver operator characteristic (ROC) curve analysis being 0.89 for discriminating superficial from invasive bladder tumors and the sensitivity and specificity being 100 and 78%, respectively. This is a very promising biomarker, but needs to be validated in larger cohorts (Table 10.1).

Interestingly, this approach is not limited to utilizing fresh or frozen tumors, as specific techniques can be applied to urine specimens to effectively capture RNA and distinguish cancerous from non-cancerous states via miRNA identification. Of note, Hanke et al. (2010) consistently demonstrated two particular miRNA ratios were higher in bladder cancer patients as compared to normal subjects: *miR-126:miR-152* and *miR-182:miR-152*. It is unclear at this time, however, how the modulation of these miRNAs result in invasive changes (Table 10.1).

Silencing specific miRNAs has been demonstrated to promote cell death. Based on work in melanoma and prostate cancer cell lines, Lu et al. (2009) used the T24 invasive UC cell line and found that *miR-221* was significantly up-regulated and proposed the reversal of this expression might ultimately lead to susceptibility to cell death. Like the prior utilization of combination agents to promote cell death, an antisense *miR-221* was transfected into the T24 cell line and then this UC line was exposed to tumor necrosis factor apoptosis induced ligand (TRAIL) over the ensuing 24 h. *MiR-221* expression was dramatically reduced and, when TRAIL was introduced, apoptosis was achieved at a rate of 50%.

Though most of the studies published have focused on identification of a large number of miRNAs that are differentially expressed as compared to normal, few have focused efforts on the one miRNA. Lin and colleagues (2009) confirmed prior findings of down-regulation of *miR-143* in bladder cancer tissues as compared to normal adjacent urothelium, however no differences were noted between stages of disease. Of the 26 patient tumor samples analyzed, 31% were non-invasive with the remainder muscle-invasive. To further characterize the effects of *miR-143*, the mesenchymal and invasive cell lines, T24 and EJ, were evaluated as to the baseline expression of *miR-143* as well as the end results from transfection with *miR-143*. As compared to control *U6*, T24 and EJ cells had little to no expression of *miR-143*. Once transfected with the mature form of *miR-143*, however, significant growth

inhibition occurred suggesting a negative regulation for cell proliferation. Further, *KRAS*, a gene that has been associated with bladder cancer, has several *miR-143* binding sites. When assessing the amount of RAS protein produced by T24 and EJ cells transfected with *miR-143*, the *KRAS* mRNA remained stable, but the protein product was significantly decreased leading to a second methodology for regulation by *miR-143* (Lin et al. 2009). Overall, *miR-143* has decreased expression in bladder cancer and this can yield both lack of growth inhibition as well as negative regulation of the protein product from *KRAS* mRNA.

Evaluation of mechanistic targets of the down-regulated *miR-145* in urothelial carcinoma has also begun to shed some light as to the variability and wide range of possible targets for a given miRNA. Chiyomaru and colleagues performed a microarray analysis of 3 *miR-145* transfected UC cell lines as compared to the normal UC cell lines and performed a genome wide evaluation of possible targets. Ultimately, the focus was on the most down-regulated target, *FSCN1* (Chiyomaru et al. 2010), which is essential in forming filopodia (Vignjevic et al. 2006) and is highly expressed in cells that have increased motility (Pelosi et al. 2003). Identification was made of 2 complementary sites for *miR-145* on *FSCN1* and when transfected with *miR-145*, T24 cell lines had a significant growth inhibition and inhibition of migration as compared to the wt controls, however, matrigel invasion was not prevented by the transfected cell with T24. Of note, the other cell line investigated, BOY, ceased invasion with transfection of *miR-145*. Which raised the possibility for other mechanistic events promoting invasion based on the given cell line. Finally, immunohistochemistry staining for *FSCN1* demonstrated increased intensity with advanced stage and is consistent with prior studies (Tong et al. 2009). These two studies demonstrate the style of mechanistic work that needs assessment in urothelial carcinoma.

10.2.4 Genetic Variability in MiRNA Machinery

Based on previously published findings that the majority of tumors have decreased expression of miRNAs as compared to normal (Lu et al. 2005), this premise was applied to bladder cancer and DNA copy number (Lamy et al. 2006). Evaluation of thirty superficial (Ta or T1) UC tumors based on single nucleotide polymorphism (SNP) and subsequent gain or loss of DNA copy number in those regions was performed. Interestingly, patients with T1 UC with a gain in DNA copy number had decreased expression of miRNAs, whereas, an increased expression of miRNAs has been demonstrated in prostate and colon tumors (Lamy et al. 2006; Volinia et al. 2006).

The effect of miRNA biogenesis in relation to genetic variants in bladder cancer outcome was recently assessed by the MD Anderson Cancer Center group (Yang et al. 2008). In this large case control study, 41 SNPs with potential targeting of miRNA functionality underwent relational assessment to bladder cancer risk. With nearly 1,500 patients evaluated and half with newly diagnosed disease, 7 of the 41 SNPs had at least a borderline statistical increased risk of bladder cancer. Two genes

and their regulatory SNPs stand out as prominently increasing the risk of bladder cancer: *GEMIN3* (2.5-fold increased risk) and *GEMIN4* (1.25-fold increased risk) (Yang et al. 2008). Both genes are important in the processing of miRNA precursors (Hutvagner and Zamore 2002) and the proteins made by transcription of these genes interact with the survivor proteins involved with pre-mRNA splicing (Nelson et al. 2004). Beyond this, a decreased bladder cancer risk was also associated with 4 miRNA genes including *miR-423*, *miR-492*, *miR-26a-1*, and *miR-124-1*. In other epithelial cancers, the chromosome responsible for production of *miR-26a-1* (Chr3p21) is deleted (Protopopov et al. 2003) whereas *CpG* island methylation has been attributed to the decreased expression for *miR-124-1* (Lujambio et al. 2007). When the 7 SNPs were then taken together as markers of unfavorable genotypes, the higher the number of unfavorable SNPs led to an increased bladder cancer risk ($p < 0.0001$) and looking at individual numbers of unfavorable genotypes, 3 or more led to an almost 2-fold increased bladder cancer risk (OR 1.92, $p < 0.0001$) (Yang et al. 2008). In summary, this is the first to identify specific genetic variants of miRNA biogenesis machinery and increased risk in bladder cancer.

Another gene adversely regulated in bladder cancer is the tumor suppressor gene, *Rb*. Further, the protein formed by transcription of the *E2F3* oncogene is bound to the *Rb* protein under normal cellular processes. However, with phosphorylation of *Rb*, *E2F3* becomes free to target several different sites with ultimate mitotic promotion. Huang and colleagues (2010) published their results of the interaction of the *miR-125b* with the *E2F3* oncogene. Utilizing miRNA microarray technology, they demonstrated in a prior study *miR-125b* was significantly down-regulated in bladder cancers and was related to cell proliferation in cancer cells (Lee et al. 2005; Lin et al. 2009). Utilizing the tissues from 25 bladder cancer tumors that had never undergone local or systemic treatment prior to operation, they assessed the amount of *miR-125b* and *E2F3* expression along with comparison to eight UC cell lines including T24 and UMUC3 (Huang et al. 2010). *MiR-125b* was decreased across all bladder tumors as compared to adjacent normal tissues, but did not correlate to stage or grade of disease. When stable transfectants of *miR-125b* were placed into mesenchymal UC cell lines (T24 and 5637), cell proliferation was significantly depressed as compared to the wt cells. *E2F3* was identified as a possible target based on several complementary sites for *miR-125b* and, utilizing luciferase reporter, *E2F3* was significantly reduced and protein expression of the two was noted to be inversely correlated ($p < 0.001$). However, mRNA for *E2F3* was not affected by the transfectants, suggesting a role of *miR-125b* for post-translational modification. Finally, *cyclin A2* is inhibited in both T24 and 5687 UC cell lines with induction *miR-125b*. Globally, the repression of the oncogene *E2F3* by *miR-125b* is post-translational and affects cell proliferation by decreasing *cyclin A2* (Huang et al. 2010).

10.3 Prognostic Implications of MiRNAs

Determining the changes between different tumor types allows the ability to evaluate the extent of disease and could lead to determining when progression might occur on a molecular level (Table 10.2). An excellent study evaluating these different tumor

Table 10.2 MicroRNAs in bladder cancer

MicroRNAs	Cancer regulation	Target discovery	Mechanistic discovery	Prognostic discovery	References
miR-124-1	Down-regulated	–	CpG island hyper-methylation	–	Lujambio et al. (2007)
miR-125b	Down-regulated	E2F3	Decreased protein expression; Cyclin A2 inhibition	Increased IHC intensity in high grade urothelial carcinoma	Huang et al. (2010)
miR-127	Down-regulated	BCL6	Decreases BCL6 protein expression	–	Saito et al. (2006)
miR-129	Down-regulated	–	–	Worse progression and stage	Dyrskjot et al. (2009)
miR-133b	Down-regulated	–	–	Worse progression and stage	Dyrskjot et al. (2009)
miR-137	Down-regulated	–	–	Decreased expression from epithelial tumor to mesenchymal tumor	Liu et al. (2009)
miR-143	Down-regulated	KRAS, FSCN1	Decreases protein expression	No differences between stages; IHC of FSCN1 increased with stage	Lin et al. (2009); Tong et al. (2009); Wang et al. (2010)
miR-145	Down-regulated	CBFB, PPP3CA, CLINT1	Apoptosis	–	Ostenfeld et al. (2010)
miR-199a	Down-regulated	KRT7	Inversely correlated with miRNA	–	Kawakami et al. (2006); Wang et al. (2010)
miR-200a/b/c, 141, 429	Down-regulated	Zeb1, Zeb2	EMT prevention with increased expression	Loss of miR-200c expression correlated to progression; miR-141 up-regulated in T1 tumors as compared to ANT	Gregory et al. (2008); Neely et al. (2010); Park et al. (2008); Wiklund et al. (2010); Wszolek et al. (2010); Wang et al. (2010)
miR-205	Up-regulated	–	–	Up-regulated as compared to normal tissue	Gottardo et al. (2007)

Table 10.2 (continued)

MicroRNAs	Cancer regulation	Target discovery	Mechanistic discovery	Prognostic discovery	References
miR-21	Up-regulated/down-regulated (one study)	Caspase, PTEN	Prevention of apoptosis	Only detected in cancerous tissue; more likely to progress in disease	Chan et al. (2005); Dyrskjot et al. (2009); Gottardo et al. (2007); Ichimi et al. (2009); Neely et al. (2010); Wang et al. (2010); Meng et al. (2007)
miR-221	Up-regulated	–	Knockdown of microRNA increased susceptibility to TRAIL	–	Gottardo et al. (2007); Lu et al. (2010)
miR-26a-1	Down-regulated	–	–	Compared to ANT	Wang et al. (2010)
miR-26b	Up-/down-regulated	–	–	Up-regulated as compared to normal tissue; down-regulated with increasing stage	Richer et al. (1999); Gottardo et al. (2007)
miR-29c	Down-regulated	–	–	Disease progression and worsened stage as compared to ANT	Dyrskjot et al. (2009); Wang et al. (2010)
miR-518c	Down-regulated	–	–	Worse disease progression and stage	Dyrskjot et al. (2009)

ANT, Adjacent normal tissue; TRAIL, TNF-related apoptosis inducing ligand.

types was published in this past year, where assessment was made of the following disease states: low-grade non-muscle invasive, high-grade non-muscle invasive, muscle invasive, normal urothelium from patients with bladder cancer, and normal urothelium without evidence of bladder cancer (Catto et al. 2009). RNA was isolated and the expression of 354 known mature miRNAs assessed. Mean follow-up for the entire cohort was just over 3 years (range 0.2–7.34). Of note, patients with bladder cancer had an up-regulation of the adjacent normal urothelium miRNAs as compared to disease free controls and unsupervised cluster analysis demonstrated two branches corresponding to tissue state. Specific miRNAs include the *let-7* family, *miR-492*, *miR-221*, *miR-492*, and *miR-141*. When specifically evaluating urothelial carcinoma tissues only, twelve miRNAs were identified with sensitivity and specificity for identifying cancer of 90–100 and 80–100%, respectively. A similar process was found in regard to cancer tissues where there was up-regulation of miRNA expression from tissues with low-grade tumor to those of invasive disease (Catto et al. 2009).

The gene targets of the up-regulated miRNAs included *RBAK*, *LATS2*, *RAB6c*, and *FGF2*, while down-regulated targets included *SOX4*, *RUNX2*, and *ANGPT1*. Further, *Dicer*, *Drosha*, and *Exportin5* were significantly up-regulated in the normal urothelium adjacent to bladder cancer as compared to the malignant tissue where these three processing genes were down-regulated. Finally, patients with up-regulated *miR-21* and normally expressed *miR-100* and *miR-99a* were more likely to progress to advanced disease (Catto et al. 2009).

In another smaller evaluation of 14 bladder cancers as compared to 5 samples of normal urothelium, seven miRNAs were identified as being down-regulated in bladder cancers (Ichimi et al. 2009). ROC analysis revealed that these seven miRNAs could distinguish normal from cancerous tissues with at least 70% sensitivity and 75% specificity for each miRNA. Of the down-regulated miRNAs, *miR-199a* was significantly reduced in the cancerous state. Further, based on prior research demonstrating the cytokeratin 7 (*KRT7*) as elevated in bladder cancer (Kawakami et al. 2006) and this finding was recapitulated in further study. Of particular interest was the finding that *KRT7* was inversely associated with the amount of each of the 7 miRNAs suggesting *KRT7* as a target of the miRNAs.

Returning to the *miR-200* family, which, as outlined earlier is mechanistically involved in the EMT, while the reduction of *miR-200* yielding increasing expression of the mesenchymal nuclear factors of *Zeb1* and *Zeb2*, clinical application of the expression of *miR-200* has demonstrated similar results in the clinical arena. Utilizing a panel of 57 urothelial carcinoma tumors (45% $\leq$ T1) with a mean follow-up of survivors being 92 months, 12 miRNAs were found to be differentially expressed between invasive and non-invasive tumors (Wszolek et al. 2010. In particular, *miR-200c*, *miR-141*, and *miR-30b* demonstrated significantly worse cancer specific survival when they were reduced in expression as compared to normal expression patterns. Five-year survival for *miR-200c* high and low expression was 90 and 36% ($p < 0.001$), respectively, with *miR-141* high and low expression of 95 and 31% ($p < 0.0001$), and *miR-30b* high and low expression being 100 and 26% ($p < 0.0001$). The expression of these three miRNAs had a predictive capacity for distinguishing invasive from non-invasive disease with a sensitivity of 100% and

specificity of 96.2%. This results needs to be clarified in a larger cohort of patients (Wszolek et al. 2010).

As depicted earlier, several studies demonstrate significant changes in miRNAs between normal tissues as compared to cancerous ones. Studying adjacent normal tissue with T1 and T2 tumors in 7 patients, Wang and colleagues (2010) demonstrated that T1 and T2 tumors had different miRNA up-regulation and down-regulation between them. In T1 tumors, six known miRNAs were up-regulated (*miR-129, 141, 494, 498, 500*) and 13 miRNAs were down-regulated (*let7a-d, miR-199a, 21, 24, 26a, 29c, 30a-5p, 30c, 30e-5p*). However, in T2 tumors, none of the analyzed miRNAs were up-regulated with 4 miRNAs being down-regulated (*miR-26a, 29c, 30c*, and *30e-5p*). The differences between the T1 and T2 tumors suggest an underlying change initiated in the development of the urothelial cancer. This is depicted in the transition seen in carcinoma in situ tumors where *miR-145* was significantly reduced as compared to adjacent normal tissue (Ostenfeld et al. 2010). Further, identification was made of several gene targets (*CBFB, PPP3CA, CLINT1*) associated with caspase inhibition. Overall, each of the tumor types demonstrate different miRNA expression and may have different mechanistic progression features.

10.4 Perspective and Future Challenges

In this chapter, we have presented several new miRNAs identified as potential differentiating features for bladder cancers. In a few, the mechanistic aspects have been assessed, but many remain strictly as discovery findings of new miRNAs. Examples of these recent discoveries are the *miR-200* family (*Zeb1/2* inhibition and EMT), *miR-21* (apoptosis prevention and *PTEN*), and *miR-143* (*KRT7*). For the mechanisms that have been identified, some demonstrate post-translational protein modification rather than directed gene targeting and repression of mRNA expression (*miR-127* decreased BCL6 protein). Further, miRNAs have been identified as having numerous targets for each individual miRNA (Cho 2010; Nelson and Weiss 2008; Wu et al. 2007). The problem is to determine which of the newly discovered miRNAs should be further assessed for the mechanisms promoting bladder cancer as well with the ultimate goal of developing agents that prevent this transition in the first place. As technology continues to develop, our ability to perform large scale analysis, as demonstrated by microarray technology, will further allow us to refine these characteristics. For the current time, further evaluation of miRNAs that have already demonstrated clinical benefit need to be performed.

10.5 Conclusions

The studies enumerated here suggest that invasive bladder cancer displays panels of miRNAs with tumor-specific profiles. This could aid in discriminating among other subgroups of superficial bladder cancer that are prone to relapse or between different subgroups of invasive bladder cancers that are fatal. These findings are

of notable clinical consequence and predict unlimited potential for impacting clinical practice patterns by directing appropriate management of this disease and reducing death from bladder cancer. Understanding the biology of the differentially-expressed miRNAs, combined with the assessment of pattern similarities not only in tumors but also in surrogate tissues or in the systemic circulation (plasma, serum, urine, or saliva of patients) may represent the next step in the development of non-invasive, highly-reliable miRNA-based biomarkers. The use of reliable markers also has the potential for reducing the cost of health care delivery by improving and streamlining surveillance protocols, and by personalizing the therapy and for this, the miRNA signature profiling seems to hold great promise in bladder cancer.

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