Distinct MicroRNA Alterations Characterize High- and Low-Grade Bladder Cancer

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Abstract

Urothelial carcinoma of the bladder (UC) is a common disease that arises by at least two different molecular pathways. The biology of UC is incompletely understood, making the management of this disease difficult. Recent evidence implicates a regulatory role for microRNA in cancer. We hypothesized that altered microRNA expression contributes to UC carcinogenesis. To test this hypothesis, we examined the expression of 322 microRNAs and their processing machinery in 78 normal and malignant urothelial samples using real-time rtPCR. Genes targeted by differentially expressed microRNA were investigated using real-time quantification and microRNA knockdown. We also examined the role of aberrant DNA hypermethylation in microRNA downregulation. We found that altered microRNA expression is common in UC and occurs early in tumorogenesis. In normal urothelium from patients with UC, 11% of microRNAs had altered expression when compared with disease-free controls. This was associated with upregulation of Dicer, Drosha, and Exportin 5. In UC, microRNA alterations occur in a tumor phenotype-specific manner and can predict disease progression. High-grade UC were characterized by microRNA upregulation, including microRNA-21 that suppresses p53 function. In low-grade UC, there was downregulation of many microRNA molecules. In particular, loss of microRNAs-99a/100 leads to upregulation of FGFR3 before its mutation. Promoter hypermethylation is partly responsible for microRNA downregulation. In conclusion, distinct microRNA alterations characterize UC and target genes in a pathway-specific manner. These data reveal new insights into the disease biology and have implications regarding tumor diagnosis, prognosis and therapy. [Cancer Res 2009;69(21):8472–81]

Introduction

Bladder cancer is the fifth commonest malignancy in the United States with an incidence of 67,160 new cases and 13,750 deaths in 2007 (1). The majority of tumors are urothelial cell carcinoma (UC). Clinical and molecular evidence suggests there are at least two distinct varieties of this tumor. Most UC belong to a low-grade pathway characterized by FGFR3 mutation, chromosome 9 loss, and an indolent clinical phenotype. Around one-third of UC are high grade in differentiation and arise as lesions initially confined to the bladder mucosa (nonmuscle invasive, NMI). Progression to muscle invasion occurs in around 50% of high-grade lesions and is associated with an ominous prognosis despite radical treatment (2, 3). Although numerous genetic and epigenetic alterations are observed in high-grade UC, loss of p53 function seems most critical (4, 5).

MicroRNAs (miR) are short noncoding RNA molecules that post-transcriptionally modulate protein expression. They are transcribed as hairpin pri-miRs and processed into pre-miRs by Drosha, an RNase III endonuclease complexed with DGCR8. Pre-miRs are exported into the cytoplasm by Exportin 5 before cleavage by Dicer into mature miRs. MiRs are directed to mRNAs with a complementary seed sequence to their first 1 to 8 nucleotides (6). Alterations in mRNA expression seem important for carcinogenesis. To test this hypothesis, we investigated the expression of numerous miRs and their processing molecules in urothelial tissues. Our studies reveal that UC are characterized by widespread alterations in miR expression. Changes in expression occur early in the tumorogenic pathway, in a phenotype-specific manner, and are associated with altered expression of their processing machinery. Of particular note, low-grade tumors are characterized by miR-mediated FGFR3 upregulation before its mutation.

Materials and Methods

Patients, samples, and cell lines. We studied 72 urothelial samples and six cell lines (Table 1). Tumors were classified using the 2004 WHO/ISUP criteria, and selected from three cancer groups: (a) low-grade NMI, (b) high-grade NMI, and (c) invasive UC. Twenty normal urothelial samples were obtained from 10 patients with UC [NU(UCC) taken distant to any tumor] and 10 disease-free controls [NU(Controls)]. Tissues were immediately frozen in liquid nitrogen and histologic confirmation was obtained before use. Patients were treated according to tumor stage and grade following endoscopic resection (14). Adjuvant intravesical chemotherapy was used for low-grade and maintenance intravesical Bacillus Calmette-Guéruin (BCG) for high-grade NMI tumors. Patients underwent surveillance stratified by their disease. Radical cystectomy with pelvic lymphadenectomy was used for invasive or BCG-refractory high-grade NMI tumors (4, 14). We analyzed UCC cell lines representing the three tumor groups (RT4, RT112, and...
EJ/T24, respectively, purchased from American Type Culture Collection) grown in Dulbecco’s medium with 10% FCS, and three normal human urothelial (NHU) cell lines maintained in keratinocyte serum–free medium containing bovine pituitary extract, epidermal growth factor (bovine), and cholera toxin. Nonimmortalized NHU cells were derived from histologically confirmed normal urothelium obtained from patients without a history of UCC, using standard methods (15). DNA methyltransferase inhibition experiments were performed in quadruplicate by adding 2 μmol/L 5-azacytidine to the media for 5 to 7 d before harvesting.

**miR expression profiling.** For both normal and malignant samples, ten 10 μmol/L sections were microdissected to obtain >90% pure cell populations. Enriched small and total RNA were extracted using the mirVana kit (Ambion) according to manufacturer’s protocol. RNA concentrations were measured using a 2100 Bioanalyzer (Agilent, Cheshire). The expression of 354 mature miRs and 3 small nuclear RNA molecules was determined using real-time PCR with preprinted microfluidic cards (Human miR v1.0, Applied Biosystems; ref. 16). Initially, reverse transcription using stem loop primers was performed with 50 ng RNA, MultiScribe Reverse Transcriptase (Applied Biosystems), RNase inhibitor, 100 nm deoxynucleotide triphosphates (dNTP) and nuclease-free water. To generate 357 miR and reference rTPCR products, 8 multiplex pools were used per sample [each pool containing ingredients and primers for 48 mature miR, total volume 10 μL (Human Multiplex RT Pool 1-8 v1.0, Applied Biosystems)]. The multiplexed RT assays were cooled to 16°C for 30 min, heated to 42°C for 30 min, and 86°C for 5 min before diluting 62.5-fold in nuclease-free water. For each diluted assay, 50 μL were mixed equally with 2× Taqman Universal PCR MasterMix and loaded separately into eight reservoirs of a microfluidic card (Human miR v1.0, Applied Biosystems). Following careful centrifugation (2 min at 331 grams) and preparation (well sealing and reservoir removal), the loaded card was analyzed on an ABI 7900HT real-time PCR system using the recommended PCR conditions. Each microfluidic card contained MGB-labeled probes specific to 354 mature miRs, 11 duplicate assays, 2 empty wells, and 17 replicates of 3 endogenous small nucleolar RNA molecules for relative quantification (RNU6B, RNU4H, and RNU48). MiR concentrations were calculated from the cycle number at which each reaction crossed an arbitrarily threshold (Ct = 0.2). The amplification plots were checked manually (SDS 2.2.1, Applied Biosystems) to confirm the Ct value corresponded with the midpoint of logarithmic amplification. To test the reproducibility of this real-time quantification, we analyzed each cell line and 10 tissue samples in duplicate.

**Identification of genes targeted by aberrantly expressed miR.** We identified potential protein targets of miRs using TargetScan8 (version 4.2; refs. 17, 18). Target genes were ranked according to context score and the average was used for those with multiple targeting miRs. mRNA transcripts of target and miR-processing genes were measured in those samples with sufficient material [n = 71 (93%)]. cDNA synthesis from 100 ng whole RNA was performed using random primers, RT buffer, dNTP (100 mmol/L), RNase inhibitor, and MultiScribe Reverse Transcriptase (cDNA Reverse Transcription kit, Applied Biosystems). Real-time quantified PCR using 2 μL of synthesized cDNA, gene-specific primers with FAM-TAMRA–labeled probes (Supplementary Table S1), water, and 2× Taqman Universal PCR MasterMix (Applied Biosystems) in a total volume of 10 μL was performed on the ABI 7900HT system according to manufacturers guidelines. Relative mRNA quantification was measured with respect to the mean of glyceraldehyde-3-phosphate dehydrogenase and β-actin (and PCNA as a proliferation reference gene).

**miR knockdown in NHU cells.** MiRs-99a/100 targeting of FGFR3 was investigated using protein expression following miR knockdown and a Luciferase reporter assay. All experiments were replicated between three and six times using nonimmortalized NHU cell lines at 70% confluence reverse transfected with anti–miR molecules specific to miR-99a, miR-100, and a negative control (scrambled sequence). To determine target protein expression, NHU cells were transfected with 50 nmol/L anti-miR or control sequence using siPORT NeoFX transfection reagent (Ambion). The mixture was dispensed into a six-well dish, seeded at 2 × 105 cells per well, and incubated for 48 h. Transfection was determined by real-time-rPCR (Applied Biosystems) and FGFR3 expression measured by Western Blotting using anti-FGFR3 primary antibody (1:1,000; Cell Signaling Technologies, Inc.). Cells were lysed in radioimmunoprecipitation assay buffer (20 mmol/L Tris-HCl, 135 mmol/L NaCl, 10% glycerol, 1% Igepal, 0.1% SDS, 0.5% deoxycholic acid, 2 mmol/L EDTA) containing protease and phosphatase inhibitors (Complete EDTA-free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail; Roche), and protein content was quantified using the DC-protein assay reagent (Bio-Rad). Protein lysates (50 μg) were loaded onto 8% gels, fractionated, and electropholotted onto nitrocellulose membranes. After blocking with 5% nonfat milk powder and 0.1% Tween, the membranes were incubated overnight with anti-FGFR3 primary antibody (1:1,000; Cell Signaling Technologies, Inc.) at 4°C, washed, and incubated at room temperature for 1 h with goat anti-rabbit peroxidase-labeled secondary antibody (1:1,000; Cell Signaling Technologies, Inc.). β-Actin expression was also measured as a loading control (Sigma, 1:2,000). Immune complexes were quantified using ImageJ for band densitometry. To validate direct targeting of FGFR3, NHU cells were cotransfected with either anti–miR-99a, anti–miR-100, or negative control, and the pSG3.3 untranslated region (UTR) plasmid containing the luc2P Luciferase reporter and either the partial 3′UTR for FGFR3 (from 27′–1,762 bp downstream of the gene) or a control (scrambled sequence; SwitchGear Genomics). Cotransfection was performed in opaque 96-well plates using 40% to 80% confluent cells and FuGene transfection reagent (Roche) and Opti-MEM serum–free media. After incubation for 48 h, luciferase activity was determined following the addition of Promega Steady-Glo Luciferase Assay Reagent (Promega) in a luminometer. Negative controls using scrambled anti-miR sequences or a scrambled 3′ UTR sequence were included.

**Generation of FGFR3 mutant and wild-type paired cell lines.** Telomerase-immortalized NHU cells were used to investigate miR expression following FGFR3 mutation (19). Site-directed mutagenesis on FGFR3 IIIb cDNA created the S249C mutation. The presence of this mutation was verified by sequencing. Wild-type and mutant FGFR3 were cloned into a retroviral expression vector (pFB; Stratagene) containing a hygromycin resistance cassette (20). The expression vectors were transfected into Phoenix A cells using siPORT XP-1 (Ambion). Telomerase-immortalized NHU cells were used to investigate miR expression following FGFR3 mutation (19). Site-directed mutagenesis on FGFR3 IIIb cDNA created the S249C mutation. The presence of this mutation was verified by sequencing. Wild-type and mutant FGFR3 were cloned into a retroviral expression vector (pFB; Stratagene) containing a hygromycin resistance cassette (20). The expression vectors were transfected into Phoenix A cells using siPORT XP-1 (Ambion). Telomerase-immortalized NHU cells were used to investigate FGFR3, NHU cells were cotransfected with either anti–miR-99a, anti–miR-100, or negative control, and the pSG3.3 untranslated region (UTR) plasmid containing the luc2P Luciferase reporter and either the partial 3′UTR for FGFR3 (from 27′–1,762 bp downstream of the gene) or a control (scrambled sequence; SwitchGear Genomics). Cotransfection was performed in opaque 96-well plates using 40% to 80% confluent cells and FuGene transfection reagent (Roche) and Opti-MEM serum–free media. After incubation for 48 h, luciferase activity was determined following the addition of Promega Steady-Glo Luciferase Assay Reagent (Promega) in a luminometer. Negative controls using scrambled anti-miR sequences or a scrambled 3′ UTR sequence were included.

**Statistical methods.** Relative miR concentrations were calculated with respect to the median of three reference RNA molecules (ΔCt = Ct miR – Ct median control). The median was chosen after analysis revealed variation in concentrations between samples (data not shown). Expression fold changes were computed using 2^ ΔΔCt calculations (21). Median data centering was performed before analysis with Significance of Analysis of Microarray software (22) and BRB-ArrayTools8 (version 3.7) developed by Dr. Richard Simon and BRB-ArrayTools Development Team. Class-specific miR signatures were established using Prediction of Microarray software (23). MiR expression and tumor outcome were investigated using the log-rank test and plotted by the Kaplan-Meier method within SPSS (version 14.0, SPSS, Inc.). Tumor progression was defined as the presence of pathologic, radiological, or clinical evidence of an increase in tumor stage and measured from the time of surgery to the time of proven event. Changes in miR expression and statistical significance were illustrated using Volcano plots. Significant expression difference was defined as both a P value of < 0.05 and a false discovery rate of <0.05. Hierarchical clustering was performed after removal of those miRs with a low detection frequencies in all groups.
Unsupervised hierarchical clustering revealed two branches corresponding to the two groups (Supplementary Fig. S1). Three miRs were detected only in NU(UCC) [miR-569 (detected in 50% of samples), miR-633 (40%), miR-507(30%)] and two by only the NU(control) [miR-412 (50%), miR551a (30%); data not shown].

Differential miR expression in malignant urothelium. We compared malignant miR expression with that from disease-free normal urothelium (Fig. 1). The annotated raw data can be downloaded from our website.10 Twelve miRs were expressed only in the malignant tissues and five to seven of these could identify cancer with a sensitivity of 90% to 100% and a specificity of 100% to 80% (Table 2), suggesting roles as diagnostic biomarkers. Quantitative analyses revealed that 16 miRs were differentially expressed in the malignant and normal urothelia.

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10 http://www.shef.ac.uk/medicine/research/sections/oncology/medicine-urology/data.html

<20%: defined as “noise”) using Cluster 3.0 and visualized in Tree view (Eisen Lab9). Correlation between variables was assessed using Pearson’s correlation coefficient within SPSS.

Results

MiR expression in normal urothelium. Following assay optimization (Supplementary Results), we compared the expression of 322 miRs in normal urothelium from patients with UCC and disease-free controls. Thirty-six miRs were differentially expressed between the two groups (P < 0.05 and FDR 0.0; Supplementary Table S2) and were always upregulated in the NU(UCC). There was clustering of differential expression according to RNA family, e.g., let-7a/b/c/d/e/g, miRs-382/487b, miRs-17/93, miRs-181b/d, miRs-34a/c, and chromosomal location, e.g., 9q22.32 (let-7a/d), 14q32.31 (miRs-376a/487b/382/412), and 19q13.41 (miRs-520a/b/c/d/e/518a/519e).

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*Cancer Research 2009; 69: (21). November 1, 2009 www.aacrjournals.org Published OnlineFirst October 20, 2009; DOI: 10.1158/0008-5472.CAN-09-0744

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Table 1. Details of patients and tissue samples analyzed in this study

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*RC, radical cystectomy; RP, radical prostatectomy; TURP/BT, transurethral resection of prostate/bladder tumor.
†Details of associated tumour.
‡Ex-smoking status defined as stopping >1 y before tumor.

(9) http://rana.lbl.gov/eisen/
Thirteen of these were up-regulated in cancer and seven of these were also aberrantly expressed in the normal urothelium from UCC cases, suggesting early alteration in the disease pathway. Hierarchical clustering revealed miR expression stratified tissues mostly according to phenotype.

**Genes targeted by aberrantly expressed miR.** With TargetScan, we identified potential target genes for the 16 differentially
expressed miRs and selected the top one-third, as this fraction is most associated with altered protein expression (18). Of 1,095 predicted genes, 276 were targeted by more than one miR, including PLAG1 by 8 miRs, and E2F7 and DMTF1 by 5. Target ranking by context score revealed many carcinoenic genes with high binding affinity (Supplementary Fig. S2). We selected nine genes, from those with highest binding affinity and potentially carcinogenic roles, and measured their expression in our urothelial samples. For six of nine genes, there was differential expression between normal and malignant urothelial (Supplementary Fig. S2, P < 0.02 t test). For five of these genes, this differential expression was inverse to that of their targeting miR, suggesting they play a regulatory role in expression (17).

Expression of miR with respect to tumor phenotype. Comparisons of the three tumor groups with disease-free normal urothelium revealed global and specific differential miR expression (Figs. 2 and 3). In general, there was a downregulation of miR in the low-grade samples (18 of 25 differentially expressed miRs) and an upregulation in high-grade and invasive tumors (28 of 30 and 20 of 20 miRs, respectively; χ² P < 0.0001). Few aberrantly expressed miRs were common to the low-grade and high-grade/invasive cohorts [4 of 59 (6%)], whereas many were shared between the high-grade and invasive tumors [12 of 38 (32%), χ² P = 0.003]. The magnitude of differential miR expression varied among tumor groups [average fold change, 4.32 (low grade), 28.24 (high-grade NMI), and 50.18 (invasive); ANOVA P = 0.006]. When the three groups were compared with each other, the low-grade tumors were distinct from the high-grade NMI (n = 9 miRs differentially expressed) and invasive cohorts (n = 45 miRs differentially expressed), whereas no significant differences in expression were present between the latter two (Fig. 3A).

We defined a specific signature for each tumor group by selecting those miRs that were differentially expressed when compared with both normal urothelium and one or more of the other two tumor groups (Fig. 3B). As many miRs were shared between
high-grade NMI and invasive tumor groups, we merged these to create a single defining signature. We also identified four miRs that were altered in UCC regardless of tumor phenotype.

**Phenotype-specific miR targets.** We were interested by the difference in miR expression between the low-grade and high-grade tumors. MiR upregulation was a feature of the high-grade pathway, and miRs-21/373 were among those with most prominence. Recent data reveal that miR-21 negatively regulates the p53 tumor suppressor pathway, leading to a loss of cellular control (25). MiR-373 is known to regulate prometastatic pathways (26), possibly through LATS2 suppression (of note, LATS2 was downregulated in our tumors; Supplementary Fig. S2; ref. 8).

Low-grade tumors were characterized by downregulation of many miRs. Of the 16 miRs with significant downregulation, 7 are predicted to target FGFR3 (miRs-99a/100/214/145/30a/125b/507) and 1 is the only highly conserved miR to target H-Ras (miR-218). We focused upon miRs-99a/100 as they target a highly conserved 8mer region within the FGFR3 UTR and their expression was inversely correlated with FGFR3 mRNA ($r = -0.48$ and $-0.52$, respectively; $P < 0.0004$; Fig. 4A). To investigate whether FGFR3 is a target of miRs-99a/100, we manipulated their expression in NHU cells. These cells represent normal urothelium, have a 200-fold higher miR99a/100 expression than RT4 cells and a low FGFR3 expression (data not shown). Following transfection with anti-miRs, a 70% to 80% reduction in miR expression (data not shown) resulted in an average 3-fold (miR-99a) and 6-fold (miR-100) upregulation of FGFR3 protein (Fig. 4). Direct targeting of the 3′ UTR region of the FGFR3 gene was confirmed by a Luciferase reporter assay (Fig. 4). Transfection of NHU cells with anti-miRs to 99a/100 and a plasmid containing Luciferase and a partial FGFR3 3′UTR resulted in a 2-fold (miR-99a) and 3.4-fold (miR-100) increase in Luciferase expression, when compared with cells transfected with a scrambled UTR sequence. To investigate the reverse relationship, we produced matching NHU-TERT cell lines with and without the S249C FGFR3 mutation. In each cell line regardless of FGF exposure, confluence or passage number the expression of both miRs was unchanged by the presence of the mutation (Fig. 4D), suggesting the epigenetic event is upstream of the gene mutation.

**MiR expression and tumor progression.** If miRs-99a/100 and 21/373 characterize low-grade and high-grade UCC, one would
expect their expression to be associated with tumor behavior. For each miR, there was a significant change in tumor progression rate according to their expression, in a manner similar to their associated genetic traits (log-rank \( P < 0.05 \); Fig. 3C). MiRs-99a/100 downregulation was associated with a better outcome (analogous to FGFR3 mutation), whereas miRs-21/373 upregulation was associated with a worse outcome (as with p53 mutation), when compared with contrasting tumors. Multivariate analysis revealed that miR-99a/100 expression was associated with progression when analyzed together with tumor stage, grade, and miR-21 expression (Cox MVA \( P = 0.03 \) and \( P = 0.006 \), respectively).

**Epigenetic regulation of miR.** The role of aberrant promoter hypermethylation for miR downregulation in low-grade UCC was analyzed using DNA methyltransferase inhibition. Following 5-azacytidine treatment, eight miRs (Fig. 4E) had >2-fold increase in expression, including six in the low-grade cell line (RT4). The lower rates of miR re-expression in the high-grade tumor lines (RT112 and EJ/T24) suggest that regulation by hypermethylation of these miRs is relatively low-grade specific. Both RT112 and EJ/T24 have higher rates of DNA hypermethylation than RT4 at known tumor suppressor gene promoters (27). For comparison, we analyzed four hypermethylated miRs reported in UCC (miRs-127/124a/373/517c; refs. 28, 29). For these, re-expression was usually shared between two or more cell lines rather than just isolated to RT4. MiRs-99a/100 are located at 21q21.1 and 11q24.1, respectively, together with two other miRs and one protein coding genes. Neither region contains a canonical CpG island. To investigate long-range epigenetic silencing of miRs-99a/100, we looked at the expression of neighboring genes following 5-azacytidine treatment (Supplementary Fig. S4). There was an increase in expression of mir-125b (located in duplicate genes, at each locus) but not of either protein coding gene nor the other miR in RT4.

**Processing molecules and miR expression.** We analyzed the expression of molecules known to be important for miR processing. The expression of each varied significantly between tissue type (ANOVA \( P < 0.05 \); Supplementary Fig. S5) but not between tumor phenotype. For Dicer, Drosha (RNASEN), and Exportin 5,
there was an upregulation in the normal urothelium from UCC cases that was reversed once malignancy appeared. The largest changes were for Dicer and Drosha (7.4- and 6.1-fold upregulation, \( P = 0.00006 \) and \( P = 0.00001 \), respectively), whose expression was closely correlated (Pearson's correlation = 0.79, \( P = 7 \times 10^{-19} \)). For RAN and DGCR8 the opposite was seen, with an initial downregulation followed by upregulation in the tumors. Concentrations of these two mRNA were almost identical in each tissue (Pearson's correlation = 0.48, \( P = 3 \times 10^{-6} \)). When expression of these molecules was compared with that of the 322 miRs, several associations were seen. Dicer is targeted by several miRs and expression of miR-130b (\( r = -0.28, P = 0.02 \)) and let-7g (\( r = -0.25, P = 0.05 \)) were inversely correlated with Dicer expression.

**Discussion**

Here, we have shown that altered miR expression occurs commonly in UCC, in a phenotype-specific manner and targets key pathways considered "hallmarks" of the disease. Our first finding was that protumorogenic miR alterations occur before the histologic onset of malignancy, supporting observations of promoter hypermethylation and genetic instability (27, 30). Almost half of our tumor-associated miRs were upregulated in the normal urothelium from patients with the disease, and this was associated with increased Dicer, Drosha, and Exportin 5 expression. Dicer overexpression in premalignant lesions of the prostate and lung, and reduced Dicer and Drosha expression in ovarian cancer are reported (31–33), supporting our observation. Expression of the miR machinery was not associated with specific miRs, suggesting a global miR upregulation rather than the selection of tumor-specific species.

When analyzing all UCC cases together, we found the aberrant expression of 16 miRs, including some generic to carcinogenesis [e.g., miR-21 (ref. 25), mir-133b (ref. 34)]. Numerous interesting observations were apparent. For example, reduced miR-204 expression was frequent. MiR-204 is located on chromosome
9q21.11 within an intron of TRPM3, a gene downregulated in UCC (35, 36). MiR-204 potentially targets IRS2, an important member of the FGFR3 pathway (37). Observed changes in miR expression occurred in isolation or were clustered. Isolated differences arose from solitary located miRs such as miRs-649/135b or miR-601. The latter is located within intron 1 of DENND1A, a gene upregulated in UCC (38). Isolated differences also arose when a single member of a miR cluster was differentially expressed, including downregulation of miR-133b (adjacent to mir-206) or upregulation of miR-449b (located with miR-449a) and miR-93 (clustered with miRs-25/106b within the MCM7 gene). Clustered alterations included downregulation of miRs-133a/1 (located in duplicated clusters on Chr18q11.1 and Chr20q13.33), miRs-99a/125b, and miRs-143/145. Familial clustering was also present with three members of the miR-93 family (miR-93/520b/520d), miR-133a, and 133b, and three of the miR-34 family (miR-449b/34c/34a) having aberrant expression. Bioinformatic predictions identified around 1,000 genes as putative targets of these miRs. Of these genes, 20% were targeted by more than one miR. We confirmed consistent expression changes for some of these targets including new observations suggesting a carcinogenic role for LATS2, YOD1, and RAB22a in UCC. We identified miR as a potential mechanism for SOX4 upregulation in UCC (39).

Although the comparison of UCC with normal urothelium revealed interesting data, it did not detail events within the disease well. To obtain a detailed picture, it was necessary to analyze miR expression between tumor phenotypes. High-grade tumors were characterized by miR downregulation and for most, altered expression occurred before the onset of muscle invasion. Low-grade UCCs were characterized by miR downregulation and this affected many molecules targeting FGFR3 or H-Ras. Confirmation of FGFR3 miR targeting was obtained by the inverse correlation between miR and mRNA expression, and functional miR knockdown experiments. As aberrant expression of miRs-99a/100 was almost ubiquitous in low-grade UCC, occurs more frequently than FGFR3 mutation, and the presence of the commonest FGFR3 mutation (S249C) did not alter miR expression, we concluded the miR alteration leads to FGFR3 upregulation before the acquisition of gene mutation. This is an intriguing finding and suggests that epigenetic upregulation of FGFR3 facilitates the acquisition of mutation by either increasing cell turnover, reducing regulation to make cells more susceptible to carcinogens, or providing a selection advantage for mutant cells. To investigate potential causes of reduced miR expression in low-grade UCC, we examined DNA methylation. For six miRs, there was significant upregulation in RT4 following DNA methyltransferase inhibition, including miR-1 for which aberrant hypermethylation has recently been reported in hepatocellular carcinoma (40). Our findings with respect to miR-99a/100 were less clear. There are no canonical CpG islands close to either gene, but two of the four genes clustered around miR-99a or miR-100 loci had increased expression following 5-azacytidine. This finding has been reported by others (40, 41) and suggests long-range epigenetic regulation (42). However, the expression of neighboring protein-coding genes did not alter with DNA Methyltransferase inhibition.

In summary, we have found new mechanisms related to biology and progression of UCC. These epigenetic events precede histologic changes of malignancy or disease-progression and occur in a phenotype-specific manner. These data have implications regarding out understanding of UCC tumor biology, and suggest miR as a novel diagnostic or prognostic biomarker, and therapeutic target.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 2/26/09; revised 7/21/09; accepted 8/25/09; published OnlineFirst 10/20/09.

Grant support: GSK Clinician Scientist fellowship (J.W.F. Catto) and project grants (J.W.F. Catto) from Yorkshire Cancer Research, Sheffield Hospitals Charitable trust, and the European Union (Framework 7).

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We thank Messrs. Anderson, Chapelle, Hastie, Hall, Inman, Oakley, and Smith for allowing us to study their patients; Sister Louise Goodwin and Leila Ayandi for their help in tissue collection, Richard Bryant and Paul Heath for array advice, and the staff and patients of the Royal Hallamshire hospital.

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