Expression of miRNAs Involved in Angiogenesis, Tumor Cell Proliferation, Tumor Suppressor Inhibition, Epithelial-Mesenchymal Transition and Activation of Metastasis in Bladder Cancer

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Purpose: miRNAs are noncoding RNAs that posttranscriptionally regulate gene expression. Altered expression and function have been observed in bladder cancer. We analyzed the expression profile of a group of miRNAs involved in bladder cancer angiogenesis, tumor cell proliferation, tumor suppressor inhibition, epithelial-mesenchymal transition and metastasis activation. Prognostic and diagnostic value, and validated targets were further examined.

Materials and Methods: Using quantitative real-time polymerase chain reaction 77 bladder cancer cases and 77 matched tumor associated normal samples were investigated to determine the expression of miR-10b, 19a, 19b, 21, 126, 145, 205, 210, 221, 296-5p and 378. The relationship between miRNA expression, patient survival and tumor pathological features was also examined.

Results: miR-10b, 19a, 126, 145, 221, 296-5p and 378 were significantly down-regulated in bladder cancer compared to adjacent normal urothelium. miR-145 was the most down-regulated microRNA of this group. miR-19b, 21, 205 and 210 showed no significant difference between the 2 tissue types. High miR-21 expression correlated with worse overall patient survival ($p = 0.0099$). Multivariate analysis revealed that miR-21, 210 and 378 may serve as independent prognostic factors for overall patient survival ($p = 0.005$, 0.033 and 0.012, respectively). miR-21 and 378 may serve as independent prognostic factors for recurrence ($p = 0.030$ and 0.031, respectively). miR-145, 221, 296-5p and 378 showed the best combined ROC curves for specificity and sensitivity. miRWalk analysis was used to identify validated miRNA target genes. Further Gene Ontology enrichment revealed the main classes of biological functions of these validated targets.

Conclusions: Most miRNAs analyzed are down-regulated in bladder cancer. They may serve as candidate biomarkers for diagnostic and prognostic purposes in the future.

Key Words: prognosis; bladder neoplasms; urothelium; microRNAs; tumor markers, biological

Abbreviations and Acronyms

Ct = cycle threshold
EMT = epithelial-mesenchymal transition
GO = Gene Ontology
HCL = hierarchical clustering
hsa = Homo sapiens
miR = microRNA
UCC = bladder urothelial cell carcinoma
UTR = untranslated region

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BLADDER cancer is the most common urinary tract malignancy and the fifth most common malignancy in the developed world. Each year an estimated 105,000 and 71,000 new patients are diagnosed with bladder cancer in Europe

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and the United States, and approximately 28.5% and 20%, respectively, die of the disease.

miRs are single strand, noncoding RNA molecules that regulate gene expression at the posttranscriptional level.[1] Through specific targeting of multicellular eukaryotic miR3-UTRs miRs down-regulate gene expression by inducing the degradation or impairing the translation of target mRNAs.[2] Angiogenic signaling, cell proliferation, apoptosis avoidance, EMT and tumor invasion pathways are regulated by different miRs.[3–5] The current estimate is that almost 900 unique miRs are encoded in the human genome, in part controlling the expression of more than a third of human genes.[6]

More than 40 miRs are involved in urological cancer and a number target common carcinogenic pathways.[7] Specific changes in the expression profile of miRs that regulate angiogenesis are associated with urothelial carcinoma pro-angiogenic phenotypes.[8] However, further information is required on the expression profile of miRs with key roles in the major pathways that control cellular proliferation and bladder cancer metastasis. Such miRs have invaluable prognostic value as indicators of patient survival, tumor relapse and/or metastasis as well as diagnostic value to distinguish patients with bladder cancer from healthy donors. Also, the need to identify the gene targets of such miRs is of major significance.

We explored the expression profile of 11 miRs involved in human UCC angiogenesis, tumor cell proliferation, tumor suppressor inhibition, EMT and metastasis activation.

MATERIALS AND METHODS

Patients and Tumor Samples

A total of 77 paired samples consisting of tumor and normal urothelium were obtained from 77 Greek patients with UCC. Samples were studied to determine the expression of miR-10b, 19a, 19b, 21, 126, 145, 205, 210, 221, 296-5p and 378. Written informed consent in accordance with the Institutional Committee for the Protection of Human Subjects was obtained from all patients. Study ethics approval was obtained from the Asklipieio General Hospital and University of Crete institutional review boards. All 77 patients were treated at Asklipieio General Hospital, Voula, Athens, Greece. Patients included 68 men with a mean age of 71.42 years (range 44 to 93) and 9 women with a mean age of 74.44 years (range 43 to 86). Mean age was 72.12 years in all patients. None of the 77 patients had previously received systemic chemotherapy or external radiation therapy. Of the 77 patients 56 had newly diagnosed bladder cancer and 21 had recurrent disease.

Total RNA

Total RNA extraction and reverse transcription were done as previously described.[9] Select miR primers were obtained from the miScript Primer Assay (QIAGEN®). miRs were amplified using the miScript SYBR® Green PCR Kit. Expression levels were analyzed on a Mx3000P™ thermal cycler. Endogenous control stability and ranking were calculated with the SLqPCR algorithm. RNU1A1, 5A and 6B were used for normalization by dividing the Ct of each miR by the mean Ct of the 3 normalized genes. Relative expression was determined using the $\Delta\Delta$Ct method and data were standardized by $\log_2$ transformation. Reactions were performed in triplicate. Two-way average HCL with Euclidean distance was done with Genesis 1.7.6 (Institute for Genomics and Bioinformatics, Graz University of Technology, Graz, Austria).

Analysis and Enrichment

miRWalk analysis was performed to identify validated miRNA targets.[10] GO enrichment[11] for putative miRNA targets was investigated using the WebGestalt tool.[12]

Statistical Analysis

Data distribution normality was assessed by the Kolmogorov-Smirnov test. Differences in expression levels between bladder cancer and normal tissue were evaluated using the Wilcoxon matched pairs test. Numerical values are shown as the mean ± SEM. The Kaplan-Meier method was used to estimate survival as a function of time and survival differences were assessed by the log rank test. Logistic regression analysis was done to determine potential predictors of survival, recurrence and metastasis with statistical significance considered at the 95% level ($p < 0.05$).

RESULTS

miRNA Expression

Certain miRs showed significant down-regulation in bladder cancer vs normal urothelium, including miR-10b (mean 0.0156 ± 0.004 vs 0.027 ± 0.006, $p = 0.0008$), miR-19a (0.010 ± 0.002 vs 0.021 ± 0.003, $p = 0.023$), miR-126 (0.360 ± 0.088 vs 0.731 ± 0.138, $p = 0.0057$), miR-145 (1.154 ± 0.320 vs 2.865 ± 0.500, $p < 0.0001$), miR-221 (0.128 ± 0.026 vs 0.376 ± 0.062, $p < 0.0001$), miR-296-5p (0.0068 ± 0.0020 vs 0.024 ± 0.005, $p < 0.0001$) and miR-378 (0.0039 ± 0.0008 vs 0.010 ± 0.001, $p < 0.0001$). No miR was significantly over expressed in the bladder cancer vs normal tissue. miR-19b, 21, 205 and 210 showed no significant difference in bladder cancer and normal tissue (fig. 1).

miR expression was also investigated relative to tumor stage and grade (figs. 2 and 3). No significant difference was found between low grade noninvasive papillary tumors and high grade invasive tumors. HCL for miRs regarding tumor stage and grade as well as the metastatic potential of each tumor revealed 2 main tumor sample clusters. Each cluster was further characterized by various subclusters (fig. 4). Down-regulation of these miRs in bladder cancer compared to normal urothelium was also verified by computational analysis of the 2 publicly available Gene Expression Omnibus miR data sets

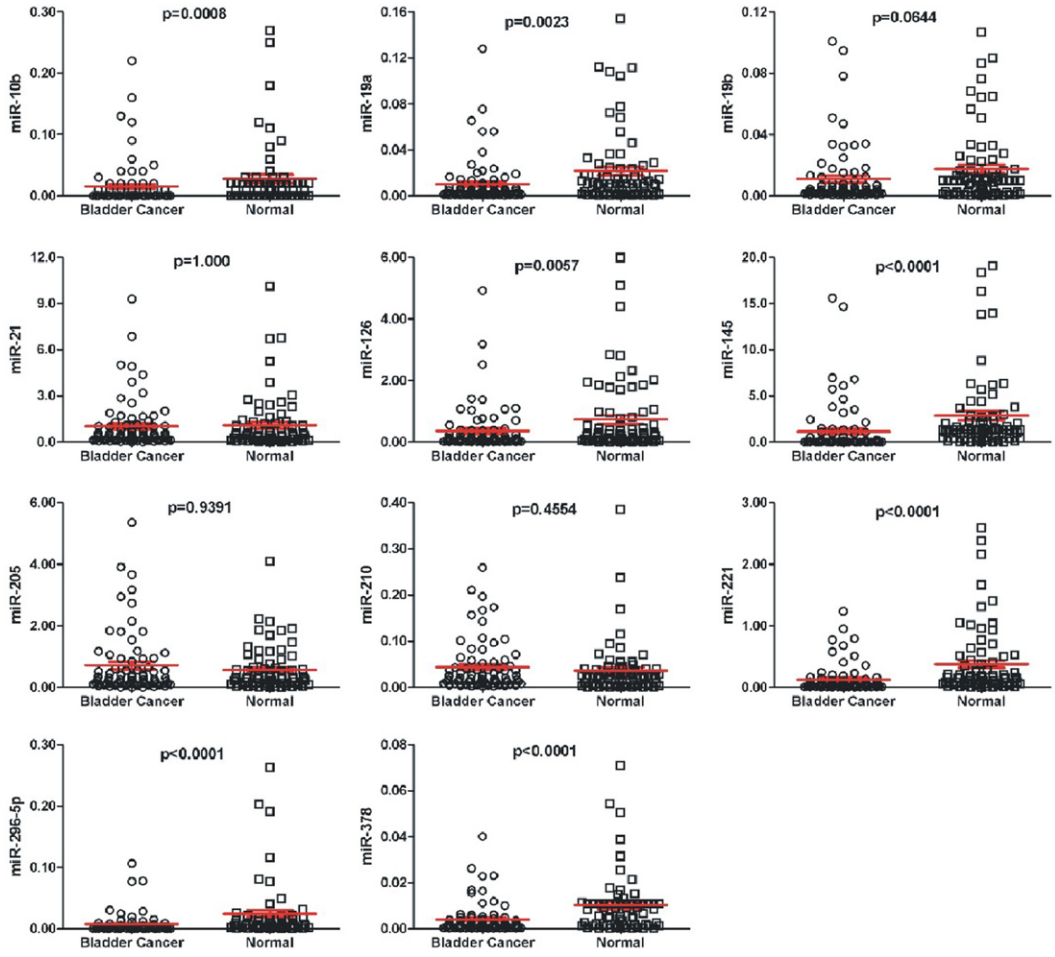

Figure 1. Mean ± SEM (red bars) bladder cancer and adjacent normal urothelium miR expression statistically compared by Wilcoxon matched pairs test.

GSE20414[13] and GSE2564[14] (fig. 5). Stronger pairwise correlations were detected among the 11 miRs for bladder cancer compared to normal urothelium.

miRNA Expression and Patient Survival

Disease-free and overall survival was evaluated in the 77 cases in relation to miR expression. Cases were divided into 2 groups with expression greater than (high expression) and less than (low expression), respectively, the median expression of each miR. High miR-21 expression was associated with worse overall survival ($p = 0.0099$, fig. 6). Univariate regression analysis showed that miR-21 and 210 could serve as prognostic factors for overall survival (relative risk 0.242, 95% CI 0.077–0.758, $p = 0.015$ and 0.335, 95% CI 0.113–0.996, $p = 0.049$, respectively). miR-21 was identified as a prognostic factor for tumor metastasis (relative risk 0.335, 95% CI 0.113–0.996, $p = 0.049$). Multivariate regression analysis also identified miR-21 (relative risk 0.119, 95% CI 0.027–0.527, $p = 0.0050$), miR-210 (relative risk 0.230, 95% CI 0.060–0.888, $p = 0.033$) and miR-378 (relative risk 7.316, 95% CI 1.544–34.661, $p = 0.012$) as good prognostic factors for overall survival. Analysis revealed that miR-21 (relative risk 0.205, 95% CI 0.049–0.856, $p = 0.0300$) and miR-378 (relative risk 5.984, 95% CI 1.176–30.447, $p = 0.031$) could serve as prognostic markers for tumor recurrence. miR-21 was also a good indicator of metastasis (relative risk 0.335, 95% CI 0.113–0.996, $p = 0.049$).

Univariate regression analysis showed that miR-145 could be used as a prognostic factor for tumor stage (relative risk 1.834, 95% CI 1.064–3.160, $p = 0.029$). Multivariate analysis showed that miR-126 and miR-378 could be used as prognostic factors for grade (relative risk 5.315, 95% CI 1.526–18.512, $p = 0.009$ and 10.799, 95% CI 3.104–37.577, $p < 0.001$), stage (relative risk 5.114, 95% CI 1.467–17.829, $p = 0.010$ and 10.366, 95% CI 2.975–36.125, $p < 0.001$) and carcinoma in situ (relative risk 5.315, 95% CI 1.526–18.512, $p = 0.009$ and 10.799, 95% CI 3.104–37.577, $p < 0.001$, respectively).

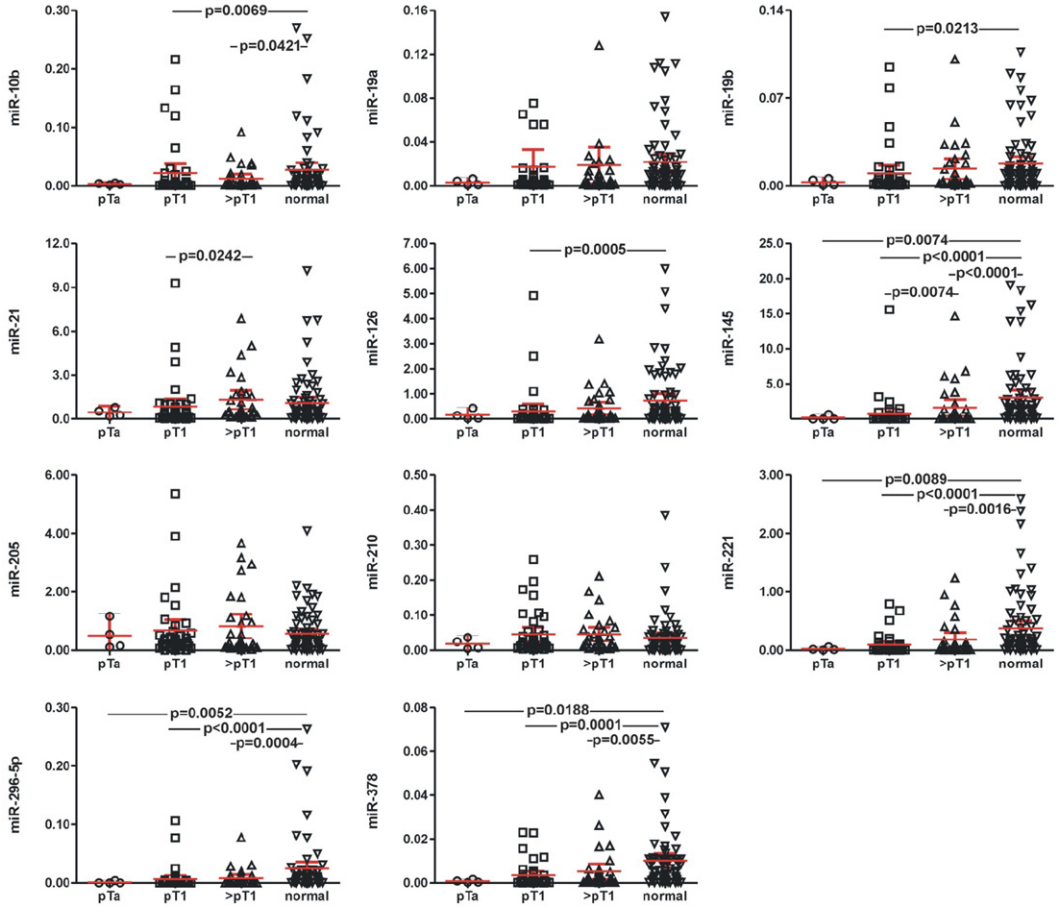

Figure 2. Mean ± SEM (red bars) miR expression by bladder cancer stage

Analysis and Enrichment

To our knowledge miRWalk is the only database that provides possible miRNA binding sites on the complete sequence (promoter, 5′UTR, coding sequences and 3′UTR) of known genes and 3 complete mitochondrial genomes.[10] miRWalk compares its identified miRNA binding sites with the results of 8 established miRNA target prediction programs, including DIANA-microT (http://diana.cslab.ece.ntua.gr/microT/), miRanda (http://www.microrna.org/microrna/getGeneForm.do), miRDB (http://mirdb.org/miRDB/), PicTar (http://pictar.mdc-berlin.de/), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) and TargetScan/TargetScanS (http://www.targetscan.org/). miRWalk incorporates all predicted miRNA binding sites produced by the miRWalk algorithm and the 8 established programs into the miRWalk relational database.

Overall we isolated 1,191 records after removing duplicate results, including 59 validated targets for hsa-miR-10b, 60 for hsa-miR-19a, 42 for hsa-miR-19b, 350 for hsa-miR-21, 101 for hsa-miR-126, 132 for hsa-miR-145, 127 for hsa-miR-205, 93 for hsa-miR-210, 155 for hsa-miR-221, 31 for hsa-miR-296-5p and 41 for hsa-miR-378. GO enrichment of these validated miR targets revealed their participation in various biological processes, molecular functions and cellular processes. The most interesting enrichment scores were those of the validated targets of miR-19a, 126, 21, 145, 205 and 221 (fig. 7).

Gene Marker Diagnostic Performance

We performed ROC analysis to evaluate miR diagnostic performance. miR-145 enabled the most sensitive (63.6%) and specific (93.5%) separation of patients with bladder cancer from healthy donors (AUC 0.788, 95% CI 0.710–0.865, $p < 0.0001$). miR-378 showed the highest sensitivity (81.6%) but decreased specificity (65.8%). The AUC was 0.738 (95% CI 0.655–0.820, $p < 0.0001$, fig. 8).

DISCUSSION

Altered miR expression in UCC, which develops early in tumorigenesis in a tumor phenotype specific manner, can predict disease progression,[15] as re-

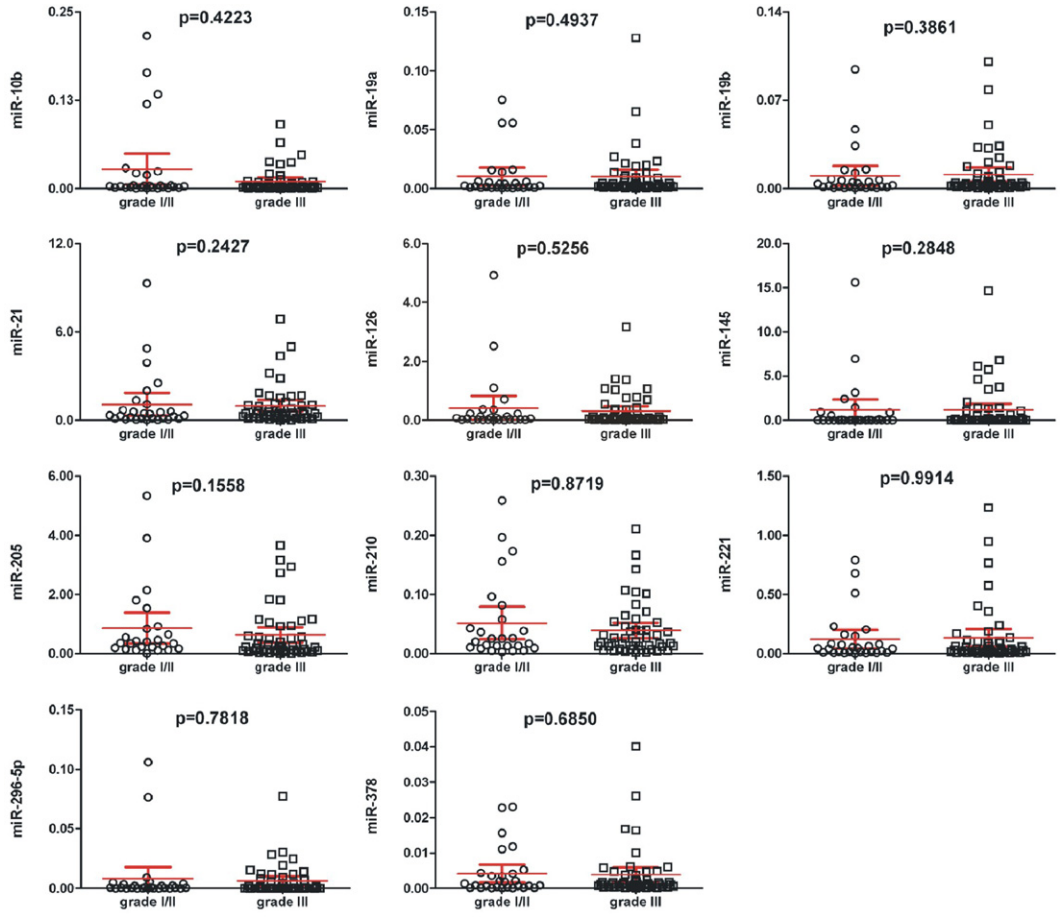

Figure 3. Mean ± SEM (red bars) miR expression by bladder cancer grade

cently reported by Dyrskjot et al.[16] They genomically profiled bladder cancer and found altered expression in various miRs, such as miR-145 down-regulation and miR-21 up-regulation. miR involvement in bladder carcinoma pathogenesis was also investigated by others.[17–19] In our study miR-10b, 19a, 126, 145, 221, 296-5p and 378 showed significant down-regulation in bladder cancer compared to that in normal

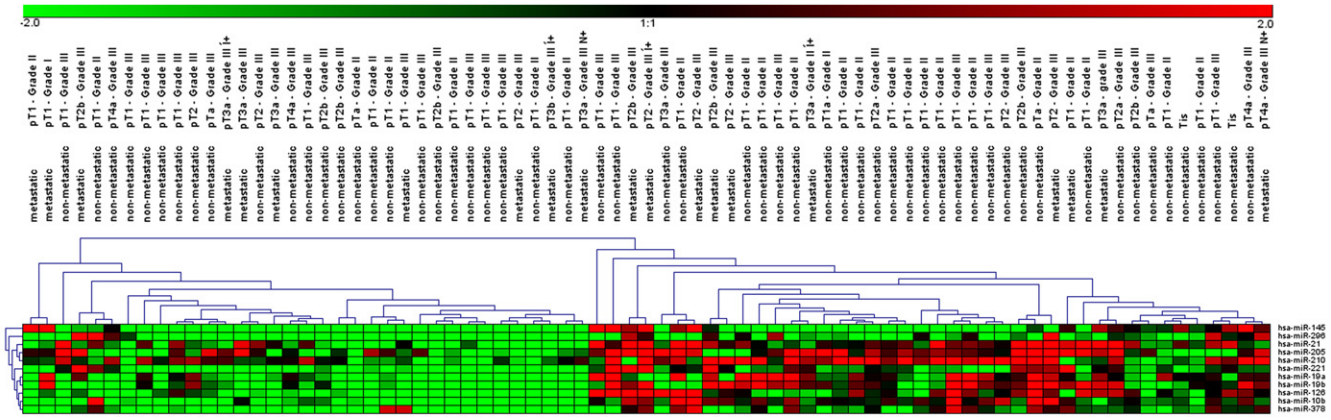

Figure 4. Two-way average linkage HCL with Euclidean distance. Neither tumor metastatic potential nor stage/grade was criterion for tumor sample clustering. miR-378 clustered with miR-10b, 126, 19b, 19a and 221, and miR-210 clustered with miR-205 and 21. Less vs greater than twofold differential expression served as threshold for HCL analysis. Rows represent miRs. Columns represent tumor vs normal sample mean fold change in miR expression. Red blocks represent signal increase. Green blocks represent signal decrease.

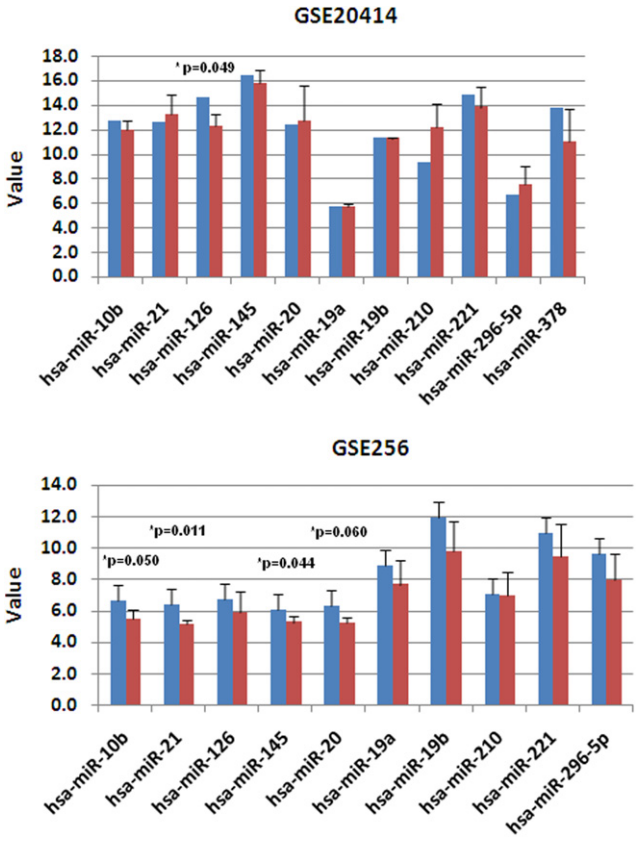

Figure 5. Computational analysis shows miR-10b, 19a, 19b, 21, 126, 145, 205, 210, 221, 296-5p and 378 expression profile in bladder cancer (red bars) and normal urothelium (blue bars). Gene Expression Omnibus data sets GSE20414 and GSE256 were analyzed. Normalized $\log_2$ signal intensity of miR group under investigation and corresponding normal counterparts were extracted from normalized data sets. Mean ± SD $\log_2$ intensity results were statistically compared by t test. Generally computational analysis verified lower expression of study miRs in bladder cancer vs normal urothelium.

urothelium. This agrees with the hypothesis that miRs are generally down-regulated in cancer.

miR-210 is a member of the hypoxia-inducible miR group, which promotes the hypoxic tumor environments that trigger the expression of hypoxically regulated genes.[20] miR-210 over expression further activates vascular endothelial growth factor and leads to the formation of capillary structures under hypoxic conditions during the early steps of tumor development.[21] Although it was found to be up-regulated,[18,19] in the current study miR-210 showed equal expression in bladder cancer and normal urothelium.

The miR-200 family of miRNAs and miR-205 regulate Zeb1 and 2 expression, and control EMT.[22] miR-21 represses the tumor suppressors phosphatase and tensin homologue deleted on chromosome 10, tropomyosin 1 and the programmed cell death gene-4.[23] It enhances tumor angiogenesis, cell growth, proliferation and invasion, and is commonly found in different human malignancies[23] as well as in invasive cancer cells and tumor metastasis.[24] The early EMT expression promoted by over expressed miR-205 in superficially invasive bladder cancer may favor the recurrent character of the disease and the early initiation of tumor metastasis, which is supported by miR-21 over expression.

In our study miR-205 and 21 showed equal expression in bladder cancer and normal urothelium, a finding that agrees with some previously reported results[16,19] but contrasts with others.[18,25] Since no metastatic tumors were included in our series, we expected to observe normal miR-21 expression. However, miR-21 levels were significantly higher in high than in low stage bladder tumors. miR-21 also significantly correlated with miR-205. Also, we noted that miR-21 could serve as a potential prognostic factor for overall patient survival, tumor recurrence and metastasis. High miR-21 levels also correlated with poor overall survival. Finally, miR-21 strongly correlated with angiogenic and cell proliferation promoting miR-19a and 19b as well as with miR-221.

Recent findings indicate that miR-126 regulates the expression of vascular cell adhesion molecule 1,[26] which appears to have a role as a tumor suppressor in the invasion process of several carcinomas. miR-126 promotes vascular integrity and angiogenesis through the response of endothelial cells to angiogenic growth factors.[27] In agreement with Han et al[19] we found that miR-126 is under expressed in low stage bladder tumors vs normal urothelium. miR-126 levels highly correlated with miR-10, 19a, 19b, 145, 221 and 378. Also, miR-126 may be used as a potential prognostic factor for tumor grade.

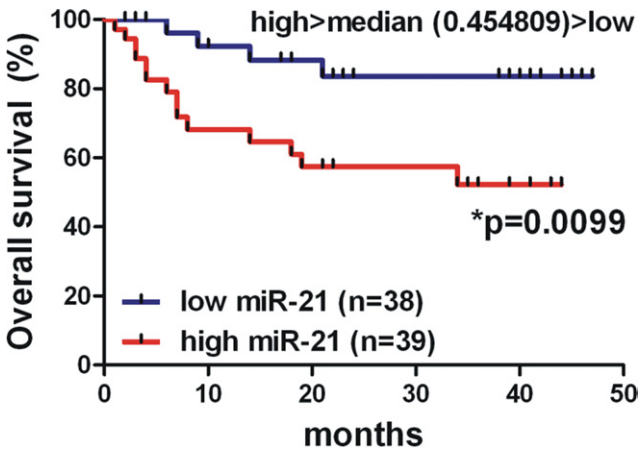

Figure 6. Kaplan-Meier curve shows that high miR-21 expression was significantly associated with worse overall survival of 77 study patients. Survival differences were assessed by log rank test with statistical significance considered at $p < 0.05$.

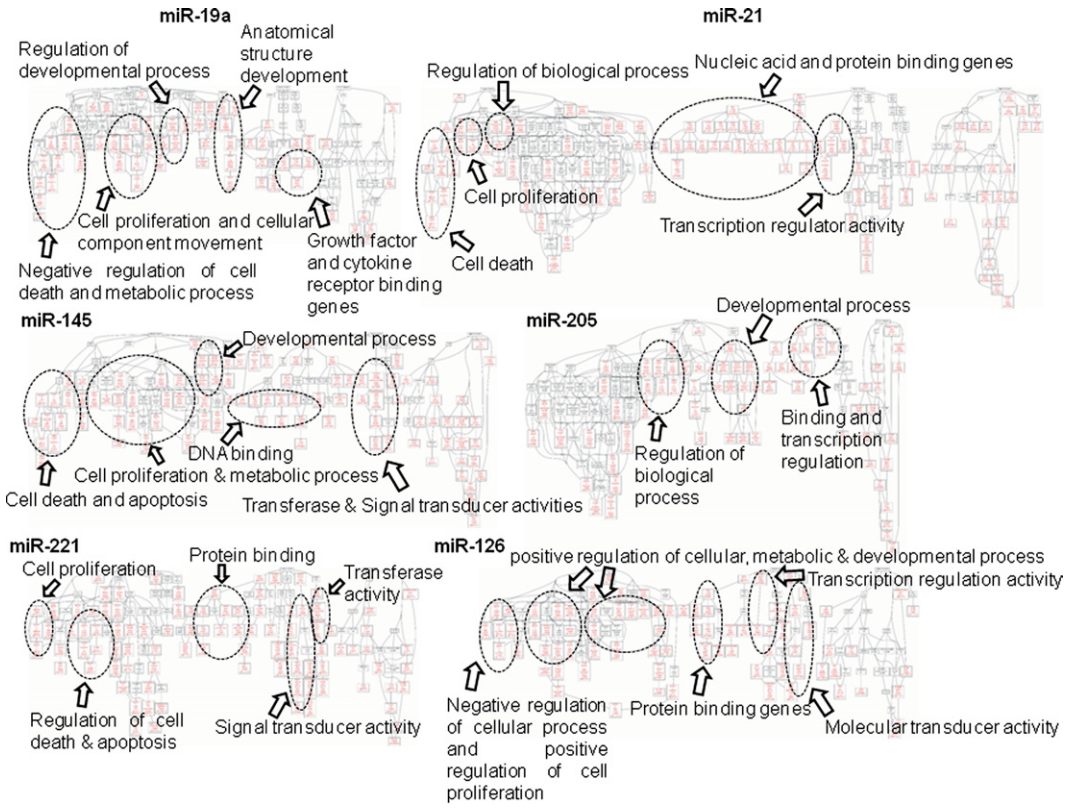

Figure 7. Significantly enriched GO categories under biological process, molecular function and cellular component with 3 directed acyclic graphs for validated targets of miR-19a, 21, 145, 205, 221 and 126. Whole genome served as reference set for enrichment analysis of validated target genes of study miR group. Hypergeometric test was used to analyze enrichment evaluation with Bonferroni adjustment and adjusted p = 0.01 as significance cutoff. Minimum of 2 genes per category was required to test. Each GO category is node in directed acyclic graph. Each node shows GO category name, number of genes and adjusted p value indicating enrichment significance. Red areas represent enriched GO categories. Black areas represent nonenriched parents. Arrows indicate significantly enriched categories.

miR-19a and 19b belong to the miR-17-92 oncogenic cluster, which is directly involved in tumor angiogenesis. The combined study of miR-19a and 19b by Olive et al yielded evidence of the oncogenic properties of the whole miR-17-92 cluster.[28] They concluded that miR-19b has an essential role in mediating the oncogenic activity of miR-17-92 and the oncogenic activity of miR-19b occurs at least in part due to inhibition of the expression of phosphatase and tensin homologue deleted on chromosome 10. miR-19a and 19b were previously reported to be up-regulated in bladder cancer.[18,19] In our study miR-19a expression was lower in bladder cancer than in normal urothelium while miR-19b showed equal expression in the 2 tissue types. The discordance between our results and those of Han et al[19] could be attributable to the strict statistical test that we used and to the different experimental methodologies followed. The 2 miRs highly correlated with other miRs involved in angiogenesis (miR-296 and 378) and vascular integrity (miR-126) as well as with those responsible for cell proliferation (miR-21, 221 and 378) and metastasis (miR-10b).

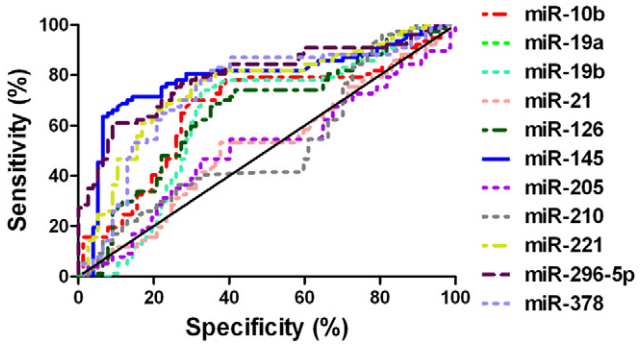

Figure 8. ROC curves were used to evaluate diagnostic performance of miRNAs. AUC helped visualize tradeoff between high sensitivity and high specificity when discriminating bladder cancer from control tissue.

The miR-143/145 cluster is down-regulated in numerous cancers, including bladder cancer and bladder cancer cell lines.[16] miR-145 inhibits cell growth, invasion and metastasis.[29] Our findings verify the

significant under expression of miR-145 reported by others,[18,19] providing more evidence to support that miR-145 suppresses bladder tumors. We also provide evidence that miR-145 could serve as a prognostic factor for tumor stage.

miR-221 along with miR-222 has an important role in the induction of cell proliferation through silencing of the cell cycle inhibitor $p27^{Kip1}$.[30] miR-296-5p enhances hepatocyte growth-promoting substance, which activates the proliferation of angiogenic endothelial cells. This miR clustered with miR-222 and 146 is predicted to interact with the KIT oncogene mRNA at a minimum of 2 sites. Our data show that miR-221 and 296-5p are significantly down-regulated in bladder cancer compared to normal urothelium, in accordance with previous reports.[19] Their expression correlated with that of the remaining miRs. High miR-221 and 296-5p levels showed a trend toward correlating with poor prognosis but no statistical significance was attained.

Lee et al noted that miR-378 promotes tumor growth and angiogenesis in vivo by targeting SUFU and FUS1 transcripts.[31] Loss of function of these tumor suppressors causes excessive tumor proliferation. In agreement with Han et al[19] we report that miR-378 is also significantly under expressed in bladder cancer and it correlates highly with miR-21 and 221. It also correlates with the angiogenic group of miR-19a, 19b, 126 and 296 as well as with the prometastatic miR-10b. miR-378 could serve as a potential prognostic factor for overall patient survival and tumor recurrence as well as tumor stage and grade, and carcinoma in situ.

miR-10b is responsible for metastasis and it positively regulates cell migration and invasion. In agreement with Han et al[19] our data indicate low miR-10b levels in bladder cancer. Since no metastatic tissue was included in our study, high miR-10b expression was rather unexpected. A high correlation of miR-10b was observed in the angiogenic miR group (miR-19a, 19b, 126, 296 and 378) and in the 2 miRs responsible for cell proliferation in bladder cancer tissue (miR-21 and 221). This correlation shows that the prometastatic character of a tumor is related to advanced angiogenesis and cell expansion.

CONCLUSIONS

In bladder cancer several miRs involved in angiogenesis, tumor cell proliferation, tumor suppressor inhibition, EMT and metastasis activation are significantly down-regulated. The miRs investigated may serve as future candidate biomarkers for diagnostic and prognostic purposes.

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