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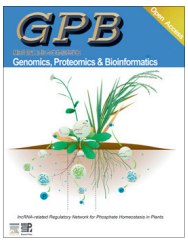

METHOD

Correlating Bladder Cancer Risk Genes with Their Targeting MicroRNAs Using *MMiRNA-Tar*

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Abstract **The Cancer Genome Atlas** (TCGA) (http://cancergenome.nih.gov) is a valuable data resource focused on an increasing number of well-characterized cancer genomes. In part, TCGA provides detailed information about cancer-dependent gene expression changes, including changes in the expression of transcription-regulating **microRNAs**. We developed a web interface tool *MMiRNA-Tar* (http://bioinf1.indstate.edu/MMiRNA-Tar) that can calculate and plot the **correlation** of expression for **mRNA–microRNA** pairs across samples or over a time course for a list of pairs under different prediction confidence cutoff criteria. Prediction confidence was established by requiring that the proposed **mRNA–microRNA** pair appears in at least one of three **target prediction** databases: TargetProfiler, TargetScan, or miRanda. We have tested our *MMiRNA-Tar* tool through analyzing 53 tumor and 11 normal samples of bladder urothelial carcinoma (BLCA) datasets obtained from TCGA and identified 204 **microRNAs**. These **microRNAs** were correlated with the **mRNAs** of five previously-reported **bladder cancer** risk genes and these selected pairs exhibited **correlations** in opposite direction between the tumor and normal samples based on the customized cutoff criterion of prediction. Furthermore, we have identified additional 496 genes (830 pairs) potentially targeted by 79 significant **microRNAs** out of 204 using three cutoff criteria, *i.e.*,

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false discovery rate (FDR) < 0.1, opposite **correlation** coefficient between the tumor and normal samples, and predicted by at least one of three **target prediction** databases. Therefore, *MMiRNA-Tar* provides researchers a convenient tool to visualize the co-relationship between **microRNAs** and **mRNAs** and to predict their targeting relationship. We believe that correlating expression profiles for **microRNAs** and **mRNAs** offers a complementary approach for elucidating their interactions.

Introduction

MicroRNAs (miRNAs) are an abundant family type of non-coding RNAs that participate in post-transcriptional regulation [1] through binding to the 3′ UTRs of mRNAs or target genes. Mature miRNAs typically are 17–24 nucleotides in length. Single-stranded mature miRNAs are generated from miRNA precursors (pre-miRNA) by the RNase III type enzyme Dicer in the cytoplasm [2].

There are many studies that demonstrate inverse correlations in the expression of specific miRNAs and their corresponding target mRNAs [3–6], although studies showing positive correlations also exist [7,8]. Aberrant miRNA expression is involved in the pathogenesis of several human diseases [9–11]. Interestingly, Miles et al [8] showed directional changes in microRNA/mRNA positive and negative correlation between the tumor and normal samples.

Urothelial carcinoma occurring in the bladder is the fourth leading type of cancer in men and the ninth most common cancer in women, with 150,000 related deaths per year in the world [12]. Many genes such as *FGFR3*, *HRAS*, *RB1*, *TSC1*, and *TP53*, have been associated with bladder cancer [13–17]. Recurrent mutations in these genes have also been reported in many studies [18,19].

The Cancer Genome Atlas (TCGA), a project initiated by the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) of the United States in 2006, continues to characterize and document a number of tumor or cancer samples. So far, more than 10 cancer tissues (breast, central nervous system, endocrine, gastrointestinal, gynecologic, head and neck, hematologic, skin, soft tissue, thoracic, and urologic) have been presented for potential study and their sequencing data are currently accessible to researchers (http://cancergenome.nih.gov).

Assuming that significant correlations between miRNA and mRNA expression levels in opposite directions between the tumor and normal samples would tend to signal the existence of demonstrable targeting relationships, we performed pairwise correlation calculations of miRNA and mRNA expression profiles of both the tumor and normal samples for the bladder urothelial carcinoma (BLCA) datasets available from the TCGA project to predict targeting relationships between specific miRNAs and mRNAs using *MMiRNA-Tar*, a tool developed in-house by us. The results from global correlation analysis of the expression data for miRNAs and mRNAs revealed potential targeting miRNAs for known bladder cancer risk genes, as well as, additional cancer risk genes apparently targeted by these miRNAs.

Methods

Data source

The test datasets were downloaded from TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/). The type of cancer studied in this paper is bladder urothelial carcinoma (BLCA). Illumina HiSeq data were acquired based on the availability of expression profile for both miRNA, which was produced by Baylor College Human Genome Sequencing Center (BCGSC), and mRNA, which was produced by University of North Carolina at Chapel Hill (UNC). Specifically, TCGA level 3 mRNASeq data were produced on Illumina HiSeq 2000 sequencers and its public release date is 04/30/2012. Read counts and reads per kilobase per million (RPKM) per composite gene (UCSC genes Dec 2009 build) were calculated using the SeqWare framework via the RNASeqAlignmentBWA workflow (http://seqware.sourceforge.net). The miRNA analyses of TCGA level 3 BLCA samples were produced by Illumina HiSeq as well. Normalized expression per miRNA gene (Reads per million miRNA mapped or RPM) was reported as miRNAs expression measurement unit. The public release date of miRNA data used in this study is 10/09/2014. To make measurement units between two sequencing data sets consistent, we converted RPKM expression values for mRNA samples into transcripts per million (TPM) values. A total of 53 tumor and 11 normal samples from seven batches (batch No. 86, 113, 128, 150, 170, 175, and 192) were downloaded and tested for both miRNA and mRNA data. The normalized mRNA and miRNA expression data of both the tumor and normal samples are shown in Tables S1 and S2, respectively.

Data pre-processing

Expression profiles of BLCA datasets for a total of 20,532 mRNAs were downloaded. We excluded 29 genes that do not have their gene symbols available (gene names marked as "?" in the annotation table) from the list. We also excluded *SLC35E2* because it is doubly reported. Thus, a total of 20,501 genes were used to check against a miRNA expression file, in which 1046 miRNAs were available.

Correlation coefficient calculation and target prediction

Calculations of linear (positive) or inverse (negative) correlation (Pearson correlation) for each miRNA−mRNA pair across samples and the prediction of miRNA and mRNA target relationship were implemented in C language. All three

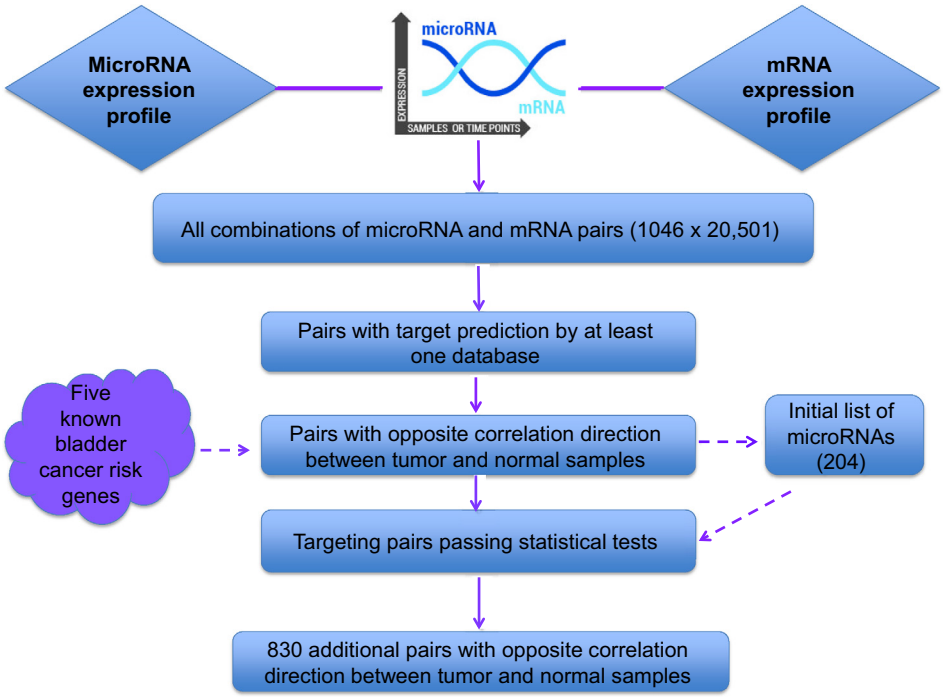

Figure 1 Workflow of selecting potential microRNAs and their gene targets

databases including TargetProfiler [20], TargetScan [21], and miRanda [22] were precompiled for the search of targeting relationship between miRNA and mRNA. We claimed the existence of the targeting relationship if a target prediction outcome is supported by at least one of the three databases mentioned above. The FDR multiple testing [23] control and normalization steps were implemented using a customized R script.

Figure 1 shows the workflow of selecting potential targeting miRNAs and additional targeted genes. *MMiRNA-Tar* is available at http://bioinf1.indstate.edu/MMiRNA-Tar and the software source code is freely available upon request for non-commercial purposes.

Results

Correlation of expression profiles of miRNAs and mRNAs

We took five genes that have been reported as common bladder cancer risk genes in multiple studies and the National Institutes of Health (NIH) Genetic Home Reference website (http://ghr.nlm.nih.gov/condition/bladder-cancer) and set out to identify their potential targeting miRNAs using three popular target prediction databases mentioned in the Method section. These genes include *FGFR3*, *HRAS*, *RB1*, *TSC1*, and *TP53*. We calculated correlations (Pearson correlation) between each of the five genes and all miRNAs reported in 53 tumor and 11 normal samples from the aforementioned TCGA datasets. We then selected the pairs with correlation values in opposite directions between the tumor and normal samples and with targeting relationship predicted by at least one of three databases using *MMiRNA-Tar*. As shown in Figure 2, three prediction databases showed similar density distribution patterns for calculated correlation values in the tumor samples, although the density distribution by miRanda was slightly different from the other two in the normal samples. We concluded that requiring a prediction outcome from any of these databases would be reasonable.

Using these five genes, 204 miRNAs in total were obtained based on the cutoff criteria of opposite correlation direction between the tumor and normal samples and by at least one database prediction (Table 1 and Table S3). These 204 miRNAs are presumed to have targeting relationships with five bladder cancer risk genes. The expression information in heatmap format for 204 miRNAs (259 pairs) across 53 tumor and 11 normal samples is shown in Figure S1. We noticed that miRNAs targeting the same gene(s) were often grouped together using hierarchical clustering with the Pearson correlation distance measure method of multiple array viewer (http://sourceforge.net/projects/mev-tm4/).

The expression profile correlation analysis for 79 selected miRNAs and their targeting mRNAs

We then calculated correlations and predicted targeting relationships for all possible pair combinations of 204 miRNAs and 20,501 mRNAs in 53 tumor and 11 normal samples of BLCA data. We obtained 830 additional miRNA–mRNA pairs (comprising of 79 miRNAs and 496 genes) showing opposite correlated relationships between the tumor and normal samples and having at least one database prediction outcome with FDR < 0.1. Figure 3 is a Venn diagram showing

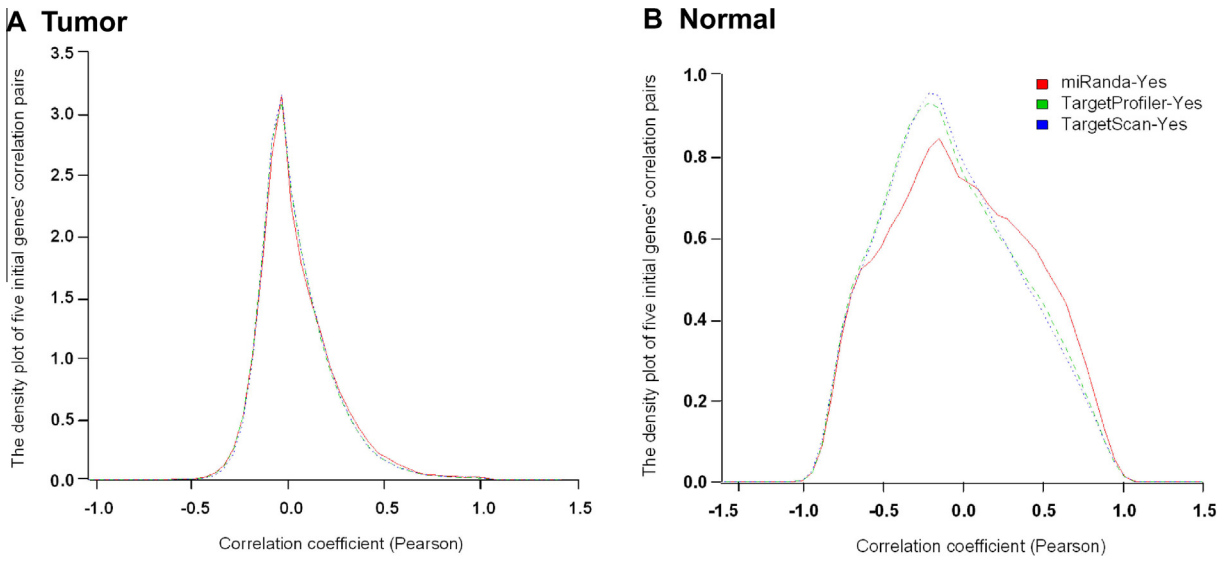

Figure 2 Density distribution of correlation of the five initial genes and their paired miRNAs for tumor and normal samples
Pearson correlation was calculated for all possible pair combinations of *FGFR3*, *HRAS*, *RB1*, *TSC1*, and *TP53* and 1046 miRNAs listed in the BLCA dataset downloaded from TCGA. Targeting relationship was then predicted using databases including TargetProfiler, TargetScan, and miRanda. The distribution of the miRNA–mRNA correlation values of the prediction results by three databases is presented for tumor samples (**A**) and normal samples (**B**).

Table 1 Correlations between five selected bladder cancer risk genes and their predicted targeting microRNAs

Gene	Chromosomal location	No. of targeting miRNAs	Average difference of correlation between tumor and normal samples
FGFR3	4p16.3	55	0.627199249
HRAS	11p15.5	10	0.714147948
RB1	13q14.2	41	0.417446885
TP53	17p13.1	31	0.327425407
TSC1	9q34	122	0.630901655

Note: Targeting relationship was predicted using Targetprofiler, TargetScan, and miRanda. Average difference of Pearson correlation for each gene was calculated for all miRNA−mRNA pairs of the respective gene between the tumor and normal samples.

prediction results derived by applying the three target prediction database filters. The additional list of miRNA-gene target pairs, along with their correlation values and target prediction result using the aforementioned cutoff criteria, is shown in Table S4. We noticed, among the 830 pairs, half of the genes seem to have targeting relationships with at least two of the 79 identified miRNAs. Thus, in addition to the five initial genes, we obtained another 496 genes having at least one predicted targeting relationship with 79 selected miRNAs.

Gene functional enrichment analysis

We searched the Database for Annotation, Visualization and Integrated Discovery (DAVID) [24,25] for functional information about the 496 genes with their predicted targeting miRNAs identified above. Enrichment of these genes was found in several GO biological processes. Some of genes are involved in chromatin remodeling complex, some of genes are associated with cell cycle regulation, and some genes are involved in protein kinase signaling pathways. These biological processes (cell cycle regulation, kinase signaling, chromatin remodeling) are frequently dysregulated in bladder cancer [26]. Genes associated with aforementioned biological processes and their associated GO terms are shown in Table 2.

Discussion

In this study, we computed the correlation coefficients for all available combinations of miRNA and mRNA pairs using TCGA BLCA sequencing data. Performing multivariable correlation analysis on a genome scale would be our future research strategy. Under the assumption of an opposite correlation of miRNA and mRNA (gene) expression levels between the tumor and normal samples as an indicator for the miRNA–mRNA target relationship, we used five previously reported bladder cancer risk genes to obtain a list of 204 potential targeting miRNAs by applying several state-of-the-art target prediction algorithms. We then used this list of miRNAs to identify other potential targeted pairs (genes), which could be bladder cancer risk candidate genes, and perform GO functional analysis on these genes. Fewer pairs with negative

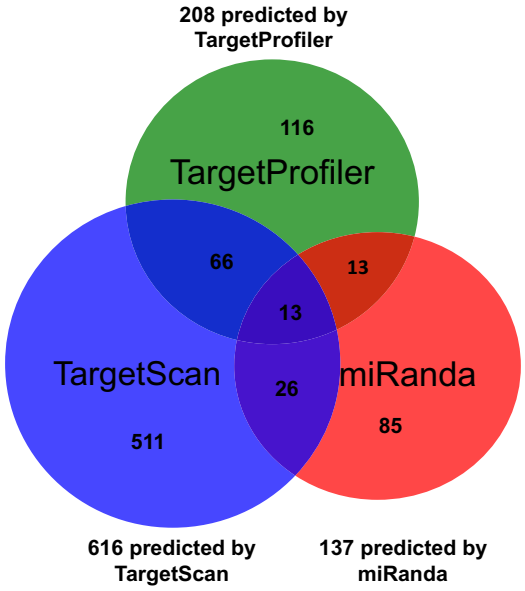

Figure 3 Venn diagram of miRNA–mRNA pairs of BLCA dataset predicted by difference databases

Correlation was calculated for all possible pair combinations of 204 miRNAs (targeting the initial five genes) and 20,501 mRNAs of the BLCA dataset. Targeting relationship was predicted with the criteria: (1) opposite correlation between the tumor and normal samples, (2) prediction by at least one database of TargetProfiler, TargetScan, and miRanda, and (3) false discovery rate < 0.1.

correlation were reported in tumor samples than in normal samples, suggesting that these miRNAs possibly lose their functions in tumor samples, under the assumption that miRNAs often anti-correlate with their gene targets.

Target prediction tools employed in our study for predicting miRNA targets likely contain false positives since the intersection of the predictions by Targetprofiler, TargetScan, and miRanda are low (Figure 3). In our effort, to identify more targets, further analysis with at least one prediction selection criteria was performed.

Conclusion

We have developed a web-based tool, *MMiRNA-Tar,* to plot the correlation relationships and to report target prediction outcomes between miRNAs and mRNAs across multiple samples and time course data. We used the complete TCGA BLCA dataset currently available to test the tool and identified 204 potential targeting miRNAs and many additional targeted genes by 79 selected miRNAs. We believe our tool is the first to utilize miRNA and mRNA correlation plotting combined with multiple target prediction tools for the analysis of miRNA contributions to transcription regulation in cancer. Although the current work was limited to BLCA, the tool developed in this study should also be valuable for studies of functional miRNAs for other cancer datasets as well. The future work will be extended to enhance our web-based tool by incorporating the functionality of matching seed regions of miRNA to the mRNA targets. We would also like to incorporate other

Table 2 Predicted target genes along with their associated GO terms enriched

Gene	GO ID	Biological process
SHPRH, RSF1, MLL, NAP1L1, WRN, MLH3, SIRT1, TAF5L, HUWE1, BRPF3, SUPT16H, PHF21A, KDM3B, PARP1, USP16, MYSM1, RERE, EP400, APC	0051276	Chromosome organization
TAF5L, HUWE1, BRPF3, USP16, SIRT1, MYSM1, EP400	0016570	Histone modification
TAF5L, MLL, RSF1, HUWE1, BRPF3, PHF21A, KDM3B, USP16, SIRT1, MYSM1, EP400, RERE	0016568	Chromatin modification
TAF5L, HUWE1, BRPF3, USP16, SIRT1, MYSM1, EP400	0016569	Covalent chromatin modification
SHPRH, RSF1, MLL, NAP1L1, SIRT1, TAF5L, BRPF3, HUWE1, SUPT16H, PHF21A, KDM3B, USP16, RERE, EP400, MYSM1	0006325	Chromatin organization
UHRF2, ZAK, SMAD3, PPP1CB, PTPN11, APC	0051726	Regulation of cell cycle
BCAT1, TAF1, MLL, ZAK, SMAD3, MLH3, PPP1CB, TACC1, JMY, CUL5, PSMC6, UHRF2, HSPA2, CASP8AP2, MAPK4, PTP4A1, TUBE1, TNKS, MAPRE2, MAPRE1, USP16, DST, APC	0007049	Cell cycle
BCAT1, TAF1, ZAK, SMAD3, MLH3, PPP1CB, JMY, CUL5, PSMC6, HSPA2, TUBE1, MAPRE2, TNKS, MAPRE1, USP16, DST, APC	0022402	Cell cycle process
BCAT1, CUL5, PPP1CB	0000082	G1/S transition of mitotic cell cycle
BCAT1, TAF1, CUL5, PPP1CB	0051329	Interphase of mitotic cell cycle
BCAT1, TAF1, CUL5, PPP1CB	0051325	Interphase
BCAT1, TAF1, PSMC6, CUL5, TNKS, MAPRE2, MAPRE1, USP16, PPP1CB, APC	0000278	Mitotic cell cycle
BCAT1, TAF1, CUL5, HSPA2, TNKS, MAPRE2, MAPRE1, MLH3, USP16, PPP1CB, APC	0022403	Cell cycle phase
TNIK, ZAK, MAPK8IP1	0031098	Stress-activated protein kinase signaling pathway
PHIP, UTP11L, EPHA7, GRB10, BAIAP2, SOCS7, RAF1, SOCS5, PTPN11	0007169	Transmembrane receptor protein tyrosine kinase signaling pathway
TWSG1, SMAD9, ID1, SMAD5, SMAD3	0007178	Transmembrane receptor protein serine/threonine kinase signaling pathway

available TCGA cancer datasets and identify interesting signatures of miRNA–mRNA pairs for other datasets as well. We also plan to develop a visualization tool to present the relationships between miRNAs and mRNAs for comparing tumor and normal expression data sets.

Authors' contributions

YL designed the web interface and deployed the software on the server. SB wrote C code to perform pairwise correlation calculation and target database prediction. HJ wrote R code for statistical filtering and offered statistical advice. GS was involved in interpreting findings and critically reviewing this manuscript for content and accuracy. YB designed and supervised the project, performed the analysis, provided biological interpretation, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gpb.2015.05.003.

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