LEF1 Targeting EMT in Prostate Cancer Invasion is Regulated by miR-34a

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# Equal contribution

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Running title: LEF1 in PCa EMT by miR-34a

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Abstract

The microRNA-34a (miR-34a), a tumor suppressive microRNA (miRNA), is implicated in epithelial-mesenchymal transition (EMT) and cancer stem cells. Lymphoid enhancer-binding factor-1 (LEF1) is a key transcription factor in the Wnt signaling pathway, and has been suggested to be involved in regulation of cell proliferation and invasion. Here, the molecular mechanism of miR-34a and LEF1 in cooperatively regulating prostate cancer (PCa) cell invasion is described. Molecular profiling analysis of miRNA levels in PCa cells revealed a negative correlation between miR-34a and LEF1 expression, and the downregulation of LEF1 by miR-34a was confirmed by luciferase assays. Further, miR-34a specifically repressed LEF1 expression through direct binding to its 3'-untranslated (3’-UTR) regions. miR-34a modulated the levels of LEF1 to regulate EMT in PCa cells. Functionally, miR-34a negatively correlated with the migration and invasion of PCa cells through LEF1. An analysis of miR-34a expression levels in matched human tumor and benign tissues demonstrated consistent and statistically significant downregulation of miR-34a in primary PCa specimens. These data strongly suggest that miR-34a/LEF1 regulation of EMT plays an important role in PCa migration and invasion.

Implications: The miR-34a/LEF1 axis represents a potential molecular target for novel therapeutic strategies in PCa.
Introduction

Prostate cancer (PCa) is the most common malignancy among men in the United States (US) and one of the leading causes of cancer death (1). Early-stage PCa can be treated with prostatectomy or radiation therapy, whereas there are no effective treatments for metastatic or recurrent tumors (2, 3). It has been estimated that 233,000 new prostate cancer cases will be diagnosed and approximately 29,480 men will die from PCa in the US in 2014 (4).

Epithelial-mesenchymal transition (EMT) is essential during cell differentiation and embryo development (5). In cancer, accumulating evidence indicates that EMT modulates malignant characteristics of cancer cells such as inter-cellular adhesion, migration potential and invasive capacity (6, 7). In PCa, Twist1 and other transcriptional factors of EMT such as Slug and Snail promote invasive properties via different upstream regulation pathways.

Lymphoid enhancer-binding factor-1 (LEF1) also modulates EMT through WNT signaling pathway or interaction with other EMT transcriptional factors such as Twist1, Slug and Snail (8-12). LEF1 is one element of the LEF1/T-cell factor (TCF) family, and mediates a nuclear response to the canonical Wnt signaling pathway by interacting with β-catenin. In the nucleus, LEF1/TCF protein directly interacts with β-catenin to target downstream genes, such as Cyclin D1, c-myc and MMP 7, involved in cell cycle, cell proliferation and metastasis (13-15). Our previous papers demonstrated that LEF1 positively regulates Androgen Receptor (AR) expression, PCa growth, and invasion (16, 17). Our studies on the role of LEF1 in PCa are in line with recent reports that LEF1 mediated signaling is critical for PCa progression (18, 19). It the present study, we aimed to determine how LEF1 expression is regulated in PCa cells.

MicroRNAs (miRNAs) are a type of short, noncoding single-stranded RNA, consisting of 22-25 nucleotides in length. Mature miRNAs negatively regulate gene expression at post-
transcriptional level by binding to the 3’ untranslated regions (UTRs) of target messenger RNAs (mRNAs), and exerting their function as oncogenes or tumor suppressor genes during tumor development and progression (20, 21). miR-34a is located on the region of chromosome 1p and is commonly mutated or deleted in many tumor types. Ectopic expression of miR-34a induces apoptosis, senescence and cell cycle arrest (22-24). More recently, Siemens et al. reported that miR-34a promoter methylation predicts distant metastasis of colon cancer (25). As tumor suppressor microRNA, miR-34a is a cell-fate determinant in early-stage dividing colon cancer stem cells (26). Through its interaction with the genome guardian p53, miR-34 exerts profound actions in suppressing human cancers. Several studies have implicated that p53 suppresses canonical Wnt and the Snail-mediated EMT program through the transactivation of the miR-34 family (27-29). In PCa, miR-34 suppresses prostate cancer metastasis by directly targeting prostate cancer stem cells (30, 31) and plays an important role in AR-dependent, p53-mediated apoptosis (32). The functional overlap between miR-34 and LEF1 in regulating PCa, especially in its initiation and metastasis, prompted us to study the relation of these two important regulators in PCa. Relevant to this study, recent reports show that miR-34a downregulates LEF1 to exert an anti-oncogenic miRNA function in lung, colon and breast cancer cell lines (29, 33, 34).

In this study, we found a negative correlation between miR-34a and LEF1 expression in various PCa cell lines and clinical PCa samples. In addition, we demonstrated that miR-34a regulated PCa cell EMT through direct binding to LEF1 mRNA 3’ UTR region and silencing its translation. Our data highlight the miR-34a/LEF1 axis as a potential molecular target for the development of novel therapeutic strategies in PCa.
Materials and methods

Cell culture, migration and matrigel invasion assays
LNCaP, LNCaP-LEF1, C4-2B and DU145 cells were maintained in RPMI 1640 (Gibco) and PC-3 cells were cultured in 50% RPMI 1640 and 50% F2 Gibco) with 10% heat-inactivated FBS, 1% penicillin and streptomycin (PS). The androgen-independent LNCaP-AI and LNCaP-AILEF1shRNA cells were maintained in RPMI 1640 medium containing 10% charcoal-stripped, heat-inactivated FBS and 1% PS (CSFBS; Hyclone Laboratories, Inc.). RC165 and RC170 were maintained in DMEM with 10% heat-inactivated FBS, 1% PS. BD matrigel Chamber Assay and migration assay were performed as previously described (35).

miRNA array and miRNA quantification by qPCR
The four cell lines, LNCaP, LNCaP-LEF1, LNCaP-AI and LNCaP-AILEF1shRNA, were used for miRNA array analysis. The HTG Molecular qDiscovery miRNA Whole Transcriptome Array (WTA, including 687 human miRNAs) was used to compare the expression profiles. miRNA hybridization and scanning were performed by HTG (34). Cell lysis and microRNA profiling was conducted and analyzed by High Throughput Genomics, Inc (www.htgenomics.com) using the HTG platform (miRNA on the qNPA ArrayPlate) with a total of 770 microRNAs.

Total RNA was extracted with mirVana miRNA Isolation kit (AM1560, Ambion). Taqman MicroRNA Reverse Transcription or RetroScript kit was used for cDNA synthesis with isolated RNA by following the manufacturer’s instructions. PCR was performed using the TaqMan Universal PCR Master Mix or Fast SYBR Green Master Mix and BioRad CFX96 machine. The endogenous reference gene RNU6B (MS00014000) or GAPDH was used for RNA quantification. The PCR primers used were: 5’-GTCTCCTCTGACTTCAACAGCG-3’ and 5’-ACCACCCTGTTGCTGTAGCCAA-3’ (GAPDH); 5’-CTACCCATCCTCAGCTCATGTCAGTC-3’
and 5’-GGATGTTCCTGTTTGACCTGAGG-3’ (LEF1). All miRNA TaqMan primers were purchased from Ambion.

**Western blot analysis**

50 μg whole-cell extract was subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. Immunoblots were blocked for 30 min and then incubated with primary antibodies (LEF1, 1:200; E-cadherin, 1:1,000; N-cadherin, 1:1,000; anti-GAPDH, 1:5,000; CD44, 1:1,000; Beta-integrin, 1:1,000; Snail1, 1:1,000) for 2 h at room temperature and incubated for 1.5 h with the horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) at 1:5,000 dilution in 5% nonfat dry milk. The protein bands were detected by an enhanced chemiluminescence kit (Amersham Biosciences).

**Luciferase assays**

The pEZX-MT05 carrying miRNA binding sequence of 3’ untranslated region (UTR) of LEF1 gene or control sequence (Genecopoeia) was co-transfected with 30 nmol/L has-miR-34a using Lipofectamine 2000 (Invitrogen). After 24 hours, Gaussian luciferase and alkaline phosphatase activities were measured from culture medium using the secreted pair dual luminescence kit followed manufacturer’s instructions (Genecopoeia). Gaussian luciferase activity was normalized to alkaline phosphatase activity.

**miR-34a expression in human PCa**

All specimens were acquired under an Institutional Review Board (IRB) approved protocol at the Johns Hopkins Hospital. RNA samples from matched tumor and benign tissues were obtained from 30 radical prostatectomy specimens using the standard operating procedure (SOP) protocols for the Prostate Cancer Biorepository Network (PCBN) as previously described in detail (36). The grade and stage of each case are listed in Supplemental Table 1. Each case
consisted of fresh frozen tumor and benign tissues obtained at radical prostatectomy. For RNA isolation, tissues containing cancer were macrodissected such that they contained at least 70-90% tumor cells. Taqman assays for miR-34a were performed as described above using the endogenous reference gene RNU6B.

**Statistical analysis**

The data was shown as the mean ± SEM. An unpaired two-tailed Student's t test was used for comparisons, with P<0.05 considered to be significant. * denotes P<0.05, ** denotes P<0.01, *** denotes P<0.001. The linear correlation test was used for LEF1 and miR-34a.
Results

Negative correlation between miR-34a and LEF1 in PCa cells

We previously reported that LEF1 regulates cancer growth and invasion in androgen-independent prostate cancer (16). Two stable cell lines LNCaP-LEF1 (stably overexpressing LEF1 in LNCaP cells) and LNCaP-AI-LEF1shRNA (stably silencing LEF1 by shRNA in LNCaP-AI cells) have been established with retrovirus system. To identify miRNAs associated with LEF1 expression, we performed miRNA microarray analysis in LNCaP, LNCaP-LEF1, LNCaP-AI and LNCaP-AI-LEF1shRNA cells. The paired comparison of miRNA expression of LNCaP-AI/LNCaP (left column) and LNCaP-AI/LNCaP-AI-LEF1shRNA by a clustering analysis revealed distinct patterns of miRNAs potentially associated with LEF1 expression (Fig. 1A). Of the tested 687 miRNAs when comparing LNCaP-AI to LNCaP, 8 miRNAs showed changes of at least 2-fold, we confirmed some of miRNAs in Table 1 by RT-PCR (Supplemental Fig. 1), suggesting that these miRNAs were associated with LEF1 expression. Among them, miR-34a was decreased 6.97-fold in LNCaP-AI cells (Table 1), indicating that miR-34a may negatively correlate with LEF1 expression. To further evaluate the relationship between miR-34a and LEF1, we analyzed the levels of miR-34a and LEF1 expression in 7 prostate cell lines including 2 immortalized benign prostate cell lines (RC165 and RC170) and 5 PCa cell lines (LNCaP, LNCaP-AI, C4-2B, PC-3 and DU145) by quantitative RT-PCR (Fig. 1B, C). LEF1 was expressed at high level in LNCaP-AI cells, moderate level in C4-2B cells and low level in LNCaP cells. In contrast to LEF1 expression, miR-34a is expressed at highest level in LNCaP cells. Analysis of the expression data confirmed a negative correlation between miR-34a and LEF1 expression in the 7 cell lines with $r = -0.6349$ (Fig. 1D).
Interestingly, our data (Table 1) indicates that there may be a reciprocal regulation of miR-34a expression by LEF1. We performed additional studies to examine the effect of LEF1 on miR-34a in LNCaP, LNCaP-LEF1, LNCaP-AI and LNCaP-AI-LEF1shRNA cells by qRT-PCR. The results suggest that LEF1 does negatively regulate miR-34a expression (Supplemental Fig. 4).

**miR-34a negatively regulates LEF1 via targeting 3’ UTR**

To investigate if miR34a directly regulates LEF1 expression, miRNA target gene prediction softwares (TargetScanHuman and miRWalk) were applied to predict the potential miR-34a binding site on LEF1 3’UTR. The search revealed a miR-34a potential binding site within the LEF1 3’-UTR (Supplemental Fig. 2). To determine the dynamics of miR-34a in regulation of LEF1, miR-34a mimic was transfected to LNCaP-AI and LNCaP-LEF1 cells followed by western blot to detect LEF1 protein level. As shown in Fig. 2A, successful transfection of miR-34a mimic in both LNCaP-AI and LNCaP-LEF1 cells was confirmed. The transfection of miR-34a mimic lead to approximately a 2-fold decrease of LEF1 expression in LNCaP-AI cells, suggesting that miR-34a mimics may exert regulation on 3’-UTR of LEF1 (Fig.2B). Meanwhile, LEF1 expression in LNCaP-LEF1 cells was not affected by transfection of miR-34a mimic due to lack of LEF1 3’-UTR in the plasmid used to stably overexpress LEF1 (Fig.2B). Consistently, miR-34a inhibited LEF1 expression with co-transfection of miR-34a and a plasmid of LEF1 containing 3’-UTR in LNCaP-AI cells (Fig. 2C).

To further confirm that miR-34a negatively regulates LEF1 expression, we performed luciferase assay with a reporter (HMI) containing 3’-UTR of LEF1 gene (Supplemental Fig. 3). The luciferase activity of the construct (HMI) was significantly decreased in a dosage-dependent
manner by co-transfection of miR-34a mimic in a dosage-dependent manner (Fig. 2D). On the contrary, miR-34a transfection had no effect on luciferase activity in control plasmid (CMI) lacking the LEF1 3’UTR fusion (Fig. 2D).

To confirm miR-34a negatively regulates LEF1 at the protein level, we examined LEF1 expression with western blot. Similarly, transfection of miR-34a mimic inhibited LEF1 in LNCaP-AI and C4-2B cells (Fig. 2E, G), and transfection of anti-miR-34a increased LEF1 expression in LNCaP and C4-2B (Fig. 2F, H).

**miR-34a-LEF1-EMT in PCa cells**

Several reports have indicated that LEF1 induces EMT in PCa cells (18, 37, 38). Here we sought to determine whether miR-34a, via LEF1, regulates EMT, migration and invasion of PCa cells. Three PCa cell lines, LNCaP-AI (high LEF1 and low miR-34a), LNCaP (low LEF1 and high miR-34a) and C4-2B (moderate LEF1 and low miR-34a), were used to examine EMT markers after transfection with either miR-34a mimic or anti-miR-34a. Increased E-cadherin and decreased N-cadherin were observed upon miR-34a overexpression in LNCaP-AI and C4-2B cell lines (Fig. 2E, G). Reciprocally, decreased E-cadherin and increased N-cadherin were observed with anti-miR-34a transfection in LNCaP and C4-2B (Fig. 2F, H). These data show miR-34a mediated suppression of LEF1 as well as EMT markers in PCa cells.

To investigate if miR-34a regulates EMT associated migration and invasion, we performed BD transwell migration and matrigel invasion assays. As shown in Fig. 3A and B, migration and invasion was significantly decreased more than 3 fold upon transfection of miR-34a in a dosage-dependent manner in LNCaP-AI cells. On the contrary, migration and invasion were enhanced with anti-miR-34a treatment in LNCaP cells (Fig. 3C, D). In C4-2B cells,
transfection of miR-34a decreased while anti-miR-34a treatment increased the migration and invasion (Fig. 3E, F, G, and H). Taken together, the data supported that miR-34a is involved in regulation of migration and invasion in PCa cells.

**Decreased miR-34a expression in human PCa**

To determine whether miR-34a is downregulated in human primary prostate cancer, we obtained a series of RNA samples from macrodissected fresh-frozen cancer and matched benign tissues from 30 radical prostatectomy specimens. The samples represented a range of Gleason grades and pathological stages (Supplemental Table 1). As shown in Fig. 4, an overall trend for downregulation of miR-34a was observed in primary PCas relative to matched benign tissues, in 20 of 30 cases. The decrease is statistically significant with p value as 0.013 using one side t test. These data confirm our *in vitro* data that miR-34a is dysregulated in association with human PCa and would support a putative function of miR-34a as a tumor suppressor in PCa.
Discussion

LEF1 is a transcription factor in the canonical Wnt pathway that activates downstream transcriptional programs via interaction with β-catenin. The importance of LEF1 in cancer invasion and metastasis is supported by the report that the level of LEF1 is associated with lymph node and distant metastases in colon cancers (39). A recent study revealed that LEF1 promotes EMT associated invasion in PCa, however it did not investigate the mechanism regulated, in particular, by miRNAs (18). In this study, we sought to identify whether LEF1 promotion of tumor invasion and metastasis is mediated through its regulation by miRNAs in PCa. Using miRNA microarray analysis, we discovered a subset of miRNAs that are significantly dysregulated in associated with LEF1 in PCa cells. Among these miRNAs, decreased miR-34a association with high LEF1 expression in PCa were selected for further investigation as it showed the most significant difference.

We confirmed the direct negative regulatory mechanism between miR-34a and LEF1 by several experiments. First, LEF1 expression is decreased with transfection of miR-34a mimic in LNCaP-AI cells, which have low levels of miR-34a and high levels of LEF1. The direct regulation was confirmed by luciferase assays with luciferase reporter genes bearing LEF1 3’UTR region miR34a binding sites. Conversely miR-34a knockdown in LNCaP cells can increase the levels of LEF1 and was also observed in C4-2B cells. The results of these experiments indicate that miR-34a directly targets and inhibits LEF1 expression. Taken together, these findings provide a detailed molecular link between miR-34a/LEF1/EMT in PCa, similar to previous studies that showed a link between miR-34a and LEF1 in other cancers (33, 40).

miR-34a functions as a tumor suppressor and plays a very important role in many human cancers (24). Consistently, our data here show miR-34a is reduced in prostate cancer compared
to adjacent benign prostate. It will be of great importance to further assess the relationship between miR-34a and LEF1, as well as EMT markers such as E-cadherin and N-cadherin on a larger patient cohort with adequate follow up in future studies to determine the potential clinical utility of miR-34a as a PCa prognostic biomarker.

The reduced levels of miR-34a in the prostate cancer can be regulated by epigenetics (40). miR-34a is closely related with Wnt signaling through specific targeting of a panel of Wnt target genes, including WNT1, WNT3, LRP6, AXIN2, β-catenin, SNAIL and LEF1, resulting in repression of TCF/LEF transcriptional activity and the EMT program (27). Our study reveals miR-34a regulation of EMT, migration, and invasion in PCa is modulated through LEF1, as the decrease of EMT marker, migration and invasion by miR34a was rescued by LEF1 overexpression. Other molecules besides LEF1 may also contribute to the miR-34a regulation of tumor cell EMT. Silencing of miR-34a leads to upregulation of c-Met, SNAIL, and β-catenin expression in liver metastases of colon cancer (25). Interestingly, AR as a downstream effector of LEF1 (16) is also involved in the process of EMT (41). Androgen has a capacity to enhance prostate cancer cell migration and invasion. It has been reported that only a low level of AR is required for androgen induced EMT phenotype. In another study, high expression level of β-catenin is observed in human PCa tissue samples accompanied with low AR expression. These findings demonstrate that only low AR expression can lead to androgen induced EMT alteration (42).

In squamous (43) and colorectal cancer cells (44), repression of miR-34a was required for IL-6-induced cell differentiation, EMT and invasion. Collectively, these results strongly support the potential of miR-34a as a therapeutic target for tumors. Notably, miR-34a may enhance EMT phenotype via increased EMT proteins in different types of tumor cells}

A recent study has shown
miR-34a could regulate EMT via targeting SNAIL, in breast and lung cancer cell lines (29). Our data did not show that miR-34a regulates EMT SNAIL pathway in PCa as previously reported (29). Nevertheless, the importance of miR-34a in inhibition of EMT and presumably, suppression of the early phases of metastasis warrant further exploration focusing on its application in the clinical setting for human cancers. In addition, miR-34a has been suggested as a radiosensitizer and biomarker for cancer prognosis (26, 45-48). Most recently, miR-34a was successfully co-encapsulated with doxorubicin into hyaluronic acid-chitosan nanoparticles and simultaneously delivered into breast cancer cells for improved therapeutic drug effects (49). Synergistic effects on tumor suppression as demonstrated both in in vitro and in vivo systems could be a promising combined therapeutic strategy that can be extended to other types of tumors such as prostate cancer for enhanced anti-tumor therapy. It would be of significant value to demonstrate its anti-tumor effects in preclinical animal models of PCa.

In summary, we report that miR-34a regulates LEF1, subsequently affecting EMT and contributes to PCa cell migration and invasion (Supplemental Fig. 5). Taken together, these data suggest that miRNA-34a and LEF1 may be therapeutic targets to inhibit the initiation, proliferation, EMT and invasion in PCa.
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FIGURE LEGENDS

Figure 1. miR-34a and LEF1 expression level in different prostate cancer cell lines. (A) A clustering analysis between paired miRNA expression comparison of LNCaP-AI/LNCaP and LNCaP-AI/LNCaP-AI-LEF1shRNA, top 9 downregulated are listed. (B and C) LEF1 and miR-34a expression level in five (LNCaP, LNCaP-AI, C4-2B, DU-145 and PC-3) PCa cell lines and two normal prostate cell lines (RC-170, RC-165). (D) The line correlation between miR-34a and LEF1 expression level in the seven cell lines. GAPDH and U6 were used for normalization.

Figure 2. miR-34a restrains EMT progress via targeting LEF1 3′-UTR in prostate cancer. (A) Increased miR-34a expression level after miR-34a mimic transfection. (B and C) miR-34a decreased endogenous LEF1 in LNCaP-AI cells but not in LNCaP-LEF1 cells. (D) Decreased luciferase activity upon transfection of miR-34a in dosage-dependent manner with the luciferase reporter containing LEF1 3′UTR. (E, F, G, and H) miR-34a regulated the LEF1 and EMT markers by western blotting in PCa cells.

Figure 3. miR-34a negatively regulates migration and invasion of PCa through LEF1. (A and B) miR-34a inhibited migration and invasion in LNCaP-AI cells. (C and D) Anti-miR-34a treatment increased migration and invasion in LNCaP in a dosage-dependent manner. (E and F) Transfection of miR-34a decreased migration and invasion in C4-2B cells. (G and H) Anti- miR-34a treatment increased migration and invasion in C4-2B cells.

Figure 4. miR-34a relative expression level in prostate cancer tissue compared to matched benign tissue. miR-34a expression levels were normalized to U6 and then tumor was compared to benign using the 2^−ΔΔCT method.
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1. LNCaP-AI/LNCaP
2. LNCaP-AI/LNCaP-AILEF1shRNA

miR-34a expression normalized with U6

ΔCt Value miR-34a/U6

ΔCt Value LEF1/GAPDH

r=-0.6349
P=0.0908
n=7
Figure 2

A. miR-34a relative expression

B. LEF1 relative expression

C. LEF1 relative expression

D. Luciferase activity

E. Western blot analysis of LNCaP-AI and LNCaP-LEF1

F. Western blot analysis of LNCaP-AI and LNCaP-LEF1

G. Western blot analysis of C4-2B

H. Western blot analysis of C4-2B

MiR34a mimic (nM)
Figure 3

A
LNCaP-AI

Migration
NC | miR-34a

Invasion
NC | miR-34a

B
LNCaP-AI

% migration
NC | 10nM | 60nM | 120nM

% invasion
NC | 10nM | 60nM | 120nM

p=0.07 | p=0.08 | p=0.002

p=0.003 | p=0.001 | p=0.002

C
LNCaP

Migration
NC | anti-miR-34a

Invasion
NC | anti-miR-34a

D
LNCaP

% migration
NC | 10nM | 60nM | 120nM

% invasion
NC | 10nM | 60nM | 120nM

p=0.03 | p=0.06 | p=0.005

p=0.08 | p=0.004 | p=0.001
Figure 3 (continued)

E

Migration

C4-2B

NC

miR-34a

Invasion

NC

miR-34a

F

C4-2B

% migration

% invasion

NC

miR-34a

NC

miR-34a

p=0.03

p=0.01

p<0.01

p=0.02

p=0.01

p<0.01

G

Migration

C4-2B

NC

anti-miR-34a

Invasion

NC

anti-miR-34a

H

C4-2B

% migration

% invasion

NC

anti-miR-34a

NC

anti-miR-34a

p=0.01

p<0.001

p<0.001

p=0.01

p<0.001

p=0.07

p=0.05

p<0.001

p=0.007

p<0.001

p=0.007

p<0.001

p=0.007

p<0.001
Figure 4: miR-34a fold difference (tumor relative to benign)
### Table 1

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Molecular Cancer Research

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Jiaqian Liang, Yirong Li, Garrett Daniels, et al.

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