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# MicroRNA-33a levels do not correlate with the expression of its host gene SREBF2 and its isoforms in prostate cancer cell lines

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## ABSTRACT

***Objective.*** Prostate cancer is currently the most frequently diagnosed malignant neoplasm and the second leading cause of cancer related mortality in men over the age of 50 years in the developed countries. MicroRNA-33a (miR-33a), localized within the intron 16 of SREBF2, has been reported to have tumor suppressive properties in some cancers including prostate cancer, whereas its host gene, SREBF2, has been shown to be elevated in prostate cancer and to act as an oncogene. Due to the paradoxical expression of an oncogene and a tumor suppressor from a single genetic locus, there is a need for evaluation of miR-33a and SREBF2 expression status in prostate cancer cells to help understanding their roles in prostate carcinogenesis. ***Methods.*** In this study, we aimed at investigating the link between the expressions of miR-33a and its host gene SREBF2 and its isoforms in prostate cancer cell lines using quantitative real time PCR. We evaluated the relative expression levels with using $2^{-\Delta\Delta CT}$ method and tested the correlations of microRNA and gene expressions with Pearson's Correlation test using GraphPad Prism 6. ***Results.*** Our results demonstrated variable expression levels for SREBF2 mRNA and miR-33a expression levels in prostate cancer cell lines, with some decreased, some increased and some unchanged. Further analysis showed a strong correlation among expressions of SREBF2 isoforms though we could not find a significant association between levels of SREBF2 isoforms and miR-33a expression. ***Conclusion.*** This data suggest possible posttranscriptional regulation of miR-33a expression in prostate cancer.

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***Keywords:*** Prostate cancer, miR-33a, SREBF2, correlation

## Introduction

Prostate cancer (PCa) is the most commonly diagnosed non-skin cancer and the second leading cause of cancer deaths in males over the age of 50 years in the developed countries [1]. Emerging evidences suggest a high-cholesterol Western diet as an importantrisk factor for several solid tumors

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including PCa [2]. Although contradictory findings are present about the link between serum cholesterol levels and PCa risk, accumulating data supports a critical role for HDL, LDL and, total cholesterol in PCa development and progression [3-6]. In line with those studies, prostate tumor cells have been postulated to acquire castration-resistance via reactivating intrinsic androgen biosynthesis pathway, which might be through acquisition ofthe ability to synthesize androgens from its precursor, cholesterol [7].

The sterol regulatory element-binding protein (SREBP) transcription factors, SREBP1 and SREBF2, are among the crucial modulators of cholesterol/lipid homeostasis, and of those, SREBF2 upregulate genes associated with cholesterol synthesis (e.g. HMGCR) and cholesterol uptake (e.g. LDLR) [8]. In addition, microRNA-33a (miR-33a), localized within the intron 16 of the SREBF2 gene (Figure 1), which encodes SREBF2 protein, has been also reported to play important roles in cholesterol synthesis and uptake through targeting 3' untranslated regions of ABCA1, a cholesterol efflux protein, and several other mRNAs for proteins implicated in β-oxidation of fatty acids including CPT1A and HADHB [9].

Paradoxically, increase in SREBF2 but decrease in miR-33a level lead to increased cholesterol synthesis and uptake, although their expressions are controlled by the same promoter. Furthermore, SREBP2 has been reported to be increased in PCa and our recent findings imply tumor suppressive activities for miR-33a with decreased expression in PCa tissues [10]. Therefore, a comprehensive evaluation of this paradoxical expression pattern of miR-33a and SREBF2 in prostate cancer cells is necessary to help understanding their roles in prostate carcinogenesis.
In this study, we aimed at investigating the link between the expressions of miR-33a and its host gene SREBF2 and its isoforms in PCa cell lines.

## Methods

*Cell Culture*

Immortalized non-tumorigenic prostate cell line PNT1a cells, LNCaP, DU145, 22RV1 and PC3 cells were grown using RPMI medium (GenDepot) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Invitrogen). VCaP cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were cultured at 37°C in a humidified and 5% CO2 incubator. Cell lines were obtained from American Type Culture Collection and routinely authenticated by STR analysis at MD Anderson Cancer Center Characterized Cell Line Core Facility.

*cDNA Synthesis and Quantitative Real-Time PCR*

For microRNA first strand DNA (cDNA) synthesis, equal amounts of total RNA were reverse transcribed using microRNA specific primers (Applied Biosystems) and TaqMan MicroRNA reverse transcription Kit (Applied Biosystems) following the manufacturer's instructions. cDNA synthesis from mRNA was carried out with "amfiRivert cDNA Synthesis Platinum Master Mix" (GenDepot) following the manufacturer's protocol.

For microRNA expression analysis, microRNA specific probes (Applied Biosystems) and TaqMan Fast Advanced Master Mix (Applied Biosystems) were used. MicroRNA expression data were normalized to RNU43. For gene expression analysis, SYBR Green PCR Master Mix (Applied Biosystems) was used. Expression data were normalized to ь-actin. Primer sequences used for quantitative real time PCR (qRT-PCR) are provided in Supplementary Table1.

qRT-PCR was performed in a StepOnePlus™ real-time thermal cycler (Applied Biosystems) using standard parameters. Each experiment was performed

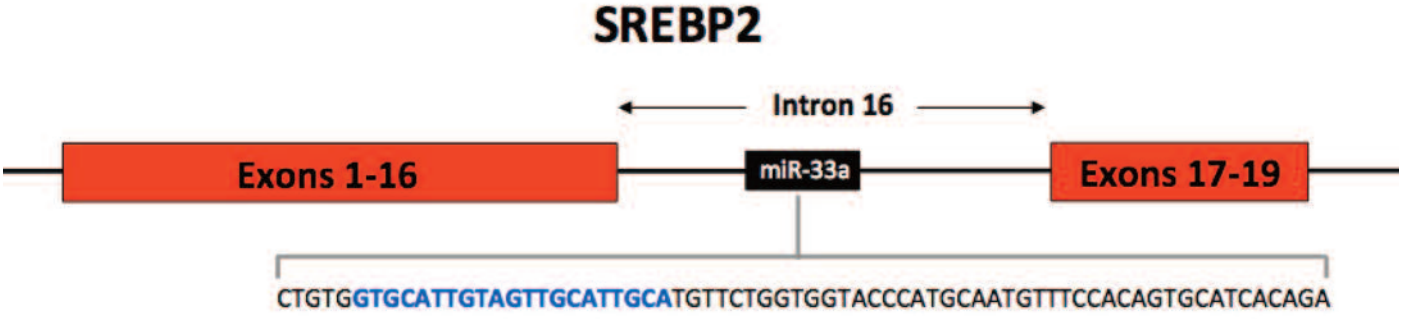


**Figure 1.** Schematic representation of the localization of miR-33a within 16th intron of SREBF2

in triplicates and the differences in expression levels were evaluated using $2^{-\Delta\Delta CT}$ method.

*Statistical Analysis*

Data were plotted as mean ± standard error and statistical significances were evaluatedusing Student's t test. Correlations of microRNA and gene expressions were analyzed with Pearson's Correlation testusing GraphPad Prism 6. A p value of 0.05 or below was accepted as significant.

## Results

*MiR-33a and SREBF2 have variable expression in PCa cell lines*

To evaluate the correlation of miR-33a and SREBF2 expression, we initially measured their levels in PNT1a and PCa cell lines using qRT-PCR. MiR-33a expression was significantly reduced in LNCaP and VCaP cells and was significantly increased in 22RV1 and PC3 cells (Figure 2A). Its expression in DU145 cells was similar to that of PNT1a cells (Figure 2A). We also found variable expression levels for SREBF2 mRNA in PCa cell lines, with some decreased (PC3; Figure 2B), some increased (VCaP, DU145, and 22RV1; Figure 2B) and some unchanged (LNCaP; Figure 2B). However, there was no correlation of SREBF2 and miR-33a levels in the same cancer cell lines (Table 1; p >.1, Pearson).

*Expression of MiR-33a and SREBF2 isoforms do not correlate in PCa cell lines*

We then examined the expression levels of SREBF2 isoforms (See Supplementary Table 1), which include intron 16 in their premature unspliced forms, in PCa cells lines to look for a specific isoform,

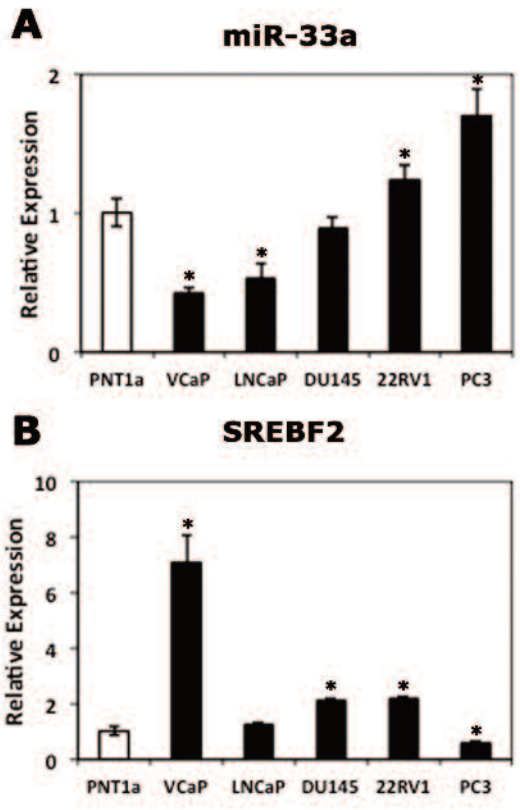


**Figure 2.** Relative expression of (A) miR-33a and (B) SREBF2 in PCa cells. Mean +/- SEM is shown.*$p < 0.05$

whose expression might be correlated with miR-33a expression. These isoforms represented similar expression profiles with total SREBF2 level (compare Figure 2B and Figure 3) and correlation analysis demonstrated that the levels of SREBF2 isoforms strongly related with each other in the same cancer cell lines, although they lack a significant correlation with miR-33a (Table 1).

Furthermore, we searched for the retained introns that include intron 16 and analyzed the expression level of a retained intron in PCa cell lines (See Supplementary Table 1). We designed a primer pair

**Table 1.** Correlation of SREBF2 mRNA and miR-33a levels

| | SREBF2 | SREBF2 001 | SREBF2 002 | SREBF2 005 | SREBF2 001+201 | Retained Intron |
|---|---|---|---|---|---|---|
| miR-33a | R = -0.616<br>*p* = 0.192 | R = -0.662<br>*p* = 0.151 | R = -0.450<br>*p* = 0.369 | R = -0.671<br>*p* = 0.144 | R = -0.617<br>*p* = 0.191 | R = -0.648<br>*p* = 0.163 |
| SREBF2 | | **R = -0.970**<br>***p* = 0.001** | **R = -0.933**<br>***p* = 0.006** | **R = -0.957**<br>***p* = 0.002** | **R = -0.993**<br>***p* = 0.0001** | **R = -0.959**<br>***p* = 0.002** |
| SREBF2 001 | | | **R = -0.950**<br>***p* = 0.003** | **R = -0.997**<br>***p* = 0.0001** | **R = -0.993**<br>***p* = 0.0001** | **R = -0.959**<br>***p* = 0.002** |
| SREBF2 002 | | | | **R = -0.935**<br>***p* = 0.006** | **R = -0.949,**<br>***p* = 0.003** | **R = -0.945**<br>***p* = 0.004** |
| SREBF2 005 | | | | | **R = -0.980**<br>***p* = 0.0005** | **R = -0.999**<br>***p* = 0.0001** |
| SREBF2 001+201 | | | | | | **R = -0.982**<br>***p* = 0.0005** |

targeting intron 1 as control to exclude the possibility of amplifying genomic DNA. qRT-PCR results demonstrated no correlation of the retained introns expression to miR-33a levels (Figure 4).

Our overall results demonstrated a strong correlation among expression of SREBF2 isoforms althoughthere was nolink between expressions of SREBF2 isoforms and miR-33a level (Figure 5), which suggested possible posttranscriptional regulation of miR-33a expression in PCa.

## Discussion

Numerous studies suggest a critical role for cholesterol in PCa development and progression in recent years [3-6]. In advanced prostate tumors, castration-resistance of tumor cells might occur via reactivation of intrinsic androgen biosynthesis pathways, where cholesterol might serve as an important precursor for synthesis of androgens [7].

There is strong evidence that in normal tissues,

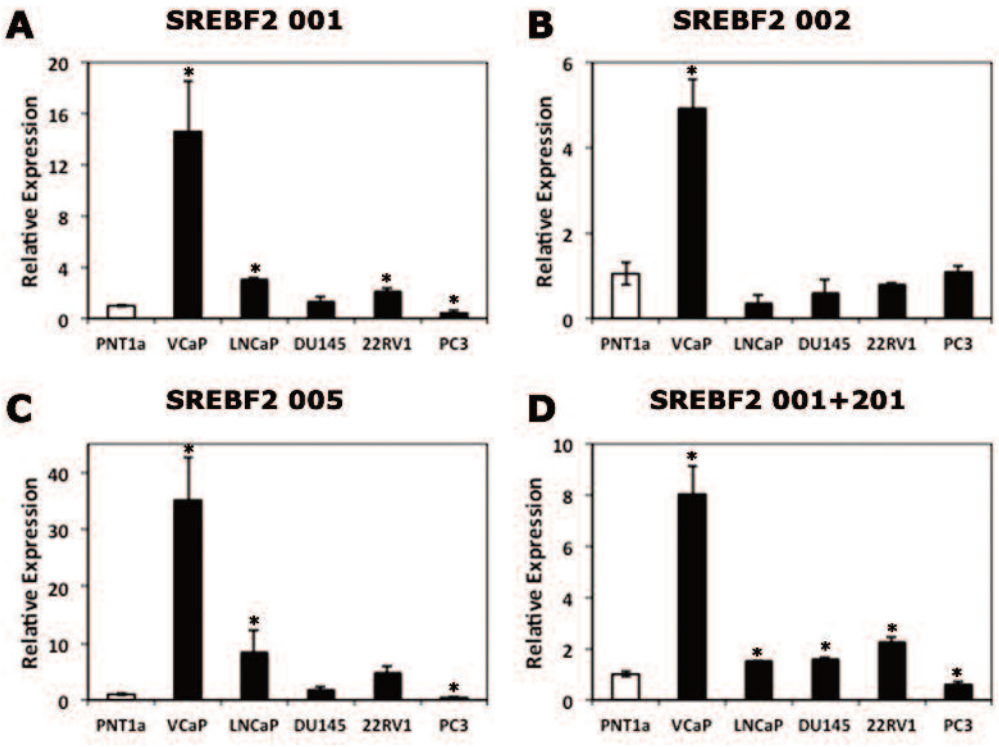


**Figure 3.** Relative expression of (A) SREBF2 001, (B) SREBF2 002, (C) SREBF2 005, and (D) SREBF2 001+201 in PCa cells. Mean +/- SEM is shown. *$p < 0.05$

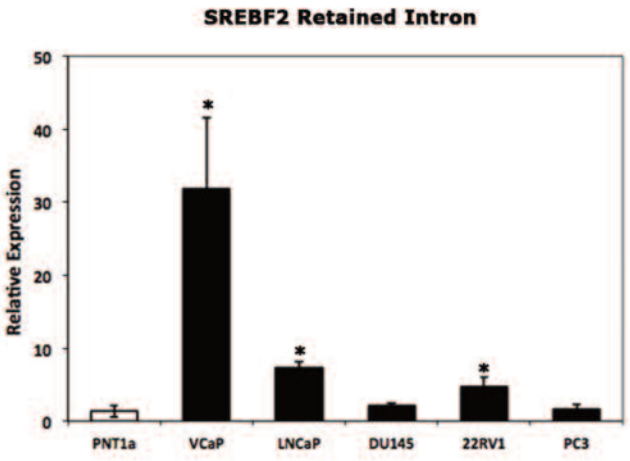


**Figure 4.** Relative expression of SREBF2 retained intron in PCa cells. Mean +/- SEM is shown. *$p < 0.05$

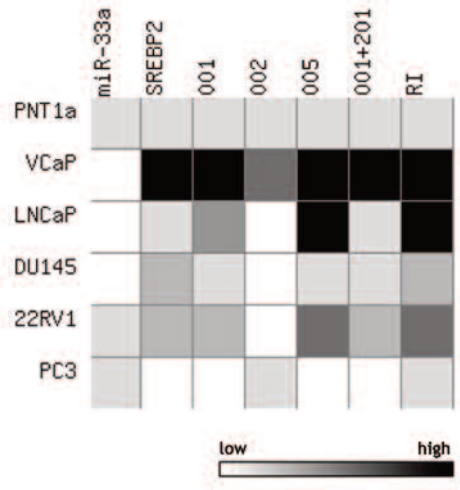


**Figure 5.** Heat-map representation of SREBF2 isoform and miR-33a relative expression levels in PCa cell lines. Mean +/- SEM is shown. *$p < 0.05$

miR-33a levels are elevated in parallel to increased SREBF2 transcription, leading to collaborative regulation of cholesterol and other lipid levels by SREBF2 and miR-33a [11]. In contrast to this finding, miR-33a, localized within the intron 16 of SREBF2, has been reported to have tumor suppressive properties in some cancers including PCa [10, 12-18], whereas its host gene, SREBF2, has recently been shown to be increased in PCa and to act as an oncogene [19]. Due to these paradoxical findings, there is a need for evaluation of miR-33a and SREBF2 expression status in PCa cells to help understanding their roles in prostate carcinogenesis.

Therefore, in this study, we investigated the association between the levels of miR-33a and its host gene SREBF2 and its isoforms in PCa cell lines and found that there is no correlation of SREBF2 isoform mRNA levels with its intronic microRNA miR-33a in PCa unlike the correlation seen in normal tissues.

In normal tissues, SREBF2 increase cholesterol by increasing transcription of multiple genes that increase levels of cholesterol. Elevated levels of SREBF2 in cancer tissues more profoundly induce upregulation of those genes associated with cholesterol synthesis and cholesterol uptake. Increased synthesis and uptake of fatty acids can pave the way for reactivation of intrinsic androgen biosynthesis pathways but also provide an energy source for PCa, which are known to have low glucose uptake. Besides, apart from lipid biogenesis, SREBP-2 was found to induce c-Myc expression via directly interacting with c-Myc promoter region to drive stemness and metastasis [19]. In addition, downregulation of miR-33a allows both upregulation of oncogenic genes such as PIM1 [10] and promotes β-oxidation of fatty acids through overexpression of genes like HADHB and CPT1A [9]. Such increased β-oxidation might contribute to providing of energy to PCa cells. Another potential association of miR-33a to cholesterol metabolism is its targets that are involved in cholesterol transport such as ABCA1, ABCG1, and NPC1 [9]. Several studies showed that upregulation of miR-33a in vitro profoundly suppressed cholesterol export in various cell culture models [20-23]. Further in vivo studies also demonstrated significant elevation in serum HDL cholesterol in miR-33a -/- mice [21]. However, it is worth mentioning that ABCA1, a cholesterol efflux protein, which is targeted by miR-33a, is significantly methylated in PCa [24], which would abolish the potential deleterious effects of elevated cholesterol efflux secondary to reduced miR-33a.

Furthermore, interestingly in 2 of the androgen receptor positive cell lines tested, LNCaP and VCaP cells, miR-33a expressions were lower compared to that of PNT1a. On the other hand, its expression was either unchanged or elevated in androgen receptor negative DU-145 and PC3 cells, implying a possible androgen receptor related mechanism for differential expression of miR-33a in PCa cells.

*The Limitations of the Study*

Our study focuses on the cell lines for the evaluation of miR-33a and SREPF2 isoforms' expression. Lack of the correlation of miR-33a and SREPF2 isoforms' expression in tumor and normal prostate samples obtained from PCa patients is one of the important limitations of our study. In addition, SREBF2 expression in protein was not evaluated in regards to its correlation with miR-33a expression.

## Conclusions

The potential expression of an oncogene and a tumor suppressor from a single genetic locus creates a paradox in PCa. In this study, we show that miR-33a expression is not correlated with SREBF2 mRNA levels, implying post-transcriptional mechanisms of control of miR-33a levels in PCa, leading to decreased miR33a levels. We demonstrated a strong correlation among expressions of SREBF2 isoforms though we could not find a significant correlation between expressions of SREBF2 isoforms and miR-33a expression, which suggested possible post-transcriptional regulation of miR-33a expression in PCa. Further studies should be carried out to better understand the possible mechanisms of differential expression of miR-33a and SREPF2 isoforms in PCa cells although transcribed from a single locus. Also, further in vivo research is needed to clarify the roles of miR-33a and SREBF2 in PCa tumorigenesis process.

*Authorship declaration*

All authors listed meet the authorship criteria according to the latest guidelines of the International Committee of Medical Journal Editors, and all authors are in agreement with the manuscript.

*Conflict of interest*

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

**Supplementary Table 1.** QRT-PCR primer sequences and PIM1 3'UTR cloning and mutagenesis primer sequences

| Primer | Ensembl Transcript ID | Sequence |
|---|---|---|
| **Beta-actin-F** | | 5'-GCCTCGCCTTTGCCGATC-3' |
| **Beta-actin-R** | | 5'-CCCACGATGGAGGGGAAG-3' |
| **SREBF2-F** | | 5'-CAGCCTCAAGTCCAAAGCCT-3' |
| **SREBF2-R** | | 5'-TGTCTTGATGATCTGAGGCTGG-3' |
| **SREBF2-001-F** | ENST00000361204 | 5'-CTCGCCAGAGGAGATTTTGC-3' |
| **SREBF2-001-R** | | 5'-TGGAAGACTTTCTTGAGCAGC-3' |
| **SREBF2-002-F** | ENST00000424354, | 5'-TGTGCGCTCTCATTTTACCA-3' |
| **SREBF2-002-R** | | 5'-CGCAGACATGAATCTCCAAA-3' |
| **SREBF2-005-F** | ENST00000435061 | 5'-GTCCAGGGCTTTCTTGTCAC-3' |
| **SREBF2-005-R** | | 5'-CAGGCTGTGTTCCAGCAG-3' |
| **SREBF2-001 + 201-F** | ENST00000361204 + ENST00000612482 | 5'-TGGAAGTGACAGAGAGCCC-3' |
| **SREBF2-001 + 201-R** | | 5'-GTTGAGGGCAGGGTCAGAG-3' |
| **Retained Intron-F** | ENST00000490262 | 5'-GGCACACAAACAGAGCTGAA-3' |
| **Retained Intron-R** | | 5'-CCTTCAGTCAGGGCAGTCTC-3' |
| **Control Intron-F** | | 5'-GGCGGTCCTCAACCCTTC-3' |
| **Control Intron-R** | | 5'-AGAGCGGACCACGGAAAC-3' |

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