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**TITLE:** Role of microRNA in aggressive prostate cancer

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Role of microRNA in aggressive prostate cancer

The majority of mortality of prostate cancer (PCa) is due to the recurrent metastasized castration resistance PCa. The acquisition of epithelial–to-mesenchymal transition (EMT) in PCa signifies the initial process of cancer metastasis. Our previous findings unveiled that DAB2IP is down-regulated in high-grade PCa specimens and this novel tumor suppressor can block EMT leading to lymph node metastasis. It has recently been associated with the onset of cancer stem cell (CSC) that is considered as cancer initiating cell with a survival advantage during the course of cancer therapy. However, the mechanism of action is not fully characterized. Using microRNA microarray screening, we found microRNA-363 (miR363) is significantly down regulated in several DAB2IP knockdown (KD) prostate cells. In particular, miR363 is predominately expressed in normal prostate and belongs to the miR106a-363 cluster that is closely resembled to the oncogenic miR17-92 cluster in their seed sequence. It appears that DAB2IP significantly regulates the expression of a unique miR-363; the profile of miR-363 expression appears to be highly specific in normal prostate. The objective of this project is to delineate the functional links of miR-363 with the appearance of CSC and its clinical correlation in aggressive PCa.
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INTRODUCTION

DAB2IP (DOC-2/DAB2 interactive protein) was first identified as a new member of RAS-GTPase activating protein (RAS-GAP) family with growth inhibitory activity in PCa. Loss of DAB2IP expression, mainly due to altered epigenetic regulation of its promoter, is often associated with PCa (1-3) as well as other cancer types (4-7). Loss of endogenous DAB2IP also initiated epithelial-to-mesenchymal transition (EMT) (8-10); tumor cells become highly metastatic in various lymph nodes from several orthotopic mouse models (1, 2). However, the underlying mechanism of DAB2IP in inhibiting EMT is not fully understood.

MicroRNAs (miRs) are endogenous non-coding small RNAs. By base pairing between its seed region and the complementary sequence at the 3’UTR of target mRNA, miRs mediate gene silencing by (i) site-specific cleavage, (ii) enhanced mRNA degradation and (iii) translational inhibition. The post-transcriptional regulation of miRs is widely involved in proliferation, differentiation, angiogenesis, and tumorigenesis. In particular, about 50% of miR genes are located at the fragile chromosomal regions and hence susceptible to amplification, deletion and translocation during oncogenesis. MiRs can either function as tumor suppressor or oncogenes. Therefore, unveiling the mechanism of miR-mediated EMT signaling can provide more insights into the fundamental role of miRs contribute to tumorigenesis. Based on miR array screening, DAB2IP appears to regulate the expression of a unique miR363. The function of miR363 is largely unknown. Thus, this project is to determine the functional role of miR363 in prostate cancer (PCa).

BODY

During the second year we have identified the mechanism of action of miR363 in the EMT process and unveil a possible effect of DAB2IP on increasing miR363 levels. The progress for each aim is summarized as follows:

Aim 1. Study the regulation of miR363 gene expression by DAB2IP.

It is known that human miR363 gene is located in chromosome X (X chr 133131074 - 133131148) (11). By combining nucleosome mapping with linker sequences analyses of chromatin signature for promoters, it is predicted the transcriptional initiation site of miR-363 is at the 3’end of this gene (Fig. 1A). We performed genomic PCR using several primer sets to amplify this region into several fragments then cloned them into pGL3 basic luciferase promoter vector. So far, we have three clones: P1 (X chr 133135131-133135805), P2 (X chr 133135123-133135396), P3 (X chr 133134520-133134886). By transfecting these clones into LAPC4-Con (i.e., shControl) and KD cells separately, we observed higher luciferase activity in KD than Con cells (Fig. 1B). In addition, the luciferase activities of P3 were not induced by the increment of DAB2IP expression (Fig. 1C), indicating that DAB2IP-elicited miR363 elevation is not regulated at the gene transcriptional level. We then hypothesized that DAB2IP may modulate miR363 biogenesis, i.e., miR processing from primary, precursor to mature form. Thus,
we determined the levels of primary, precursor and mature form of miR363 in a variety of cells with or without DAB2IP expression. Noticeably, only the levels of mature form of miR363 correlated with DAB2IP expression, suggesting that DAB2IP can facilitate the maturation of miR363 in prostate cell. Currently, we are analyzing the possible interaction of DAB2IP with miR biogenesis machinery.

**Aim 2. Delineate the functional role of miR363 in modulating CSC phenotype**
Recent study indicated that cancer cells undergoing EMT changes also expression stem cell phenotype. DAB2IP-KD cells often express EMT phenotypes (2). Thus, using gene transfection technique, increased expression of miR363 in two different DAB2IP-KD prostate cells (LAPC4 [Fig. 3] and RWPE-1 [Fig. 4]) can reverse EMT phenotypes in these cells based on (a) remarkable elevation of E-cadherin and reduction of vimentin, (b) significant reduction of in vitro migration motility of these cells through Transwell membrane. Also, microarray data indicated that reduced SNAI2/Slug mRNA was found in miR363-expressing cells. Thus, SNAI2/Slug appeared to be a target gene of miR-363, which in turn suppresses the transcriptional activity of E-Cadherin gene expression (Fig. 5). On the other hand, by restoring Slug expression in PCa cells can antagonize the effect of miR363 on EMT phenotypes (Fig. 6). Taken together, miR363 as a downstream effector of DAB2IP appears to be a tumor suppressor miR to suppress EMT process via targeting Slug in PCa.

**Aim 3. Study the correlation of DAB2IP and miR363 in PCa progression.**
We have collected 20 more specimens this year and stored at -135°C freezer; the collection is right on schedule. We expect to start preparing miR from these specimens at the end of Year 2. Currently, we are standardizing miR extraction procedure and qRT-PCR for detection. However, the limitation of qRT-PCR is only detecting overall miR363 from a given sample; this method does not provide information for miR363 in each individual cell. In addition, we develop in situ hybridization (ISH) technique using locked nucleic acid probe for miR363. We first determine miR363 expression level in prostate gland derived from either wild type (DAB2IP+/+) or DAB2IP knockout (DAB2IP−/−) animal because we have known loss of miR363 expression in the prostate of DAB2IP−/− animal using qRT-PCR. Indeed, the data from ISH (Fig. 7) exhibited the similar observation from qRT-PCR.

In order to profile the expression of miR363 in both adjacent benign tissue and cancer lesion, we applied ISH on formalin-fixed paraffin embedded (FFPE) tissue. We first confirmed the specificity of miR363 probe using benign prostatic hyperplasia (BPH) specimens (Fig. 8A and B) because BPH is considered benign tissue detected with DAB2IP expression. Furthermore, the preliminary data demonstrated miR363 (Fig. 8C and D)

**KEY RESEARCH ACCOMPLISHMENTS**
- miR363, within the miR106a-363 cluster, is significantly down-regulated following DAB2IP knockdown in both RWPE1-KD and LAPC4-KD cells.
- miR363 can suppress SNAI2/Slug expression by targeting the 3’UTR of Slug mRNA.
• Slug is the transcriptional suppressor for E-Cadherin gene expression.
• Overexpression of miR363 in DAB2IP-KD cells can restore the epithelial characteristics by enhancing E-cadherin upregulation and suppressing vimentin expression.
• Loss of miR363 expression is associated with prostate cancer development.

REPORTABLE OUTCOMES


CONCLUSION
miR363 is a key effector for the inhibitory effect of DAB2IP on EMT. The mechanism of action of miR363 is to decrease SNAI2/slug mRNA stability that lead to increase E-cadherin mRNA and protein expression level, reduce vimentin at both transcriptional and translational level, and suppress cell motility associated with EMT. In summary, our study indicates that miR-363 is an anti-metastatic microRNA and modulates the EMT-associated signaling mechanisms contribute to PCa metastasis.

REFERENCES
Appendices

Figure 1 Cloning of miR363 promoter and determination of the effect of DAB2IP on miR363 promoter activity. (A) Schematic representation of each clone in the 5’-upstream regulatory region of miR363 gene. (B) Luciferase reporter gene activity of different clones (P1, P2, and P3) of miR363 promoter region in LAPC4-KD and its control (LAPC4-shControl). (c) The effect of DAB2IP on luciferase reporter gene activity of P3 clone. RLU: Relative luciferase unit.

Figure 2 Determination of primary, precursor and mature form of miR363 in cells with or without DAB2IP expression. Both total RNA and enriched small RNA were prepared from each cell and subjected to qRT-PCR. qRT-PCR was employed to determine primary, precursor and mature form of miR363 with different primer sets. U6 was used as an internal control. Each cell line has a pair: DAB2IP knockdown (KD) vs. control (Con) or DAB2IP-expressing cell (D2 or D) vs. control cell (Neo or pcDNA).
Figure 3 miR363 reverse EMT in LAPC4-KD Cells. (A) miR363 level in LAPC4-KD cells transfected with pCMV-miR363 plasmid. (B) E-Cadherin and Vimentin mRNA level in stable clones of LAPC4-KD cells-expressing miR363. (C) E-Cadherin and Vimentin protein expression in LAPC4-KD cells-expressing miR363. (D) Transwell migration assay in LAPC4-Con and LAPC4-KD cells-expressing miR363. Transmigrated LAPC4-KD cells carried Green Fluorescent Protein were observed under microscope and quantified by crystal violet staining. Each bar represents mean ± SEM of three replicated experiments. *P<0.05.

Figure 4 miR363 reverse EMT in RWPE-KD Cells. (A) miR363 level in RWPE-KD cells transfected with pCMV-miR363 plasmid, compared to empty pCMV vector. (B) E-Cadherin and Vimentin mRNA level in RWPE-KD cells-expressing miR363. (C) E-Cadherin and Vimentin protein expression in RWPE-KD cells-expressing miR363. (D) Transwell migration assay in RWPE-Con and RWPE-KD cells-expressing miR363. Transmigrated RWPE-KD cells at the lower chamber were stained and quantified using crystal violet. Each bar represents mean ± SEM of three replicated experiments. * P<0.05.
Figure 5 miR363 targets the 3'UTR of Slug mRNA and suppresses its expression. 
(A) Slug mRNA level in stable clones of LAPC4-KD cells with miR363 overexpression.
(B) Slug protein level in LAPC4-KD cells expressing miR363. (C) Luciferase reporter assay in LAPC4-KD cells co-transfected with siCHECK2-slug-WT 3'UTR or siCHECK2-Slug Mut363 3'UTR and pCMV-miR363 or empty pCMV vector. (D) Slug mRNA level in stable clones of RWPE-KD cells expressing miR363. (E) Slug protein level in RWPE-KD cells with stabilized miR363 overexpression. (F) Luciferase reporter assay in RWPE-KD cells co-transfected with siCHECK2-slug-WT 3'UTR or siCHECK2-Slug Mut363 3'UTR and pCMV-miR363 or empty pCMV vector. Luciferase activity unit is plotted as Renilla to Firefly luciferase activity (RFU). Each bar represents mean ± SEM of four replicated experiments. *P<0.05. CL: Clones of DAB2IP-knockdown cells with stabilized miR363 overexpression.

Figure 6 Slug antagonize the effect of miR363 on EMT in PCa cells. (A) E-cadherin and Vimentin mRNA level after restoration of Slug in LAPC4-KD cells expressing miR363. (B) E-cadherin and Vimentin protein expression after restoration of Slug in LAPC4-KD cells expressing miR363. (C) E-cadherin and Vimentin mRNA level after restoration of Slug in RWPE-KD cells expressing miR363. (B) E-cadherin and Vimentin protein expression after restoration of Slug in RWPE-KD cells expressing miR363.
Figure 7 Expression of miR363 in mouse prostate gland. Prostate glands were harvested from either wild type (DAB2IP+/+; A, B) or DAB2IP knockout (DAB2IP−/−; C, D) mouse with 6-8 weeks old and subjected to in situ hybridization of miR363.

Figure 8 Expression of miR363 in BPH, adjacent benign and PCa tissues. The consecutive sections of BPH tissues from the same patient were subjected to ISH (A) with excess “cold” probe for competition (B). A section from FFPE tissue block of PCa patients was subjected to ISH. From the same section, adjacent benign tissue (C) exhibited higher expression of miR363 than tumor area (D).