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Repression of TSC2 Expression by miR-296 in Human Normal and Tumor Tissues

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Tuberous sclerosis complex (TSC) genes are tumor suppressors and are ubiquitous and expressed in almost all human tissues. Loss or inactivation of TSC genes contributes to a broad spectrum of diseases, including multiorgan neoplasia, and virtually any part of the body can be affected. Loss of the wild type allele and second somatic mutations is the major molecular mechanism identified in some but not all TSC-associated tumors. We proposed that microRNA miR-296 may be the native molecule regulating TSC2 expression during development and tumorigenesis in some TSC-associated tumors. To explore whether TSC2 can be regulated by the endogenous miRNAs during development and tumorigenesis, we will firstly study whether miR-296 is a native molecule that can specifically repress TSC2 expression in vitro. This can be accomplished by examination of TSC2 repression by exogenous miR-296 in cell models. Particularly we will examine whether presence of TSC2 3’ untranslated region is critical for miR-296 regulation. We will then examine the expression correlation between TSC2 and miR-296 in human normal and tumor tissue samples. Identification of repression of TSC2 by microRNA miR-296 will provide a novel molecular mechanism of TSC gene regulations in development and TSC associated lesions. The findings will greatly impact on the differential diagnosis and potential therapeutic modality.

No subject terms provided.
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INTRODUCTION

Tuberous sclerosis complex (TSC) genes are tumor suppressors and are ubiquitous and expressed in almost all human tissues. Loss or inactivation of TSC genes contributes to a broad spectrum of diseases, including multiorgan neoplasia, and virtually any part of the body can be affected. There are a few normal tissues and cell types (such as Glomeruli, pneumocytes, pituitary gland and thymus) that either do not express or express at an undetectable level of TSC genes or gene products. Genetic mutations of TSC genes are the initial events in familial TSC patients. To induce TSC-associated tumors, the second wild type allele must be inactivated. Loss of the wild type allele and second somatic mutations is the major molecular mechanism identified in all types of TSC-associated tumors, except for subependymal giant-cell astrocytomas (1) and rhabdomyomas (2). All of these findings suggest that there is(are) still another, more universal, mechanism(s) responsible for TSC-associated tumorigenesis.

Several alternative mechanisms that could potentially trigger TSC-associated neoplastic transformation have been proposed. AKT- and ERK-mediated inactivation of the TSC gene product (tuberin) is characterized to be a cellular mechanism leading to post translation modification of tuberin in TSC pathogenesis, even when one or both alleles of TSC2 remains intact. However AKT-mediated tuberin inactivation seems to be less important, since the active AKT is not high in many TSC-associated tumors, such as subependymal giant-cell astrocytomas, angiomylipomas, skin tumors and rhabdomyomas, but is at the same or even lower concentrations than healthy tissues (3). The role of pERK in regulating tuberin is still under active study. Epigenetic silencing of TSC genes is suggested but no methylation of the TSC2 promoter region has been noted (4). Recently, it has been proposed that there may be a third protein in the TSC complex (putative TSC3) (5).

TSC-associated cardiac rhabdomyomas develop in the specific developmental stages (during the first years of life), and are not closely related to the germ cell or somatic cell TSC mutations (2). If TSC genes are indeed to play a critical role in this tumor, developmental regulation of TSC gene expression at the transcriptional and/or translational levels may exist. We proposed that microRNA (miRNA) miR-296 may be the native molecule regulating TSC2 expression during development and tumorigenesis in some TSC-associated tumors. The hypothesis is largely based on the observations that: 1) differential expression of miRNAs at development can regulate cell proliferation and differentiation; 2) miRNA-mediated gene regulation is commonly associated with benign and malignant neoplasms; 3) there is a complementary site of miR-296 at the TSC2 3' untranslation region (3'UTR), which is evolutionarily highly conservative (Figure 1); and 4) most importantly, there is an inverse association between tuberin and miR296 expression, as observed in a pilot study of 36 smooth muscle tumors (unpublished data).

TSC2 contains 41 exons with a very short 3’ untranslation region (3’UTR, <110 nt). The short TSC2 3’UTR may prevent from miRNA regulation. However, in the TSC2 3’UTR immediately adjacent to the stop code, there is a highly conserved sequence that harbors the complementary sites of miR-296 and a few other miRNAs (Figure 1). MiR-296 is over-expressed in many solid epithelial and mesenchymal neoplasms. In HeLa cells, inhibition of miR-296 causes a decrease in cell growth. Characterization of the molecular pairing between miR-296 and TSC2 currently has not been studied. The result will greatly impact our current understanding of whether TSC2, a tumor suppressor gene, can be regulated by miRNAs.

BODY

TSC2 plays a key role in the Akt-mTOR signaling pathway and is associated with many benign and malignant neoplasms. The hypothesis is that miR-296 is the negative regulator of TSC2 in some normal or tumor cells. To explore whether TSC2 can be regulated by the endogenous miRNAs during development and tumorigenesis, the relationship between miR-296 and TSC2 will be analyzed in two different approaches, both in vitro and in vivo.
Aim 1. To characterize whether miR-296 is a native molecule that can specifically repress TSC2 expression in vitro. The molecular pairing of miR-296::TSC2 will be tested on several different levels. First, it will be examined whether exogenous miR-296 and its inhibitor can repress endogenous TSC2 expression. Next, it will be determined whether presence of the TSC2 3’UTR sequence is required for miR-296-mediated TSC2/luciferase activities. Finally, the correlation between TSC2, the mTOR pathway and cell proliferation in cells with constant overexpression of miR-296 will be examined.

Aim 2. To examine the correlation between levels of endogenous TSC2 and endogenous miR-296 expression in vivo. The levels of TSC2 and miR-296 expression may be cell-type-specific; examination of these two molecules by in situ hybridization (ISH) and immunohistochemistry (IHC) will be conducted in adult normal tissues as well as the selected solid tumor tissues.

Key Research Accomplishments

Experiment 1. Transient transfection analysis of miR-296 in HEK293T cell line. HEK293T cell line (which has high TSC2 and low miR-296, data not shown) were used for this study. Transfection were performed in Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells (5x10^4) were plated onto each of 12-well plates. To estimate transfection efficiency, the Block-iT (Fluorescent double-stranded random 22mer RNA from Invitrogen, Carlsbad, CA) were used as a negative control. HEK293T cells were transfected with 20, 40, 60 pmol of miR-296 mimic (Dharmacon Inc., Lafayette, CO) in triplicate. Cells treated with the miR-296 inhibitor were used as a control to block the effects of endogenous miR-296. Cells were harvested at 48 hours post-transfection and total RNA and proteins were prepared according to standard protocols, as described previously (6-8) The efficacy of transfection was validated by mirVana qRT-PCR of miR-296 and U6 (control) with the mirVana qRT-PCR Kits (Ambion, Austin, TX). As seen in Figure 2B, by transient transfection of TSC2 siRNA, significant reduction of TSC protein was noted. In the same experiment, application of miR-296 mimic and inhibitor has minimal effect on TSC2 expression (not shown) and translation (Figure 2B). The experiment was repeated in different cell line showing the similar finding (Figure 2B right panel).

Experiment 2. Luciferase expression analysis in the presence and absence of TSC2 3’UTR.

We previously found that TSC2 was significantly down regulated in leiomyomas (9-10). Down regulation of TSC2 was also found in this study. We found that miR-296 was inversely correlated with TSC2 protein in 36 leiomyomas.
TSC2 contains 41 exons with a very short 3’ untranslation region (3’UTR, <110 nt). The short TSC2 3’UTR may prevent from miRNA regulation. However, in the TSC2 3’UTR immediately adjacent to the stop code, there is a highly conserved sequence that harbors the complementary sites of miR-296 and a few other miRNAs (Figure 1). To study whether TSC2 is the target of miR-296, we prepared TSC2 3’UTR construct and examined the luciferase activity by treated cells with control, miR-296 mimic and inhibitor.

PC3 cell line has moderate levels of miR-296 and low levels of TSC2 expression (data not shown). Luciferase constructs of psiCHECK2-TSC2 3’UTR vectors containing a 110 bp fragment of the TSC2 3’UTR with or without putative miR-296 complementary site were introduced into PC3 cells with the aid of Lipofectamine 2000. Luciferase activities were measured using the Dual-Glo™ Luciferase Assay System (Promega) following slightly modified manufacturer’s instructions.

In triplicate cell lines treated with miR-296 mimic and inhibitor, there was no reduction of luciferase expression in cells (Figure 2A). The findings indicated that TSC2 was not the direct target of miR-296. The inverse correlation of TSC2::miR-296 pair may be related to some other molecular mechanisms in leiomyomas. This finding was published as part of our study in uterine leiomyoma in PloS One (11).

Experiment 3. Functional analysis of TSC-mTOR pathway by disrupting miR-296::TSC2 pairs in HEK293T cell line. Although computer analysis revealed that TSC2 was the predicted target gene (Figure 1), but we found that TSC2 was not regulated by miR-296 (see experiment 1 and 2) in molecular level. It apparently was lack of basis for further analysis of how miR-296 mediated TSC dysregulation in mTOR pathway. To better use our system and resource to characterize the role of miR-296 in regulation of other oncogenic factors, we turned our attention to a new target of miR-296: HMGA1. We established stable miR-296 cell lines in prostate cancer cell lines and examined and characterized that HMGA1 was the direct target of miR-296 (Figure 4). We further analyzed the functional role of miR-296 in association with HMGA1 mediated tumorigenesis in prostate cancer (Figure 5). The study was published in Clinical Cancer Research (12).

Experiment 4. In situ hybridization (ISH) analysis of TSC2 cRNA and miR-296 and Immunohistochemistry (IHC) analyses of tuberin in tissue microarray (TMA). Again, when we found miR-296 did not regulate TSC2, further comparison of miR-296 and TSC2 in the candidate tumors and normal tissues listed in the proposal will not yield the expected results. We therefore turn our research direction to more specific target of miR-296. We prepare human tumor samples which more relevant to our newly identified molecular pairs of miR-296 and HMGA1, including leiomyomata, prostate cancer and ovarian cancer. We collected and prepared high density tissue microarrays from 36 uterine leiomyomas (9-10, 13), 30 prostate cancer (unpublished data), 115 cases of ovarian cancer (6 different types) (14). A high-density TMA (0.6-1.0 mm tissue cores) were prepared by the MTA-1 manual tissue arrayer (BeecherInstruments, Sun Prairie, WI).

TMA blocks were sectioned at 4 microns. The detailed procedure for ISH followed the manufacturer’s protocol. miRCURY LNA probes of miR-296 (test) and U6 (control) were purchased from Exiqon (Vedbaek, Denmark). The expression levels of miR-296 were scored based on the intensity of ISH signals on each core by a density photometry (NIH Scion). IHC stains for TSC2 and HMGA1 (the tumorigenic factor for uterine leiomyoma and prostate cancer) were performed on a Ventana Nexus automated system (Tucson, Arizona) with antibodies of tuberin and other control markers (including hamartin, cytokeratin, vimentin and gFAP). Immunoreactivity were scored semiquantitatively based on intensity and percentage.

Correlation analysis Between TSC2 protein tuberin, HMGA1 and miR-296 were performed by a case matched analysis. Statistical significance was analyzed by a paired t-test. We found that there significant increase of miR-296 in all types of tumors including leiomyomas, prostate cancer and ovarian cancer. In contrast, downregulation of TSC2 were found in the same groups of tumors. However, inverse correlation of TSC2 and miR-296 were only found in leiomyomas, not in prostate cancer and ovarian cancer. In contrast, we found that HMGA1 was the direct target of miR-296 and these two paired molecules showed moderate correlation in prostate cancer (Figure 4) and leiomyoma (data not shown).
Reportable Outcomes
See above results and two publications listed below.

Conclusion
We found that TSC2 was downregulated in leiomyomata and prostate cancer. Although TSC2 was inversely correlated with miR-296 expression in leiomyomata, but this correlation could not be established in prostate cancer (data not shown). We found that miR-296 could not directly repress TSC2 expression based on expression analysis and luciferase analysis (Figure 2). This negative results force us to adjust our research direction into new miR-296 targets in association with solitary tumors. We would be able to identify HMGA1, a novel target of miR-296 in prostate cancer. Further infestation revealed that impairing of miR-296::HMGA1 expression contributed to prostate carcinogenesis.

References

Appendices
Two manuscripts related to this study have been published listed below.