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THE MIR-503 CLUSTER IS COORDINATELY UNDER-EXPRESSED IN ENDOMETRIAL CANCER

by

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A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Health and Human Physiology

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Dr. Eric J. Devor
Thesis Mentor

Fall 2018

All requirements for graduation with Honors in the
Health and Human Physiology have been completed.

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The mir-503 cluster is coordinately under-expressed in endometrial cancer

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Abstract

The microRNA-503 cluster, composed of six microRNAs (miR-424, miR503, miR-542, miR-450a-1, miR450a-2 and miR-450b), is located on the human X-chromosome at Xq26. This location is very near the gene encoding placenta-specific protein 1 (PLAC1) that we have shown is significantly over-expressed in several gynecologic cancers. We examined expression of the miR-503 cluster in a panel of endometrial endometrioid adenocarcinomas (EEAs) and found that all of the miRNAs are significantly under-expressed compared with benign tissue. Expression of the miRNAs is highly correlated suggesting that they are regulated from a single promoter. We have used DNA editing (CRISPR) of the miR-503 cluster promoter to down-regulate the entire cluster together. Additionally, known and validated targets of cluster members include a number of important oncogenes. Thus, suppression of miR-503 cluster members contributes to the establishment and maintenance of endometrial cancer. We further suggest that decreased miR-503 cluster expression is due to hyper-methylation of the promoter in tumor cells. Thus, we conclude that the miR-503 cluster is a potential therapeutic target in endometrial cancer.

Introduction

MicroRNA-503 cluster, composed of six microRNAs (miRs): miR-424, miR-503, miR-542, miR-450a-1, miR-450a-2 and miR-450b, is located on chromosome Xq26.3 in close proximity (26.5kb) to placenta-specific protein 1 (PLAC1) [1]. PLAC1 is normally exclusively expressed in the trophoblast cells of the placenta. However, PLAC1 has been shown to be expressed in a variety of cancers, including breast, prostate, ovarian and uterine. Expression of PLAC1 promotes invasiveness, proliferation, and migration, all of which are characteristics of cancer cells. Expression of PLAC1 in tumor cells increases with aggressiveness and advanced stage. PLAC1 may serve as a biomarker for endometrial cancers with the potential to be both a prognostic indicator and a therapeutic target [2]. The close proximity of the miR-503 cluster to PLAC1 makes it an area of interest in endometrial cancer.

Nearly 11,000 women in the US will die of endometrial cancer this year alone [3] and it is projected that by 2030, there will be a 55% increase in the number of women with this cancer [4]. Endometrial cancer is the most common gynecologic cancer and is the 6th most common cancer among women [5]. It is thus imperative to understand as much as possible about the genetics of endometrial cancer. Consequently, the miR-503 cluster and its chromosome region are the subject of this study.

As noted, the mir-503 cluster is composed of six microRNAs but miR-450a-1 and miR-450a-2 are identical so, for the purposes of this study, they are considered as a single

locus: miR-450a. We report here that all five microRNAs in the 503 cluster are significantly down-regulated in endometrial cancer compared with benign uterine tissue and that their expression patterns are highly correlated. Further, we show that members of the cluster target a number of well-known oncogenes as well as DNA repair genes. Our hypothesis is that the entire cluster is transcribed as a single transcript and that the observed down-regulation in endometrial cancer is due to methylation of the tumor's promoter. This model, in turn, suggests that targets of miR-503 cluster members are up-regulated in endometrial tumors. The objective of our research is to detail the regulation of the mir-503 cluster and its relationship to oncogene and DNA repair gene targets as this could lead to developing it as a potential therapeutic target in endometrial cancer.

Methods

We created a tumor panel composed of 20 endometrial endometrioid adenocarcinomas, the most common form of the cancer, and 4 benign uterine tissues (table 1). These 24 tissue samples were obtained with informed consent from the Gynecologic Tumor Bank which is part of the Women's Health Tissue Repository maintained in the Department of Obstetrics and Gynecology [6].

Total cellular RNA was purified from these tissue specimens using the mirVana miRNA isolation kit following manufacturer's (Thermo Fisher) recommendations. Equal mass amounts of the purified RNAs (250ng each) were then reverse transcribed using miRNA-specific primers and MultiScribe Reverse Transcriptase (Thermo Fisher). The resulting cDNAs were then amplified using miRNA-specific TaqMan expression assays (Thermo Fisher). All experiments were carried out in triplicate.

TABLE 1: Tumor Panel used in study with clinical characteristics. Estrogen Receptor (ER) and Progesterone Receptor (PR) status were determined by Immunohistochemistry in the Department of Pathology. We determined the TP53 tumor suppressor mutation through direct sequencing of tissue genomic DNAs

ID #	Age	Histology	Stage	Grade	ER+	PR+	TP53
13	84	Endometrioid	IA	2	N	N	wt
46	63	Endometrioid	IIIC1	2, 3	N	N	R213ter
43	41	Endometrioid	IIIA	2	N	N	wt
11	76	Endometrioid	IA	3	N	N	wt
64	51	Endometrioid	II	1	Y	N	wt
20	57	Endometrioid	IIIC1	3	N	N	wt
61	62	Endometrioid	IB	1	N	N	wt
84	41	Endometrioid	IA	3	Y	Y	wt
35	34	Endometrioid	II	2,3	N	N	I50 ter
51	56	Endometrioid	IA		N	N	wt
50	62	Endometrioid	IA	2	N	N	wt
38	62	Endometrioid	IIIC1	1	Y	Y	wt
68	65	Endometrioid	IB	3	N	N	wt
73	43	Endometrioid	IB	2	Y	Y	wt
83	83	Endometrioid	IIIA	2	Y	Y	wt
55	58	Endometrioid	IA	1	Y	Y	wt
09	62	Endometrioid	IVB	3	N	N	wt
69	57	Endometrioid	IIIB	2	N	Y	wt
39	65	Endometrioid	IIIC2	3	N	N	wt
19	54	Endometrioid	II	2	Y	Y	wt
BE129	61	Benign					wt
BE253	83	Benign					wt
BE311	64	Benign					wt
BE2190	63	Benign					wt

Over-expression of each member of the miR-503 cluster in Ishikawa H Cells, a well-validated endometrioid adenocarcinoma model cell line [7], was accomplished with miRNA-specific mimics (Thermo Fisher). Over-expression was confirmed using the same miRNA-specific assays as above. Each miRNA mimic transfection as well as the un-transfected control was carried out in triplicate. Total cellular RNA was purified as above and the RNA submitted for next generation RNA sequencing to identify additional gene targets.

Previous identification of a large CpG island in the miR-503 cluster promoter and a recent study of miR-424 in glioma [8] suggested that expression is regulated by methylation (Figure 1). We exposed Ishikawa cells to a cytidine analog (5-aza-2' deoxycytidine), a de-methylating agent, in order to assess the effect of de-methylation on miRNA expression and selected target gene expression. Cells were exposed to 1 μM 5-aza-2' deoxycytidine for 72 hours before

harvesting. Control cells were grown in parallel. The treated and un-treated media was changed every 24 hours. Again, total cellular RNA was purified and all treatments and controls were maintained in triplicate.

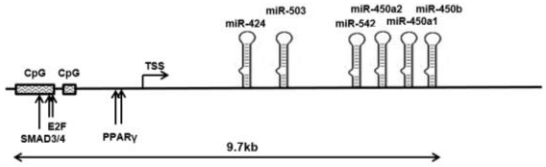

Figure 1. The miR-503 cluster on X-Chromosome with identified promoter, CpG islands (methylation sites) and the transcription start site

In all cases, miRNA and gene expression was measured as normalized fold change relative to controls. Expression values (Ct) for every RNA sample and assay are normalized as ΔCt using an endogenous control; RNU48 for miRNAs and 18S rRNA for mRNAs. Fold change is then determined by the standard ΔΔCt method in which $\Delta\Delta Ct = \Delta Ct_{experiment} - \Delta Ct_{control}$ and fold change is computed as $2^{-\Delta\Delta Ct}$ [9,10]. Significance is assessed on grouped ΔCt values via a two-sided t-test with unequal variances with $p < 0.05$ being taken as statistically significant [11].

Correlations among normalized miRNA ΔCt values were computed as standard Pearson Correlation and significance assigned from a standard look-up table at df = 22. Again, $p < 0.05$ was taken as statistically significant.

Results

Coordinated under-expression of the miR-503 cluster in Endometrioid Adenocarcinoma

Results from miRNA-specific QPCR are seen in Table 2. Expression of miR-503 cluster members in endometrioid adenocarcinoma relative to benign endometrium is presented in Figure 2. Each cluster member is significantly under-expressed in the tumors compared with benign endometrium. The magnitude of under-expression ranges from around 5-fold in miR-503 to nearly 25-fold in miR-424. Using normalized expression values (ΔCt) for each miRNA in all 24 individuals in the panel, including the benign tissues, pairwise correlations among all five members of the cluster reveals a highly significant pattern of coordinated expression across the entire cluster (Figure 2A). Correlation coefficients range from 0.76 to 0.94 and all are statistically significant at $p < 0.001$.

Table 2. Pearson correlation coefficients among the five miRNAs in the miR-503 cluster. All are significant to $p < 0.0001$ at df = 22

	miR-503	miR-542	miR-450a	miR-450b
miR-424	0.81***	0.91***	0.90***	0.92***
miR-503		0.83***	0.76***	0.86***
miR-542			0.85***	0.94***
miR-450a				0.85***

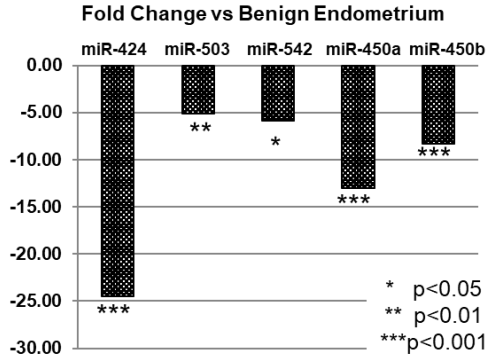

Figure 2. Relative expression differences (fold change) between endometrial tumors and benign uterine tissue. Fold change is calculated as 2-ΔΔCt where ΔΔCt is the difference between mean normalized expression in the tumors and mean normalized expression in the controls

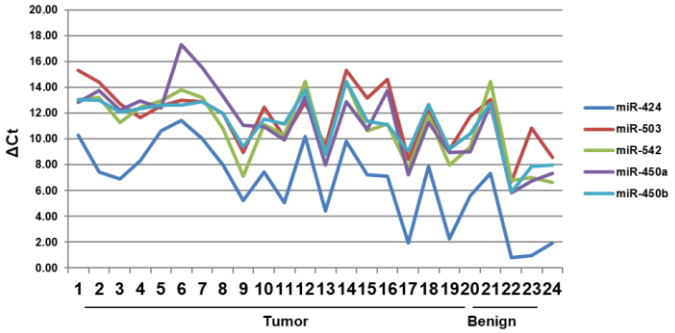

Figure 2a. Correlation between members of mir-503 with ΔCt values for each member for each of the 24 individuals on the panel

Coordinate expression is confirmed in The Cancer Genome Atlas (TCGA) where expression of the five cluster members is also uniformly significant at $p < 0.001$. Correlation coefficients ranged from 0.56 to 0.88. Comparing the pairwise correlation coefficients between our sample and TCGA is also statistically significant ($r = 0.62$, $p < 0.05$, $df = 8$). Thus, the pattern of expression in the two analyses are is the same.

Catalog of experimentally validated miR-503 cluster targets

Using miRTarBase 7.0 a total of 55 unique experimentally validated targets were identified for members of the miR-503 cluster (Table 3). Among these are numerous genes commonly regarded as oncogenes such as FGFR1, MYB, BCL2, PI3K, and MYCN. Additionally present are genes involved in DNA repair such as CHK1 and WEE1; cell cycle including several cyclins, RUNX2, and CDC25A; anti-apoptosis genes IGF1R and BIRC5 (survivin). Also, chemotherapy response inhibitors such as TRAF5 (cisplatin), ZNF217 (paclitaxel), and ERBB3 (gefitinib) are also present.

Table 3. miR-503 cluster member experimentally validated targets (miRTarBase 7.0). Targets classified in the COSMIC census (www.sanger.ac.uk/science/data/cancer-gene-census) as Tier 1 oncogenes are shown in red. Targets validated for more than one member of the cluster are shown in Bold type.

miR-424-5p	miR-503-5p	miR-542-3p	miR-450a-5p	miR-450b-5p
CCND1	BCL2	AKT	**DNMT3A**	**DNMT3A**
CCND3	**CCND1**	ANGPT2	**ERBB3**[f]	ENOX2
CCNE1	CDH1	BIRC5[d]	HRNPK2	**ERBB3**[f]
CCNF	DDHD2	BMP7	**IRF2**	**IRF2**
CDC14A	E2F3	COL1A1	STAT1	PAX9
CDC25A	FANCA	CTTN		
CDK6	IGF1R	EGFR		
CHEK1[a]	IKBKB	FTSJ2		
CUL2	PI3K	FZD7		
E2F7	**RICTOR**	ILK		
EPAS1	SMAD2	MTDH		
FASN1	TRAF5[b]	MYCN		
FGFR1	ZNF217[c]	OTUB1[e]		
HIF1α		PIK3R1		
KIF23		RUNX2		
MAP2K1				
MYB				
PLAG1				
RICTOR				
SMAD3				
SOC6				
TNFAIP1				
WEE1[a]				

[a]DNA damage response/DNA repair
[b]implicated in cisplatin resistance
[c]implicated in paclitaxel resistance
[d]apoptosis inhibition, survivin
[e]inhibits ubiquitination of FOXM1
[f]implicated in gefintinib resistance

The targets currently validated for the miR-503 cluster members represent a rich array of genes whose dysregulation in cancer would be advantageous for carcinogenesis, recurrence, and metastasis.

Potential Target Identification

The number of oncogenes, cell cycle, DNA repair, and drug resistance genes seen in the validated targets of the miR-503 cluster initiated the investigation into a number of other potentially target genes that may exist. Transient transfection with the miR-specific mimics at 10µM resulted in an average expression increase of 33,000-fold compared with mock transfected cells. We chose to set a False Discovery Rate (FDR) of 10% which placed the corrected acceptable significance level at $p < 0.001$. Loci whose expression was significantly influenced, either up or down, relative to untreated cells in response to miRNA mimic transfection (Figure 3). As can be seen there are hundreds of potential additional targets for members of the miR-503 cluster and many of these are genes known to be involved in carcinogenesis.

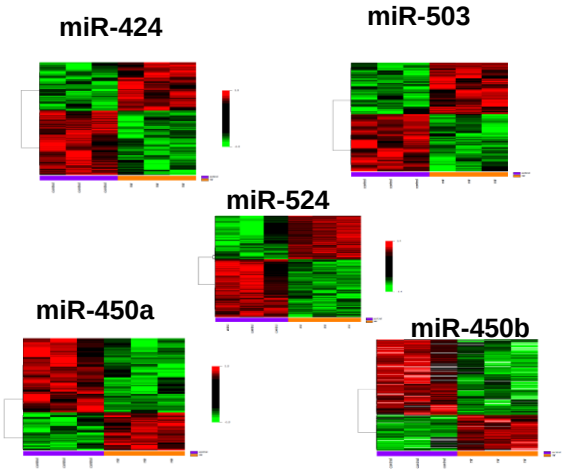

Figure 3. Heat Maps of the target genes from RNA sequencing data. The areas above the purple bars belong to the untreated cells and the areas above the orange belong to the cells treated with mimics. Red is overexpressed and green represents genes that are under expressed.

Promoter De-methylation

Changes in expression of miR-503 cluster members as a result of treating Ishikawa cells with Decitabine are shown in Figure 4. Though the fold changes are modest, three of the five cluster members did reach statistical significance. The pattern of alteration of expression due to de-methylation in vitro is exactly the inverse of the expression pattern of the cluster members seen previously in Figure 2 from the primary tumor tissues.

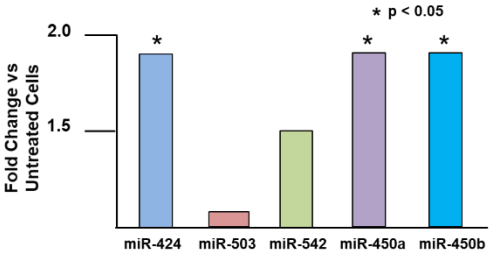

Figure 4. Fold Change from Decitabine treatment on Ishikawa cells vs. untreated cells.

DISCUSSION

The miR-503 miRNA cluster, located at Xq23.1 between HPRT and PLAC1, consists of six miRNAs (miR-424, miR-503, miR-542, miR-450a-1, miR-450a-2 and miR-450b). We have shown that this cluster is transcribed as a polycistron and that the entire cluster is significantly down-regulated in endometrial adenocarcinoma. Considering the cluster as a whole and assembling experimentally validated targets reveals a wide range of loci involved in carcinogenesis and the consequences of carcinogenesis which includes DNA repair, DNA damage response, cell cycle maintenance and chemotherapy response. Further, we have provided evidence consistent with suggestions from other cancers [12] that down-regulation of the cluster in endometrial adenocarcinomas is accomplished via methylation. Given all of this data, we suggest that the miR-503 cluster, as a whole, functions as a tumor suppressor complex and is, therefore, a potentially important therapeutic target in endometrial adenocarcinoma.

It is well documented that high PLAC1 expression leads to more aggressive tumors and a poor prognosis [13]. Similarly, several studies have linked down-regulation of members of the miR-503 cluster to carcinogenesis and poor prognosis. Most of these studies have focused on miR-424 and/or miR-503 [14, 15-18] but miR-542 has also been implicated including a specific relationship with endometrial carcinoma [19]. While there are fewer studies involving the miR-450 family, their role in carcinogenesis has also been confirmed [20, 21].

Future directions include continuing to archive and evaluate the genes revealed through RNA sequencing of over-expression of members of the miR-503 cluster.

Finally, the fact that up-regulation of expression of miR-503 cluster members, possibly through use of cytidine analogs, will negatively affect targets whose actions are oncogenic, but such an effect will not apply to neighboring PLAC1 where up-regulation is contrary to good outcomes. Thus, the focus in the future should be to find an appropriate balance between the two to further explore and exploit it.

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