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ORIGINAL RESEARCH

The miR-503 cluster is coordinately under-expressed in endometrial endometrioid adenocarcinoma and targets many oncogenes, cell cycle genes, DNA repair genes and chemotherapy response genes

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Background: The miR-503 miRNA cluster, located at Xq23.1, is composed of six miRNAs; miR-424, miR-503, miR-542, miR-450a-1, miR-450a-2 and miR-450b. Numerous studies have focused on the relationship of one or two members of the cluster and various human cancers. Here, we suggest that the entire cluster as a single coordinately expressed polycistron transcribed from a single promoter in endometrial endometrioid adenocarcinoma (EEA).

Subjects and methods: A tissue panel composed of twenty histologically confirmed endometrial endometrioid adenocarcinomas (EEA) and four benign endometrium was assembled under informed consent. Expression of each member of the miR-503 cluster was determined by quantitative PCR and differences in expression between EEA and benign tissues were assessed via the standard ΔΔCt method. In addition, the role of promoter methylation status in miRNA expression was examined in Ishikawa H cells following exposure to the cytidine analog Decitabine.

Results: Expression of each member of the miR-503 cluster is significantly down-regulated in EEA in our tumor sample. Both in our tumor sample and in The Cancer Genome Atlas (TCGA) there is evidence of highly correlated expression further supporting the idea that the miR-503 cluster is a polycistron. Looking at each member of the miR-503 cluster we were able to identify 55 unique experimentally validated target genes which include a substantial number of genes involved in carcinogenesis, DNA damage response, cell cycle regulation and chemotherapeutic response. We also found preliminary evidence that regulation of the miR-503 cluster is governed by methylation of the promoter in EEA.

Conclusion: The totality of the data presented here strongly suggest that the miR-503 cluster as a whole merits further investigation as an important potential therapeutic target in EEA.

Keywords: microRNA-503 cluster, polycistron, endometrial cancer, methylation, decitabine, TCGA, correlated expression

Introduction

Endometrial cancer is the most common gynecologic cancer and the fourth most common cancer among women worldwide.[1] In 2017, there were >60,000 new cases in the USA and nearly 11,000 deaths.[2] It is estimated that, at any one time, in excess of 700,000 women are living with endometrial cancer in the USA alone.[2] Although the overall prognosis for endometrial cancer is relatively favorable, particularly in the developed world, there are disturbing indications that endometrial cancer incidence is on the rise

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as is the recurrence risk.[3] Indeed, one estimate projects a staggering 55% increase in endometrial cancer by the year 2030.[3] In addition, data collected since the turn of the twenty-first century indicate that there is a growing disparity in outcomes among racial/ethnic groups and that African-American women suffer nearly twice the mortality rate of other women.[4] Thus, it is important to expand our understanding of the underlying mechanisms of endometrial carcinogenesis and recurrence in order to develop more effective prevention and treatment.

We have reported that expression of *PLAC1*, located on chromosome Xq23.1, is highly expressed in gynecologic cancers and is an indicator of both advanced disease and poor outcomes.[5–8] Recently, a cluster of miRNAs collectively referred to as the miR-503 cluster was shown not only to be located very close to the *PLAC1* gene locus but also to have expression patterns very similar to *PLAC1*.[9] The miR-503 cluster is home to long noncoding RNAs, linc00629 and miR503HG, and six miRNAs; miR-424, miR-503, miR-542, miR-450a-1, miR-450a-2, and miR-450b. In the human genome, miR-450a-1 and miR-450a-2 are identical and will be referred to here collectively as miR-450a. Given the proximity of the miR-503 cluster to *PLAC1* and the expression pattern of its members, we have assessed expression levels of the cluster member miRNAs in endometrial endometrioid adenocarcinoma (EEA), the most common type of endometrial cancer. We observed that all five unique miRNAs in the cluster are significantly underexpressed in EEA compared with benign endometrium. In addition, expression of cluster members is highly and significantly correlated across samples suggesting that the miR-503 cluster may be a polycistron transcribed from a single source in a manner similar to the classic miR-17 polycistron.[10] A compilation of experimentally validated miR-503 cluster target genes includes a number of well-known oncogenes as well as several loci implicated in DNA repair, DNA damage response, cell cycle, and chemotherapy response. Thus, down-regulation of the miR-503 cluster frees expression of a variety of genes involved in the initiation and maintenance of endometrial carcinogenesis. Finally, consistent with evidence from other cancers, we suggest that the mechanism of miR-503 cluster suppression in endometrial cancer is hypermethylation. Taken together, these observations nominate the miR-503 cluster as a locus of interest in endometrial carcinogenesis and an inviting therapeutic target.

Subjects and methods

Tissue procurement

A screening panel composed of 20 histology-confirmed EEAs and four benign endometrial tissues was assembled for this study. Tissues were obtained under written informed consent from patients undergoing surgery at the University of Iowa Hospitals and Clinics and who were enrolled in the Gynecologic Tissue Bank (Institutional Review Board [IRB]#200209010) that is part of the Women's Health Tissue Repository (WHTR; IRB#200910784) maintained in the Department of Obstetrics and Gynecology of the University of Iowa Carver College of Medicine.[11] In addition to the archived tissue samples, the WHTR provides complete clinical information and outcomes for each patient (Table 1).

Nucleic acid purification

Total cellular RNA was purified from 20 tumor and four control tissue samples using the mirVana miRNA isolation kit also according to manufacturer's (Thermo Fisher, Waltham, MA, USA) instructions. RNA yield and quality were assessed using an Agilent Model 2100 bioanalyzer and a Trinean DropSense 16 spectrophotometer. Only RNAs with an RNA integrity number (RIN)[12] >7.0 were used in these studies. The average RIN was 8.2 with a range from 7.0 to 9.5.

Table 1 The endometrioid adenocarcinoma panel used in this study

ID #	Age (years)	Histology[a]	Stage	Grade	ER+[b]	PR+[b]	TP53[c]
13	84	EEA	IIIB	2	Y	N	WT
46	63	EEA	IIIC1	2	Y	Y	R213ter
43	41	EEA	IA	2	Y	Y	WT
11	76	EEA	IA	3	N	Y	WT
64	51	EEA	IIIA	2	Y	Y	WT
20	57	EEA	IB	2	Y	Y	WT
61	62	EEA	IIIC1	1	Y	Y	WT
84	41	EEA	IA	3			WT
35	34	EEA	IA	1	Y	N	150ter
51	56	EEA	IVB	1	Y	N	WT
50	62	EEA	IIIA	3	Y	N	WT
38	62	EEA	IIIC1	2	Y	Y	WT
68	65	EEA	IA	2	Y	N	WT
73	43	EEA	IA	1	Y	Y	WT
83	83	EEA	II	2	Y	Y	WT
55	58	EEA	IA	2	Y	Y	WT
09	62	EEA	IVB	3	N	N	WT
69	57	EEA	IA	3	Y	Y	WT
39	65	EEA	IB	2	Y	Y	WT
19	54	EEA	IB	1	Y	Y	WT
129	61	BENIGN					WT
253	83	BENIGN					WT
311	64	BENIGN					WT
2190	63	BENIGN					WT

Notes: [a]Pathology confirmed; [b]Immunohistochemistry; [c]Direct sequencing.
Abbreviations: EEA, endometrial endometrioid adenocarcinoma; ER, estrogen receptor; PR, progesterone receptor; N, no; WT, wild type; Y, yes.

Cell culture

For in vitro experiments, we selected the well-known and validated endometrial adenocarcinoma model cell line Ishikawa H.[13] Cells were authenticated with CODIS VNTR markers by Bio-Synthesis (Lewisville, TX, USA) and comparison with published data.[14] CODIS typing confirmed that our cells are from the 3-H-4 subline established in 1993 and distributed between 1993 and 1996.[15,16] In addition, these cells are ER+, PR+, contain a TP53 mutant of unknown significance (M247V, rs483352695), a terminal PTEN mutant (E91fs ter), a nonactivating POLE mutant (P102S), and a PIK3R1 mutant (L570P) also of uncertain significance. This combination of molecular features as well as the endometrioid histology of mouse explants makes these cells a prime model of a Type I endometrial cancer.[13] However, their placement within the new four class system[17,18] is less certain as they display features of both Class 2 and 3 tumors. Cells were grown in DMEM media supplemented with 10% FBS and 1% antibiotic (Sigma, St Louis, MO, USA; Pen-Strep). All cell culture experiments were carried out in triplicate. Whole-cell RNA purifications and quality control (QC) were performed as previously stated.

miRNA expression

miRNA-specific expression assays were carried out on fixed mass RNA inputs of 250 ng. Total cellular RNAs were reverse transcribed using miRNA-specific RT primers (Thermo Fisher) in the presence of MultiScribe Reverse Transcriptase (Thermo Fisher). Resulting cDNAs were then amplified in miRNA-specific TaqMan fluorescence assays (Thermo Fisher). For miRNA assays, the standard, well-validated endogenous RNA control RNU48 (Thermo Fisher) was used for normalizing. miRNA expression (Ct) was normalized (ΔCt) and tumor miRNA expression compared with benign tissue expression via the standard ΔΔCt method[19,20] where $\Delta\Delta Ct=\Delta Ct_{tumor}-\Delta Ct_{control}$ and fold change is $2^{-\Delta\Delta Ct}$. Statistical significance was assessed by a two-tailed *t*-test with unequal variance.[21] A P-value<0.05 was taken as statistically significant.

miRNA expression correlations were carried out pairwise across all five unique members of the miR-503 cluster on all 24 tissues in the sample using Pearson product–moment correlation. Normalized expression values (ΔCt) were the input data. Statistical significance was assessed using a standard look-up table with n–2 degrees of freedom. A P-value<0.05 was taken as statistically significant.

Target validation

Experimentally validated targets were culled from the extant literature as well as from the miRTarBase database, Version 7.0 (http://miRTarBase.mbc.nctu.edu.tw/).[22] Only those targets identified in miRTarBase by reporter assay, Western blot, quantitative PCR (qPCR), or any combination of these methods were accepted by us as experimentally validated.

Promoter de-methylation

In order to assess the methylation status of the miR-503 cluster promoter, Ishikawa H cells were treated with the cytidine analog, 5-aza-2′ deoxycytidine (Sigma, Decitabine). Cells were grown in optimum media for 24 hours and then treated for 72 hours with 1 μM Decitabine in dimethyl sulfoxide (DMSO). Decitabine-containing media was replaced every 24 hours and cells were harvested on day 5 for RNA purification. Control cells were treated in parallel with vehicle (DMSO) only. The entire process was carried out in triplicate.

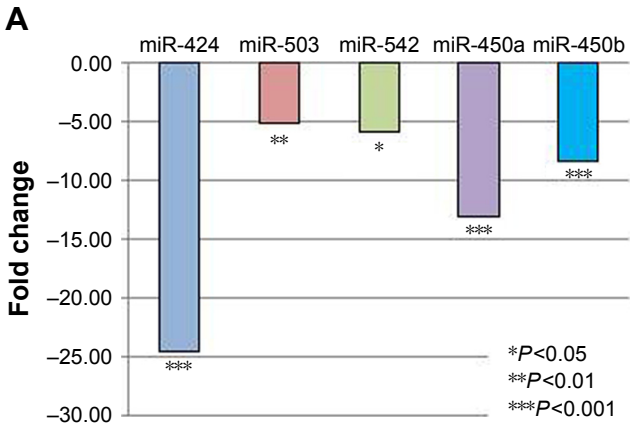
Total cellular RNA was purified and QC validated as previously. MiR-503 cluster member expression levels between treated and un-treated cells were assessed via miR-specific TaqMan assays as previously. Again, differences in expression between Decitabine-treated and un-treated cells were evaluated via the ΔΔCt method described previously with a P-value<0.05 taken as statistically significant.

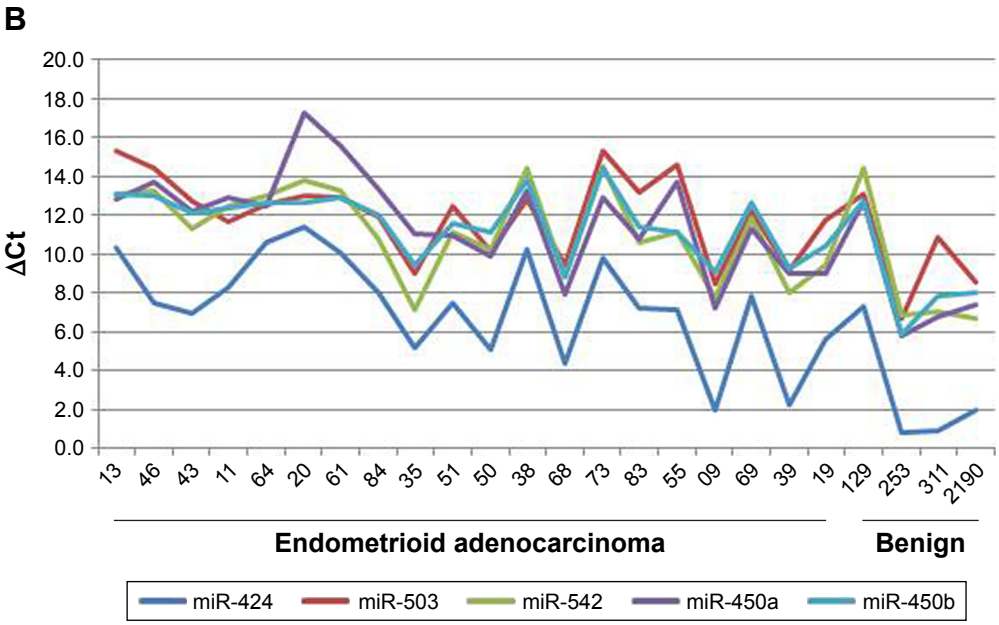
Results

Coordinated under-expression of the miR-503 cluster in EEA

Expression of miR-503 cluster members in endometrioid adenocarcinoma relative to benign endometrium is presented in Figure 1A. As can be seen, each cluster member is significantly under-expressed in the tumors compared with benign endometrium. The magnitude of under-expression ranges from 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 correlation among all five members of the cluster reveals a highly significant pattern of coordinated expression across the entire cluster. As can be seen, correlation coefficients range from 0.76 to 0.94 m and all are statistically significant at $P<0.001$ (Figure 1B).

Coordinate expression is confirmed in The Cancer Genome Atlas (TCGA) where expression of the five cluster members is also uniformly significant at $P<0.001$ with correlation coefficients ranging from 0.56 to 0.88. Moreover, 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 is the same in the two analyses.





Pairwise miR-503 cluster expression correlations

	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***

***P<0.0001

Figure 1 Expression of members of the miR-503 cluster.
Notes: (**A**) Fold change in expression of each member of the cluster in endometrial endometrioid adenocarcinomas vs benign endometrium. (**B**) Pairwise correlation of normalized miRNA expression values (ΔCt) across the entire panel (n=24). Statistical significance was assessed at *df*=22.

Catalog of experimentally validated miR-503 cluster targets

Using the miRTarBase database (Version 7.0) and the extant literature, a total of 55 unique experimentally validated targets were identified for members of the miR-503 cluster (Table 2). Among these are numerous genes commonly regarded as oncogenes, such as FGFR1, MYB, BCL2, PI3K, and MYCN. In addition, 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); and chemotherapy response inhibitors, such as TRAF5 (cisplatin), ZNF217 (paclitaxel), and

Table 2 Experimentally validated targets of members of the miR-503 cluster

miR-424-5p	miR-503-5p	miR-542-3p	miR-450a-5p	miR-450b-5p
CCND1	BCL2	AKT	**DNMT3A**	**DNMT3A**
CCND3	**CCND1**	ANGPT2	**ERBB3**[a]	ENOX2
CCNE1	CDH1	BIRC5[b]	HRNPK2	**ERBB3**[a]
CCNF	DDHD2	BMP7	**IRF2**	**IRF2**
CDC14A	E2F3	COL1A1	STAT1PAX9	
CDC25A	FANCA	CTTN		
CDK6	IGF1R	EGFR		
CHEK1[c]	IKBKB	FTSJ2		
CUL2	PI3K	FZD7		
E2F7	**RICTOR**	ILK		
EPAS1	SMAD2	MTDH		
FASN1	TRAF5[d]	MYCN		
FGFR1	ZNF217[e]	OTUB1[f]		
HIF1α		PIK3R1		
KIF23		RUNX2		
MAP2K1				
MYB				
PLAG1				
RICTOR				
SMAD3				
SOC6				
TNFAIP1				
WEE1[c]				

Notes: Targets defined in the literature as oncogenes regardless of the cancer in which they were reported are shown in bold type. This list was compiled from miRTarBase 7.0 and extant literature. Gene symbols are the most accepted version reported in Gene Cards (www.genecards.org). Targets validated for more than one member of the cluster are shown in bold type. miR-450a-1 and miR-450a-2 are reported as miR-450a only. [a]Implicated in gefintinib resistance. [b]Apoptosis inhibition, survivin. [c]DNA damage response/DNA repair. [d]Implicated in cisplatin resistance. [e]Implicated in paclitaxel resistance. [f]Inhibits ubiquitination of FOXM1.

ERBB3 (gefitinib) are also present. Overall, the targets so far validated for miR-503 cluster members represent a rich array of genes whose dysregulation in cancer would be fortuitous for carcinogenesis, recurrence, and metastasis.

Promoter de-methylation

Changes in expression of miR-503 cluster members as a result of treating Ishikawa cells with Decitabine are shown in Figure 2. Though the fold changes are modest, three of the five cluster members did reach statistical significance. Moreover, 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 in Figure 1 from the primary tumor tissues.

Discussion

The miR-503 miRNA cluster, located at Xq23.1 between hypoxanthine-guanine phosphoribosyltransferase and *PLAC1*, consists of six miRNAs (miR-424, miR-503,

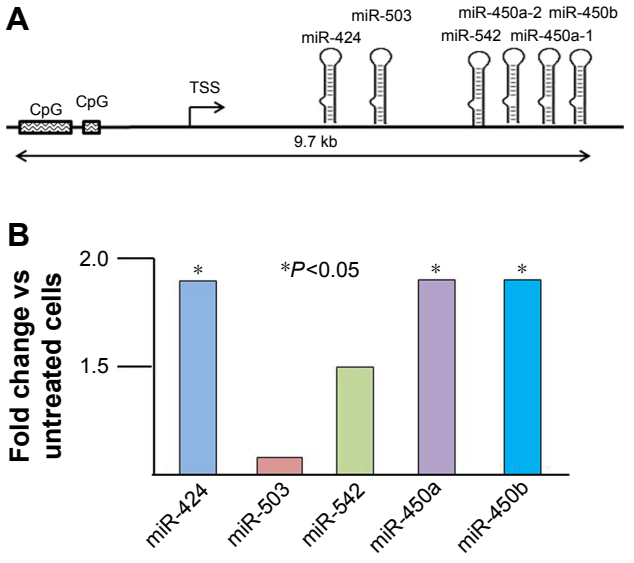


Figure 2 (**A**) Map of the miR-503 cluster locus showing the location of the cluster members, the transcription start site and the CpG islands. (**B**) Change in expression of each member of the miR-503 cluster in Ishikawa H cells treated with the cytidine analog 5-aza-2′ deoxycytidine (Decitabine) compared with vehicle only (untreated) cells.

miR-542, miR-450a-1, miR-450a-2, and miR-450b). We have provided evidence 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. Several studies of individual members of the cluster reported elsewhere have linked down-regulation to carcinogenesis and poor prognosis. Suppression of miR-503 expression in cervical cancer has been linked to significantly reduced progression-free and overall survival in a case–control study.[23] An examination of endometrial cancer progression from normal tissue to hyperplasia and finally, to endometrioid carcinomas showed consistently decreasing levels of miR-503 expression.[24] Moreover, in that same study, overall survival among the cancer patients was positively correlated with miR-503 expression levels.[24] Similarly, miR-424 expression has been shown to be suppressed in endometrial cancers and that de-repression of miR-424 expression inhibits the growth of endometrial cancer cells in vitro.[25] An important observation involving miR-542 is that, not only is it significantly down-regulated in endometrial cancer[26] but also that down-regulation enhances morphological transformation of endometrial stromal cells,[27] which might contribute to the rarer endometrial sarcomas. Finally, while there are fewer studies involving the miR-450 family, their down-regulation in carcinogenesis has also been confirmed albeit not in endometrial cancers until now.[28,29]

We have provided evidence that down-regulation of the cluster in endometrial adenocarcinomas is accomplished via methylation. We acknowledge that the in vitro reactivation of the miR-503 cluster by the cytidine analog Decitabine in Ishikawa cells is, at best, modest but it is consistent with similar suggestions offered in studies in other cancers.[30] Even provisional acceptance of this mechanism opens the possibility of restoring the tumor suppression function of the miR-503 cluster through employing agents, such as cytidine analogs as adjuvants to chemotherapy.

The subtle in vitro data are limitation of this study as is the relatively small patient sample size that was determined by our self-imposed RNA quality threshold. In spite of this, however, further investigation of the miR-503 cluster is warranted by the data presented here. We sought additional support in TCGA and found that expression of four of the five miR-503 cluster members, specifically miR-424, miR-503, miR-450a, and miR-450b, are significantly inversely associated with endometrial cancer grade (OR=0.61, 0.81, 0.75, and 0.81, respectively) and that miR-450b is negatively associated with survival (OR=0.57). The significantly lower expression of miR-503 cluster members seen in our patients compared with controls is consistent with a prediction supported by TCGA data in which both survival and recurrence, implied from tumor grade, are linked with expression of the cluster members. Therefore, we believe that the miR-503 cluster as a whole functions as a tumor suppressor complex in human endometrial cancers. We also believe that this tumor suppressor function is likely disrupted in a coordinated manner in endometrial cancers by hyper-methylation. The list of experimentally validated miR-503 cluster targets reinforces this tumor suppressor role in which up-regulation of many of the targets by methylation inhibition of miRNA expression would be beneficial to establishing, maintaining, and expanding an endometrial cancer. This makes the entire miR-503 cluster an attractive subject for further research and an attractive therapeutic target in endometrial adenocarcinomas for adjuvant use of cytidine analogs along with conventional chemotherapies.

IRB approval

Tumor and control tissues were obtained under informed consent from patients in the University of Iowa Hospitals and Clinics Department of Obstetrics and Gynecology (IRB#200209010) and archived along with complete medical record information in the WHTR (IRB #200910784).

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Author contributions

EJD and JGB conceived, designed, and managed the project under the mentorship of KKL. EC, AW, and MDM carried out all the nucleic acid purifications, cell culture, in vitro cell treatments, and miR-specific qPCR assays. EJD and JGB performed all data analyses. All authors contributed to writing the manuscript and approved the submission. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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