

Global dysregulation of the chromosome 14q32 imprinted region in uterine carcinosarcoma

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Abstract. Uterine carcinosarcoma (UCS) is a rare but very aggressive cancer of the female reproductive tract with an extremely poor prognosis. With the goal of understanding the role of microRNA (miRNA) dysregulation in these tumors, we profiled the expression of 667 human miRNAs in a panel of eight UCS patients and five benign control primary tissue samples. These expression profiles revealed two important characteristics of UCS. First, compared with the two most common uterine cancers, endometrial endometrioid adenocarcinoma and endometrial serous adenocarcinoma, UCS samples display a virtually unique pattern of miRNA dysregulation with an overlap of only 5% among the three tumor types. In addition, nearly one-third of the miRNAs significantly dysregulated in UCS tissues compared with benign endometrium (32 of 114) lie in a single small (250-kb) imprinted region of chromosome 14q32. These data suggest that the presence of such a global, region-specific disruption substantially contributes to the unique histology and poor outcome of this type of cancer.

Introduction

Uterine carcinosarcoma (UCS), also known as malignant mixed Müllerian tumor (MMMT), is a rare and aggressive neoplasia characterized histologically by both carcinomatous/epithelial and sarcomatous/mesenchymal elements (1). These tumors are generally thought to account for 3-7% of uterine cancers (2). In general, UCSs present in the late seventh decade of life, are quite advanced and have a very poor prognosis due to the aggressive nature of the tumors (3). An epidemiological study revealed that UCS and endometrial carcinoma share a similar risk factor profile including obesity and unopposed estrogen exposure (4). Moreover, the behavior of UCS more closely resembles that

of the highly aggressive endometrial carcinomas (e.g. serous adenocarcinomas) than that of other uterine sarcomas (5,6). For these reasons, the 2009 revised FIGO staging for uterine sarcomas recommends that UCS be staged as a carcinoma of the endometrium (1). Collectively, these clinical data reflect a need to better understand the molecular characteristics of UCS, particularly the similarities and differences with other endometrial carcinomas.

The most contentious aspect of UCS in the past has been the origin of the tumor itself. Certain investigators have argued that UCS tumors are actually two independent tumors (the collision model), while others contend that UCS begins as a single tumor that differentiates into carcinomatous and sarcomatous elements (the monoclonal model). While the collision model has not been completely excluded, an increasing number of molecular studies support the monoclonal model. For example, patterns of X-inactivation in most UCSs are identical in the epithelial and mesenchymal components (7,8). Similar findings have been reported for loss of heterozygosity (LOH), the tumor suppressor TP53, the oncogene KRAS and microsatellite instability (MSI) (8-11). Thus, the emerging consensus is that the majority of UCSs are monoclonal.

One aspect of UCS that has received little attention to date is the pattern of microRNA (miRNA) expression in these tumors compared either with benign endometrium or with other endometrial cancers. miRNAs are small (21 to 23 nt) regulatory RNAs whose known role in oncogenesis and cancer progression is rapidly expanding (12,13). We determined the expression of 667 miRNAs in a panel of eight UCS tissue samples and compared the expression patterns with a panel of five benign endometrium, four endometrial endometrioid adenocarcinoma and four endometrial serous adenocarcinoma tissue samples. We found that the expression of 114 miRNAs was significantly dysregulated (106 overexpressed and eight underexpressed) as compared with benign endometrium, but only nine of these miRNAs were found to overlap with those significantly dysregulated in endometrial adenocarcinomas. We also found that, among the 105 significantly dysregulated miRNAs that do not overlap with the adenocarcinomas, nearly one-third (32 miRNAs) lie in the 250-kb imprinted region in chromosome 14q32. Moreover, all but three of the 61 miRNAs in the region whose expression we measured showed at least two-fold overexpression as compared with benign endometrium.

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Global dysregulation of this region in UCS is further evidenced by the altered expression of the reciprocally imprinted δ -like homolog 1 (*Drosophila*) (DKL1)/non-coding RNA locus maternally expressed 3 (GTL2) (also known as MEG3) locus that also lies in the same chromosome 14q32 imprinted region (14,15). The dysregulation of expression of such a large number of miRNAs in such a small region suggests the possibility of the disruption of a single regulatory factor, particularly in view of the evidence that the entire region is imprinted.

Materials and methods

Carcinosarcomas and benign endometrium. Primary carcinosarcoma tissues (n=8) and samples of benign endometrium (n=5) were obtained from patients undergoing surgery in the Gynecologic Oncology Division of the Department of Obstetrics and Gynecology at the University of Iowa Hospitals and Clinics. The study was approved by the Institutional Review Board and informed consent was obtained from all patients. All carcinoma patients presented as grade 3 in stages IB to IIIC. Benign endometrium was obtained from post-menopausal patients who were cancer-free. All tissue specimens were snap-frozen and stored at -80°C following histological evaluation.

RNA purification. Tissue samples were transitioned from -80 to -20°C following perfusion in RNAlater ICE (Ambion, Life Technologies) according to the supplier's instructions. Total RNA was extracted from homogenized tissue using the miRvana miRNA isolation kit (Ambion, Life Technologies). RNA yield and quality were assessed on a NanoDrop M-1000 spectrophotometer and an Agilent 2100 Bioanalyzer. An RNA quality cut-off value at RIN ≥ 7.0 was adhered to and RNAs meeting that cut-off value were standardized to 200 ng/ μl (NanoDrop confirmed) for all subsequent analyses.

qPCR assays. miRNA expression levels were determined on miRNA TaqMan Low Density Arrays (TLDA; Applied Biosystems, Life Technologies). The A-set (v. 2.0, 377 miRNAs) and the B-set (v. 2.0, 290 miRNAs) TLDA cards were used for these assays. Reverse transcription of 600 ng of total RNA was carried out for each tissue and TLDA card using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Life Technologies) and either the Human A-pool (v. 2.1) or Human B-pool (v. 2.0) Megaplex RT primers (Applied Biosystems, Life Technologies).

All 26 TLDA cards were processed on an Applied Biosystems Model 7900 Genetic Analyzer and the resulting data analyzed using Applied Biosystems StatMiner software. The expression of all 667 miRNAs in all samples was normalized against the expression of the RNU48 endogenous control RNA. Comparison of individual, normalized miRNA expression levels was performed using the $\Delta\Delta\text{Ct}$ method where fold change between carcinosarcoma and benign endometrium is given as $2^{-\Delta\Delta\text{Ct}}$ for each miRNA (16). Statistical significance of the fold change estimates were assessed using two-tailed t-tests with unequal variances and potential false discovery was adjusted via Bonferroni correction of the p-values. A p-value of ≤ 0.05 was considered to indicate a statistically significant difference.

mRNA expression assays. Expression of the reciprocally imprinted DKL1/GTL2 locus was examined using 500 ng of RNA from each tissue using Applied Biosystems (Life Technologies) gene-specific TaqMan Assays. RNAs were reverse-transcribed with random hexamers using the Applied Biosystems (Life Technologies) High Capacity cDNA Reverse Transcription Kit. Expression differences between carcinosarcomas and benign endometrium were calculated using the $\Delta\Delta\text{Ct}$ method as above. The expressions of DLK1 and GTL2 were normalized against 18S rRNA prior to analysis.

Bisulfite sequencing. To assess methylation status, bisulfite sequencing was performed on the differentially methylated region (DMR) lying between DLK1 and GTL2 in the carcinosarcomas. DNA was purified from tumor tissue and 2 μg aliquots were bisulfite converted using the EZ DNA Methylation-Direct Kit (Zymo Research) according to the manufacturer's instructions. The DKL1/GTL2 DMR was then amplified from converted DNA using the following primer pair, DMRFOR: 5'-GTGGATTTGTGAGAAATGATTTTGT-3' and DMRREV: 5'-CCATTATAACCAATTACAATACCAC-3' as specified by Guens *et al* (15). Amplicons were cloned into pGEM T-EASY (Promega), multiple colonies were selected for each tumor and DNA was prepared using the QIAprep Spin Miniprep Kit (Qiagen). These DNAs were then sequenced in both directions on an Applied Biosystems Model 3730xl DNA Sequencer using vector M13 primers.

Results

miRNA expression. The results of the TLDA array comparisons of the eight UCS samples with the five benign endometrium samples are presented in Table I. A total of 114 miRNAs were significantly altered in the UCSs versus benign endometrium after the p-values were adjusted by Bonferroni correction to reduce the false discovery rate (FDR) (17). The majority of these differences are in the direction of overexpression among the UCSs (n=106) as opposed to underexpression among the UCSs (n=8). A number of miRNAs, including *miR-431*, *miR-888* and *miR-206*, are very highly expressed in UCS tissues as compared with benign samples.

When compared with the 71 miRNAs found to be significantly dysregulated among endometrial adenocarcinomas (18), only nine were found to overlap with altered UCS miRNAs (Table I). These nine miRNAs are the underexpressed *miR-133a* and *let-7c* and the overexpressed *miR-107*, *miR-181c**, *miR-205*, *miR-210*, *miR-516a-3p*, *miR-888* and *let-7f-2**.

Among the twenty most highly expressed miRNAs are *miR-431*, *miR-432*, *miR-541*, *miR-543* and *miR-369-3p*. These five miRNAs are all from the cluster of miRNAs found in the imprinted region of chromosome 14q32, demonstrating that a quarter of the most highly expressed miRNAs in UCS are found in this small (250 kb) region. A complete scan of the A-set and B-set miRNAs on the TLDA arrays revealed that 43 miRNA loci in this chromosome 14q32 region, accounting for a total of 61 miRNAs (9.2%), were represented. The current miRNA content of the chromosome 14q32 cluster is 57 loci accounting for 71 miRNAs (miRBase, release 17 April 2011). Of the 61 chromosome 14q32 cluster miRNAs represented on the TLDA cards, all but three (*miR-154*, *miR-329* and

Table I. Fold change and adjusted p-values of the 114 microRNAs significantly dysregulated in UCSs compared with benign endometrium.

MicroRNA	Fold change	Adjusted p-value	MicroRNA	Fold change	Adjusted p-value	MicroRNA	Fold change	Adjusted p-value
Underexpressed								
miR-29c	-10.6	0.009	miR-143	-11.7	0.009	miR-145	-8.4	0.02
miR-195*	-7.1	0.03	<i>let-7c</i>	-7.5	0.03	<i>miR-133a</i>	-8.2	0.04
let-7b	-6.2	0.04	miR-29a	-6.0	0.04			
Overexpressed								
miR-431	1525.3	0.0001	<i>miR-888</i>	132.7	0.0001	miR-216b	91.5	0.0001
miR-543	65.8	0.0002	miR-206	135.1	0.0003	miR-330-5p	46.9	0.0003
miR-15b*	56.4	0.0003	miR-153	30.1	0.0004	miR-183	43.7	0.0004
miR-33b	30.2	0.0004	let-7f-1*	79.4	0.0004	miR-432	70.2	0.0007
miR-541	32.9	0.0008	miR-369-3p	67.9	0.0008	miR-20b*	92.9	0.001
miR-622	20.9	0.001	<i>miR-205</i>	26.4	0.002	miR-769-3p	86.7	0.002
miR-433	36.1	0.002	miR-182	23.3	0.003	miR-183*	26.4	0.003
miR-656	20.3	0.004	miR-605	23.0	0.004	miR-493*	31.2	0.004
miR-376a*	33.0	0.004	<i>miR-516a-3p</i>	17.1	0.005	miR-296-3p	18.1	0.005
miR-615-3p	53.8	0.006	miR-551a	43.7	0.006	miR-192*	17.5	0.006
miR-376b	32.6	0.006	miR-892a	58.3	0.006	miR-130b*	15.8	0.006
miR-488*	38.7	0.006	miR-154*	32.9	0.006	miR-377	46.8	0.006
miR-889	15.4	0.007	miR-496	40.9	0.007	miR-668	32.8	0.007
miR-541*	35.7	0.007	miR-18a*	12.4	0.008	miR-493	15.4	0.008
miR-518d-3p	14.2	0.008	miR-323-3p	13.4	0.009	miR-155*	28.9	0.009
miR-7	13.3	0.009	miR-641	25.8	0.01	miR-135b*	17.5	0.01
miR-223*	12.6	0.01	miR-654-5p	17.3	0.01	miR-922	26.7	0.01
miR-217	14.1	0.01	miR-551b*	9.7	0.01	miR-539	13.3	0.01
miR-147b	12.8	0.01	miR-499-5p	13.7	0.01	miR-382	18.4	0.01
miR-887	10.7	0.01	miR-195*	21.1	0.02	miR-639	11.0	0.02
miR-373	22.5	0.02	<i>miR-107</i>	10.0	0.02	miR-582-3p	10.2	0.02
miR-891a	14.6	0.02	miR-409-5p	10.5	0.02	miR-138-1*	9.6	0.02
<i>miR-210</i>	8.1	0.02	<i>let-7f-2*</i>	10.1	0.02	let-7a*	13.5	0.02
miR-651	10.7	0.02	miR-198	9.8	0.02	miR-346	8.2	0.02
miR-146a*	17.5	0.02	miR-937	12.0	0.02	miR-450b-3p	16.6	0.02
miR-505	9.4	0.02	miR-491-3p	18.0	0.02	miR-501-3p	13.4	0.02
miR-369-5p	8.5	0.02	miR-630	7.6	0.02	<i>miR-181c*</i>	8.3	0.03
miR-18b	8.4	0.03	miR-643	7.6	0.03	miR-506	15.7	0.03
miR-943	16.1	0.03	miR-570	7.7	0.03	miR-188-3p	14.0	0.03
miR-299-3p	8.7	0.03	miR-941	8.7	0.03	miR-584	8.5	0.03
miR-545*	7.9	0.03	miR-638	8.0	0.04	miR-409-3p	9.6	0.04
miR-875-5p	7.5	0.04	miR-485-3p	8.9	0.04	miR-379*	7.5	0.04
miR-561	7.2	0.04	miR-661	7.2	0.04	miR-432*	7.1	0.04
miR-223	7.0	0.04	miR-487b	8.6	0.05	miR-301b	8.2	0.05
miR-127-5p	6.7	0.05	miR-654-3p	7.4	0.05	miR-376a	7.2	0.05
miR-372	6.8	0.05						

MicroRNAs in the chromosome 14q32 imprinted cluster are shown in bold type. MicroRNAs shared between UCSs and endometrioid adenocarcinomas are italicized. UCS, uterine carcinosarcoma.

miR-412) were overexpressed in the UCSs by at least two-fold and, of these, 32 achieved statistical significance (Fig. 1). Thus, while chromosome 14q32 miRNAs represent 9.2% of

the miRNAs present on the TLDA arrays, they account for a statistically significant 30.5% ($\chi^2=56.9$, $p<0.001$) proportion of the significantly overexpressed miRNAs. Moreover, among

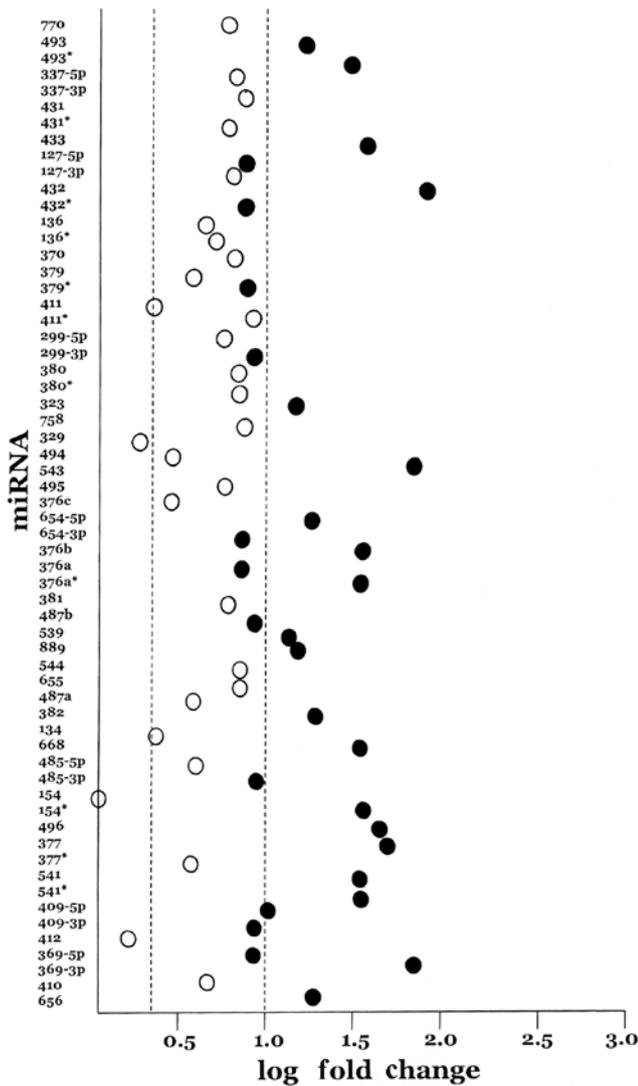


Figure 1. Log fold change of 61 microRNAs in the chromosome 14q32 imprinted region. The microRNAs are shown in their physical order from 5' (*miR-770*) to 3' (*miR-656*). The dashed lines indicate 2-fold and 10-fold increased expression in UCSs compared with benign endometrium. Closed circles represent those microRNAs reaching at least $p \leq 0.05$ statistical significance and open circles indicate those microRNAs at $p > 0.05$.

the 18 miRNAs in which mature sequences from both sides of the hairpin are expressed, that is, both the 5p and the 3p arms, eight show nearly equal expression.

DLK1/GTL2 expression in UCS. On the centromeric flank of the chromosome 14q32 miRNA cluster is the reciprocally imprinted locus composed of *DLK1* and *GTL2*. In order to discover whether the global dysregulation of the miRNAs in chromosome 14q32 extends to other genes, the expression of both *DLK1* and *GTL2* was assessed in the UCS tumor panel as well as the benign endometrium group. *DLK1/GTL2* expression in UCSs and benign endometrium, normalized against 18S rRNA transcription, is shown in Fig. 2. These data show that *GTL2* expression is higher (lower ΔCt) than *DLK1* expression (higher ΔCt) in the benign endometrium panel whereas levels are nearly equal in the UCS panel. In a reciprocally imprinted locus a modest negative correlation of expression levels of the two transcripts is anticipated. In the

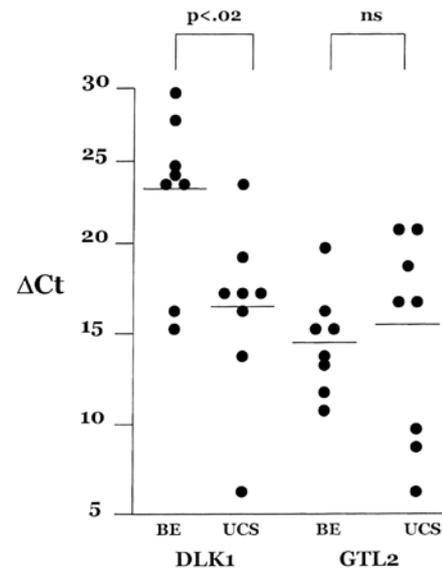


Figure 2. Expression of *DLK1* and *GTL2* mRNA in uterine carcinosarcoma (UCS) and in benign endometrium (BE). Three additional benign samples were added to the panel to equalize sample sizes. *DLK1* shows a significant increase in expression in UCS compared with benign endometrium while *GTL2* shows no change in expression. ns, not significant.

benign endometrium panel, the correlation between *DLK1* and *GTL2* is $r = -0.21$ [not significant (ns)] whereas we see a positive correlation in the UCS panel, $r = 0.52$ (ns). While *GTL2* expression levels were not altered in the UCSs as compared to benign endometrium, the *DLK1* expression level increased 92-fold ($p < 0.02$) in the UCSs. This result suggests that paternal chromosome expression (*DLK1*) has become uncoupled from maternal chromosome expression (*GTL2*).

Previous studies have shown that reciprocal expression of *DLK1* and *GTL2* is regulated in part by the methylation status of a DMR located between the two loci (14). Bisulfite sequencing of the *DLK1/GTL2* DMR in the eight carcinosarcomas and five benign endometrium samples revealed no change in methylation patterns. This implies that the change in expression of *DLK1* in UCS tissues versus benign endometrium is not attributable to altered methylation.

Discussion

UCSs display a unique miRNA expression signature. A recent TLDA-based miRNA expression survey of uterine cancers including a small number of UCSs concluded that these tumors display a unique miRNA dysregulation signature compared with other uterine cancers, specifically endometrial adenocarcinomas (19). Our data reinforce and expand upon this observation. Specifically, we show from our larger panel of miRNAs that, among the same 667 miRNAs surveyed by us in both endometrial adenocarcinoma (18) and UCS, only nine of the 71 miRNAs significantly dysregulated in endometrial adenocarcinoma compared with benign endometrium overlap with the 114 miRNAs significantly dysregulated in UCS compared with benign endometrium. Apart from these few miRNAs, it is the unique miRNA signature of UCSs that dominates the data presented here. A recent TLDA-based miRNA study of UCSs focused upon

the biphasic nature of these tumors (20). Using the A-set miRNAs, the present study separated the epithelial from the mesenchymal components of 23 tumors and reported on a modest differential miRNA expression signature observed in the two components. Not surprisingly, a number of the miRNAs reported in that study also appear in the present study. Among these is *miR-29c* which is more highly expressed in the epithelial component but significantly underexpressed overall compared with benign endometrium. This miRNA has been shown to be involved in the regulation of the extracellular matrix marker SPARC and other markers associated with invasion and metastasis (21). Another miRNA shown to be differentially expressed in UCSs and underexpressed overall in UCSs compared with benign endometrium is *miR-23b* which is also negatively associated with the proliferation of cancer cells (22). One notable finding is that none of the five members of the *miR-200* family, known to be intimately involved in epithelial to mesenchymal transition (19,23-25) and significantly overexpressed in the epithelial component of UCSs (20) as well as in endometrial adenocarcinomas (18), achieved statistical significance in our data, although all five *miR-200* family members were more highly expressed in UCSs than in benign endometrium.

miRNAs in the chromosome 14q32 imprinted region are globally dysregulated. In the above-mentioned study on miRNA expression in the epithelial and mesenchymal components of UCSs (20), 24 miRNAs were shown to be more highly expressed in the mesenchymal component than in the epithelial component. Of these, seven (29.2%) are from the imprinted region at chromosome 14q32. This is a statistically significant over-representation of the chromosome 14q32 imprinted region miRNAs present on the A-set TLDA card ($\chi^2=7.7$, $p<0.05$) and is consistent with the statistically significant overrepresentation of miRNAs from this region in UCSs compared with benign endometrium that we report here. Our data also demonstrate that the dysregulation of miRNA expression among the UCSs is distributed across the entire 251 kb breadth of the region. However, the extent of this phenomenon is clearly contained within the boundaries of the imprinted region as three miRNAs at the 5' boundary, *miR-342-5p*, *miR-342-3p* and *miR-345*, and the miRNA closest to the 3' boundary, *miR-203*, are all expressed at the same level in the UCSs as in the benign endometrium. Future studies are necessary to understand the mechanisms by which miRNA expression in this region is coordinately regulated and dysregulated.

The correlated expression of miRNAs in the chromosome 14q32 imprinted region has previously been reported. For example, correlated overexpression was observed in acute myeloid leukemia (26) and in gastrointestinal stromal tumors without loss of chromosome 14q (27). Cytogenetic effects, either amplifications or deletions, are not at issue here as no such events have ever been reported on chromosome 14 in uterine cancers (28,29).

miRNAs in the chromosome 14q32 imprinted cluster show uncharacteristically low complementarity with cellular mRNA targets, suggesting that they are involved in a shared mechanism of action involving general inhibition of translation rather than degradation of specific mRNA targets (27). In mice, coordinated expression of the entire cluster has

been linked with a DMR between the imprinted non-miRNA loci *DLK1/GTL2* (30). Our examination of the methylation status of this DMR in relation to *DLK1/GTL2* expression in our UCSs and benign endometrium clearly shows that this *cis*-acting regulation is disrupted, and it is likely that this regulatory disruption extends throughout the region and results in the observed miRNA expression patterns as well.

We have carried out an miRNA expression survey of UCSs which confirms that these tumors display a largely unique miRNA signature. Most striking is the observation that the entire imprinted region at chromosome 14q32 is dysregulated with all but one of the 61 miRNAs assayed overexpressed in UCSs compared with benign endometrium and more than half (32 of 60) reaching statistical significance. We find that this dysregulation includes the reciprocally imprinted gene pair *DLK1/GTL2* lying at the upstream boundary of the region where, in spite of a 'normal' methylation pattern in the intergenic DMR, both members of the pair are highly expressed. It is possible that this DMR regulates expression of the entire region in *cis* (30). Coordinated overexpression of such a large block of miRNAs may contribute to the unique nature and aggressive behavior of these tumors. In addition, the significant increase in *DLK1* expression may also play a role, as *DLK1* has been shown to inhibit Notch signaling in mesenchymal multipotent cells (31). Whatever the effect of increased *DLK1* expression coupled with the global overexpression of the chromosome 14q32 miRNA cluster may turn out to be, understanding the mechanism that leads to the dysregulation of multiple miRNAs at once may present a unique opportunity to identify a therapeutic target whose effects are widespread.

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