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Epigenetic silencing of monoallelically methylated miRNA loci in precancerous colorectal lesions

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ORIGINAL ARTICLE

Epigenetic silencing of monoallelically methylated miRNA loci in precancerous colorectal lesions

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Epigenetic silencing of protein-encoding genes is common in early-stage colorectal tumorigenesis. Less is known about the methylation-mediated silencing of genes encoding microRNAs (miRNAs), which are also important epigenetic modulators of gene expression. Using quantitative PCR, we identified 56 miRNAs that were expressed in normal colorectal mucosa and in HT29 colorectal cancer cells treated with demethylating agents but not in untreated HT29 cells, suggesting that they probably undergo methylation-induced silencing during colorectal tumorigenesis. One of these, *miR-195,* had recently been reported to be underexpressed in colorectal cancers and to exert tumor-suppressor effects in colorectal cancer cells. We identified the transcription start site (TSS) for primary miRNA (*pri-miR)-497/195*, the primary precursor that yields *miR-195* and another candidate on our list, *miR-497*, and a single CpG island upstream to the TSS, which controls expression of both miRNAs. Combined bisulfite restriction analysis and bisulfite genomic sequencing studies revealed monoallelic methylation of this island in normal colorectal mucosa (50/50 samples) and full methylation in most colorectal adenomas (38/50; 76%). The hypermethylated precancerous lesions displayed significantly downregulated expression of both miRNAs. Similar methylation patterns were observed at two known imprinted genes, *MEG3* and *GNAS-AS1,* which encode several of the 56 miRNAs on our list. Imprinting at these loci was lost in over half the adenomas (62% at *MEG3* and 52% at *GNAS-AS1*). Copy-number alterations at *MEG3*, *GNAS-AS1* and *pri-miR-497/195,* which are frequent in colorectal cancers, were less common in adenomas and confined to tumors displaying differential methylation at the involved locus. Our data show that somatically acquired, epigenetic changes at monoallelically methylated regions encoding miRNAs are relatively frequent in sporadic colorectal adenomas and might contribute to the onset and progression of these tumors.

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Keywords: *miR-497*; *miR-195*; CpG-island methylation; allele-specific DNA methylation

INTRODUCTION

Discovered almost 30 years ago,[1] cancer-related epigenetic alterations in gene expression patterns have now been found in almost every component of human chromatin.[2] The most widely studied changes of this type involve DNA methylation at CpG dinucleotides, which can affect the expression of protein-coding genes and noncoding RNAs, such as microRNAs (miRNAs).[3] The latter bind to messenger RNAs (mRNAs) with base-pair sequences more or less complementary to their own, causing degradation of the target transcript or repressing its translation.[4] miRNAs are believed to regulate the translation of over 60% of all protein-coding genes,[5] including many known oncogenes and tumor suppressors.[6] Epigenetic silencing of protein-encoding genes is a well-documented feature of colorectal tumorigenesis,[7–10] but less is known about the epigenetic regulation of miRNA genes in this setting.

We recently screened 742 human miRNAs to identify those whose expression in the colon might be silenced by DNA methylation during the course of malignant transformation. Unexpectedly, the candidates that emerged included several miRNA genes that were monoallelically methylated in normal colorectal mucosa, including some located in imprinted loci of the genome. The parent-of-origin-specific monoallelic expression that characterizes imprinting[11] has crucial roles in normal growth and development.[12] Genetic and/or epigenetic alterations can activate the normally silenced allele or silence the one that is normally expressed,[13] and this loss of imprinting (LOI) has been described in several types of cancer.[14] To date, ~80 imprinted human genes have been identified (http://www.geneimprint.com/), but bioinformatic predictions indicate that the true number is probably higher.[11,15] Allele-specific DNA methylation (ASM) of nonimprinted genes also seems to be widespread in the human genome,[16] and loci of this type are also potential targets of epigenetic changes relevant for tumorigenesis.

Here, we show that a single CpG island controls the expression of two miRNAs, *miR-497* and *miR-195*, which appear to undergo silencing during colorectal tumorigenesis. In normal colorectal mucosa, this island was found to be monoallelically methylated, but in precancerous colorectal lesions, biallelic methylation was more common, a pattern similar to that observed at the CpG islands of two other multi-miRNA-encoding loci (*MEG3* and *GNAS-AS1*), which are known to be imprinted.

RESULTS

Using quantitative real-time PCR (qPCR), we measured the levels of 742 human miRNAs in HT29 colorectal cancer cells before and

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after treatment with the DNA-demethylating agent 5-aza-2-deoxycytidine and the histone deacetylase inhibitor trichostatin A and in a single sample of normal colonic mucosa. Fifty-six miRNA genes had expression patterns suggestive of methylation-induced silencing during colorectal tumorigenesis, that is, constitutive expression in normal mucosa, loss of expression in HT29 cells and restored expression in HT29 cells treated with 5-aza-2-deoxycytidine/trichostatin A (Table 1, left column).

Shortly after we completed these experiments, one of the candidate miRNAs, *miR-195*, was reported to be underexpressed in colorectal cancers (73% of the 81 carcinomas analyzed) and to exert tumor-suppressor activity in HT29 and LoVo colon cancer cells involving the reduction of BCL2 levels.[17] These findings, along with reports of miR-195-induced tumor suppression in hepatocellular,[18,19] adrenocortical[20] and peritoneal tumorigenesis,[21] prompted us to investigate the mechanism underlying *miR-195* downregulation during colorectal tumorigenesis.

Given the criteria used in our screening study, the most likely cause seemed to be cytosine methylation at CpG island(s) in the *miR-195* promoter region. *MiR-195* and *miR-497*, another miRNA gene that appeared to be epigenetically silenced during colorectal-cell transformation (Table 1), are both encoded in the first intron of the *MIR497HG* gene (Gene ID: 100506755) on chromosome 17p13.1. The transcription start site (TSS) for the primary precursor that yields both these miRNAs (primary miRNA (*pri-miR)-497/195*) had not been identified, but it was predicted to be independent of its host-gene promoter.[22,23] We identified this site by subjecting total RNA from the normal mucosa specimen to rapid amplification of cDNA ends (5′ RACE) (Figure 1a) and explored a CpG island flanking this site as a potential target of cytosine methylation (Figure 1b).

Using combined bisulfite restriction analysis (COBRA), we investigated the methylation status of this island in HT29 cells and five other colorectal cancer lines and in 50 paired adenoma-normal mucosa samples (Table 2). The extensive methylation of this island documented in all six cell lines (Figure 1c) suggests that this phenomenon is quite common in advanced colorectal cancers with different genetic and epigenetic backgrounds and/or in cultured cells in general. In all 50 samples of normal mucosa (controls), methylation of this CpG island was observed in roughly half of the alleles (see representative example in Figure 1c). The presence of methylated and unmethylated alleles in these samples might have reflected differences between the epithelial and mesenchymal components of the mucosa, as described for *miR-200b*.[24] To explore this possibility, we isolated lamina propria and epithelial crypts from a single surgical specimen of normal mucosa and subjected the DNA from each tissue component to COBRA. Both contained methylated and unmethylated *pri-miR-497/195* alleles (Figure 1d). RT–PCR confirmed the purity of the isolated tissue components and the presence in both of *miR-497* and *miR-195* (although they were more abundantly expressed in the lamina propria) (Figure 1e).

As for the 50 colorectal adenomas, 38 (76%) exhibited extensive methylation of *pri-miR-497/195* alleles. Representative COBRA experiments are shown in Figure 2a. In the remaining 12 (24%), *pri-miR-497/195* allele methylation resembled that observed in the corresponding samples of normal mucosa (for example, the one shown for patient 9 in Figure 2a). A similar picture emerged when subcloned PCR products of bisulfite-converted DNA from these tissues were sequenced: heavy methylation was observed in almost all the *pri-miR-497/195* alleles in adenomas but in only ~50% of those in the normal mucosal specimens (Figure 2b).

Using RT–PCR, we compared *miR-497* and *miR-195* expression in five adenomas with hypermethylation at *pri-miR-497/195* and five others with methylation levels at this locus comparable to those in the normal mucosal samples. The significantly lower transcript levels of both mRNAs found in the hypermethylated tumors (Figure 2c) support our view that the CpG island we analyzed has a role in the epigenetic control of *miR-497/195* cluster transcription.

Table 1. MicroRNAs that were re-expressed in HT29 cells after treatment with 5-aza-2-deoxycytidine and trichostatin A

Expressed in normal colorectal mucosa	*Not expressed in normal colorectal mucosa*
miR-199a-3p	*miR-603*
miR-664	*miR-518f*
miR-9	*miR-520b*
miR-942	*miR-891a*
miR-28-3p	*miR-526b**
miR-138	*miR-523*
miR-95	*miR-525-5p*
miR-145	*miR-518e*
miR-218	*miR-512-5p*
miR-133b	*miR-518e**
miR-30a	*miR-518d-5p*
miR-148a	*miR-520h*
miR-489	*miR-517a*
*miR-550**	*miR-524-3p*
miR-595	*miR-373*
miR-124	*miR-519a*
*miR-126**	*miR-182**
*let-7i**	*miR-105*
*let-7f-1**	*miR-34c-5p*
*miR-23b**	*miR-1908*
miR-146b-5p	*miR-515-3p*
miR-146b-3p	*miR-122*
miR-130a	*miR-891b*
miR-129-3p	*miR-520e*
*miR-616**	*miR-371-5p*
miR-342-3p	*miR-769-3p*
miR-136	*miR-767-5p*
miR-432	*miR-519e*
miR-370	*miR-520c-3p*
miR-127-3p	*miR-519d*
miR-376c	*miR-517c*
miR-154	*miR-518b*
miR-409-3p	*miR-525-3p*
miR-495	*miR-520f*
miR-411	*miR-520d-3p*
miR-376a	*miR-515-5p*
miR-543	*miR-372*
miR-382	*miR-518c**
miR-485-3p	*miR-520g*
miR-487b	*miR-518c*
miR-323-3p	*miR-526b*
miR-497	*miR-519c-3p*
miR-195	*miR-512-3p*
miR-144	*miR-517**
miR-142-5p	*miR-557*
miR-1	*miR-498*
miR-1539	*miR-371-3p*
miR-524-5p	*miR-519b-3p*
miR-517b	*miR-129-5p*
miR-518a-3p	*miR-516a-5p*
miR-296-5p	*miR-509-3-5p*
miR-502-3p	
miR-766	
miR-188-3p	
miR-363	
miR-450a	

Boldface represents miRNA cluster in chromosome 14q32 (*MEG3* locus).

The asymmetric methylation of this island observed in normal mucosal DNA was suggestive of genomic imprinting, a phenomenon known to affect loci encoding several miRNAs listed in Table 1. We examined the monoallelically methylated CpG islands of two of these imprinted loci in DNA from our paired adenoma-normal mucosa samples: *GNAS-AS1,* which encodes *miR-296*,[25,26]

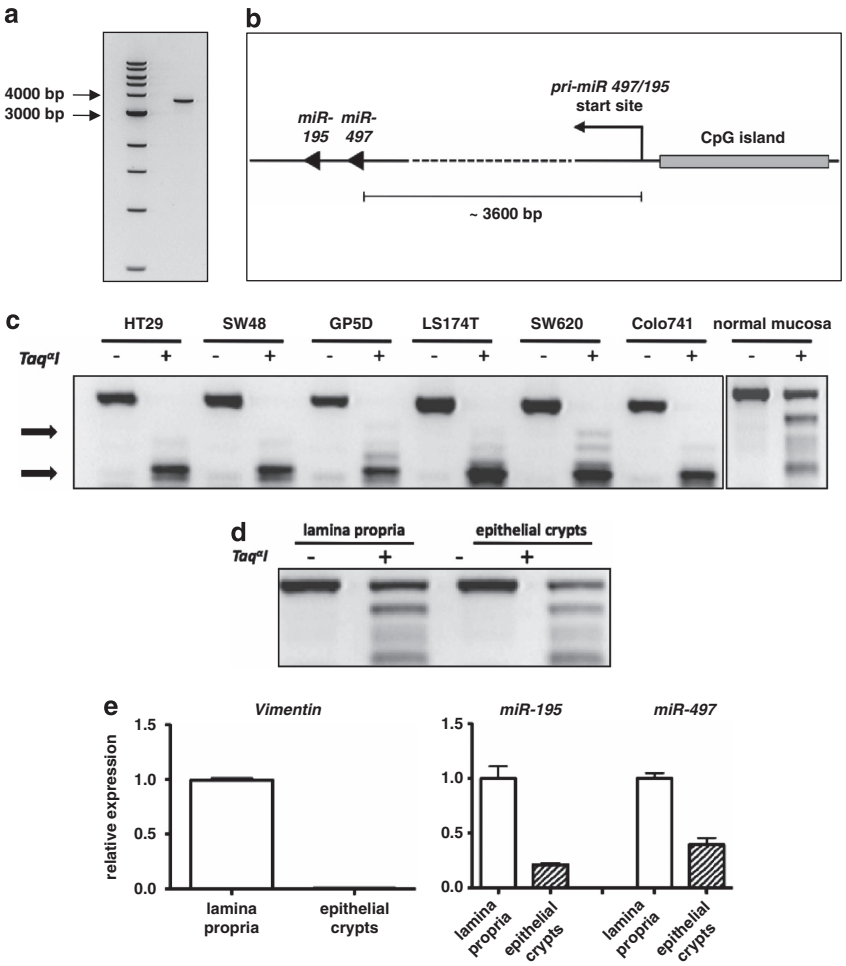

Figure 1. Identification of the *pri-miR-497/195* TSS in normal human colorectal mucosa and assessment of the methylation status of the CpG island located upstream from this site. (**a**) 5′ RACE: a 3638-bp product was obtained by nested PCR with the gene-specific outer and inner primers listed in Material and methods. (**b**) Schematic showing locations of *miR-497* and *miR-195*, the *pri-miR-497/195* TSS and the CpG island located upstream from the latter (genome coordinates of *pri-miR-497/195* from its TSS to *miR-497* according to the Human Reference Sequence GRCh37/hg19: chr17, 6 921 342–6 924 948). The position of the 294-bp COBRA amplicon is 6 926 487–6 926 780. (**c**) COBRA analysis of six colorectal cancer cell lines and a control sample of normal colon mucosa. Arrows indicate *Taq*$^{\alpha}$*I*-digested DNA fragments representing methylated alleles; slower-migrating fragments represent undigested, unmethylated DNA. (**d**) COBRA revealed methylated and unmethylated *pri-miR-497/195* alleles in isolated epithelial cells and isolated stromal (lamina propria) cells from the normal mucosa. (**e**) Left panel: the purity of the epithelial cell preparation was confirmed by qPCR exclusion of the expression of *Vimentin*, a stromal marker that was abundant in the lamina propria specimens. Right panel: RT–PCR revealed higher expression of *miR-195* and *miR-497* in the stromal component.

and *MEG3*, which encodes 16 miRNAs in our list.[27,28] The results recalled those obtained for *pri-miR-497/195* CpG island. Methylated and unmethylated alleles of *GNAS-AS1* and *MEG3* were found in all 50 specimens of normal mucosa, whereas methylated alleles of both loci predominated in most adenomas (Figures 3a and b), reflecting tumor-related LOI. Twenty-six (52%) of the 50 adenomas presented LOI at *GNAS-AS1* (involving hypermethylation of the active allele in 24 and hypomethylation of the silenced allele in 2). LOI at *MEG3* was even more common: hypermethylation of this locus was documented in 31 (62%) of the adenomas.

RT–PCR confirmed that *miR-296-5p* expression was significantly downregulated in adenomas with LOI at *GNAS-AS1* (Figure 3a). For the *MEG3* locus, which encodes over 50 miRNAs,[29] we limited our analysis to three of those that appeared to undergo epigenetic silencing during colorectal tumorigenesis (Table 1). As shown in Figure 3b, two of these, *miR-127-3p* and *miR-154*, displayed underexpression (statistically significant in the latter case) in the adenomas with LOI at *MEG3*, whereas the third, *miR-495,* was slightly but not significantly overexpressed in these tumors.

Collectively, these data suggest that *pri-miR-497/195* is—like *GNAS-AS1* and *MEG3*—an imprinted locus, and that imprinting at all three loci is frequently lost during colorectal tumorigenesis. As shown in Figure 4, 20 (40%) of the 50 adenomas we examined exhibited hypermethylation at all three loci, and 12 others (24%) were methylated at two of the three. Only seven adenomas (14%) exhibited methylation at all three loci that was similar to that of their paired samples of normal mucosa.

LOI can also stem—wholly or in part—from genetic events, such as copy-number alterations (CNAs). Using qPCR, we assessed CNA frequencies at *GNAS-AS1* (chromosome 20q13.3), *MEG3* (14q32) and *pri-miR-497/195* (17p13.1) in our 50 adenomas. As reported in Figure 4, nine of the tumors presented amplifications, which involved *GNAS-AS1* in seven tumors, *pri-miR-497/195* in one and *MEG3* in another. Deletions were found at *pri-miR-497/195* in four adenomas and at *MEG3* in one. Interestingly, all the CNAs were found in adenomas with COBRA-documented differential methylation of the involved locus (compared with normal mucosa from the same patient), including the two that appeared to be hypomethylated at the *GNAS-AS1* locus.

Table 2. Characteristics of the 50 patients with precancerous colorectal lesions included in the study

Patient no.	*Sex*	*Age*	*Colon segment involved*[a]	*Maximum lesion diameter (mm)*	*Macroscopic appearance*[b]	*Pit pattern*[c]	*Microscopic appearance*	*Dysplasia*[d]	*No. of lesions present*[e]
1	F	75	Tra	20	IIa	IIIS-IIIL	VA	High	7
2	F	75	Tra	40	IIa-IIc	IIIS-IIIL	TA	High	1
3	F	54	Cec	45	IIa	IIIL	TVA	Low	16
4	M	73	Asc	30	Is	nr	TVA	High	1
5	F	69	Asc	45	Ip	nr	TVA	high	1
6	M	74	Rec	40	Is	IV	VA	high	2
7	M	80	Asc	40	IIa	IIIL	TVA	Low	1
8	M	77	Cec	25	IIa	IIIL	VA	Low	1
9	M	71	Sig	30	IIa	IIIL	TVA	Low	2
10	M	89	Rec	30	Is	IV	TA	Low	3
11	F	85	Sig	25	Is-IIa	IV	TA	Low	1
12	F	63	Sig	35	Ip	IV	VA	Low	1
13	M	82	Asc	15	IIa	IIIL	VA	Low	2
14	F	73	Asc	25	IIa-IIc	IIIs-IIIL	TA	Low	1
15	F	70	Cec	25	IIa	IIIL	TVA	Low	2
16	M	70	Asc	15	Is	IV	TVA	Low	7
17	M	63	Asc	45	Is	IIIL-IV	TVA	Low	1
18	F	73	Sig	20	Ip	IV	TVA	Low	1
19	M	60	Des	30	Is	IV-Vi	TVA	High	1
20	M	68	Asc	30	Is-IIa	IIIL-IV	TVA	High	1
21	M	55	Cec	25	Is-IIa	IIIL-IV	SSA	Low	1
22	M	64	Sig	12	Ip	IIIL	TA	Low	1
23	M	78	Asc	50	Is	IV-Vi	TA	Low	1
24	F	69	Rec	90	Is-IIa	IV	TVA	Low	1
25	F	78	Rec	60	Is-IIa	IV	TVA	Low	1
26	M	72	Asc	30	Is	IV	TVA	High	2
27	M	76	Sig	30	Is	IV-Vi	TA	High	1
28	M	75	Rec	25	Is	IV-Vn	TVA	High	6
29	M	66	Asc	30	IIa	IIIL	TA	High	2
30	F	66	Asc	30	Is-IIa	IV	TA	High	2
31	M	75	Tra	18	IIa	IIIL	TA	Low	1
32	M	61	Asc	40	Is-IIa	IV	TVA	Low	20
33	M	84	Cec	30	Is	Vn	TVA	High	1
34	M	59	Tra	30	IIa-IIc	IIIs-IIIL	TA	Low	1
35	F	73	Rec	50	Is	IV	VA	Low	1
36	F	73	Des	25	Is	IV	TA	Low	1
37	M	59	Rec	60	Is	IV-Vi	TVA	High	1
38	M	75	Asc	40	IIa	II	TA	Low	6
39	M	75	Asc	50	Is	Vn	TVA	High	7
40	M	77	Sig	25	IIa-IIc	IIIs-IIIL	TA	Low	1
41	F	72	Asc	20	Is	IV	TVA	Low	1
42	F	66	Sig	35	Ip	IV	TA	Low	1
43	F	78	Rec	45	Ip	IV	TVA	High	1
44	M	74	Sig	25	Ip	IIIs-IV	TA	Low	1
45	M	60	Cec	30	Is	IV	TVA	Low	2
46	M	62	Des	22	Ip	nr	TA	Low	1
47	F	68	Asc	16	IIa	nr	TA	High	1
48	F	46	Des	30	Ip	nr	TVA	High	2
49	M	75	Cec	20	Is	nr	TVA	Low	1
50	M	59	Sig	8	Ip	nr	TA	Low	2

[a]Abbreviations: Asc, ascending colon; Cec, cecum; Des, descending colon; nr, pit pattern not reported by the endoscopist; Rec, rectum; Sig, sigma; SSA, sessile serrated adenoma; TA, tubular adenoma; Tra, transversum; TVA, tubulovillous adenoma; VA, villous adenoma. [b]Classified according to the Paris Endoscopic Classification of Superficial Neoplastic Lesions.[60] [c]Kudo classification of colonic crypt morphology.[61] [d]Highest degree of dysplasia in the lesion based on the WHO classification of tumors of the digestive system.[62] [e]Total number of lesions noted during the study colonoscopy, including those used in the present study.

This is the first evidence suggesting that the *pri-miR-497/195* locus might be imprinted in colorectal tissues. To validate this hypothesis in our colorectal tissue samples, we analyzed the transcribed *pri-miR-497/195* sequence identified by 5′ RACE for single-nucleotide polymorphisms (SNPs) that could be employed to distinguish alleles. Unfortunately, the only candidate that emerged, SNP rs412999 (dbSNP ID: 412999), is extremely rare in individuals of northern and western European ancestry (CEU samples of The HapMap 3 collection) and was therefore unsuitable for use in our tissue series. As an alternative, we sequenced RNA from B-lymphocytes from three Nigerian individuals known to be heterozygous for this SNP, but as shown in Supplementary Table 1, none of the samples exhibited any evidence of preferential allelic expression.

Our final set of experiments focused on putative or validated mRNA targets of *miR-497* and *miR-195* identified in public databases (mirTarBase,[30] miRecords[31] and miRWalk[32]). In a data set previously collected by our group,[33] 35 of these mRNAs had presented significantly upregulated expression in precancerous colorectal lesions (relative to normal mucosa samples from the

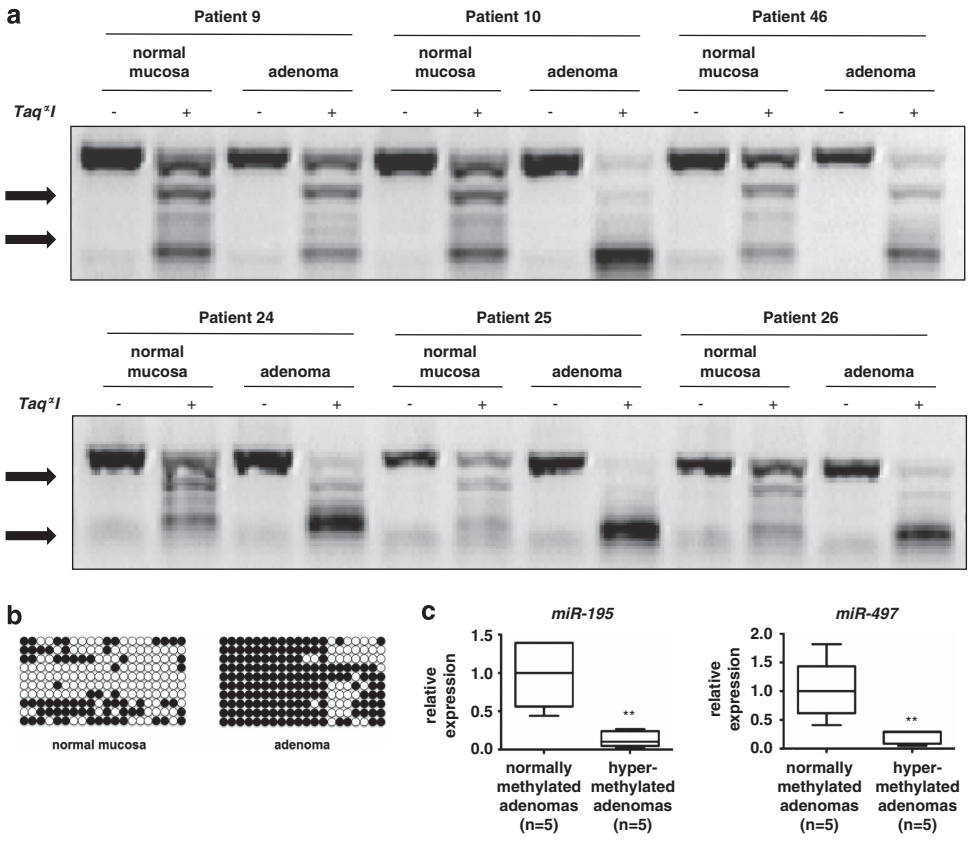

Figure 2. *Pri-miR-497/195* CpG island methylation in paired samples of normal colorectal mucosa and adenomas (six exemplary pairs from the 50 patients) and differential expression of *miR-195* and *miR-497* in the dysplastic mucosa of adenomas with hypermethylated or normally methylated *pri-miR-497/195* CpG island. (**a**) COBRA of colorectal adenomas and corresponding normal mucosa samples. Case 9: a normally methylated sample; the other cases: hypermethylated samples. Arrows indicate *Taq*$^{\alpha}$*I*-digested DNA fragments representing methylated alleles; and slower-migrating fragments represent undigested, unmethylated DNA. The weak residual band corresponding to *Taq*$^{\alpha}$*I*-undigested alleles reflects low-level stromal contamination in these endoscopic biopsy specimens, which is absent in the epithelial cell lines shown in Figure 1c. (**b**) An example of BGS from a normal/adenoma tissue pair. Each row shows the methylation status of a cloned target sequence. Circles represent unmethylated (white) and methylated (black) CpG dinucleotides. (**c**) Relative *miR-195* and *miR-497* expression in colorectal adenomas that were normally methylated ($n = 5$) or hypermethylated ($n = 5$) at *the pri-miR-497/195* CpG island. These samples were chosen over 40 other adenomas (7 normally methylated and 33 hypermethylated) because of their high epithelial cell contents reflected by low *Vimentin* expression (data not shown). $^{**}P = 0.0025$ (*miR-195*) and $P = 0.0087$ (*miR-497*) vs expression in the normally methylated tumors.

same patient) (Supplementary Table 2). To determine whether this overexpression was caused by *miR-497* and/or *miR-195* downregulation, we measured transcript levels in our more recent set of adenomas for four of these 35 mRNA targets and for *BCL2* as well (which was recently identified as a putative *miR-195* target[17] although it appeared to be downregulated rather than upregulated in our adenomas) (Supplementary Table 2). None of the five mRNAs exhibited significantly different expression in the five adenomas with hypermethylation at the *pri-miR-497/195* CpG island (compared with that observed in the five that were normally methylated at this locus) (Supplementary Figure 1). Immunohistochemistry experiments revealed no BCL2 protein expression in epithelial cells of either normal or adenomatous colorectal tissues (Supplementary Figure 2), where its expression was limited almost exclusively to stromal lymphocytes.

DISCUSSION

Our attempt to identify miRNAs whose expression is epigenetically downregulated in colorectal adenomas revealed that changes of this type were surprisingly frequent in miRNA-cluster loci that were monoallelically methylated in normal colorectal mucosa. We investigated the methylation statuses of CpG islands located upstream from the TSSs of three of these loci (*MEG3, GNAS-AS1* and *pri-miR-497-195*) in 50 paired samples of colorectal adenoma-normal mucosa. All three displayed biallelic methylation in a high percentage of the tumors.

For *MEG3* and *GNAS-AS1,* which are imprinted loci, this tumor-related hypermethylation of the CpG islands that control their transcription results in LOI. The link between this phenomenon and cancer was first noted in 1993, when two laboratories independently reported biallelic expression of the imprinted gene *IGF2* in Wilms tumors.[34,35] Later, *IGF2* LOI was also found in normal and lesional tissues from patients with colorectal cancer,[36] and subsequent studies pointed to hypomethylation-mediated activation of the normally silent, maternally inherited *IGF2* allele as a risk factor for familial forms of colorectal cancer.[37,38] Interestingly, an miRNA gene embedded within *IGF2*, *miR-483*, also appears to be frequently overexpressed in different human cancers, including those of the colon.[39–41] Thus far, *KCNQ1OT1* is the only other gene known to undergo LOI in colorectal cancers,[42] but its expression in the precancerous phase of colon tumorigenesis has not been characterized.

The high frequency of LOI at the *MEG3* and *GNAS-AS1* miRNA-cluster loci in colorectal adenomas has never been reported, but LOI at *MEG3* has been associated with hematologic malignancies,[43] meningiomas[44] and pituitary adenomas.[45] Hypermethylation of the CpG island at *MEG3* leading to LOI was

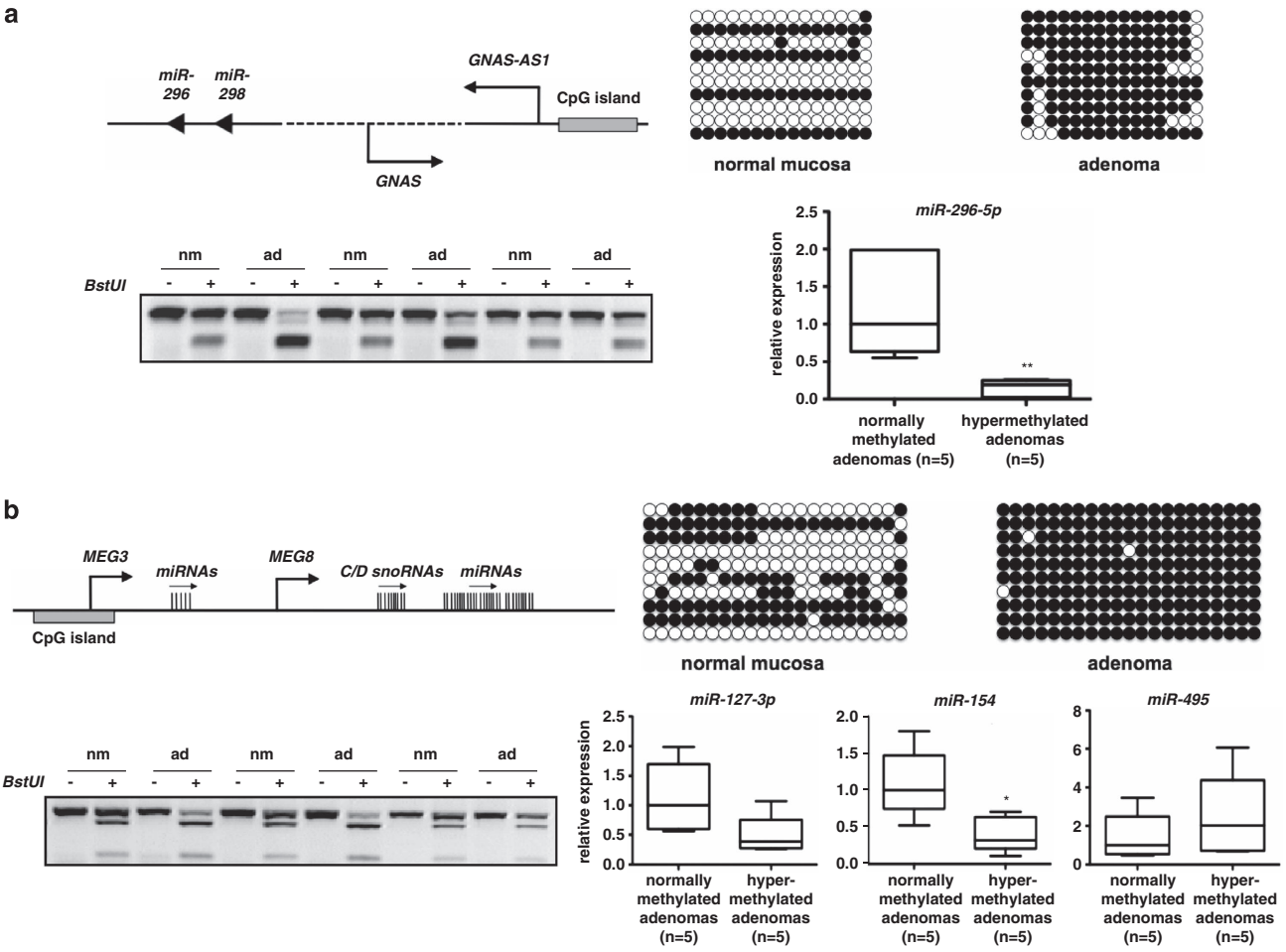

Figure 3. Methylation analysis of regions of *GNAS-AS1* (**a**) and *MEG3* (**b**) that are differentially methylated in adenomas and normal mucosal samples. Both panels include schematic depictions of the imprinted locus (upper left); examples of COBRA (lower left) and BGS results (upper right); and expression levels for miRNAs encoded in the locus measured in adenomas in which the locus was hypermethylated or normally methylated. (Each panel includes representative COBRA results for two adenomas with hypermethylation at the locus and one normally methylated adenoma.) (**a**) *MiR-296-5p* expression was significantly downregulated ($P = 0.0080$) in adenomas with hypermethylation of the *GNAS-AS1* CpG island. (**b**) Adenomas whose *MEG3* CpG island was hypermethylated displayed significantly downregulated *miR-154* expression ($P = 0.0183$) and substantial but nonsignificant downregulation of *miR-127-3p* expression ($P = 0.0717$). ad, adenoma; nm, normal mucosa.

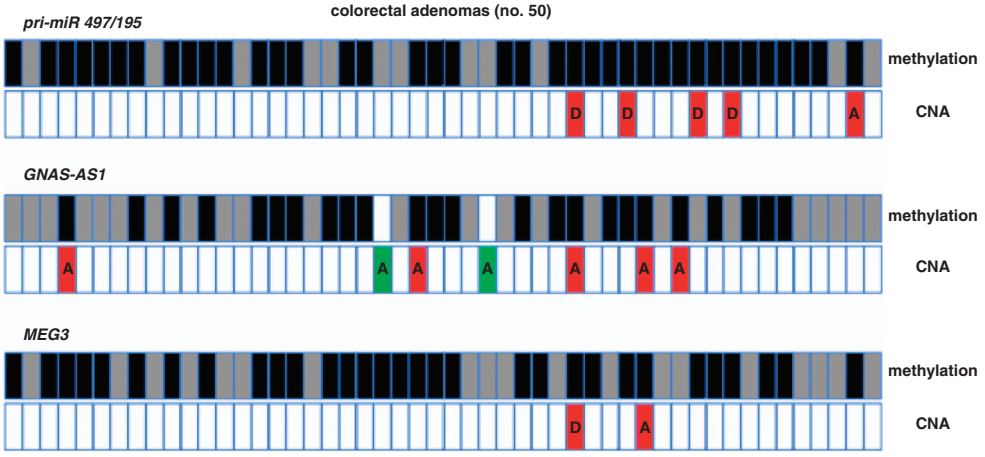

Figure 4. Epigenetic and genetic variations at the three loci investigated in our 50 adenomas. For each locus, the upper row of squares shows the methylation status of each tumor (black, hypermethylation; gray, normal methylation; white, hypomethylation), and the lower row indicates the CNA status (A, amplification; D, deletion; red, loss of the active (unmethylated) allele; green, gain in the number of active (unmethylated) alleles; white, no CNA).

detected in over 60% of our colorectal adenomas. However, the transcriptional regulation of this locus is highly complex (Figure 3b). Discordance between *MEG3* expression and the expression of certain miRNAs included in this locus has been reported in pituitary adenomas,[45] and a similar picture emerged in the colorectal adenomas we analyzed. In tumors with LOI at *MEG3*, *miR-154* and *miR-127-3p* expression was clearly downregulated ($P = 0.01$ and $P = 0.07$, respectively) but *miR-495* expression was not. MEG3 encodes over 50 miRNAs,[29] so extensive transcriptional analysis will be necessary to delineate the consequences of epigenetic alterations at this locus in colorectal adenomas.

Over half of our adenomas displayed LOI at the *GNAS-AS1* locus on chromosome 20q13.3.[46] *GNAS-AS1* contains imprinted protein-coding genes and antisense transcripts,[47] and imprinting of the two miRNA genes included in this locus, *miR-296* and *miR-298*, has also been recently confirmed.[26] Underexpression of *miR-296* has been documented in various types of cancers,[48,49] including metastatic lesions from colorectal malignancies,[50] but its cause has not been explored. Our findings indicate that the reduced expression associated with human colorectal tumorigenesis may well be due to *miR-296* LOI, which appears to be a frequent, early event in this process that generally stems from epigenetic alterations (Figure 4).

As for the third gene cluster we tested, *miR-497*/*miR-195* on chromosome band 17p13.1, its imprinting status is unknown. We identified the *pri-miR-497/195* TSS and found that the CpG island located upstream from this site is monoallelically methylated in the normal colon mucosa. In contrast, the island was hypermethylated in roughly 75% of the colorectal adenomas we tested (Figure 4), and this change was associated with significant downregulated expression of both *miR-497* and *miR-195* (Figure 2c).

This methylation pattern and its similarity to those of *GNAS-AS1* and *MEG3* (Figure 3) suggest that the *pri-miR-497/195* locus might be a novel imprinted region of the genome. This hypothesis would have been strengthened by findings of preferential allelic expression in the lymphoblast cell DNA we analyzed, but it cannot be rejected solely on the basis of the absence of such evidence. Indeed, *pri-miR-497/195* imprinting might simply be a tissue-specific phenomenon that occurs in colorectal cells (and possibly other cells as well) but not in lymphocytes.[51] Interestingly, a review of the COBRA and bisulfite genomic sequencing (BGS) data published recently by Li *et al.*[52] reveals that this locus also tends to be monoallelically methylated in normal human breast tissues and hypermethylated in breast cancers. An alternative hypothesis is that the phenomenon we observed at *pri-miR-497/195* represents ASM of a nonimprinted locus,[16] but this seems less likely for two reasons: (1) Nonimprinted ASM is usually an individual-specific phenomenon,[16] whereas ASM at *pri-miR-497/195* was observed in *all 50* of the normal colorectal mucosal samples we examined; and (2) the *pri-miR-497/195* ASM involves a canonical CpG island—a typical feature of *bona fide* imprinting—whereas nonimprinted ASM generally affects other DNA sequences.[53]

Regardless of whether it is imprinted or not, the fact that *pri-miR-497/195* is monoallelically methylated in normal colorectal mucosa and biallelically methylated in most preinvasive tumors of this tissue is potentially important in terms of the development and clinical outcome of colorectal cancer. Guo *et al.*[54] showed that *miR-497* exerts tumor-suppressor activity in colorectal cancer by downregulating the expression of insulin-like growth factor 1. Downregulation of *miR-195* expression has been significantly linked to increased mortality in patients with this type of cancer.[55] Furthermore, Liu *et al.*[17] found that this miRNA is frequently underexpressed in colorectal cancers (73% of those they examined) and that it exerts tumor-suppressor effects in colon cancer cell lines, which are mediated by downregulation of BCL2 expression. The fact that *BCL2* mRNA levels were not increased in the adenomas of our series that exhibited *pri-miR-497/195* hypermethylation might reflect differences between the dynamics of miRNA/target regulation in tissues and those observed in cell lines, as suggested by our preliminary data on BCL2 protein expression (Supplementary Figure 2). This finding, however, together with the lack of negative correlation between transcript levels of *pri-miR-497/195* and those of four other possible *miR-497/195* targets (Supplementary Figure 1), also suggests that the regulatory effects of *pri-miR-497/195* may be exerted mainly during mRNA translation.

Underexpression of *miR-497* and *miR-195* was also associated with DNA copy-number reductions in 93 (71%) of the 131 colorectal cancers analyzed by Guo *et al.*[54], suggesting that genetic alterations at this locus of chromosome 17p13.1 are common in advanced-stage colorectal malignancies. These findings are consistent with our own, which suggest that epigenetic alterations may precede CNAs at this locus. Hypermethylation at 17p13.1 was observed in roughly 75% of our adenomas, but only 10% of these tumors exhibited CNAs. In contrast, CNAs at this locus were detected in 24.3% of the 833 colorectal cancers included in the *Progenetix* database.[56] The *Progenetix* tumors were also frequently characterized by CNAs at *MEG3* (16.4%) and *GNAS-A1* (48.1%). These patterns suggest that early epigenetic alterations might 'prime' certain regions for subsequent loss or gain of genetic material, which—depending on the genes affected—could have important implications for tumorigenesis (for example, deletion of the tumor-suppressor gene *TP53,* which is located close to *miR-497/miR-195* on chromosome 17p13.1).

In conclusion, our findings reopen the debate on the importance of LOI in the early stages of colorectal tumorigenesis. Sporadic colorectal adenomas seem to be characterized by a relatively high frequency of epigenetic alterations affecting miRNA-cluster loci that are monoallelically methylated in normal mucosa, and some of these are known to be imprinted. Somatically acquired changes of this type could drive transformation by directly affecting gene expression, but there is also reason to suspect that they predispose neoplastic cells to genomic alterations. Additional work is needed to determine whether *pri-miR-497/195* is also, as we suspect, an imprinted locus and to clarify its mRNA targets.

MATERIALS AND METHODS

Cell lines and tissue samples

Colorectal cancer cell lines (HT29, SW48, GP5D, LS174T, SW620, Colo741, SW480, HCT116 and SW837) were obtained from the Zurich Cancer Network's Cell Line Repository. Cells from this repository have undergone only a few passages since purchase (from the American Tissue Culture Collection, Teddington, UK) and are free from mycoplasma infection.

Human colorectal tissues were collected with local ethics committee approval from patients undergoing colonoscopy in Cremona, Italy (Istituti Ospitalieri) or Zurich, Switzerland (University Hospital). They were used in accordance with the Declaration of Helsinki, and each donor provided written informed consent to sample collection, analysis of data and publication of the findings. We obtained 50 precancerous lesions (adenomas), each with a paired sample of normal mucosa from the same colon segment, >2 cm from the lesion. As these tissues were collected endoscopically, they were relatively superficial samples with consistently high epithelial contents.

Comparative studies of the epithelial and stromal components of the mucosa were carried out on a single, freshly excised specimen of normal colon from a patient undergoing surgery for diverticulitis. (This donor provided written informed consent, as described above for patients with adenomas.) Epithelial crypts and lamina propria were isolated from this specimen as previously described.[57]

Genomic DNA and total RNA (including small RNAs) were purified with commercially available kits. In brief, samples were lysed and homogenized in RTL Plus Buffer (Qiagen, Basel, Switzerland). The DNA was purified on spin columns (AllPrep DNA/RNA Mini Kit, Qiagen), and the total RNA was purified from the AllPrep column flow-through with Mini Spin Columns (miRCURY RNA Isolation Kit, Exiqon, Vedbaek, Denmark).

DNA demethylation

HT29 cells were subjected to DNA demethylation treatment the day after seeding. Cells were grown in medium containing 5-aza-2-deoxycytidine (Sigma, Buchs SG, Switzerland), which was renewed every 24 h. After 72 h, they were transferred to medium containing trichostatin A (final concentration 300 nM) (Sigma) for an additional 16-h growth.

miRNA profiling

miRNA levels were measured in HT29 cells, before and after demethylation, and in normal colorectal mucosa. We used Exiqon's MicroRNA Ready-to-Use PCR, Human panel I + II, V2.R, which includes assays for 742 mature miRNAs and 6 reference genes. The reverse-transcribed RNA (obtained with the miRCURY LNA Universal RT microRNA PCR, Polyadenylation and cDNA Synthesis kit from Exiqon) was added to the PCR panels with SYBR Green Master Mix (Exiqon), and real-time PCR was performed with a Roche Lightcycler 480 system (Roche, Rotkreutz, Switzerland). miRNAs with a Cp of >40 were classified as nonexpressed. Supplementary Table 3 shows the LNA PCR primer sets used to quantify individual miRNAs.

5′ RACE

To identify the TSS for *pri-miR-497/195*, we used 5′ RACE (Ambion's FirstChoice RLM-RACE Kit, Ambion, Zug, Switzerland) to analyze 10 μg of total RNA from normal human colonic mucosa. Two specific primers (Supplementary Table 3) were designed to perform nested-PCR amplification of *Homo sapiens* cDNA FLJ25640 fis, clone STM04823 (GeneBank Accession AK098506) encompassing the mature forms of *miR-497* and *miR-195* (miRBase 19). The miR-RACE amplicon was then cloned (InsTAclone PCR Cloning kit, Fermentas, St Leon-Rot, Germany) and its specificity confirmed by sequencing.

Bisulfite conversion, COBRA and BGS

Sodium bisulfite conversion of genomic DNA was done with Qiagen's EpiTect Bisulfite kit. COBRA was performed as previously described.[8] (See Supplementary Table 3 for primer sequences and PCR conditions.) Amplicons were digested with BstUI (*GNAS-AS1* and *MEG3* CpG islands) and Taq$^{\alpha}$I (*pri-miR-497/195* 5′ CpG island) restriction enzymes (New England Biolabs, Beverly, MA, USA). For BGS, PCR products were cloned with the InsTAclone PCR Cloning kit (Fermentas), and individual clones were sequenced.

qPCR copy-number analysis

Copy numbers at the *pri-miR-497/195*, *MEG3* and *GNAS-AS1* loci were determined with predesigned assays (SABiosciences, Frederick, MD, USA) and qBiomarker SYBR Green qPCR Mastermix (Qiagen). Gene copy numbers were averaged with the ΔΔCT method after PCR efficiency correction.[58] The Human Multicopy Reference PCR Assay (SABiosciences, Assay ID: VPH000-0000000A) was used to normalize input DNA. (Locus-specific assay IDs are listed in Supplementary Table 3.) Pooled DNA from three samples of normal colonic mucosa was used as the calibrator, and assay specificity was validated on DNAs from colon cancer cell lines (HT29, SW480, SW837, HCT116 and SW48) with known genome-wide copy-number alteration profiles.[59] Loci were classified as amplified or deleted when their estimated copy numbers were >2.5 or <1.5, respectively.

Analysis of pri-miR-497/195 allelic expression in human white blood cells

Three lymphoblast cell lines were obtained from the Coriell Institute for Medical Research. Each line came from a member of the Yoruba population in Ibadan, Nigeria, who was heterozygous for the SNP rs412999 (dbSNP ID: 412999) in the *pri-miR-497/195* locus. Individual subcloned PCR products from retrotranscribed RNA isolated from these cells were sequenced to assess differential allelic expression of *pri-miR-497/195*. Primers used for genotyping are listed in Supplementary Table 3.

Quantitative real-time reverse-transcription PCR

First-strand cDNA synthesis, qPCR and relative quantification of transcript levels were performed as previously described.[8] All primer sequences are reported in Supplementary Table 3.

BCL2 immunohistochemistry

Immunostaining was performed with BCL2 antibody (no. 790-4464; Ventana, Tucson, AZ, USA) as described elsewhere.[7]

Statistical analysis

Two-tailed *P*-values were calculated with the unpaired *t*-test to compare differences between sample miRNA and mRNA expression means. Analyses were performed using the GraphPad Prism 5.0 statistical software package for Macintosh computers (GraphPad, La Jolla, CA, USA).

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