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MicroRNA Expression Profile Reveals miR-17-92 and miR-143-145 Cluster in Synchronous Colorectal Cancer

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Abstract: The expression of abnormal microRNA (miRNA, miR) is a ubiquitous feature of colorectal cancer (CRC). The pathological features and clinical behaviors of synchronous CRC have been comprehensively described; however, the expression profile of miRNA and small nucleolar RNA (snoRNA) in synchronous CRC has not been elucidated. In the present study, the expression profile of miRNA and snoRNA in 5 synchronous CRCs, along with the matched normal colorectal tissue was evaluated by microarray. Function and pathway analyses of putative targets, predicted from miRNA–mRNA interaction, were performed. Moreover, we analyzed clinicopathological and molecular characteristics of 22 patients with synchronous CRC and 579 solitary CRCs in a retrospective cohort study. We found a global dysregulation of miRNAs, including an oncogenic miR-17-92 cluster and oncosuppressive miR-143-145 cluster, and snoRNAs in synchronous CRC. Differential miRNA rather than snoRNA expression was robust enough to distinguish synchronous cancer from normal mucosa. Function analysis of putative targets suggested that miRNA clusters may modulate multiple effectors of oncogenic pathways involved in the pathogenesis of synchronous CRC. A comparison of normal mucosa between synchronous and solitary CRC suggested a differential genetic background of synchronous CRC from solitary CRC during carcinogenesis. Compared with solitary cancer patients, synchronous cases exhibited multiple extra-colonic cancers ($P = 0.012$), coexistence of adenoma ($P = 0.012$), microsatellite instability ($P = 0.024$), and less glucose transporter 1 ($P = 0.037$). Aberrant miRNA expression profiles could potentially be used as a diagnostic tool for synchronous CRC. Our findings represent the first comprehensive miRNA and snoRNA expression signatures for synchronous CRC, implicating that the miRNAs and snoRNAs may present therapeutic targets for synchronous CRC.

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Abbreviations: CIMP = CpG island methylator phenotype, CIN = chromosomal instability, CRC = colorectal cancer, GEO = Gene Expression Omnibus, GLUT1 = glucose transporter 1, IPA = ingenuity pathway analysis, LOH = loss of heterozygosity, miRNA, miR = microRNA, MMR = mismatch repair, MSI = microsatellite instability, MSS = microsatellite stability, ncRNA = noncoding RNA, PCA = principal component analysis, snoRNA = small nucleolar RNA.

INTRODUCTION

Patients with sporadic colorectal cancer (CRC) are at risk of synchronous CRC at the time of diagnosis. Synchronous CRC cases are rare, with the overall incidence of 2% to 10%.[1] As a heterogeneous disease, CRC has various pathological features and clinical behaviors, which are mainly associated with genetic and epigenetic instability. Genetic instability includes chromosomal instability (CIN) and microsatellite instability (MSI). CIN denotes tumors with frequent karyotypic abnormalities and chromosomal gain and loss, while MSI phenotype is caused by a defect in the mismatch repair (MMR) system and characterized by repetitive DNA alterations. Each of them plays a significant role in the pathological and biological characteristics of solitary CRC; however, the molecular mechanism responsible for the underlying genetic phenotype in synchronous CRC has not been well described.

In recent years, epigenetics has also attracted much attention. This field broadly encompasses changes in cytosine methylation of DNA, changes in histone and chromatin structure, and alterations in microRNA (miRNA, miR) expression. The CpG island methylator phenotype (CIMP) may lead to transcriptional inactivation of specific tumor suppressor and DNA repair genes and has been shown to be associated with synchronous CRC,[2] suggesting that transcriptional silencing by CIMP is an important mechanism in synchronous CRC.

miRNAs, a class of small noncoding RNAs (ncRNAs), have essential regulatory roles in cellular development, differentiation, proliferation, and apoptosis, and therefore act as tumor suppressors or oncogenes in human cancers.[3] Differential expression of miRNAs and their role in pathogenesis of solitary CRC has been extensively evaluated;[4–7] however, the global miRNA expression profile in synchronous CRC has not been reported to date. Besides miRNAs, small nucleolar RNAs

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(snoRNAs) might also contribute to the etiology of CRC. snoRNAs are well-conserved, abundant, short ncRNA molecules that guide the modification of particular RNAs. Recent evidence suggests that alterations of snoRNAs play important functions in cellular proliferation and ribosome biogenesis in CRC.[8] Therefore, the identification of new functions of snoRNAs and in-depth understanding of the roles of their dysregulations in synchronous CRC will help comprehend tumorigenesis, and hence provide potential biomarkers and therapeutic targets for cancer.

In the present study, we analyzed the expression profile of miRNAs and snoRNAs, as well as the retrospective analysis on 601 CRC patients. The patient cohort consisted of 22 synchronous and 579 solitary cancers together with the clinicopathological and molecular characteristics including MSI status, MMR expression, p53 status, deleted in colorectal cancer expression, *KRAS* mutation, loss of heterozygosity (LOH), and CIMP status. To our knowledge, this is the first study aimed to understand the molecular basis of synchronous CRC by studying the global expression profile of miRNAs and snoRNAs combined with the clinicopathological and molecular characteristics.

METHODS

Study Population

Tumor and matched normal tissue samples were collected from 5 patients with synchronous CRC and 7 patients with solitary CRC diagnosed at West China Hospital of Sichuan University (Chengdu, China) between October 2007 and March 2008 were used for miRNA array. Synchronous CRC was defined as having more than 1 primary adenocarcinoma in the colorectum when surgery or within 6 months. For patients with synchronous CRC, a tissue sample was obtained from the tumor with a higher stage or a larger tumor mass if the synchronous tumors were the same stage. Histological confirmation was performed after surgery. Informed consent was obtained from all patients, and the study was approved by the Regional Ethical Review Board.

Besides, a total of 601 primary CRC patients with available pathological and follow-up data were consecutively collected from January 1972 to January 2001 at Linköping University Hospital (Linköping, Sweden). The information of patients and tumors was taken from medical and pathological records, and survival data were extracted from follow-up system. Among them, 22 patients with synchronous CRC were identified. The remaining 579 patients had solitary CRC at the first diagnosis, which had arisen in the same population as synchronous CRC cases and thus constituted an optimal comparison group (Table 1). Informed consent was obtained from all 601 subjects. The study was approved by the Regional Review Board. An overview of the experimental design is depicted in Figure 1.

miRNA Microarrays and Data Analysis

Affymetrix GeneChip miRNA 1.0 array comprising 7815 probe sets representing 847 human mature miRNAs and 922 human snoRNAs (snoRNAs are inclusive of scaRNAs) was utilized to detect the expression pattern of miRNAs and snoRNAs by a service provider (Shanghai Biotechnology Corporation, Shanghai, China). Briefly, RNA spike control oligos and poly(A) tails were added to each RNA sample, and a biotinylated signaling molecule was ligated to the RNA. Labeled samples were subsequently added to a hybridization cocktail, incubated, and injected into miRNA arrays according to manufacturer's instructions. After 16 hours of hybridization at 48 °C, the arrays were washed and stained in the fluidics station 450, then scanned with GeneChip scanner and detected with GCOS1.4 software. Then, the hybridization data (CEL files) was normalized by miRNA QC tool and filtered by flags (absent or present). The raw array data was analyzed by unpaired *t*-test by GeneSpring Software 10.0 (Agilent Technologies, Santa Clara, CA, USA). The miRNAs with fold change > 1.0 and $P < 0.05$ were selected as significantly differential expression. Hierarchical cluster analysis was performed by Gene Clustering 3.0 to cluster genes and samples. Principal component analysis (PCA) was used to visualize the expression pattern of all samples. The data discussed in this publication was deposited in NCBI's Gene Expression Omnibus (GEO) database and accessible through GEO accession number GSE54632 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54632) and GSE67895 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67895).

The mRNA microarray data of colorectal tumor and the matched normal tissue was obtained from GEO database (accession number GSE15960).[9] The raw data (CEL files) was normalized and then analyzed using GeneSpring Software 10.0. Unpaired *t*-test was applied to identify dysregulated mRNA in tumor versus normal tissue with fold change ≥ 2.0 and $P < 0.05$.

Functional Enrichment Analysis of Differentially Expressed miRNAs

TAM, a web-accessible program, was used to functionally classify differentially expressed miRNAs.[10] TAM organizes miRNAs into different categories according to miRNA conservation, genome locations, functions, associated diseases, and tissue specificity. Then, the statistically significant over representation of each miRNA category among lists of miRNAs was evaluated by the hypergeometric test. The *P* values for all miRNA sets were adjusted by Bonferroni and false discovery rate corrections.

miRNA Target Prediction and Intersection Analysis

Furthermore, miR-17-92 and miR-143-145 clusters were selected for further analysis because of their significant dysregulation in synchronous tumor tissues. The potential targets of the miRNA clusters were predicted using ingenuity pathway analysis (IPA, http://www.ingenuity.com), whose miRNA Target Filter functionality enables prioritization of experimentally validated mRNA targets from TarBase and miRecords, peer-reviewed biomedical literature, as well as predicted miRNA–mRNA interactions from TargetScan. The experimentally validated along with the highly and moderately predicted targets were retrieved for the 2 miRNA clusters. The targets for miR-20a were not retrieved from the database in IPA because it shared extensive sequence similarity with miR-17, which is reflected in the almost identical targets predicted.

For intersection analysis of the miRNA clusters and the reported mRNAs (GSE15960), potential targets of upregulated miRNAs were identified by comparing their predicted target mRNAs with downregulated mRNAs derived from the mRNA microarray. Similarly, the predicted targets for downregulated miRNAs were compared with the upregulated mRNAs in the microarray.

TABLE 1. Clinicopathological and Molecular Features of Colorectal Cancer Patients According to Cancer Status

	N	Solitary Cancer Patients (Controls)	Synchronous Cancer Patients (Cases)*	*P* Value
Clinicopathological features				
Sex				
Male	329	304 (92.4)	12 (3.7)	0.851
Female	297	275 (92.6)	10 (3.4)	
Age, year	626	70.69 ± 10.21	71.09 ± 11.43	0.857
Tumor location				
Proximal colon‡	228	203 (89.0)	12 (5.3)	0.161
Distal colon§	389	368 (94.6)	10 (2.6)	
Unknown	9	8 (88.9)	0 (0)	
TNM stage				
I	85	77 (90.6)	4 (4.7)	0.497
II	161	148 (91.9)	5 (3.1)	
III	121	112 (92.6)	5 (4.1)	
IV	68	67 (98.5)	0 (0)	
Unknown	191	175 (91.6)	8 (4.2)	
Tumor differentiation				
Well	31	26 (83.9)	3 (9.7)	0.073
Moderate	424	392 (92.4)	16 (3.8)	
Poor + others	169	159 (94.1)	3 (1.8)	
Tumor growth				
Expansive	209	191 (91.4)	8 (3.8)	0.378
Infiltrative	222	204 (91.9)	10 (4.5)	
Others	195	184 (94.3)	4 (2.1)	
Multiple extra-colonic cancers				
Yes	120	116 (96.7)	0 (0)	0.012
No	506	463 (91.5)	22 (4.3)	
Coexistence of adenoma				
Yes	5	3 (60.0)	2 (40.0)	0.012
No	621	576 (92.8)	20 (3.2)	
Perioperative therapy				
Yes	106	100 (94.3)	5 (4.7)	0.441
No	459	421 (91.7)	14 (3.1)	
Surgery				
Palliative	19	18 (94.7)	1 (5.3)	0.481
Radical	177	162 (91.6)	5 (2.8)	
Complications				
Yes	69	62 (89.9)	4 (5.8)	0.188
No	127	118 (92.9)	2 (1.6)	
Local recurrence				
Yes	16	14 (87.4)	1 (1.3)	0.399
No	181	167 (92.3)	5 (2.7)	
Distant recurrence				
Yes	40	37 (92.5)	2 (5.0)	0.606
No	157	144 (91.7)	4 (2.5)	
Molecular features				
RAS				
Negative	96	92 (95.8)	4 (4.2)	0.187
Weak	64	64 (100)	0 (0)	
Strong	73	69 (94.5)	4 (5.5)	
c-erbB				
Negative + weak	111	108 (97.3)	3 (2.7)	0.745
Moderate + strong	166	160 (96.4)	6 (3.6)	
p53				
Negative	139	131 (94.2)	6 (5.8)	0.331
Positive	140	137 (97.9)	3 (2.1)	
p73				
Negative	70	68 (97.1)	2 (2.9)	0.721
Positive	144	137 (95.1)	7 (4.9)	
p27				

TABLE 1. (Continued)

	N	Solitary Cancer Patients (Controls)	Synchronous Cancer Patients (Cases)*	*P* Value
Negative	88	84 (95.5)	4 (4.5)	0.138
Positive	85	85 (100)	0 (0)	
DCC				
Negative	51	49 (96.1)	2 (3.9)	0.617
Positive	134	131 (97.8)	3 (2.2)	
COX-2				
Negative	26	23 (88.5)	3 (115)	0.091
Positive	81	79 (97.5)	2 (2.5)	
PCNA				
Weak	162	155 (95.7)	7 (4.3)	0.314
Strong	115	113 (98.3)	2 (1.7)	
Ki-67				
Weak	93	91 (97.8)	2 (2.2)	0.714
Strong	149	143 (96.0)	6 (4.0)	
GLUT1				
≤25%	22	19 (86.4)	3 (13.6)	0.037
>25%	74	73 (98.6)	1 (1.4)	
Apoptosis				
≤mean	131	128 (97.7)	3 (2.3)	0.406
>mean	18	17 (94.4)	1 (5.6)	
KRAS mutation				
Wild	106	100 (94.3)	6 (5.7)	0.674
Mutational	41	40 (97.6)	1 (2.4)	
p53 mutation				
Wild	62	60 (96.8)	2 (3.2)	0.519
Mutational	44	44 (100)	0 (0)	
DCC codon 201				
Wild	21	19 (90.5)	2 (9.5)	0.152
Mutational	32	32 (100)	0 (0)	
p73 mutation				
Wild	105	102 (97.1)	3 (2.9)	0.431
Mutational	66	62 (93.9)	4 (6.1)	
APC (8636 C>A)				
Wild	155	151 (97.4)	4 (2.6)	1
Mutational	4	4 (100)	0 (0)	
DCC-LOH				
Negative	13	11 (84.6)	2 (15.4)	0.157
Positive	19	19 (100)	0 (0)	
MSI status				
MSI	53	48 (90.6)	5 (9.4)	0.024
MSS	362	351 (97.0)	11 (3.0)	
hMLH1				
Negative	97	96 (99.0)	1 (1.0)	0.297
Strong	55	53 (96.4)	2 (3.6)	
hMSH2				
Weak	75	74 (94.9)	1 (1.3)	0.397
Strong	102	98 (96.1)	4 (3.9)	
hMSH3				
Weak	130	122 (93.8)	8 (6.2)	0.171
Strong	67	66 (98.5)	1 (1.5)	
hMSH6				
Weak	108	103 (95.4)	5 (4.6)	1
Strong	97	93 (95.9)	4 (4.1)	
O^6-MGMT				
(−)	44	42 (95.5)	2 (4.5)	1
(+)	29	28 (96.6)	1 (3.4)	
$p14^{ARF}$				
(−)	50	48 (96.0)	2 (4.0)	1
(+)	23	22 (95.7)	1 (4.3)	

TABLE 1. (Continued)

	N	Solitary Cancer Patients (Controls)	Synchronous Cancer Patients (Cases)*	*P* Value
$p16^{INK4a}$				
(−)	53	51 (96.2)	2 (3.8)	1
(+)	20	19 (95.0)	1 (5.0)	
RASSF1A				
(−)	61	58 (95.1)	3 (4.9)	1
(+)	12	12 (100)	0 (0)	
APC1A				
(−)	52	50 (96.2)	2 (3.8)	1
(+)	21	20 (95.2)	1 (4.8)	

APC = adenomatous polyposis coli, COX-2 = cyclooxygenase-2, CRC = colorectal cancer, DCC = deleted in colorectal cancer, GLUT1 = glucose transporter 1, LOH = loss of heterozygosity, MSI = microsatellite instability, MSS = microsatellite stability, O^6-MGMT = O^6-methylguanine DNA methyltransferase, PCNA = proliferating cell nuclear antigen, RASSF1A = Ras association domain family 1 isoform A.
* For synchronous cases, results were obtained from a tumor at a higher stage or a larger tumor if the 2 synchronous tumors were at the same stage.
‡ Proximal colon refers to cecum to transverse colon.
§ Distal colon refers to splenic flexure to rectum.

Network and Gene Ontology Analysis

The intersecting targets of both miRNA clusters were imported into IPA to identify the molecular functions and canonical pathways. Fisher exact test was used to determine the probability that the association between the targets in the dataset and the canonical pathway could be explained by chance alone. Then, the miRNA–mRNA network was constructed by IPA based on 2 respective expression profiles.

Immunohistochemistry and Apoptosis Analysis

Methods of immunohistochemistry were previously described as follows: p73,[11] p53, RAS, c-erbB-2, proliferating cell nuclear antigen,[12] p27, deleted in colorectal cancer,[13] cyclooxygenase-2,[14] glucose transporter 1 (GLUT1, also named SLC2A1),[15] and Ki-67.[16] All stained slides for each marker were independently reviewed by 2 investigators and interpreted by 1 of the investigators unaware of other data. The apoptosis was examined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.[17]

MSI Status and MMR Expression Analysis

MSI status was determined by BAT26.[18] MSI was defined as the presence of at least 1 altered BAT-26 allele, and microsatellite stability (MSS) as the absence of altered BAT-26 allele.

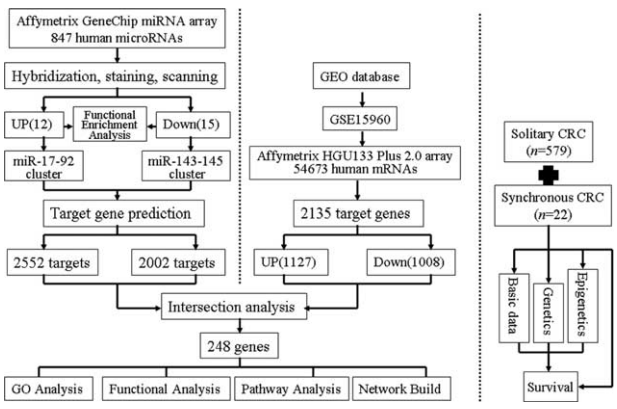

FIGURE 1. An overview of our experimental design.

The expression of human *MMR* gene, *hMLH1*, *hMSH2*, *hMSH3*, and *hMSH6*, was determined by immunohistochemistry.[19]

Gene Polymorphism and LOH Analysis

DNA from paraffin-embedded tissue was extracted, and allele-specific PCR for *KRAS* codons 12 and 13[20] and *p73* polymorphism,[21] DNA sequencing for *p53* mutation,[22] and *APC* (adenomatous polyposis coli, 8636 C > A) polymorphism[23] were performed. For 18q LOH analysis by PCR-restriction fragment length polymorphism, the lack of the larger or 2 smaller fragments of PCR amplified products in tumor DNA relative to normal DNA was considered as LOH.[24]

Methylation Assay

Bisulfite DNA treatment and real-time PCR assays were performed. We quantified methylation at 6 loci (O^6-methylguanine DNA methyltransferase, hMLH1, $p14^{ARF}$, $p16^{INK4a}$, ras association domain family 1 isoform A, and APC1A) by pyrosequencing.[25] Each site is analyzed as a C/T-polymorphism where a 100% C-reading denotes a fully methylated C, and a 100% T-reading denotes that this locus is unmethylated in the original genomic DNA. Intermediate C/T percentages denote partial methylation level of the sample.[25]

Statistical Analysis

Fisher exact test or Pearson χ^2 test was used as appropriate to examine an association between categorical variables. Student's *t*-test and Wilcoxon rank-sum test were used when performing two-group comparison using parametric and nonparametric data, respectively. Kaplan–Meier method with log-rank test was used to analyze the correlation with survival. Data analysis was performed using SPSS 17.0. *P* values less than 0.05 were considered statistically significant.

RESULTS

miRNA and snoRNA Expression Profile of Synchronous CRC

When tumor and the matched normal tissue were compared, 24 miRNA transcripts representing 27 mature miRNAs

were significantly dysregulated. Of these, 12 were upregulated and 15 downregulated (Table 2). Strikingly, an oncogenic miR-17-92 cluster including miR-17, miR-20a, miR-92a-1, and miR-92a-2 was significantly upregulated, and an oncosuppressive miR-143-145 cluster composed of miR-143 and miR-145 was most significantly downregulated in tumor tissue compared with normal tissue. Additionally, many oncogenic miRNAs, such as miR-21, miR-93, and miR-182, were upregulated, and

TABLE 2. The List of Differentially Expressed miRNAs and snoRNAs in Synchronous Cancer Tissues Compared With Normal Tissues

ProbeSet Name	Fold Change	*P* Value	Regulation	Chromosome	ProbeSet Type	Transcript ID (Array Design)
miRNA						
hsa-miR-182_st	2.921426	0.005567	Up	7	miRNA	miR-182
hsa-miR-17_st	2.074103	0.042797	Up	13	miRNA	miR-17
hsa-miR-203_st	1.842922	0.006146	Up	14	miRNA	miR-203
hsa-miR-93_st	1.836569	0.041822	Up	7	miRNA	miR-93
hsa-miR-92a_st	1.700699	0.013897	Up	13	miRNA	miR-92a-1//miR-92a-2
hsa-miR-155_st	1.641582	0.038797	Up	21	miRNA	miR-155
hsa-miR-20a_st	1.543397	0.042784	Up	13	miRNA	miR-20a
hsa-miR-191_st	1.513297	0.014014	Up	3	miRNA	miR-191
hsa-miR-21_st	1.382739	0.041057	Up	17	miRNA	miR-21
hsa-miR-141_st	1.363241	0.033744	Up	12	miRNA	miR-141
hsa-miR-532-5p_st	1.336633	0.009162	Up	X	miRNA	miR-532-5p
hsa-miR-145_st	3.506072	0.02212	Down	5	miRNA	miR-145
hsa-miR-143_st	3.383716	0.025076	Down	5	miRNA	miR-143
hsa-miR-195_st	2.706363	0.012001	Down	17	miRNA	miR-195
hsa-miR-125b_st	2.258891	0.041881	Down	11	miRNA	miR-125b-1//miR-125b-2
hsa-miR-378_st	1.857824	0.030989	Down	5	miRNA	miR-378
hsa-miR-30a_st	1.641447	0.010191	Down	6	miRNA	miR-30a
hsa-miR-140-3p_st	1.631755	0.002536	Down	16	miRNA	miR-140-3p
hsa-miR-30c_st	1.504937	0.029445	Down	1	miRNA	miR-30c-1//miR-30c-2
hsa-miR-181c-star_st	1.449835	0.039439	Down	19	miRNA	miR-181c*
hsa-miR-10a_st	1.422717	0.025791	Down	17	miRNA	miR-10a
hsa-miR-222-star_st	1.196781	0.01665	Down	X	miRNA	miR-222*
hsa-miR-1275_st	1.169236	0.028297	Down	6	miRNA	miR-1275
hsa-miR-508-3p_st	1.092042	0.019402	Down	X	miRNA	miR-508-3p
snoRNA						
U78_x_st	1.756061	0.001739	Up	1	CDBox	U78
U55_st	1.731131	0.038207	Up	1	CDBox	U55
U55_x_st	1.702426	0.038404	Up	1	CDBox	U55
U29_st	1.676609	0.022739	Up	11	CDBox	U29
U78_s_st	1.61288	0.03094	Up	1	CDBox	U78
U34_st	1.383936	0.009672	Up	19	CDBox	U34
U30_st	1.354333	0.038152	Up	11	CDBox	U30
HBII-55_st	1.316304	0.024868	Up	20	CDBox	HBII-55
U56_st	1.305295	0.04185	Up	20	CDBox	U56
U74_x_st	1.265221	0.040094	Up	1	CDBox	U74
snR38C_st	1.250882	0.030176	Up	17	CDBox	snR38C
U3-2_s_st	1.244579	0.048233	Up	17	CDBox	U3-2
U42A_st	1.239371	0.002561	Up	17	CDBox	U42A
U57_st	1.229814	0.009529	Up	20	CDBox	U57
U83B_st	1.151721	0.044253	Up	22	CDBox	U83B
U96a_x_st	1.150129	0.030142	Up	5	CDBox	U96a
U68_x_st	1.131481	0.043647	Up	19	HAcaBox	U68
HBI-61_st	1.117102	0.049732	Up	3	HAcaBox	HBI-61
U56_x_st	1.111402	0.027149	Up	20	CDBox	U56
U20_st	1.091105	0.029403	Up	2	CDBox	U20
U97_st	1.07852	0.045841	Up	11	CDBox	U97
ACA41_x_st	1.06634	0.041158	Up	2	HAcaBox	ACA41
HBII-85-4_x_st	1.100252	0.012749	Down	15	CDBox	HBII-85-4
ENSG00000202370_st	1.126566	0.040022	Down	16	snoRNA	ENSG00000202370

miRNA, miR = microRNA, snoRNA = small nucleolar RNA.

many oncosuppressive miRNAs, such as miR-195 and miR-125b, were downregulated in tumor tissue compared with normal tissue.

Besides, a total of 24 snoRNAs were significantly dysregulated in tumor tissue compared with normal tissue (Table 2). Of the 127 HAC-Box snoRNAs, 3 were upregulated. Of the 274 CD-box snoRNAs, 19 were upregulated and 1 was downregulated. Of the additional 499 snoRNAs, only 1 was downregulated. Interestingly, none of the 22 scaRNAs were dysregulated.

Hierarchical clustering analysis based on the 27 differential miRNAs expression was shown in Figure 2A. The samples were separated into 2 clusters. The left cluster was comprised of 5 tumors (T1-T5), while the right cluster contained 5 normal samples (N1-N5), which was accordant with the results from 2-dimensional PCA. Hierarchical clustering of tumor and normal samples also revealed that all components of miR-17-92 and miR-143-145 cluster were classified in the same cluster, respectively (Figure 2B). However, cluster and 2-dimensional PCA analysis of differentially expressed snoRNAs show that the expression profile of snoRNAs does not accurately define tissue type, normal samples from tumor samples (Figure 2C, D).

Differential Expression of miRNA and snoRNA of Normal and Tumor Tissue Between Synchronous and Solitary CRC

Comparing normal mucosa in the patients of synchronous CRC with those of solitary CRC, there were 24 differentially expressed miRNA transcripts representing 29 mature miRNAs. Of these, 21 were upregulated and 8 were downregulated (see Table S1, Supplemental Digital Content http://links.lww.com/MD/A364, which describes the list of differentially expressed miRNAs and snoRNAs of normal mucosa between synchronous and solitary CRC in detail). A total of 44 snoRNAs were significantly differentially expressed in normal tissue of synchronous cancer when compared with those of solitary cancer (see Table S1, Supplemental Digital Content http://links.lww.com/MD/A364, which describes the list of differentially expressed miRNAs and snoRNAs of normal mucosa between synchronous and solitary CRC in detail). Of the 127 HAC-Box snoRNAs, only ACA31 was upregulated and ACA3-2 was downregulated. Fourteen of 274 CD-box snoRNAs were upregulated and 9 were downregulated, and strikingly 19 of the other 499 snoRNAs were upregulated. Of the 22 scaRNAs, none was dysregulated.

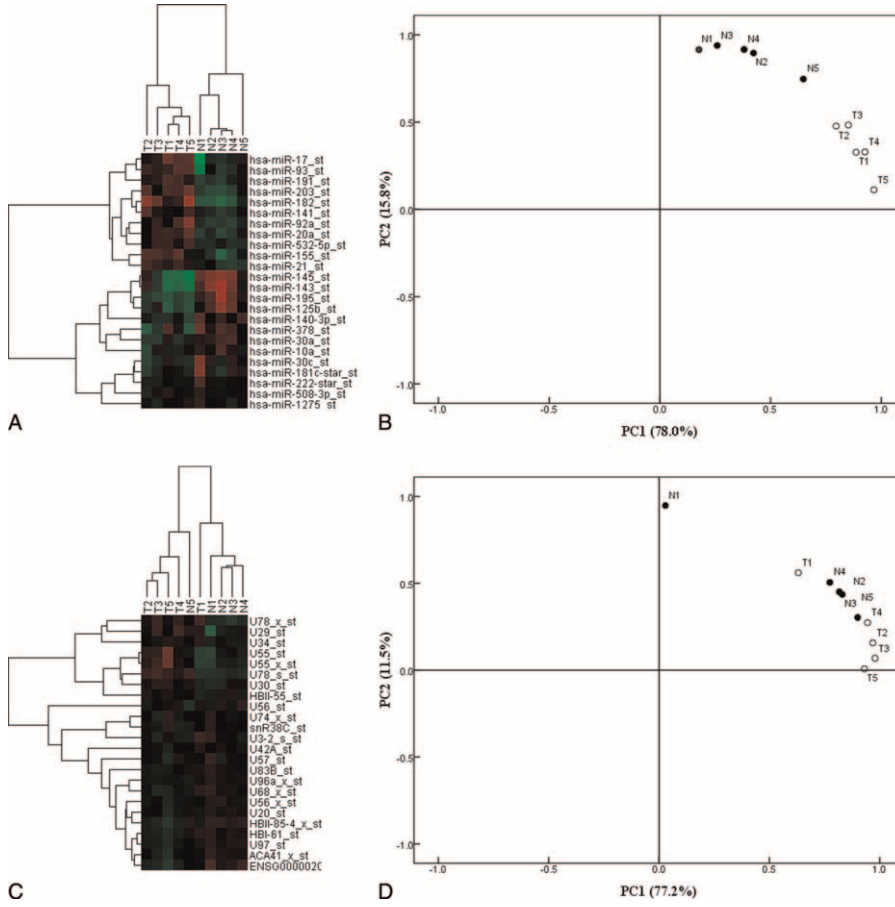

FIGURE 2. Unsupervised hierarchical clustering and PCA of miRNA and snoRNA expression. (A) Hierarchical clustering of 10 samples and 24 differentially expressed miRNA transcripts. (B) PCA of dysregulated miRNA transcripts. (C) Hierarchical clustering of 10 samples and 24 differentially expressed snoRNAs. (D) PCA of dysregulated snoRNAs. In clustering analysis, colored bars indicate the range of normalized log2-based signals, with red indicating high expression and green low expression. PCA = principal component analysis, snoRNA = small nucleolar RNA, miRNA, miR = microRNA.

When comparing with solitary cancer, only 1 miRNA (miR-1234) and 2 snoRNAs (U17a and U36A) were upregulated in the tumor tissue of synchronous CRC.

Functional Enrichment Analysis of Differentially Expressed miRNAs

The functional enrichment analysis of differentially expressed miRNAs by TAM showed that upregulated miRNAs were enriched in the following functions; immune system, cell proliferation, Akt pathway, and angiogenesis, while downregulated miRNAs were significantly involved in cell proliferation, smooth muscle cell fate, cell motility, and cell differentiation (see Table S2, Supplemental Digital Content http://links.lww.com/MD/A365, which describes functional enrichment analysis of differentially expressed miRNAs in detail). However, this category was not completely representative of the exact role of these dysregulated miRNAs in synchronous CRC because each miRNA acts in a tissue-specific manner.[26] Therefore, further analysis on the targets in CRC will be of paramount importance to provide insight into the function of differentially expressed miRNAs in synchronous CRC.

Potential Targets Regulated by miR-17-92 and miR-143-145 Cluster

We computationally identified mRNA targets of miRNAs using IPA. miR-17-92 and miR-143-145 clusters were significantly dysregulated in synchronous CRC and therefore used for further functional analysis. To reduce the false targets in the analysis, only experimentally validated and highly and moderately predicted targets were retrieved for 2 miRNA clusters from the database as mRNA targets. Altogether, 4554 targets were identified, with 2552 downregulated and 2002 upregulated (see Table S3, Supplemental Digital Content http://links.lww.com/MD/A366, which describes the targets of 2 miRNA clusters predicted by IPA in detail). The expression of the identified mRNA targets of the miR-17-92 and miR-143-145 clusters were analyzed from the GEO database, GSE15960, and totally 2135 genes with differential expression were identified, with 1008 downregulated and 1127 upregulated (see Table S4, Supplemental Digital Content http://links.lww.com/MD/A367, which describes the list of differentially expressed mRNAs in detail).

Intersecting analysis was performed using IPA between the miRNA targets and the mRNA obtained from the microarray study. This comparison revealed 130 genes potentially upregulated due to an under-expression of miRNAs, and 118 genes potentially downregulated due to an over-expression of miRNAs (see Table S5, Supplemental Digital Content http://links.lww.com/MD/A368, which describes the list of intersecting targets in detail).

Molecular Functions and Biological Pathways Associated With the Intersecting Targets in Synchronous CRC

To further understand the biological and molecular functions of these intersecting targets, an IPA Functional Analysis was performed. A total of 75 molecular functions were significantly associated with these intersecting targets ($P < 0.05$). The top 5 associated functions were cancer, organismal injury and abnormalities, developmental disorder, embryonic, and cellular development (Figure 3A and see Table S6, Supplemental Digital Content http://links.lww.com/MD/A369, which describes enrichment functional analysis of intersecting targets by IPA in detail).

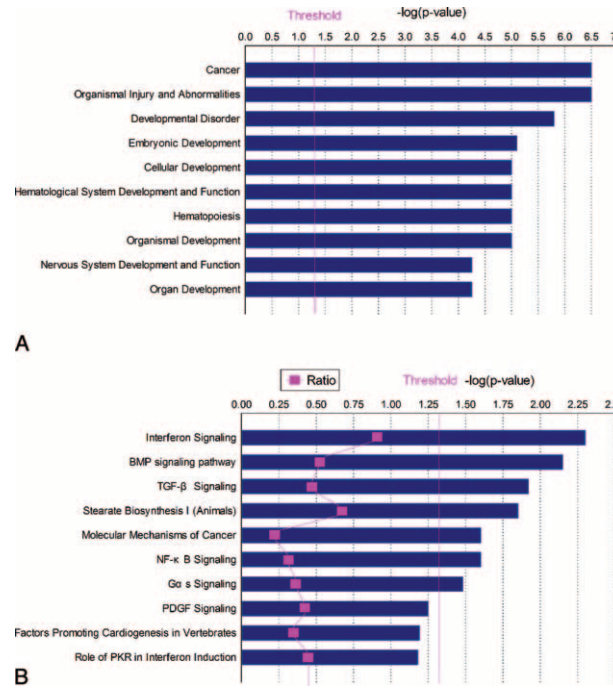

FIGURE 3. Molecular functions and canonical pathways were associated with the intersecting targets derived from the comparison of predicted targets of microRNA (miRNA) clusters with mRNA expression profiles. The top 10 molecular functions (A) and 10 pathways (B) are shown. A larger value on the X-axis indicates a higher degree of significance. The pink vertical line crossing all the bars indicates the threshold of significance, bars above this line represents $P < 0.05$.

In order to understand the specific signaling pathways involved, a pathway analysis was performed using IPA. Top-associated pathways for the intersecting targets included interferon signaling, bone morphogenetic proteins signaling, transforming growth factor-β signaling, molecular mechanisms of cancer, NF-κB signaling, and Gαs signaling (Figure 3B and see Table S7, Supplemental Digital Content http://links.lww.com/MD/A370, which describes enrichment pathway analysis of intersecting targets by IPA in detail). Finally, we compiled the miRNA–mRNA network, in which miRNA clusters may concurrently target multiple effectors of oncogenic pathways involved in the pathogenesis of synchronous CRC (Figure 4).

Clinicopathological Features of Synchronous CRC Patients

We assessed the clinicopathological features of 22 synchronous CRC cases and 579 solitary cancer controls (Table 1). Compared with solitary cancer patients, synchronous cases had more multiple extra-colonic cancers ($P = 0.012$) and coexistence of adenoma ($P = 0.012$). Additionally, synchronous cancers tended to be well differentiated compared with solitary cancers ($P = 0.073$).

Cancer Synchronicity Status and Patient Survival

During the follow-up period, there were 246 cancer-specific deaths for the 601 patients. No cancer-specific survival benefit was observed in synchronous cancer patients when compared with solitary cancer cases (log-rank, $P = 0.893$;

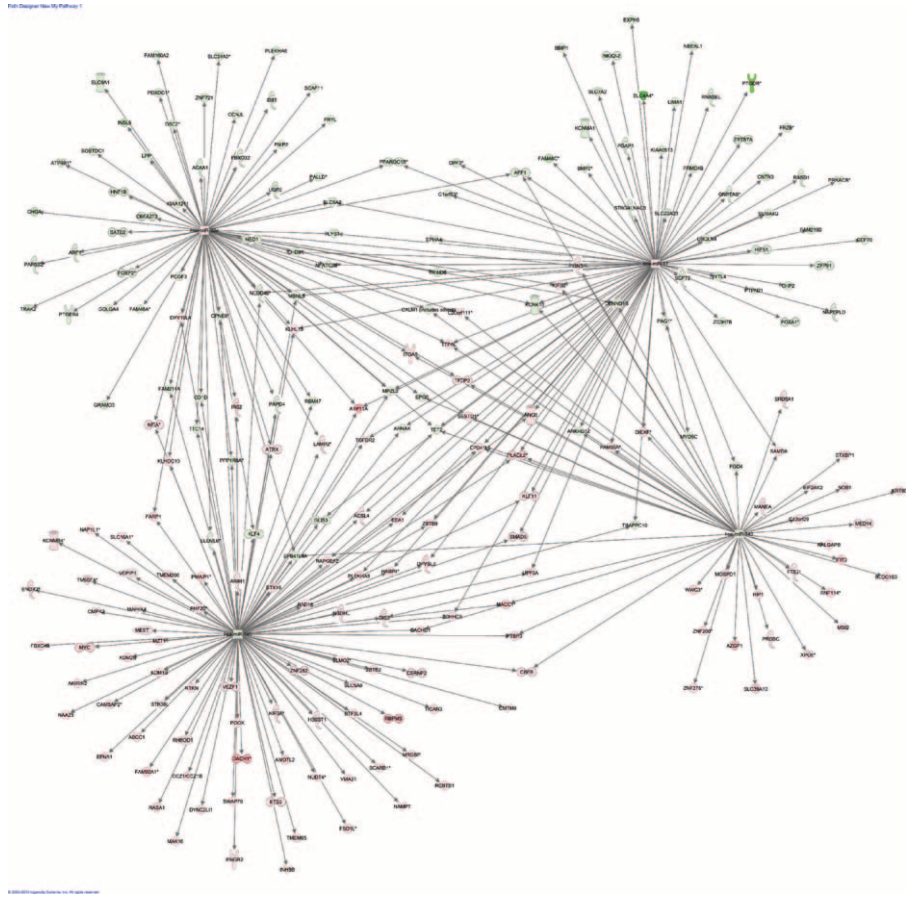

FIGURE 4. Network interaction map based on combined microRNA (miRNA) cluster expression, target prediction, and mRNA expression data for tumor and normal samples. Red symbols are assigned for upregulated and green symbols are for downregulated genes.

Figure 5A). Because MSI status is closely associated with the survival of CRC patients, we stratified synchronous cancer patients based on MSI phenotype. Within the synchronous cancer group, patients with MSI cancer had a worst clinical outcome compared with MSS patients, although not a significant difference ($P = 0.065$; Figure 5B). No difference of recurrence was observed in synchronous cancer patients compared with solitary cancer patients ($P > 0.05$; Table 1).

Pathological and Molecular Characteristics in Synchronous Versus Solitary Cancers

Compared with solitary cancers, synchronous cancer cases showed less *GLUT1* ($P = 0.037$) and MSI ($P = 0.024$). We did not find any difference in methylation patterns when comparing synchronous cancers with solitary cancers (Table 1).

As MSI status is closely associated with tumor location (ie, proximal colon) in CRC, we created a logistic regression model including tumor location, MSI status, and cancer synchronicity status as an outcome variable. The association between MSI and cancer synchronicity status persisted (adjusted odds ratio, 3.475; 95% CI: 1.007–11.984; $P = 0.049$).

DISCUSSION

ncRNAs play a major role in regulating almost every level of gene expression,[27] in which miRNAs are the most widely studied and characterized. Differential expression of miRNAs and their potential roles in pathogenesis of CRC have been extensively evaluated;[4–7] however, global miRNA expression profile from microarray has not been reported in synchronous CRC to date. In the present study, we identified 12 upregulated and 15 downregulated miRNAs in synchronous tumor tissue compared with normal tissue. Strikingly, an oncogenic miR-17-92 cluster including miR-17, miR-20a, miR-92a-1, and miR-92a-2 was significantly upregulated, and an oncosuppressive miR-143-145 cluster composed of miR-143 and miR-145 was significantly downregulated.

Previous reports showed that miR-17-92 cluster was upregulated in various human cancers, including CRC.[28] miR-17-92 cluster was associated with the progression of colorectal adenoma to adenocarcinoma.[26] Similarly, the enhanced expression of miR-143 and miR-145 in CRC acts as tumor suppressive by altering cell proliferation, migration, growth, and undergo apoptosis on genotoxic stimulation.[29]

Accumulating evidence strongly suggested that the members of a particular miRNA cluster were likely to be processed as cotranscribed units.[30] In the present study, 2 miRNA clusters were significantly dysregulated, and therefore highly representative of miRNAs for further functional analysis. Since individual miRNA may regulate multiple targets and act as tumor suppressors or oncogenes depending on their targets, we performed genome-wide analysis of miRNA:mRNA expression to

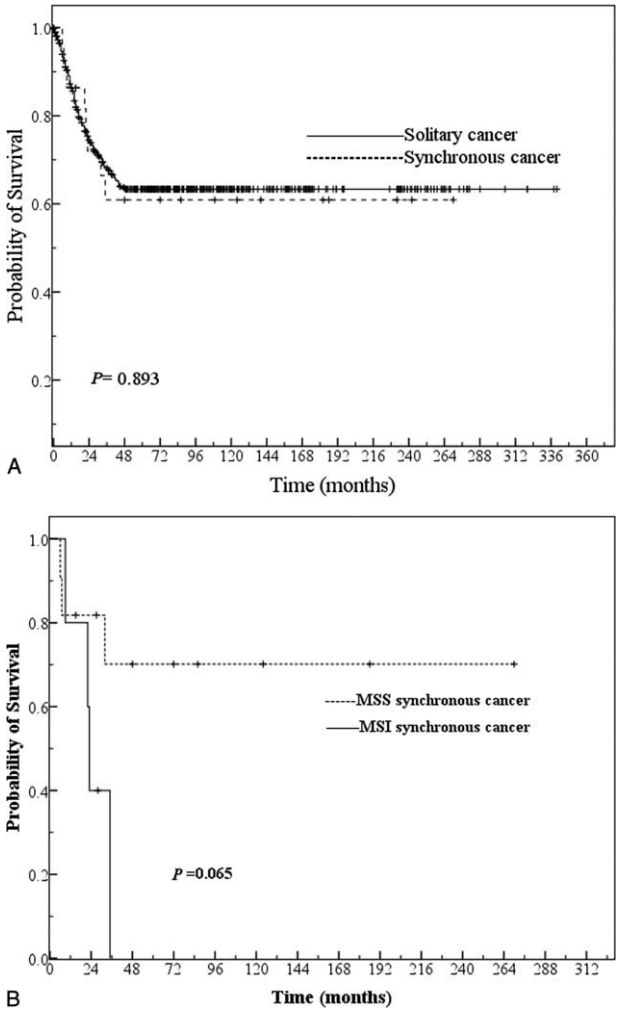

FIGURE 5. Kaplan–Meier curves for overall survival of CRC patients according to synchronicity status (A) and of synchronous CRC patients according to MSI status (B). CRC = colorectal cancer, MSI = microsatellite instability.

reveal the mechanisms of how these significantly dysregulated miRNAs clusters regulate gene expression in synchronous CRC.

As miRNA represses the expression of its targets, the first step was to obtain the intersecting targets based on the inverse relationship between miRNA expression and those of its potential targets. Thus, gene enrichment analysis on the combined expression data from our study on the miRNA clusters and the GSE15960 dataset showed that a total of 75 molecular functions were significantly associated with these targets, and the top 5 were cancer, organismal injury and abnormalities, developmental disorder, embryonic development, and cellular development. Furthermore, pathway analysis revealed that interferon signaling, bone morphogenetic proteins signaling pathway, transforming growth factor-β signaling, NF-κB signaling, and Gαs signaling were highly represented.

In the present study, MSI phenotype is more common in synchronous CRC than solitary cancer, which is consistent with other study.[31] Tumor multiplicity is a hallmark of hereditary cancers, and multiple tumors represent 5% to 10% of all CRC cases in the colorectum. It has been well documented that MSI phenotype is the result of a germline mutation in one of *MMR* genes (typically *MLH1* or *MSH2*) in most hereditary nonpolyposis CRC,[32] and the result of hypermethylation of MLH1 promoter in sporadic CRC.[33] In the current series, we did not find a significant loss of MLH1 or MSH2 or MSH6 expression, but the more multiple extra-colonic cancers and coexistence of adenoma, 2 important characteristics of hereditary nonpolyposis CRC, were found in synchronous CRC patients compared with solitary cancer cases, implying that most synchronous MSI tumors result from hereditary predisposition syndromes in our patient population. These findings were also supported by other studies.[34,35]

We did not find a difference in survival between patients with synchronous cancer and those with solitary cancer, as reported by others.[1,31] However, when we stratified synchronous cancer patients based on MSI status, a tendency of survival benefit was revealed in patients with MSS cancer rather than MSI cancer, indicating that MSI pathway plays an important role in the development of synchronous CRCs, and that information regarding MSI phenotype might help to predict clinical outcome of synchronous CRC. The consistency of our results with those previously reported suggested that current findings based on these observations are robust although they derived from a small sample size due to the rarity of synchronous CRC.

Since both MSI phenotype and miRNA activity can profoundly influence cancer cell behavior, determination of the relationship between them would be helpful for the understanding of miRNA function in synchronous CRC. Recently, genome-wide miRNA profiling showed that various miR-17-92 family members were significantly upregulated in MSS cancers, reliably distinguishing MSI from MSS CRCs.[36] Likewise, Valeri et al[37]found that overexpression of miR-155 downregulated the core MMR proteins (hMSH2, hMSH6, and hMLH1). These findings provide support for miRNA clusters modulation of MSI pathway as a mechanism of synchronous cancer pathogenesis.

Synchronous cancers showed less *GLUT1* expression when compared with solitary cancers in the present study. *GLUT1* is overexpressed and associated with poor prognosis in various cancers including CRC,[15] suggesting that *GLUT1* acts as an oncogene in human cancers. Although many miRNAs have been predicted to regulate *GLUT1*, none of those have been experimentally validated. In fact, *GLUT1* is predicted by TargetScan to be a candidate target of some upregulated miRNAs (miR-21, miR-93, and miR-203) and downregulated miRNAs (miR-143, miR-378, miR-181c, and miR-140) in the present study (data not shown). Therefore, the identification of potential regulation between *GLUT1* and dysregulated miRNAs, especially these upregulated miRNAs, will be helpful for the in-depth understanding of the roles of their dysregulations in synchronous CRC. The need for appropriate cell lines representing the molecular background of synchronous CRC is still in high demand.

Besides miRNAs, we observed a global upregulation of snoRNAs in tumor tissue in the present study. U56, U57, and HBII-55 are situated in chromosomal regions 20p13 that is frequently amplified in CRC,[38] while U74 and U78 are located in chromosome 1q25.1, which is one of the most frequently amplified chromosomal segments in solid tumors.[39] These results implied that snoRNAs might be specifically regulated in a background of CIN. U74 and U78 belong to the growth arrest-specific transcript 5 gene snoRNA cluster and their dysregulation correlated with growth arrest of breast cancer

cells.[40] Therefore, global upregulation of snoRNAs in synchronous CRC may have an important role in oncogenesis, similarly to miRNAs. Future investigations aiming at characterizing the role of specific snoRNAs in colorectal carcinogenesis are imperative in the cancer research field.

To further understand the molecular mechanism of synchronous CRC, we performed the comparison in miRNA and snoRNA expression of normal and tumor tissue between synchronous and solitary CRCs. The results showed that normal colorectal tissue from synchronous CRC patients were significantly different from those from solitary CRC patients in the context of miRNA and snoRNA expression, indicating the potentially differential genetic background of synchronous CRC from solitary CRC during carcinogenesis. When miRNA and snoRNA expression profile was compared in tumor tissue between synchronous and solitary CRC; however, only 1 miRNA and 2 snoRNAs were dysregulated. We speculate that although synchronous and solitary CRC have different genetic background during carcinogenesis, the difference in miRNAs and snoRNAs expression after tumor formation was subtle except miR-1234 and snoRNA U17a and U36A. Further study is needed to validate this speculation and determine whether these 3 ncRNAs could potentially be used to differentiate synchronous CRC from solitary CRC.

We are aware, however, of some limitations of this study. First, relatively small sample sizes in retrospective cohort study were not enough to verify the biological significance of epigenetics in the pathogenesis of synchronous CRC. Second, the relative limitations are, to a certain point, the difficulties when clarifying the specific role of individual snoRNA in synchronous colorectal carcinogenesis because of the paucity of advanced methods.

In conclusion, the dysregulated miRNAs rather than snoRNAs can differentiate synchronous cancer from normal tissue, indicating that miRNA could potentially be used as a diagnostic tool for synchronous CRC. Our findings suggest the potentially differential genetic background of synchronous CRC from solitary CRC during carcinogenesis, and the dysregulated miRNAs including miR-17-92 and miR-143-145 clusters, as well as the aberrant expressed snoRNAs, may be functionally associated with the pathogenesis of synchronous CRC, and hence may serve as therapeutic targets for cancer intervention.

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REFERENCES

1. Latournerie M, Jooste V, Cottet V, et al. Epidemiology and prognosis of synchronous colorectal cancers. *Br J Surg.* 2008;95:1528–1533.
2. Nosho K, Kure S, Irahara N, et al. A prospective cohort study shows unique epigenetic, genetic, and prognostic features of synchronous colorectal cancers. *Gastroenterology.* 2009;137:1609–1620e1-3.
3. Farazi TA, Spitzer JI, Morozov P, Tuschl T. miRNAs in human cancer. *J Pathol.* 2011;223:102–115.
4. Schetter AJ, Leung SY, Sohn JJ, et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA.* 2008;299:425–436.
5. Faltejskova P, Svoboda M, Srutova K, et al. Identification and functional screening of microRNAs highly deregulated in colorectal cancer. *J Cell Mol Med.* 2012;16:2655–2666.
6. Yan H, Choi AJ, Lee BH, et al. Identification and functional analysis of epigenetically silenced microRNAs in colorectal cancer cells. *PLoS One.* 2011;6:e20628.
7. Hamfjord J, Stangeland AM, Hughes T, et al. Differential expression of miRNAs in colorectal cancer: comparison of paired tumor tissue and adjacent normal mucosa using high-throughput sequencing. *PLoS One.* 2012;13:e34150.
8. Pacilli A, Ceccarelli C, Treré D, et al. SnoRNA U50 levels are regulated by cell proliferation and rRNA transcription. *Int J Mol Sci.* 2013;14:14923–14935.
9. Galamb O, Spisák S, Sipos F, et al. Reversal of gene expression changes in the colorectal normal-adenoma pathway by NS398 selective COX2 inhibitor. *Br J Cancer.* 2010;102:765–773.
10. Lu M, Shi B, Wang J, et al. TAM: a method for enrichment and depletion analysis of a microRNA category in a list of microRNAs. *BMC Bioinformatics.* 2010;11:419.
11. Sun XF. p73 overexpression is a prognostic factor in patients with colorectal adenocarcinoma. *Clin Cancer Res.* 2002;8:165–170.
12. Sun XF, Carstensen JM, Stål O, et al. Proliferating cell nuclear antigen (PCNA) in relation to ras, c-erbB-2, p53, clinico-pathological variables and prognosis in colorectal adenocarcinoma. *Int J Cancer.* 1996;69:5–8.
13. Zhang H, Sun XF. Loss of p27 expression predicts poor prognosis in patients with Dukes' B stage or proximal colorectal cancer. *Int J Oncol.* 2001;19:49–52.
14. Zhang H, Sun XF. Overexpression of cyclooxygenase-2 correlates with advanced stages of colorectal cancer. *Am J Gastroenterol.* 2002;97:1037–1041.
15. Shen YM, Arbman G, Olsson B, et al. Overexpression of GLUT1 in colorectal cancer is independently associated with poor prognosis. *Int J Biol Markers.* 2011;26:166–172.
16. Jansson A, Sun XF. Ki-67 expression in relation to clinicopathological variables and prognosis in colorectal adenocarcinomas. *APMIS.* 1997;105:730–734.
17. Evertsson S, Bartik Z, Zhang H, et al. Apoptosis in relation to proliferating cell nuclear antigen and Dukes' stage in colorectal adenocarcinoma. *Int J Oncol.* 1999;15:53–58.
18. Emterling A, Wallin A, Arbman G, et al. Clinicopathological significance of microsatellite instability and mutated RIZ in colorectal cancer. *Ann Oncol.* 2004;15:242–246.
19. Jansson A, Arbman G, Zhang H, et al. Combined deficiency of hMLH1, hMSH2, hMSH3 and hMSH6 is an independent prognostic factor in colorectal cancer. *Int J Oncol.* 2003;22:41–49.
20. Zhang H, Nordenskjöld B, Dufmats M, et al. K-ras mutations in colorectal adenocarcinomas and neighbouring transitional mucosa. *Eur J Cancer.* 1998;34:2053–2057.
21. Pfeifer D, Arbman G, Sun XF. Polymorphism of the p73 gene in relation to colorectal cancer risk and survival. *Carcinogenesis.* 2005;26:103–107.
22. Jansson A, Gentile M, Sun XF. p53 mutations are present in colorectal cancer with cytoplasmic p53 accumulation. *Int J Cancer.* 2001;92:338–341.
23. Zhou XL, Eriksson U, Werelius B, et al. Definition of candidate low risk APC alleles in a Swedish population. *Int J Cancer.* 2004;110:550–557.

24. Zhang H, Arbman G, Sun XF. Codon 201 polymorphism of DCC gene is a prognostic factor in patients with colorectal cancer. *Cancer Detect Prev.* 2003;27:216–221.
25. Löf-Öhlin ZM, Nilsson TK. Pyrosequencing assays to study promoter CpG site methylation of the O^6-MGMT, hMLH1, $p14^{ARF}$, $p16^{INK4a}$, RASSF1A and APC1A genes. *Oncol Rep.* 2009;21: 721–729.
26. Babak T, Zhang W, Morris Q, et al. Probing microRNAs with microarrays: tissue specificity and functional inference. *RNA.* 2004;10:1813–1819.
27. Galasso M, Sana ME, Volinia S. Non-coding RNAs: a key to future personalized molecular therapy? *Genome Med.* 2010;2:12.
28. Nicoloso MS, Spizzo R, Shimizu M, et al. MicroRNAs – the microsteering wheel of tumour metastases. *Nat Rev Cancer.* 2009;9:293–302.
29. Pagliuca A, Valvo C, Fabrizi E, et al. Analysis of the combined action of miR-143 and miR-145 on oncogenic pathways in colorectal cancer cells reveals a coordinate program of gene repression. *Oncogene.* 2013;32:4806–4813.
30. Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA.* 2005;11:241–247.
31. Bae JM, Cho NY, Kim TY, et al. Clinicopathologic and molecular characteristics of synchronous colorectal cancers: heterogeneity of clinical outcome depending on microsatellite instability status of individual tumors. *Dis Colon Rectum.* 2012;55:181–190.
32. Peltomäki P, Vasen HF. Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. *Gastroenterology.* 1997;113:1146–1158.
33. Herman JG, Umar A, Polyak K, et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci U S A.* 1998;95:6870–6875.
34. Pedroni M, Tamassia MG, Percesepe A, et al. Microsatellite instability in multiple colorectal tumors. *Int J Cancer.* 1999;81:1–5.
35. Abe Y, Masuda H. Genetic alterations of sporadic colorectal cancer with microsatellite instability, especially characteristics of primary multiple colorectal cancers. *J Surg Oncol.* 2000;74:249–256.
36. Lanza G, Ferracin M, Gafà R, et al. mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. *Mol Cancer.* 2007;6:54.
37. Valeri N, Gasparini P, Fabbri M, et al. Modulation of mismatch repair and genomic stability by miR-155. *Proc Natl Acad Sci U S A.* 2010;107:6982–6987.
38. Tsafrir D, Bacolod M, Selvanayagam Z, et al. Relationship of gene expression and chromosomal abnormalities in colorectal cancer. *Cancer Res.* 2006;66:2129–2137.
39. Mannoor K, Liao J, Jiang F. Small nucleolar RNAs in cancer. *Biochim Biophys Acta.* 2012;1826:121–128.
40. Mourtada-Maarabouni M, Pickard MR, Hedge VL, et al. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. *Oncogene.* 2009;28:195–208.