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. Herein, 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

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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

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.
(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. 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 miRNA microarray data of colorectal tumor and the matched normal tissue was obtained from GEO database (accession number GSE15960). The raw data (CEL files) was normalized and then analyzed using GeneSpring Software 10.0. Unpaired t-test was applied to identify dysregulated miRNA 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. 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 miRNA 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 miRNA microarray. Similarly, the predicted targets for downregulated miRNAs were compared with the upregulated mRNAs in the microarray.
| Clinicopathological features | N     | Solitary Cancer Patients (Controls) | Synchronous Cancer Patients (Cases)* | P Value |
|-----------------------------|-------|------------------------------------|-------------------------------------|---------|
|                            |       | 304 (92.4)                         | 12 (3.7)                            | 0.851   |
| Solitary Cancer Patients    |       | 275 (92.6)                         | 10 (3.4)                            |         |
|                            | 329   |                                    | 12 (3.7)                            |         |
|                            | 277   |                                    | 10 (3.4)                            |         |
|                            | 54    |                                    |                                     |         |
|                            | 626   | 70.69 ± 10.21                      | 71.09 ± 11.43                       | 0.857   |
|                            |       |                                    |                                     |         |
| Tumor location             |       |                                    |                                     |         |
|                            | 228   | 203 (89.0)                         | 12 (5.3)                            | 0.161   |
|                            | 389   | 368 (94.6)                         | 10 (2.6)                            |         |
|                            | 9     | 8 (88.9)                           | 0 (0)                               |         |
|                            |       |                                    |                                     |         |
| TNM stage                  |       |                                    |                                     |         |
|                            | 85    | 77 (90.6)                          | 4 (4.7)                             | 0.497   |
|                            | 161   | 148 (91.9)                         | 5 (3.1)                             |         |
|                            | 121   | 112 (92.6)                         | 5 (4.1)                             |         |
|                            | 68    | 67 (98.5)                          | 0 (0)                               |         |
|                            | 191   | 175 (91.6)                         | 8 (4.2)                             |         |
| Tumor differentiation      |       |                                    |                                     |         |
|                            | 31    | 26 (83.9)                          | 3 (9.7)                             | 0.073   |
|                            | 424   | 392 (92.4)                         | 16 (3.8)                            |         |
|                            | 169   | 159 (94.1)                         | 3 (1.8)                             |         |
| Multiple extra-colonic cancers |   |                                    |                                     |         |
|                            | 120   | 116 (96.7)                         | 0 (0)                               | 0.012   |
|                            | 506   | 463 (91.5)                         | 22 (4.3)                            |         |
| Coexistence of adenoma     |       |                                    |                                     |         |
|                            | 5     | 3 (60.0)                           | 2 (40.0)                            | 0.012   |
|                            | 621   | 576 (92.8)                         | 20 (3.2)                            |         |
| Perioperative therapy      |       |                                    |                                     |         |
|                            | 106   | 100 (94.3)                         | 5 (4.7)                             | 0.441   |
|                            | 459   | 421 (91.7)                         | 14 (3.1)                            |         |
| Surgery                    |       |                                    |                                     |         |
|                            | 19    | 18 (94.7)                          | 1 (5.3)                             | 0.481   |
|                            | 177   | 162 (91.6)                         | 5 (2.8)                             |         |
| Complications              |       |                                    |                                     |         |
|                            | 69    | 62 (89.9)                          | 4 (5.8)                             | 0.188   |
|                            | 127   | 118 (92.9)                         | 2 (1.6)                             |         |
| Local recurrence           |       |                                    |                                     |         |
|                            | 16    | 14 (87.4)                          | 1 (1.3)                             | 0.399   |
|                            | 181   | 167 (92.3)                         | 5 (2.7)                             |         |
| Distant recurrence         |       |                                    |                                     |         |
|                            | 40    | 37 (92.5)                          | 2 (5.0)                             | 0.606   |
|                            | 157   | 144 (91.7)                         | 4 (2.5)                             |         |
| Molecular features         |       |                                    |                                     |         |
|                            | 96    | 92 (95.8)                          | 4 (4.2)                             | 0.187   |
|                            | 64    | 64 (100)                           | 0 (0)                               |         |
|                            | 73    | 69 (94.5)                          | 4 (5.5)                             |         |
| RAS                        |       |                                    |                                     |         |
|                            | 111   | 108 (97.3)                         | 3 (2.7)                             | 0.745   |
|                            | 166   | 160 (96.4)                         | 6 (3.6)                             |         |
| e-erbB                     |       |                                    |                                     |         |
|                            | 139   | 131 (94.2)                         | 6 (5.8)                             | 0.331   |
|                            | 140   | 137 (97.9)                         | 3 (2.1)                             |         |
| p53                        |       |                                    |                                     |         |
|                            | 70    | 68 (97.1)                          | 2 (2.9)                             | 0.721   |
|                            | 144   | 137 (95.1)                         | 7 (4.9)                             |         |
| p73                        |       |                                    |                                     |         |
|                            |       |                                    |                                     |         |
| p27                        |       |                                    |                                     |         |
|                        | 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)                            |         |
| **O6-MGMT**            |       |                                   |                                    |         |
| (–)                    | 44    | 42 (95.5)                         | 2 (4.5)                            | 1       |
| (+)                    | 29    | 28 (96.6)                         | 1 (3.4)                            |         |
| **p14ARF**             |       |                                   |                                    |         |
| (–)                    | 50    | 48 (96.0)                         | 2 (4.0)                            | 1       |
| (+)                    | 23    | 22 (95.7)                         | 1 (4.3)                            |         |
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 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. 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 1320 and p73 polymorphism,21 DNA sequencing for p53 mutation,22 and APC (adenomatous polyposis coli, 8636 C > A) polymorphism23 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 (O6-methylguanine DNA methyltransferase, hMLH1, p14ARF, p16INK4a, 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 $\chi^2$ 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 non-parametric 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

| 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| 1.842222    | 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    | 0.041041| Up         | 3          | miRNA         | miR-191                       |
| hsa-miR-21_1_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    | 0.022122| Down       | 5          | miRNA         | miR-145                       |
| hsa-miR-92a_st| 3.387116    | 0.025076| Down       | 5          | miRNA         | miR-143                       |
| hsa-miR-143_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        | 1.731311    | 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| Down       | 11         | CDBox         | U30                           |
| HBBI-55_st    | 1.316304    | 0.024868| Up         | 20         | CDBox         | HBBI-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         | HAcBox        | U68                           |
| HBII-61_st    | 1.117102    | 0.049732| Up         | 3          | HAcBox        | HBII-61                       |
| U56_x_st      | 1.114402    | 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          | HAcBox        | 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.
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. 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 miRNAs 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 over-expression of miRNAs, and 118 genes potentially downregulated due to an under-expression of miRNAs, and 1127 upregulated (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).

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-$\beta$ signaling, molecular mechanisms of cancer, NF-$\kappa$B signaling, and G$\alpha$s 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, $P = 0.893$; $P = 0.893$).
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 worse 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, 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; 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. miR-17-92 cluster was associated with the progression of colorectal adenoma to adenocarcinoma. 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.

Accumulating evidence strongly suggested that the members of a particular miRNA cluster were likely to be processed as cotranscribed units. 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
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 Gox signaling were highly represented.

In the present study, MSI phenotype is more common in synchronous CRC than solitary cancer, which is consistent with other studies.1,31 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 al37 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. U65, U57, and HBII-55 are situated in chromosome 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

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.
cells. 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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