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Genes involved in the transition from normal epithelium to intraepithelial neoplasia are associated with colorectal cancer patient survival

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ABSTRACT

Whether the heterogeneity in tumor cell morphology and behavior is the consequence of a progressive accumulation of genetic alterations or an intrinsic property of cancer-initiating cells established at initiation remains controversial. The hypothesis of biological predetermination in human cancer was proposed many years ago and states that the biological potency of cancer cells is predestinated in the precancerous stage. The present study aimed to investigate whether the aberrant molecular events occurring in initial cancer stages could eventually influence colorectal cancer (CRC) progression. We analyzed the mRNA and miRNA expression profiles of colorectal normal mucosa, low-grade intraepithelial neoplasia (LIN), high-grade intraepithelial neoplasia (HIN), and adenocarcinoma tissues. Compared with the transitions from LIN to HIN to invasive carcinoma, the transition from normal epithelium to LIN appeared to be associated with greater changes in the number and expression levels of mRNAs and miRNAs, with a differential expression of 2322 mRNAs and 71 miRNAs detected. Utilizing these early molecular changes, a miRNA-hub network analysis showed that 166 genes were identified as targets regulated by 30 miRNAs. Among these genes, a 55-gene signature regulated by 5 miRNAs was shown to be associated with overall survival or disease-free survival in three independent sample sets. Thus, the molecular changes in the transcriptome associated with the transition from normal to intraepithelial neoplasm may influence CRC progression.

1. Introduction

Based on the multistage and multistep model of colorectal carcinogenesis [1], early detection and excision of intraepithelial lesions may reduce CRC morbidity and mortality. However, this clinical strategy appears to be ineffective for some patients with initially aggressive cancers. Hence, the conceptual stepwise framework of tumorigenesis does not fully reflect the natural history of cancer. Early in the 1950s, MacDonald proposed the notion of biological predeterminism in human cancers based on a clinical survey suggesting that the clinical outcome could be defined by the intrinsic or destined natural history of cancer [2]. This theory challenges the widely accepted sequential model of carcinogenesis. Utilizing the mammary intraepithelial neoplasia outgrowth mouse model of ductal carcinoma *in situ*, researchers found that mammary precancerous tissue contains specific cells that possess the ability to reconstruct the precancerous tissue and that these cells exhibit malignant potential independent of the accumulation of genetic alterations [3]. Invasive behavior was discovered in pancreatic intraepithelial neoplasias through *in vivo* lineage tracing, indicating that cancer dissemination precedes pancreatic tumor formation [4]. Husemann et al. provided evidence that early invasive behavior is irrelevant to tumor size in mouse models of breast cancer [5]. These observations imply that certain capabilities of cancer cells may be fully developed in intraepithelial neoplasms and that the risk for intraepithelial neoplasm progression is predictable. If correct, this hypothesis has profound implications for researchers who should pay more attention to the initial and predisposing alterations in intraepithelial neoplasms and reconsider current guidelines for cancer diagnosis and treatment. However, a limited number of studies have focused

Abbreviations: LIN, low-grade intraepithelial neoplasia; HIN, high-grade intraepithelial neoplasia; CRC, colorectal cancer; GO, gene ontology; PCA, principal component analysis; PC, principal component; FDR, false-discovery rate.

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on the association of genes involved in intraepithelial neoplasms and the clinical outcome of cancer. These genes may be promising for the early detection and prognosis prediction of CRC developing from intraepithelial neoplasm.

In the present study, to explore whether the molecular alterations in the transcriptome in the transition from normal to LIN can eventually affect the subsequent progression of colorectal carcinogenesis, mRNA and miRNA expression profiles were analyzed in human colorectal normal mucosa tissues as well as LIN, HIN, and adenocarcinoma tissues. Through integrative analysis of miRNA and mRNA expression profiles, the association between the molecular events involved in the transition from colorectal normal mucosa to intraepithelial neoplasia and the clinical outcome of CRC was evaluated.

2. Materials and methods

2.1. Specimens

Biopsy samples of colorectal LINs, HINs, and adenocarcinomas were obtained from patients undergoing colonoscopy in the Department of Endoscopy, Cancer Hospital, Chinese Academy of Medical Sciences, between 2008 and 2011. Patients with a history of familial adenomatous polyposis, hereditary non-polyposis colorectal cancer, or inflammatory bowel disease were excluded. The LIN is defined as low-grade adenoma/dysplasia; the HIN includes high-grade adenoma/dysplasia, carcinoma *in situ*, suspicion for invasive carcinoma and intramucosal carcinoma; the adenocarcinoma is defined as submucosal invasion by adenocarcinoma [6]. According to ASGE guideline [7], biopsy specimens of colorectal neoplasia were excised from 4 to 6 different areas, including the edges and the center of the lesion. Normal colorectal mucosae samples were detached from the surgical specimens obtained from patients with hemorrhoids undergoing surgical excision in the Department of Colon and Rectal Surgery of Beijing Shi Ji Tan Hospital between 2009 and 2010. The tissue samples were snap frozen in liquid nitrogen immediately after biopsy or surgery and stored at −80 °C. A portion of the same tissue was subjected to pathological analysis. Histopathological assessment of all samples was performed by two independent, blinded and experienced pathologists. Samples satisfied with the diagnostic criteria of normal mucosa and neoplasia (neoplastic cells >70%) were enrolled. If more than one biopsy tissue from the same patient was enrolled, these samples were pooled. This study was approved by the Ethics Committee of the Cancer Institute, Chinese Academy of Medical Sciences, and informed consent was acquired from all patients. Information regarding enrolled patients and the relevant characteristics of the subjects are presented in Supplementary Table 1.

2.2. RNA isolation

Total RNA was extracted from frozen tissue using the TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. RNA integrity was determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). If the RNA integrity number was greater than or equal to five, the total RNA was purified using the RNeasy Mini Kit (Cat No. 74106, Qiagen, Germany). The RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.3. Microarray expression profiling

After histopathological evaluation and RNA integrity analysis, 12 colorectal normal mucosa, 21 LIN, 30 HIN, and 25 adenocarcinoma samples were analyzed using mRNA microarrays, and 15 normal mucosa, 39 LIN, 20 HIN, and 33 adenocarcinoma tissues were analyzed using miRNA microarrays. Purified RNA samples were labeled and hybridized to Agilent 4×44K Whole Human Genome Oligo Microarrays (G4112F) according to the manufacturer's protocol. Total RNA was analyzed with an Agilent 8×15K Human miRNA Microarray V3 (G4470C). The microarray data have been deposited in NCBI's Gene Expression Omnibus with the series accession numbers GSE41657 and GSE41655.

2.4. Microarray statistical analyses

2.4.1. Microarray data normalization

The mRNA microarray raw data were global median normalized with the GeneSpring GX software, version 11.5 (Silicon Genetics, Redwood City, CA, USA). A total of 19,503 single probes were obtained based on GeneID, reserving the probe with the largest frequency for the flag-P (present) probe. For the miRNA microarray raw data, quantile normalization was performed using R project software (R Foundation for Statistical Computing, Vienna, Austria). Measured miRNAs were estimated as present if a miRNA was present in more than 50% of all samples or more than 80% of a specific group of samples. Finally, the expression profiles of 214 miRNAs were acquired.

2.4.2. Differentially expressed mRNA and miRNA screening,GO enrichment and KEGG pathway enrichment analyses

The assessment of differentially expressed mRNAs or miRNAs between different stages of colorectal carcinogenesis was performed using a generalized linear model (*Bonferroni*-corrected $P < 0.05$) in R project software. Gene Ontology (GO) enrichment was performed using the software DAVID [8,9] (False-Discovery Rate-corrected $P < 0.05$). The interested genes were mapped to the KEGG database using the SubpathwayMiner tool [10], an R-based software package, for pathway enrichment analysis. We used hypergeometric distribution test to calculate the P value and adjusted the P value using the FDR method.

2.4.3. Construction of a miRNA-mRNA regulatory network

In total, 65 cases were subjected to both mRNA and miRNA microarray analysis, including 11 normal mucosae, 17 LINs, 16 HINs, and 21 adenocarcinomas; thus, a combination of profiles was analyzed. First, we searched five miRNA databases (DIANA-microT 3.0 [11], miRanda [12], TargetScan [13], PicTar [14] and RNAhybrid [15]) and selected miRNA-mRNA interactions that were present in at least two databases; 770 miRNAs and 14,545 target genes were obtained. Among the 770 miRNAs, 189 miRNAs were found in filtered 214 miRNAs for further analysis. Second, the negative *Pearson* correlations between miRNAs and mRNAs in 65 samples of all stages were calculated (*Bonferroni*-corrected $P < 0.05$). Third, we integrated the miRNA-mRNA interaction results that emerged both in database searching and microarray data calculations to determine the miRNA–mRNA regulatory network. The data analysis described above was performed with R project software.

2.4.4. Kaplan–Meier survival analysis and Cox regression analysis

The mRNA expression profiles of CRC with prognosis information were retrieved from the Gene Expression Omnibus, accession numbers GSE17536, GSE14333, and GSE17537. All survival data were extracted from the original publication. Principal component analysis (PCA) was conducted using interested genes in each dataset. The first principal component (PC1) captures the greatest amount of total variance in the profiles, and the patients were divided into two groups with equal size based on the rank order of PC1 across their tumor profiles. Kaplan–Meier survival analyses were performed to evaluate the association between the PCA-as-

signed groups and survival time [16]. A log-rank test was applied to assess significance. The Cox proportional hazards regression model was used to evaluate the independence of the prognostic factors in a stepwise manner using SPSS 15.0 (SPSS Inc., Chicago, USA). A value of $P < 0.05$ was regarded as significant.

3. Results

3.1. mRNA and miRNA expression profiles during colorectal carcinogenesis

Compared with the mRNA expression profiles of normal mucosa, a set of 2322 differentially expressed genes were identified in LIN, including 756 up-regulated genes and 1566 down-regulated genes. The expression variations of these two gene clusters over the entire course of CRC development are shown in Fig. 1A. The two gene clusters displayed an inverse expression pattern, and the expression levels of the two clusters changed from normal to LIN remarkably and then maintained relatively stable expression levels from LIN to HIN to adenocarcinoma. GO enrichment analysis revealed that the up-regulated genes were involved in RNA processing and mRNA metabolic processes, whereas the down-regulated genes were enriched in immune responses, positive regulation of immune system processes, leukocyte activation, cellular ion homeostasis, and homeostatic processes (Fig. 1B). Ninety-seven differentially expressed genes were screened between LIN and HIN (Fig. S1A), and no significant GO categories were enriched. From HIN to adenocarcinoma, 204 genes were up-regulated and 91 genes were down-regulated (Fig. S1B). The functional categories of these 295 genes exhibited cancer-related characteristics involving the cell cycle, extracellular matrix organization, collagen fibril organization, and vasculature development (Fig. S1C). The overlap of the gene sets in the three carcinogenesis phases depicted above is illustrated as a Venn diagram (Fig. S2A) showing that only 84 genes of the 2322 altered genes from normal to LIN also changed in the other two phases. The three gene sets with the fold changes and *Bonferroni*-corrected *P* values are listed in Supplementary Excel File 1.

To investigate the miRNA regulatory mechanism behind the mRNA expression of colorectal carcinogenesis, miRNA expression levels were profiled. Compared with the miRNA expression profiles of normal mucosa, 71 miRNAs were differentially expressed in LIN, among which 18 miRNAs were over-expressed and 53 miRNAs were under-expressed. As shown in Fig. 1C, the expression levels of these two miRNA clusters also varied significantly from normal to LIN and presented mild variations during the subsequent processes, which was similar to the expression pattern of altered mRNAs in this initial phase. Only 3 differentially expressed miRNAs were screened between LIN and HIN (Fig. S1D). When HIN transitioned to adenocarcinoma, 10 up-regulated and 5 down-regulated miRNAs were significantly changed (Fig. S1E). An intersectional analysis of the three miRNA sets in different stages revealed that 6 of the 71 miRNAs altered from normal to LIN changed in the subsequent processes (Fig. S2B). The three miRNA sets with the fold changes and *Bonferroni*-corrected *P* values are shown in Supplementary Excel File 2. Collectively, both mRNA and miRNA expression levels showed prominent alterations during the transition from normal epithelium to LIN; therefore, integration of the data in two levels was more effective in investigating the molecular events involved in early CRC development.

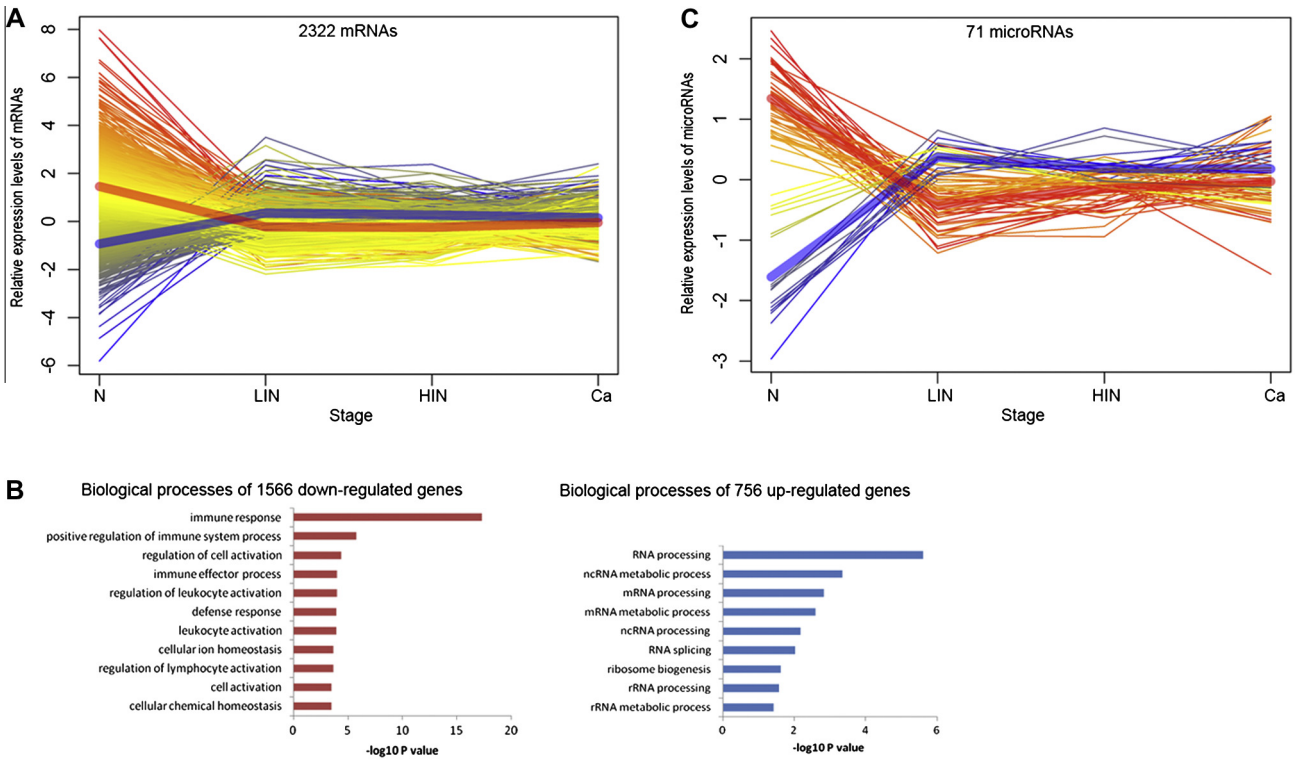

Fig. 1. The relative expression patterns of differentially expressed mRNAs (A) and microRNAs (C) between normal mucosa and LIN throughout the process of colorectal carcinogenesis. A line represents the relative expression level of a gene across four stages of colorectal carcinogenesis. A red or blue bold line represents the average expression level of a cluster of down-regulated or up-regulated genes. N, normal mucosa; LIN, low-grade intraepithelial neoplasia; HIN, high-grade intraepithelial neoplasia; Ca, adenocarcinoma. (B) GO enrichment analysis of differentially expressed mRNAs from normal mucosa to LIN. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. MicroRNA–mRNA regulatory networks of colorectal carcinogenesis

Based on the mRNA and miRNA interaction mechanisms, a miRNA–mRNA regulatory network was established using the miRNAs and mRNAs altered from normal to LIN. In the network, 5 elevated miRNAs and 55 down-regulated target genes composed 5 sub-networks (named module A), and 25 reduced miRNAs that targeted 111 up-regulated mRNAs composed 25 sub-networks (named module B). The miRNAs and their target mRNAs in the network are listed in Supplementary Table 2. Because the negative correlation of miRNAs and mRNAs was computed based on the microarray data of samples in all stages, this network existed throughout the process of CRC development but with different expression levels. The expression levels of the miRNAs and mRNAs in the network differed substantially between normal mucosa and LIN (Fig. 2), whereas there were only slight fluctuations in HIN (Fig. S3A) and adenocarcinoma (Fig. S3B) relative to LIN. Therefore, this miRNA–mRNA interaction network changed expression levels in the process of LIN formation and sustained aberrant functioning throughout carcinogenesis, which may affect adenocarcinoma outcome.

3.3. The association between the expression of miRNA-targeted genes and the clinical outcome of CRC

To determine whether the expression levels of miRNA-targeted genes in the miRNA–mRNA regulatory network are associated with the clinical outcome of CRC patients, three independent sample sets from published CRC databases were used. In dataset GSE17536, 177 patients with colorectal adenocarcinoma were divided into two groups based on the rank order of the first principal component (PC1) of the 55 down-regulated genes in module A across their tumor expression profiles. Kaplan–Meier analysis showed that the median overall survival time in the PC1-group 2 was significantly longer than that in the PC1-group 1 ($P = 0.001$; Fig. 3A). In addition, the expression of the 55-gene signature was associated with early stage (I and II) CRC survival in this dataset ($P = 0.003$; Fig. 3B). The prognostic value of this gene signature was validated in two additional datasets: GSE17537, with overall survival information ($P = 0.031$; Fig. 3C), and GSE14333, with disease-free survival information ($P = 0.013$; Fig. 3D). The Cox regression model showed that the 55-gene signature was an independent prognostic factor for CRC in all three datasets (the details of the

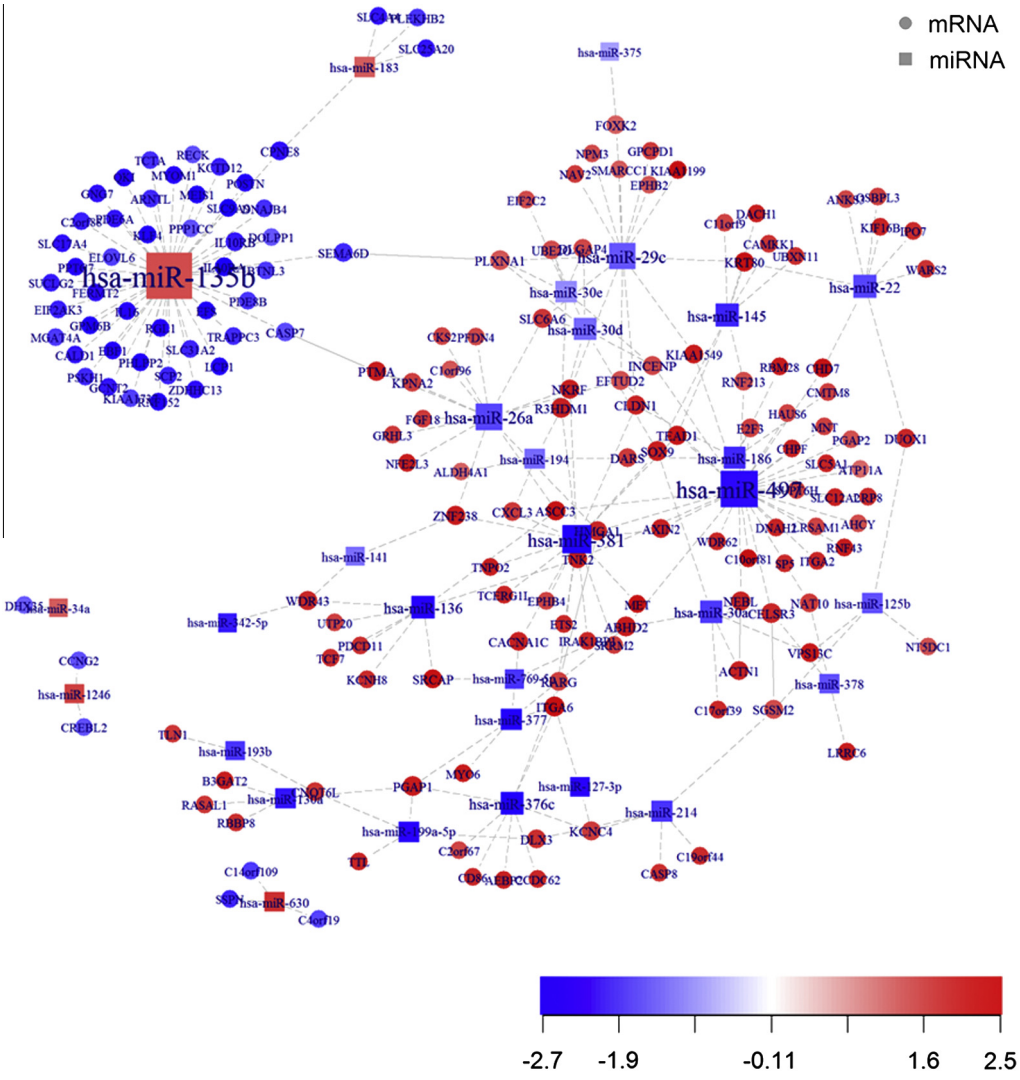

Fig. 2. The relative expression of the miRNA-mRNA regulatory network in colorectal low-grade intraepithelial neoplasia compared to normal mucosa. The degree of relatively low to high expression levels is represented by color depth from blue to red. Gray dotted lines connect miRNAs to their predicted target genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

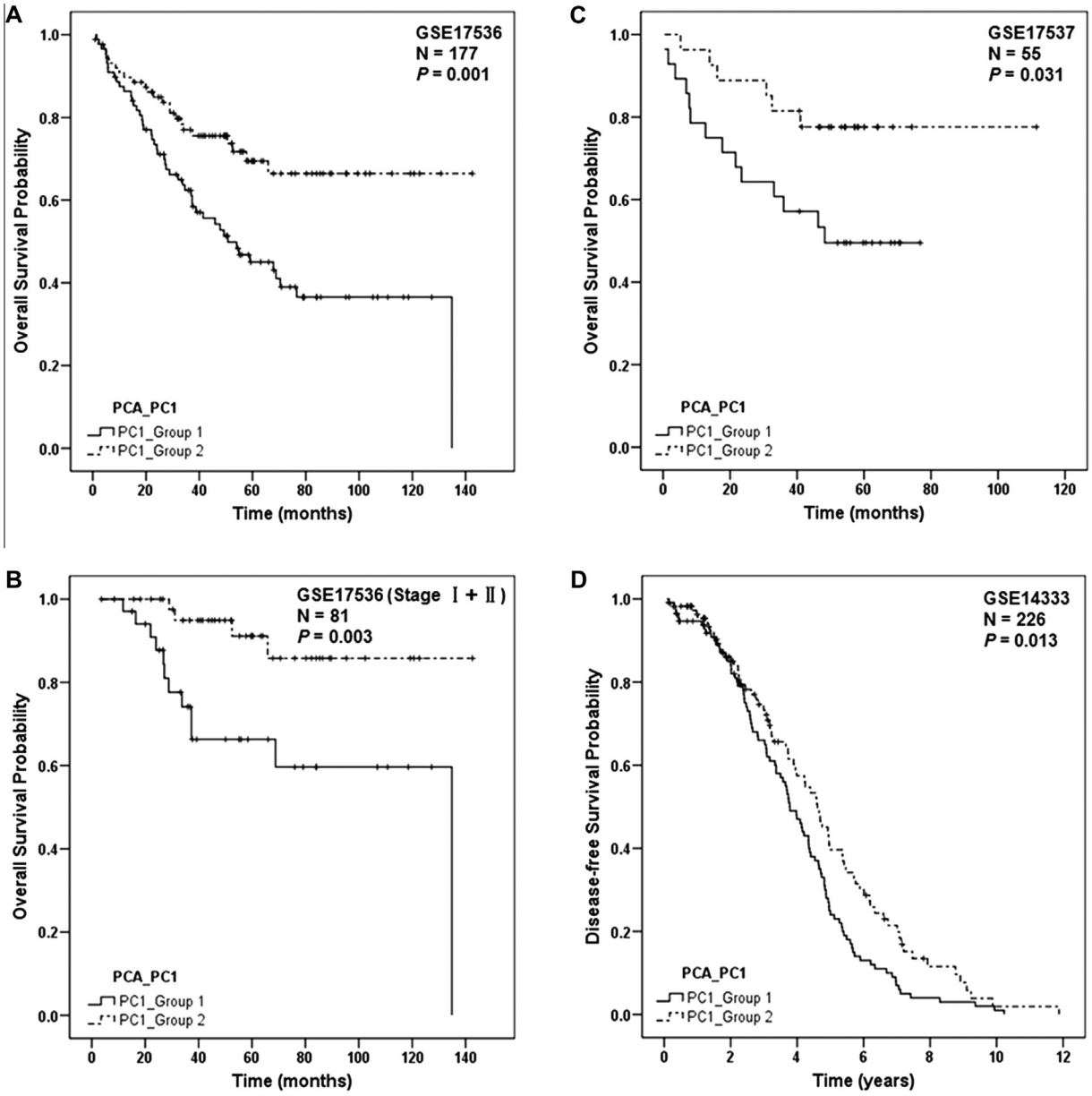

Fig. 3. Kaplan–Meier survival analyses of overall survival time or disease-free survival time defined by a 55-gene set in three published gene expression datasets of CRC. Kaplan–Meier analyses were performed using the PCA-assigned Group 1 and Group 2. (A) GSE17536. (B) Early stage (I and II) samples of GSE17536. (C) GSE17537. (D) GSE14333.

univariate and multivariate analyses are shown in Table 1). The detailed information of the expression level of the 55 genes in CRCs with poor or good prognosis of three datasets was shown in Fig. S4. However, the expression levels of the 111 up-regulated target genes in module B did not correlate with CRC overall survival in dataset GSE17536 ($P = 0.88$; Fig. S5A) or GSE17537 ($P = 0.46$; Fig. S5B), whereas they were associated with disease-free survival in dataset GSE14333 ($P = 6.6e\text{-}05$; Fig. S5C).

4. Discussion

In this study, the expression levels of mRNAs in normal colorectal mucosa, LIN, HIN, and adenocarcinoma tissues were profiled. A substantial change in a number of differentially expressed mRNAs occurred in the transition from normal to LIN, with 756 up-regulated genes and 1566 down-regulated genes. Interestingly, compared with the tremendous change in expression in the transition from normal to LIN, only a few of these differentially expressed genes varied in subsequent phases of colorectal carcinogenesis. Tang et al. screened 2950 differentially expressed genes between normal adjacent tissues and adenomas and 742 genes from adenomas to carcinomas, and 463 genes were overlapped between the two gene sets [17]. In addition, we screened the differentially expressed genes from normal to LIN (2322 genes), normal to HIN (2789 genes), and normal to adenocarcinoma (2784 genes). Intersectional analysis of these gene lists revealed that 1139 genes coincided in all three lists (Fig. S6A), indicating that most of the initially changed 2322 genes maintained a constant status from LIN to adenocarcinoma. As shown in Fig. 1, the highly expressed genes in normal mucosa were dramatically down-regulated in LIN and subsequently remained in HIN and adenocarcinoma without significant fluctuations. Similarly, the low-expressed genes in normal tissues displayed an inverse expression pattern. Notterman et al. also found similar variation tendency that

Table 1
Univariate and multivariate analyses of overall survival or disease-free survival (Cox proportional hazards regression model) in patients with CRC in three published datasets according to clinicopathologic factors and the 55-gene signature expression.

Factors	Univariate Cox regression				Multivariate Cox regression			
	HR	95% CI		*P* value	HR	95% CI		*P* value
GSE17536								
Age	1.01	0.99	1.02	0.492	–	–	–	–
Sex (Male/Female)	1.10	0.69	1.76	0.675	–	–	–	–
Stage [a] (I + II/III + IV)	4.22	2.39	7.46	<0.001[d]	3.56	1.99	6.38	<0.001[d]
Grade[b] (W + M/P)	2.19	1.25	3.83	0.006[d]	1.64	0.93	2.88	0.085
PCA_PC1[c]	0.45	0.28	0.73	0.001[d]	0.56	0.34	0.92	0.022[d]
GSE17537								
Age	1.01	0.98	1.05	0.442	–	–	–	–
Sex (Male/Female)	0.68	0.28	1.66	0.395	–	–	–	–
Stage[a] (I + II/III + IV)	2.51	0.84	7.52	0.100	2.86	0.95	8.61	0.061
PCA_PC1[c]	0.36	0.14	0.95	0.039[d]	0.33	0.13	0.86	0.023[d]
GSE14333								
Age	1.02	1.00	1.03	0.020[d]	1.01	1.00	1.03	0.068
Sex (Male/Female)	0.88	0.65	1.18	0.390	–	–	–	–
Dukes Stage A	1.00	–	–	–	1.00	–	–	–
B	1.56	1.05	2.32	0.029[d]	1.81	1.2	2.73	0.004[d]
C	1.17	0.77	1.78	0.468	1.48	0.96	2.28	0.076
Adj (XRT)	0.43	0.21	0.88	0.021[d]	0.47	0.23	0.99	0.046[d]
Adj (CTX)	0.85	0.62	1.16	0.300	–	–	–	–
PCA_PC1[c]	0.68	0.51	0.92	0.013[d]	0.70	0.51	0.96	0.028[d]

Abbreviations: HR, hazard ratio; CI, confidence interval; Adj, adjuvant therapy; XRT, radiation therapy; CTX, cyclophosphamide.
[a] The American Joint Committee (AJCC) staging system for colorectal cancer.
[b] Well differentiated (W), moderately differentiated (M), poorly differentiated (P).
[c] Based on the rank order of the first principal component (PC1) of the 55 prognostic genes to divide samples into Group 1 and Group 2.
[d] $P < 0.05$.

many mRNA expression abnormalities observed in CRC were already present in adenomas [18]. These results raise questions regarding whether the molecular events occurring in the initiation stage are necessary and sufficient for the progression of colorectal tumorigenesis and whether the LIN stage is a critical pathological state in which a potentially malignancy-related molecular event already exists.

Multiple genetic and epigenetic alterations are involved in colorectal carcinogenesis. The genomic events occurred in premalignant and malignant colorectal tumors are both considerably abundant, implying that genomic instability and resulting gene alterations are key molecular steps occurred early in CRC development [19]. There was a significant increase in the number of methylated genes from normal to adenoma, whereas there was no increase from adenoma to carcinoma [20]. MicroRNA alterations also play important roles early in colorectal carcinogenesis [21]. These variations at genomic or epigenetic levels may lead to early mRNA expression alterations in colorectal carcinogenesis. To partially answer the above questions, we first attempted to determine the possible regulatory mechanism behind the characteristic expression patterns by profiling miRNA expression levels in the four sample groups. Intriguingly, similar to the mRNA expression patterns, the miRNA profiles also exhibited major changes in the transition from normal to LIN (Figs. S2B and S6B). It is well known that aberrant expression of miRNAs contributes to carcinogenesis by promoting or inhibiting the expression of target genes and that the biological functions of miRNAs and their regulation mechanisms are encoded in interaction network properties [22]. Therefore, in this study, a miRNA–mRNA interaction network containing two modules was constructed utilizing the molecular changes in two levels from normal to LIN: module A (5 up-regulated miRNAs and 55 down-regulated target genes) and module B (25 down-regulated miRNAs and 111 up-regulated target genes). In module A, *hsa-miR-135b* was documented to be up-regulated and function as an oncogene in CRC pathogenesis [23], and its expression is associated with disease-free survival in CRC patients [24]. *Hsa-miR-1246*, *hsa-miR-34a*, and *hsa-miR-183* were also identified as over-expressed in CRC compared with adjacent normal tissues [25]. The 55 target genes were enriched in Jak-STAT signaling pathway, p53 signaling pathway, insulin signaling pathway, chemokine signaling pathway, apoptosis, metabolic pathways, etc. (Supplementary Table 3A). In module B, the down-regulated *hsa-miR-497*, *hsa-miR-29c*, and *hsa-miR-26a* have been shown to act as tumor-suppressive miRNAs in carcinogenesis [26–28]. The 111 target genes were enriched in Wnt signaling pathway, MAPK signaling pathway, focal adhesion, pathways in cancer, etc. (Supplementary Table 3B), also exerting vital roles in tumorigenesis. Most signaling pathways enriched in module A and B are classic tumor related pathways and some are correlated with cancer prognosis [29–31]. Therefore, the alteration of miRNAs and mRNAs in this network may make an early and significant contribution to colorectal carcinogenesis.

Finally, to determine whether the network modules concerned with the initiation stage can influence subsequent progression in carcinogenesis, the prognostic values of the expression of miRNA target genes in two modules were estimated in three independent CRC sample sets. The 55-gene signature in module A was found to be associated with overall survival or disease-free survival. Furthermore, this gene expression signature was an independent prognostic variable for overall or disease-free survival of patients with adenocarcinoma. These data indicated that (1) the 5-miRNA-hub interaction network displayed a molecular phenotype that reflected the pathological transition from normal to LINs; (2) the regulation of the miRNAs on their target genes was stable and appeared undisturbed in the process of colorectal carcinogenesis; and (3) the sustained aberrant expression of target genes may be an important event that directly or indirectly influences the clinical outcome of patients with colorectal adenocarcinoma. However, the 111-gene signature from module B was discovered to correlate with only disease-free survival of patients with CRC in one dataset.

The reason the two distinct signatures differed in prognostic value is complex and requires further exploration; however, their critical role in colorectal carcinogenesis should still be appreciated.

In summary, due to the heterogeneity of cancer, some cancer subtypes begin with aggressive or high mortality-risk cancer phenotypes. Whether these phenotypes are the consequence of the accumulation of genetic alterations or an intrinsic property of cancer-initiating cells is debatable. In this study, we provided a clue that potentially malignancy-related molecular events might occur in the transition from normal to intraepithelial neoplasm and may affect cancer progression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.063.

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