Diagnostic and Prognostic MicroRNAs in Stage II Colon Cancer

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Abstract

MicroRNAs (miRNA) are a class of small noncoding RNAs with important posttranscriptional regulatory functions. Recent data suggest that miRNAs are aberrantly expressed in many human cancers and that they may play significant roles in carcinogenesis. Here, we used microarrays to profile the expression of 315 human miRNAs in 10 normal mucosa samples and 49 stage II colon cancers differing with regard to microsatellite status and recurrence of disease. Several miRNAs were differentially expressed between normal tissue and tumor microsatellite subtypes, with miR-145 showing the lowest expression in cancer relative to normal tissue. Microsatellite status for the majority of cancers could be correctly predicted based on miRNA expression profiles. Furthermore, a biomarker based on miRNA expression profiles could predict recurrence of disease with an overall performance accuracy of 81%, indicating a potential role of miRNAs in determining tumor aggressiveness. The expression levels of miR-320 and miR-498, both included in the predictive biomarker, correlated with the probability of recurrence-free survival by multivariate analysis. We successfully verified the expression of selected miRNAs using real-time reverse transcription-PCR assays for mature miRNAs, whereas *in situ* hybridization was used to detect the accumulation of miR-145 and miR-320 in normal epithelial cells and adenocarcinoma cells. Functional studies showed that miR-145 potently suppressed growth of three different colon carcinoma cell lines. In conclusion, our results suggest that perturbed expression of numerous miRNAs in colon cancer may have a functional effect on tumor cell behavior, and, furthermore, that some miRNAs with prognostic potential could be of clinical importance. [Cancer Res 2008;68(15):6416–24]

Introduction

Colorectal cancer (CRC) accounts for ~13% of all cancers and is the second most common cause of cancer death in the Western world (1–3). CRC is staged according to the extent of primary organ involvement and metastatic spread to lymph nodes or distant organs (4–7). Stage II (no lymph node or distant metastases) cancer patients have a 5-year survival of 75% to 80% when surgically treated. Despite surgical resection being highly effective for localized disease, a significant proportion of stage II patients (20–25%) develops recurrence and dies from the disease. At present, it is not possible to accurately differentiate between good and poor prognosis stage II patients. Consequently, there is an acute need for markers capable of distinguishing these two groups (8) as the poor prognosis group should receive chemotherapy. Molecularly, CRC can be discerned into two major subgroups: microsatellite stable (MSS) and microsatellite unstable cancers (MSI), respectively. Histologically, the two molecular subgroups are similar, but, clinically, they behave differently. The MSI cancers have a better prognosis: In general, they follow a more benign disease course; however, they respond less well to chemotherapy (9–11).

Interestingly, recent studies have documented a link between the aberrant expression of a class of small noncoding RNAs (17–28 nucleotides), termed microRNAs (miRNA), and the pathogenesis of several cancer types including CRC (12–14). MiRNAs negatively regulate gene expression at the posttranscriptional level by cleavage and/or translational repression of their mRNA targets and their dysregulation is believed to promote the malignant behavior of tumors (12). Although exciting, the association between miRNAs and CRC warrants further investigation as most studies have been limited to a modest number of cancer tissue samples (15–20) and uncertainty exist as to the precise nature of the disturbed miRNA expression pattern observed in cancer tissue, which has been reported to be severely skewed toward an overall up- or down-regulation relative to normal tissue (14, 20). Differences between methodologies (14, 18–20) and clinicopathologic characteristics of the tissue used may explain this discrepancy. This latter issue, however, is difficult to account for because only a minority of the articles provides information such as tumor stage and location (16, 17). Accordingly, the association between miRNA expression and clinicopathologic variables, including metastatic relapse, remains to be investigated. In addition, no reports have described how the expression of miRNAs in MSS and MSI tumor subgroups relates to the expression of miRNAs in normal mucosa. Finally, in contrast to crude differences between cancerous and healthy colon tissue, virtually nothing is known about which specific cells contribute to the miRNA expression patterns observed as the location of only one miRNA, miR-143, has been described in colon tissue sections (21). All of these issues are important for providing a deeper understanding of the roles of miRNAs in CRC pathogenesis and as potential prognostic and diagnostic markers in CRC.

In this study, we have begun to address the issues raised above. We profiled the expression of 315 unique human miRNAs in 49 colon cancers and 10 normal mucosa samples using spotted locked nucleic acid (LNA)–based oligonucleotide microarrays and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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identified several miRNAs that may serve as potential diagnostic and prognostic disease markers. We confirmed the expression of selected miRNAs using real-time reverse transcription-PCR (RT-PCR) and *in situ* hybridization (ISH), and provide data suggesting that altered expression levels of miRNAs have a functional consequence in colon cancer.

Materials and Methods

Patient material. Biological materials from primary tumors were obtained directly from surgery. All tumors were classified according to the Unio Internationale Contra Cancrum tumor-node-metastasis staging system. Informed written consent was obtained from all patients and research protocols were approved by the ethical committee of Aarhus County. The tumor biopsies used for expression profiling were immediately lysed in a guanidinium thiocyanate solution and snap frozen in liquid nitrogen. RNA was extracted from the samples using a standard Trizol RNA extraction method (Invitrogen). The quality of the RNA samples was assessed using an Agilent Bioanalyzer (criteria, 28S/18S > 1 and RIN > 5). The tumor biopsies used for ISH were immediately embedded in Tissue Tek (Sakura Prohosp) and snap frozen in liquid nitrogen. For this study, we only included patients with stage II colon cancers. The MSI and MSS status were available for all included cancers. We included 30 patients (MSS/MSI) without distant recurrence of disease. The median follow-up time for nonrecurrent cases was 75 mo (range, 13–148 mo). Nineteen patients (MSS/MSI) with distant recurrence were included. Median time to recurrence was 24 mo (range, 5–80 mo). Only patients with histologically verified recurrence to liver and/or lung were included. Patients with recurrence to other organ sites were not included. Clinical and histopathologic variables for the patients included in this study are listed in Supplementary Table S1*A* and *B*.

MiRNA microarray expression profiling and data analysis. Microarrays were produced using a LNA-based oligonucleotide probe library (miRCURY LNA array ready to spot v.7.1; Exiqon A/S). Oligonucleotides were spotted in duplicates in a 10 μmol/L phosphate buffer on CodeLink microarray glass slides (GE Health Care) using a VersArray Chipwriter Pro system (Bio-Rad) as previously described (22). Two micrograms of sample RNA were directly labeled with Hy3 using the miRCURY LNA array labeling kit (Exiqon). As reference, we used a pool of RNA extracted from bladder, prostate, and colon tumors. For each experiment, we labeled 2 μg of the reference RNA with Hy5 using the LNA array labeling kit (Exiqon). Hybridization and washing of the microarray slides were performed as recommended by Exiqon. Scanning was performed using a ScanArray 4000 scanner (GSI Lumonics). After scanning of the microarrays, we used TIGR Spotfinder 2.23 software to generate raw intensity data, which were LOWESS (global) normalized using TIGR MIDAS 2.19 software (23). Average log 2 ratios were calculated from the normalized data based on the two measurements of each miRNA.

Hierarchical cluster analysis. For hierarchical cluster analysis, human miRNA probes on the array were median centered and normalized. Samples were then clustered hierarchically using Cluster 2.0. To evaluate the robustness of the formed clusters, the clustering analysis was performed using three different similarity metrics (correlation centered, correlation uncentered, and Spearman's rank) and two different clustering algorithms (average linkage clustering and complete linkage clustering). Treeview 2.0 was used for visualization. The cluster dendrograms shown in this article were produced using the correlation-centered metric and average linkage clustering algorithm.

Statistical analysis. For statistical analysis of microarray data, we used the significance analysis of microarrays (SAM) implemented in TIGR MEV 3.1 software (23). For all analyses, we performed 1,000 permutations of the data to select differentially expressed miRNAs. The significance cutoff, δ value, was adjusted so as to set the median number of falsely discovered miRNAs to <0.00001, resulting in a median false discovery rate of <0.0001%.

For recurrence-free survival analysis, patients were dichotomized according to the expression level of a given miRNA. High and low expressions were defined according to the median expression of the miRNA in the tumor samples. Censored recurrence-free survival curves were plotted according to Kaplan-Meier. Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model. The software used for survival analysis was STATA 9.2 (StataCorp).

Construction of predictive biomarkers based on miRNA expression. Molecular predictors were built using the Prophet tool (24) with a forward filtering approach for gene selection based on the *F* ratio (between-group to within-group sums of squares). Three prediction algorithms were chosen for the analyses: prediction analysis of microarrays (PAM), diagonal linear discriminant analysis (DLDA), and *k*-nearest neighbor (k-NN). For the k-NN algorithm, the value of *k* (number of neighbors) was varied to achieve more robustness against noise; specifically, *k* was varied across the following values: 1, 2, 3, 4, and 5. The average of sensitivity, TP / (TP + FN), and specificity, TN / (TN + FP), was used to evaluate the performance of the predictors in leave-one-out cross-validation. TP, TN, FN, and FP denote true positives, true negatives, false negatives, and false positives, respectively. Accuracy was defined as (TP + TN) / (TP + FP + FN + TN).

Real-time RT-PCR. cDNA was synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA Assay protocol (Applied Biosystems) in a 15 μL reaction volume with 10 ng of RNA template. Reverse transcription was performed using the following program: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. Reverse transcription products were diluted 20-fold, and 2 μL were used in a total reaction volume of 20 μL for relative quantification by real-time PCR using an Applied Biosystems 7500 Sequence Detection system. Thermal cycling program used for quantification was as follows: 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60° for 1 min. For normalization, random nonamer-primed cDNA synthesis was made in parallel, and the relative expression of the Ubiquitin gene (*UBC*) was quantified as previously described (25). Each measurement was performed in triplicate and no-template controls were included for each assay. Relative expression values were obtained using 5-point 10-fold dilution curves. The dilution curve was created using a cDNA pool of each of the test cDNAs.

***In situ* detection of miRNAs.** The *in situ* detection was performed on 5-μm paraffin sections from matched normal mucosa and adenocarcinoma material. Sections were deparaffinized and subsequently treated with 1 mol/L HCl for 15 min at room temperature to inhibit endogenous alkaline phosphatases. The sections were then digested with proteinase K [50 μg/mL in 50 mmol/L Tris-HCl (pH 7.5)] for 30 min at room temperature. After proteinase K digestion, the sections were fixed in 4% paraformaldehyde at 4°C for 10 min and prehybridized in prehybridization buffer [50% formamide, 50 μg/mL heparin, 5 mmol/L EDTA, 5× SSC, 50 μg/mL yeast tRNA, 0.1% Tween 20, and 2% blocking reagent (Roche)] for 3 h and 15 min at 57°C. Subsequently, the slides were hybridized with 10 pmol probe (LNA-modified and DIG-labeled oligonucleotide; Exiqon) complementary to miR-145 and miR-320 in hybridization buffer [50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 10 mmol/L $NaPO_4$ (pH 8.0), 10% dextran sulfate, 0.5 mg/mL yeast tRNA, and 1× Denhardt's solution] overnight at 57°C. After incubation with anti–DIG-AP Fab fragments conjugated to alkaline phosphatase diluted 1:250 in blocking solution (Roche), the hybridized probes were detected by applying nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color substrate (Roche) to the slides. Slides were mounted in AquaTex (Merck) and analyzed using a Zeiss Axiovert 40 CFL microscope. No-probe controls were included for each hybridization procedure, and specificity was verified using probes that were mismatched at two (miR-145) or three (miR-320) nucleotide positions.

Cell culture and transfection. LS174T and DLD1 cell lines were maintained in RPMI 1640 (+ 25 mmol/L HEPES; Invitrogen/Life Technologies), whereas McCoy's 5A medium was used for HCT116 cells. The medium was supplemented with 10% fetal bovine serum (FBS; Invitrogen/Life Technologies) and 1% penicillin-streptomycin (Invitrogen/Life Technologies) for all cell lines but DLD1 cells, which grew in 5% FBS.

Antisense LNA oligonucleotides (Exiqon) and precursor miRNAs (pre-miRs; Ambion/Applied Biosystems) were transfected using Lipofectamine 2000 reagent (Invitrogen), whereas cells were in suspension (reverse

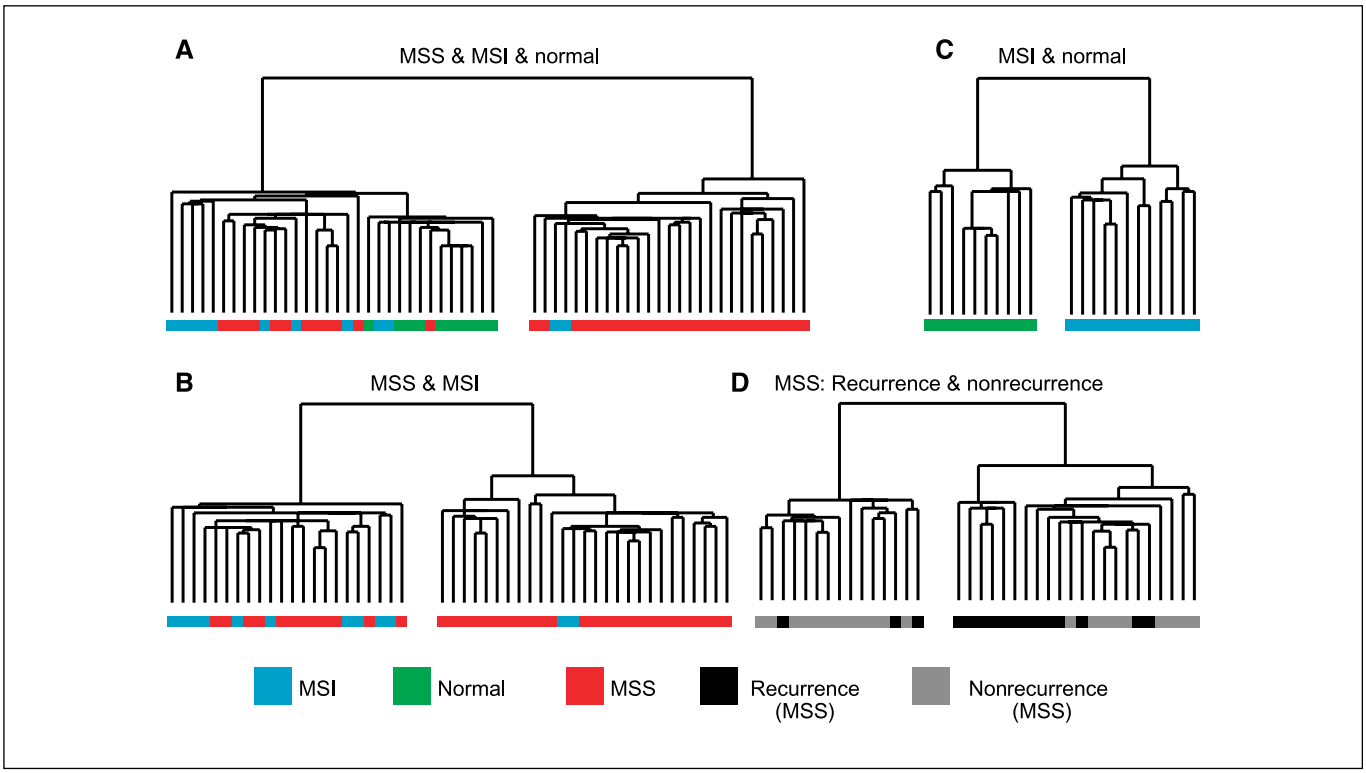

Figure 1. Unsupervised hierarchical cluster analysis based on the expression levels of 315 miRNAs. MiRNA microarray expression profiles from 59 colon tissue samples (10 normal mucosa and 49 stage II colon cancers consisting of 37 MSS and 12 MSI) were clustered using the average linkage clustering algorithm with correlation-centered similarity metric. Clustering dendrograms from analyses of different sample groups. *A,* all samples composed of three groups—normal, MSI, and MSS. *B,* cancer samples composed of two groups—MSS and MSI. *C,* two groups, normal mucosa and MSI cancers. *D,* MSS cancers being either recurrent or nonrecurrent.

transfection) according to the manufacturer's instructions. Transfected cells were seeded in 96-well plates, and the medium was replaced 12 h posttransfection. Final concentrations of antisense LNA oligonucleotides were 10, 20, or 50 nmol/L, whereas final concentrations of 2, 5, and 20 nmol/L were used for pre-miRs. The following LNA oligonucleotides and pre-miRs were used for the experiments: LNA-miR-92, LNA-miR-20a, LNA-scrambled, pre-miR-145, and scrambled pre-miR.

Cell viability and cell death assays. Cell viability was assessed using the Cell Proliferation kit I [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] from Sigma-Aldrich according to the manufacturer's instructions. Cell death was quantified using the Cytotoxicity Detection kit [lactate dehydrogenase (LDH)] from Roche essentially as described by the manufacturer; however, cells were lysed in a buffer consisting of 0.5% Triton X-100, 25 mmol/L HEPES (pH 7.0), 5 mmol/L $MgCl_2$, 1 mol/L EGTA supplemented with serine protease inhibitor, Pefabloc (Roche) 0.5 mmol/L. Transfected cells were seeded in quadruplicate wells for each condition in 96-well plates and assayed 72 h posttransfection. Spectrophotometry for the MTT and LDH assays was performed on a microplate reader (Labsystems Multiscan MCC/340) at 540 and 492 nm, respectively. Absorbance at 690 nm was used as reference.

Results

Unsupervised cluster analysis of miRNA expression profiles. We used LNA-based oligonucleotide microarrays to examine the expression of 315 unique human miRNAs in 10 normal mucosa biopsies and 49 primary stage II colon carcinomas comprising 37 MSS and 12 MSI.

To investigate whether miRNA profiles were informative with regard to tissue type and tumor subgroup, we performed an unsupervised hierarchical cluster analysis. Samples were essentially partitioned into two groups—the first containing the normal mucosa biopsies and the vast majority of the MSI tumors and the other containing most of the MSS tumors (Fig. 1*A*). When clustering tumor samples separately, the MSS and MSI tumor subtypes segregated into two clusters with statistical significance (Fig. 1*B*; Fisher's exact test, $P < 0.005$). Collectively, these observations indicated that the clinically relevant MSS and MSI tumor subtypes differed with regard to miRNA expression patterns, and that MSI tumors seemed to resemble normal mucosa samples more so than MSS tumors. Yet, MSI tumors were readily distinguishable from normal tissue as indicated by a perfect split between the two groups when restricting the cluster analysis to normal mucosa and MSI samples only (Fig. 1*C*).

Identification of a miRNA-based biomarker associated with microsatellite status of colon cancer. To identify the most predictive miRNAs associated with tumor microsatellite status, we used three widely accepted prediction algorithms (DLDA, k-NN, and PAM) in combination with a leave-one-out cross-validation approach. By varying the number of miRNAs to be included in the predictors, an optimal molecular signature composed of only four miRNAs (miR-142-3p, miR-212, miR-151, and miR-144) was identified, which showed a cross-validated performance of 84% accuracy, 81% specificity, and 92% sensitivity when using the DLDA algorithm. The other algorithms exhibited a similar performance (Supplementary Fig. S1*A*). Thus, the microsatellite status of colon cancer is reflected at the miRNA level, and, accordingly, miRNAs may potentially be used to classify colon cancers as either MSI or MSS. These results are pending validation on independent sample sets before firm conclusions can be reached.

Differentially expressed miRNAs between normal mucosa and colon cancer subtypes, MSS and MSI. We next used SAM to identify which specific miRNAs were differentially expressed between normal mucosa and each tumor subtype, MSI and MSS. When comparing normal mucosa to MSI colon cancers, we found 25 miRNAs to be differentially expressed (7 down; 18 up), and 54 miRNAs were differentially expressed when comparison was made to MSS colon cancers (29 down; 25 up). The most significantly dysregulated miRNAs are listed in Table 1 (for a complete list, see Supplementary Table S2). In total, 60 miRNAs were dysregulated in cancer with 19 miRNAs being dysregulated in both MSS and MSI subtypes. Notably, the majority of dysregulated miRNAs, 63% (38 of 60), had not previously been reported to be dysregulated in colon cancer, thus providing a novel association of several miRNAs with this disease. Of the miRNAs previously reported to be dysregulated in colon cancer, 59% (13 of 22) exhibited concordant patterns of dysregulation (Supplementary Table S3). Therefore, in addition to identifying numerous novel miRNAs associated with colon cancer, we successfully validated several miRNAs previously reported to be dysregulated in colon cancer. When comparing MSS and MSI subtypes directly using SAM, 11 miRNAs were differentially expressed with the average expression being highest in MSI tumors (Supplementary Table S2; Table 1). In agreement with MSI cancers exhibiting a miRNA expression pattern closer to normal mucosa than MSS cancers, most of these miRNAs 91% (10 of 11) were not significantly differentially expressed between MSI and normal mucosa, whereas the majority 64% (7 of 11) of the same miRNAs were significantly down-regulated in MSS cancers relative to normal mucosa.

Table 1. MiRNAs dysregulated in stage II colon cancer

MiRNA	Regulation*	Fold (log 2) †
Normal vs MSI comparison		
hsa-miR-145	Down	−1.9
hsa-miR-455	Down	−1.3
hsa-miR-484	Down	−1.2
hsa-miR-101	Down	−1.0
hsa-miR-492	Up	1.2
hsa-miR-20a	Up	1.1
hsa-miR-432*	Up	1.1
hsa-miR-21	Up	1.0
Normal vs MSS comparison		
hsa-miR-142-3p	Down	−2.4
hsa-miR-212	Down	−2.3
hsa-miR-146b	Down	−1.9
hsa-miR-217	Down	−1.7
hsa-miR-20a	Up	1.6
hsa-miR-510	Up	1.5
hsa-miR-92	Up	1.3
hsa-miR-513	Up	1.2
MSS vs MSI comparison		
hsa-miR-212	Down	−2.3
hsa-miR-142-3p	Down	−2.1
hsa-miR-144	Down	−1.5
hsa-miR-151	Down	−1.4
MiRNAs dysregulated in both MSI and MSS		
hsa-miR-145	Down	−1.7
hsa-miR-455	Down	−1.4
hsa-miR-484	Down	−1.2
hsa-miR-101	Down	−1.1
hsa-miR-20a	Up	1.4
hsa-miR-510	Up	1.3
hsa-miR-92	Up	1.0
hsa-miR-513	Up	1.0
Recurrence vs nonrecurrence comparison (MSS only)		
hsa-let-7d	Up	0.7
hsa-let-7b	Up	0.7
hsa-miR-126	Up	0.5
hsa-miR-1	Up	0.5
hsa-miR-320	Down	−1.1
hsa-miR-498	Down	−0.7
hsa-miR-492	Down	−0.7
hsa-miR-526c	Down	−0.7

*Up- or down-regulation is stated as miRNA expression in cancer relative to normal, recurrence relative to nonrecurrence, or MSS relative to MSI.

† Median log2 fold change between the two groups compared.

MiRNAs associated with recurrence of stage II MSS cancer. MiRNA expression levels have, for some cancers, been reported to be associated with clinical disease course (26–28). Hence, we were interested in evaluating the prognostic potential of miRNAs in stage II colon cancer. Initially, we performed an unsupervised hierarchical cluster analysis of all MSS cancers. The resulting clusters significantly ($P < 0.05$, Fisher's exact test) separated the tumors according to their metastatic recurrence status (Fig. 1*D*), suggesting that miRNA expression profiles of primary stage II MSS colon cancers hold promise in predicting the future disease course. Inspired by this finding, we next sought to identify the most informative miRNAs associated with recurrence of disease using the same approach as described for predicting tumor microsatellite status. For this analysis, we selected a subset of the MSS samples using the following criteria: nonrecurrence, >53 months of disease-free survival after surgery; recurrence, occurrence of histologically verified liver and/or lung metastases latest 53 months after surgery. These criteria ensured that the time to recurrence of the recurrence samples was shorter than the shortest follow-up time of the nonrecurrence samples. Thirty-one of the 37 MSS samples satisfied the criteria (Supplementary Table S1*A*). The three different prediction algorithms yielded very similar results (Supplementary Fig. S1*B*). The best performance was reached using 17 miRNAs and the k-NN algorithm ($k = 2$), resulting in 81% accuracy, 83% specificity, and 77% sensitivity. Repetition of the analyses, including all MSS samples, produced outcomes highly similar to the analysis performed on the selected samples (Supplementary Fig. S1*C*). The two highest ranked miRNAs based on the F ratio (between-group to within-group sums of squares) from the molecular predictor were miR-320 and miR-498. Kaplan-Meier survival curves showed that stage II colon tumors with high expression of miR-320 or miR-498 showed a significant difference in progression-free survival compared with tumors with low expression (log-rank test, $P = 0.03$ and $P = 0.002$, respectively). The probability of recurrence-free survival as a function of miR-320 and miR-498 expression is plotted in Fig. 2. When stratifying for age, gender, pT stadium, differentiation grade, and histologic subtype in multivariate Cox regression analysis, we observed a hazard ratio of 6.6 (95% confidence interval, 1.5-28.1; $P = 0.011$) for miR-320 and 11.5 (95% confidence interval, 2.3-59.0; $P < 0.003$) for miR-498. This indicates that miR-320 and miR-498 provide independent information and may prove to be of value when tested in a validation set. By using SAM, 12 miRNAs were found to be significantly differentially expressed between recurrence and disease-free samples, and 11 of

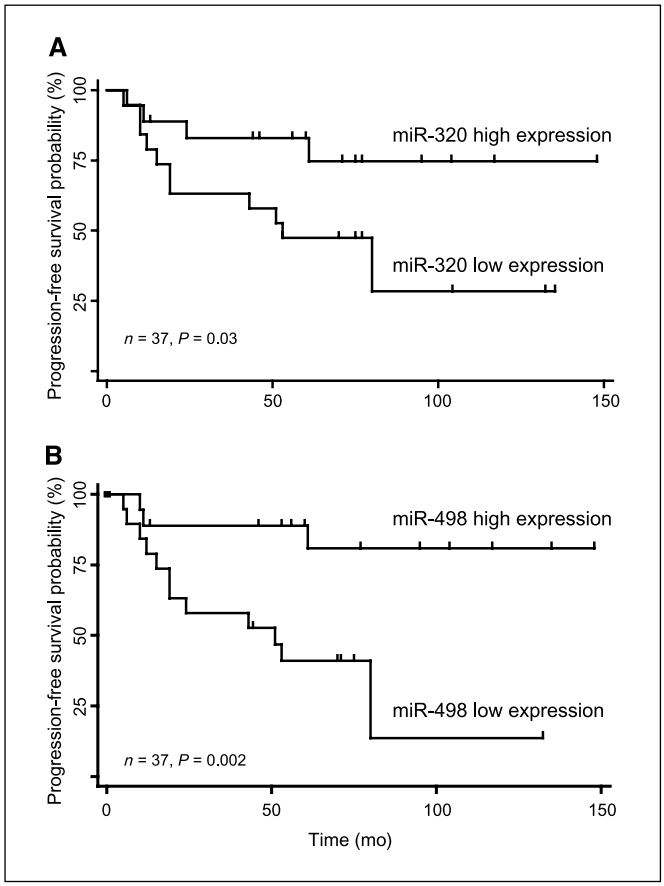

Figure 2. Recurrence-free survival curves as a function of miR-498 and miR-320 expression. Censored recurrence-free survival curves (Kaplan-Meier) as functions of miR-320 and miR-498 expression on miRNA microarrays in stage II MSS colon cancers. Median expression level was used for grouping the samples according to high and low expression. *A,* recurrence-free survival curves based on miR-320 expression. *B,* recurrence-free survival curves based on miR-498 expression.

these overlapped with the miRNAs used in the recurrence prediction signature (Supplementary Table S4). Taken together, these data provide a novel example of how differences in miRNA expression levels are associated with the recurrence-free survival of patients suffering from stage II colon cancer.

Assessment of miRNA expression by real-time RT-PCR. The LNA probes on the miRNA microarray have been designed to hybridize to mature miRNA sequences that are also included in the pri-miRNA and pre-miRNA forms. Accordingly, the microarray has the potential to detect all precursor transcripts in the miRNA biogenesis pathway. To investigate the relationship between the data obtained from the microarray platform and the expression of the mature and biologically active miRNA forms, we quantified the expression of several miRNAs using real-time RT-PCR assays designed to only detect the mature miRNA form. Specifically, we measured the expression of 13 miRNAs (miR-142-3p, miR-512-5p, miR-92, miR-151, miR-212, miR-24, miR-29a, miR-21, miR-7f, miR-7d, miR-126*, miR-141, and miR-145) in 10 tissue samples each also analyzed by microarray expression profiling. Of the 13 miRNAs, 2 assays failed to amplify. Of the remaining 11 assays, 7 (64%) showed a Pearson correlation coefficient to the microarray data equal or above 0.50 with an average of 0.71 (range, 0.5-0.92). Four assays had correlation coefficients less than 0.50 (Table 2). Thus, the majority of the working assays exhibited a relatively good correlation to the microarray data, suggesting that mature miRNAs comprise the predominating form in the cell or, alternatively, that the levels of miRNA precursors are tightly coupled to the level of the mature form. In contrast, the poor correlations for a subset of miRNAs may, to some extent, be explained by technical challenges but may very well also be rooted in biology. Thus, for at least a subset of miRNAs, the cell may contain very complex dynamic pools of different precursor miRNA forms. This notion is supported by published observations indicating that the ratio between different miRNA precursor and mature forms are not closely linked and that certain groups of miRNAs may be processed by distinct enzymes or pathways (29, 30).

Detection of miRNA accumulation by *in situ* hybridization. Microarray and real-time RT-PCR analyses showed that several miRNAs were differentially expressed between normal and cancerous tissue. However, these results were based on crude tissue biopsies and could possibly reflect differences in cellular compositions rather than true differences between cancerous cells and their normal epithelial counterparts. Therefore, to investigate which specific cell types expressed miR-145 and miR-320, we conducted ISH using 5′-DIG–labeled LNA probes that were detected using a colorimetric detection system (Fig. 3). Both miRNAs were expressed by normal epithelial and adenocarcinoma cells. Although miR-145 expression in the normal mucosa was observed throughout the colonic crypts, miR-320 expression was confined to the basal two thirds of the crypts, corresponding to the proliferative compartment. miR-145 expression was also observed in the normal stroma, especially by cells surrounding blood vessels. Neither the normal nor the tumor stroma showed any miR-320 expression. The ISH analyses of miR-145 and miR-320 were repeated using fluorescence-based detection, which showed highly similar results (Supplementary Fig. S2), and the specificity of the ISH procedure was verified using mismatch probes (Supplementary Fig. S3). Consistent with our observations using the microarray platform, miR-145 expression seemed to be reduced in the majority of tumors investigated (four of five) relative to normal mucosa.

Table 2. Correlation of real-time RT-PCR and microarray data

miRNA TaqMan assay	Pearson*
hsa-miR-141	0.92
hsa-miR-142-3p	0.86
hsa-miR-145	0.80
hsa-miR-29a	0.72
hsa-let-7d	0.65
hsa-let-7f	0.53
hsa-miR-92	0.50
hsa-miR-212	0.14
hsa-miR-21	−0.11
hsa-miR-151	−0.15
hsa-miR-24	−0.56
hsa-miR-126*	NA
hsa-miR-512-5p	NA

Abbreviation: NA, not available.
*Pearson correlation coefficient of real-time RT-PCR and microarray expression data.

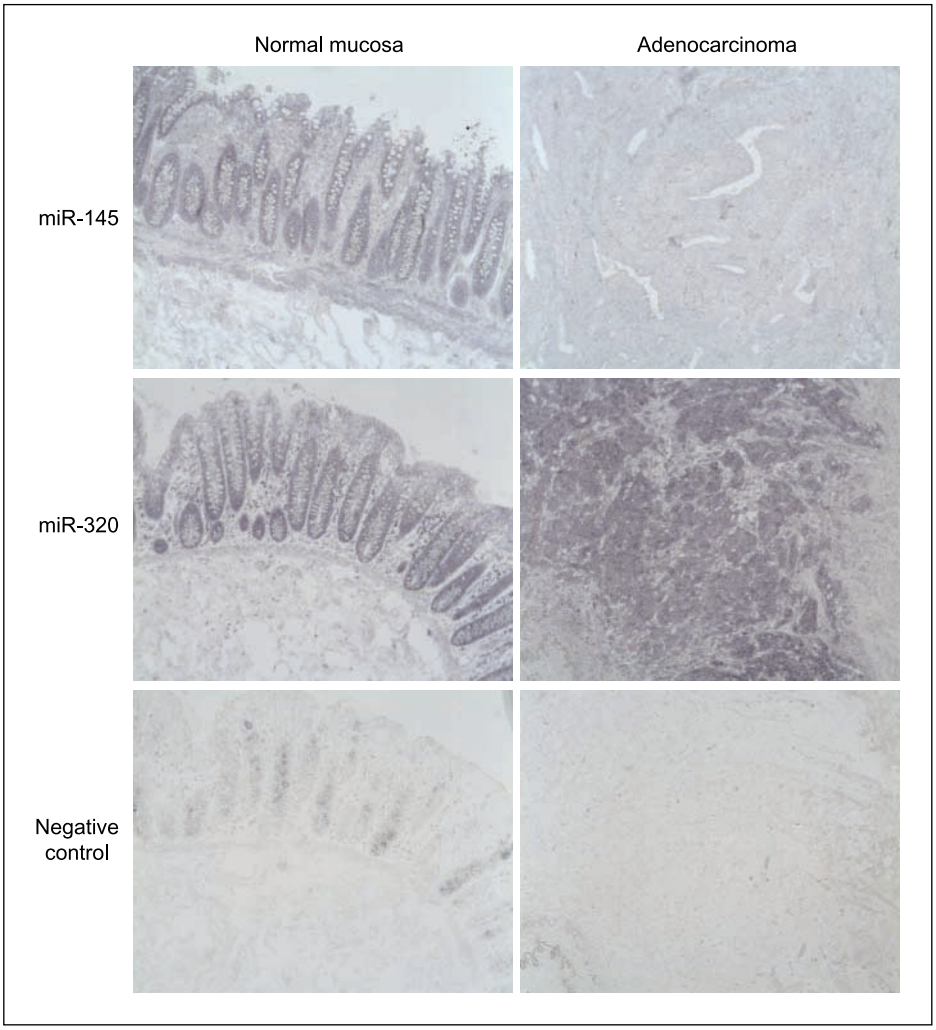

Figure 3. ISH analysis of miR-145 and miR-320 expression in normal colon mucosa and adenocarcinoma. miR-145, miR-320, and negative control (no probe) ISH analyses were performed on matched normal colon mucosa and adenocarcinoma tissue. Typical staining patterns observed in a screen of five patients. miR-145 expression was observed in colonocytes throughout the crypts of Lieberkühn in the normal mucosa. In four of the five matched adenocarcinoma samples, the cancer cells showed a dramatic reduction in miR-145 expression compared with the normal colonocytes. In normal mucosa, miR-320 expression was observed as a gradient declining from the bottom to the top of the crypts. miR-320 was highly expressed in tumors (four of four), at least matching the levels of expression observed in the proliferative compartments of the normal colonic crypts. The weak staining observed in the negative control of the normal mucosa represents remnant endogenous alkaline phosphatase activity. Images were captured at ×10 magnification.

Conversely, miR-320 was highly expressed in tumors (four of four), at least matching the levels of expression observed in the proliferative compartments of the normal colonic crypts.

Functional characterization of miRNAs. To evaluate the functional consequence of the alterations observed in miRNA expression levels between normal mucosa and cancer tissue, we selected three of the most dysregulated miRNAs in both MSI and MSS subtypes as measured by log2 fold changes. One miRNA, miR-145, showed reduced expression levels in tumors, whereas miR-20a and miR-92 were up-regulated. The highly expressed miRNAs in tumor tissue were antagonized *in vitro* by LNA antisense oligonucleotides in three different colon carcinoma cell lines, and metabolic activity, reflecting cell viability, was assessed 72 h posttransfection. Transfection efficiency was >90% as evaluated by fluorescence-tagged marker oligonucleotides using fluorescence microscopy, and the specific antisense LNAs effectively reduced the real-time RT-PCR signal from both miRNAs (Supplementary Fig. S4*A* and *B*). Compared with control scrambled LNAs, however, no consistent phenotypic change was observed when cells were transfected with LNAs against miR-20a and miR-92 (Supplementary Fig. S4*C*). More strikingly, the viability of all three cell lines was lowered in a dose-dependent manner when transfecting them with precursor miR-145 (Fig. 4*B*). A real-time RT-PCR assay specific for mature miR-145 was used to verify that the synthetic miR-145 precursors were indeed processed into mature miRNAs (Fig. 4*A*). No strict correlation between the abundance of exogenous miR-145 and the reduction of cell viability was apparent between cell lines, which may be attributable to a combination of experimental variation and differences in the intrinsic apoptotic and antiproliferative capabilities of the individual cell lines, and/or expression of yet unidentified miR-145 target genes. Notably, endogenous miR-145 levels of all three cell lines were below the detection limit of the real-time RT-PCR assay, indicating none or very low expression of miR-145 (nontransfected cells; Fig. 4*A*), which is in complete agreement with our observations in the tumor tissue (Supplementary Fig. S2). Inspection of the precursor miR-145–transfected cells by light microscopy revealed abundant apoptotic morphology, especially in the LS174T cell line, and this was confirmed quantitatively using a cell death assay measuring the release of the cytoplasmic enzyme, LDH (Fig. 4*C* and *D*). Our data indicate that altered expression levels of miRNA-145 in colon cancer has a functional effect on transformed cells, and that miR-145 may act as a potent suppressor of tumor growth through an increased rate of cell death.

Discussion

In agreement with previous reports, our findings show that numerous miRNAs are aberrantly expressed in colon cancer

relative to normal tissue. Despite fundamental methodologic differences between previously published studies, we still found that most dysregulated miRNAs overlapping with previous reports exhibited concordant expression. A group of eight miRNAs specifically dysregulated in MSS cancers, however, showed discrepant patterns of expression compared with previous reports. This may, in addition to technical differences, be explained by biological differences of tissue material such as tumor stage. Also, one study analyzed only four cancer samples, making the data more susceptible to stochastic effects (19).

Intriguingly, tumor subtypes distinguished by microsatellite status, MSI or MSS, exhibited distinct miRNA expression patterns, and, accordingly, the expression of specific miRNAs could be used to correctly classify the majority of colon cancers as either MSI or MSS. These observations are consistent with a recent study by Lanza and colleagues (31) who also found that miRNA profiles could accurately predict microsatellite status in a set of 39 colon cancers. Together, these findings support the notion that the type of genetic instability in cancer tissue is reflected at the miRNA level, which is also consistent with results from our unsupervised cluster analyses showing that miRNA expression profiles from MSI tumors, known to generally be near diploid, have a tendency to cluster more closely with normal tissue than do MSS tumors, known to exhibit aneuploidy with varying chromosome numbers (32). Thus, chromosomal abnormalities may directly or indirectly contribute to the altered expression of several miRNAs in colon cancer. It is noteworthy, however, that we did not identify the same specific miRNAs that distinguished MSI from MSS cancers as Lanza and colleagues (Supplementary Table S3; ref. 31). Proper comparison of the results is obscured by differences in analytic platforms. Specifically, the probes on the array platform used by Lanza and colleagues (31) were designed against precursor miRNAs, and this platform has previously been reported to yield data that do not correlate well with real-time RT-PCR data measuring the expression of mature miRNAs (33). By contrast, our array platform with probes designed against mature miRNAs produces data that for the majority of investigated miRNAs correlate well with real-time RT-PCR data.

We found several miRNAs to be associated with metastatic recurrence of stage II MSS colon cancers. By predicting recurrence status of the primary tumor samples based on miRNA expression levels, we found 17 miRNAs to give the best performance of 81% accuracy, 77% sensitivity, and 83% specificity. These results are highly similar to the predictive power obtained by other investigators when classification was based on mRNA microarray expression profiles (34). It may prove beneficial to combine miRNA/mRNA gene expression data, thereby possibly enhancing the accuracy of the molecular predictors, as shown by Lanza and colleagues (31), when predicting microsatellite status. Our analysis of the probability of recurrence-free survival as a function of the

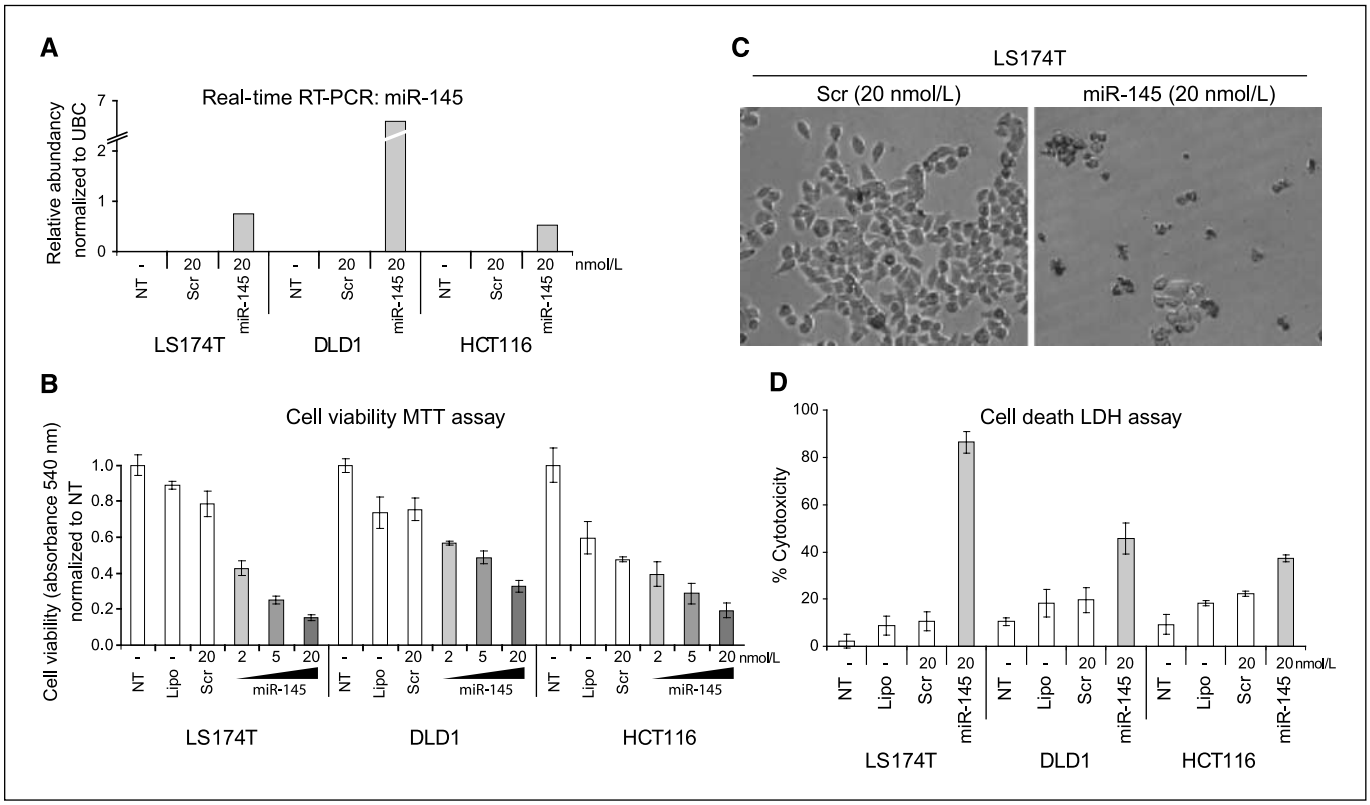

Figure 4. Overexpression of miR-145 in colon carcinoma cell lines. Three colon carcinoma cell lines were transfected with precursor miR-145 or a scrambled (nontargeting) control precursor miRNA. Cell death and cell viability were assessed 72 h posttransfection. *A,* real-time RT-PCR was used to determine the relative abundance of mature miR-145 in nontransfected cells and in cells transfected with precursor miR-145 and scrambled control precursor miRNA. miR-145 abundance was quantified 20 h posttransfection and normalized to the abundance of the UBC transcript. *B,* cell viability was quantified using MTT assay. *C,* light microscopy images at ×5 magnification showing marked inhibition of cell growth in LS174T cells upon precursor miR-145 transfection. *D,* cell death was quantified by measuring the activity of released cytoplasmic LDH enzyme in the supernatant. Representative data are shown from at least two independent experiments performed in quadruplicate. *Columns,* mean; *bars,* SD. *NT,* nontransfected; *Lipo,* Lipofectamine 2000 transfection reagent alone; *Scr,* transfected with scrambled (nontargeting) control precursor miRNA; *miR-145,* transfected with precursor miR-145.

expression levels of either of the two top-ranked predictive miRNAs from our predictive recurrence signature, miR-320 and miR-498, underpins the prognostic potential of miRNAs in relation to stage II colon cancer. Both miRNAs independently of known risk factors such as age, pT stadium, differentiation grade, and histologic subtype significantly predict metastatic recurrence. Ultimately, it may be possible to use miRNA expression profiles, either alone or in combination with other biomarkers including mRNA expression data, to identify the stage II colon cancer patients who are at high risk of metastatic recurrence and thus likely to benefit from adjuvant chemotherapy. Although our study is the first to investigate whether miRNA expression levels can predict recurrence of stage II colon cancer, the prognostic value of miRNAs have also been suggested from other studies investigating disease course in lung cancer, neuroblastoma, and leukemia (26–28). It is interesting to note that, in general, a few hundred miRNAs seem to be at least as informative with respect to diagnostic and prognostic characteristics as the abundant class of mRNAs containing thousands of variants when analyzing microarray expression profiles. However, as has been suggested, an effort to achieve universal standards for miRNA arrays would be required to allow optimal comparison of miRNA array data from multiple laboratories (35). Furthermore, an independent validation study is needed before firm conclusions can be made on clinical utilization.

Although the literature describing miRNA expression and function is exponentially growing, little is still known about the *in vivo* localization of miRNAs. This issue is important, not only for assessing whether differences in miRNA expression levels between crude tissue biopsies arise from variations in cellular composition, or true differences between normal and transformed cells, but also for understanding the biological roles of miRNAs linked to particular cell types. By performing ISH with probes against miR-320 and miR-145, we found that both miRNAs are expressed in the epithelial cells of normal colon tissue. Interestingly, miR-320 expression was highest in the proliferative compartment of the colonic crypts, and in the small panel of tumors investigated, miR-320 was highly expressed in cancer cells. These observations are consistent with our microarray data showing that miR-320 is more abundantly expressed in cancer relative to normal tissue. It may therefore be speculated that miR-320 expression is involved in maintaining the undifferentiated and proliferative tumor phenotype. Conversely, microarray expression data for miR-145 showed that expression levels varied across cancer samples, but, overall, miR-145 expression was drastically reduced relative to normal tissue. ISH analysis showed that in four of five tumors, miR-145 expression seemed to be reduced compared with healthy colon tissue.

Strikingly, by restoring miR-145 levels in a panel of three different colon carcinoma cell lines, we found cell growth to be markedly retarded and the reduction in cell viability was subsequently found to be caused by an increased rate of cell death. In contrast, *in vitro* inhibition of two miRNAs highly expressed in tumor tissue, miR-20a and miR-92, did not affect cell viability. This was unexpected, considering that these miRNAs are part of the miR-17-92 cluster reported to be oncogenic in other cancer types (36, 37). Although our data indicated that the viability of colon cancer cell lines were unaffected by inhibition of miR-20a and miR-92, other cancer hall marks such as motility, invasion, and angiogenesis might have been influenced. This, however, was beyond the scope of this study to investigate. Importantly, the growth inhibition we observed by miR-145 are in agreement with a recent study reporting miR-143 and miR-145 to negatively influence cell growth in two colon carcinoma cell lines, of which one overlapped with the ones we tested (17). This miRNA has also been reported to be down-regulated in colon carcinomas by other investigators (15, 18), and recent work by Shi and colleagues (38) showed that one target of miR-145 is the insulin receptor substrate-1 conveying mitogenic and antiapoptotic signaling. Thus, increasing the levels of miR-145 in transformed cells may prove to be an efficient way of inhibiting tumor growth, and our results support the suggestion that miR-145 is an interesting candidate for the development of future RNA medicine against cancer as has been put forward for hematopoietic malignancies (39) and colon cancer (38).

In conclusion, we showed significant correlations between the expression of miRNAs and histologic as well as clinical variables in colon cancer. Our findings provide the basis for an in-depth study of the role of certain miRNAs as clinical prospective markers and as new pharmaceutical entities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. CA Cancer J Clin 2001;51:15–36.
2. Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–43.
3. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
4. Compton CC, Fielding LP, Burgart LJ, et al. Prognostic factors in colorectal cancer. College of American Pathologists Consensus Statement 1999. Arch Pathol Lab Med 2000;124:979–94.
5. Guerra A, Borda F, Javier Jimenez F, Martinez-Penuela JM, Larrinaga B. Multivariate analysis of prognostic factors in resected colorectal cancer: a new prognostic index. Eur J Gastroenterol Hepatol 1998;10:51–8.
6. Steinberg SM, Barwick KW, Stablein DM. Importance of tumor pathology and morphology in patients with surgically resected colon cancer. Findings from the Gastrointestinal Tumor Study Group. Cancer 1986;58:1340–5.
7. Wolters U, Stutzer H, Keller HW, Schroder U, Pichlmaier H. Colorectal cancer-a multivariate analysis of prognostic factors. Eur J Surg Oncol 1996;22:592–7.
8. Wang Y, Jatkoe T, Zhang Y, et al. Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. J Clin Oncol 2004;22:1564–71.
9. Ribic CM, Sargent DJ, Moore MJ, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med 2003;349:247–57.
10. Benatti P, Gafa R, Barana D, et al. Microsatellite instability and colorectal cancer prognosis. Clin Cancer Res 2005;11:8332–40.
11. Soreide K, Janssen EA, Soiland H, Korner H, Baak JP. Microsatellite instability in colorectal cancer. Br J Surg 2006;93:395–406.
12. Bandres E, Agirre X, Ramirez N, Zarate R, Garcia-Foncillas J. MicroRNAs as cancer players: potential clinical and biological effects. DNA Cell Biol 2007;26:273–82.

13. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol 2007;302: 1–12.
14. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 2006;103: 2257–61.
15. Michael MZ, SM OC, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 2003; 1:882–91.
16. Akao Y, Nakagawa Y, Naoe T. let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. Biol Pharm Bull 2006;29:903–6.
17. Akao Y, Nakagawa Y, Naoe T. MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers. Oncol Rep 2006;16:845–50.
18. Bandres E, Cubedo E, Agirre X, et al. Identification by real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. Mol Cancer 2006;5:29.
19. Cummins JM, He Y, Leary RJ, et al. The colorectal microRNAome. Proc Natl Acad Sci U S A 2006;103: 3687–92.
20. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–8.
21. Akao Y, Nakagawa Y, Naoe T. MicroRNA-143 and -145 in colon cancer. DNA Cell Biol 2007;26:311–20.
22. Dyrskjot L, Zieger K, Kruhoffer M, et al. A molecular signature in superficial bladder carcinoma predicts clinical outcome. Clin Cancer Res 2005;11:4029–36.
23. Saeed AI, Sharov V, White J, et al. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 2003;34:374–8.
24. Medina I, Montaner D, Tarraga J, Dopazo J. Prophet, a web-based tool for class prediction using microarray data. Bioinformatics 2007;23:390–1.
25. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 2004; 64:5245–50.
26. Yanaihara N, Caplen N, Bowman E, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006;9:189–98.
27. Chen Y, Stallings RL. Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. Cancer Res 2007;67:976–83.
28. Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 2005;353: 1793–801.
29. Fukuda T, Yamagata K, Fujiyama S, et al. DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. Nat Cell Biol 2007;9:604–11.
30. Thomson JM, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev 2006;20:2202–7.
31. Lanza G, Ferracin M, Gafa R, et al. mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. Mol Cancer 2007;6:54.
32. Atkin NB. Microsatellite instability. Cytogenet Cell Genet 2001;92:177–81.
33. Liu CG, Calin GA, Meloon B, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. Proc Natl Acad Sci U S A 2004;101:9740–4.
34. Barrier A, Boelle PY, Roser F, et al. Stage II colon cancer prognosis prediction by tumor gene expression profiling. J Clin Oncol 2006;24:4685–91.
35. Jay C, Nemunaitis J, Chen P, Fulgham P, Tong AW. miRNA profiling for diagnosis and prognosis of human cancer. DNA Cell Biol 2007;26:293–300.
36. Matsubara H, Takeuchi T, Nishikawa E, et al. Apoptosis induction by antisense oligonucleotides against miR-17-5p and miR-20a in lung cancers overexpressing miR-17–92. Oncogene 2007;26:6099–105. Epub 2007 Mar 26.
37. Venturini L, Battmer K, Castoldi M, et al. Expression of the miR-17–92 polycistron in chronic myeloid leukemia (CML) CD34+ cells. Blood 2007;109:4399–405.
38. Shi B, Sepp-Lorenzino L, Prisco M, Linsley P, deAngelis T, Baserga R. Micro RNA 145 targets the insulin receptor substrate-1 and inhibits the growth of colon cancer cells. J Biol Chem 2007;282:32582–90.
39. Akao Y, Nakagawa Y, Kitade Y, Kinoshita T, Naoe T. Downregulation of microRNAs-143 and -145 in B-cell malignancies. Cancer Sci 2007;98:1914–20. Epub 2007 Sep 24.