Detection of miR-34a Promoter Methylation in Combination with Elevated Expression of c-Met and β-Catenin Predicts Distant Metastasis of Colon Cancer

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Abstract

**Purpose:** Here, we determined whether epigenetic inactivation of miR-34a and miR-34b/c genes may serve as a prognostic marker for distant metastases in colon cancer.

**Experimental Design:** Using a case–control study design of 94 primary colon cancer samples with and without liver metastases, we determined CpG methylation frequencies of miR-34a and miR-34b/c promoters, expression of miR-34a, and its targets c-Met, Snail, and β-catenin and their prognostic value.

**Results:** miR-34a methylation was detected in 45.1% (n = 42 of 93) of the samples and strongly associated with metastases to the liver (P = 0.003) and lymph nodes (P = 0.006). miR-34b/c methylation was detected in 91.9% of the samples (n = 79/86). A significant inverse correlation between miR-34a methylation and expression of mature miR-34a (P = 0.018) was detected. Decreased miR-34a expression was associated with upregulation of c-Met, Snail, and β-catenin protein levels (P = 0.031, 0.132, and 0.004), which were associated with distant metastases (P = 0.001, 0.017, and 0.005). In a confounder-adjusted multivariate regression model miR-34a methylation, high c-Met and β-catenin levels provided the most significant prognostic information about metastases to the liver (P = 0.014, 0.031, and 0.058) and matched pairs showed a higher prevalence of these risk factors in the samples with distant spread (P = 0.029). Finally, we obtained statistical evidence indicating that the simultaneous detection of these three markers has the highest prognostic value.

**Conclusions:** Silencing of miR-34a and upregulation of c-Met, Snail, and β-catenin is associated with liver metastases of colon cancer. Detection of miR-34a silencing in resected primary colon cancer may be of prognostic value, especially in combination with detection of c-Met and β-catenin expression. Clin Cancer Res; 19(3); 710–20. ©2012 AACR.

Introduction

Colon cancer is one of the most frequent solid tumors and a public health problem worldwide. For the United States, 103,170 new cases of colon cancer and 51,690 colon cancer–related deaths are predicted for 2012 (1). For patients who have undergone potentially curative resection, prognosis mainly depends on disease recurrence, which is associated with distant metastasis (2). Therefore, prognostic markers, which identify patients with high risk of recurrence and metastasis may have an important impact on the clinical management of patients with colon cancer. Within the last years, several prognostic markers associated with distant spread could be identified, and more recently, the expression of several miRNAs, such as miR-17-92 and miR-21, was associated with metastatic disease in colon cancer (3).

miRNAs are small molecules of 20 to 24 nucleotides which specifically downregulate target mRNAs by binding to complementary seed sequences within their 3′-untranslated regions (UTR; ref. 4). The miR-34a and miR-34b/c genes represent direct p53 targets and presumably mediate multiple tumor-suppressive effects of p53 (5). Furthermore, they may represent tumor suppressor genes themselves, as they are commonly silenced by CpG methylation in tumors (5–7). miR-34a, which resides on chromosome 1p36, may also be a target for deletion in neuroblastoma (8). Ectopic expression of miR-34a/b/c induces apoptosis, cell-cycle arrest, senescence and inhibits migration and invasion (5). Furthermore, systemic administration of miR-34...
Translational Relevance
After potentially curative resection, colon cancer–related death and prognosis mainly depend on occurrence of distant metastases. To prevent distant metastases and to improve survival, adjuvant chemotherapy is applied. However, a subset of patients does not display distant spread. Therefore, reliable prognostic markers are needed to avoid unnecessary chemotherapy. Here, we show that the p53-independent inactivation of miR-34a by promoter methylation in primary tumors is associated with locoregional and distant spread of colon cancer at a high frequency. Detection of elevated expression of the miR-34 targets c-Met and β-catenin in combination miR-34a Cpg methylation resulted in a further increased prognostic value; in fact, all primary tumors that were positive for these three markers had developed distant metastases. Hence, detection of miR-34a promoter methylation in combination with c-Met and β-catenin expression identifies patients with an increased risk of distant metastases. This approach may therefore improve the clinical management of patients with colon cancer.

In this study, we found that the formation of distant metastases is associated with epigenetic silencing of miR-34a in primary tumors. Furthermore, we detected a preferential upregulation of the miR-34a targets Snail, c-Met, and β-catenin, which have prometastatic functions, in primary tumors of patients which subsequently developed distant metastases. Notably, our analyses indicate that the detection of enhanced c-Met and β-catenin expression, in combination with miR-34a Cpg methylation may have prognostic value. The potential applications of these findings for the management of colon cancer are discussed.

Materials and Methods
Tissue samples
Formalin-fixed, paraffin-embedded (FFPE) colon cancer samples from 94 patients who underwent surgical tumor resection at the Ludwig-Maximilians-Universität München (LMU; Munich, Germany) between 1994 and 2005 were obtained from the archives of the Institute of Pathology. Clinicopathologic data were retrieved from the Munich Cancer Registry. All tumors were located on the right side of the colon. The collection was assembled using a case-control study design. Half of these samples were colonic adenocarcinomas with synchronous liver metastases (M1). Colonic adenocarcinomas without distant metastases at the time of diagnosis from patients with disease-free survival of at least 5 years after primary surgical resection served as controls (M0). To exclude potential confounders, controls and cases were matched with respect to tumor grade (according to WHO 2000; ref. 20), T classification (according to TNM Classification of Malignant Tumors 2009; ref. 21), and tumor localization, resulting in 47 matched pairs. The study was conducted according to the recommendations of the local ethics committee of the Medical Faculty of the LMU.

Isolation and bisulfite treatment of genomic DNA
Genomic DNA was isolated from 5-μm paraffin sections after overnight proteinase K digestion (0.1 mg/mL) in 0.1% SDS (Sigma) at 58°C with subsequent phenol/chloroform extraction (pH 8) and precipitation. About 400 ng of DNA was treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research).

Methylation-specific PCR
Methylation-specific PCR (MSP) analyses were conducted as described before (6, 7). PCRs were carried out in a total volume of 20 μL using 1.5 units Platinum Taq-polymerase (Invitrogen) per reaction and 30 ng of bisulfite-converted DNA as template. The reaction conditions for miR-34a were the following: 95°C for 10 minutes, followed by 35 cycles of 95°C for 30 seconds, 64°C for 30 seconds, and 72°C for 30 seconds. The final elongation step was conducted for 5 minutes at 72°C. Amplification conditions for miR-34a were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The final elongation step was conducted for 5 minutes at 72°C. The oligonucleotide sequences are...
shown in Supplementary Table S1. Addition of two inosines at the 5’-end of the primer was used to increase the annealing temperature and hence achieve a decrease in unspecific PCR products. Amplified fragments were separated by electrophoresis on 10% PAGE and visualized by staining with ethidium bromide. Pictures were taken using a Multimaging Light Cabinet (Alpha Innotech).

miRNAs quantification

miRNAs were isolated from 5-μm sections of FFPE tumor tissue samples after microdissection using the High Pure miRNA Isolation Kit (Roche). cDNA was synthesized from 15 to 20 ng of RNA enriched for small RNAs using the TaqMan Reverse Transcription Kit (Applied Biosystems). For determination of mature miR-34a expression, the TaqMan qPCR Detection Kit was used in a Light Cycler 480 System (Roche) with normalization to RNU48.

Immunohistochemistry

For immunohistochemistry, 5-μm serial tissue sections of FFPE tumor samples were used. For detailed information on the antibodies and protocols used here, see Supplementary Table S2. To avoid unspecific reactions of antibodies and/or reagents, isotype and system controls were conducted (see Supplementary Fig. S2A and S2B). Furthermore, antibody specificity was confirmed by Western blot analysis with lysates of cells with defined expression of the respective proteins (see Supplementary Fig. S2C and S2D).

Scoring of immunohistochemistry signals

For quantification of the c-Met expression, the previously published H-score was applied (22, 23). Each region of the tumor was assigned an intensity score from 0 to 3 (where 0 indicates no staining, 1 an incomplete and 2, and 3 complete staining with ascending intensity) and proportion of the tumor staining for that intensity was recorded as 5% increments from a range of 0 to 100. A final H-score (possible range, 0–300) was obtained by adding the sum of scores obtained for each intensity and proportion of area stained. Cases showing an H-score above the median (median = 176.5) were categorized as high-grade expression, whereas cases with a lower score were classified as low-grade expression. Nuclear Snail expression was evaluated using the following score: 0, no staining; 1, weak; 2, moderate; and 3, strong staining. Subsequently, the samples were assigned to 2 groups with either low (scores 0 and 1) or high Snail expression (scores 2 and 3). As transcriptionally active Snail is only localized in the nucleus, the detection of cytoplasmic Snail expression was not considered in this analysis. For evaluation of p53 immunostaining, the frequency and intensity of stained nuclei was evaluated. Intensity values were categorized as follows: 0, no staining; 1, weak; 2, moderate; and 3, strong staining. When more than 70% of tumor cell nuclei were stained and the intensity was at least moderate (score 2) or strong (score 3), p53 expression was considered to be “high.” Cytoplasmic p53 staining was not included in further statistical analysis.

The staining score for nuclear expression of β-catenin was based only on the quantity of stained tumor nuclei throughout the whole tumor, whereas intensity of staining was not considered. The score was as follows: 0, negative; 1+, <30%; 2+, 30%–60%; 3+, >60% positive cells. The samples were grouped into subsets expressing low (scores 0 and 1) and high (scores 2 and 3) β-catenin levels. All immunohistochemical detections were evaluated independently by 2 observers (H. Siemens and J. Neumann for c-Met, J. Neumann and R. Jackstadt for Snail, and J. Neumann and T. Kirchner for p53 and β-catenin). Discrepant evaluations were discussed and a consensus was reached.

Statistical analysis

A paired t-test was used to assess differences in continuous outcomes between cases and controls. Comparisons between multiple groups were made by one-factorial ANOVA. Pearson correlation coefficient was used to study the correlation between 2 continuous variables. Correlation analyses were stratified between cases and controls. Conditional logistic regression was used to determine the simultaneous prognostic potential of several factors with respect to distant metastases. Age and gender were added as nuisance parameters to adjust for corresponding influences (24). We used the algorithms for Cox regression to calculate the conditional logistic regression as described previously (25). The significant regression coefficients determined by multivariate analysis were used to derive a prognostic score. A log-linear model was used to assess the relevance of the score with respect to case-control matching. All calculations were conducted using SPSS software 19 (SPSS Inc.).

Results

miR-34a promoter methylation correlates with distant metastases in colon cancer

To determine the frequency of CpG methylation of the miR-34a and miR-34b/c promoters, a collection of primary colon cancer samples was subjected to analysis with MSP (Fig. 1A and B). The MSP amplicon used for detection of miR-34a CpG methylation is located within the only CpG island present in the promoter and transcribed region of miR-34a (Supplementary Fig. S1). Therefore, the MSP amplicon used here is likely to comprehensively represent relevant CpG methylation affecting miR-34a expression. Further supporting this notion, only the region surrounding the transcriptional start site adjacent to the CpG island and a region in the middle of the 30-kbp large intron, lacking a CpG island, displayed histone H3K27 acetylation (Supplementary Fig. S1). Therefore, the miR-34a gene presumably does not contain additional regulatory elements, which may be subject to silencing by CpG methylation. In addition, we have previously shown that the methylation detected using this PCR amplicon corresponds to results obtained with bisulfite sequencing of the promoter region of miR-34a (6). Of the 94 tumor samples analyzed here, 93 showed an miR-34a-specific and 86 an miR-34b/c-specific MSP product. Not all of the MSP analyses resulted in a
detectable PCR product, presumably due to the limited amount of template that could be isolated from slides by dissection of tumor areas. Methylation of miR-34a and miR-34b/c was observed in 45.1% (42 of 93) and 91.9% (79 of 86) of the samples, respectively. CpG methylation of miR-34a was significantly increased in primary tumors, which had formed liver metastases (M1 samples; Fig. 1A and Table 1): in 63.8% (30 of 46) of these miR-34a promoters methylation was observed, whereas only 25.5% (12 of 47) of the samples without distant spread (M0) showed miR-34a methylation. Moreover, miR-34a methylation significantly correlated with the nodal status ($P = 0.006$; Supplementary Table S3). While more than 54% of the cases with lymph node metastases showed an miR-34a methylation, only about 31% of the lymph node metastasis-free samples did (data not shown). miR-34b/c methylation did not significantly correlate with any of the analyzed parameters (data not shown), presumably due to the high frequency of miR-34b/c methylation (91.9%). Furthermore, the expression of mature miR-34c was about 300-fold lower than miR-34a ($n = 6$, data not shown). As miR-34b and miR-34c are encoded by the same precursor RNA, which has a single transcriptional start site, these 2 miRNAs generally have similar expression levels. Furthermore, the common promoter for both miRNAs was found to be consistently methylated in colon cancer in this and other studies (7, 26, 27). Therefore, we focused on the analyses on miR-34a silencing. To determine whether CpG island hypermethylation results in transcriptional downregulation of miR-34a in the colon cancer samples, the abundance of mature miR-34a was determined by quantitative, real-time PCR (qPCR). miR-34a expression levels showed a strong inverse correlation with miR-34a methylation ($P = 0.018$; Fig. 2A and B). In addition, elevated miR-34a expression correlated significantly with the absence of distant metastases ($P = 0.001$) in primary colon cancer (Fig. 2C and D). Moreover, the M0 samples showed higher miR-34a expression than the M1 samples in 68.1% (32 of 47) of the matched M0/M1-pairs (Fig. 2E). In accordance with the positive correlation between miR-34a methylation and lymph node metastasis, the expression of miR-34a showed a significant inverse correlation with the occurrence of
Figure 2. Correlative analysis of miR-34a expression, CpG methylation, and clinicopathologic features. A and B, correlation of mature miR-34a expression with miR-34a methylation. C and D, correlation of mature miR-34a expression with M0 and M1. E, expression levels of mature miR-34a in matched pairs of M0 and M1 samples. Correlation of mature miR-34a expression with (F) hMLH1 expression, (G) MSI-H versus MSS, (H) V600E mutation of BRAF, and (I) age (using the median of the overall age as a cutoff for high and low age). Results are shown for single cases (A, C, and E) or as a box plot representation of the entire cohort (B, D, and F–I). y-axes in B and D–I are given in a log_{10} scale.
metastatic lymph nodes as well (P = 0.007; Supplementary Table S3).

Next, we determined whether miR-34a expression correlates with parameters previously studied in this cohort of patients (28): the protein expression of the DNA mismatch repair gene product hMLH1, high-grade microsatellite instability (MSI-H) and BRAF V600E mutation. A significant correlation of decreased miR-34a expression and preserved hMLH1-protein expression was observed (P = 0.002; Fig. 2F). We could also detect a correlation between MSI-H and elevated miR-34a expression (P = 0.001; Fig. 2G). These results are in line with the notion that loss of hMLH1 expression causes MSI-H in colon cancer (29, 30).

In addition, miR-34a expression correlated with the presence of the activating V600E BRAF mutation as well as advanced patient age (Fig. 2H and I). Interestingly, neither expression nor methylation of miR-34a was associated with mutations in the p53 gene (ref. 31; data not shown).

Inverse correlation between miR-34a and its targets c-Met, Snail, and β-catenin

Next, we analyzed whether the silencing of miR-34a affects known miR-34a targets previously implicated in the regulation of metastasis, such as the RTK c-Met, the EMT-inducing transcription factor Snail, and β-catenin, using immunohistochemistry (Fig. 3A). c-Met expression showed a weak but significant inverse correlation with the expression of mature miR-34a (P = 0.031; Fig. 3B) and was significantly increased in M1 samples (P = 0.001; Fig. 3C). Snail expression did not correlate significantly with miR-34a expression when the Pearson algorithm was applied (P = 0.132; Fig. 3D). However, a weak correlation was determined using the Spearman correlation algorithm (P = 0.044, data not shown). Furthermore, Snail expression correlated with distant metastases (P = 0.017; Fig. 3E). In addition, decreased miR-34a expression was significantly associated with elevated expression of β-catenin (P = 0.004; Fig. 3F), which was increased in M1 samples (P = 0.005; Fig. 3G).

A marker combination predicting liver metastases

Finally, we determined whether the different markers studied here may have a prognostic value for predicting distant metastases in colon cancer. For this, all data were categorized into subsets with either high or low expression of the respective markers using the median of the total population as a threshold. Then, we first analyzed the data using conditional logistic regression. In accordance with the analyses described above, Snail was not significantly associated with the occurrence of distant metastases. However, miR-34a methylation, high c-Met and β-catenin levels showed the highest ORs for liver metastases (3.571, 4, and 3.2) and these associations were all statistically significant (P = 0.003, 0.006, and 0.023; Table 1). In contrast to our previous analysis, miR-34a expression was not significantly associated with distant spread. This difference was probably due to the assignment of the tumors into subpopulations of high and low miR-34a expression.

Next, we generated a multivariate regression model to exclude influences of the confounding variables gender and age. Thereby, miR-34a methylation and high c-Met expression were significantly associated with distant metastases, whereas high β-catenin expression showed a strong trend toward an association with distant metastases (P = 0.014, 0.031, and 0.058; Table 2). Remarkably, miR-34a methylation, c-Met expression, and β-catenin expression displayed similar ORs indicating a similar prognostic power for distant metastases. Subsequently, we evaluated the prognostic value of combinations of the markers analyzed here by generating an additive score (Fig. 3H). For this, the value of one was assigned to the detection of either miR-34a methylation, high c-Met, or high β-catenin levels. In about 66% (31 of 47) of the matched pairs, the M1 sample displayed a higher score value than the respective M0 counterpart, whereas only in 6 of the 47 studied pairs (12.8%), the M0 patient showed a higher marker score. For 10 pairs, no difference between case and control was determined. The difference of the combined score between case and control samples was statistically significant (P = 0.029). Noteworthy, none of the M0 samples displayed a score larger than two and only three M1 samples had a score of zero. Notably, 13 of the 94 (13.8%) primary tumor samples showed the maximum score of three, thereby representing the group of patients with the highest risk of distant metastases. To illustrate the practical relevance of combined marker detections, we conducted a hypothetical calculation of the risk for distant metastasis (Table 3). Assuming a baseline risk of 0.2 (20%) if none of the markers is positive, the risk increases to about 0.48 (48%) if only 1 of the 3 markers is positive, to about 0.77 (77%) if only 2 of the 3 markers are positive, and to about 0.92 (92%) if all 3 markers are positive. Taken together, the combined detection of miR-34a CpG methylation, elevated c-Met and β-catenin expression emerged as the combination with the highest prognostic power for distant metastasis in this cohort of patients with colon cancer.

Discussion

In the present study, we could show that miR-34a is preferentially downregulated via hypermethylation in primary colon cancers, which are associated with distant metastases, whereas miR-34b/c seems to be silenced irrespective of tumor stage. In 2 previous studies it was shown that miR-34a mediates tumor-suppressive effects of p53 by decreasing metastatic properties such as invasion and migration in colon cancer cell lines (16, 32). Furthermore, ectopic miR-34a prevented prostate cancer metastasis in a mouse model (9, 33). Therefore, the inactivation of miR-34a expression in colon cancer may be causally linked to increased metastatic potential of colon cancer. Unexpectedly, miR-34a methylation and p53 mutation were not mutually exclusive. This finding was unexpected, as the miR-34 genes are direct p53 target genes and p53 mutation would presumably cause a downregulation or
Figure 3. Correlative analysis of c-Met, Snail, β-catenin, and miR-34a expression in colon cancer with and without distant metastases. A, representative immunohistochemical detection of c-Met, Snail, and β-catenin protein expression in primary colon cancer samples. Scale bars indicate 50 μm. Correlations of mature miR-34a with c-Met (B), Snail (D), and β-catenin (F) protein expression are shown as scatter (B, D) or box plot (F) representations. Analysis of c-Met, Snail, and β-catenin expression in M0 versus M1 samples (C, E, and G). In the box plots (first, third quartile, median), whiskers indicate maximum/minimum values and dots indicate outliers. x-axes of B and D as well as y-axes of F are given in a log10 scale. H, combinatorial distribution of markers within the matched M0/M1 pairs. Numbers in the fields indicate number of pairs with the respective M0 and M1 score. Increased darkening of the grey tone indicates elevated abundance of the respective combination; white fields indicate the absence of adequate pairs.
inability of inducing miR-34a. Therefore, one would assume that miR-34a methylation would preferentially occur in samples with an intact p53 pathway. In a previous study of colorectal cancer samples, we found a mutual exclusiveness of miR-34a CpG methylation and p53 mutation (7). One possible explanation for this discrepancy may lie within the different locations of the analyzed tumor samples: in this study, tumors from the right-sided colon were analyzed, whereas the previous study included mainly left-sided colorectal cancer. Another reason may be the existence of p53-independent inducers of miR-34a expression. For example, activation of BRAF may cause a p53-independent induction of miR-34a expression via upregulation of the transcription factor ELK1 (18). In line with this observation, we detected a significantly higher miR-34a expression in tumors harboring activating BRAF mutations. Therefore, it is conceivable that p53-independent, oncogenic, cellular events exert a selective pressure, which favors the inactivation of miR-34a genes. Moreover, it was previously shown that promoter methylation often occurs early during tumor progression in the colon (34). Therefore, the inactivation of miR-34a genes may precede the inactivation of p53, which is known to occur late during the progression of colon cancer (35). Interestingly, recent findings indicate the involvement of CpG-methylation–mediated gene silencing in the progression of local tumors toward metastatic disease (36).

In a previous study, we detected methylation of miR-34a in 75% of colorectal cancer samples (7). The difference is presumably due to patient selection for the case–control study and the analyses of right-sided colon cancer in this study instead of colorectal cancer in the previous study. Our analysis is based on a case–control design. Therefore, the chosen patients do not represent a population-based clinical collective. As endpoint, we defined the presence or absence of distant metastases within a follow-up period of 5 years after surgical resection of the primary tumor. Because of the design of the study, the analyzed markers and marker combinations could not be correlated with the overall and disease-free survival of the patients. The relevance of our findings for overall and disease-free survival of the patients will be the subject of future analyses. Interestingly, low levels of miR-34a expression correlate with relapse of non–small cell lung cancer (14), as well as recurrence of breast cancer and poor patient survival (12). Therefore, it is likely that CpG methylation of miR-34a in colon cancer is also associated with shorter survival and recurrence, presumably due to the associated increase of distant metastases detected here.

As discussed above, miR-34a mediates the downregulation of several factors, besides c-Met, Snail, and β-catenin, which may promote metastasis, such as CD44, c-Myc, Axl, TPD52, Lef1, and MTA2. The relatively weak correlation between the downregulation of miR-34a expression and elevated expression of its targets detected in this study may be due to other tumor-specific signaling events, which contribute to enhanced expression of c-Met, Snail, and β-catenin.

The assessment of methylated genes in colorectal cancer has revealed a unique molecular subgroup of tumors with the so-called CpG island methylator phenotype (CIMP). These tumors have a particularly high frequency of methylated genes and represent 30% to 40% of right-sided colon cancer (37). In sporadic colon cancer, CIMP has distinct epidemiologic and clinical features and is associated with
most cases of MSI-H caused by hMLH1 inactivation via CpG methylation (38). Furthermore, CIMP-positive colon cancers usually show either mutation of \textit{BRAF} or \textit{KRAS}. Our finding that high miR-34a expression, and therefore the absence of CpG methylation at \textit{miR-34a} promoters correlates with loss of hMLH1 protein expression, MSI-H, and the presence of \textit{BRAF}-mutations, indicates that \textit{miR-34a} may not be a ‘target’ of CIMP-associated hypermethylation. Interestingly, a further analysis of the subgroups revealed that the combination of microsatellite stability (MSS) and \textit{miR-34a} promoter methylation is associated with a higher risk of distant metastasis (80.6%) than the combination MSS and unmethylated \textit{miR-34a} promoter (35.9%). MSI-H cases had a 16.7% risk for metastases whether the \textit{miR-34a} promoter was methylated or not (data not shown). However, the case numbers for these subanalyses were relatively low and thus prevent a statistically robust interpretation. Further studies on larger cohorts will be necessary to address the role of \textit{miR-34a} methylation in specific colon cancer subgroups. Moreover, we noticed an overall increase of \textit{miR-34a} expression in tumors of older patients. This is consistent with recent studies, which reported elevated expression of \textit{miR-34a} during aging in rat liver, human diploid fibroblast, and fruit flies (39–41). However, as other variables also correlated with age, this link may be indirect. Ectopic expression of \textit{miR-34a} was shown to prevent lymph node metastasis in a murine model of hepatocellular carcinoma (42). In line with this finding, we found a

| Table 2. Confounder-adjusted multivariate regression model for quantitative analysis of factors associated with metastasis formation in colon cancer |
|---------------------------------|-----------------|----------------|-----------------|
| Variable                        | Categorization  | OR (95% CI)    | P               | Adjusted OR (95% CI) | P          |
| \textit{miR-34a} methylation    | No product = 0  | 3.182 (1.117–9.058) | 0.03            | 3.522 (1.286–9.645) | 0.014      |
|                                 | Product = 1     |                |                 |                  |            |
| Low \textit{miR-34a} expression | Value < median  | 1.835 (0.456–7.379) | 0.393           |                  |            |
|                                 | Value > median  |                |                 |                  |            |
| \textit{c-Met} expression       | Value > median  | 3.992 (1.044–15.191) | 0.043           | 3.685 (1.125–12.059) | 0.031      |
|                                 | Value < median  |                |                 |                  |            |
| \textit{Snail} expression       | Value > median  | 1.113 (0.406–3.048) | 0.834           |                  |            |
|                                 | Value < median  |                |                 |                  |            |
| \textit{b-Catenin} expression   | Value > median  | 3.519 (0.899–13.765) | 0.71            | 3.762 (0.954–14.835) | 0.058      |
|                                 | Value < median  |                |                 |                  |            |
| Gender                          | Male = 1        | 2.316 (0.732–7.334) | 0.154           |                  |            |
|                                 | Female = 0      |                |                 |                  |            |
| Age                             | Value > median  | 1.155 (0.333–4.001) | 0.82            |                  |            |
|                                 | Value < median  |                |                 |                  |            |

NOTE: Unmatched confounders age and gender were added to the multivariate analysis to estimate their potential respective effects on the analyzed data. A multivariate conditional logistic regression was applied to calculate the respective ORs, P values, and the 95% confidence intervals (CI).

| Table 3. Theoretical evaluation of the prognostic value of combining markers for the prediction of metastases in colon cancer |
|-------------------------------------------------|-------|-------|-------|-------|
| \textit{miR-34a} methylation                    | \textit{c-Met} high | \textit{b-Catenin} high | OR (by components) | OR (total) | Odds | M1 risk |
| No                                              | No    | No    | 1     | 1     | 0.25  | 0.2    |
| Yes                                             | No    | No    | 3.522 | 3.522 | 0.881 | 0.48   |
| No                                              | Yes   | No    | 3.685 | 3.685 | 0.921 | 0.48   |
| Yes                                             | No    | Yes   | 3.762 | 3.762 | 0.941 | 0.485  |
| No                                              | Yes   | No    | 3.522 × 3.685 | 12.98 | 3.245 | 0.764  |
| Yes                                             | No    | Yes   | 3.522 × 3.762 | 13.25 | 3.313 | 0.768  |
| No                                              | Yes   | Yes   | 3.685 × 3.762 | 13.86 | 3.465 | 0.776  |
| Yes                                             | Yes   | Yes   | 3.522 × 3.685 × 3.762 | 48.83 | 12.208 | 0.924 |

NOTE: ORs of single markers (see also Table 2) and ORs for combinations of 2 or 3 markers are listed. The column M1 risk indicates the result of a hypothetical calculation. For this, the baseline risk for metastases in the absence of detection of any of the 3 markers was set to 20% (=0.2). The increasing darkness of grey shading indicates the number of detected markers (0–3).
significantly decreased expression of miR-34a and increased miR-34a promoter methylation in colon cancer samples with lymph node metastases.

As the samples analyzed in a case–control study constitute pairs of patients with and without distant metastases, the selected 94 patients do not faithfully represent a clinical population of patients with colon cancer. But the design allows to draw insightful conclusions about the association between factors and the clinical condition (in this study presence or absence of distant metastases). Furthermore, it is possible to identify biologically relevant differences between 2 groups using a relative small number of investigated cases. In our study, patients with miR-34a methylation in combination with high c-MET and β-catenin expression showed the highest risk for distant metastasis. Although this subgroup represented only a small proportion of patients with colon cancer (13 of 94; Fig. 3H) an intensified follow-up should be implemented. However, further studies of larger patient cohorts should be conducted in the future to elaborate on the prognostic power of the marker combinations proposed here.

Our results suggest a possible clinical value of detecting miR-34a methylation in released tumor cells for the prognostication of patients with colon cancer with a higher risk for distant metastases. The prognostic value of miR-34a methylation may be increased by the analysis of selected miR-34 targets as shown here. One possible application would be the detection of miR-34a methylation in combination with other methylation and mutation markers in stool DNA as it is in principle currently tested in clinical trials (43). Here, miR-34b/c was methylated in almost all analyzed samples. Similarly high miR-34b/c methylation rates in colorectal cancer have been previously reported by others: 90% by Toyota and colleagues (27), 99% by Vogt and colleagues (7), and 97.5% by Kalimuthu and colleagues (26). Obviously, the extremely high frequency of miR-34b/c methylation in colon cancer did not allow any association with other parameters of lower frequency. Although detection of miR-34b/c methylation cannot be used for prognostic purposes, it may allow early detection of colon cancer. For example, it was shown that miR-34b/c methylation in stool DNA can be used for early detection of colorectal cancer (26). The reasons for the previously reported lower frequency (~35%) of miR-34b/c methylation in colorectal cancer samples are currently unknown (10). Potentially, differences in the sensitivity of the used MSP assay are causing these differences, although the same MSP primer pairs were used. The low frequency allowed to correlate miR-34b/c methylation with metastases formation in the study by Lujambio and colleagues (10).

Similarly, miR-34a methylation in DNA released by colorectal cancer cells may also be detected in body fluids, as blood and mucosal washes. Recently, miR-34a methylation was detected in DNA present in mucosal wash fluid from patients undergoing colonoscopy (44). Moreover, systemic delivery of miR-34a was shown to reduce tumor burden and growth of xenografts in mice and led to reduced metastasis (9, 45–47). Therefore, a combination of detecting miR-34a methylation and treatment of tumors with miR-34a mimetics may be advantageous.

Disclosure of Potential Conflicts of Interest
The authors have no relevant financial interest to disclose.

Authors’ Contributions
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Acknowledgments
The authors thank Andrea Sendelhofert, Anja Heier, and Mona Melz for technical assistance.

Grant Support
The work in Heiko Hermeking’s laboratory is supported by the German-Israeli Science Foundation (GIF), the Rudolf-Bartling-Stiftung, the Deutsche Krebsforschungszentrum (DKFZ), and the Deutsche Forschungsgemeinschaft (DFG). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 23, 2012; revised December 3, 2012; accepted December 4, 2012; published OnlineFirst December 14, 2012.

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