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Differential expression of microRNA-320a, -145, and -192 along the continuum of normal mucosa to high-grade dysplastic adenomas of the colorectum

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Abstract

BACKGROUND—MicroRNA (miR)-320a, miR-145, and miR-192 have been shown to play a role in colorectal carcinogenesis and metastasis. We examined if there is a difference in expression during the histologic progression from normal mucosa (NM) to high-grade dysplastic adenomas (HG).

METHODS—Genome-wide miRNA expression profiling was performed on 113 colon adenomas. Information included histologic type, tumor grade, location, sex, age, family, and smoking history.

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Discussion

Lloyd Mack, M.D.: In this study, genomic-wide miR expression profiling was performed on 113 colonic adenomas. Clinical information including histologic type, tumor grade, location, patient gender, age, family history, and smoking history was collected. Analysis was performed to assess how miR expressions of miR-320a, -145, and -192 varied by polyp grade and these clinical features. Interestingly, the authors note differential expression of all 3 miRs depending on tumor histology and grade. Furthermore, tumor location seemed to have differential expression of miR-320a.

There are a few questions regarding methodology. A total of 825 human miR probe sets on the array. After processing, 3 miR probes were selected for further analysis. Although these miR probes have been studied in invasive colon cancer as described, why were only 3 selected for further analysis? Were there any other promising areas of research for other miR probe sets?

The authors also noted that lesions located in the left colon had a higher overall level of expression of miR-320a, whereas adenomas located in the right colon had higher expression levels of miR-145 and miR-192. Can the authors give possible theories as to why this may be? What is the potential relationship between these different miR expression levels and the commonly described molecular pathways in colorectal cancer, whether the adenoma–carcinoma sequence (CIN), microsatellite instability with defective mismatch repair genes, or CpG island methylator phenotype pathways?

Because all adenomas were found by and removed at screening colonoscopy, what are the possible next steps or possible clinical applications of this line of research? Although further confirmatory studies are needed, does the differential expression of miR levels found give further information on possible interventions or targets to prevent progression of these molecular pathways?

A 2-way ANOVA was performed to evaluate the effect of the following factors adjusted for scan dates: location, sex, age, family history, smoking, and histology.

RESULTS—The expression of miR-320a increased; miR-145 and miR-192 expression decreased ($P < .0001$), with higher histologic grade, and were independent of age, sex, family history, and smoking status.

CONCLUSIONS—The miRs studied had statistically significant changes in expression with progression of histologic grade. These changes may signify progression of normal mucosa to HG and potentially serve as early markers for disease progression and differentiating high- from low-risk adenomas.

Keywords

MicroRNAs; High-grade dysplastic adenomas; Colorectal carcinogenesis and metastasis; Adenocarcinomas; miR expression

Colorectal cancer (CRC) is thought to be the result of sequential mucosal changes from normal colonic mucosa to adenoma and then to adenocarcinoma (NM-A-AC). This sequence is characterized by a progressive acquisition of genetic and epigenetic mutations in the epithelial lining of the colon.¹ Although a few studies² have reported differences in the microRNA (miR) profile between adenomatous polyps of the colorectum and the normal mucosa (NM), sequential changes in miR expression during the histologic progression from normal colonic mucosa to adenoma with high-grade dysplasia have not been described. MicroRNA (miR) are short, single-stranded, noncoding RNA that regulate gene expression on both the pre- and post-transcriptional level via direct inhibitory interaction with target messenger RNA.³ Through their regulatory role of messenger RNA expression, they directly influence normal cellular functions, including cell proliferation and differentiation.⁴ An increasing body of evidence suggests that miR are aberrantly expressed in CRC. Deregulated miRs have been shown to modulate a tumor's ability to evade apoptosis, migrate, invade, and undergo epithelial to mesenchymal transition.^{5,6} For colorectal cancer, miR-320a, -145, and -192 have been implicated as potential key players in the colorectal carcinogenesis and metastasis. In particular, miR-145 appears to act as a potent suppressor of tumor growth, where expression is associated with an increased rate of cell death.⁷ In contrast, miR-320a is involved in maintaining the undifferentiated and proliferative tumor phenotype.⁸ The miR-192 is less characterized and appears to play a role in the p53 tumor suppressor network. The p53 tumor suppressor gene is commonly mutated in CRC carcinogenesis, and miR-192 is dependent on the presence of the wild-type functional p53.^{9,10} The aim of this study was to gain additional insight on when in the NM-A-AC sequence these miRs are deregulated in human carcinogenesis and whether these miRs differentiate between transitional points in benign and high-risk adenomatous polyps.

Methods

Patient and sample selection

We examined colonic adenomas that were collected from patients undergoing screening colonoscopy during 1990 to 1999 and who were participants of 2 large polyp prevention

studies for the prevention of metachronous colorectal adenomas at the University of Arizona —The Wheat Bran Fiber Trial¹¹ and the Ursodeoxycholic Acid Trial.¹² We would like to clarify that all samples examined in our study are baseline samples from the subjects that were recruited later in the aforementioned trials. Therefore, subjects were not on any drugs nor had any dietary modifications when those samples were retrieved. We excluded all patients with a familial colorectal cancer syndrome or with evidence of hyperplastic polyposis. The Wheat Bran Fiber Trial and Ursodeoxycholic Acid Trial studies were approved by the University of Arizona Human Subjects Committee and local hospital committees, and written informed consent was obtained from each participant before study enrollment. The institutional review board obtained for our study is part of the initial institutional review board that had included permission to review any colonoscopy sample at a later date. Patient information quarried from the database included age, gender, family history of colorectal cancer, location of lesion, history of smoking, and histology of the adenoma. We examined a total of 113 unique colonic adenoma samples from 108 patients (with 4 patients providing 2 different adenoma samples).

All tissue samples were formalin fixed and then paraffin embedded as part of routine clinical pathology. Normal colonic mucosa was obtained distant to the adenoma at the time of biopsy. Polyps were evaluated for and categorized based on their histologic type and reviewed by 2 GI specialty study pathologists. Polyp types were defined hyperplastic + normal mucosa (HPNM), tubular adenoma, sessile serrated adenoma, traditional serrated adenoma (sessile serrated adenomas with dysplasia), and high grade + tubular villous adenoma (HG). The classification was determined per the Guidelines for Colonoscopy Surveillance after Screening and Polypectomy: A Consensus Update by the US Multi-Society Task Force on Colorectal Cancer.¹³

miR analysis

For miR extraction, 3 micron slides with an hematoxylin and eosin guidewere used to guide macrodissection of regions of interest containing that defined the predominant histologic features on which the tissue was classified and deparaffinized. Total RNA, including miR, was isolated and extracted, labeled, and hybridized to the Affymetrix miR v. 1.0 microarray. There are a total of 825 human miR probe sets on this array. Sample processing dates and batch dates were annotated. After preprocessing and normalization, 3 high-interest CRC candidate miR probes (miR-320, miR-145, and miR-192) were selected for further analysis, based on a prior knowledge and investigator interest.

Statistical analysis

The analysis set consisted of data from 113 adenoma samples from 113 unique patients. Variables examined included histologic types, location, sex, age, family history, and smoking history. Each sample is classified into one of 5 histologic types: HPNM, tubular adenoma, sessile serrated adenoma, traditional serrated adenoma (sessile serrated adenomas with dysplasia), and HG.

Individual array data were preprocessed and normalized using robust microarray analysis routine from Partek Genomics Suite 6.5. The following options were specified: PM values

only, quantile normalization, log2 transformation, and median polish summarization. For each probe set, we performed a 2-way ANOVA to evaluate the main effect of the factor of interest (eg, histology) while adjusting for 2 different scan dates. Least squares means (model-predicted means) and standard errors were also estimated from the ANOVA models.

Results

All 3 miRNA probes, miR-320a, miR-145, and miR-192, were highly expressed among the 113 different tissue samples. The miR-320a had the highest level of relative expression, with miR-145 demonstrating the lowest level of overall expression. Table 1 shows descriptive statistics of the normalized log2 intensity levels. Fig. 1 demonstrates least squares mean and its 95% confidence interval for each histology type for each miR.

All 3 miR probes showed statistically significant differences in expression level across the 5 histologic subgroups (Table 2; all P values $< .0001$). Among the HG tissue sample group, miR-320a had the highest overall level of expression, with miR-145 having the lowest overall expression level. Conversely, miR-192 had the highest levels of expression in the HPNM (normal and or benign) tissue, with miR-145 demonstrating the lowest levels of expression. The P value from ANOVA only detects significant differences among any of the 5 histologic groups. Although there is overlap among some groups, there is also significant difference in expression depicted for miRNA with the ANOVA (P value $< .0001$) as seen in Table 1.

All 3 miR probes showed a trend toward expression differences by anatomic location (left vs right sided), with 2 (miR-320a and miR-192) reaching significance. Lesions located in the left colon had overall higher levels of expression of miR-320a ($P = .023$), whereas those adenomas located in the right colon had higher expression levels of both miR-145 and miR-192 ($P = .063$ and $.033$, respectively).

The expression levels of the 3 described miRs differed for other variables, including smoking history, age group, and family history. None of these differences, however, reached statistical significance (Table 2).

Comments

Colonic adenomas are precursors to adenocarcinomas, and this progression, although considered stepwise, is considerably heterogeneous.¹⁴ It is thought that this heterogeneity in disease progression is secondary to aberrant genetic expression that is regulated by miRs. Although downstream targets of miR have been increasingly understood and characterized, the changes in miR expression and when they occur are largely unknown. This study examined the difference in miR expression during the histologic progression from normal colonic mucosa to tubular and then to tubular villous adenomas and histologically advanced high-grade dysplastic polyps. Using 3 distinct miR probes, miR-320a, miR-145, and miR-192, we found progressive upregulation of miR-320a and a progressive downregulation of miR-145 and miR-192 during the transition from NM to high-grade dysplasia.

Our findings of the downregulation of miR-192 in more histologically advanced adenomas tubular villous high grade dysplastic adenoma is in agreement with the findings by Chiang et al,¹⁵ who showed an overall decreased level of expression of miR-192 in both CRC tissue samples and colon cancer cell lines. Lower levels of miR-192 were further associated with increased size in primary tumors. These findings, in conjunction with our own, suggest that miR-192 plays a significant role as a tumor suppressor and that loss of function in this miR-192 and downregulation of its downstream targets may occur early in tumorigenesis. Furthermore, cell cycle analysis data have demonstrated that the function on miR-192 is dependent on the presence of wild-type p53.⁹ Ectopic expression of miR-192 specifically results in a more profound inhibition of cellular proliferation in cells containing wild type than that of mutant p53. Loss of p53 has been thought to be a key step in colon carcinogenesis.¹⁰

Although the differential expression of miR-320a among colon cancer samples has been suggested to play a role in CRC cancer recurrence and metastasis, its role is not completely understood. In 1 study, miR-320a was shown to be frequently downregulated in both tumor tissue and colon cancer cell lines.¹⁶ The authors further suggested that one of the miR-320a downstream targets, β -catenin, is suppressed when miR-320a expression levels are restored in CRC cell lines and concluded that restoration of miR-320a leads to inhibition of cell proliferation. In support of these findings, Shepeler et al⁸ demonstrated that in stage II colon microsatellite stable tumors, when stratifying on age, gender, and T stage, the progressive free survival was 6.6 times higher for patients with high expression of miR-320a, compared with tumors with low expression. Our findings indicate that with colonic adenoma progression to high-grade dysplasia and more advanced histology, miR-320a is overexpressed. We speculate that overexpression of miR-320a may be a key defense mechanisms against transition to invasiveness. We speculate that progression from high-grade dysplasia to carcinoma may require negative feedback inhibition on miR-320a and downregulation for progression to carcinoma. This hypothesis is supported by findings that tumor metastasis is associated with a decrease and or loss of miR-320a expression. Zhang et al¹⁷ reported loss of miR-320a expression in CRCs that metastasized to the liver, compared with their corresponding primary tumor. Arndt et al¹⁸ showed that although a large proportion of metastatic CRC lesions retain the same epigenetic profile as their primary lesions, there is still enough variance between metastatic lesions and their corresponding primaries to suggest that perhaps downregulation of miR-320a may act late, after the lesion had metastasized. Collectively, the data suggest that miR-320a is an important molecule in disease progression.

Of the 3 probes examined, miR-145 is the best characterized as having tumor suppressor qualities.¹⁹ It is decreased in a variety of human epithelial cancers, including lung and breast.^{20,21} It has downregulated in CRC, which is in agreement with our findings.⁷ Furthermore, restoration of miR-145 levels in CRC cells has been shown to decrease proliferation, migration, and enhance chemoresistance.²² Based on this study, it appears that deregulation of miR-145 is an early event in the NMA-AC progression, and its loss of function may lead progression to adenocarcinoma.

Overall, we did not examine how the differential expression of these 3 miRs in our study could be associated with the differing molecular pathways of colon carcinogenesis described in the literature. For example, the serrated neoplastic pathway that describes the progression of serrated polyps, including sessile serrated adenomas and traditional serrated adenomas, to colorectal cancer has been associated with the CpG island methylator phenotype.²³ Furthermore, the traditional chromosomal instability (CIN) pathway, which is characterized by widespread imbalances in chromosome number (aneuploidy) and loss of heterozygosity, is considered the most frequent pathway to colon carcinogenesis. The CIN pathway is associated with loss of the APC gene and formation of early tubular adenomas.²⁴ Although we saw differing expressions of the miRs, 320a, 145, and 192, in sessile serrated and in tubular adenomas, future studies would have to take place to examine how these miRs may be associated with the CIN and CpG island methylator phenotype pathways of colorectal carcinogenesis.

Conclusions

In summary, we have found unique alterations in miRs-145, -192, and -320a expression levels during progression of normal/benign colorectal mucosa to tubular villous or high-grade dysplasia. It appears that miR-145 expression, a well-characterized tumor suppressor, is lost early in the NM-A-AC progression. Similarly, miR-192 exhibits a behavior consistent with tumor suppression with decreasing levels as the disease progresses. The role of miR-320a expression increases with premalignant disease progression. The role of miR-320a is still not well understood; our data and published studies suggest that miR-320a may act as a defense mechanism against “invasiveness” and metastasis in CRC. Further study of miR-320 may provide insight on local defense mechanism against invasion. Overall, our findings support progressive epigenetic changes at the level of the miRs in the NM-A-AC progression that might provide unique biomarkers for patient risk stratification and may, through future mechanism studies, guide prevention efforts at specific events in early carcinogenesis in the colorectum.

Acknowledgments

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Figure 1.
Least squares mean and its 95% confidence interval for each histology type for each miR.

Table 1

Expressions of miR-320a, miR-145, and miR-192 among 123 colon adenoma samples

Probe set	Mean	SD	Median	Minimum	Maximum
miR-320a	7.85	.79	7.86	3.82	9.45
miR-145	4.74	1.84	5.45	1.81	7.55
miR-192	6.56	1.71	7.30	1.85	9.14

Table 2

Summary of 2-way ANOVA results

Variable	Level	n (%)	miR-320a		miR-145		miR-192	
			LS mean (SE)	P value	LS mean (SE)	P value	LS mean (SE)	P value
Histology	HPNM	23 (20)	7.46 (.15)	<.0001	6.07 (.27)	<.0001	7.38 (.32)	<.0001
	TA	26 (23)	7.43 (.14)		5.50 (.26)		7.18 (.30)	
	SSA	13 (12)	7.96 (.20)		6.31 (.37)		7.22 (.45)	
	TSA	11 (10)	8.10 (.21)		4.22 (.40)		6.18 (.46)	
	TVHG	40 (35)	8.25 (.11)		3.12 (.21)		5.53 (.24)	
Location	Left	74 (65)	7.98 (.09)	.023	4.50 (.21)	.063	6.29 (.19)	.033
	Right	39 (35)	7.62 (.12)		5.18 (.29)		7.01 (.27)	
Sex	Female	40 (35)	7.83 (.13)	.84	5.10 (.29)	.12	6.69 (.27)	.48
	Male	73 (65)	7.87 (.09)		4.54 (.22)		6.46 (.20)	
Family history	No	78 (69)	7.82 (.09)	.64	4.87 (.21)	.51	6.70 (.19)	.34
	Yes	17 (15)	7.81 (.19)		4.44 (.46)		6.16 (.42)	
	Unknown	18 (16)	8.02 (.19)		4.42 (.44)		6.21 (.41)	
Smoking history	No	84 (74)	7.82 (.09)	.65	4.60 (.20)	.13	6.49 (.19)	.70
	Yes	15 (13)	7.85 (.20)		5.64 (.47)		6.89 (.45)	
	Unknown	14 (12)	8.04 (.21)		4.59 (.50)		6.49 (.46)	
Age group	<55	14 (12)	7.99 (.21)	.25	4.80 (.50)	.61	6.56 (.46)	.47
	55-64	19 (17)	7.72 (.18)		5.24 (.43)		7.07 (.39)	
	65-74	54 (48)	7.97 (.11)		4.57 (.26)		6.34 (.23)	
	75	26 (23)	7.64 (.15)		4.65 (.37)		6.56 (.33)	

HPNM = hyperplastic + normal mucosa; LS = least squares; miR = microRNA; SSA = sessile serrated adenoma; TA = tubular adenoma; TSA = traditional serrated adenoma; TVHG = tubular villous high grade dysplastic adenoma.