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MicroRNA Expression Profiles of Esophageal Cancer

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Abstract

Objectives—Expression of micro RNAs (miRNAs) by array analysis has identified unique profiles for classifying tissues and tumors. The purpose of our study was to examine miRNA expression in Barrett's esophagus (BE) and esophageal cancer to identify potential markers for disease progression.

Methods—miRNA was isolated from 35 frozen specimens (10 adenocarcinomas [AC], 10 squamous cell carcinomas [SCC], 9 normal epithelium [NSE], 5 BE and 1 high grade dysplasia [HGD]). miRNA expression was analyzed using Ambion Bioarrays containing 328 human miRNA probes.

Results—Unsupervised hierarchical clustering resulted in four major branches corresponding with four histologic groups. One branch consisted of 7 NSE and 1 SCC sample. The second branch of 7 SCC and 1 NSE. Third branch contained 4 BE and 1 SCC, and the fourth all the AC, and 1 each BE, NSE, SCC and the HGD. Supervised classification with Principal Component Analysis determined the NSE samples were more similar to the SCC tumors, and the BE more similar to AC. Pair-wise comparisons between sample types revealed miRNA's which may be markers of tumor progression. mir_203 and mir_205 were expressed 2–10 fold lower in SCC and AC compared with NSE. mir_21 expression was 3–5 fold higher in both tumors versus NSE. Prediction analysis of microarray (PAM) classified 3 BE samples as BE, 1 as AC and 1 as NSE.

Conclusions—miRNA expression profiles distinguish esophageal tumor histologies and discriminate normal tissue from tumorMicroRNA expression may prove useful for identifying BE patients at high risk for progression to AC.

Keywords

Esophagus Cancer; MicroRNA; Array Profiling

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Introduction

Esophageal adenocarcinoma (AC) has the fastest increasing incidence of any solid tumor in the United States (1) and the 5-year survival rate for this disease is dismal, ranging from 14–22%(2–4). While the incidence of esophageal squamous cell carcinoma has been declining or has remained constant in the United States, that of esophageal AC has increased >300% in the past 30 years and continues to rise (1;5–7). The most common risk factor for AC is chronic gastroesophageal reflux disease (GERD) which is associated with an approximately 16-fold increased risk of AC and occurs in up to 60% of patients diagnosed with this tumor (8). GERD causes inflammation in the distal esophagus resulting in development of the premalignant lesion known as Barrett's esophagus (BE), or intestinal metaplasia (9), and the presence of BE confers a 0.5% per patient-year risk of developing AC (10;11). The progression of BE to AC develops through established histologic changes: intestinal metaplasia (BE) to low grade dysplasia (LGD) to high grade dysplasia (HGD) then to AC. HGD represents an unstable epithelium that is a marker for concurrent occult carcinoma or for subsequent development of carcinoma with such risk ranging from 25–80% in multiple series (12–15). The current standard of care for patients with HGD and low operative risk factors is to treat with an esophagectomy. If the patient chooses not to undergo surgical resection or is deemed a poor surgical candidate, alternatives such as endomucosal resection, photodynamic therapy or other local therapies may be proposed (16–18). Patients then are advised to undergo endoscopies and multiple random biopsies every three months.. Similarly, patients with LGD are advised to undergo serial endoscopies, albeit less frequently, to identify development of HGD (19).

Dysplasia alone as a marker of malignant progression is mired by pathologists' differential interpretation of degrees of dysplasia (20). As only a subset of dysplastic lesions will progress to cancer, the challenge lies in sub-stratifying biopsies based on the probability of malignancy development. If molecular markers of premalignancy can be identified on small pieces of esophageal tissue obtained endoscopically, this may lead to improved early detection and provide objective criteria for selection of patients who may benefit from aggressive surgical treatment.

MicroRNAs (miRNAs), a class of small non-coding RNAs first reported in 1993 (21) are post-transcriptional regulators of gene expression and have been shown to be involved in cell differentiation, proliferation and apoptosis. Furthermore, it has recently been demonstrated that some miRNAs may function as either oncogenes or tumor suppressor genes (oncomirs) (22). Altered expression of oncomirs has now been found in a variety of human tumors including lung, breast and prostate cancer (23–27) and recent reports demonstrate a role for miRNA expression in disease progression and outcome (24;26). While protein-coding gene expression studies have shown differences between normal esophagus and BE and between BE and AC (28;29) only one recent report on seven patients has determined miRNA expression in esophageal lesions (30).

We hypothesized that unique miRNA expression profiles exist to distinguish normal esophagus, BE, esophageal dysplasia and esophageal cancer. Furthermore, since miRNA expression is associated with differentiation, specific miRNA's may become deregulated in the progression from BE to dysplasia to AC. Identification of these differences in miRNA expression may then identify patients at high risk for progression to cancer and who need to be followed more closely or treated more aggressively.

Materials and Methods

Specimens

Esophageal specimens were obtained from patients undergoing esophagectomy for malignant disease during the years 1999–2005, at the University of Pittsburgh Medical Center and Mount Sinai Medical Center. This study involving human tissue was approved by the Institutional Review Boards at both institutions. Histologic confirmation of the esophageal diagnosis was confirmed by the pathologist at the Mount Sinai Medical Center (M.W.).

Thirty-five frozen specimens were chosen for analysis. There were 10 adenocarcinomas (AC), 10 squamous cell carcinomas (SCC), 5 Barrett's esophagus (BE), 1 high grade dysplasia (HGD) lesion and 9 normal squamous epithelia (NSE). NSE specimens were obtained from individuals undergoing esophagectomy for HGD or cancer and were taken from as far from the malignancy as possible.

miRNA isolation

Enriched miRNA was isolated from tissue specimens using the mirVana miRNA Isolation Kit (Ambion, Austin, Texas) which allows for capture of both enriched miRNA and larger RNA species separately. Both the enriched miRNA and large RNA were quantified using a NanoDrop spectrophotometer and integrity of the large RNA fraction was determined using an Agilent Bioanalyzer as a surrogate for miRNA quality control. miRNA samples were only used in microarray experiments if the RNA integrity number (RIN) was 6 for the large RNA from the same specimen.

miRNA labeling, hybridization, scanning and data processing

miRNA samples from 35 esophageal specimens were labeled using the mirVana miRNA Labeling Kit (Ambion) based on the manufacturer's protocol. The Cy5 labeled miRNA was then hybridized at 42°C for 16 hours on mirVana miRNA Bioarrays (Ambion) which contain 796 probes representing duplicate spots for each of 287 human miRNAs, 65 mouse and rat miRNAs, 33 Ambion predicted miRNAs, and 4 positive controls (20 replicate spots for each positive control). The slides were then washed and scanned using an Axon 4000B (Molecular Devices, Sunnyvale, CA) scanner at 100% power and a PMT gain of 750. The microarray images were analyzed using Genepix PRO (Version 5.1) and normalization was performed with CyclicLoess (31) in R program downloaded from BioConductors (http://www.bioconductor.org/). Unsupervised hierarchical clustering, differentially expressed gene analysis among the groups, and prediction analysis of microarrays (PAM) were performed with the NCI BRB-ArrayTools Version 3.4 (32). For differentially expressed gene analysis among four histologies (AC, SCC, NSE, BE), class comparison between groups of arrays was performed using a random-variance F-test. Genes were considered statistically significant if their p value was less than 0.001. A stringent significance threshold was used to limit the number of false positive findings. This tool also used the multivariate permutation test to provide 90% confidence that the false discovery rate (FDR) was less than 10%. The FDR is the proportion of the list of genes claimed to be differentially expressed that are expected to be false positives. For PAM, 10-fold cross validation was performed using samples from four histologies (AC, SCC, NSE and BE). The comparison of PAM with histology was facilitated by transforming the continuous PAM output into binary data using a probability of 0.5 as the cut off. Principal Component Analysis (PCA) was performed with Partek Genomics Suite software (Partek, Inc., St. Louis, MO).

Results

Sample classification with unsupervised hierarchical clustering

We analyzed miRNA expression in 10 AC, 10 SCC, 5 BE, 1 HGD and 9 NSE samples. Unsupervised hierarchical clustering of samples with all miRNAs was able to separate NSE, SCC, BE and AC into four major groups with a few exceptions (Figure 1A). SCC sample MS305 was exceptional as the miRNA pattern clustered in the AC group. The histology of MS305 was an AC on frozen section but was then interpreted as SCC on the fixed tissue for final pathology. After the miRNA profile was obtained, the fixed tissue slides were reviewed again by a pathologist at UPMC who determined that this was a basaloid squamous cell carcinoma with focally undifferentiated areas which could look like a poorly differentiated adenocarcinoma.

Significantly Differentially expressed miRNAs

In addition to unsupervised sample classification, we also performed analysis to identify miRNAs that were differentially expressed among different histologic groups. Our results indicated that 13 human miRNAs are significantly, differentially expressed among AC, SCC and NSE (Table 1). miR_194, miR_192 and miR_200c are significantly up-regulated in AC but not in SCC. Compared with NSE, miR_342 is differentially expressed in SCC but not AC, and others including miR_21, miR_205, miR_203 and miR_93 are differentially expressed between tumor and normal but not necessarily between the two tumor histologies.

Supervised Sample classification with Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was performed with significantly differentially expressed genes (described above) on the 35 esophageal specimens. As with the cluster analysis, PCA was able to separate the four main types of samples (NSE, AC, SCC and BE) into distinct groups. This analysis also provides a clear visual representation of similarity between the histologies (Figure 1B). The NSE and SCC samples are more similar to each other than to the AC samples. The BE samples sit between the AC and NSE and the one HGD specimen has a miRNA expression profile similar to the AC.

Sample classification with prediction analysis of microarray (PAM)

PAM identified a set of miRNA’s, the expression of which was able to accurately classify samples according to histologic type. All 10 AC and 10 SCC were accurately classified into the correct histologic groups while 2 NSE samples were classified incorrectly; one as BE and one as SCC. Of the 5 BE samples, 3 were classified as BE while one was called AC and the other was NSE. Using the same miRNA classifier, the single HGD sample was determined to be more similar to AC than to the other three histologies. These data are shown in Table 2.

Discussion

The incidence of esophageal cancer has increased >300% in the United States since the 1970’s and the overall cure rate is dismal and failing to improve. Barrett’s esophagus is the earliest known pre-malignant lesion for this cancer and is associated with an 0.5% per patient-year risk of cancer (11;33). Current national guidelines from the American College of Gastroenterology include serial endoscopies and random biopsies to identify dysplasia and cancer in this small but high risk population of reflux patients(10;11). In addition to the potential sampling error with random endoscopic biopsies, the dysplasia interpretation is subject to inter-observer variability. Thus, the best clinical management of these patients is still limited by our current diagnostic approach and our understanding of the disease process.

Global gene expression (mRNA) profiling of Barrett's metaplasia and esophageal cancer has previously been used to identify differentially expressed genes in Barrett's compared with esophageal AC(34). However, in non-esophageal tissues micro RNA expression profiling has been shown to provide more accurate classification of tissue and tumor types than global mRNA, expression profiles (35) (23). For example, differential miRNA expression has been used to discriminate lung cancer from benign lung tissue, lung adenocarcinoma from squamous cell cancer(26) as well as malignant from benign tissues of the prostate and thyroid (27) (36). In addition, miRNA plays a role in lineage specific tissue differentiation and as such is particularly good at identifying the source of poorly differentiated tumors of unknown origin (23). Therefore, we hypothesized that 1. tumor-specific miRNA expression profiles exist for malignant and normal esophagus and 2. miRNA expression profiles may be associated with progression from BE to AC.

In order to test our hypotheses we used miRNA expression arrays from Ambion to determine the miRNA profiles for normal esophagus, Barrett's esophagus, one HGD lesion and the two primary types of esophageal cancer, AC and SCC. In this series we demonstrated that miRNA expression profiles distinguish different esophageal tissue types and also discriminate malignant tissue from normal esophagus. Furthermore, the normal squamous epithelium and squamous cancer samples are much more similar to each other than to the adenocarcinoma samples. Similarly, Barrett's esophagus and adenocarcinoma miRNA expression profiles are more similar to each other than to tissues of squamous origin (either benign or malignant). Given our current understanding of the development of esophageal adenocarcinoma, this makes good biological sense since adenocarcinoma is believed to arise from Barrett's columnar epithelium, not from squamous epithelium.

In addition to identifying differential expression of particular miRNAs in normal, BE, AC and SCC of the esophagus, we also used the miRNA data to predict into which groups the Barrett's specimens would be genotyped based on miRNA expression instead of histology. This analysis identified possible subtypes of miRNA expression within the five BE patients: one with a "normal" profile, one with an "AC" profile and three with BE-specific miRNA expression. These results support future studies in a larger sample of BE patients with cancer follow-up to answer the hypothesis that miRNA expression may identify a subgroup of BE patients most likely to develop esophagus cancer.

Finally, there are reports in the literature associating some of the differentially expressed miRNAs reported here with other cancers. For example, miR_21 is up-regulated in both esophageal adenocarcinoma and SCC and this has also been reported in tumors of the breast, lung, prostate, colon and stomach as well as in glioblastomas (25;26;37) (38). In addition, in lung cancer tissue miR_192 and miR_203 expression was upregulated compared to normal lung (37) and inhibition of miR_192 has been shown to down-regulate cell growth in a lung adenocarcinoma cell line (39). In our data we have shown higher expression of miR_192 in esophageal adenocarcinoma but lower expression of miR_203 in both cancers versus normal esophagus.

Conclusions

The differential miRNA expression of BE and esophageal adenocarcinoma may lead to identification of specific markers for progression, additional molecular classification of dysplastic lesions and a better understanding of the biology of progression of Barrett's and dysplasia. Improvement in the survival rate of patients with AC will most likely result from new therapeutics based on an increased understanding of the tumor biology and identification of biomarkers for earlier detection.

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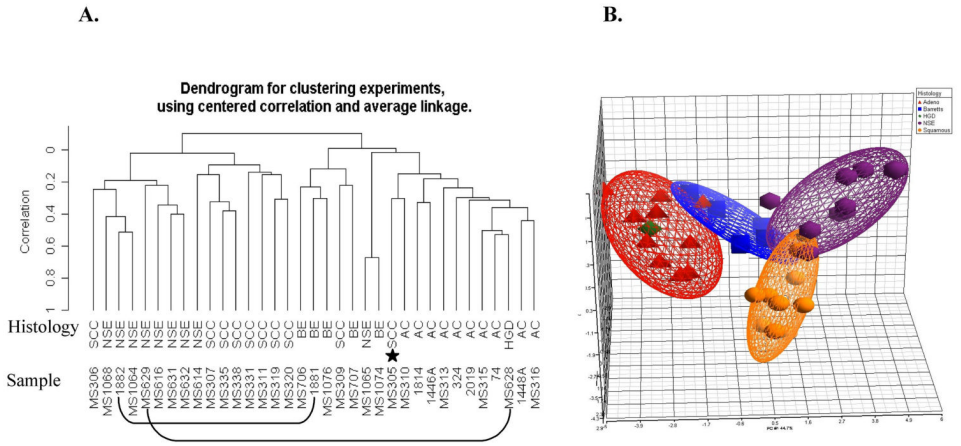

Figure 1.

miRNA expression patterns distinguish esophageal adenocarcinoma (AC), squamous cell carcinoma (SCC), normal squamous epithelium (NSE), Barrett's esophagus (BE) and high grade dysplasia (HGD). **A**. Unsupervised hierarchical clustering of esophageal specimens using all expressed miRNAs. Linked samples (1882/1881 and MS629/MS628) were obtained from the same patient. **B**. PCA mapping based on 14 miRNAs that exhibit differential expression between AC, SCC and NSE. Samples are coloured and shaped by histological subtypes.

Table 1

Differentially expressed miRNAs among four histological types (Adenocarcinoma, Squamous Cell Carcinoma, Barrett's esophagus, and Normal Squamous Epithelium)

	Fold change				
MiRNA	**AC vs BE**	**AC vs NSE**	**SCC vs NSE**	**AC vs SCC**	**FDR**
hsa_miR_192	1.7	3.9	0.9	4.4	< 1e-07
hsa_miR_194	2.0	4.7	0.8	5.6	< 1e-07
mmu_miR_192	1.4	3.0	0.9	3.4	< 1e-07
hsa_miR_21	3.7	5.2	2.9	1.8	0.0003384
hsa_miR_27b	0.7	0.4	0.6	0.7	0.0003384
hsa_miR_205	0.9	0.1	0.5	0.3	0.0005452
hsa_miR_203	0.9	0.2	0.4	0.6	0.0009346
hsa_miR_200c	1.9	1.9	0.8	2.4	0.0014706
hsa_miR_342	0.8	0.8	1.8	0.4	0.0014706
hsa_let_7c	1.1	0.6	0.5	1.2	0.006298
hsa_miR_93	1.3	1.4	1.3	1.1	0.0107505
hsa_miR_125b	0.5	0.5	0.8	0.6	0.0107505
hsa_miR_100	0.8	0.5	0.7	0.7	0.0112511
hsa_miR_152	1.0	0.8	1.1	0.8	0.0147661

Table 2

Prediction analysis of microarray (PAM) classification of samples based on miRNA expression. Training was performed on all samples except the single HGD using 10-fold internal cross validation. The HGD sample classification was then predicted based on the training analysis but is forced into one of the histologies used in the training. Adenocarcinoma, AC; squamous cell carcinoma, SCC; normal squamous epithelium, NSE; Barrett's esophagus (BE); High grade dysplasia (HGD).

Histology	Number of samples	PAM Classification			
		AC	SCC	NSE	BE
AC	10	10	0	0	0
SCC	10	0	10	0	0
NSE	9	0	1	7	1
BE	5	1	0	1	3
HGD	1	1	0	0	0