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Original Article

MicroRNA analysis of microdissected normal squamous esophageal epithelium and tumor cells

Liang Zhu[1], Wusheng Yan[1], Jaime Rodriguez-Canales[1], Alex M. Rosenberg[1], Nan Hu[2], Alisa M. Goldstein[2], Philip R. Taylor[2], Heidi S. Erickson[3], Michael R. Emmert-Buck[1], Michael A. Tangrea[1]

[1]Pathogenetics Unit, Laboratory of Pathology, Center for Cancer Research, Bethesda, MD, USA; [2]Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; [3]Department of Thoracic/Head and Neck Medical Oncology, UT MD Anderson Cancer Center, Houston, TX, USA.

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Abstract: Previous studies have identified several dysregulated microRNAs in esophageal squamous cell carcinoma (ESCC); however, to date there are no *ex vivo* analyses comparing expression levels of these regulatory molecules in esophageal squamous cell tumors versus patient-matched normal epithelium. We describe here a technical strategy to evaluate microRNAs in normal esophageal basal cells (NB), normal esophageal differentiated cells (ND), and tumor cells (T). Laser capture microdissection was used to procure target populations from five cases and 18 ESCC-associated microRNAs were measured by RT-qPCR. Five microRNAs (miR-25, miR-106b, miR-21, miR-203, and miR-145) demonstrated consistent differential expression in at least one of the three comparisons: T vs. NB, T vs. ND, or NB vs. ND. The potential regulatory role of the microRNAs in ESCC was further evaluated by correlating their expression with a matched mRNA dataset, which included the same five cases and cell populations. In conclusion, the present work demonstrates the feasibility of studying microRNA levels in precisely dissected cell populations from clinical samples, and sheds light on the molecular mechanisms associated with ESCC.

Keywords: Esophageal squamous cell carcinoma, laser capture microdissection, microRNA, basal layer, differentiated layer, miR-25, miR-106b, miR-21, miR-203, miR-145

Introduction

Esophageal cancer is the sixth most common cause of cancer-related deaths worldwide with the majority of cases being esophageal squamous cell carcinoma (ESCC) [1]. The overall survival rate remains low with < 20% of diagnosed patients surviving for five years [2, 3]. In contrast, the survival rate increases to 90% in patients who are diagnosed with Stage I disease (T1N0M0) and undergo surgical resection [4]. ESCC develops when the basal cells of the normal epithelium lose the ability to properly differentiate and expand to fill the entire epithelial compartment, ultimately breaking through the basal lamina and invading into sub-epithelial structures [5]. The histopathological changes associated with this process have been thoroughly described; however, the molecular underpinnings are less well understood. In order to improve survival rates in patients with ESCC there is a critical need to understand the molecular etiology towards design of improved therapeutics, and to identify new biomarkers that are clinically useful for early detection of pre-malignant lesions and localized cancer.

MicroRNAs are small non-coding RNAs involved in temporal and tissue-specific eukaryotic gene regulation and several recent studies have identified candidates that may be important in esophageal cancer [6-10]. These microRNAs were discovered through comparisons of expression profiles of bulk normal and tumor tissue specimens, or from serum samples of people with or without esophageal cancer [11-14]. The goals of the current study were to: a) Evaluate an innovative laser capture microdissection (LCM) approach to studying microRNA levels in microdissected normal epithelial cell populations and

Table 1. Clinical annotation of the five ESCC cases studied

Case Number	Age/Sex	Stage/ Grade	Smoking	Alcohol Drinking	Pickled Vegetables	Survival	Survival months
1	68/M	3/2	Y	<weekly	Never	N	16.2
2	52/M	3/2	Y	<weekly	Daily	Y	42.5
3	55/F	2/2	N	Never	<monthly	N	2.9
4	49/F	2/2	N	Never	<monthly	N	29.6
5	61/M	2/2	N	Weekly	<monthly	N	19.8

tumor cells; b) Measure the expression level of 18 microRNAs known to be associated with ESCC; and, c) Integrate the microRNA results with mRNA expression data from the same patient cases.

Materials and methods

Clinical tissue specimens

Table 1 lists the clinical annotation of the five ESCC cases from subjects residing in the Taihang mountain region of north central China. The cases were evaluated and selected by a pathologist (J.R.-C.). Specimens were fresh frozen, blocked, and stored at -80° C according to standard practices [15], and the study was approved by the Institutional Review Boards of the collaborating institutions: Shanxi Cancer Hospital and Institute, Taiyuan, Shanxi Province, China; and the National Cancer Institute, Bethesda, MD, USA.

Tissue processing and laser capture microdissection

The tissue specimens were cryo-cut into 8 micrometer thick sections, placed on glass slides, and stored for less than two weeks at -80° C. Before use, each section was individually removed from storage and immediately stained and dehydrated using an abbreviated hematoxylin and eosin (H&E) staining protocol for microdissection [16-18]. After H&E staining, the normal basal layer or differentiated layer of the epithelium was morphologically identified and procured using laser capture microdissection (LCM) with the PixCell IIe (Arcturus Engineering, Inc., Mountain View, CA) [19], according to standard protocols [17, 20] (**Figure 1**). The area of overlap between the two normal subtypes was strictly avoided during microdissection. Corresponding tumor cells were microdissected from matched tissue blocks for each case using the same dissection procedure. Cell populations were procured from each sample using 4,000-8,000 laser shots.

RNA isolation and assessment

Total RNA was isolated with the mirVana miRNA Isolation kit (Life Technologies Co., Carlsbad, CA) following the manufacturer's protocol. Care was taken to limit the time needed to remove the slide from the freezer and to complete the microdissection procedure in less than 30 minutes. RNA concentrations were measured with a NanoDrop ND-100 Spectrophotomer (NanoDrop Technologies, Wilmington, DE), according to the manufacturer's instruction.

Reverse transcription reactions, pre-amplification and real-time PCR

The Megaplex Primer Pool Human Set A v2.1 (Life Technologies Corporation, Carlsbad, CA) was used for the reverse transcription (RT) reactions and pre-amplification. Briefly, 0.8 μl Megaplex RT Primers, 0.2 μl dNTPs with dTTP, 1.5 μl reverse transcriptase, 0.8 μl RT buffer, 0.9 μl $MgCl_2$, 0.1 μl RNase inhibitor, and 0.2 μl nuclease-free water were mixed with 3 μl total RNA (150 ng). The RT mixtures went through 40 cycles of 16°C for 2 min, 42°C for 1 min and 50°C for 1 sec, followed by 85°C for 5 min and 4°C thereafter. 2.5 μl of the RT product was mixed with 12.5 μl Taqman PreAmp Master Mix, 2.5 μl Megaplex PreAmp Primers, and 7.5 μl nuclease-free water. The mixture was held at 95°C for 10 min, 55°C for 2 min, 72°C for 2 min followed by 12 cycles of 95°C for 15 sec and 60°C for 4 min. The samples were then incubated at 99.9°C for 10 min and 4°C thereafter. After pre-amplification, the product was mixed with 75 μl 0.1 × TE pH 8.0 and stored at -20°C for less than a week before performing real-time PCR.

Real-time PCR was performed using Taqman microRNA assay kits (Life Technologies Corpora-

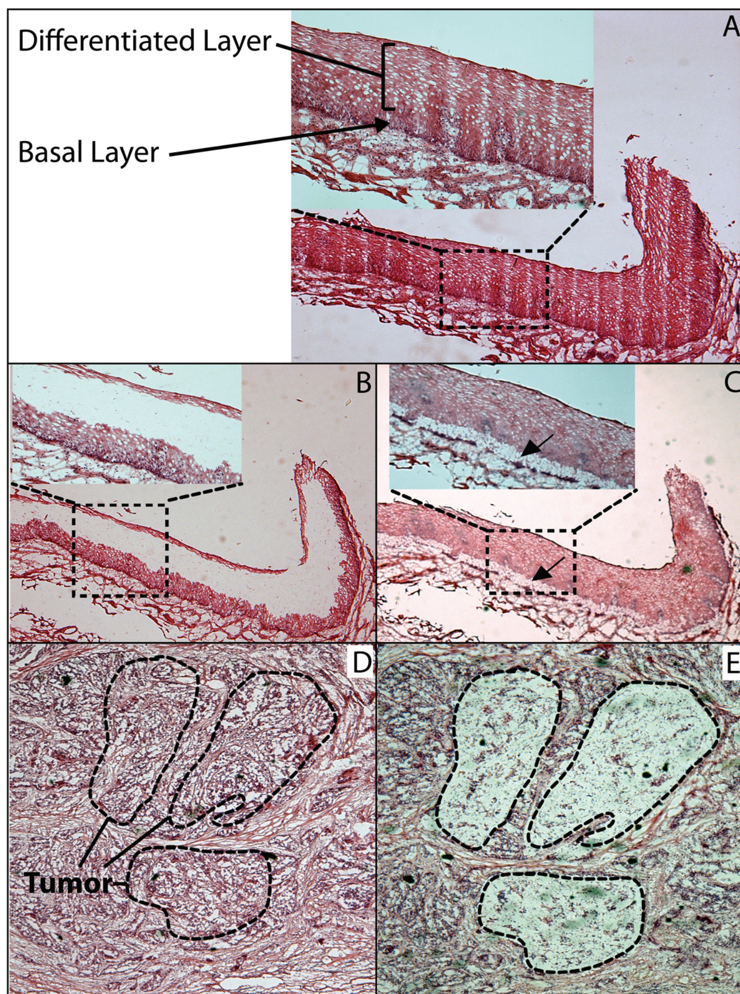

Figure 1. Histology of normal esophageal epithelium and ESCC. **A.** Hematoxylin and eosin (H&E) staining of normal esophageal epithelium. Arrows highlight the differentiated and basal layers. **B.** Microdissection of normal esophageal differentiated epithelium. **C.** Microdissection of normal esophageal basal epithelium. Arrows highlight basal layer. **D.** ESCC tumor foci before microdissection highlighted by dashed lines. **E.** Tissue following tumor microdissection. Magnification: A, B, C (inset) = 4X (10x), D = 10x, E = 10x.

tion, Carlsbad, CA). The pre-amplified product was further diluted ten times with 0.1 × TE pH 8.0. Then, 1.33 μl of the diluted product was mixed with 1 μl of the Taqman microRNA assay, 10 μl Taqman universal PCR master mix, and 7.67 μl nuclease-free water. The mixture was incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All of the reactions were run in triplicate along with no-RT and no template controls on an Applied Biosystems 7500 Real-time PCR system.

Normalization and data analysis

Four control RNA (U6, RNU44, RNU48, and ath-miR 159a) contained in the Megaplex Primer Pool (Life Technologies Corporation, Carlsbad, CA) were used for normalization. Relative quantification of microRNA expression was calculated using the ΔΔCt method [21]. The Wilcoxon matched-pairs signed-rank test was used to assess the differences between the variables (http://www.fon.hum.uva.nl/Service/Statistics/Signed_Rank_Test.html).

Examination of mRNA expression

A detailed protocol for the examination of mRNA expression in the samples is described in a previous report [22]. Briefly, following microdissection, total RNA was isolated with the PicoPure RNA Isolation kit (Arcturus Engineering, Inc. Mountain View, CA) and amplified with MessageAmp™ II-Biotin Enhanced Single Round aRNA Amplification Kit (Ambion, Inc., Austin, TX) and MessageAmp™ II aRNA Amplification Kit (Ambion, Inc., Austin, TX). Fifty ng of total RNA from each sample was subjected to two rounds of linear amplification and 15 μg of biotin-labeled cRNA was fragmented and processed for hybridization to Human Genome U133A 2.0 Genechips (Affymetrix Inc., Santa Clara, CA), following the Expression Manual from Affymetrix. In this study, microRNA and mRNA correlations were considered only in the comparison groups in which the microRNA demonstrated differential expression.

Results

Technical parameters

A total of 15 microdissected samples from five patients were generated, using laser microdissection to capture NB, ND, and tumor cell popu-

Table 2. Average fold changes of the microRNA differential expression in microdissected normal basal (NB), normal differentiated (ND) and tumor samples from five ESCC cases. The Wilcoxon Matched-Pairs Signed-Ranks Test was used for the statistical assessment. Table includes fold changes of microRNA with the lowest p-value (p = 0.06). Positive values indicate up-regulation while negative values indicate down-regulation.

microRNA	NB vs. ND	T vs. NB	T vs. ND
miR-21	--	+4.91	+4.53
miR-25	--	+3.70	+20.29
miR-106B	--	+3.22	+2.99
miR-145	-2.19	--	--
miR-203	-5.27	--	-17.46

lations (**Figure 1**). Eighteen microRNAs were selected for RT-qPCR evaluations based on previous reports describing either up-regulation (miR-21, miR-25, miR-106b, miR-199a, miR-373 and miR-424) or down-regulation (miR-27b, miR-99a, miR-100, miR-125b, miR-133a, miR-133b, miR-145, miR-192, miR-194, miR-200c, miR-203 and miR-205) in esophageal tumors [6, 8, 12-14, 23].

Normalization

We evaluated four RNAs as potential normalizers (U6, RNU44, RNU48 and ath-miR-159a). The combined mean expression of U6, RNU44 and RNU48 was used in the analyses since no Ct values were obtained for ath-miR-159a after 40 cycles of PCR in any of the five cases studied.

MicroRNA expression

The ΔΔCt method was used to identify differentially expressed microRNAs in the three comparisons: T vs. ND, T vs. NB, and NB vs. ND. The average fold change was calculated by $2^{\Delta\Delta Ct}$ assuming 100% PCR efficiency. None of the differentially expressed microRNAs reached statistical significance ($p < 0.05$), due in part to the small number of cases in the study. Therefore, we focused the data analysis on the microRNAs that demonstrated consistent changes across all five cases ($p = 0.06$). Of the 18 microRNAs that were assayed, five demonstrated consistent differential expression in at least one of the three comparisons (**Table 2**). For example, miR-21, miR-25 and miR-106b were all up-regulated in T vs. NB and in T vs. ND. miR-203 was down-regulated in NB vs. ND and T vs. ND, whereas miR-145 was uniquely down-regulated only in NB vs. ND (**Table 2**). In contrast, the following microRNA demonstrated no detectable expression differences between the groups; miR-27b, miR-99a, miR-100, miR-125b, miR-133a, miR-192, miR-194, miR-200c, miR-205. The remaining microRNA; miR-133b, miR-199a, miR-373 and miR-424 did not consistently demonstrate expression throughout all five cases and hence were excluded from the analysis.

Correlation of microRNA and mRNA expression

The correlation of microRNA and mRNA was established through three steps: 1) Individual microRNAs from **Table 2** were searched against PubMed to find associated mRNAs; 2) The expression patterns of these mRNAs were identified from our previous study [22]; 3) If both the microRNA and its associated mRNA were found to be dysregulated in the same comparison (NB vs. ND, T vs. NB or T vs. ND), they were presented in **Figure 2** as correlated pairs [12, 24-43]. The mRNA dataset consisted of 12 ESCC cases, which included the five cases presented in the microRNA analysis [22].

As an example, a PubMed search indicated that miR-25 was associated with Krüppel-like factor 4 (KLF4) [44], C-C Chemokines (RANTES and eotaxin) [44], Tumor Necrosis Factor-alpha (TNF-α) [44], Microphthalmia-Associated Transcription Factor (MITF) [45], Mitogen-Activated Protein Kinase Kinase 4 (MKK4) [46] and Transforming Growth Factor-beta (TGF-β) [47]. However, only KLF4 was differentially expressed in the T vs. ND and T vs. NB comparisons of the mRNA data [22], thus KLF4 was correlated with miR-25 in **Figure 2**. Similarly, miR-203 in **Figure 2** correlates with transcription factor JunB [27], and with ATP-binding cassette sub-family E

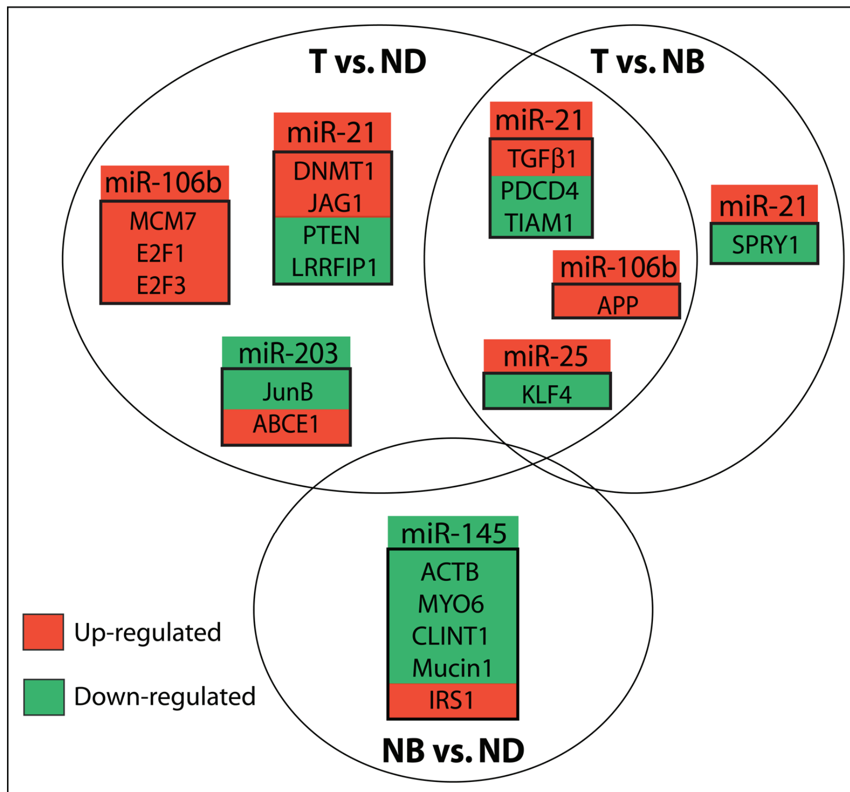

Figure 2. microRNAs and their associated mRNAs that were differentially expressed in microdissected normal basal (NB), normal differentiated (ND) and tumor (T) samples from matched ESCC cases [22].

(OABP), member 1 (ABCE1) [25].

Discussion

We employed a novel microdissection strategy to study microRNA levels in ESCC patient-matched normal squamous epithelium and tumor cells (**Figure 1**). By specifically dissecting two distinct epithelial populations, differentiated and basal, we aimed to more closely define microRNA expression levels in the normal tissue compartments as compared to bulk specimen analysis. Previously, it has been shown that LCM procured material displays a significantly different expression profile as compared to undissected whole tissue specimens from patients with either colorectal cancer [48] or Barrett's esophagus [49]. Thus, the present analysis provided the first precise measurement of microRNA levels in individual cell populations and allowed us to explore both normal-tumor and normal basal-normal differentiated cell differences.

At the outset of the study, identification of a high-throughput yet robust method to measure microRNA expression from a relatively small number of dissected cells was challenging. We first excluded methods that required large amounts of total RNA/microRNA input such as hybridization-based microarrays. We then reviewed the technical aspects of available assays and selected the ABI 7500 RT-qPCR platform (ABI 7500) approach with full control of the 96-well plate design, employing a pre-amplification RT-qPCR protocol to enhance the detection sensitivity as well as maximize the number of microRNAs investigated.

Also of importance in the study were the development of a consistent microdissection protocol, the use of uniform inputs of total RNA, and the selection of control microRNAs for normalization [16]. To date, there are few recognized microRNAs that are expressed evenly across all tissue and cell types, thus several normalization strategies have been suggested, including the use of individual microRNAs such as miR-191 or miR-103 [50], or the mean expression value of all expressed microRNAs in a given sample [51]. However, individual microRNAs have not been widely applied due to the lack of extensive validation, and generating a mean expression value from a large number of microRNAs is not easily

accomplished using laser microdissected samples. Therefore, we averaged the RT-qPCR data for three commonly used microRNA normalizers, U6, RNU44, and RNU48 with the assumption that they are biologically consistent across the samples studied.

The list of potential microRNA biomarkers that distinguish normal and diseased esophagus is beginning to accumulate [6, 8, 12-14, 23]. To further understand the role of these molecules in esophageal development and the formation of ESCC, we studied the expression levels of 18 microRNA known to be associated with ESCC within microdissected subpopulations of normal squamous epithelial cells and matched tumor. Five of the 18 microRNAs demonstrated a consistent change in expression (Table 2). Only miR-145 was differentially expressed (down-regulated) in the NB vs. ND comparison alone, suggesting it may play a role in the normal differentiation process. Previous studies have indicated that miR-145 may inhibit cell proliferation in ESCC and act as a tumor suppressor [13, 40, 52, 53]. However, our data indicate that miR-145 expression was down-regulated in the NB dissections and not in the tumor population (Table 2). Recently, Shi *et al* presented data that indicates an association between miR-145 and the insulin receptor substrate-1 (IRS1) in colon cancer cell lines [54]. In their study, over-expression of miR-145 prevented cell proliferation via IRS1 down-regulation [54]. Our data indicate that in the NB vs. ND comparison, the opposite is occurring as IRS1 is up-regulated and miR-145 is down-regulated (Figure 2). Therefore, the normal basal epithelial layer may express cell proliferation factors with the down-regulation of miR-145.

Four other microRNAs (miR-25, miR-106b, miR-21, and miR-203) were differentially expressed in one or more tumor comparisons (Table 2). miR-25, miR-106b and miR-21 were up-regulated in the T vs. NB and T vs. ND comparisons, while miR-203 was down-regulated in the T vs. ND comparison. These data implicate miR-25, miR-21, miR-106b, and miR-203 as involved in ESCC development.

Consistent with these findings, other studies have shown that miR-25 is up-regulated in tumors, including; lung cancer, hepatocellular carcinoma, pediatric brain tumors, acute myeloid leukemia, prostate cancer, and gastric cancer [55-61]. Interestingly, a recent study by Kuhn *et al* correlated the increased expression of the tumor suppressor, KLF4, with inhibition of miR-25 in airway smooth muscle cells of the lung [44]. A similar relationship was observed in ESCC as KLF4 was down-regulated and miR-25 up-regulated in both the T vs. NB and T vs. ND comparisons (Figure 2).

Furthermore, miR-25 and miR-106b have been shown to be overexpressed as a polycistron in a number of tumor tissues [12, 57, 62-64]. Both miR-25 and miR-106b exhibited increased expression in the ESCC tumor samples studied, although at different levels (Table 2). As expected, the miR-106b-25 host gene, minichromosome maintenance protein 7 (MCM7) was also upregulated in the T vs. ND comparison of our mRNA study (Figure 2) [22, 64]. The miR-106b-25 polycistron has been shown to play a key role in the development of esophageal adenocarcinoma via degradation of the p21 mRNA [12]. However, the predicted down-regulation of p21 was not observed in our mRNA study, indicating a potentially novel role of the miR-106b-25 polycistron in ESCC.

The widely studied microRNA, miR-21, is over-expressed in the majority of tumors [23, 29, 31, 32, 65-80]. ESCC is no exception and our study showed this over-expression in the T vs. NB and T vs. ND comparisons (Table 2). Interestingly, the analysis of microRNA-mRNA associations indicates that several mRNA are uniquely expressed in either the normal basal (SPRY1) or normal differentiated (DNMT1, JAG1, PTEN, and LRRFIP1) epithelial layers when compared to tumor (Figure 2). The Sprouty1 (SPRY1) and miR-21 relationship has been demonstrated in cardiovascular disease, but this is the first report of a possible association in ESCC [28].

Unlike miR-25, miR-106b, and miR-21, miR-203 is down-regulated in the majority of tumors [6, 66, 70, 78, 79, 81-83]. The present study is consistent with those findings, as miR-203 was down-regulated by greater than seventeen fold in the T vs. ND comparison and more than 5 fold in the NB vs. ND comparison (Table 2). The effect of miR-203 down-regulation in the NB vs. ND comparison is unclear as no correlating mRNA were found to be differentially expressed in the related mRNA dataset. However, two miR-203-associated mRNA, JunB and ABCE1, were differentially expressed in the T vs. ND compari-

son (**Figure 2**). A direct relationship between miR-203 and ABCE1, a known RNase L inhibitor [84, 85] is possible since they show opposite directionality in their expression changes and related microRNAs and mRNA are typically inversely correlated.

In summary, the current study presents a technical advance for quantitatively measuring microRNA levels in defined cell populations using laser capture microdissection, and provides the first analysis of tumor-associated microRNA levels in sub-populations of normal squamous epithelium and matched esophageal tumors. The investigation into the correlation of consistently differentiated microRNA and their related mRNA identified relationships unique to the microdissected subpopulations of ESCC. miR-25, miR-106b, miR-21, miR-203 and miR-145 were identified as key dysregulated microRNAs in ESCC and warrant additional study in this cancer type.

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Please address correspondence to: Michael R. Emmert-Buck, MD, PhD, Pathogenetics Unit, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA. E-mail: buckm@mail.nih.gov; Michael A. Tangrea, PhD, Pathogenetics Unit, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. E-mail: tangream@mail.nih.gov

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