

Gastric adenocarcinoma microRNA profiles in fixed tissue and in plasma reveal cancer-associated and Epstein-Barr virus-related expression patterns

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MicroRNA expression in formalin-fixed paraffin-embedded tissue (FFPE) or plasma may add value for cancer management. The *GastroGenus miR* Panel was developed to measure 55 cancer-specific human microRNAs, Epstein-Barr virus (EBV)-encoded microRNAs, and controls. This Q-rtPCR panel was applied to 100 FFPEs enriched for adenocarcinoma or adjacent non-malignant mucosa, and to plasma of 31 patients. In FFPE, microRNAs upregulated in malignant *versus* adjacent benign gastric mucosa were hsa-miR-21, -155, -196a, -196b, -185, and -let-7i. Hsa-miR-18a, 34a, 187, -200a, -423-3p, -484, and -744 were downregulated. Plasma of cancer *versus* non-cancer controls had upregulated hsa-miR-23a, -103, and -221 and downregulated hsa-miR-378, -346, -486-5p, -200b, -196a, -141, and -484. EBV-infected *versus* uninfected cancers expressed multiple EBV-encoded microRNAs, and concomitant dysregulation of four human microRNAs suggests that viral infection may alter cellular biochemical pathways. Human microRNAs were dysregulated between malignant and benign gastric mucosa and between plasma of cancer patients and non-cancer controls. Strong association of EBV microRNA expression with known EBV status underscores the ability of microRNA technology to reflect disease biology. Expression of viral microRNAs in concert with unique human microRNAs provides novel insights into viral oncogenesis and reinforces the potential for microRNA profiles to aid in classifying gastric cancer subtypes. Pilot studies of plasma suggest the potential for a noninvasive addition to cancer diagnostics.

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Gastric adenocarcinoma is the leading cause of infection-related cancer mortality and is projected to soon rise to eighth in all-cause mortality globally.^{1,2} Emerging data suggest that gastric adenocarcinoma is not one disease but rather has distinct molecular subtypes, including the Epstein-Barr virus (EBV)-infected subtype that comprises ~10% of all cases.³ In this subset of cancers, EBV DNA is localized within malignant cells. EBV was the first virus recognized to encode its own microRNAs. Like human microRNAs, viral microRNAs function post-transcriptionally to interfere with translation or promote mRNA degradation.

MicroRNAs are relatively stable in stored tissue or plasma specimens,^{4,5} and microRNA profiles are increasingly reported as ancillary markers of disease status. Formalin-fixed, paraffin-embedded tissue (FFPE) and blood plasma are the practical specimen types in which to examine a tumor's biochemical

profile because, unlike fresh or frozen tissue, these specimens are available for nearly every cancer patient. Blood plasma has recently been exploited in 'liquid biopsy' assays of circulating microRNAs.^{6–9} Pertinent genomic technology such as Q-rtPCR can characterize microRNAs in tumor tissue and plasma, and clinical-grade quality assurance efforts are increasingly bringing RNA-based assays into the clinical arena.¹⁰

Prior discovery work found significantly altered microRNA expression profiles in gastric cancer compared with normal mucosa,^{11–18} and in blood of gastric cancer patients versus controls.^{7,8,11,19–24} In those studies, hsa-miR-21, hsa-miR-196a, and hsa-miR-196b were some of the most consistently altered microRNAs in gastric cancer despite marked variability in study designs and specimen types. Very few studies have taken into account the different molecular subtypes of gastric cancer, including EBV-positive cancers. In

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Table 1 Target microRNAs in the *GastroGenus miR* Panel

| Target microRNA | References | Target microRNA | References |
|----------------------------|------------|---|------------|
| hsa-let-7e | 25 | hsa-miR-196a | 11 |
| hsa-let-7f | 26 | hsa-miR-196b | 27 |
| hsa-miR-16 | 19, 28 | hsa-miR-200a | 29 |
| hsa-miR-17 | 23, 24 | hsa-miR-200b | 29, 30 |
| hsa-miR-18a | 31 | hsa-miR-221 | 7, 20 |
| hsa-miR-19a | 17 | hsa-miR-222 | 20 |
| hsa-miR-20a | 7, 23, 32 | hsa-miR-370 | 12 |
| hsa-miR-21 | 24, 33 | hsa-miR-371-5p | 21 |
| hsa-miR-23a | 6, 26 | hsa-miR-376c | 20 |
| hsa-miR-23b | 26 | hsa-miR-378 | 21 |
| hsa-miR-25 | 19, 23 | hsa-miR-423-3p | 6 |
| hsa-miR-27a | 32 | hsa-miR-425 | 26 |
| hsa-miR-27b | 20 | hsa-miR-451 | 19, 34 |
| hsa-miR-30e | 26 | hsa-miR-484 | 23 |
| hsa-miR-346 | 35 | hsa-miR-486-5p | 19, 23 |
| hsa-miR-34a | 36 | hsa-miR-744 | 20 |
| hsa-miR-93 | 26, 28 | hsa-miR-1203 | 23 |
| hsa-miR-103 | 37 | ebv-miR-BART 1-5p | 38 |
| hsa-miR-106a | 23, 24 | ebv-miR-BART 10-5p | 38 |
| hsa-miR-106b | 7, 24 | ebv-miR-BART 12 | 38 |
| hsa-miR-141 | 36, 39 | ebv-miR-BART 17-3p | 38 |
| hsa-miR-146a | 40 | ebv-miR-BART 7-3p | 38 |
| hsa-miR-155 | 41 | ebv-miR-BART 7-5p | 38 |
| hsa-miR-185 | 26 | ebv-miR-BHRF 1-2-5p | 38 |
| hsa-miR-187 | 21 | UniSp2 negative control | 6 |
| hsa-miR-191 | 20 | UniSp3 reagent positive control | 6 |
| hsa-miR-191 (replicate) | 20 | UniSp6 spiked control Cel-miR-39-3p negative control | 6 |

the current study, we designed a literature-based panel of the most dysregulated microRNAs in gastric cancer tissue or plasma, and then devised an analytic approach to measure these microRNAs in both fixed tissue and plasma, to examine suitability of this technology for distinguishing cancer, non-cancer, and EBV-infected cancer patients.

MATERIALS AND METHODS

DNA Extraction

Formalin-fixed, paraffin-embedded gastric adenocarcinoma tissues from the clinical archives of three hospitals in disparate parts of the world were assembled. They included 21 from the University of North Carolina Hospitals in Chapel Hill (UNC), 55 from the Western Regional Hospital in

Santa de Rosa de Copán, Honduras, and 2 from Wakayama Medical University in Wakayama, Japan. Case selection was based on adequate FFPE tissue from either resection or biopsy specimens, with preference given to EBV-infected cancers in order to explore viral microRNA expression. In addition, EDTA blood samples from 17 untreated UNC gastric adenocarcinoma patients (ages 51–88), and 14 blood samples from patients with no history of gastric cancer (ages 47–83, one of whom had a remote history of prostate cancer and the rest of whom had no cancer history) were used to prepare plasma for analysis of circulating cancer-related microRNAs. Plasma was not available from enough EBV-positive gastric cancer patients to justify studies of circulating viral microRNAs. The study was approved by the Institutional Review Boards of the testing facilities.

Nine FFPE tissue sections, each 10 μ M thick, were prepared from each block. A pathologist marked areas of tumor and benign mucosa (when available) on hematoxylin and eosin-stained sections, and tumor and benign mucosa were separately macrodissected. When feasible, a separate tissue block was used as a source of benign mucosa. Total nucleic acid was extracted using the HighPure miR Isolation kit according to manufacturer instructions (Roche Applied Science, Mannheim, Germany). Following proteinase K treatment, the UniSp6 Exiqon control microRNA was spiked in. Eluate was treated with DNase and cDNA was prepared and stored at -20°C until analysis.

Plasma was prepared by centrifugation (1500 g for 10 min) of EDTA-anticoagulated blood from gastric adenocarcinoma patients and non-carcinoma controls. A 200 μ l aliquot of plasma was spiked with the Sp6 Exiqon control microRNA before nucleic acid extraction using the Exiqon miRCURY RNA Isolation Kit- Biofluids (Exiqon, Woburn, MA, USA). Eluate was treated with DNase, and cDNA was prepared and stored at -20°C until analysis.

Q-rtPCR of MicroRNAs

A custom microRNA expression panel dubbed the *GastroGenus miR* Panel was devised to measure 51 unique target microRNAs chosen because they are reportedly gastric cancer-specific, EBV-specific, or serve as endogenous markers of specimen quality (Table 1). Spiked and no-template controls were also included to assess system performance.

Locked nucleic acid (LNA) technology is reported to yield precise and reproducible microRNA profiles.²⁵ In this study, microRNA was measured by Q-rtPCR using 96-well plates preloaded with lyophilized miRCURY LNA Universal RT miR rtPCR primers (Exiqon) targeting 43 human microRNAs, one replicate, and four controls. A separate 10-well panel of Exiqon Q-rtPCRs was used to measure seven EBV microRNAs and three human microRNAs in the subset of cases with known EBV status. Expression was measured in real-time using SYBR Green detection on a Roche Lightcycler 480. Recovery of Sp6 microRNA that was spiked in prior to extraction served as a control for recovery and integrity of the

stored nucleic acid and as a check for efficacy of downstream cDNA preparation and amplification. Lack of product in the no-spike controls (Sp2 and Cel-miR-39–3p) on each plate, and consistent expression of Sp3 that is included in Exiqon reagents, demonstrated that the analytic test system performed as expected. Levels of Sp3 also served as an interplate comparator, and expression of hsa-miR-191 served as a reproducibility control as it was tested twice on each panel.

Prior to panel-based testing, cDNA prepared using the Exiqon Universal cDNA synthesis kit on 10 μ l of eluate was vetted using individual Exiqon Q-rtPCRs targeting three human housekeeping microRNAs. Analytic capability to distinguish EBV-infected from uninfected cells, and lymphoid from epithelial cell lineage, was proven using FFPE cell pellets prepared from cultured cell lines (data not shown).

EBV Q-PCR and EBER *In Situ* Hybridization

EBV status was assigned in tumor tissue based on EBV DNA viral load exceeding a pre-established threshold, or EBV-encoded RNA (EBER) localization to malignant cells. Q-PCR of the *BamHI*W segment of the EBV genome served to quantify EBV DNA viral load in plasma and in fixed tissue as previously described.^{3,26} EBER *in situ* hybridization (BOND assay, Leica Microsystems, Nussloch, Germany) results were used to localize EBV to malignant cells or to background lymphocytes, and oligo-dT control hybridization assured RNA was preserved in fixed tissues.²⁷

Statistical Analysis

Raw microRNA expression data were uploaded to the GenEx software platform (version 6.0.1.612, MultiD Analyses AB). Raw data were calibrated using interplate calibrators. The NormFinder algorithm (in GenEx software) was applied to identify the most stably expressed subset of microRNAs in the data sets.²⁸ These microRNAs with relatively stable expression across all samples allowed for data normalization, which helps control for differences in quantity and quality of amplifiable cDNA. In the human microRNA data set, 32 microRNAs were identified as normalizers in FFPE, and 33 were identified in plasma. For the EBV microRNA data set, expression data were normalized to hsa-mir-423–3p, as it had the lowest variance of the three human microRNAs included in the 10-well test panel along with the seven EBV microRNAs. The seven EBV microRNAs had highly variable expression and could not be used as normalizers. Raw data were converted to relative expression by log₂ transformation. Significant differential expression between groups was determined using unpaired two-tailed *t*-tests. To maintain a type I error rate below 0.05, Dunn–Bonferroni correction was used to establish a significance threshold of $P < 0.00125$ for the human miR panel, and $P < 0.00568$ for the EBV-specific miRs. Unsupervised hierarchical clustering was performed using Ward's algorithm with Pearson correlation as the

distance metric for human microRNAs, and Euclidean distance for EBV microRNAs.

RESULTS

The *GastroGenus miR* Panel was applied to formalin-fixed tissue from 78 gastric adenocarcinoma specimens and 22 non-malignant gastric mucosa specimens. Three microRNAs (hsa-miR-1203, hsa-miR-371-1p, and hsa-miR-30e) were excluded from analysis owing to low expression rates. After housekeeper microRNA normalization, 13 human microRNAs were significantly differentially expressed in tumor compared with non-malignant gastric mucosa ($P < 0.00125$) (Figure 1a). Of those, six were upregulated and seven were downregulated in tumor tissue. The upregulated microRNAs were hsa-miR-21, hsa-miR-196a, hsa-miR-196b, hsa-miR-185, hsa-miR-155, and hsa-miR-let-7i. The downregulated microRNAs were hsa-miR-187, hsa-miR-200a, hsa-miR-744, hsa-miR-423–3p, hsa-miR-484, hsa-miR-34a, and hsa-miR-18a.

A total of 32 microRNAs were identified as normalizers using the NormFinder algorithm on tissue specimens (Table 2). Because 11 of these were also identified as being dysregulated in disease (10 of them altered in tumor compared with benign, and one additional altered in EBV-positive compared with EBV-negative tumors) a reanalysis of the data was performed excluding those 11 microRNAs as normalizers. Using the modified normalization strategy, the resulting lists of significantly dysregulated microRNAs were unchanged.

Unsupervised hierarchical clustering using the 13 significant microRNAs showed 90% accuracy in separation of cancer and non-malignant tissues (Figure 2). Among the 10 tissues that segregated incorrectly, 8 were non-malignant tissues exhibiting a cancer-like profile, and 2 were cancers exhibiting a benign-like profile. Four of the eight misclassified benign samples were macrodissected from the same slide as their adenocarcinoma counterparts, suggesting occult tumor involvement of benign-appearing mucosa, or 'field effect' whereby adjacent benign-appearing tissue has biochemical features of malignancy. The remaining four misclassified benign samples contained chronic gastritis and were from gastric resection specimens located at a distance ranging from 1 to 7 cm from the corresponding adenocarcinoma. Both of the adenocarcinoma samples that clustered with the benign samples had low tumor content. One was a small focus of adenocarcinoma (2 mm square) flanked by gastric mucosa. The other was a small gastric biopsy (4 mm square) with ~50% adenocarcinoma cellularity. It is feasible that the surrounding mucosa in these two cases diluted adenocarcinoma-related microRNA signals.

Pilot Studies of Plasma MicroRNA Profiles

The *GastroGenus miR* Panel was applied to plasma samples from 17 patients with untreated gastric cancer and to 14 plasmas from patients without a history of gastric cancer.

After housekeeper microRNA normalization, 11 human microRNAs were significantly differentially expressed in

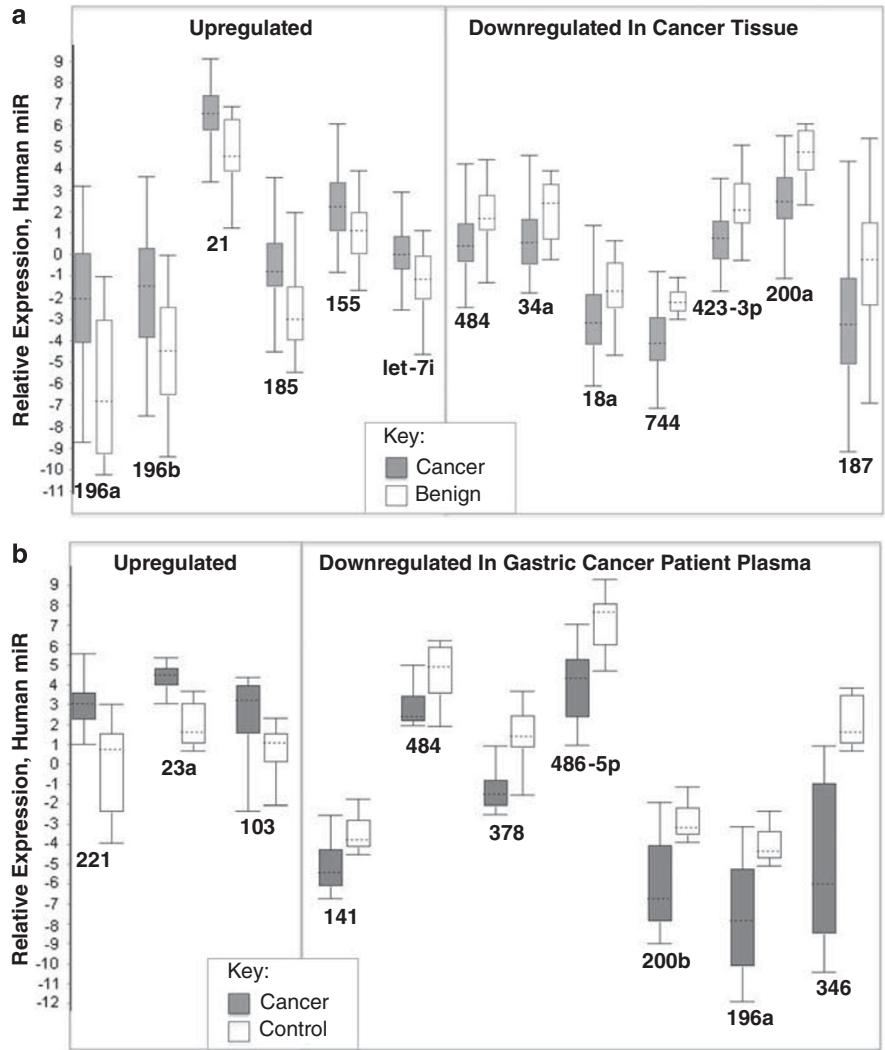


Figure 1 Differential expression of microRNAs in cancer tissue compared with benign mucosa, and in cancer patient plasma compared with control plasma. **(a)** In formalin-fixed tissues, thirteen microRNAs were significantly dysregulated (adjusted $P < 0.00125$) in gastric cancer tissue compared with non-cancer. **(b)** In plasma, 10 microRNAs were significantly dysregulated (adjusted $P < 0.00125$) in gastric cancer patients compared with the control cohort lacking gastric cancer. Box plots represent the median (dotted line) and middle quartiles; whiskers are $1.5 \times$ interquartile range.

cancer versus non-cancer plasma ($P < 0.00125$). Of the 11 dysregulated microRNAs, 4 were upregulated and 7 were downregulated in cancer patient plasma. The four upregulated microRNAs were hsa-miR-23a, -103, -21, and -221, whereas the seven downregulated ones were -196a, -200b, -346, -378, -486-5p, 141, and -484.

A total of 33 microRNAs were identified as normalizers using the NormFinder algorithm on plasma specimens. Because eight of these were dysregulated in cancer compared with non-cancer patients, these eight microRNAs were excluded in a reanalysis of the plasma data. This renormalization resulted in loss of significance of miR-21; however, the remaining 10 dysregulated microRNAs remained significant (Figure 1b).

Unsupervised hierarchical clustering using the eight most significant microRNAs showed excellent separation of tumor and non-tumor patients, with an accuracy of 100% (Figure 3). Only two microRNAs, hsa-miR-196a and hsa-miR-484, were dysregulated in both cancer tissue and cancer patient plasma. Although hsa-miR-484 was downregulated in both tissue and plasma, hsa-miR-196a was upregulated in cancer tissue and downregulated in plasma of cancer patients. This finding implies that tumor-derived microRNA is not necessarily informative when measured in plasma.

EBV-Positive Versus EBV-Negative Cancers

Twenty gastric adenocarcinomas were EBV-positive as defined by localization of EBV to malignant cells using *EBER*

Table 2 MicroRNAs identified as normalizers, ranked from major to minor in terms of stability across all tissue or plasma specimens

| Normalizers in tissue | Average expression | Normalizers in plasma | Average expression |
|-----------------------------|--------------------|-----------------------------|--------------------|
| hsa-miR-191 | Medium | hsa-miR-106a | High |
| hsa-miR-191 (replicate) | Medium | hsa-miR-25 | Medium |
| hsa-let-7i ^a | Medium | hsa-miR-191 | Medium |
| hsa-miR-423-3p ^a | Medium | hsa-miR-106b | Low |
| hsa-miR-25 | Medium | hsa-miR-141 ^a | Low |
| hsa-miR-93 | Medium | hsa-miR-370 | Low |
| hsa-miR-27a | Medium | hsa-miR-20a | High |
| hsa-let-7e | Medium | hsa-miR-191 (replicate) | Medium |
| hsa-miR-222 | High | hsa-miR-222 | Medium |
| hsa-miR-425 | Medium | hsa-miR-23b | Medium |
| hsa-miR-376c | Low | hsa-miR-484 ^a | High |
| hsa-miR-484 ^a | Medium | hsa-miR-146a | Medium |
| hsa-miR-146a | Medium | hsa-let-7i | Medium |
| hsa-miR-221 | Medium | hsa-miR-155 | Medium |
| hsa-miR-27b | Medium | hsa-miR-93 | High |
| hsa-miR-744 ^a | Low | hsa-miR-196b | Medium |
| hsa-miR-20a | Medium | hsa-miR-378 ^a | Medium |
| hsa-miR-103 | Medium | hsa-miR-34a | Low |
| hsa-miR-18a ^a | Medium | hsa-miR-16 | High |
| hsa-miR-370 | Low | hsa-miR-23a ^a | High |
| hsa-miR-23a | High | hsa-miR-17 | Medium |
| hsa-miR-16 | Medium | hsa-miR-423-3p | Medium |
| hsa-miR-23b | High | hsa-miR-425 | Medium |
| hsa-miR-378 ^a | Medium | hsa-miR-200a | Low |
| hsa-miR-106b | Low | hsa-miR-486-5p ^a | High |
| hsa-miR-21 ^a | High | hsa-miR-21 ^a | Medium |
| hsa-miR-185 ^a | Medium | hsa-let-7e | Medium |
| hsa-miR-106a | Medium | hsa-miR-27a | Medium |
| hsa-miR-17 | Low | hsa-miR-744 | Medium |
| hsa-miR-34a ^a | Medium | hsa-miR-376c | Medium |
| hsa-miR-155 ^a | Medium | hsa-miR-103 ^a | Medium |
| hsa-miR-196b ^a | Medium | hsa-miR-200b ^a | Low |
| | | hsa-miR-27b | Medium |

^aMicroRNAs identified as normalizers and also associated with disease status in the corresponding sample type.

in situ hybridization or by high EBV DNA viral load using a validated Q-PCR of tumor tissue. Expression of seven EBV microRNAs was examined in 17 EBV-positive and 32 EBV-negative tumors for which sufficient tissue was available.

All seven EBV microRNAs tested were upregulated in the EBV-infected tumors ($P < 0.00568$). The pattern of viral microRNA expression was consistent across all infected cancer tissues (Figure 4). These findings are strong evidence that the analytic methods used to measure viral microRNAs in this study are sensitive and specific for identifying virus-infected cancers.

Four human microRNAs were significantly differentially expressed in EBV-positive compared with the EBV-negative tumors ($P < 0.00125$): hsa-miR-196b was downregulated, whereas hsa-miR-155, hsa-miR-185, and hsa-miR-378 were upregulated in infected tumors (Figure 4).

DISCUSSION

This study used modern molecular methods to show that selected human and viral microRNAs were differentially expressed in gastric cancer compared with non-cancer control tissues, and that EBV-infected cancers could be distinguished from uninfected cancers by virtue of viral miR expression. Circulating cell-free microRNA exhibited somewhat different patterns of microRNA expression than was found in primary tissues, yet the plasma profiles were found to distinguish cancer from non-cancer patient groups.

Benign and malignant tissues tended to cluster separately, with some exceptions that might be explained by field effect,²⁹ by exosome or by other means of transfer of microRNA across cells in the microenvironment,^{30,31} by imperfect macrodissection, or by features such as inflammation that are common to both malignant and benign tissues.

Among the six microRNAs upregulated in our gastric cancer cohort compared with adjacent non-malignant gastric mucosa was hsa-miR-21, which was previously reported as overexpressed in gastric cancer tissues and in plasma of affected patients.^{18,32–39} The Cancer Genome Atlas Network showed that, compared with expression in non-malignant gastric mucosa, hsa-miR-21 was upregulated in all gastric cancer classes except for the ‘microsatellite instability’ class.^{40,41} Utility in screening for recurrence is under investigation. Interestingly, hsa-miR-21 is induced by *Helicobacter pylori* co-culture with gastric epithelial cells.¹⁸

Hsa-miR-196a and -196b are strongly upregulated in gastric cancer tissues.^{11,42–45} In fact, The Cancer Genome Atlas Network showed that, compared with normal gastric mucosa, hsa-miR-196a and -196b were the top two most upregulated microRNAs in all molecular classes of gastric cancer except for the ‘EBV-positive’ class.^{40,41}

The remaining three of our study’s upregulated microRNAs (hsa-miR-155, hsa-miR-185, and hsa-miR-let7i) have discordant reports in the literature suggesting either downregulation, utility as normalizers, or upregulation in gastric cancer cell lines and tissues.^{28,40,46–50} The Cancer Genome Atlas Network showed that, compared with normal mucosa, hsa-miR-155, and -185 are upregulated in the EBV-positive molecular class of cancer, whereas hsa-miR-185 is upregulated in the ‘chromosome instability’

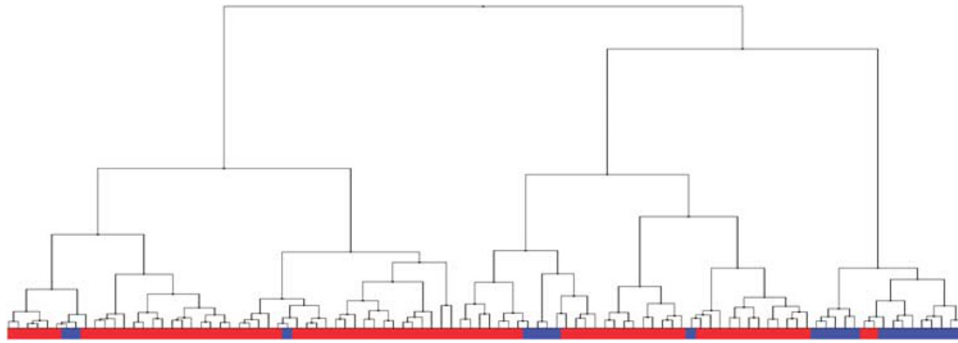


Figure 2 MicroRNA expression patterns differ between malignant tissue and benign mucosa. Unsupervised hierarchical clustering of 78 gastric cancers (red) and 22 benign gastric mucosal (blue) reveal that expression profiles of 13 significantly dysregulated microRNAs discriminate benign from malignant tissue with 90% accuracy.

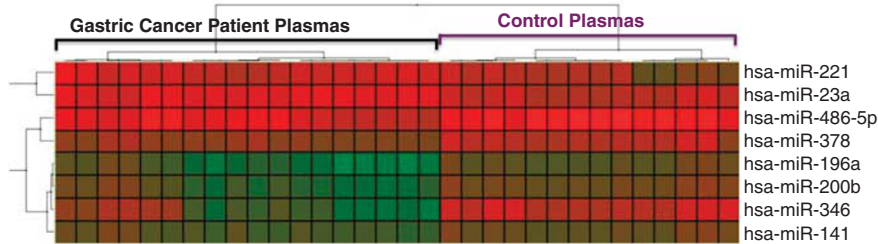


Figure 3 Plasma microRNA expression patterns differ in gastric cancer patients and in non-cancer controls. A heat map created by unsupervised hierarchical clustering of plasma levels for the eight most significantly differentially expressed microRNAs reveals perfect discrimination of disease categories. Each column is a different patient with either gastric cancer or no history of gastric cancer. Red color intensity corresponds to increased expression and green color intensity corresponds to decreased expression.

class of cancer that comprises 50% of all cases.⁴⁰ Hsa-miR-155 reportedly acts to downregulate toll-like receptors after either bacterial or viral infection,⁵¹ raising the question of whether this miR contributes to *Helicobacter pylori* or EBV pathogenicity in gastric tissue. Of note, both of these pathogens as well as hsa-miR-155 upregulation are implicated in B-lymphocyte proliferation and lymphomagenesis.⁵²

Among the seven microRNAs downregulated in our gastric cancer cohort compared with adjacent non-malignant gastric mucosa were hsa-miR-200a and hsa-miR-34a, which were previously shown to be downregulated in gastric cancer cell lines and tissues.^{53–56} However, hsa-miR-18a has been reported to be upregulated in gastric cancer, and hsa-miR-187, hsa-miR-484, and hsa-miR-744 are reportedly upregulated in sera of gastric cancer patients.^{20,21,23,57–60} Hsa-miR-423-3p was previously described as a normalizer.⁶

Eleven microRNAs were significantly dysregulated in our cohort of plasma samples. Hsa-miR-221 and hsa-miR-23a were significantly upregulated, whereas hsa-miR-200b and hsa-miR-141 were downregulated, consistent with reported literature.^{7,20,61–71} The miR-200 family, which includes miR-141 and miR-200b, are tumor suppressors implicated in epithelial to mesenchymal transition. They are known to be downregulated in association with EBV infection of epithelial

cells.⁷² Hsa-miR-378 reportedly acts as a tumor suppressor and was downregulated in gastric cancer cell lines and in our cohort of plasmas, but a separate study reported upregulation in gastric cancer patient plasma.^{21,73,74} Association of hsa-miR-103 with gastric cancer is inconsistent in the literature, and it was also reported as a normalizer.^{75,76} Hsa-miR-486-5p was reportedly upregulated in the sera of gastric cancer patients, whereas it was downregulated in our cohort of plasmas.^{19,23} Hsa-miR-346 has not been described in gastric cancer, although it is reportedly induced by EBV infection in B lymphocytes.⁷⁷

Only hsa-miR-21, -196a, and -484 were significantly dysregulated in both gastric cancer tissue and in patient plasma. Hsa-miR-21 was increased, whereas hsa-miR-484 was diminished in both cancer tissue and plasma; however, hsa-miR-21 was considered borderline significant for dysregulation in plasma, as it was impacted by choice of normalization strategy.

Surprisingly, the directionality of hsa-miR-196a was different in the two specimen types, with upregulation in cancer tissue but relatively low levels found in cancer patient plasma. A recent review⁷⁸ of published studies demonstrated that, out of 154 expression profiles reported, only 7% showed statistically significant dysregulation in the same direction in

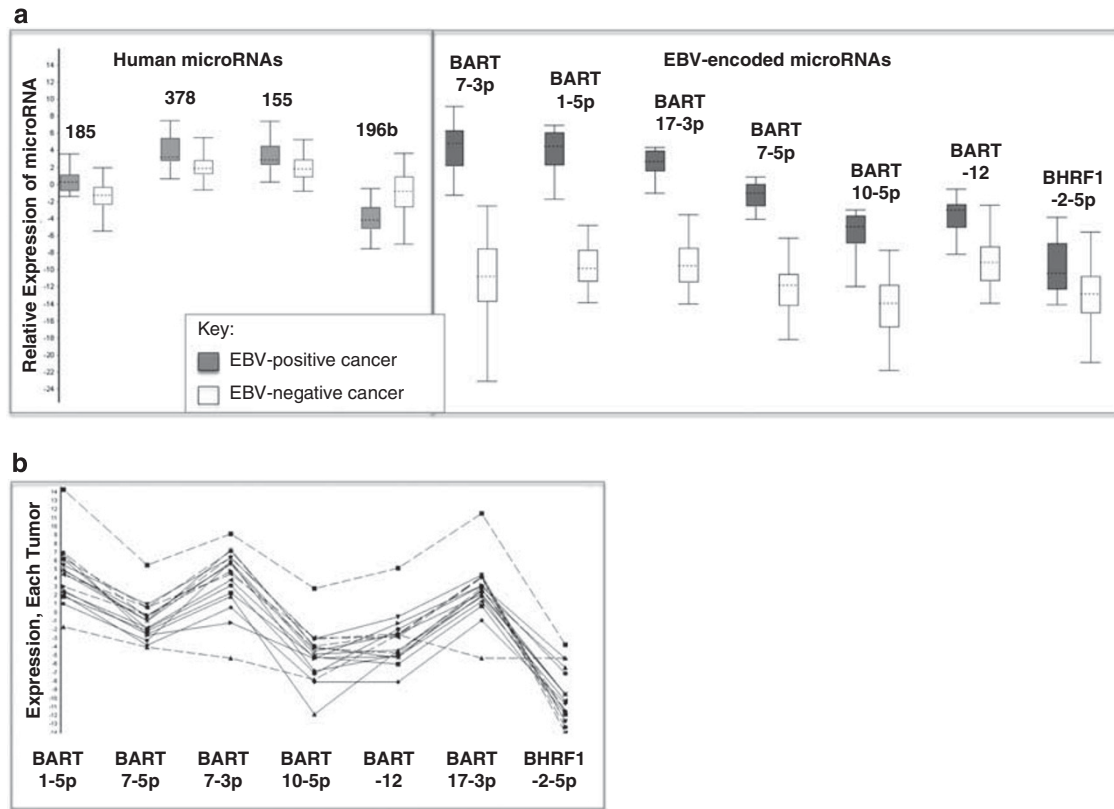


Figure 4 Differential expression of viral and associated human microRNAs suggests that Epstein-Barr virus (EBV) infected cancers are biologically distinct from uninfected cancers. **(a)** EBV-positive compared with EBV-negative cancer tissue has significant dysregulation of four human microRNAs ($P < 0.00125$). All seven EBV microRNAs that were examined were expressed at higher levels in EBV-positive compared to EBV-negative cancers (adjusted $P < 0.00568$). Note the wide dynamic range of expression for the viral microRNAs, signifying high levels of expression of several viral microRNAs in the tissues of infected cancer patients. Box plots represent the median (dotted line) and middle quartiles; whiskers are $1.5 \times$ interquartile range. **(b)** Patterns of viral microRNA expression were similar across the 17 infected gastric cancers.

both tissue and the circulation, whereas an additional 7% showed dysregulation in the opposite direction between these specimen types. The data challenge the notion that microRNA signatures in the circulation correspond to those of tumor tissue. Though the reasons for such discrepancies are not fully understood, possible explanations include selective release of microRNAs from cells, inflammatory and other systemic responses to disease, or differences in stability of a microRNA that could relate to whether it is protected inside exosomes. These discrepancies have implications for the development of clinical assays, reinforcing that each specimen type must be separately validated.

Hsa-miR-21 is one of the most thoroughly studied microRNAs and has been reported in numerous cancer types. Both hsa-miR-21 and -155 are associated with inflammation.⁷⁹ In our study, these two microRNAs were upregulated in cancer tissue only, not in plasma. Prior studies have confirmed the expression of hsa-miR-21 in gastric cancer, whereas hsa-miR-155 upregulation is associated with EBV-positive gastric cancers.^{18,40} None of the microRNAs we

report as dysregulated in plasma has been consistently associated with inflammation.^{79,80}

EBV-infected gastric cancer tissue had exceedingly high levels of most EBV microRNAs. Of the seven viral microRNAs tested, those most highly overexpressed in infected cancers were EBV-miR-BART 7-3p, 1-5p, and 17-3p. Even EBV-miR-BHRF 1-2-5p, not previously reported to be expressed in gastric cancer, was revealed to be differentially overexpressed in infected compared with uninfected cancer tissues, albeit at lower levels than the six microRNAs encoded from the BART region of the viral genome. The findings may indicate low-level viral replication in cancer tissues having viral *EBNA1* expression from the Cp or Wp promoters.⁸¹ Marquitz *et al*⁷² reported expression of the same group of viral microRNAs in infected epithelial cell lines and xenografts, although BART 10-5p expression appears to be low or absent in laboratory models. Our demonstration of similar viral expression profiles across all 17 infected cancer tissues suggest the potential to use viral microRNA expression patterns as a means of identifying infected cancers.

In addition to EBV-encoded microRNAs, we identified four human microRNAs that were differentially expressed in the EBV-infected subset of gastric cancer tissues. Hsa-miR-155, -185 and -378 were upregulated in infected cancer, whereas hsa-miR-196b was downregulated in infected cancer. All four of these microRNAs have been described as being dysregulated in gastric cancer tissue or plasma, and hsa-miR-155 is known to be induced by EBV.^{21,44,45,47,48,73,74,82,83} Hsa-miR-200a was previously reported to be downregulated by EBV,⁸⁴ and we observed this same trend, but this association did not reach significance following Bonferroni correction. Nevertheless, the unique pattern of human microRNA dysregulation seen in association with EBV infection suggests that the virus is not an innocent bystander when it is present within malignant cells.

Identification of infected cancers is important because of their favorable prognosis⁸⁵ and because of the potential for enrollment in clinical trials exploring virus-targeted therapy (NCT00982449 or NCT02080416 at clinicaltrials.gov). Anti-neoplastic strategies include (1) infused EBV-specific cytotoxic T cells,^{86–89} (2) reversing EBV-related hypermethylation,⁹⁰ (3) inciting the body's innate and adaptive antiviral immune responses,^{91–99} and (4) capitalizing on expression of a viral enzyme to convert a drug (such as gancyclovir) to its cytotoxic form.^{100–107} Laboratory support for such clinical trials requires assays to predict which candidates are likely to respond, and serial assays that reflect tumor burden or that assess biochemical effects on pertinent pathways. MicroRNA profiling shows promise for these purposes.

Cell-free microRNAs in plasma might serve as noninvasive indicators of disease status that could form the basis for cancer-screening programs. Proof of principle is already established for EBV DNA measurement as a harbinger of progression to EBV-driven post-transplant lymphoproliferations. Emerging data suggest that viral microRNA profiling might add value beyond EBV DNA viral load based on the spectrum of microRNA expression in benign versus malignant infections.¹⁰⁸ Furthermore, levels of viral microRNA appear to be much higher than viral DNA, potentially yielding improved sensitivity for low-level disease.

Review of the literature reveals marked variability in specimen type, study design, methods, and identification of candidate microRNAs. Accurate interpretation of the body of experimental microRNA data is further complicated by the fact that gastric adenocarcinoma is not one disease but rather is divided into histologic subtypes, with varying degree of cytologic and neuroendocrine differentiation and varying levels of inflammatory infiltrates. Though studies of single microRNAs have shown some promise for diagnosis and prognosis, the use of a panel of microRNAs will likely yield increased sensitivity and specificity. Our findings encourage further work to validate such a panel on patients with cancer and relevant comparator populations.

This study demonstrates the potential for microRNA profiling to distinguish cancer from normal tissue, and to subclassify cancer based on virus-encoded and associated human microRNA expression patterns. This study reinforces applicability of microRNA profiling to practical clinical specimens including small biopsies and plasma, thus highlighting its appeal for practice of laboratory medicine. Q-rtPCR profiling is facilitated by commercial availability of preloaded 96-well plates containing lyophilized primers that can be customized for the analytes of interest. Large panels of tests, including multiple controls, promote confidence in the findings. The strong association between EBV microRNA expression and known EBV status by clinical-grade assays verifies that the test system performs well. The protocols described herein reflect the type of technology that could support clinical trials and, once validated as adding value in patient management, could be implemented in routine patient care.

We verified a subset of the previously reported human microRNA associations. Varying analytic methods undoubtedly contribute to divergent data in the literature, which underscores the need to continue to gather evidence of analytic and clinical performance of specified microRNA test protocols in large groups of subjects.¹⁰⁹ In paraffin-embedded tissue, we envision that ancillary microRNA profiling could help resolve whether invasive cancer is present. This technology may add value in small biopsies or when the tissue is poorly oriented in a paraffin block. In plasma, future work should explore microRNA profiling as a means of monitoring tumor burden during therapy, or as a harbinger for initial diagnosis or for relapse.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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