View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Carolina Digital Repository

HHS Public Access

Author manuscript

Lab Invest. Author manuscript; available in PMC 2018 January 14.

Published in final edited form as:

Lab Invest. 2016 June ; 96(6): 661–671. doi:10.1038/labinvest.2016.33.

Gastric Adenocarcinoma MicroRNA Profiles in Fixed Tissue and in Plasma Reveal Cancer-Associated and Epstein-Barr Virus-Related Expression Patterns

Amanda L Treece, MD[1,7], **Daniel L Duncan, MD**[1,7], **Weihua Tang, MD, PhD**[1,7], **Sandra Elmore, MT(ASCP)**[1,7], **Douglas R Morgan, MD**[2,3,6,7], **Michael O Meyers, MD**[2,4,7], **Ricardo L Dominguez, MD**[5], **Olga Speck, MD, PhD**[1,7], and **Margaret L Gulley, MD**[1,2,7]

[1]Pathology and Laboratory Medicine

[2]Lineberger Comprehensive Cancer Center

[3]Gastroenterology

[4]Surgical Oncology

[5]Western Regional Hospital, Santa Rosa de Copan, Honduras

[6]Vanderbilt University, Nashville, TN

[7]University of North Carolina at Chapel Hill, Chapel Hill, NC

Abstract

Objective—MicroRNA expression in formalin fixed paraffin embedded tissue (FFPE) or plasma may add value for cancer management.

Methods—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.

Results—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.

Conclusion—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

Corresponding Author: Margaret L. Gulley, MD, Department of Pathology & Laboratory Medicine, Lineberger Comprehensive Cancer Center, University of North Carolina, 913 Brinkhous-Bullitt Building, Chapel Hill, NC 27599-7525, Telephone: (919) 843-4595, margaret_gulley@med.unc.edu.

Disclosure: The authors have no conflicts to disclose.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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 non-invasive addition to cancer diagnostics.

Keywords

Gastric adenocarcinoma; microRNA expression profile; Epstein-Barr virus; formalin fixed tissue; plasma; biomarker; Honduras

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 about 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 to 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 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

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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. Additionally, 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 μM 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 −20C until analysis.

Plasma was prepared by centrifugation (1500g for 10 minutes) of EDTA anticoagulated blood from gastric adenocarcinoma patients and non-carcinoma controls. A 200 μL 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, Massachusets). Eluate was treated with DNase, and cDNA was prepared and stored at −20C 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. (See 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, Woburn, MA) targeting 43 human microRNAs, one replicate, and 4 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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Prior to panel-based testing, cDNA prepared using the Exiqon Universal cDNA synthesis kit on 10 μL 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 EBER localization to malignant cells. Q-PCR of the *BamH1W* segment of the EBV genome served to quantify EBV DNA viral load in plasma and in fixed tissue as previously described.[3,26] EBV-encoded RNA (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 3 human microRNAs included in the 10-well test panel along with the 7 EBV microRNAs. The 7 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 due to low expression rates. After housekeeper microRNA normalization, 13 human microRNAs were significantly differentially expressed in tumor compared with non-malignant gastric mucosa ($p<0.00125$). (See Figure 1A.) Of those, 6 were upregulated and 7 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

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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. (See Table 2.) Because 11 of these were also identified as being dysregulated in disease (10 of them altered in tumor compared to benign, and one additional altered in EBV-positive compared to 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 (See 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 8 misclassified benign samples were macrodissected from the same slide as their adenocarcinoma counterparts, suggesting occult tumor involvement of benign-appearing mucosa, or else "field effect" whereby adjacent benign-appearing tissue has biochemical features of malignancy. The remaining 4 misclassified benign samples were chronic gastritis lesions 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 (2mm square) flanked by gastric mucosa. The other was a small gastric biopsy (4mm square) with approximately 50% adenocarcinoma cellularity. It is feasible that the surrounding mucosa in these 2 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 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, while 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 8 of these were dysregulated in cancer compared to non-cancer patients, these 8 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. (See Figure 1B.)

Unsupervised hierarchical clustering using the 8 most significant microRNAs showed excellent separation of tumor and non-tumor patients, with an accuracy of 100%. (See Figure 3.) Only two microRNAs, hsa-miR-196a and hsa-miR-484, were dysregulated in both cancer tissue and cancer patient plasma. While hsa-miR-484 was downregulated in both tissue and plasma, hsa-miR-196a was upregulated in cancer tissue and downregulated in

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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

20 gastric adenocarcinomas were EBV-positive as defined by localization of EBV to malignant cells using *EBER in situ* hybridization or by high EBV DNA viral load using a validated Q-PCR of tumor tissue. Expression of 7 EBV microRNAs was examined in 17 EBV-positive and 32 EBV-negative tumors for which sufficient tissue was available. All 7 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. (See 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 ($p<.00125$): hsa-miR-196b was downregulated while hsa-miR-155, hsa-miR-185 and hsa-miR-378 were upregulated in infected tumors. (See 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 6 microRNAs upregulated in our gastric cancer cohort compared to 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 to 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]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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 to normal mucosa, hsa-miR-155 and -185 are upregulated in the EBV-positive molecular class of cancer, while hsa-miR-185 is upregulated in the "chromosome instability" 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 7 microRNAs downregulated in our gastric cancer cohort compared to 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 while 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, while hsa-miR-484 was diminished in both cancer tissue and plasma; however, hsa-miR-21 was considered borderline significant for dysregulation in plasma since 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 both tissue and the circulation, while 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

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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 expression of hsa-miR-21 in gastric cancer, while 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-BHRF1-2-5p, not previously reported to be expressed in gastric cancer, was revealed to be differentially overexpressed in infected compared to 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* 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.[72] 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 4 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, while 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 prognosis[85] 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

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

assess biochemical effects on pertinent pathways. MicroRNA profiling shows promise for these purposes.

Cell-free microRNAs in plasma might serve as non-invasive 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 a 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 pre-loaded 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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Acknowledgments

The authors thank Kennichi Kakudo MD PhD and Takashi Ozaki MD for providing tissues. This study was sponsored by the University of North Carolina (UNC) Department of Pathology and Laboratory Medicine, the University Cancer Research Fund, UNC Clinical Translational Science Award (NIH U54 RR024383), an award for Innovative Technologies for Molecular Analysis of Cancer (NCI R21 CA155543), and the Western Honduras Gastric Cancer Initiative (NCI CA125588 and CA197773, HHS N261200800001).

ABBREVIATIONS

EBER	EBV-encoded RNA
EBV	Epstein Barr Virus
FFPE	Formalin-fixed paraffin-embedded
LNA	Locked nucleic acid
PCR	Polymerase chain reaction
RNA	Ribonucleic acid

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010; 127:2893–2917. [PubMed: 21351269]
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61:69–90. [PubMed: 21296855]
3. Tang W, Morgan DR, Meyers MO, et al. Epstein-barr virus infected gastric adenocarcinoma expresses latent and lytic viral transcripts and has a distinct human gene expression profile. Infect Agent Cancer. 2012; 7:21. [PubMed: 22929309]
4. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008; 105:10513–10518. [PubMed: 18663219]
5. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods. 2010; 50:298–301. [PubMed: 20146939]
6. Blondal T, Jensby Nielsen S, Baker A, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods. 2013; 59:S1–6. [PubMed: 23036329]
7. Cai H, Yuan Y, Hao YF, Guo TK, Wei X, Zhang YM. Plasma microRNAs serve as novel potential biomarkers for early detection of gastric cancer. Med Oncol. 2013; 30:452. [PubMed: 23307259]
8. Gorur A, Balci Fidanci S, Dogruer Unal N, et al. Determination of plasma microRNA for early detection of gastric cancer. Mol Biol Rep. 2013; 40:2091–2096. [PubMed: 23212612]
9. Kodahl AR, Lyng MB, Binder H, et al. Novel circulating microRNA signature as a potential non-invasive multi-marker test in ER-positive early-stage breast cancer: A case control study. Mol Oncol. 2014
10. Tang W, Hu Z, Muallem H, Gulley ML. Quality assurance of RNA expression profiling in clinical laboratories. J Mol Diagn. 2012; 14:1–11. [PubMed: 22020152]
11. Tsai KW, Liao YL, Wu CW, et al. Aberrant expression of miR-196a in gastric cancers and correlation with recurrence. Genes Chromosomes Cancer. 2012; 51:394–401. [PubMed: 22420029]
12. Lo SS, Hung PS, Chen JH, et al. Overexpression of miR-370 and downregulation of its novel target TGFbeta-RII contribute to the progression of gastric carcinoma. Oncogene. 2012; 31:226–237. [PubMed: 21666718]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

13. Oh HK, Tan AL, Das K, et al. Genomic Loss of miR-486 Regulates Tumor Progression and the OLFM4 Antiapoptotic Factor in Gastric Cancer. Clin Cancer Res. 2011; 17:2657–2667. [PubMed: 21415212]
14. Ueda T, Volinia S, Okumura H, et al. Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. Lancet Oncol. 2010; 11:136–146. [PubMed: 20022810]
15. Suzuki H, Yamamoto E, Nojima M, et al. Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis. 2010; 31:2066–2073. [PubMed: 20924086]
16. Yao Y, Suo AL, Li ZF, et al. MicroRNA profiling of human gastric cancer. Mol Med Report. 2009; 2:963–970.
17. Guo J, Miao Y, Xiao B, et al. Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues. J Gastroenterol Hepatol. 2009; 24:652–657. [PubMed: 19175831]
18. Zhang Z, Li Z, Gao C, et al. miR-21 plays a pivotal role in gastric cancer pathogenesis and progression. Lab Invest. 2008; 88:1358–1366. [PubMed: 18794849]
19. Zhu C, Ren C, Han J, et al. A five-microRNA panel in plasma was identified as potential biomarker for early detection of gastric cancer. Br J Cancer. 2014; 110:2291–2299. [PubMed: 24595006]
20. Song MY, Pan KF, Su HJ, et al. Identification of serum microRNAs as novel non-invasive biomarkers for early detection of gastric cancer. PLoS One. 2012; 7:e33608. [PubMed: 22432036]
21. Liu H, Zhu L, Liu B, et al. Genome-wide microRNA profiles identify miR-378 as a serum biomarker for early detection of gastric cancer. Cancer Lett. 2012; 316:196–203. [PubMed: 22169097]
22. Li BS, Zhao YL, Guo G, et al. Plasma microRNAs, miR-223, miR-21 and miR-218, as novel potential biomarkers for gastric cancer detection. PLoS One. 2012; 7:e41629. [PubMed: 22860003]
23. Konishi H, Ichikawa D, Komatsu S, et al. Detection of gastric cancer-associated microRNAs on microRNA microarray comparing pre- and post-operative plasma. Br J Cancer. 2012; 106:740–747. [PubMed: 22262318]
24. Tsujiura M, Ichikawa D, Komatsu S, et al. Circulating microRNAs in plasma of patients with gastric cancers. Br J Cancer. 2010; 102:1174–1179. [PubMed: 20234369]
25. Redshaw N, Wilkes T, Whale A, Cowen S, Huggett J, Foy CA. A comparison of miRNA isolation and RT-qPCR technologies and their effects on quantification accuracy and repeatability. Biotechniques. 2013; 54:155–164. [PubMed: 23477383]
26. Ryan JL, Fan H, Glaser SL, Schichman SA, Raab-Traub N, Gulley ML. Epstein-Barr virus quantitation by real-time PCR targeting multiple gene segments: a novel approach to screen for the virus in paraffin-embedded tissue and plasma. J Mol Diagn. 2004; 6:378–385. [PubMed: 15507678]
27. Ryan JL, Morgan DR, Dominguez RL, et al. High levels of Epstein-Barr virus DNA in latently infected gastric adenocarcinoma. Lab Invest. 2009; 89:80–90. [PubMed: 19002111]
28. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004; 64:5245–5250. [PubMed: 15289330]
29. Chai H, Brown RE. Field effect in cancer-an update. Ann Clin Lab Sci. 2009; 39:331–337. [PubMed: 19880759]
30. Meckes DG Jr, Shair KH, Marquitz AR, Kung CP, Edwards RH, Raab-Traub N. Human tumor virus utilizes exosomes for intercellular communication. Proc Natl Acad Sci U S A. 2010; 107:20370–20375. [PubMed: 21059916]
31. Meckes DG Jr, Raab-Traub N. Microvesicles and viral infection. J Virol. 2011; 85:12844–12854. [PubMed: 21976651]
32. Chan SH, Wu CW, Li AF, Chi CW, Lin WC. miR-21 microRNA expression in human gastric carcinomas and its clinical association. Anticancer Res. 2008; 28:907–911. [PubMed: 18507035]
33. Yang SM, Huang C, Li XF, Yu MZ, He Y, Li J. miR-21 confers cisplatin resistance in gastric cancer cells by regulating PTEN. Toxicology. 2013; 306:162–168. [PubMed: 23466500]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

34. Zheng Y, Cui L, Sun W, et al. MicroRNA-21 is a new marker of circulating tumor cells in gastric cancer patients. Cancer Biomark. 2011; 10:71–77. [PubMed: 22430134]
35. Xu Y, Sun J, Xu J, Li Q, Guo Y, Zhang Q. miR-21 Is a Promising Novel Biomarker for Lymph Node Metastasis in Patients with Gastric Cancer. Gastroenterol Res Pract. 2012; 2012:640168. [PubMed: 22792096]
36. Komatsu S, Ichikawa D, Tsujiura M, et al. Prognostic impact of circulating miR-21 in the plasma of patients with gastric carcinoma. Anticancer Res. 2013; 33:271–276. [PubMed: 23267156]
37. Zeng Z, Wang J, Zhao L, et al. Potential role of microRNA-21 in the diagnosis of gastric cancer: a meta-analysis. PLoS One. 2013; 8:e73278. [PubMed: 24023850]
38. Song J, Bai Z, Zhang J, et al. Serum microRNA-21 levels are related to tumor size in gastric cancer patients but cannot predict prognosis. Oncol Lett. 2013; 6:1733–1737. [PubMed: 24260069]
39. Ma GJ, Gu RM, Zhu M, et al. Plasma post-operative miR-21 expression in the prognosis of gastric cancers. Asian Pac J Cancer Prev. 2013; 14:7551–7554. [PubMed: 24460332]
40. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014; 513:202–209. [PubMed: 25079317]
41. Gulley ML. Genomic assays for Epstein-Barr virus-positive gastric adenocarcinoma. Experimental & molecular medicine. 2015; 47:e134. [PubMed: 25613731]
42. Sun M, Liu XH, Li JH, et al. MiR-196a is upregulated in gastric cancer and promotes cell proliferation by downregulating p27(kip1). Mol Cancer Ther. 2012; 11:842–852. [PubMed: 22343731]
43. Zheng G, Xiong Y, Xu W, et al. A two-microRNA signature as a potential biomarker for early gastric cancer. Oncol Lett. 2014; 7:679–684. [PubMed: 24527072]
44. Lim JY, Yoon SO, Seol SY, et al. Overexpression of miR-196b and HOXA10 characterize a poor-prognosis gastric cancer subtype. World J Gastroenterol. 2013; 19:7078–7088. [PubMed: 24222951]
45. Liao YL, Hu LY, Tsai KW, et al. Transcriptional regulation of miR-196b by ETS2 in gastric cancer cells. Carcinogenesis. 2012; 33:760–769. [PubMed: 22298639]
46. Rather MI, Nagashri MN, Swamy SS, Gopinath KS, Kumar A. Oncogenic microRNA-155 down-regulates tumor suppressor CDC73 and promotes oral squamous cell carcinoma cell proliferation: implications for cancer therapeutics. J Biol Chem. 2013; 288:608–618. [PubMed: 23166327]
47. Skalsky RL, Corcoran DL, Gottwein E, et al. The viral and cellular microRNA targetome in lymphoblastoid cell lines. PLoS Pathog. 2012; 8:e1002484. [PubMed: 22291592]
48. Tan Z, Jiang H, Wu Y, et al. miR-185 is an independent prognosis factor and suppresses tumor metastasis in gastric cancer. Mol Cell Biochem. 2014; 386:223–231. [PubMed: 24352663]
49. Ohshima K, Inoue K, Fujiwara A, et al. Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. PLoS One. 2010; 5:e13247. [PubMed: 20949044]
50. Liu K, Qian T, Tang L, Wang J, Yang H, Ren J. Decreased expression of microRNA let-7i and its association with chemotherapeutic response in human gastric cancer. World J Surg Oncol. 2012; 10:225. [PubMed: 23107361]
51. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A. 2007; 104:1604–1609. [PubMed: 17242365]
52. Belair C, Darfeuille F, Staedel C. Helicobacter pylori and gastric cancer: possible role of microRNAs in this intimate relationship. Clin Microbiol Infect. 2009; 15:806–812. [PubMed: 19702585]
53. Cao W, Yang W, Fan R, et al. miR-34a regulates cisplatin-induce gastric cancer cell death by modulating PI3K/AKT/survivin pathway. Tumour Biol. 2014; 35:1287–1295. [PubMed: 24068565]
54. Hao Q, Lu X, Liu N, et al. Posttranscriptional deregulation of Src due to aberrant miR34a and miR203 contributes to gastric cancer development. BMB Rep. 2013; 46:316–321. [PubMed: 23790975]
55. Cao W, Fan R, Wang L, et al. Expression and regulatory function of miRNA-34a in targeting survivin in gastric cancer cells. Tumour Biol. 2013; 34:963–971. [PubMed: 23264087]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

56. Cong N, Du P, Zhang A, et al. Downregulated microRNA-200a promotes EMT and tumor growth through the wnt/beta-catenin pathway by targeting the E-cadherin repressors ZEB1/ZEB2 in gastric adenocarcinoma. Oncol Rep. 2013; 29:1579–1587. [PubMed: 23381389]
57. Wu W, Takanashi M, Borjigin N, et al. MicroRNA-18a modulates STAT3 activity through negative regulation of PIAS3 during gastric adenocarcinogenesis. Br J Cancer. 2013; 108:653–661. [PubMed: 23322197]
58. Tsujiura M, Komatsu S, Ichikawa D, et al. Circulating miR-18a in plasma contributes to cancer detection and monitoring in patients with gastric cancer. Gastric Cancer. 2014
59. Zheng Y, Li S, Ding Y, et al. The role of miR-18a in gastric cancer angiogenesis. Hepatogastroenterology. 2013; 60:1809–1813. [PubMed: 24624454]
60. Wang JL, Hu Y, Kong X, et al. Candidate microRNA biomarkers in human gastric cancer: a systematic review and validation study. PLoS One. 2013; 8:e73683. [PubMed: 24040025]
61. Liu K, Li G, Fan C, Diao Y, Wu B, Li J. Increased Expression of MicroRNA-221 in gastric cancer and its clinical significance. J Int Med Res. 2012; 40:467–474. [PubMed: 22613407]
62. Chun-Zhi Z, Lei H, An-Ling Z, et al. MicroRNA-221 and microRNA-222 regulate gastric carcinoma cell proliferation and radioresistance by targeting PTEN. BMC Cancer. 2010; 10:367. [PubMed: 20618998]
63. Wang M, Zhao C, Shi H, et al. Deregulated microRNAs in gastric cancer tissue-derived mesenchymal stem cells: novel biomarkers and a mechanism for gastric cancer. Br J Cancer. 2014; 110:1199–1210. [PubMed: 24473397]
64. Zhu LH, Liu T, Tang H, et al. MicroRNA-23a promotes the growth of gastric adenocarcinoma cell line MGC803 and downregulates interleukin-6 receptor. FEBS J. 2010; 277:3726–3734. [PubMed: 20698883]
65. Liu X, Ru J, Zhang J, et al. miR-23a targets interferon regulatory factor 1 and modulates cellular proliferation and paclitaxel-induced apoptosis in gastric adenocarcinoma cells. PLoS One. 2013; 8:e64707. [PubMed: 23785404]
66. An J, Pan Y, Yan Z, et al. MiR-23a in amplified 19p13.13 loci targets metallothionein 2A and promotes growth in gastric cancer cells. J Cell Biochem. 2013; 114:2160–2169. [PubMed: 23553990]
67. Tang H, Kong Y, Guo J, et al. Diallyl disulfide suppresses proliferation and induces apoptosis in human gastric cancer through Wnt-1 signaling pathway by up-regulation of miR-200b and miR-22. Cancer Lett. 2013; 340:72–81. [PubMed: 23851184]
68. Kurashige J, Kamohara H, Watanabe M, et al. MicroRNA-200b regulates cell proliferation, invasion, and migration by directly targeting ZEB2 in gastric carcinoma. Ann Surg Oncol. 2012; 19(Suppl 3):S656–664. [PubMed: 22311119]
69. Song F, Yang D, Liu B, et al. Integrated microRNA network analyses identify a poor-prognosis subtype of gastric cancer characterized by the miR-200 family. Clin Cancer Res. 2014; 20:878–889. [PubMed: 24352645]
70. Zhou X, Su J, Zhu L, Zhang G. Helicobacter pylori Modulates Cisplatin Sensitivity in Gastric Cancer by Down-Regulating miR-141 Expression. Helicobacter. 2014; 19:174–181. [PubMed: 24628843]
71. Du Y, Xu Y, Ding L, et al. Down-regulation of miR-141 in gastric cancer and its involvement in cell growth. J Gastroenterol. 2009; 44:556–561. [PubMed: 19363643]
72. Marquitz AR, Mathur A, Chugh PE, Dittmer DP, Raab-Traub N. Expression profile of microRNAs in Epstein-Barr virus-infected AGS gastric carcinoma cells. J Virol. 2014; 88:1389–1393. [PubMed: 24227849]
73. Deng H, Guo Y, Song H, et al. MicroRNA-195 and microRNA-378 mediate tumor growth suppression by epigenetical regulation in gastric cancer. Gene. 2013; 518:351–359. [PubMed: 23333942]
74. Fei B, Wu H. MiR-378 inhibits progression of human gastric cancer MGC-803 cells by targeting MAPK1 in vitro. Oncol Res. 2012; 20:557–564. [PubMed: 24139413]
75. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA. 2008; 14:844–852. [PubMed: 18375788]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

76. Shrestha S, Hsu SD, Huang WY, et al. A systematic review of microRNA expression profiling studies in human gastric cancer. Cancer Med. 2014
77. Lu F, Weidmer A, Liu CG, Volinia S, Croce CM, Lieberman PM. Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. J Virol. 2008; 82:10436–10443. [PubMed: 18753206]
78. Jarry J, Schadendorf D, Greenwood C, Spatz A, van Kempen LC. The validity of circulating microRNAs in oncology: five years of challenges and contradictions. Mol Oncol. 2014; 8:819–829. [PubMed: 24656978]
79. Olivieri F, Rippo MR, Procopio AD, Fazioli F. Circulating inflamma-miRs in aging and age-related diseases. Frontiers in genetics. 2013; 4:121. [PubMed: 23805154]
80. Ranjha R, Paul J. Micro-RNAs in inflammatory diseases and as a link between inflammation and cancer. Inflammation research : official journal of the European Histamine Research Society... [et al.]. 2013; 62:343–355.
81. Amoroso R, Fitzsimmons L, Thomas WA, Kelly GL, Rowe M, Bell AI. Quantitative studies of Epstein-Barr virus-encoded microRNAs provide novel insights into their regulation. J Virol. 2011; 85:996–1010. [PubMed: 21068248]
82. Kim BH, Hong SW, Kim A, Choi SH, Yoon SO. Prognostic implications for high expression of oncogenic microRNAs in advanced gastric carcinoma. J Surg Oncol. 2013; 107:505–510. [PubMed: 22996433]
83. Yu BQ, Su LP, Li JF, et al. microrna expression signature of gastric cancer cells relative to normal gastric mucosa. Mol Med Rep. 2012; 6:821–826. [PubMed: 22842726]
84. Shinozaki A, Sakatani T, Ushiku T, et al. Downregulation of microRNA-200 in EBV-associated gastric carcinoma. Cancer Res. 2010; 70:4719–4727. [PubMed: 20484038]
85. Song HJ, Srivastava A, Lee J, et al. Host inflammatory response predicts survival of patients with Epstein-Barr virus-associated gastric carcinoma. Gastroenterology. 2010; 139:84–92. e82. [PubMed: 20398662]
86. Merlo A, Turrini R, Dolcetti R, Zanovello P, Rosato A. Immunotherapy for EBV-associated malignancies. Int J Hematol. 2011; 93:281–293. [PubMed: 21336546]
87. Louis CU, Straathof K, Bollard CM, et al. Adoptive transfer of EBV-specific T cells results in sustained clinical responses in patients with locoregional nasopharyngeal carcinoma. J Immunother. 2010; 33:983–990. [PubMed: 20948438]
88. Kim YJ, Lim J, Kang JS, et al. Adoptive immunotherapy of human gastric cancer with ex vivo expanded T cells. Arch Pharm Res. 2010; 33:1789–1795. [PubMed: 21116782]
89. Lundqvist A, Berg M, Smith A, Childs RW. Bortezomib Treatment to Potentiate the Anti-tumor Immunity of Ex-vivo Expanded Adoptively Infused Autologous Natural Killer Cells. J Cancer. 2011; 2:383–385. [PubMed: 21750690]
90. Fukayama M, Ushiku T. Epstein-Barr virus-associated gastric carcinoma. Pathol Res Pract. 2011; 207:529–537. [PubMed: 21944426]
91. Claerhout S, Lim JY, Choi W, et al. Gene expression signature analysis identifies vorinostat as a candidate therapy for gastric cancer. PLoS One. 2011; 6:e24662. [PubMed: 21931799]
92. Daigle D, Megyola C, El-Guindy A, et al. Upregulation of STAT3 marks Burkitt lymphoma cells refractory to Epstein-Barr virus lytic cycle induction by HDAC inhibitors. J Virol. 2010; 84:993–1004. [PubMed: 19889776]
93. Destro F, Sforza F, Sicurella M, et al. Proteasome inhibitors induce the presentation of an Epstein-Barr virus nuclear antigen 1-derived cytotoxic T lymphocyte epitope in Burkitt's lymphoma cells. Immunology. 2011; 133:105–114. [PubMed: 21342184]
94. Shirley CM, Chen J, Shamay M, et al. Bortezomib induction of C/EBPbeta mediates Epstein-Barr virus lytic activation in Burkitt lymphoma. Blood. 2011; 117:6297–6303. [PubMed: 21447826]
95. Kenney S, Theodore E. Woodward Award: Development of Novel, EBV-Targeted Therapies for EBV-Positive Tumors. Trans Am Clin Climatol Assoc. 2006; 117:55–74. [PubMed: 18528464]
96. Schwarzmann F, Jager M, Prang N, Wolf H. The control of lytic replication of Epstein-Barr virus in B lymphocytes (Review). Int J Mol Med. 1998; 1:137–142. [PubMed: 9852211]
97. Laichalk LL, Thorley-Lawson DA. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. J Virol. 2005; 79:1296–1307. [PubMed: 15613356]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

98. Feng WH, Kenney SC. Valproic acid enhances the efficacy of chemotherapy in EBV-positive tumors by increasing lytic viral gene expression. Cancer Res. 2006; 66:8762–8769. [PubMed: 16951192]

99. Hui KF, Chiang AK. Suberoylanilide hydroxamic acid induces viral lytic cycle in Epstein-Barr virus-positive epithelial malignancies and mediates enhanced cell death. Int J Cancer. 2010; 126:2479–2489. [PubMed: 19816947]

100. Feng WH, Israel B, Raab-Traub N, Busson P, Kenney SC. Chemotherapy induces lytic EBV replication and confers ganciclovir susceptibility to EBV-positive epithelial cell tumors. Cancer Res. 2002; 62:1920–1926. [PubMed: 11912175]

101. Feng WH, Westphal E, Mauser A, et al. Use of adenovirus vectors expressing Epstein-Barr virus (EBV) immediate-early protein BZLF1 or BRLF1 to treat EBV-positive tumors. J Virol. 2002; 76:10951–10959. [PubMed: 12368338]

102. Feng WH, Cohen JI, Fischer S, et al. Reactivation of latent Epstein-Barr virus by methotrexate: a potential contributor to methotrexate-associated lymphomas. J Natl Cancer Inst. 2004; 96:1691–1702. [PubMed: 15547182]

103. Feng WH, Hong G, Delecluse HJ, Kenney SC. Lytic induction therapy for Epstein-Barr virus-positive B-cell lymphomas. J Virol. 2004; 78:1893–1902. [PubMed: 14747554]

104. Jones K, Nourse J, Corbett G, Gandhi MK. Sodium valproate in combination with ganciclovir induces lysis of EBV-infected lymphoma cells without impairing EBV-specific T-cell immunity. Int J Lab Hematol. 2010; 32:e169–174. [PubMed: 19196381]

105. Westphal EM, Blackstock W, Feng W, Israel B, Kenney SC. Activation of lytic Epstein-Barr virus (EBV) infection by radiation and sodium butyrate in vitro and in vivo: a potential method for treating EBV-positive malignancies. Cancer Res. 2000; 60:5781–5788. [PubMed: 11059774]

106. Zhao J, Jin H, Cheung KF, et al. Zinc finger E-box binding factor 1 plays a central role in regulating Epstein-Barr virus (EBV) latent-lytic switch and acts as a therapeutic target in EBV-associated gastric cancer. Cancer. 2012; 118:924–936. [PubMed: 21717425]

107. Meng Q, Hagemeier SR, Fingeroth JD, Gershburg E, Pagano JS, Kenney SC. The Epstein-Barr virus (EBV)-encoded protein kinase, EBV-PK, but not the thymidine kinase (EBV-TK), is required for ganciclovir and acyclovir inhibition of lytic viral production. J Virol. 2010; 84:4534–4542. [PubMed: 20181711]

108. Lung RW, Tong JH, To KF. Emerging roles of small Epstein-Barr virus derived non-coding RNAs in epithelial malignancy. Int J Mol Sci. 2013; 14:17378–17409. [PubMed: 23979421]

109. Sun Y, Zhang K, Fan G, Li J. Identification of circulating microRNAs as biomarkers in cancers: what have we got? Clin Chem Lab Med. 2012; 50:2121–2126. [PubMed: 23087086]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

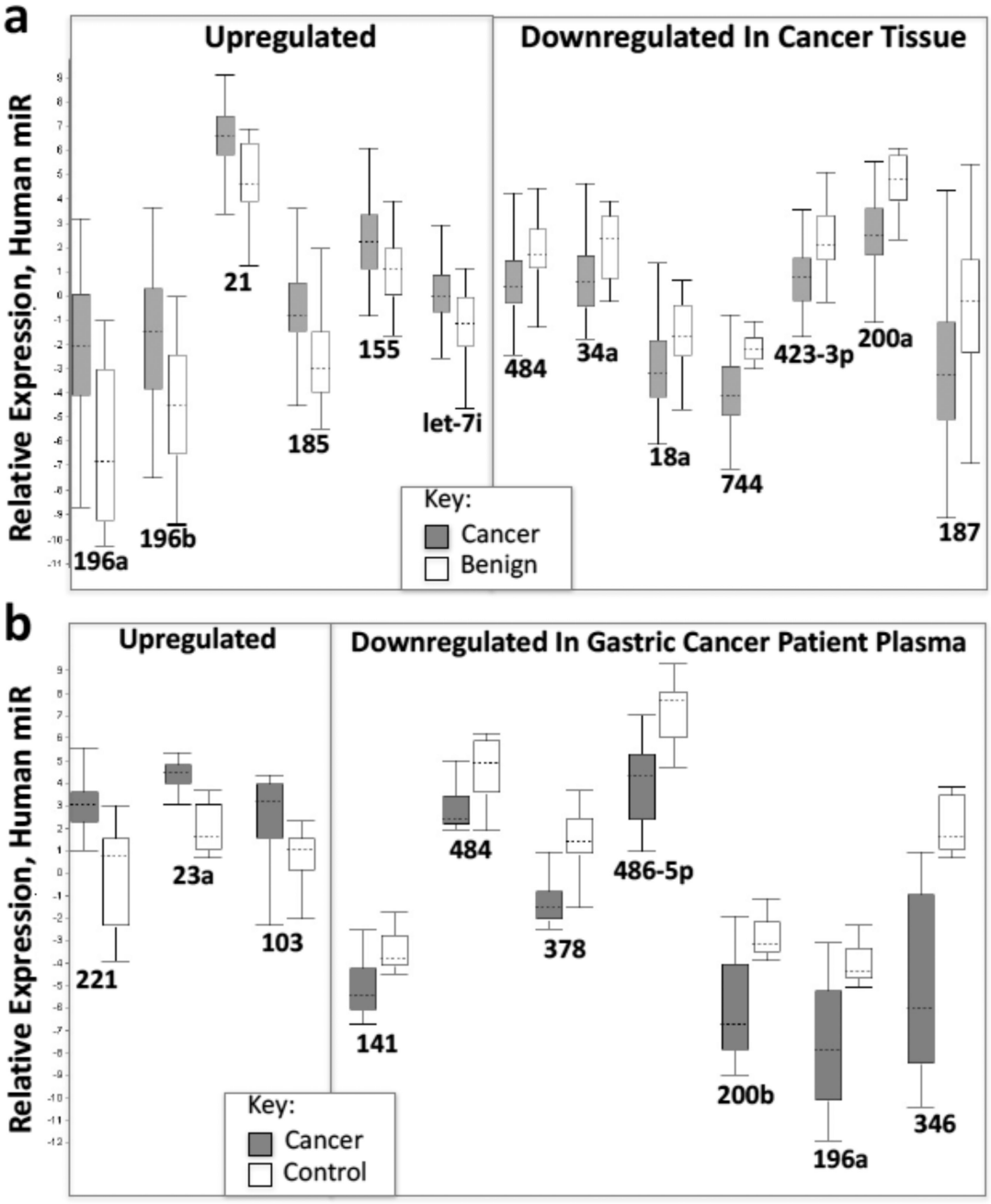

Figure 1.

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, ten 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*interquartile range.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

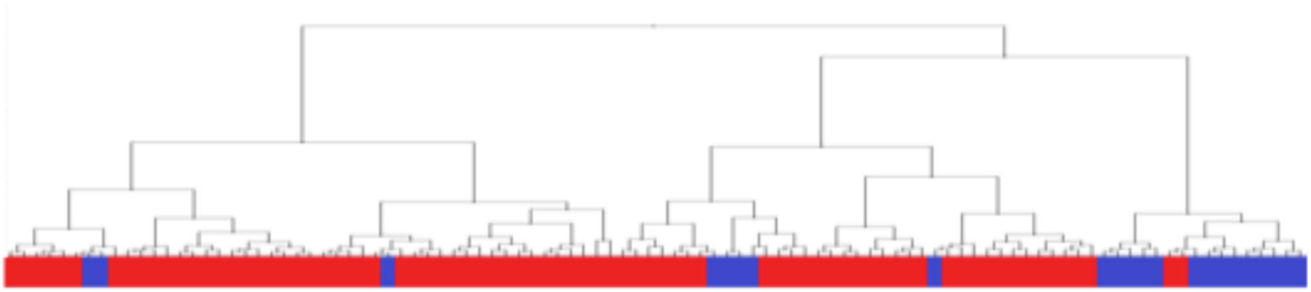

Figure 2.

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.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

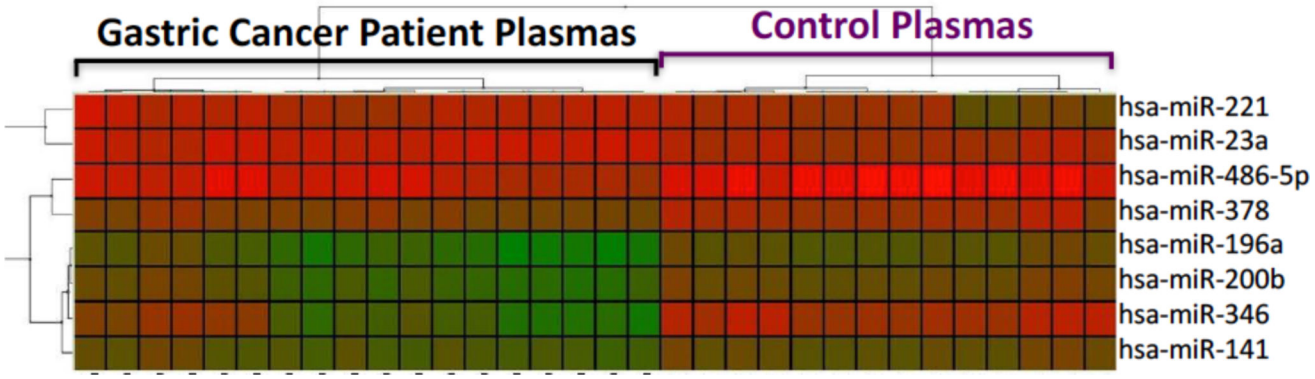

Figure 3.

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.

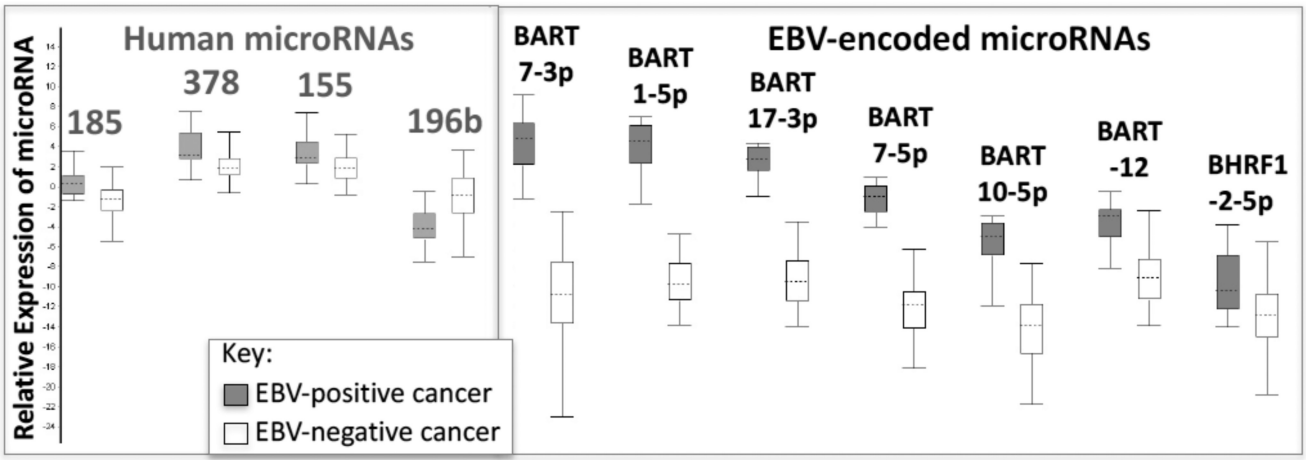

Figure 4.

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 circulation of infected cancer patients. Box plots represent the median (dotted line) and middle quartiles; whiskers are 1.5*interquartile range. (B) Patterns of viral microRNA expression were similar across the 17 infected gastric cancers.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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-7i	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	6
		Cel-miR-39-3p negative control	

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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[1]	Medium	hsa-miR-191	Medium
hsa-miR-423-3p[1]	Medium	hsa-miR-106b	Low
hsa-miR-25	Medium	hsa-miR-141[1]	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[1]	High
hsa-miR-484[1]	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[1]	Low	hsa-miR-196b	Medium
hsa-miR-20a	Medium	hsa-miR-378[1]	Medium
hsa-miR-103	Medium	hsa-miR-34a	Low
hsa-miR-18a[1]	Medium	hsa-miR-16	High
hsa-miR-370	Low	hsa-miR-23a[1]	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[1]	Medium	hsa-miR-200a	Low
hsa-miR-106b	Low	hsa-miR-486-5p[1]	High
hsa-miR-21[1]	High	hsa-miR-21[1]	Medium
hsa-miR-185[1]	Medium	hsa-let-7e	Medium
hsa-miR-106a	Medium	hsa-miR-27a	Medium
hsa-miR-17	Low	hsa-miR-744	Medium
hsa-miR-34a[1]	Medium	hsa-miR-376c	Medium
hsa-miR-155[1]	Medium	hsa-miR-103[1]	Medium
hsa-miR-196b[1]	Medium	hsa-miR-200b[1]	Low
		hsa-miR-27b	Medium

[1] microRNAs identified as nomalizers and also associated with disease status in the corresponding sample type