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Gastric adenocarcinoma has a unique microRNA signature not present in esophageal adenocarcinoma

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Abstract

Background—MicroRNAs (miRNAs) play critical roles in tumor development and progression. The fact that a single miRNA can regulate hundreds of genes places miRNAs at critical hubs of signaling pathways. In this study, we investigated the miRNA expression profile in gastric adenocarcinomas and compared it to esophageal adenocarcinomas to better identify a unique miRNA signature of gastric adenocarcinoma.

Methods and Results—The miRNA expression profile was obtained using Agilent and Exiqon microarray platforms on primary gastric adenocarcinoma tissue samples. The cross comparison of results identified 17 up-regulated and 12 down-regulated miRNAs that overlapped in both platforms. Quantitative real-time RT-PCR was performed for independent validation of a representative set of 8 miRNAs in gastric and esophageal adenocarcinomas as compared to normal gastric mucosa or esophageal mucosa, respectively. The de-regulation of miR-146b-5p, -375, -148a, -31, and -451 was significantly associated with gastric adenocarcinomas. On the other hand, de-regulation of miR-21 (up-regulation) and miR-133b (down-regulation) was detectable in both gastric and esophageal adenocarcinomas. Interestingly, miR-200a was significantly down-regulated in gastric adenocarcinoma ($p=0.04$) but up-regulated in esophageal adenocarcinoma samples ($p=0.001$). In addition, the expression level of miR-146b-5p displayed a strong correlation with the tumor staging of gastric cancer.

Conclusion—Gastric adenocarcinoma displays a unique miRNA signature that distinguishes it from esophageal adenocarcinoma. This specific signature could reflect differences in the etiology and/or molecular signaling in these two closely related cancers. Our findings suggest important

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miRNA candidates that can be investigated for their molecular functions and possible diagnostic, prognostic, and therapeutic role in gastric adenocarcinoma.

Keywords

miRNA; esophageal adenocarcinoma; gastric adenocarcinoma; microarray; prognosis

Introduction

Gastric adenocarcinoma remains a major health problem and is the second largest cause of cancer deaths worldwide[1, 2]. Moreover, the prognosis for gastric adenocarcinoma patients remains poor, especially in advanced stages[3]. Approximately 80% of patients in the United States present with regional or distant metastases and are poorly responsive to therapy, with an unfavorable outcome. Infection with *Helicobacter pylori*, classified as a class 1 carcinogen by the World Health Organization[4], has been strongly correlated with gastric tumorigenesis through a pathway involving atrophic gastritis and intestinal metaplasia[5]. Over the past few decades, there has been a change in trends of gastric adenocarcinoma in the USA and in Northern and Western Europe. While a significant decline in the incidence of distal adenocarcinomas has been noted in these regions, there has been a surprising increase in the incidence involving the gastric cardia, gastro-esophageal junction, and distal esophagus[6]. Both gastric and esophageal adenocarcinomas are characterized by poor response to chemotherapeutics with a 5-year survival below 20%[7, 8]. This poor clinical outcome can be attributed to lack of effective strategies for early detection, weak prognostic value of histological indicators, limited effect of surgery or cytotoxic treatment in advanced disease, and lack of molecular markers utilized for targeted therapy[7, 9]. Although gastric and esophageal adenocarcinomas share some common biological behaviors, they display distinct risk factors, molecular mechanisms and histological types[10–13]. Therefore, it is necessary to have a better understanding of the unique molecular signature of gastric adenocarcinoma as a critical step towards understanding the biology and improving our currently limited management approaches[14].

microRNAs (miRNAs), short non-coding RNAs, are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression or target degradation and gene silencing[15]. miRNAs have emerged in the past few years as significant regulators of cellular activities, with a significant implication in cancer development and progression[16]. De-regulation of miRNAs has been identified in cancers from different organs with different histological types[17, 18]. The expression pattern of miRNAs in tumors could provide useful information about molecular biology and signaling pathways in cancer[19, 20]. miRNAs have been suggested as novel powerful diagnostic, prognostic and possibly therapeutic tools in cancer[21, 22]. In the present study, we detected the gastric cancer-related miRNAs and identified differences in the microRNA expression patterns between gastric and esophageal adenocarcinomas.

Material and methods

MicroRNA microarray analysis

For microarray analysis, total enriched RNA was purified from six tissue samples that included three advanced gastric adenocarcinomas (stage III–IV) and three non-tumor histologically normal tissue samples using miRNeasy kit (Qiagen). All the samples were processed and hybridized on two independent microarray platforms, Exiqon miRCURY LNA™ microRNA Array, v.11.0 – human, that contains 1434 unique miRNAs on the chip (Exiqon Life Science, Woburn, MA) and Agilent Human microRNA Microarray V2 that represents 961 unique records with 723 human microRNAs (Agilent Technology, Santa

Clara, CA), following the manufacturer's protocol. To minimize variations, the normal samples were pooled into a single normal reference sample that was used for all hybridizations. This approach was considered to minimize technical variations and to allow us to focus on the most reproducible changes that are cross-validated between the two platforms. Preliminary analysis of the Agilent miRNA microarray data was performed using the Agilent Feature Extraction (AFE) program to obtain the miRNA expression signal status. Subsequent analysis was carried out by using R language (http://www.r-project.org). 136–230 miRNAs were detected per sample. Following data transformation, inter-array normalization was performed using a quantile normalization method. Data were log2 transformed and the log2 ratio for each miRNA was computed by subtracting the log2 value in the pooled control sample from the average log2 values in three cancer samples. We employed the limma R package to process the Exiqon miRNA expression data. The following steps were executed sequentially: (1) background correction, (2) intra-array normalization using the loess method, (3) for replicate probes, the median value was calculated to represent the miR intensity value, and (4) quantile normalization to allow comparison among arrays. To identify the differentially expressed miRNAs between the cancer and normal groups, we then performed a two-class un-paired comparison, and the p values were adjusted using the false discovery rate (FDR) method[23]. The miRNAs that were detected by both platforms to exhibit differential expression between normal and cancer tissues were subject to subsequent validation by qRT-PCR.

Quantitative Real-time PCR (qRT-PCR)

A total of 145 tissue samples (33 gastric adenocarcinomas, 37 esophageal adenocarcinomas, 47 normal gastric, 28 normal esophagus) were collected from the NCI Cooperative Human Tissue Network and the archives of pathology at Vanderbilt University Medical Center. All tissue samples were collected, coded and de-identified in accordance with the Vanderbilt University Institutional Review Board's approved protocols. Total RNA was isolated using the miRNeasy kit (Qiagen, Valencia, CA), and single-stranded cDNA was subsequently synthesized. cDNA was synthesized using a 3-step protocol that includes poly(A) tail synthesis using 2 ug RNA in the presence of 1.5 units of poly(A) polymerase, 10X poly(A) buffer, and 10X ATP in a reaction volume of 15 ul incubated at 37°C for 30 minutes, followed by the annealing of a universal poly(dT)-adaptor at 60°C for 5 minutes. Reverse transcription was carried out by means of iScript kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Primers for qRT-PCR were designed by means of the online database miRbase (http://www.miRbase.org/); Table 1 lists the primers' sequences. qRT-PCR was carried out using the CFX Connect Real-Time System (Bio-Rad, Hercules, CA). The reactions were carried out in a 96-well plate following a thermal protocol that includes the initial incubation at 95°C for 3 minutes followed by 40 cycles of a 2-step annealing at 95°C for 10 seconds and 60°C for 30 seconds. Normalization was made to two reference miRs, miR-191 and miR-103a-5p[24]. Fold change was calculated based on the formula $2^{(Rt-Et)}/2^{(Rn-En)}$, where Rt is the threshold cycle number of the reference gene in the tumor sample, Et is the threshold cycle number of the experimental gene in the tumor sample, Rn is the threshold cycle number for the reference gene in the normal sample, and En is the threshold cycle number of the experimental gene in the normal sample[25]. A heatmap representing the relative fold changes of the tumor and normal tissue was constructed using Treeview® software. Student's t-test was used for the evaluation of statistical significance with a p value cutoff set at ≤0.05.

Results

De-regulation of miRNAs in gastric adenocarcinoma

We identified 17 up-regulated and 12 down-regulated miRNAs that were consistently de-regulated in both microarray platforms (Figure 1). The details of these miRNAs are shown in Table 2. Eight miRNAs (up-regulated; miR-146b-5p and -21, and down-regulated; miR-375, -133b, -148a, -31, -200a, and -451) were selected for validation using qRT-PCR, based on their expression level (more than two-fold change) and known cancer-related molecular functions such as regulating cancer cell proliferation, apoptosis, and invasion. We confirmed that these miRNAs were significantly de-regulated in gastric adenocarcinoma samples, as compared to normal gastric mucosae. Our next step was to find out if any of these miRNAs is also de-regulated in the closely located esophageal adenocarcinomas. Analyses of these miRNAs in esophageal adenocarcinoma samples indicated that miRNA-21 and miRNA-133b were similarly de-regulated in both gastric and esophageal adenocarcinoma (Figure 3). On the other hand, 5 of the 8 miRNAs were not significantly altered in esophageal adenocarcinomas. In addition, miR-200a, which was down-regulated in gastric adenocarcinomas, was up-regulated in esophageal adenocarcinomas (Figure 4). These data suggest the presence of unique differences between gastric and esophageal adenocarcinomas. Linear regression analysis of miRNA alterations indicated that the miR-146b-5p expression level showed strong correlation with gastric adenocarcinoma stage classification (Figure 5, r^2=0.46).

Discussion

miRNAs are non-coding RNA molecules widely expressed in human tissues with significant power to regulate several biological activities[26]. In this context, miRNAs provide a way to explore the complicated mechanisms within the disease status, including cancer. Given the fact that one miRNA could regulate hundreds of mRNA with the similar function, it has evolved as a powerful target for understanding the biology of cancer[15]. Studies have shown that miRNA expression levels can have a diagnostic and/or prognostic value for cancer[27, 28]. Gastric and esophageal adenocarcinomas share several common features, including close proximity in the anatomical position, histology types, and poor prognosis in the late stages[29, 30]. However, they also have some distinguishing features such as etiology, risk factors, and molecular biological mechanisms[31, 32]. In this study, we investigated the miRNA signature of gastric adenocarcinoma and examined whether there are miRNAs that are uniquely expressed in gastric adenocarcinoma but not in esophageal adenocarcinoma. The differences between the two cancers could provide a novel insight into understanding the mechanisms within the development and progression of these cancers.

miR-191 and miR-103a-5p were found to be stably expressed and highly consistent in human normal and tumor tissues. These two miRNAs were statistically superior to the most commonly used reference RNAs in miRNA qRT-PCR experiments, such as 5S rRNA, U6 snRNA, or total RNA[24]. In this study, miR-191 and miR-103a-5p were used for the normalization controls. Our finding that miR-21 is up-regulated in both gastric and esophageal adenocarcinomas is in agreement with several recent findings that have documented overexpression of miR-21 in several malignancies such as lung cancer, lymphoma, hepatocellular cancer, and colon cancer[33–36]. Recent studies have shown several oncogenic functions for miR-21 demonstrating its role in inhibiting apoptosis by suppressing the inhibitors of Ras/MEK/ERK[36]. It has also been shown that miR-21 overexpression mediates activation of NF-kB signaling, providing a link between inflammation and cancer[33, 37]. These findings suggest that miR-21 is a common oncomiR in human malignancies. Similar to miR-21, we detected down-regulation of miR-133b in both gastric and esophageal adenocarcinomas. Earlier studies have shown that miR-133b is down-

regulated in esophageal squamous cell carcinoma and bladder cancer[38, 39]. miR-133b regulates colorectal cancer cell proliferation and apoptosis by targeting the receptor tyrosine kinase MET signaling pathway[40]. It can also directly target the pro-survival gene MCL-1, thus regulating cell survival and sensitivity of lung cancer cells to chemotherapeutic agents[41]. Our results, together with the reported studies, suggest that the de-regulation of miR-21 and miR-133b regulate important pathways that control tumorigenicity, irrespective of organ type.

Through our approach, we were able to identify and validate miRNAs that were uniquely de-regulated in gastric but not esophageal adenocarcinomas. miR-146b-5p was among the miRNAs that were uniquely overexpressed in gastric but not esophageal adenocarcinomas. We found that the expression level of miR-146b-5p had strong correlation with the TNM stages in gastric adenocarcinoma. A similar finding was observed in lung cancer where miR-146b overexpression was predictive of patients' outcome[42]. miR-146b-5p is also up-regulated from non-neoplastic tissue to dysplasia in inflammatory bowel disease patients with associated colorectal cancer[43]. At the functional level, a recent report has confirmed the direct binding of miR-146b-5p on the SMAD4 3'UTR. The overexpression of miR-146b-5p decreases the SMAD4 levels and disrupts TGF-beta signal transduction suggesting an oncogenic role of miR-146b-5p in thyroid follicular cells[44]. Taken together, these data suggest that miR-146b expression, although tissue specific, could play a role in tumor progression. However, whether miR-146b targets the same pathways in gastric cancer as identified in other cancers, remains to be investigated.

Our results demonstrated down-regulation of miR-375, -148a, -31, and -451 in gastric but not esophageal adenocarcinomas. The down-regulation of miR-375 is consistent with previous reports that have shown its role in targeting the JAK2 oncogene to suppress gastric cancer cell proliferation[45]. Expression of miR-375 in gastric adenocarcinoma inhibits expression of PDK1, which is a direct target of miR-375, followed by suppression of AKT phosphorylation. These findings explain miR-375 as a tumor suppressor in gastric adenocarcinoma[46]. We have also shown down-regulation of miR-148a in gastric adenocarcinoma. Earlier reports have shown that miR-148a suppresses tumor cell invasion and metastasis by down-regulating Rho-associated coiled-coil containing protein kinase 1 (ROCK1). Down-regulated miR-148a was also significantly associated with tumor-node-metastasis (TNM) stage and lymph node-metastasis[47]. miR-148a also promotes apoptosis by targeting BCL-2 in colorectal cancer and induces apoptosis[48]. Our results further support the role of miR-148a in tumorigenesis. We also detected down-regulation of miR-31 in gastric cancer. Our finding is consistent with another study showing that the expression levels of miR-31 in gastric cancer tissues were significantly lower than those in non-tumor tissues[49]. miR-31 expression correlates inversely with metastasis in breast cancer[50]. Silencing of miR-31 is also implicated in the aberrant activation of NF-kappaB signaling in tumors[51]. Loss of miR-31 has been associated with defects in the p53 pathway and functions in serous ovarian cancer and other cancers[52]. Whether down-regulation of miR-31 in gastric cancer is associated with these functions or different targets remains to be investigated. miR-451 is down-regulated in non-small cell lung carcinoma (NSCLC) and is also associated with shorter overall survival of NSCLC patients[53]. Ectopic miR-451 expression suppresses the *in vitro* proliferation and colony formation of NSCLC cells and the development of tumors in nude mice by enhancing apoptosis, which may be associated with the inactivation of the AKT signal pathway[53]. miR-451 is also involved in the self-renewal, tumorigenicity and chemo resistance of colorectal cancer stem cells[54]. Down-regulation of miR-451 induces the expression of cyclooxygenase-2 (COX-2) and activates the Wnt pathway, which is essential for cancer stem cell growth[54]. The fact that we only detected significant down-regulation of miR-375, -148a, -31, and -451 in gastric adenocarcinoma tissues, but not in esophageal

adenocarcinomas may indicate the presence of distinct molecular mechanisms driving the development and progression of these two types of cancers.

One of our most interesting findings is related to miR-200a that was down-regulated in gastric but up-regulated in esophageal adenocarcinoma. miR-200a has been shown to be overexpressed or down-regulated in different cancer types[55, 56]. Expression of miR-200a mimics p38alpha deficiency and increases tumor growth in mouse models, but it also improves the response to chemotherapeutic agents. miR-200a targets p38alpha and modulates the oxidative stress response in ovarian cancer[57]. The role of miR-200a in stress response could be a predictive marker for clinical outcome in ovarian cancer[57]. On the other hand, all five members of the microRNA-200 family (miR-200a, -200b, -200c, -141, and -429) were markedly down-regulated in cells that had undergone epithelial to mesenchymal transition (EMT) in response to transforming growth factor (TGF)-beta[58]. Overexpression of the miR-200 family miRNAs in mesenchymal cells initiated mesenchymal to epithelial transition (MET). Consistent with their role in regulating EMT, expression of these miRNAs was found to be lost in invasive breast cancer cell lines with mesenchymal phenotype[58] suggesting that down-regulation of miR-200a may be an important step in tumor progression. Taken together, it is possible that the up- or down-regulation of miR-200a in different cancers is a reflection of its tissue-specific complex role in tumorigenesis. There are also reports showing down-regulation of miR-143, miR-145 and miR-215 in esophageal adenocarcinomas. In addition, miR-23b and let-7b were involved in the progression from low-grade dysplasia Barrett's esophagus to esophageal adenocarcinoma[59, 60]. Additional studies that address this complexity could achieve further understanding the different roles of miRNAs in gastric versus esophageal adenocarcinomas.

In summary, our findings demonstrate a unique miRNA expression profile in gastric tumors. While few miRNAs shared similar expression patterns in gastric and esophageal adenocarcinomas, we have identified and validated 6 miRNAs that are uniquely de-regulated in gastric adenocarcinomas. These miRNAs could provide a new approach to understanding the specific molecular mechanisms that are relevant to gastric tumorigenesis with the possibility of improving our currently limited diagnostic, prognostic, and therapeutic options in this devastating worldwide disease.

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Abbreviations

miRNA	microRNA
NG	normal gastric
GC	gastric adenocarcinomas
NS	normal esophagus
EAC	esophageal adenocarcinomas

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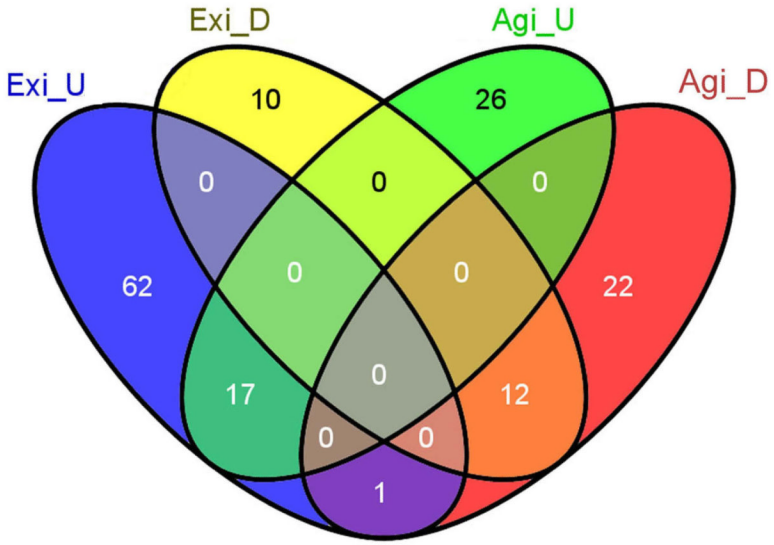

Figure 1. Venn diagrams of miRNA microarray analysis

The Venn diagram was used to identify overlapping and non-overlapping miRNA in the analysis Exiqon and Agilent microarrays. miRNAs with ≥2 fold change difference (up or down) were included. Exi, Exiqon; Agi, Agilent; D, down-regulated; U, up-regulated.

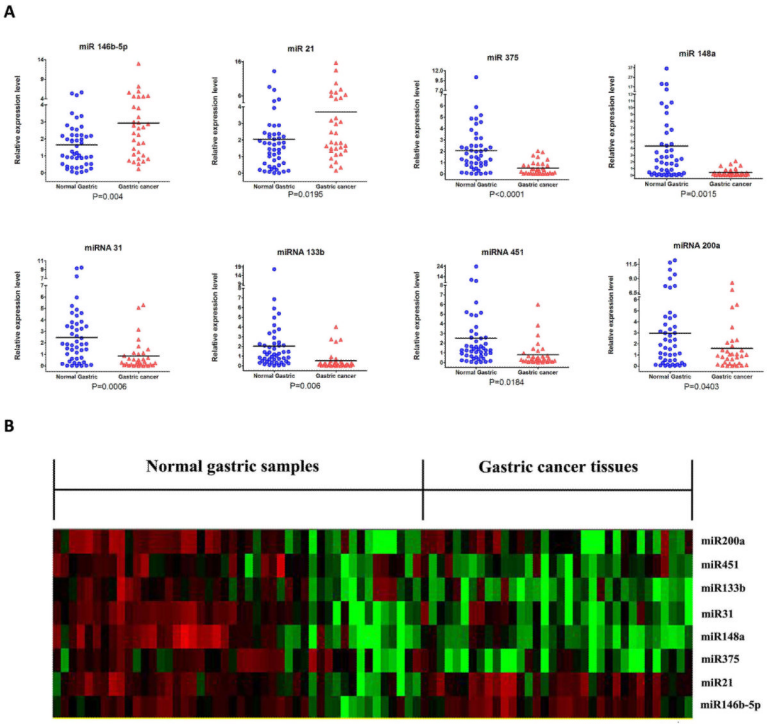

Figure 2. Validation of expression of 8 miRNAs using qRT-PCR

A) The expression of 8 miRNAs (miR-146b-5p, miR-21, miR-375, miR-148a, miR-31, miR-133b, miR-451, and miR-200a) was evaluated by qRT-PCR using 33 gastric adenocarcinoma and 47 normal gastric tissues. miR-191 and miR-103a-5p were used for normalization and the data are shown as relative expression level. The horizontal bars indicate the arithmetic mean. Statistical analysis was performed using Student's t-test and a $p \leq 0.05$ was considered statistically significant. **B)** Heat map analysis of the relative expression level of the eight miRNAs in gastric adenocarcinoma and normal gastric tissues.

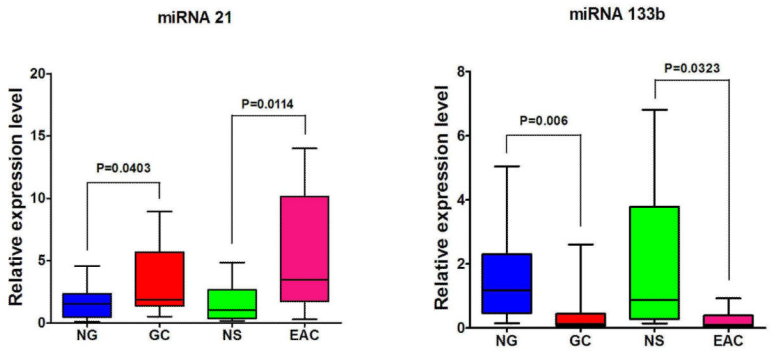

Figure 3. qRT-PCR analysis of 2 miRNAs in gastric and esophageal tissues

The expression of 2 miRNAs (miR-21, miR-133b) was evaluated by qRT-PCR using a total of 145 tissue samples (33 gastric adenocarcinomas, 37 esophageal adenocarcinomas, 47 normal gastric, 28 normal esophagus). miR-191 and miR-103a-5p were used for normalization and the data are shown as relative fold change. The box-and-whisker plots are used to demonstrate the data. The bottom and the top edges of the box mark the 25th and 75th percentile, respectively. The areas between the box and the whisker extend to the 10th and 90th percentile. $p \leq 0.05$ was considered statistically significant. NG, normal gastric; GC, gastric adenocarcinomas; NS, normal esophagus; EAC, esophageal adenocarcinomas.

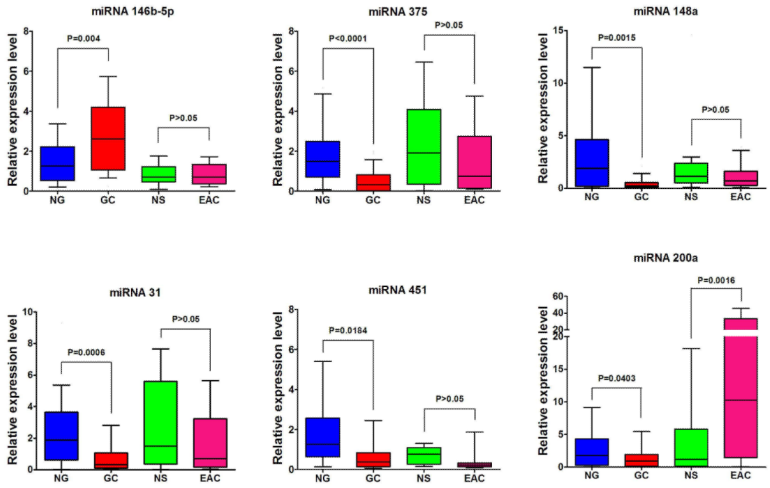

Figure 4. Differences in expression of miRNAs in gastric and esophageal adenocarcinomas

The expression of 6 miRNAs (miR-146b-5p, miR-375, miR-148a, miR-31, miR-451, miR-200a) was evaluated by qRT-PCR using a total of 145 tissue samples (33 gastric adenocarcinomas, 37 esophageal adenocarcinomas, 47 normal gastric, 28 normal esophagus). miR-191 and miR-103a-5p were used for normalization and the data are shown as relative fold change. The horizontal line represents the median value. The box-and-whisker plots are used to demonstrate the data. The bottom and the top edges of the box mark the 25th and 75th percentile, respectively. The areas between the box and the whisker extend to the 10th and 90th percentile. $p \leq 0.05$ was considered statistically significant. NG, normal gastric; GC, gastric adenocarcinomas; NS, normal esophagus; EAC, esophageal adenocarcinomas.

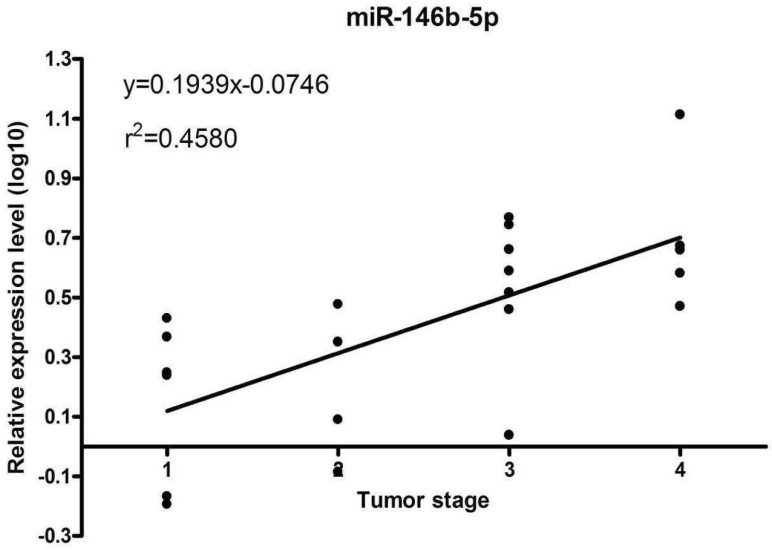

Figure 5. Correlation between miR-146b-5p and tumor TNM stage

Linear regression analysis of miRNA expression indicated that miR-146b-5p expression level had a strong correlation with gastric adenocarcinoma stage classification (Figure 5, r^2=0.4580). miR-191 and miR-103a-5p were used for normalization and the data are shown as log10 value of relative fold change. Patients were staged in accordance with the 7th Edition of the AJCC Cancer's TNM Classification. 1=stage I, 2=stage II, 3=stage III, 4=stage IV.

Table 1

Primers used in qRT-PCR analysis of miRNAs

miRNA	Primer's sequence
Universal 5' primer	GCGAGCACAGAATTAATACGAC
miR-191	CAACGGAATCCCAAAAGCAGCTG
miR-21	TAGCTTATCAGACTGATGTTGA
miR-146b-5p	TGAGAACTGAATTCCATAGGCT
miR-375	TTTGTTCGTTCGGCTCGCGTGA
miR-148a	TCAGTGCACTACAGAACTTTGT
miR-31	AGGCAAGATGCTGGCATAGCT
miR-133b	TTTGGTCCCCTTCAACCAGCTA
miR-451	AAACCGTTACCATTACTGAGTT
miR-200a	TAACACTGTCTGGTAACGATGT

Table 2

Fold change expression values of miRNAs in gastric adenocarcinomas *

Down-regulated			Up-regulated		
miRNA	**Agilent**	**Exiqon**	**miRNA**	**Agilent**	**Exiqon**
hsa-miR-1	−6.5	−2.4	ebv-miR-BART16	2.0	2.9
hsa-miR-133b	−6.1	−3.0	ebv-miR-BART3	2.1	3.0
hsa-miR-143*	−5.5	−2.5	ebv-miR-BART7	3.2	4.4
hsa-miR-145	−4.3	−2.5	hsa-miR-142-3p	20.5	3.8
hsa-miR-145*	−2.9	−3.3	hsa-miR-142-5p	3.6	3.9
hsa-miR-148a	−23.6	−2.4	hsa-miR-146a	5.8	3.7
hsa-miR-203	−41.2	−19.6	hsa-miR-146b-5p	2.3	4.5
hsa-miR-205	−63.2	−93.3	hsa-miR-155	3.7	2.7
hsa-miR-31	−5.0	−4.7	hsa-miR-192	3.4	5.8
hsa-miR-365	−8.2	−2.3	hsa-miR-194	32.2	6.7
hsa-miR-375	−15.1	−4.8	hsa-miR-20a	2.5	2.1
hsa-miR-451	−18.3	−3.7	hsa-miR-21	2.6	3.0
			hsa-miR-214	5.7	2.1
			hsa-miR-215	4.1	5.8
			hsa-miR-223	5.1	3.3
			hsa-miR-342-3p	4.2	2.3
			hsa-miR-765	5.8	3.1

* Fold change (FC) expression levels of miRNAs in Agilent and Exiqon platforms. miRNAs were selected if their FC values were ≤ −2 (down-regulated) or ≥ 2 (up-regulated) in both platforms.

Table 3

Comparison of miRNA expression between gastric and esophageal adenocarcinoma

miRNA	Gastric adenocarcinoma		Esophageal adenocarcinoma	
	Status	P value	Status	P value
146b-5p	Up-regulated	0.004	Not significant	0.71
21	Up-regulated	0.019	Significant	0.01
375	Down-regulated	<0.0001	Not significant	0.06
148a	Down-regulated	0.0015	Not significant	0.40
31	Down-regulated	0.0006	Not significant	0.07
133b	Down-regulated	0.0048	Significant	0.03
451	Down-regulated	0.017	Not significant	0.33
200a	Down-regulated	0.040	Significant	0.001