

Evidence that dysplasia related microRNAs in Barrett's esophagus target PD-L1 expression and contribute to the development of esophageal adenocarcinoma

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ABSTRACT

Esophageal adenocarcinoma (EAC) is the cancer arising from the esophagus, which frequently develop from Barrett's esophagus (BE). Extracellular vesicles (EVs), particularly exosomes, are nanosized vesicles of endosomal origin released from various types of cells that have been implicated in cancers. However, the significance of circulating exosomes during the progression of BE to EAC remains unknown. Sera exosomal microRNAs were profiled from 13 EAC and 12BE patients compared to 12 healthy controls. We found a substantial dysregulation of exosomal miRNA levels in BE compared to healthy control, and identified a unique signature of 24 up regulated and 14 down regulated miRNAs. Further validation showed exosomal miR-196a, -26b, -21, and -143 expression was significantly higher in BE and continued to have higher levels in EAC compared to healthy controls; while sera exosomal miR-378, -210, -205, and -200c-3p were significantly lower expressed in BE patients compared to compared to controls. Further, miR-378, -210, -205, and -200c-3p continue to have even lower levels in EAC patients compared to BE. Interestingly, sera expression levels of exosomal miR-15a, -16, and -193a-3p were significantly down regulated in BE PD-L1(+) patients; Sera exosomal miR-15a, -15b, -16, and -193a-3p expression levels in EAC PD-L1(+) patients were significantly lower (all $p < 0.01$) when compared to EAC PD-L1(-) patients. More importantly, the BE-EAC group had longitudinally decreased exosomal expression levels of miR-15a, -15b, -16, and -193a-3p from BE status to their EAC progression. In conclusion, distinct microRNA expression patterns were demonstrated in circulating exosomes from Barrett's esophagus and esophageal adenocarcinoma; Furthermore exosomal microRNAs potentially targeting PD-L1 mRNA were down regulated in PD-L1 (+) BE and EAC patients.

INTRODUCTION

Esophageal adenocarcinoma (EAC) is the cancer arising from the esophagus. EAC is also known as one of the

most common types of tumors among the esophageal cancers. Barrett's esophagus (BE) is one kind of intestinal metaplasia in the distal esophagus and also a precursor cause of EAC [1]. BE is more common in

developed countries, affecting 2% of the general adult population [2]. One study with a cohort of 961 patients undergoing colonoscopy who were offered an additional endoscopy, and found an overall prevalence of 6.8%, with 5.5% for short-segment BE in persons aged 40 years or older [3]. In another similar colonoscopy-based study of 300 patients over the age of 65 years, the prevalence was 4% and 15% for long- and short-segment BE, respectively [4]. Therefore BE is common in older men and women undergoing screening colonoscopy regardless of reflux symptoms [4]. In other population-based studies, the prevalence of BE in the general population ranged between 1.3%, 1.6% and 1.9% [5–7]. The length of BE is greater in men than in women, but other features are similar [8]. Incidence rates for high-grade dysplasia/cancer are similar in men and women, although the number of cases is small [8]. BE is clinically linked to the development of EAC by a 0.5% risk each year. Dysplasia is now used as the major biomarker to classify patients with Barrett’s esophagus at high risk to develop EAC [9, 10]. The transition from BE to EAC was implicated as a progress through low grade to high-grade dysplasia [11, 12]. BE included non-dysplastic Barrett’s intestinal metaplasia, low-grade and high-grade dysplasia [9, 13]. The malignant progression rate varies according to the presence of dysplasia in esophagus [9, 10, 13]. However, it remains unclear that how BE mediated dysplasia contributes to EAC development.

Among the esophagus cancer patients PD-L1 is reported to be over expressed nearly 40% and associated with the worse overall survival [14]. In clinical trials, immune check point inhibitors reported to have a response rate 9.9-33.3% in esophagus cancer [15]. Our group recently reported the identification of biomarkers, microRNAs,

to determine which patients are more likely to respond PD-L1 inhibitor treatment [16]. Recent studies indicated that exosomes (~50-200nm), membrane-bound extracellular vesicles, coordinate intercellular communication between different cells [17]. Exosomes could potentially regulate cancer progression and metastasis by transferring DNA, RNA or proteins between various cells in the local and distant sites [18]. MicroRNAs (miRNAs) are small non-coding RNAs that involved in messenger RNA degradation and translation [19], which also implicated in its ability to inhibit translation of tumor associated genes [20–23]. Numerous documents have implicated in miRNAs’ significance on development of BE as well as EAC [24–27], which further demonstrated the potential of miRNA profiling to distinguish BE from EAC [28–30]. However, circulating exosomal miRNAs during the disease progression of BE to EAC has yet been examined. We, therefore, aimed to characterize miRNA content of exosomes in both BE and EAC patients, further validated that circulating exosomes contain differential level of microRNAs targeting Programmed death-ligand 1 (PD-L1) mRNA in Barrett’s esophagus and esophageal adenocarcinoma.

RESULTS

Serum miRNAs were differentially expressed in BE and EAC

Sera exosomes isolated from BE and EAC were firstly characterized. Isolated exosomes were shown a spherical morphology with an average size of 133 ± 25 nm (Figure 1A, 1B). Exosomal markers, CD63, CD9 and CD81 were present (Figure 1C), which clearly indicate that this vesicles are mostly exosomes. No

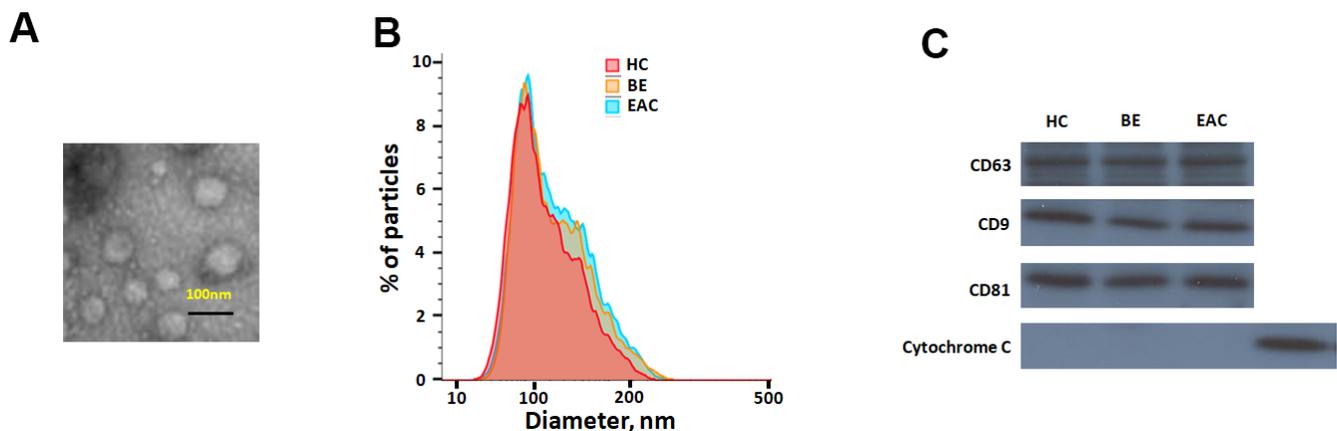


Figure 1. Characterisation of exosome-like vesicles released from serum by (A) transmission electron microscopy, (B) dynamic light scattering analysis (133 ± 25 nm) and (C) western blotting. Presence of exosomal markers, CD63, CD9, CD81 and mitochondrial protein cytochrome c in lysates from sera-derived exosomes and cell lysate.

significant differences were seen among healthy subjects, BE, and EAC.

A pilot microRNA profiling was further performed to identify the differentially expressed exosome miRNAs in 13 EAC and 12BE patients compared to 12 healthy controls. Comparison shown that 38 exosomal miRNAs were differentially expressed in EAC patients compared to those of BE patients. Among them, we found that 24 exosomal miRNAs were significantly up regulated (including miR-4508, miR-145, miR-106b, miR-30a-5p, miR-125b-5p, miR-382-5p, miR-196a, miR-100-5p, miR-151a-3p, miR-26b, miR-127-3p, miR-4488, miR-21, miR-25, miR-143, miR-320e, miR-215, miR-4286, miR-186-5p, miR-181a-5p, miR-219-5p, miR-28-3p, miR-601, miR-194) and 14 miRNAs (miR-378, miR-192-5p, miR-210, miR-15b-5p, miR-205, miR-31-5p, miR-720, let-7a-5p, miR-133a-3p, miR-200c-3p, miR-200a-3p, miR-203, miR-375, miR-1) were down regulated significantly (fold change>2, $p<0.05$) in sera exosomes of EAC patients with compared to BE (Table 1 and Figure 2A). Moreover, principal component analysis (PCA) were performed for Healthy, BE and EAC based on miRNA profiles (Figure 2B). EAC was correlated with the first component ($p<0.01$). These results indicated that aberrantly expressed serum exosomal miRNA profiles were implicated in BE to EAC progression. We further identified which biologic pathways were affected during the sequential progression of BE to EAC, we applied DIANA-mirPath on the dysregulated serum miRNAs, and 52 KEGG pathways were significantly enriched ($p<0.05$) after false discovery rate was corrected. Fatty acid biosynthesis (2.238E-12), Hippo signaling pathway (2.604E-06), ErbB signaling pathway (1.001E-04) were top ranked as the most prominent pathways enriched in quantiles with the serum miRNA signature (Supplementary Table 1), suggesting that these biologic pathways were involved in the sequential progression of BE to EAC.

We further identified and assessed a miRNA combination that could classify BE or EAC from healthy controls. A miRNA classifier based on the differential miRNA pattern was built to classify BE and EAC patients and from healthy individuals. The miRNA classifier showed higher accuracy to distinguish individuals with BE and EAC from controls (EAC vs. Controls, area under the curve (AUC) = 0.887 [0.799–0.935]; BE vs. Controls AUC = 0.723 [0.675–0.788], $p<0.001$) (Figure 2C). The miRNA classifier had higher sensitivity (range 75.4-88.9%) and specificity (82.1-93.4%) to detect EAC. These data further supported a notion that the potential mechanisms regulated by differentially expressed microRNAs would play important roles during BE and EAC development.

Validation of dysregulated miRNAs with independent larger sample-size cohort

To further validate the dysregulated exosomal miRNAs during BE-EAC sequential progression, TaqMan Real-Time PCR was employed to validate the differential expression levels of eight miRNAs including miR-196a, -26b, -21, -143, -378, -210, -205, and -200c-3p. These miRNA candidates were selected from the original microRNA assay screening according to their functional significance of previous investigations. An independent larger cohort of 79 EAC patients, 56 BE patients and 66 healthy controls was included here for further validation (Table 2). We found that exosomal miR-196a, -26b, -21, and -143 were significantly increased in individual BE patients when compared with healthy controls (Figure 3). In addition, the results demonstrated that exosomal miR-196a, -26b, -21, and -143 continued to have higher levels in EAC compared to BE (Figure 3); while miR-378, -210, -205, and -200c-3p were significantly under expressed in individual BE patients compared to healthy controls (Figure 3). Further exosomal miR-378, -210, -205, and -200c-3p continued to show lower levels in EAC patients (Figure 3). These findings confirmed that extracellular/circulating exosomal miRNAs are potentially important regulators influencing the sequential progression of Barrett's esophagus to esophageal adenocarcinoma.

MiRNAs targeting PD-L1 were down regulated in BE and EAC patients with PD-L1 positive expression

The upregulation of PD-L1 is found in cancers and contributes to evasion of the host immune defense. Some previous studies suggested that PD-L1 was regulated by miR-15a, -15b, -16, and -193a-3p targeting PD-L1 mRNA in human malignancies [31]. We analyzed the association between the expression of PD-L1 and that of sera exosomal miR-15a, -15b, -16, and -193a-3p in 25 BE and 48 EAC patients with PD-L1 positive expression compared to those PD-L1 negative patients (43 BE and 44 EAC). Healthy controls with positive PD-L1 individuals (n=22) and negative PD-L1 individuals (n=56) were also included for comparison. The median expression levels of sera exosomal miR-15a, -16, and -193a-3p were significantly down regulated (all $p < 0.05$) in BE PD-L1(+) patients when compared to BE PD-L1(-) patients (Figure 4); further sera exosomal miR-15a, -15b, -16, and -193a-3p expression levels in EAC PD-L1(+) patients were significantly lower (all $p < 0.01$) when compared to EAC PD-L1(-) patients (Figure 4). However, there were no significant differentially expressed between healthy controls with positive PD-L1 patients and negative PD-L1 patients (Figure 4).

Table 1. Differential microRNAs expression in serum during the sequential progression of Barrett’s esophagus to esophageal adenocarcinoma.

Barrett’s esophagus vs. Healthy Control			Esophageal adenocarcinoma vs. Barrett’s esophagus		
miR-Name	Fold change	Adjusted p-value	miR-Name	Fold change	Adjusted p-value
miR-4508	2.962	0.0700	miR-4508	4.643	0.0022
miR-145	2.928	0.0650	miR-145	4.063	0.0030
miR-106b	0.344	0.0755	miR-106b	3.125	0.0071
miR-30a-5p	1.123	0.0729	miR-30a-5p	2.881	0.0015
miR-125b-5p	1.244	0.0784	miR-125b-5p	2.811	0.0016
miR-382-5p	1.254	0.0144	miR-382-5p	2.668	0.0030
miR-196a	1.153	0.0446	miR-196a	2.651	0.0031
miR-100-5p	1.166	0.0560	miR-100-5p	2.585	0.0028
miR-151a-3p	1.099	0.0740	miR-151a-3p	2.534	0.0025
miR-26b	1.186	0.0460	miR-26b	2.495	0.0056
miR-127-3p	1.186	0.0536	miR-127-3p	2.483	0.0035
miR-4488	1.067	0.0513	miR-4488	2.429	0.0006
miR-21	1.207	0.0363	miR-21	2.422	0.0019
miR-25	0.963	0.0015	miR-25	2.408	0.0043
miR-143	1.224	0.0396	miR-143	2.334	0.0038
miR-320e	0.690	0.0615	miR-320e	2.245	0.0028
miR-215	1.211	0.0550	miR-215	2.214	0.0024
miR-4286	1.085	0.0715	miR-4286	2.209	0.0033
miR-186-5p	2.077	0.0731	miR-186-5p	2.172	0.0031
miR-181a-5p	1.435	0.0523	miR-181a-5p	2.168	0.0025
miR-219-5p	0.866	0.0880	miR-219-5p	2.153	0.0065
miR-28-3p	1.415	0.0206	miR-28-3p	2.114	0.0008
miR-601	1.245	0.0183	miR-601	2.083	0.0063
miR-194	0.819	0.0190	miR-194	2.065	0.0036
miR-378	0.731	0.0358	miR-378	0.493	0.0021
miR-192-5p	0.653	0.0517	miR-192-5p	0.393	0.0032
miR-210	0.584	0.0437	miR-210	0.250	0.0012
miR-15b-5p	0.837	0.0597	miR-15b-5p	0.162	0.0041
miR-205	0.630	0.0642	miR-205	0.093	0.0015
miR-31-5p	7.275	0.0558	miR-31-5p	0.093	0.0020
miR-720	1.678	0.0551	miR-720	0.092	0.0034
let-7a-5p	1.407	0.0344	let-7a-5p	0.089	0.0033
miR-133a-3p	2.288	0.0663	miR-133a-3p	0.088	0.0065
miR-200c-3p	0.558	0.0691	miR-200c-3p	0.087	0.0034
miR-200a-3p	0.766	0.0563	miR-200a-3p	0.073	0.0021
miR-203	0.807	0.0274	miR-203	0.053	0.0024
miR-375	0.783	0.0464	miR-375	0.048	0.0005
miR-1	0.521	0.0556	miR-1	0.033	0.0032

MiRNAs targeting PD-L1 were reduced expressed in BE patients with continually developing EAC

We then selected five BE patients continually developing EAC in 2 years from our cohorts, we tested

the sera exosomal miRNAs expression of miR-15a, -15b, -16, and -193a-3p longitudinally. 10 BE patients who did not develop EAC in 5 years were included as control group. Compared with control group, the BE-EAC group had decreased sera exosomal expression

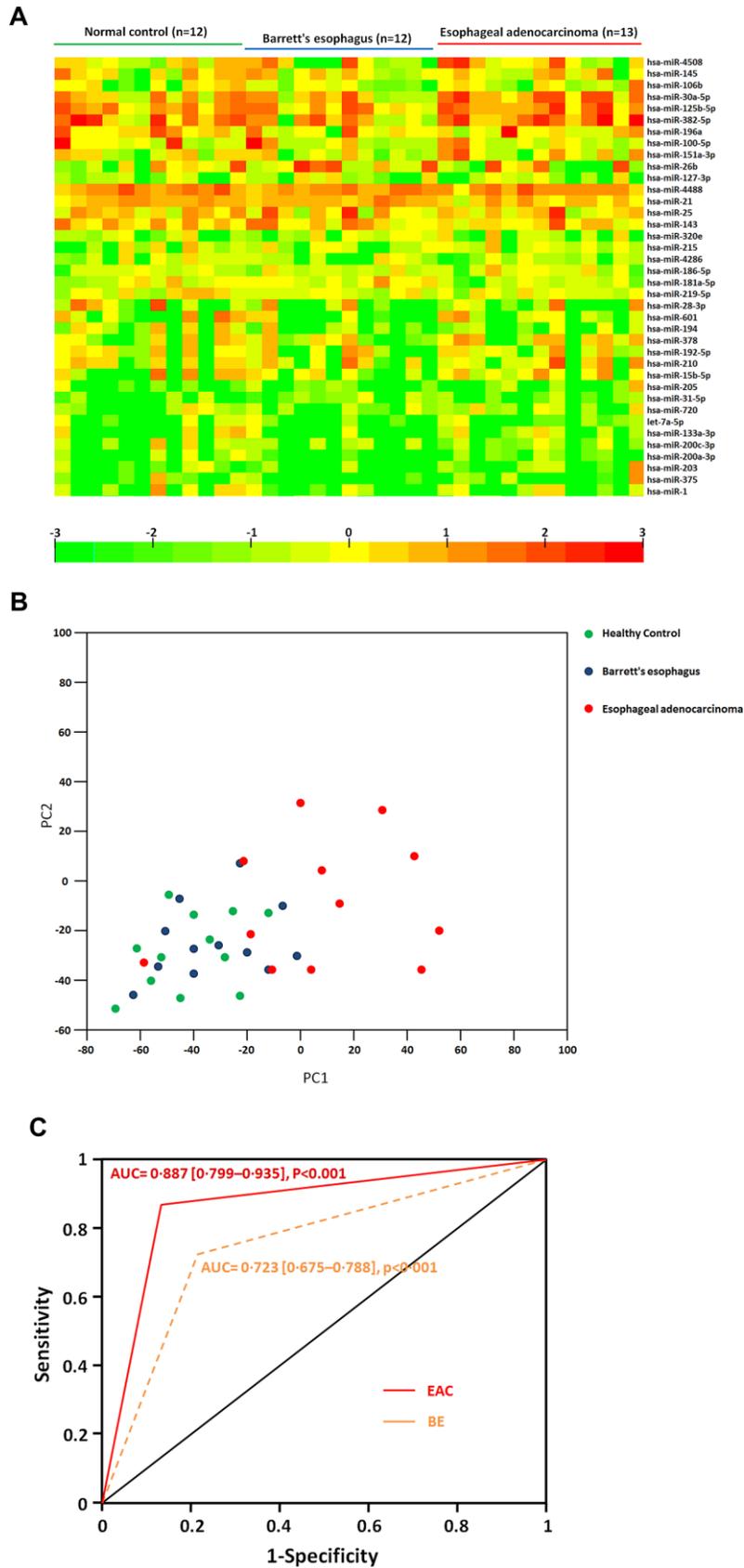


Figure 2. (A) Heatmap of differential sera miRNA profiles during the sequential progression of Barrett's esophagus to esophageal adenocarcinoma. Heatmap representation of the mean fold change in differential miRNA signature. Two-dimensional grid matrix displaying

38 serum miRNAs was obtained by the functional heat-map in R. Columns refer to time course comparison: 12 healthy controls, 12 BE and 13 EAC. Rows stand for the 38 differential miRNAs. Each entry of the grid refers to relative fold (log₂) of a given miRNA. The color of each entry is determined by the value of that fold difference, ranging from green (negative values) to red (positive values). (B) Principal component analysis. The plots for disease phenotypes (12 healthy controls, 12 BE and 13 EAC) were performed as principal component analysis among all samples based on miRNA profiles. (C) The performance of sera exosomal microRNA classifier to detect the risk of BE and EAC. ROC curves from 12 healthy controls, 12 BE and 13 EAC. Performance shown for distinguishing individuals with BE or EAC from healthy controls.

levels of miR-15a, -15b, -16, and -193a-3p from BE status to their EAC progression (Figure 5), suggesting that these exosomal microRNAs could be early biomarkers for EAC development in BE patients. Furthermore, there were 3 PD-L1(-) and 2 PD-L1 (+) patients when BE status, all turned into PD-L1 (+) patients in all 5 BE patients who progressed to EAC. However, all samples were negative expression of PD-L1 status in 10 BE patients who did not progress to EAC. However, the statistic test is not significant at $p < 0.05$ due to the small sample size.

DISCUSSION

BE patients are at a higher risk of developing EAC, which has the poor survival in its late stage [13]. In the current study, we performed a profiling of sera exosomal microRNA among BE and EAC patients. Distinct microRNA patterns were identified during the progression of BE to EAC. These differential exosomal miRNAs were validated with independent cohort, including miR-196a, -26b, -21, -143, -378, -210, -205, and -200c-3p. Expression of miR-196a, -26b, -21, and -143 were significantly higher in BE and continue to have higher levels in EAC compared to healthy controls; while miR-378, -210, -205, and -200c-3p were significantly lower expressed in BE patients compared to compared to controls. Further, miR-378, -210, -205, and -200c-3p continue to have even lower levels in EAC patients compared to BE. microRNAs-196a was found to correlate with during BE related dysplasia progression into esophagus adenocarcinoma [32]. Moreover, miR-196a levels were conversely correlated with long-term survival esophageal adenocarcinoma [33] and regulated the role of pro- and anti-apoptotic functions by targeting keratin 5, small prolinerich protein 2C, and S100 calcium-binding protein A9 [34]. MiR-21 was proven to be aberrantly increased in Barrett's oesophagus [35], which was further enhanced by the activated EGFR signaling pathway in lung cancers [36]. Up regulation of miR-143 was found in BE and EAC [35], suggesting miR-143 may play an important role in dysplasia and tumor development, possibly through targeting DNMT3A [37]. Taken all together, our findings strongly support potential roles of miRNAs in BE and sequential EAC tumorigenesis. We further confirmed exosomal miRNAs were differentially expressed at different stages of BE and EAC. We identified and assessed a miRNA combination that could detect the presence of EAC in at-

risk BE patients (Figure 2C). The miRNA classifier is a potential biomarker for esophageal adenocarcinoma in patients at risk. The miRNA classifier could be valuable to detect preclinical esophageal adenocarcinoma, providing patients with a chance of curative resection and longer survival.

In addition, Fatty acid biosynthesis ($2.238E-12$), Hippo signaling pathway ($2.604E-06$), ErbB signaling pathway ($1.001E-04$) were identified with enrichment into the serum miRNA signature (Supplementary Table 1), indicating important biological signals coordinating the sequential progression of BE to EAC. Fatty acid synthase (FASN) as a key enzyme participated in the fatty acid biosynthetic pathway, and Over expression of FASN protein was observed in over half of BE patients, especially in the intestinal mucin phenotype of Barrett's esophagus in which glandular cells display increased proliferation, angiogenesis, and COX-2 expression; Further FASN over expression pattern was retained in EAC [38]. Recent investigations demonstrated that amplification of *ERBB2* (also called *HER2*) oncogenic form was found in esophageal adenocarcinoma progression, leading to a worse outcome for EAC [39]. Combined with our current results, BE and EAC dysregulated miRNAs can regulate Fatty acid biosynthesis and ErbB signaling pathway, further contribute to development of EAC.

Up regulation of the surface expression of PD-L1 in tumor cells can interact with its receptor PD-1 on T cells, leading to evade immune surveillance and inhibit the immune checkpoint response [40]. The roles of microRNAs in transcriptional regulating the expression of PD-1/PD-L1 immune checkpoint remain elusive. In argonautes immunoprecipitation experiments, Kaso SC et al., discovered an interaction between the PD-L1 mRNA and the miR-15a or miR-16 [31]. Furthermore, Wang X et al., observed an inverse correlation between PD-L1 and miR-34a expression in acute myeloid leukemia [41]. Further, miR-93-5p and miR-106b-5p could target expression of PD-L1 mRNA in bulk cancer cells [42]. MiR-424 could regulate the PD-1/PD-L1 pathways in chemo resistant ovarian cancer through direct binding to PD-L1 mRNA 3'UTR [43]. In this study we for the first time found that microRNA expression levels of sera miR-15a, -16, and -193a-3p were significantly down regulated (all $p < 0.05$) in BE PD-L1(+) patients (Figure 5); further sera miR-15a, -15b, -16, and -193a-3p

Table 2. Characteristics of independent cohorts of healthy controls and patients with BE or EAC.

Variable	Controls (n = 66)	BE (n = 56)	EAC (n = 79)
Gender (male)	36	32	45
Age (years, mean ± SD and range)	55±15	55±10	55±6
Barrett length (mean and range)	NA	C4M5 (C1M3–C10M11)	C8M8 (C5M5–C13M13)
Tumor stage (T)			
1	NA	NA	0
2			12
3			35
4			30
Unknown			2
Lymph node status (N)			
0	NA	NA	13
1			14
2			24
3			15
4			13
Unknown			0
Metastasis (M)			
0	NA	NA	33
1			40
Unknown			6
Body mass index (mean ± SD)	25±5	26±5	25±6
Current smokers	13	14	14
Previous smokers	22	14	15
Non-smokers	31	28	50

BE Barrett’s esophagus, EAC esophageal adenocarcinoma, CM circumference length, maximum length.

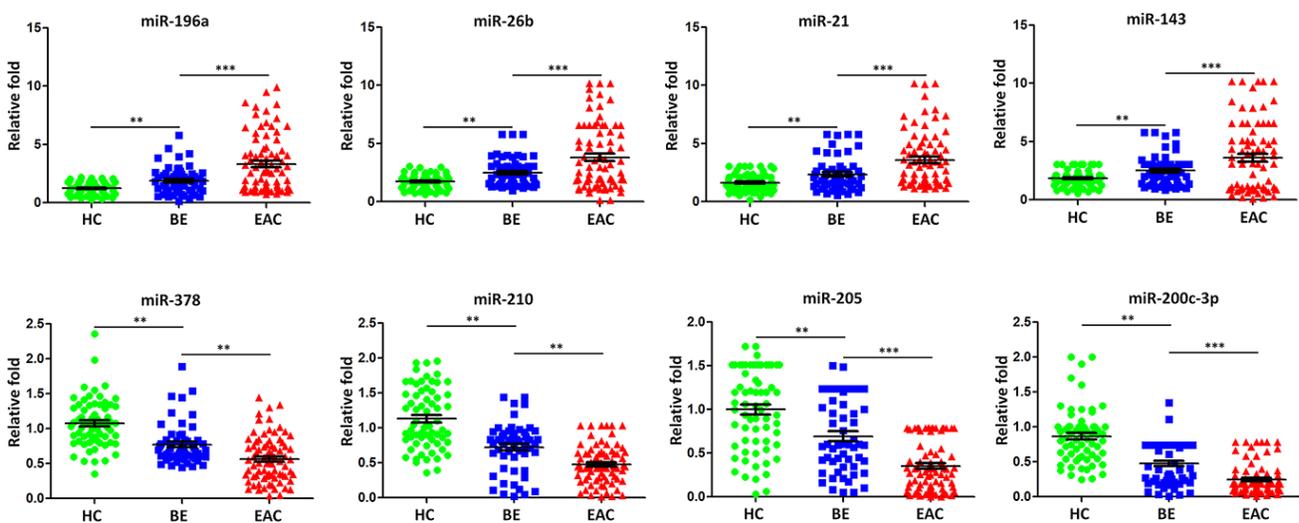


Figure 3. Validation of miRNA array expression using independent samples. TaqMan real-time RT-PCR to validate the expression levels of miR-196a, miR-26b, miR-21, miR-143, miR-378, miR-210, miR-205, and miR-200c-3p using an independent cohort of 79 EAC patients, 56 BE patients and 66 healthy controls. Data shown are as mean ± SD.

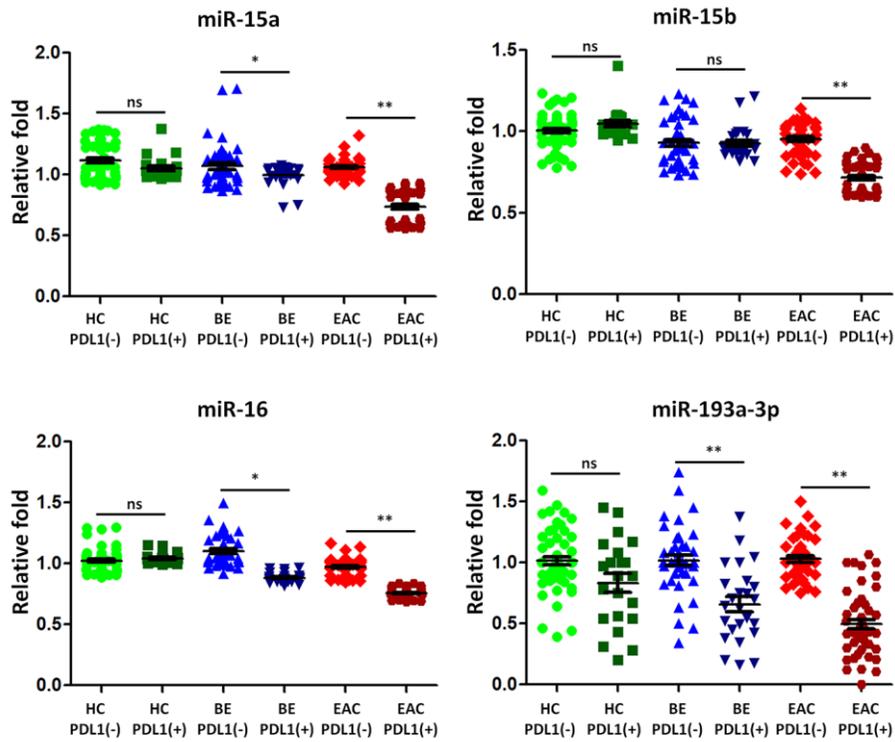


Figure 4. The expression of microRNA predicted to target programmed death ligand 1 (PD-L1) is lower in BE and EAC with PD-L1 positivity. TaqMan real-time RT-PCR to validate the expression levels of miR-15a, -15b, -16, and -193a-3p using the combined cohort of 25 BE and 48 EAC patients with PD-L1 positive samples compared to those PD-L1 negative patients (43 BE and 44 EAC). Healthy controls with positive PD-L1 patients (n=22) and negative PD-L1 patients (n=56) were also included for comparison. Data shown are as mean \pm SD.

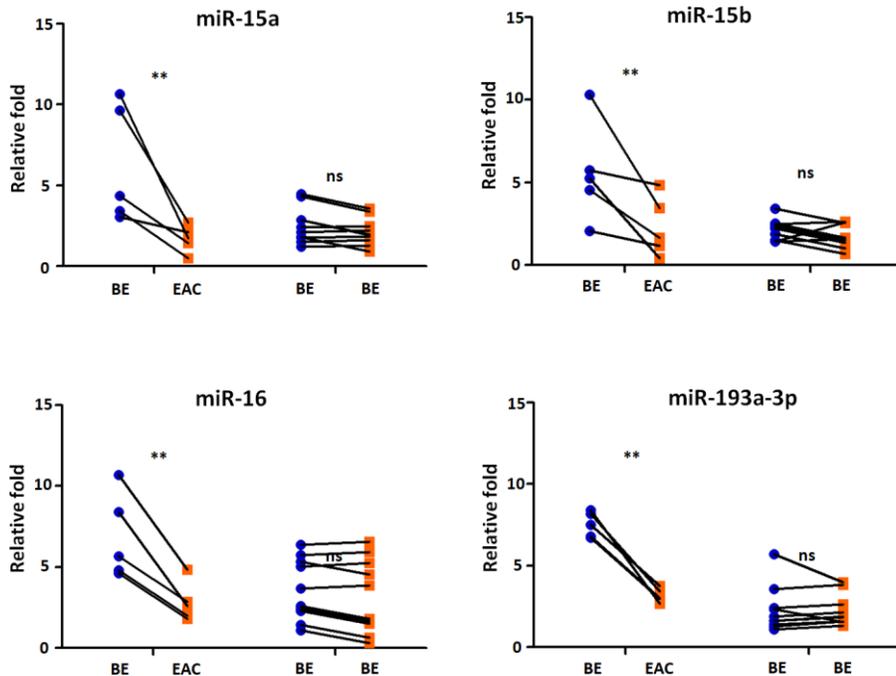


Figure 5. Exosomal miRNAs expression correlates with BE-EAC progression. TaqMan real-time RT-PCR to validate the expression levels of miR-15a, -15b, -16, and -193a-3p using 5 BE-EAC patients and 10 BE-BE patients. Data shown are as mean \pm SD.

expression levels in EAC PD-L1(+) patients were significantly lower (all $p < 0.01$) when compared to EAC PD-L1(-) patients (Figure 5). These results demonstrated that tumor suppressor microRNAs may contribute to the regulation of PD-L1 expression in BE and EAC. More importantly, compared with control group, the BE-EAC group had decreased sera exosomal expression levels of miR-15a, -15b, -16, and -193a-3p from BE status to their EAC progression (Figure 5), suggesting that these exosomal microRNAs could be not only early biomarkers for EAC development in BE patients, further differential expressed microRNAs in Barrett's esophagus may target PD-L1 expression and contribute to the development of esophageal adenocarcinoma.

It is deserved to search for changes of microRNA expression from BE to EAC as well as healthy subjects, and clarify the rising and decreasing groups of microRNA. Further investigation shows that microRNAs expression, which are likely to be involved in the progression of the parthenogenesis of EAC development from BE, compared the group that appears to have progressed in the same patient with the unchanged group. However, there are still some limitations in our study. BE and EAC samples were not paired in our study, but from separate individuals. In addition, we did not perform any functional investigation *in vivo* of the identified miRNAs. Another limitation for longitudinal analysis is that the sample size from the BE patients with continually developing EAC is small.

CONCLUSIONS

In conclusion, here we performed a retrospective study by enrolling independent and large validation set of subjects, our results demonstrated that circulating exosomal miRNAs may have a promising diagnostic performance for the detection of BE and EAC. Further circulating exosomes contain differential level of microRNAs targeting PD-L1 mRNA in Barrett's esophagus and esophageal adenocarcinoma.

MATERIALS AND METHODS

Patients

BE patients and EAC patients at Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China were enrolled between 2012 through 2019 after obtaining informed consent and approval by the Human Research Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. All participants in the first pilot study, including controls, underwent routine endoscopy [12 healthy controls, 12 BE and 13 EAC, Clinical features are displayed in Table 3]. Healthy

subjects were patients undergoing endoscopy for unexplained upper abdominal complaints, had no reflux symptoms or endoscopic abnormalities. A further validation assay included an independent larger cohort of 79 EAC patients, 56 BE patients and 66 healthy controls. No significant difference of mean BMI between healthy controls, BE and EAC patients in both cohorts. The diagnoses of BE are made on the basis of histologic specimens evaluated by two specialized GI pathologists. Patients with concurrent or previous diagnosis of low-grade dysplasia (LGD), high-grade dysplasia (HGD), or EAC were excluded from BE patients in this study. Within this cohort, we also searched the cases that were further diagnosed to develop EAC during the follow-up period. There were totally 68 BE patients recruited in this study. The median follow-up time was 4.1 years (interquartile range, 1.5 to 7.5) for the 68 BE patients. Among them, 33 BE patients had more than 5 year follow up after they were first diagnosed as BE. The tissue microarray was applied to determine PD-L1 status in this study as described previously [44]. Briefly, immunohistochemistry was performed on a Leica Bond III platform using the Bond Polymer Refine Detection Kit (Leica Biosystems). The primary antibody against PD-L1 (SP142, rabbit IgG; Spring Bioscience, Pleasanton, CA) was incubated for 30 minutes at room temperature at a 1:50 dilution. This antibody had been previously validated in a large number of NSCLCs and was shown to be satisfactory (>95% concordance). For quantitative evaluation, an average score was derived by averaging the scores from each patient. Cases of PD-L1 expression of 5% or more were considered positive. Clinical data were retrieved from the hospital's patient records. The experimental design and analysis were included in the workflow, as shown in Supplementary Figure 1.

Exosome isolation from sera by precipitation

Exosomes from sera was extracted using total exosome isolation kit (Life technologies) [45]. Briefly, 15 ml serum was centrifuged at 3,000 g for 30 min and thereafter at 10,000 g for 30 min to remove cells and debris. Sera were further diluted with PBS and centrifuged at 10,000g for 10 min at room temperature. The pelleted exosomes was solubilized with PBS and utilized for characterization.

Nanoparticle tracking analysis

Size distribution and concentration was analyzed using nanoparticle tracking analysis (NTA) on a NanoSight NS500 (Malvern Instruments Ltd.). Samples were recorded at camera level=14, detection level=3 and 5 videos of 20 seconds with a coefficient of variance <20% were used for analysis. To ensure measurement

Table 3. Characteristics of normal controls and patients with Barrett’s esophagus or esophageal adenocarcinoma used for microRNA expression profiling.

Variable	Controls (n = 12)	BE (n = 12)	EAC (n = 13)
Gender (male)	6	6	7
Age (years, mean ± SD and range)	52±13	53±9	51±16
Barrett length (mean and range)	NA	C4M5 (C1M3–C10M11)	C8M8 (C5M5–C13M13)
Tumor stage (T)			
1	NA	NA	0
2			4
3			4
4			5
Unknown			0
Lymph node status (N)			
0	NA	NA	2
1			2
2			4
3			3
4			2
Unknown			0
Metastasis (M)			
0	NA	NA	4
1			6
Unknown			3
Body mass index (mean ± SD)	26±6	25±4	25±3
Current smokers	2	3	1
Previous smokers	1	2	2
Non-smokers	9	7	10

BE Barrett’s esophagus, EAC esophageal adenocarcinoma, CM circumference length, maximum length.

accuracy all samples were analyzed during the same time frame for each cohort/experiment on a single instrument by the same operator.

Transmission electron microscopy (TEM)

Isolated exosomes were loaded onto formwar carbon-coated grids, fixed in 2% paraformaldehyde, washed and immunolabelled with anti-CD63 antibody followed by 10 nm gold-labelled secondary antibody (Sigma Aldrich). The exosomes were post-fixed in 2.5% glutaraldehyde, washed, contrasted in 2% uranyl acetate, embedded in a mixture of uranyl acetate (0.4%) and methyl cellulose (0.13%), and examined by electron microscope (Carl Zeiss NTS).

MicroRNA expression profiling

Sera exosomal RNA was extracted using the miRNeasy Mini kit (Qiagen) following the manufacturer’s protocol. RNA quality was assessed by using a Nanodrop spectrophotometer (Thermo Scientific). 25 ng

RNA was reverse transcribed for cDNA synthesis using the TaqMan Multiplex RT set (Applied Biosystems). qPCR was performed using TaqMan 2X Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). TaqMan Low-Density Array Human miRNA Panel (Applied Biosystems, Foster City, CA) was chosen for miRNA profiling.

Pathway analysis

DIANA-mirPath [46] was employed to perform the enrichment analysis of predicted target genes by one or more miRNAs in biological pathways. The software performs an enrichment analysis of multiple miRNA target genes to all known KEGG pathways based on two algorithms, microT-CDS [47, 48] and miRTarBase [49]. The software is available at <http://microrna.gr/mirpath>.

TaqMan miRNA qPCR

Sera exosome RNA was isolated from newly recruited cohort of 79 EAC, 56 BE and 66 healthy controls using

Qiagen miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA). Individual miRNA analysis was performed using TaqMan miRNA assays (Applied Biosystems, Foster City, CA). A synthetic RNA spike-in, *C. elegans* miR-39 miRNA mimic, was added prior to cDNA synthesis as a control for variations in reverse transcription efficiency. The PCR amplifications were carried out by incubation for 10 min at 95°C, followed by 40 amplification cycles at 95°C for 10 sec and 60°C for 1 min. All reactions were performed in duplicate. miRNA with more than half of the Ct values > 35 per group were excluded from the analysis. Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method. The relative miRNA levels were normalized to internal controls miR-16-5p.

Western blotting

Protein samples from lysed exosomes (5 µg) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10-15%) and transferred to polyvinylidene fluoride membranes (Millipore). Blots were blocked 1 hour with 5% milk in Tris-buffered saline (TBS) with 0.1% Tween-20, and blotted 1 hour with the following primary antibodies including CD63 (#106228D, Invitrogen), CD9 (#sc20048, Santa Cruz), CD81 (#10630D, Invitrogen), or cytochrome c (#556433, BD Pharmingen). After three washes with TBS/0.1% Tween-20, filters were incubated for 1h at room temperature with an HRP-conjugated secondary antibody before being revealed with ECL substrate (Pierce Biotechnology).

Statistical analysis

Statistical analysis was performed with the GraphPad (San Diego, CA). Group comparisons were analyzed using Kruskal-Wallis or Student's t test. Statistical data were expressed as mean ± SD. P value less than 0.05 was considered statistically significant. The microRNA classifier (MSC) test was performed by logistic regression analysis following previously reported standard operating procedures with fixed parameters [50]. The sensitivity, specificity, and area under the receiver operating characteristic curve (AUC) were used to evaluate the performance of the classifiers.

Compliance with ethics guidelines

The study was performed in accordance with relevant guidelines and regulations, following the approval of the licensing committee of Tongji Medical College, Huazhong University of Science and Technology, and conformed to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients with healthy subjects. We thank the participants of the study.

AUTHOR CONTRIBUTIONS

GHY contributed to the conception and design of the study and developed the study protocol. ZYY, JJX, LY, FW, and JSF performed the experiments and data analysis. JJX, YJ, QH and GHY collected the subject's samples and analyzed clinical data. All the authors contributed to the manuscript writing and revision.

CONFLICTS OF INTEREST

All the authors declare that they have no conflicts of interest.

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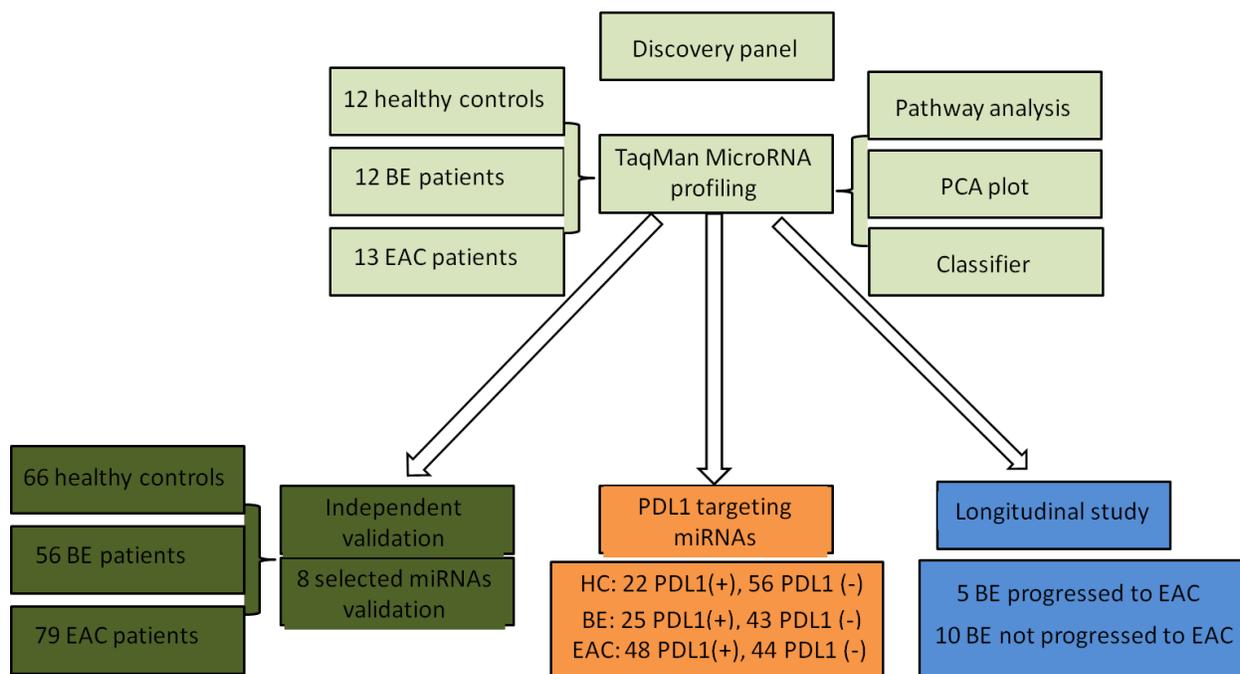
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SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. The workflow in this study.

Supplementary Table

Supplementary Table 1. Biologic pathways enriched by differentially expressed serum miRNAs.

KEGG pathway	p-value	#genes	#miRNAs
Top of Form		Top of Form	
Fatty acid biosynthesis (hsa00061) Bottom of Form	2.238E-12	7Bottom of Form	6
Top of Form		Top of Form	
Hippo signaling pathway (hsa04390) Bottom of Form	2.604E-06	70Bottom of Form	15
Top of Form		Top of Form	
Axon guidance (hsa04360) Bottom of Form	2.604E-06	69Bottom of Form	16
Top of Form		Top of Form	
Proteoglycans in cancer (hsa05205)Bottom of Form	3.331E-05	93Bottom of Form	18
Top of Form		Top of Form	
Glioma (hsa05214) Bottom of Form	5.042E-05	35Bottom of Form	15
Top of Form		Top of Form	
Adrenergic signaling in cardiomyocytes(hsa04261) Bottom of Form	9.324E-05	69Bottom of Form	17
Top of Form		Top of Form	
Neurotrophin signaling pathway (hsa04722) Bottom of Form	9.324E-05	65Bottom of Form	18
Top of Form		Top of Form	
ErbB signaling pathway (hsa04012)Bottom of Form	1.001E-04	50Bottom of Form	18
Top of Form		Top of Form	
Glutamatergic synapse (hsa04724) Bottom of Form	1.972E-04	53Bottom of Form	15
Top of Form		Top of Form	
Oxytocin signaling pathway (hsa04921)Bottom of Form	2.029E-04	79Bottom of Form	16
Top of Form		Top of Form	
FoxO signaling pathway (hsa04068) Bottom of Form	2.363E-04	67Bottom of Form	16
Top of Form		Top of Form	
Long-term potentiation (hsa04720) Bottom of Form	4.527E-04	39Bottom of Form	15
Top of Form		Top of Form	
cGMP-PKG signaling pathway (hsa04022) Bottom of Form	5.394E-04	79Bottom of Form	18
Top of Form		Top of Form	
mTOR signaling pathway (hsa04150) Bottom of Form	7.141E-04	35Bottom of Form	15
Top of Form		Top of Form	
Wnt signaling pathway (hsa04310) Bottom of Form	7.141E-04	66Bottom of Form	16
Top of Form	7.141E-04	Top of Form	18
Signaling pathways regulating pluripotency of stem cells (hsa04550) Bottom of Form		67Bottom of Form	
Top of Form		Top of Form	
PI3K-Akt signaling pathway (hsa04151) Bottom of Form	7.141E-04	146Bottom of Form	19
Top of Form		Top of Form	
Acute myeloid leukemia (hsa05221) Bottom of Form	1.070E-03	33Bottom of Form	15
Top of Form		Top of Form	
Long-term depression (hsa04730) Bottom of Form	1.318E-03	31Bottom of Form	11
Top of Form		Top of Form	
Ras signaling pathway (hsa04014) Bottom of Form	1.318E-03	100Bottom of Form	18
Top of Form		Top of Form	
Amphetamine addiction (hsa05031)Bottom of Form	1.354E-03	33Bottom of Form	16
Top of Form		Top of Form	
Prostate cancer (hsa05215) Bottom of Form	1.373E-03	46Bottom of Form	15

Top of Form		Top of Form	
Pathways in cancer (hsa05200) Bottom of Form	1.373E-03	167Bottom of Form	18
Top of Form		Top of Form	
Gap junction (hsa04540) Bottom of Form	1.412E-03	39Bottom of Form	15
Top of Form		Top of Form	
Thyroid hormone signaling pathway (hsa04919) Bottom of Form	1.412E-03	53Bottom of Form	19
Top of Form		Top of Form	
Glycosaminoglycan biosynthesis (hsa00534) Bottom of Form	1.864E-03	11Bottom of Form	10
Top of Form		Top of Form	
Non-small cell lung cancer (hsa05223) Bottom of Form	1.864E-03	28Bottom of Form	15
Top of Form		Top of Form	
Rap1 signaling pathway (hsa04015) Bottom of Form	1.864E-03	94Bottom of Form	19
Top of Form		Top of Form	
AMPK signaling pathway (hsa04152) Bottom of Form	2.614E-03	60Bottom of Form	18
Top of Form		Top of Form	
MAPK signaling pathway (hsa04010) Bottom of Form	3.176E-03	113Bottom of Form	19
