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Circulating miR-15b and miR-130b in serum as potential markers for detecting hepatocellular carcinoma: a cohort study

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Article Summary

Article focus

- More than half of the patients with hepatocellular carcinoma (HCC) are not eligible for curative treatments, because of the advanced tumor stages at the time of diagnosis.
- Serum alpha-fetoprotein (AFP) has long been used as a biomarker for HCC screening, but its sensitivity and accuracy are only modest.
- Circulating microRNAs are highly stable in blood and have a potential to become promising cancer biomarkers

Key messages

- This study identifies combined serum miR-15b and miR-130b as a classifier that provides high sensitivity and accuracy for detection of HCC.
- The classifier outperformed AFP in discriminating HCC cases from non-cancerous controls. In addition, it identified early-stage HCC cases that could not be detected by AFP.
- These findings collectively suggest that the microRNA classifier has clinical value and might be used for HCC screening program, which could increase chances of HCC patients for curative treatments.

Strengths and limitations of this study

- The proposed classifier have been validated in an independent cohort of serum samples that include HCC patients, chronic hepatitis B carriers, and healthy controls, suggesting the high positive predictive rate of the circulating microRNA classifier and could be used as non-invasive biomarkers for HCC.
- Other variables may affect the diagnostic accuracy of the classifier, for example, the present of hepatitis C virus, which is also a risk factor for HCC. The application of the classifier in HCV-related HCC is yet to be validated.

Abstract

Objective: Serum α-fetoprotein (AFP) is the most commonly used biomarker for screening hepatocellular carcinoma (HCC), but fails to detect about half of the patients. Thus, we investigated if circulating microRNAs (miRNAs) could outperform AFP for HCC detection.

Design: A retrospective cohort study.

Setting: Two clinical centers in China.

Participants: The exploration phase included 96 HCC patients who received primary curative hepatectomy, and the validation phase included 29 hepatitis B carriers, 57 HCC patients, and 30 healthy controls.

Main outcome measures: Expression of miRNAs was measured by real-time quantitative RT-PCR. Areas under receiver operating characteristic curves were used to determine the feasibility of using serum miRNA concentration as a diagnostic marker for defining HCC. A multivariate logistic regression analysis was used to evaluate performances of combined serum miRNAs.

Results: In the exploration phase, miRNA profiling on resected tumor/adjacent non-tumor tissues identified miR-15b, miR-21, miR-130b, and miR-183 highly expressed in tumors. These miRNAs were also detectable in culture supernatants of HCC cell lines and in patients' serum samples. Remarkably, these serum miRNAs were markedly reduced after surgery, indicating the tumor-derived source of these circulating miRNAs. In a cross-center validation study, combined miR-15b and miR-130b demonstrated as a classifier for HCC detection, yielding a receiver operating characteristic curve area of 0.98 (98.2% sensitivity and 91.5% specificity). The detection sensitivity of the classifier in a subgroup of HCCs with low AFP (<20 ng/mL) was 96.7%. The classifier also identified early-stage HCC cases that could not be detected by AFP.

Conclusion: The combined miR-15b and miR-130b classifier is a serum biomarker with clinical value for HCC screening.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of malignancy of liver cancer, with high incidences (>500,000 cases per year) in hepatitis B virus (HBV) endemic regions including China, Southeast Asia and Sub-Sahara Africa. [1, 2] HCC is also highly malignant and lethal, with an overall 5-year survival rate at 5-9% from the time of clinical diagnosis. The dismal prognosis is largely caused by late detection of the tumors when standard surgery is not operable, high recurrence rate of the malignancy and resistance to chemotherapy. [3, 4] Approximately 80% of HCC patients are untreatable because of advanced tumor stages at presentation; nevertheless, curative hepatectomy can improve the 5-year survival rate to 69% if the tumor is detected earlier, particularly when the tumor is single nodule and smaller than 2 cm. [5, 6]

Diagnosis of HCC is usually based on imaging techniques (abdominal ultrasound, MRI and contrast-enhanced CT scan showing a suspicious liver lesion), elevated serum α-fetoprotein (AFP) or optional biopsy. Advances in MRI and CT scan have greatly improved imaging of focal hypervascular mass consistent with HCC, but these procedures are costly and not readily available in developing countries. Ultrasonography can detect large lesion, but fails to detect small tumor, especially in obese patients and those with underlying liver cirrhosis; and the procedures are operator-dependent, varying the diagnostic accuracy. Serum AFP has long been used as a tumor marker for HCC screening — AFP < 20 ng/mL is considered as normal and AFP > 400 ng/mL as true positive in general. The EMSO guidelines also recommended elevation of AFP > 400 ng/ml can be used instead of fine needle cytology for diagnosis, especially in patients with liver cirrhosis.[7] Despite that, the sensitivity of AFP is only modest (sensitivity: 39-65% and specificity: 76-94%), leaving approximate one-third of the early-stage HCC patients with small tumors (< 3 cm) undiagnosed. [8] On the other hand, serum AFP level is elevated in benign liver diseases, such as hepatitis and cirrhosis. [9] Thus, there is still pressing need for circulating biomarkers to detect early HCC in at-risk populations (patients with chronic hepatitis and liver cirrhosis).

MicroRNAs (miRNAs) are a class of non-coding small RNAs that regulate expression of genes at post-transcriptional level. They are involved in various

biological processes including development, differentiation, signal transduction, and carcinogenesis [10, 11]. The use of miRNA as cancer biomarker is of particular interest because (1) different cancer types have distinct miRNA expression profiles; [12] (2) cancers could affect miRNA levels in the bloodstream, [13] although the exact mechanisms through which the miRNAs are being released extracellularly remain unclear; and (3) miRNAs could be detected in blood plasma or serum with high stability [13, 14]. They are well protected from RNases and remain stable after harsh conditions. To explore the clinical applicability of miRNAs as non-invasive circulating HCC biomarker, we investigated the expression profile of miRNAs in tumor tissues and selected candidate miRNA biomarkers. These biomarkers were evaluated in a set of serum samples from the same patient cohort. Last but not least, we further validated the miRNA biomarkers for detecting HCC in a separate cohort of serum samples from another clinical center.

Patients and Methods

Patients

Figure 1 shows the schematic flow chart for this miRNA biomarker discovery study. We first profiled two hundred twenty miRNAs using real-time quantitative PCR (qPCR) on frozen tumor and matched adjacent non-tumor tissues from ninety-six Chinese HCC patients who received primary curative hepatectomy at Queen Mary Hospital (Pokfulam, Hong Kong) between 1990 and 2007.[15-17] Among this cohort, we identified fifteen HCC patients having sufficient amount of matched serum samples collected before and after surgery to allow us measuring the selected miRNAs levels by qPCR. The pre-operative serum samples were collected from 1 to 4 days before surgery, whereas the post-operative serum samples were collected from 8 to 359 days after surgery. The Institutional Review Board of the University of Hong Kong / Hospital Authority Hong Kong West Cluster (HKU / HA HKW IRB) approved this study, and each patient gave his/her written informed consent on the use of the clinical specimens for research.

For the validation study, we obtained 116 serum samples from Chang Zheng Hospital and Eastern Hepatobiliary Surgery Hospital (Shanghai, China), which

included twenty-nine hepatitis B carriers, fifty-seven HCC patients, and thirty healthy controls underwent routine physical examinations with no underlying liver diseases. The hepatitis B carriers were individuals with positive serum hepatitis B surface antigen (HBsAg) for at least 6 months at the time of blood collection, and they had previously shown normal (or minimally raised) levels of liver enzymes and negative for HBsAg. The clinical-pathological characteristics of the participants are summarized in Table 1.

Cell lines

Liver cell lines (MIHA, CL-48, HepG2, Hep3B, PLC, and MHCC-97H) were obtained as previously described. [18, 19] Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Upon confluence, media were collected and spun at 1,200 g for 3 minutes to remove living cells before RNA extraction.

Samples processing and miRNA extraction

Total RNA was extracted from frozen liver tissues using TRIzol (Invitrogen, Carlsbad, CA) as previously described. [15] Blood samples were stored with EDTA, and serum specimens were stored at -80°C until use. Total RNAs from serum samples or cell culture media were extracted using TRIzol LS (Invitrogen). Each of the 500 µl of sera or media was added with 1 mL of TRIzol LS, and then added with 200 µl chloroform. One volume of the aqueous layer was precipitated with 1.5 volumes of absolute ethanol, and the mixture was transferred to RNeasy Mini spin column (Qiagen, Hilden, Germany) for purification according to manufacturer's instructions. Each sample was eluted with 30 µl RNase-free water. The concentrations of RNA extracted from the clinical samples ranged from 3.96 ng/µl to 41.95 ng/µl.

miRNA quantification by real-time qPCR

miRNAs from tumor and adjacent non-tumor tissues were profiled using custom qPCR assays as described [15]. The profiling data are available in Gene Expression Omnibus (GEO), under accession number GSE22058.

Expressions of the potential miRNA biomarkers were quantified in serum samples and HCC cell conditioned media by TaqMan miRNA Assay (Applied Biosystems, Foster City, CA). For the total RNA extracted, 10 ng of RNA was

subjected to the reverse transcription (RT) reactions using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's recommendation. The RT products were then diluted by combining 10 µl RT product with 57.8 µl water. qPCR reactions were performed in duplicate, in scaled-down 10 uL reaction volumes containing 5 µl TaqMan 2X Universal PCR Master Mix with No AmpErase UNG (Applied Biosystems), 0.5 µl 20X miRNA-specific TaqMan probe, and 5 µl diluted RT product (i.e. 1:15 final dilution in the qPCR reaction). The qPCR reactions were run in ABI StepOnePlus real-time PCR system, and data were collected and analyzed with StepOne software (Applied Biosystems).

For each of the TaqMan miRNA assays, we determined the limits of linear quantification and PCR efficiency. We prepared a standard calibration curve by a ten-fold dilution of single-stranded DNA oligonucleotides corresponding to the mature miRNA sequences (ranged from 368 to 3.7 x 10^{10} copies of RT input). The limits of linear quantification of each of the assays were determined with PCR efficiencies of 100± 8% and linearities (Pearson's correlation) > 0.99. To ensure the reliability of the data, values that were near or below the limits of the linear quantification were removed and excluded from further analyses. miRNA concentrations were normalized to the total RNA input and expressed as the number of copies per nanogram of RNA.

Statistical analysis

The statistical differences of miRNA levels between tumor and adjacent non-tumor tissues were determined by paired t-test with univariate *p*-value at < 0.001 and false-discovery rate (FDR) < 0.001. Clustering of the differentially expressed miRNAs was performed with centered correlation and average linkage. The differences of serum miRNAs before and after surgery were studied by Wilcoxon signed-rank test, and *p*-value at < 0.05 was considered statistically significant. Logistic regression analysis was performed to analyze various combinations of miRNA markers. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used to determine the feasibility of using serum miRNA concentration as a diagnostic marker for defining HCC. The Youden's index was used to identify the optimal cut-off point.

Results

Candidate miRNAs are highly expressed in AFP-low tumor tissues

To generate a comprehensive set of miRNA expression profiles for HCC, we analyzed expressions of 220 miRNAs from 96 pairs of HCC tumor and adjacent non-tumor tissues using real-time qPCR as described. [15] Clustering analysis revealed that HCC and non-tumor tissues expressed distinct patterns of miRNAs as shown in the heat map (**Fig. 2a**). Furthermore, statistical analysis using paired t-test identified that 105 miRNAs were differentially expressed with FDR < 0.001.

Given the marked miRNA alteration in tumor tissues, our next question is whether certain miRNAs could be useful for detecting HCC cases, including those with low serum AFP of <400 ng/mL. We identified the following 14 miRNAs up-regulated in the AFP-low HCC subgroup: miR-9, -9*, -15b, -21, -34c, -96, -130b, -183, -188, -196b, -216, -224, -301, and -324-5p. Subsequently, we selected those having high copy number (>100 copies/10 pg input RNA) present in the tumor tissues. Consistently, these 6 miRNAs (miR-15b, -21, -130b, -183, -224, and -301) were highly expressed in all HCC samples of varying serum AFP levels (**Fig. 2b**).

Detection of candidate miRNAs in HCC culture supernatant

Our next selection criterion is whether the miRNAs can be measured or detected in the culture supernatants from a panel of HCC cell lines. We thus assayed by qPCR the above selected miRNAs in culture media conditioned with a panel of HCC cell lines, including HepG2, Hep3B, PLC, and MHCC-97H. Two immortalized human hepatocyte cell lines, CL-48 and MIHA, were included as controls. All of the miRNAs could be detected in the culture media, except miR-301 and miR-224, whose concentrations were beyond the detection limits and thus were excluded from further analyses. We found miR-15b, miR-21, miR-183 and miR-130b had generally higher copy numbers in the medium of HCC cells when compared to CL-48 or MIHA (**Fig. 3**).

Detection of miRNAs in serum samples from HCC patients pre- and post-surgery

To determine if the circulating miRNAs were derived primarily from tumors, we measured their levels in serum samples of 15 selected HCC patients before and after surgery treatment. In agreement with the tissue data as stated above, miR-224

and miR-301 levels were very low in the sera (data not shown). For the other 4 miRNAs, there was a trend of reduction of miRNAs levels after surgery. As shown in **Fig. 4**, the median copies of miR-130b decreased significantly from 124.8 to 96.2 (p = 0.0158), and miR-183 from 153.3 to 95.2 (p = 0.0084). We also observed reductions of miR-15b and miR-21 levels after surgery, although the declination was not statistically significant. The median level of miR-15b was reduced from 177.6 to 64.1 (p = 0.0637), whereas the median level of miR-21 was decreased from 221,404.4 to 100,140.5 (p = 0.0684).

Circulating miR-15b and miR-130b classifier defines HCC cases in an independent cohort

We next verified the discriminative power of these miRNAs in identifying HCC cases in an independent cohort of thirty healthy controls, twenty-nine hepatitis B individuals, and fifty-seven HCC patients. The levels of the four miRNAs in these serum samples were measured, and ROC analysis was performed on individual miRNAs. The cut-off of each miRNA was selected with highest Youden's index. Among the four miRNAs, miR-130b showed the highest AUC of 0.913 (sensitivity of 87.7% and specificity of 81.4%) in separating the non-cancerous controls from those with HCCs, whereas miR-15b gave the highest sensitivity (98.3%), although its specificity (15.3%) was only modest (**Table 2**).

Recognizing the limitations of individual tests, we performed multivariate logistic regression analysis on various combinations of the miRNA biomarkers, and found that the combined miR-15b and miR-130b gave the best performance. It could be a potential classifier for detecting HCC, and the formula of the classifier is as follow:

-2.4966 + 0.0532(copy number of miR-130b) – 0.0210*(copy number of miR-15b)*

The ROC curve of the classifier has an AUC of 0.981 (**Fig 5a**). A sum score of the classifier at -0.61109 was chosen as a cut-off, as it has the highest Youden's index of 0.8977. At this cut-off, the classifier has enhanced sensitivity of 98.3% and specificity of 91.5% (**Fig. 5b**), when compared to the performance of individual miRNAs.

We next tested if the classifier could successfully differentiate a subset of patients with low serum AFP from non-cancerous controls. Three sub-groups were defined: (*a*) HCCs with AFP > 400 ng/mL (n = 11) and non-cancerous controls (n = 59); (*b*) HCCs with AFP between 20 and 400 ng/mL (n = 16) and non-cancerous controls (n = 59); (*c*) HCCs with AFP < 20 ng/mL (n = 30) and non-cancerous controls (n = 59). We found the classifier could accurately distinguish HCC subjects from the controls with high sensitivity and specificity in all cases (**Fig. 5c**). For the HCC subgroup with AFP < 20 ng/mL, the AUC of the classifier is 0.980, with sensitivity of 96.7% and specificity of 91.5%.

Next, we evaluated the performance of miR-15b and miR-130b classifier in detecting early-stage HCC cases. As shown in **Fig. 5d**, the miRNA classifier could accurately identify 97.8% (44 out of 45) HCC cases from both TNM stages I and II, whereas serum AFP (cut-off level at 20 ng/mL) could only detect 48.9% (22 out of 45) of the same cases. In short, the circulating miR-15b and miR-130b is a classifier outperforms the serum AFP as tumor marker in detecting HCC.

Discussion

The present study has developed an unprecedented miRNA scoring system able to identify HCC cases that could not be detected by the conventional AFP tumor marker. Our *in vitro* studies also showed that the extracellular miRNAs are good indicator of the miRNA expression in cancer cells. These finding collectively implicate the potential application of circulating miRNAs as non-invasive serological biomarker for solid malignancy like HCC.

Our tumor biomarker discovery strategy is divided into three phases: *(a) Exploration phase* – to identify candidate markers through molecular profiling of miRNAs in matched tumor and adjacent non-tumor tissues samples from HCC patients (n = 96). *(b) Selection/filtering phase* – to select those miRNAs highly expressed in both tumor tissues as well as in serum samples. In addition, we used pre- and post-operative serum samples from the same HCC patients (n = 15) to further refine that those miRNAs that were likely derived from HCC tumors. After surgical removal of the HCC tumors, serum levels of these miRNAs were significantly reduced. *(c) Cross-center validation phase* – to validate the miRNA biomarkers in an

independent cohort (n = 116) including HCC cases, hepatitis B carriers and non-malignant healthy controls from geographically distant medical center. Herein, our findings showed that the combined miR-15b and miR-130b is a robust classifier in detecting HCC cases and outperforms AFP as tumor marker with high positive predictive value.

Previous studies have suggested the potential use of circulating miRNAs in the diagnosis of HCC. [20, 21] The study of Qu *et al.* suggested that serum miR-16 could be used as a second-line test when the serum AFP and other markers are normal levels [21]. However, in our analysis, we did not find miR-16 significantly differentially expressed between tumor and adjacent normal tissues. Another study using Solexa sequencing on pooled serum samples and identified miR-375 as a candidate biomarker for HBV-positive HCC, which was further validated by TaqMan miRNA qPCR assays [20]. The majority of the HCC cases in our cohort were also HBV-positive, but we did not select miR-375 during our initial screen. This is because the difference was not significant when comparing the HCC tumors of low AFP level with the corresponding normal samples. Nevertheless, we indeed found miR-375 having an important role in regulating the YAP oncogenic signaling pathway in HCC. [22] Further investigation is under way to evaluate the application of miR-375 for identifying subtype of HCC, for which the Hippo pathway and YAP signaling are aberrantly regulated.

Limited information of miR-15b is available in HCC and its molecular mechanisms and roles in pathogenesis remain largely unknown. Consistent with the present findings, miR-15b was recently shown to be overexpressed in HCC tumors, [23] and it is in a pilot cross-sectional, phase 2 biomarker study for Barrett's esophagus progression. [24] Recently, miR-15b has been shown to be a direct transcriptional target of E2F and may be involved in preventing replicative stress in response to mitogenic signaling. [25] On the other hand, the oncogenic role of miR-130b in hepatocarcinogenesis is prominent and is considered as a cancer stem cell miRNA in HCC. First, it has been shown highly expressed in $CD133^+$ tumor-initiating cells in HCC, and transduction of miR-130b into CD133-negative cells could promote tumorigenesis and induce chemoresistance. [26] Second, miR-130b directly targets a well-known tumor suppressor — RUNX3, and regulates expression of pro-apoptotic Bim thereby enhancing cell viability. [27] Despite of these observations, it is still

unknown if miR-15b and miR-130b in circulation contribute to HCC development and tumor progression.

There are mounting evidences indicating that serum-based miRNAs are useful as non-invasive biomarkers for different cancer types. [14, 28-32] Today, circulating miRNAs in serum have been reported as potential diagnostic markers for B-cell lymphoma,[33] leukemia,[34] esophageal squamous cell carcinoma,[35] as well as lung,[36] breast,[37] colon,[38] gastric,[39] pancreatic,[40] prostate[41] and ovarian[42] cancer. A handful of studies showed that miRNAs could be released into bloodstream via active secretion from diseased tissues in forms of protein-bound complex [43] or as membrane-bound vesicles (e.g. exosomes).[44] Because the high rate of proliferation and cell lysis in tumors, non-specific passive release could also exist in cancer that contribute to the abundance of miRNAs in the blood stream. Not only reflecting physiological and pathological changes, these circulating miRNAs could be functional and correlated with tumor progression and clinical outcomes.[32, 45, 46] Most recently, miR-16, -21, -122, and -375 in serum were suggested as potential diagnostic markers for HCC.[20, 21, 47] Nevertheless, their diagnostic performance has not been robustly validated in an independent cohort. Our present findings demonstrate that the circulating miR-15b and miR-130b hold promise as valuable tumor markers for detecting HCC, in which both the early stages and low AFP group were also covered with high sensitivity and specificity. Early detection of HCC could save many lives and enhance the quality of life in patients suffering from this lethal malignancy.

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Competing Interests

No competing interests.

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47. **Xu J**, Wu C, Che X, *et al.* Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *Mol Carcinog* 2011; **50**:136-42.

Figure legend:

Figure 1. Schematic flow chart of miRNA biomarker discovery for detecting HCC. This study was divided into 3 phases: exploration, selection/filtering, and cross-center validation. Two different cohorts of patients were included – one from Hong Kong (n = 96) and one from Shanghai (n = 116).

Figure 2 (**A**) Hierarchical clustering (centered correlation and average linkage) over 105 differentially expressed miRNAs. Heat map colors represent the relative miRNA expression as indicated in the color key. (**B**) Expressions of the candidate miRNA biomarkers in HCC tumor (TU) and adjacent non-tumor (AN) tissues. The tumor tissues were sub-divided into three groups according to the serum AFP levels of the individual patients (AFP < 20 ng/ml, between 20-400 ng/ml, and > 400 ng/ml). The box plots show the median (horizontal bar), 25th and 75th percentiles, and the whiskers of the graph show the largest and smallest values.

Figure 3 TaqMan qPCR assay of extracellular levels of 4 candidate biomarkers, miR-15b, miR-21, miR-130b, and miR-183, in culture medium harvested from four HCC cell lines (HepG2, Hep3B, PLC, and MHCC-97H (97H)) and two immortalized hepatocyte cell lines (CL-48 and MIHA). The assays were repeated at least twice in different samples. The error bars represent mean ± standard error of the mean (SEM).

Figure 4. Declination of serum miRNA markers in HCC patients after surgery treatment. Changes in serum levels of miR-15b, miR-21, miR-130b, and miR-183 in HCC patients (n=15) before (pre-op) and after (post-op) surgical removal of the tumors. Statistical differences were analyzed using Wilcoxon signed-rank test. Table in the lower panel indicates the median level of the individual miRNAs before and after surgery.

Figure 5 Performance of the miR-15b and miR-130b classifier for detection of HCC in an independent cohort. **(A)** ROC curve analysis of the miRNA classifier over HCC

patients and non-cancerous control subjects (healthy and chronic hepatitis B carrier). The miRNA classifier was derived from the logistic regression model that composed of two miRNAs, i.e. miR-15b and miR-130b. **(B)** Dot plots of the sum score of the logistic regression model in non-cancerous control subjects (Control) and HCC patients (HCC). The sensitivity, specificity, and accuracy were calculated based on the cutoff at -0.611 that was determined by the Youden's Index. **(C)** ROC curve analyses of the miRNA classifier over control subjects (healthy and chronic hepatitis B individuals) and sub-group of HCC patients with AFP levels > 400, between 20 and 400 ng/mL, or < 20 ng/mL. The sensitivity, specificity, false positive rate, false negative rate, and accuracy at a cut-off of -0.611 were indicated below each ROC graph. **(D)** AFP and miRNA classifier (miR-15b and miR-130b) were compared in HCC cases between early (I and II) and late (III and IV) stages. The cut-off values were marked by grey lines.

Table 1. Demographic and clinicopathologic parameters of patients from different cohorts

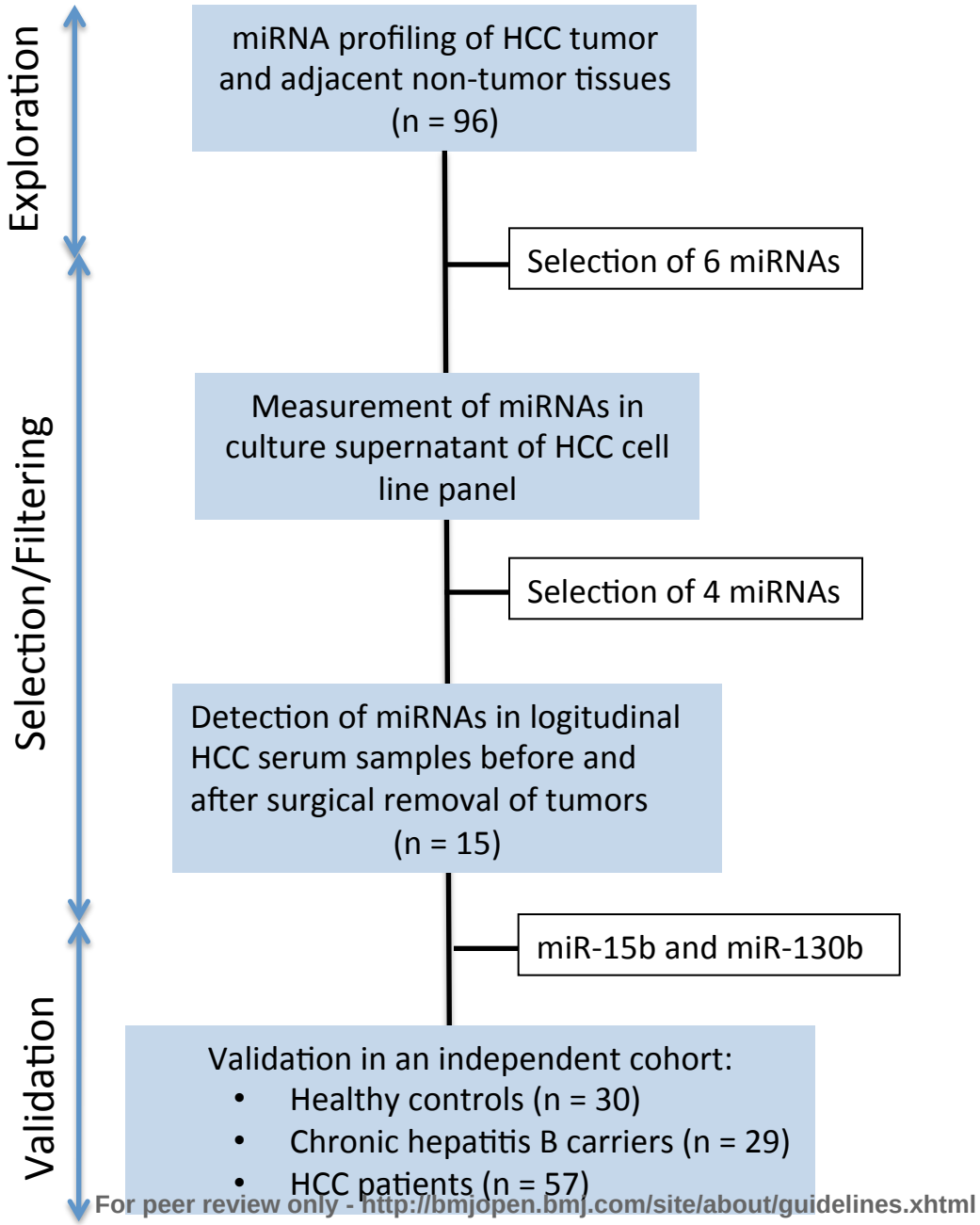
Parameters		Hong Kong cohort (n = 96)	Shanghai Cohort (n = 116)		
		HCC patients	Healthy controls	Hepatitis B carriers	HCC patients
Sex					
	Male	78	22	20	49
	Female	18	8	9	8
Age					
	< 60	60	30	28	47
	≥ 60	36	0	1	10
Alpha fetoprotein	**(ng/ml)**				
	< 20	39	-	29	30
	20-400	26	-	0	16
	> 400	31	-	0	11
Alanine aminotransferase	**(U/L)**				
	< 35	28	-	20	29
	≥ 35	68	-	9	28
Aspartate aminotransferase (U/L)					
	< 35	23	-	22	24
	≥ 35	73	-	4	33
Hepatitis B surface antigen					
	Negative	12	-	0	0
	Positive	84	-	29	57
Pathological tumor-node-metastasis (TNM) stage*					
	Early (I, II)	41	-	-	45
	Late (III, IV)	55	-	-	11
Child-Pugh§					
	A	91	-	-	54
	B	3	-	-	2
	C	0	-	-	0
Tumor size (cm)*					
	< 5 cm	26	-	-	27
	≥ 5 cm	70	-	-	29

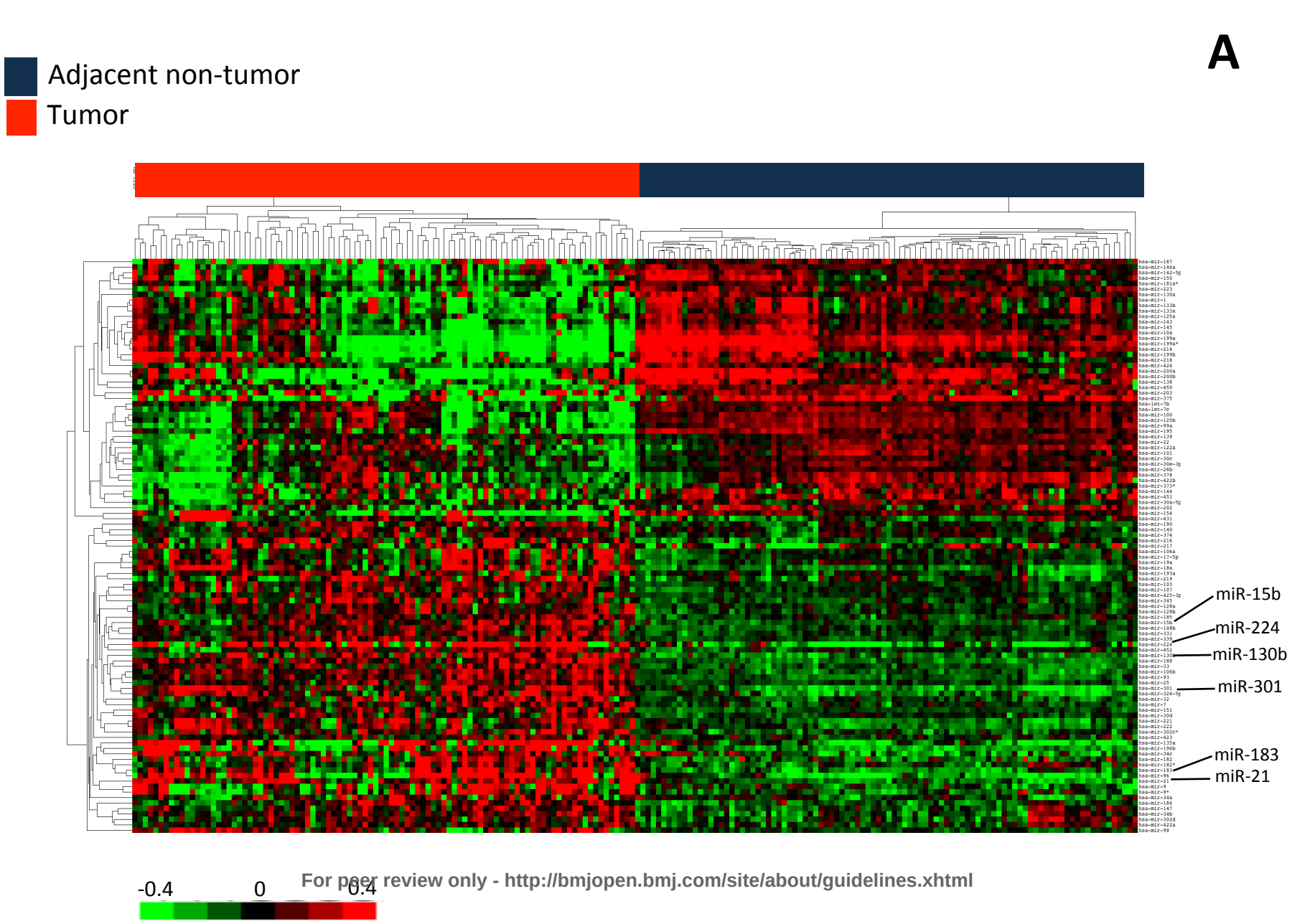
*Data indicated in the cohort from Shanghai are based on 56 cases.
§ Data indicated in the cohorts from Hong Kong and Shanghai are based on 94 and 56 cases, respectively.

Table 2. Predictive performance of individual serum miRNAs and miRNA classifier (miR-15b and miR-130b) as biomarkers for detection of HCC

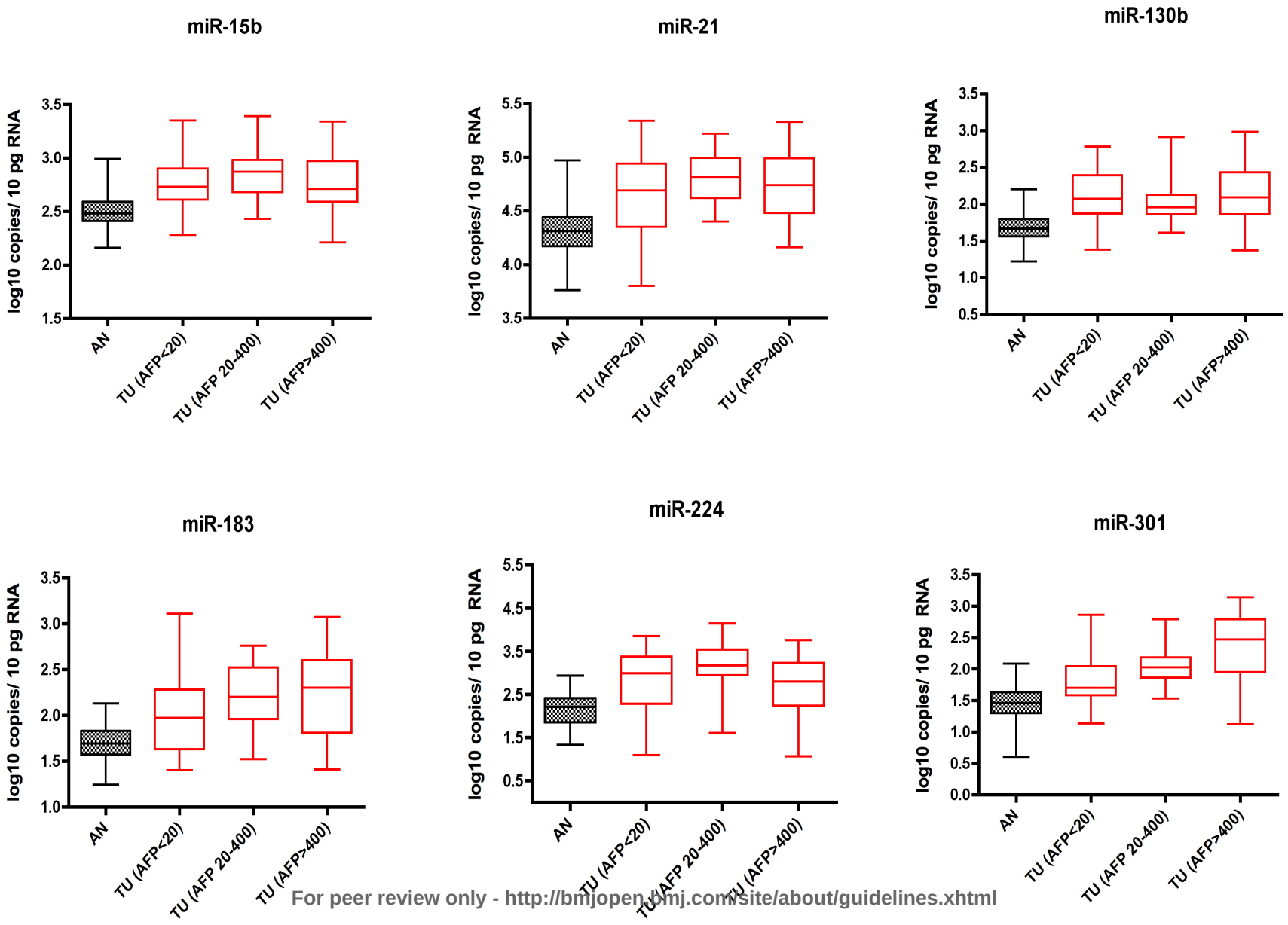
Statistical parameters	Non-cancerous controls (n=59) vs. HCCs (n =57)				
	miR-15b (≥43.485 copies/ng)	**miR-21 (≥73271.020 copies/ng)**	**miR-130b (≥97.250 copies/ng)**	**miR-183 (≥40.100 copies/ng)**	**miR-15b + miR-130b (≥ -0.6111 sum score)**
Sensitivity	98.25%	89.47%	87.72%	57.89%	98.25%
Specificity	15.25%	71.19%	81.36%	69.49%	91.53%
False positive rate	84.75%	28.81%	18.64%	30.51%	8.47%
False negative rate	1.75%	10.53%	12.28%	42.11%	1.75%
Accuracy	56.03%	80.17%	84.48%	63.79%	94.83%
Positive predictive value	52.83%	75.00%	81.97%	64.71%	91.80%
Negative predictive value	90.00%	87.50%	87.27%	63.08%	98.18%
Positive likelihood ratio	1.159	3.105	4.705	1.898	11.593
Negative likelihood ratio	0.115	0.148	0.151	0.606	0.019
Youden's index	0.135	0.607	0.691	0.274	0.898
Area under the ROC (AUC)	0.485	0.865	0.913	0.661	0.981

Figure 1





B



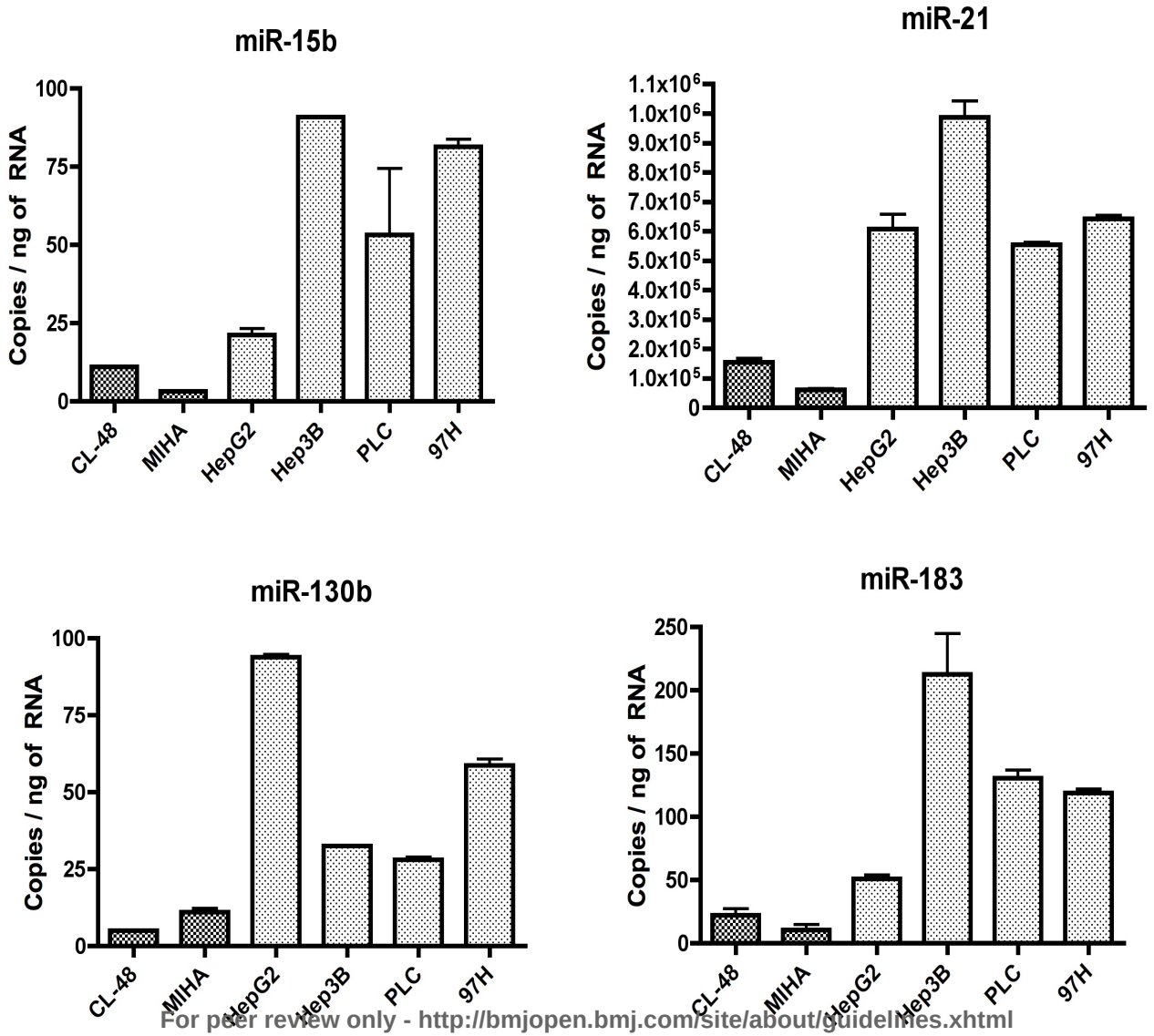
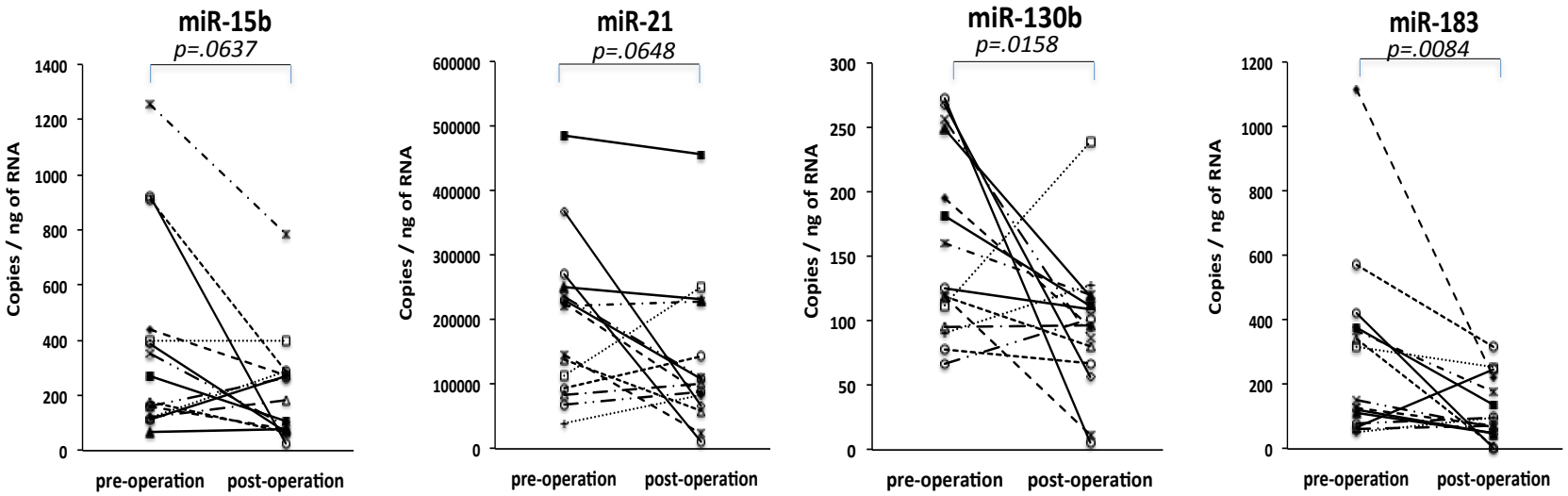


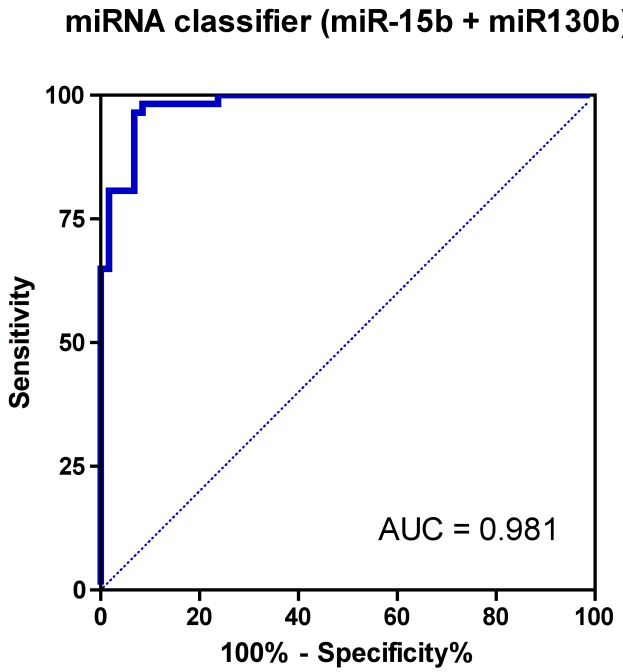
Figure 4



Median levels (copies/ ng RNA)	miR-15b	miR-21	miR-130b	miR-183
pre-operation	177.61	221404.42	124.82	153.27
post-operation	64.13	100140.49	96.18	95.19

Figure 5

A


miRNA classifier (miR-15b + miR130b)
Sensitivity
100
75
50
25
0
0
20
40
60
80
100
100% - Specificity%
AUC = 0.981

B

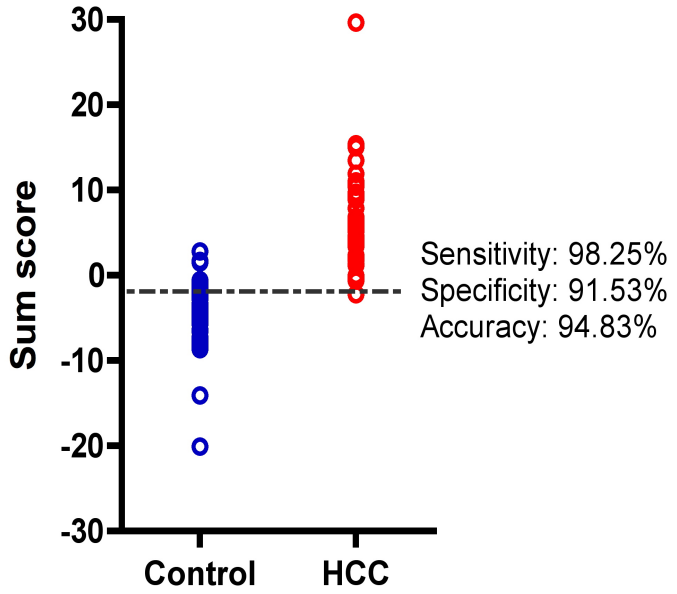
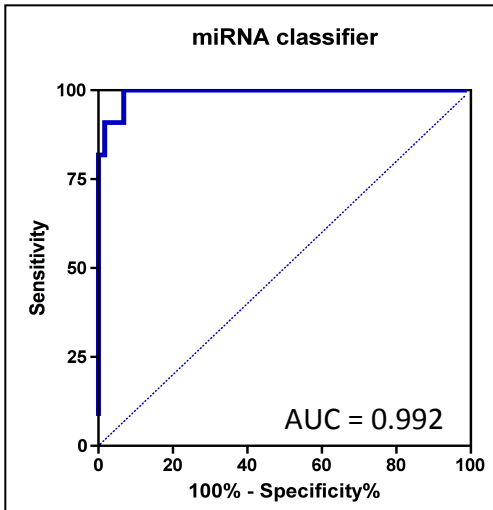
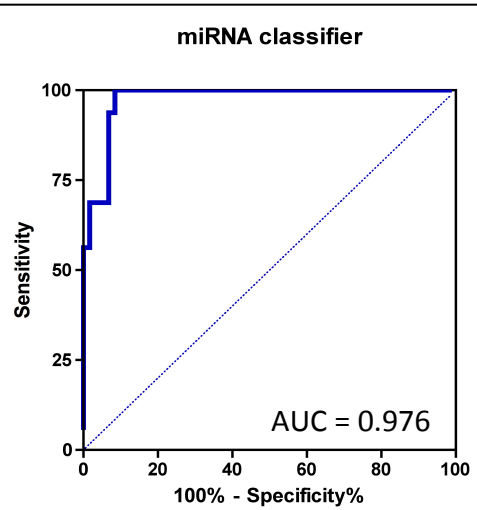
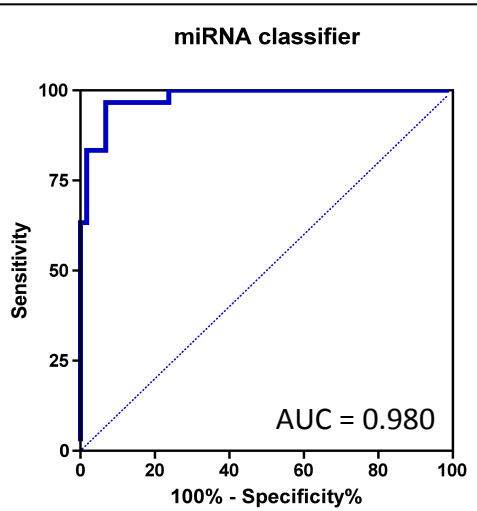

Sum score
30
20
10
0
-10
-20
-30
Control
HCC
Sensitivity: 98.25%
Specificity: 91.53%
Accuracy: 94.83%

Figure 5

C



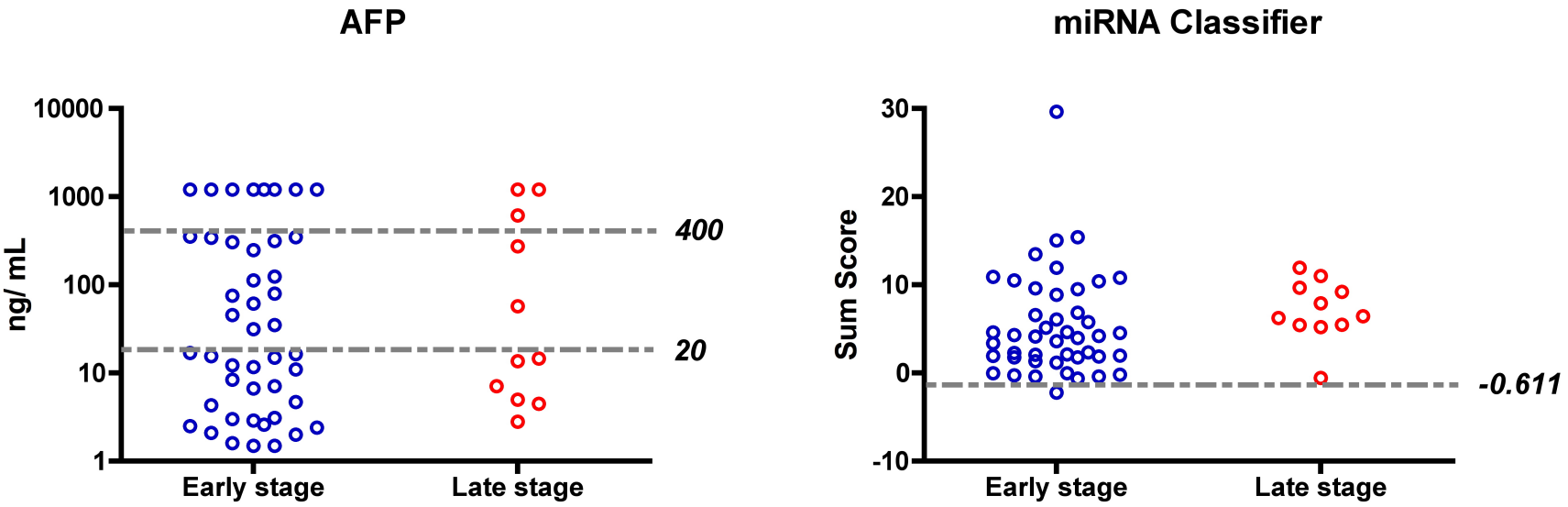




Cut-off at -0.611	Controls (n = 59) vs. HCCs with AFP > 400 (n = 11)	Controls (n = 59) vs. HCCs with AFP 20 - 400 (n = 16)	Controls (n = 59) vs. HCCs with AFP < 20 (n = 30)
Sensitivity	100.00%	100.00%	96.67%
Specificity	91.53%	91.53%	91.53%
False positive rate	8.47%	8.47%	8.47%
False negative rate	0.00%	0.00%	3.33%
Arruracy	92.86%	93.33%	93.26%

Figure 5

D


AFP
10000
1000
100
10
1
ng/ mL
400
20
Early stage
Late stage
miRNA Classifier
30
20
10
0
-10
Sum Score
-0.611
Early stage
Late stage

STARD checklist for reporting of studies of diagnostic accuracy

(version January 2003)

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	2
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	3
METHODS			
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	4
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	4
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	4-5
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	4-5
Test methods	7	The reference standard and its rationale.	4-5
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	5-6
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	6
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	6
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	6
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	6
	13	Methods for calculating test reproducibility, if done.	6
RESULTS			
Participants	14	When study was performed, including beginning and end dates of recruitment.	4-5
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	17
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	19
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	7-9
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	7-9
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	7-9
	20	Any adverse events from performing the index tests or the reference standard.	N/A
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	7-9
	22	How indeterminate results, missing data and outliers of the index tests were handled.	6
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	7-9
	24	Estimates of test reproducibility, if done.	7-9
DISCUSSION	25	Discuss the clinical applicability of the study findings.	9-11

BMJ open

Circulating miR-15b and miR-130b in serum as potential markers for detecting hepatocellular carcinoma: a cohort study

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Primary Subject Heading:	Diagnostics
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Keywords:	Hepatology < INTERNAL MEDICINE, MOLECULAR BIOLOGY, Clinical chemistry < PATHOLOGY

BMJ Open – Research article

Circulating miR-15b and miR-130b in serum as potential markers for detecting hepatocellular carcinoma: a cohort study

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Subject headings: Serum-based microRNA biomarkers for hepatocellular carcinoma

Article Summary

Article focus

- More than half of the patients with hepatocellular carcinoma (HCC) are not eligible for curative treatments, because of the advanced tumor stages at the time of diagnosis.
- Serum alpha-fetoprotein (AFP) has long been used as a biomarker for HCC screening, but its sensitivity and accuracy are only modest.
- Circulating microRNAs are highly stable in blood and have a potential to become promising cancer biomarkers

Key messages

- This study identifies combined serum miR-15b and miR-130b as a classifier that provides high sensitivity and accuracy for detection of HCC.
- The classifier outperformed AFP in discriminating HCC cases from non-cancerous controls. In addition, it identified early-stage HCC cases that could not be detected by AFP.
- These findings collectively suggest that the microRNA classifier has clinical value and might be used for HCC screening program, which could increase chances of HCC patients for curative treatments.

Strengths and limitations of this study

- The proposed classifier have been validated in an independent cohort of serum samples that include HCC patients, chronic hepatitis B carriers, and healthy controls, suggesting the high positive predictive rate of the circulating microRNA classifier and could be used as non-invasive biomarkers for HCC.
- Other variables may affect the diagnostic accuracy of the classifier, for example, the present of hepatitis C virus, which is also a risk factor for HCC. The application of the classifier in HCV-related HCC is yet to be validated.

Abstract

Objective: Serum α-fetoprotein (AFP) is the most commonly used biomarker for screening hepatocellular carcinoma (HCC), but fails to detect about half of the patients. Thus, we investigated if circulating microRNAs (miRNAs) could outperform AFP for HCC detection.

Design: A retrospective cohort study.

Setting: Two clinical centers in China.

Participants: The exploration phase included 96 HCC patients who received primary curative hepatectomy, and the validation phase included 29 hepatitis B carriers, 57 HCC patients, and 30 healthy controls.

Main outcome measures: Expression of miRNAs was measured by real-time quantitative RT-PCR. Areas under receiver operating characteristic curves were used to determine the feasibility of using serum miRNA concentration as a diagnostic marker for defining HCC. A multivariate logistic regression analysis was used to evaluate performances of combined serum miRNAs.

Results: In the exploration phase, miRNA profiling on resected tumor/adjacent non-tumor tissues identified miR-15b, miR-21, miR-130b, and miR-183 highly expressed in tumors. These miRNAs were also detectable in culture supernatants of HCC cell lines and in patients' serum samples. Remarkably, these serum miRNAs were markedly reduced after surgery, indicating the tumor-derived source of these circulating miRNAs. In a cross-center validation study, combined miR-15b and miR-130b demonstrated as a classifier for HCC detection, yielding a receiver operating characteristic curve area of 0.98 (98.2% sensitivity and 91.5% specificity). The detection sensitivity of the classifier in a subgroup of HCCs with low AFP (<20 ng/mL) was 96.7%. The classifier also identified early-stage HCC cases that could not be detected by AFP.

Conclusion: The combined miR-15b and miR-130b classifier is a serum biomarker with clinical value for HCC screening.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of malignancy of liver cancer, with high incidences (>500,000 cases per year) in hepatitis B virus (HBV) endemic regions including China, Southeast Asia and Sub-Sahara Africa. [1, 2] HCC is also highly malignant and lethal, with an overall 5-year survival rate at 5-9% from the time of clinical diagnosis. The dismal prognosis is largely caused by late detection of the tumors when standard surgery is not operable, high recurrence rate of the malignancy and resistance to chemotherapy. [3, 4] Approximately 80% of HCC patients are untreatable because of advanced tumor stages at presentation; nevertheless, curative hepatectomy can improve the 5-year survival rate to 69% if the tumor is detected earlier, particularly when the tumor is single nodule and smaller than 2 cm. [5, 6]

Diagnosis of HCC is usually based on imaging techniques (abdominal ultrasound, MRI and contrast-enhanced CT scan showing a suspicious liver lesion), elevated serum α-fetoprotein (AFP) or optional biopsy. Advances in MRI and CT scan have greatly improved imaging of focal hypervascular mass consistent with HCC, but these procedures are costly and not readily available in developing countries. Ultrasonography can detect large lesion, but fails to detect small tumor, especially in obese patients and those with underlying liver cirrhosis; and the procedures are operator-dependent, varying the diagnostic accuracy. Serum AFP has long been used as a tumor marker for HCC screening — AFP < 20 ng/mL is considered as normal and AFP > 400 ng/mL as true positive in general. The EMSO guidelines also recommended elevation of AFP > 400 ng/ml can be used instead of fine needle cytology for diagnosis, especially in patients with liver cirrhosis.[7] Despite that, the sensitivity of AFP is only modest (sensitivity: 39-65% and specificity: 76-94%), leaving approximate one-third of the early-stage HCC patients with small tumors (< 3 cm) undiagnosed. [8] On the other hand, serum AFP level is elevated in benign liver diseases, such as hepatitis and cirrhosis. [9] Thus, there is still pressing need for circulating biomarkers to detect early HCC in at-risk populations (patients with chronic hepatitis and liver cirrhosis).

MicroRNAs (miRNAs) are a class of non-coding small RNAs that regulate expression of genes at post-transcriptional level. They are involved in various

biological processes including development, differentiation, signal transduction, and carcinogenesis [10, 11]. The use of miRNA as cancer biomarker is of particular interest because (1) different cancer types have distinct miRNA expression profiles; [12] (2) cancers could affect miRNA levels in the bloodstream, [13] although the exact mechanisms through which the miRNAs are being released extracellularly remain unclear; and (3) miRNAs could be detected in blood plasma or serum with high stability [13, 14]. They are well protected from RNases and remain stable after harsh conditions. To explore the clinical applicability of miRNAs as non-invasive circulating HCC biomarker, we investigated the expression profile of miRNAs in tumor tissues and selected candidate miRNA biomarkers. These biomarkers were evaluated in a set of serum samples from the same patient cohort. Last but not least, we further validated the miRNA biomarkers for detecting HCC in a separate cohort of serum samples from another clinical center.

Patients and Methods

Patients

Figure 1 shows the schematic flow chart for this miRNA biomarker discovery study. We first profiled two hundred twenty miRNAs using real-time quantitative PCR (qPCR) on frozen tumor and matched adjacent non-tumor tissues from ninety-six Chinese HCC patients who received primary curative hepatectomy at Queen Mary Hospital (Pokfulam, Hong Kong) between 1990 and 2007.[15-17] Among this cohort, we identified fifteen HCC patients having sufficient amount of matched serum samples collected before and after surgery to allow us measuring the selected miRNAs levels by qPCR. The pre-operative serum samples were collected from 1 to 4 days before surgery, whereas the post-operative serum samples were collected from 8 to 359 days after surgery. The Institutional Review Board of the University of Hong Kong / Hospital Authority Hong Kong West Cluster (HKU / HA HKW IRB) approved this study, and each patient gave his/her written informed consent on the use of the clinical specimens for research.

For the validation study, we obtained 116 serum samples from Chang Zheng Hospital and Eastern Hepatobiliary Surgery Hospital (Shanghai, China), which

included twenty-nine hepatitis B carriers, fifty-seven HCC patients, and thirty healthy controls underwent routine physical examinations with no underlying liver diseases. The hepatitis B carriers were individuals with positive serum hepatitis B surface antigen (HBsAg) for at least 6 months at the time of blood collection, and they had previously shown normal (or minimally raised) levels of liver enzymes and negative for HBsAg. The clinical-pathological characteristics of the participants are summarized in Table 1.

Cell lines

Liver cell lines (MIHA, CL-48, HepG2, Hep3B, PLC, and MHCC-97H) were obtained as previously described. [18, 19] Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Upon confluence, media were collected and spun at 1,200 g for 3 minutes to remove living cells before RNA extraction.

Samples processing and miRNA extraction

Total RNA was extracted from frozen liver tissues using TRIzol (Invitrogen, Carlsbad, CA) as previously described. [15] Blood samples were stored with EDTA, and serum specimens were stored at -80°C until use. Total RNAs from serum samples or cell culture media were extracted using TRIzol LS (Invitrogen). Each of the 500 µl of sera or media was added with 1 mL of TRIzol LS, and then added with 200 µl chloroform. One volume of the aqueous layer was precipitated with 1.5 volumes of absolute ethanol, and the mixture was transferred to RNeasy Mini spin column (Qiagen, Hilden, Germany) for purification according to manufacturer's instructions. Each sample was eluted with 30 µl RNase-free water. The concentrations of RNA extracted from the clinical samples ranged from 3.96 ng/µl to 41.95 ng/µl.

miRNA quantification by real-time qPCR

miRNAs from tumor and adjacent non-tumor tissues were profiled using custom qPCR assays as described [15]. The profiling data are available in Gene Expression Omnibus (GEO), under accession number GSE22058.

Expressions of the potential miRNA biomarkers were quantified in serum samples and HCC cell conditioned media by TaqMan miRNA Assay (Applied Biosystems, Foster City, CA). For the total RNA extracted, 10 ng of RNA was

subjected to the reverse transcription (RT) reactions using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's recommendation. The RT products were then diluted by combining 10 µl RT product with 57.8 µl water. qPCR reactions were performed in duplicate, in scaled-down 10 uL reaction volumes containing 5 µl TaqMan 2X Universal PCR Master Mix with No AmpErase UNG (Applied Biosystems), 0.5 µl 20X miRNA-specific TaqMan probe, and 5 µl diluted RT product (i.e. 1:15 final dilution in the qPCR reaction). The qPCR reactions were run in ABI StepOnePlus real-time PCR system, and data were collected and analyzed with StepOne software (Applied Biosystems).

For each of the TaqMan miRNA assays, we determined the limits of linear quantification and PCR efficiency. We prepared a standard calibration curve by a ten-fold dilution of single-stranded DNA oligonucleotides corresponding to the mature miRNA sequences (ranged from 368 to 3.7 x 10^{10} copies of RT input). The limits of linear quantification of each of the assays were determined with PCR efficiencies of 100± 8% and linearities (Pearson's correlation) > 0.99. To ensure the reliability of the data, values that were near or below the limits of the linear quantification were removed and excluded from further analyses. miRNA concentrations were normalized to the total RNA input and expressed as the number of copies per nanogram of RNA.

Statistical analysis

The statistical differences of miRNA levels between tumor and adjacent non-tumor tissues were determined by paired t-test with univariate *p*-value at < 0.001 and false-discovery rate (FDR) < 0.001. Clustering of the differentially expressed miRNAs was performed with centered correlation and average linkage. The differences of serum miRNAs before and after surgery were studied by Wilcoxon signed-rank test, and *p*-value at < 0.05 was considered statistically significant. Logistic regression analysis was performed to analyze various combinations of miRNA markers. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used to determine the feasibility of using serum miRNA concentration as a diagnostic marker for defining HCC. The Youden's index was used to identify the optimal cut-off point.

Results

Candidate miRNAs are highly expressed in AFP-low tumor tissues

To generate a comprehensive set of miRNA expression profiles for HCC, we analyzed expressions of 220 miRNAs from 96 pairs of HCC tumor and adjacent non-tumor tissues using real-time qPCR as described. [15] Clustering analysis revealed that HCC and non-tumor tissues expressed distinct patterns of miRNAs as shown in the heat map (**Fig. 2a**). Furthermore, statistical analysis using paired t-test identified that 105 miRNAs were differentially expressed with FDR < 0.001.

Given the marked miRNA alteration in tumor tissues, our next question is whether certain miRNAs could be useful for detecting HCC cases, including those with low serum AFP of <400 ng/mL. We identified the following 14 miRNAs up-regulated in the AFP-low HCC subgroup: miR-9, -9*, -15b, -21, -34c, -96, -130b, -183, -188, -196b, -216, -224, -301, and -324-5p. Subsequently, we selected those having high copy number (>100 copies/10 pg input RNA) present in the tumor tissues. Consistently, these 6 miRNAs (miR-15b, -21, -130b, -183, -224, and -301) were highly expressed in all HCC samples of varying serum AFP levels (**Fig. 2b**).

Detection of candidate miRNAs in HCC culture supernatant

Our next selection criterion is whether the miRNAs can be measured or detected in the culture supernatants from a panel of HCC cell lines. We thus assayed by qPCR the above selected miRNAs in culture media conditioned with a panel of HCC cell lines, including HepG2, Hep3B, PLC, and MHCC-97H. Two immortalized human hepatocyte cell lines, CL-48 and MIHA, were included as controls. All of the miRNAs could be detected in the culture media, except miR-301 and miR-224, whose concentrations were beyond the detection limits and thus were excluded from further analyses. We found miR-15b, miR-21, miR-183 and miR-130b had generally higher copy numbers in the medium of HCC cells when compared to CL-48 or MIHA (**Fig. 3**).

Detection of miRNAs in serum samples from HCC patients pre- and post-surgery

To determine if the circulating miRNAs were derived primarily from tumors, we measured their levels in serum samples of 15 selected HCC patients before and after surgery treatment. In agreement with the tissue data as stated above, miR-224

and miR-301 levels were very low in the sera (data not shown). For the other 4 miRNAs, there was a trend of reduction of miRNAs levels after surgery. As shown in **Fig. 4**, the median copies of miR-130b decreased significantly from 124.8 to 96.2 (p = 0.0158), and miR-183 from 153.3 to 95.2 (p = 0.0084). We also observed reductions of miR-15b and miR-21 levels after surgery, although the declination was not statistically significant. The median level of miR-15b was reduced from 177.6 to 64.1 (p = 0.0637), whereas the median level of miR-21 was decreased from 221,404.4 to 100,140.5 (p = 0.0684).

Circulating miR-15b and miR-130b classifier defines HCC cases in an independent cohort

We next verified the discriminative power of these miRNAs in identifying HCC cases in an independent cohort of thirty healthy controls, twenty-nine hepatitis B individuals, and fifty-seven HCC patients. The levels of the four miRNAs in these serum samples were measured, and ROC analysis was performed on individual miRNAs. The cut-off of each miRNA was selected with highest Youden's index. Among the four miRNAs, miR-130b showed the highest AUC of 0.913 (sensitivity of 87.7% and specificity of 81.4%) in separating the non-cancerous controls from those with HCCs, whereas miR-15b gave the highest sensitivity (98.3%), although its specificity (15.3%) was only modest (**Table 2**).

Recognizing the limitations of individual tests, we performed multivariate logistic regression analysis on various combinations of the miRNA biomarkers, and found that the combined miR-15b and miR-130b gave the best performance. It could be a potential classifier for detecting HCC, and the formula of the classifier is as follow:

-2.4966 + 0.0532(copy number of miR-130b) – 0.0210*(copy number of miR-15b)*

The ROC curve of the classifier has an AUC of 0.981 (**Fig 5a**). A sum score of the classifier at -0.61109 was chosen as a cut-off, as it has the highest Youden's index of 0.8977. At this cut-off, the classifier has enhanced sensitivity of 98.3% and specificity of 91.5% (**Fig. 5b**), when compared to the performance of individual miRNAs.

We next tested if the classifier could successfully differentiate a subset of patients with low serum AFP from non-cancerous controls. Three sub-groups were defined: (*a*) HCCs with AFP > 400 ng/mL (n = 11) and non-cancerous controls (n = 59); (*b*) HCCs with AFP between 20 and 400 ng/mL (n = 16) and non-cancerous controls (n = 59); (*c*) HCCs with AFP < 20 ng/mL (n = 30) and non-cancerous controls (n = 59). We found the classifier could accurately distinguish HCC subjects from the controls with high sensitivity and specificity in all cases (**Fig. 5c**). For the HCC subgroup with AFP < 20 ng/mL, the AUC of the classifier is 0.980, with sensitivity of 96.7% and specificity of 91.5%.

Next, we evaluated the performance of miR-15b and miR-130b classifier in detecting early-stage HCC cases. As shown in **Fig. 5d**, the miRNA classifier could accurately identify 97.8% (44 out of 45) HCC cases from both TNM stages I and II, whereas serum AFP (cut-off level at 20 ng/mL) could only detect 48.9% (22 out of 45) of the same cases. In short, the circulating miR-15b and miR-130b is a classifier outperforms the serum AFP as tumor marker in detecting HCC.

Discussion

The present study has developed an unprecedented miRNA scoring system able to identify HCC cases that could not be detected by the conventional AFP tumor marker. Our *in vitro* studies also showed that the extracellular miRNAs are good indicator of the miRNA expression in cancer cells. These finding collectively implicate the potential application of circulating miRNAs as non-invasive serological biomarker for solid malignancy like HCC.

Our tumor biomarker discovery strategy is divided into three phases: *(a)* *Exploration phase* – to identify candidate markers through molecular profiling of miRNAs in matched tumor and adjacent non-tumor tissues samples from HCC patients (n = 96). *(b)* *Selection/filtering phase* – to select those miRNAs highly expressed in both tumor tissues as well as in serum samples. In addition, we used pre- and post-operative serum samples from the same HCC patients (n = 15) to further refine that those miRNAs that were likely derived from HCC tumors. After surgical removal of the HCC tumors, serum levels of these miRNAs were significantly reduced. *(c)* *Cross-center validation phase* – to validate the miRNA biomarkers in an

independent cohort (n = 116) including HCC cases, hepatitis B carriers and non-malignant healthy controls from geographically distant medical center. Herein, our findings showed that the combined miR-15b and miR-130b is a robust classifier in detecting HCC cases and outperforms AFP as tumor marker with high positive predictive value.

Previous studies have suggested the potential use of circulating miRNAs in the diagnosis of HCC. [20, 21] The study of Qu *et al.* suggested that serum miR-16 could be used as a second-line test when the serum AFP and other markers are normal levels [21]. However, in our analysis, we did not find miR-16 significantly differentially expressed between tumor and adjacent normal tissues. Another study using Solexa sequencing on pooled serum samples and identified miR-375 as a candidate biomarker for HBV-positive HCC, which was further validated by TaqMan miRNA qPCR assays [20]. The majority of the HCC cases in our cohort were also HBV-positive, but we did not select miR-375 during our initial screen. This is because the difference was not significant when comparing the HCC tumors of low AFP level with the corresponding normal samples. Nevertheless, we indeed found miR-375 having an important role in regulating the YAP oncogenic signaling pathway in HCC. [22] Further investigation is under way to evaluate the application of miR-375 for identifying subtype of HCC, for which the Hippo pathway and YAP signaling are aberrantly regulated.

Limited information of miR-15b is available in HCC and its molecular mechanisms and roles in pathogenesis remain largely unknown. Consistent with the present findings, miR-15b was recently shown to be overexpressed in HCC tumors, [23] and it is in a pilot cross-sectional, phase 2 biomarker study for Barrett's esophagus progression. [24] Recently, miR-15b has been shown to be a direct transcriptional target of E2F and may be involved in preventing replicative stress in response to mitogenic signaling. [25] On the other hand, the oncogenic role of miR-130b in hepatocarcinogenesis is prominent and is considered as a cancer stem cell miRNA in HCC. First, it has been shown highly expressed in $CD133^+$ tumor-initiating cells in HCC, and transduction of miR-130b into CD133-negative cells could promote tumorigenesis and induce chemoresistance. [26] Second, miR-130b directly targets a well-known tumor suppressor — RUNX3, and regulates expression of pro-apoptotic Bim thereby enhancing cell viability. [27] Despite of these observations, it is still

unknown if miR-15b and miR-130b in circulation contribute to HCC development and tumor progression.

Although the miRNA classifier gave a high positive predictive value in our HCC cohort, there are several limitations in this study. First, the classifier has yet to be validated in other ethnic populations, such as Europe and Japan in which hepatitis C virus is the major etiology of HCC. Second, the post surgical serum samples were in small sample size and varied at different time points. It would be imperative to test more longitudinal samples in order to justify the specific time or period that the circulating miRNAs return to basal levels.

There are mounting evidences indicating that serum-based miRNAs are useful as non-invasive biomarkers for different cancer types. [14, 28-32] Today, circulating miRNAs in serum have been reported as potential diagnostic markers for B-cell lymphoma,[33] leukemia,[34] esophageal squamous cell carcinoma,[35] as well as lung,[36] breast,[37] colon,[38] gastric,[39] pancreatic,[40] prostate[41] and ovarian[42] cancer. A handful of studies showed that miRNAs could be released into bloodstream via active secretion from diseased tissues in forms of protein-bound complex [43] or as membrane-bound vesicles (e.g. exosomes).[44] Because the high rate of proliferation and cell lysis in tumors, non-specific passive release could also exist in cancer that contribute to the abundance of miRNAs in the blood stream. Not only reflecting physiological and pathological changes, these circulating miRNAs could be functional and correlated with tumor progression and clinical outcomes.[32, 45, 46] Most recently, miR-16, -21, -122, and -375 in serum were suggested as potential diagnostic markers for HCC.[20, 21, 47] Nevertheless, their diagnostic performance has not been robustly validated in an independent cohort. Our present findings demonstrate that the circulating miR-15b and miR-130b hold promise as valuable tumor markers for detecting HCC, in which both the early stages and low AFP group were also covered with high sensitivity and specificity. Early detection of HCC could save many lives and enhance the quality of life in patients suffering from this lethal malignancy.

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Contributors: AL: Experimental design, experimental performance, analysis of data, drafting of the manuscript; TY: statistical analysis, revision of the manuscript; WW: experimental performance; KW: drafting and revision of the manuscript; NL: revision of the manuscript and material support; SF: acquisition of clinical samples and revision of the manuscript; RP: acquisition of clinical samples and study supervision; CG: acquisition of clinical samples and revision of the manuscript; JL: study design, study supervision, revision of the manuscript

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Figure legend:

Figure 1. Schematic flow chart of miRNA biomarker discovery for detecting HCC. This study was divided into 3 phases: exploration, selection/filtering, and cross-center validation. Two different cohorts of patients were included – one from Hong Kong (n = 96) and one from Shanghai (n = 116).

Figure 2 (**A**) Hierarchical clustering (centered correlation and average linkage) over 105 differentially expressed miRNAs. Heat map colors represent the relative miRNA expression as indicated in the color key. (**B**) Expressions of the candidate miRNA biomarkers in HCC tumor (TU) and adjacent non-tumor (AN) tissues. The tumor tissues were sub-divided into three groups according to the serum AFP levels of the individual patients (AFP < 20 ng/ml, between 20-400 ng/ml, and > 400 ng/ml). The box plots show the median (horizontal bar), 25th and 75th percentiles, and the whiskers of the graph show the largest and smallest values.

Figure 3 TaqMan qPCR assay of extracellular levels of 4 candidate biomarkers, miR-15b, miR-21, miR-130b, and miR-183, in culture medium harvested from four HCC cell lines (HepG2, Hep3B, PLC, and MHCC-97H (97H)) and two immortalized hepatocyte cell lines (CL-48 and MIHA). The assays were repeated at least twice in different samples. The error bars represent mean ± standard error of the mean (SEM).

Figure 4. Declination of serum miRNA markers in HCC patients after surgery treatment. Changes in serum levels of miR-15b, miR-21, miR-130b, and miR-183 in HCC patients (n=15) before (pre-op) and after (post-op) surgical removal of the tumors. Statistical differences were analyzed using Wilcoxon signed-rank test. Table in the lower panel indicates the median level of the individual miRNAs before and after surgery.

Figure 5 Performance of the miR-15b and miR-130b classifier for detection of HCC in an independent cohort. **(A)** ROC curve analysis of the miRNA classifier over HCC

patients and non-cancerous control subjects (healthy and chronic hepatitis B carrier). The miRNA classifier was derived from the logistic regression model that composed of two miRNAs, i.e. miR-15b and miR-130b. **(B)** Dot plots of the sum score of the logistic regression model in non-cancerous control subjects (Control) and HCC patients (HCC). The sensitivity, specificity, and accuracy were calculated based on the cutoff at -0.611 that was determined by the Youden's Index. **(C)** ROC curve analyses of the miRNA classifier over control subjects (healthy and chronic hepatitis B individuals) and sub-group of HCC patients with AFP levels > 400, between 20 and 400 ng/mL, or < 20 ng/mL. The sensitivity, specificity, false positive rate, false negative rate, and accuracy at a cut-off of -0.611 were indicated below each ROC graph. **(D)** AFP and miRNA classifier (miR-15b and miR-130b) were compared in HCC cases between early (I and II) and late (III and IV) stages. The cut-off values were marked by grey lines.

Table 1. Demographic and clinicopathologic parameters of patients from different cohorts

Parameters	Hong Kong cohort (n = 96)	Shanghai Cohort (n = 116)		
	HCC patients	Healthy controls	Hepatitis B carriers	HCC patients
Sex				
Male	78	22	20	49
Female	18	8	9	8
Age				
< 60	60	30	28	47
≥ 60	36	0	1	10
Alpha fetoprotein (ng/ml)				
< 20	39	-	29	30
20-400	26	-	0	16
> 400	31	-	0	11
Alanine aminotransferase (U/L)				
< 35	28	-	20	29
≥ 35	68	-	9	28
Aspartate aminotransferase (U/L)				
< 35	23	-	22	24
≥ 35	73	-	4	33
Hepatitis B surface antigen				
Negative	12	-	0	0
Positive	84	-	29	57
Pathological tumor-node-metastasis (TNM) stage*				
Early (I, II)	41	-	-	45
Late (III, IV)	55	-	-	11
Child-Pugh§				
A	91	-	-	54
B	3	-	-	2
C	0	-	-	0
Tumor size (cm)*				
< 5 cm	26	-	-	27
≥ 5 cm	70	-	-	29

*Data indicated in the cohort from Shanghai are based on 56 cases.
§ Data indicated in the cohorts from Hong Kong and Shanghai are based on 94 and 56 cases, respectively.

Table 2. Predictive performance of individual serum miRNAs and miRNA classifier (miR-15b and miR-130b) as biomarkers for detection of HCC

Statistical parameters	Non-cancerous controls (n=59) vs. HCCs (n =57)				
	miR-15b (≥43.485 copies/ng)	**miR-21 (≥73271.020 copies/ng)**	**miR-130b (≥97.250 copies/ng)**	**miR-183 (≥40.100 copies/ng)**	**miR-15b + miR-130b (≥ -0.6111 sum score)**
Sensitivity	98.25%	89.47%	87.72%	57.89%	98.25%
Specificity	15.25%	71.19%	81.36%	69.49%	91.53%
False positive rate	84.75%	28.81%	18.64%	30.51%	8.47%
False negative rate	1.75%	10.53%	12.28%	42.11%	1.75%
Accuracy	56.03%	80.17%	84.48%	63.79%	94.83%
Positive predictive value	52.83%	75.00%	81.97%	64.71%	91.80%
Negative predictive value	90.00%	87.50%	87.27%	63.08%	98.18%
Positive likelihood ratio	1.159	3.105	4.705	1.898	11.593
Negative likelihood ratio	0.115	0.148	0.151	0.606	0.019
Youden's index	0.135	0.607	0.691	0.274	0.898
Area under the ROC (AUC)	0.485	0.865	0.913	0.661	0.981

Figure 1

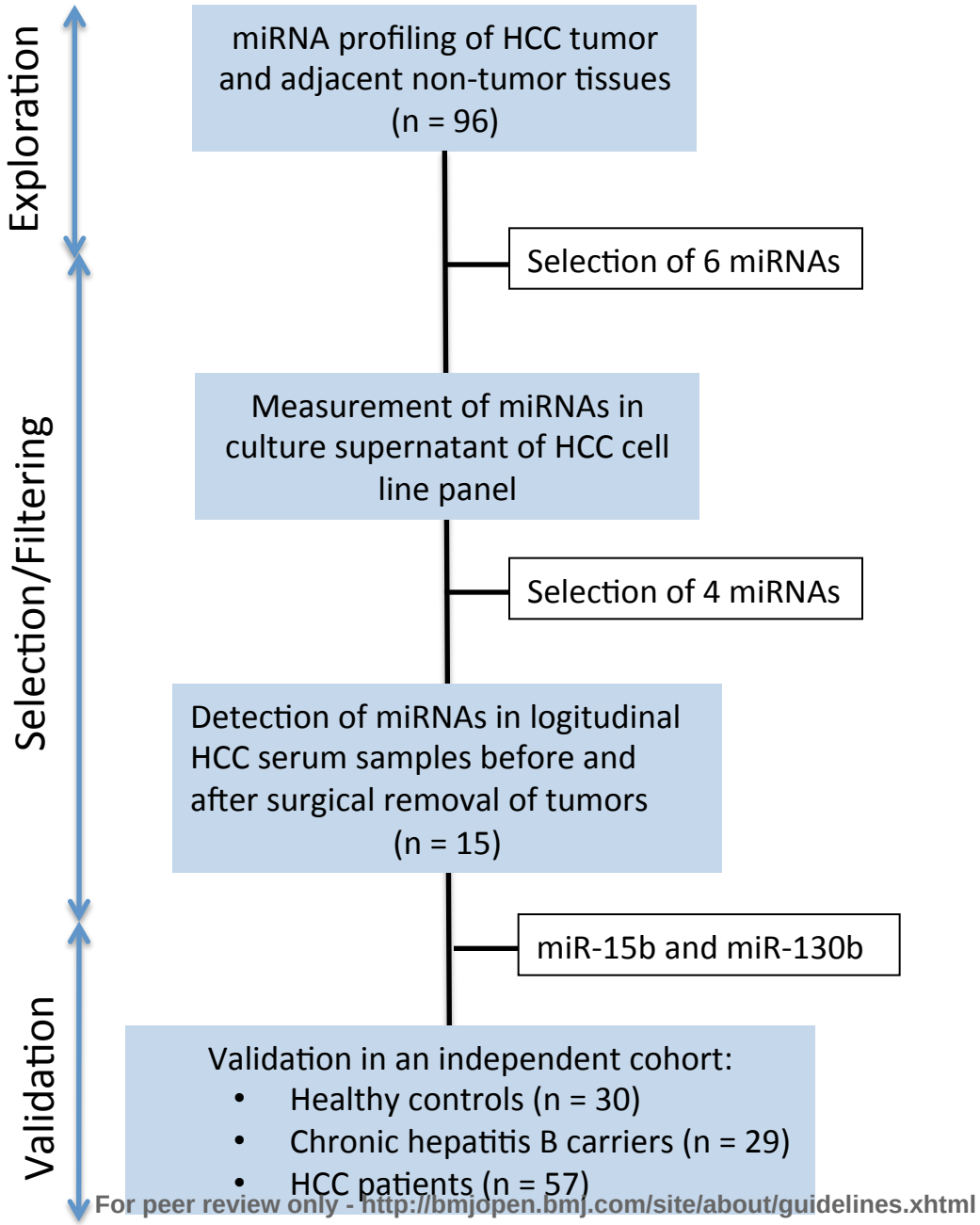
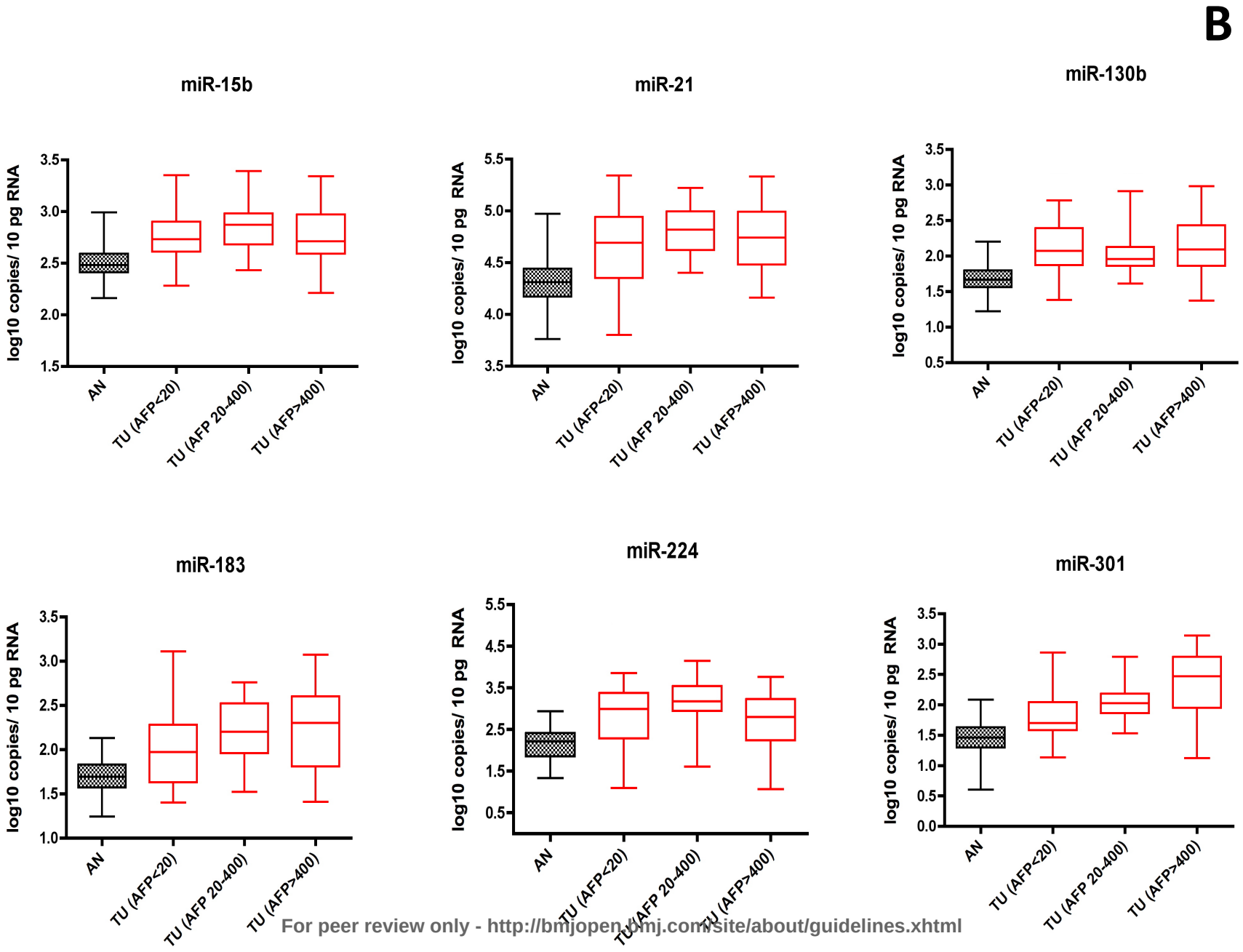


Figure 2



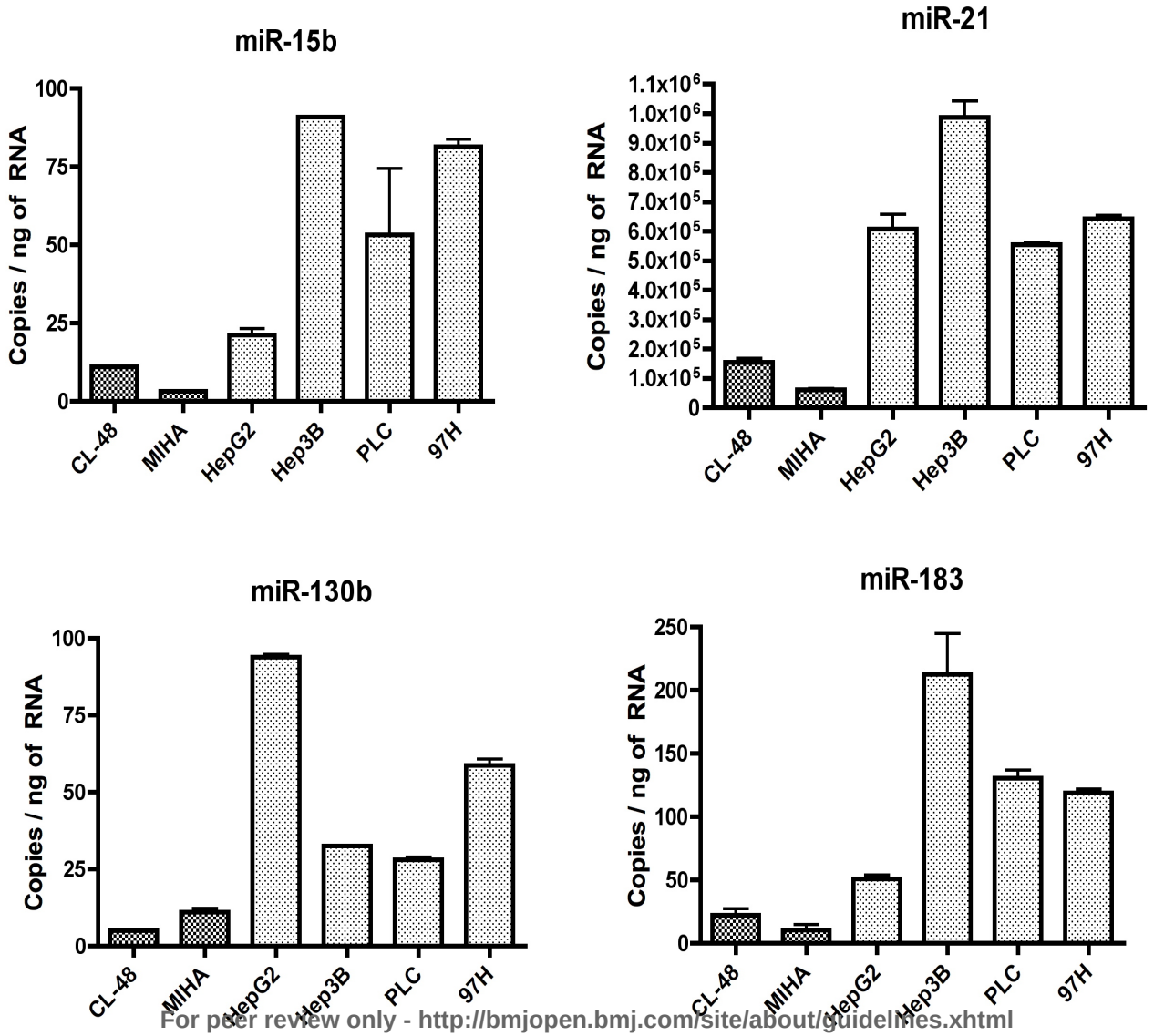
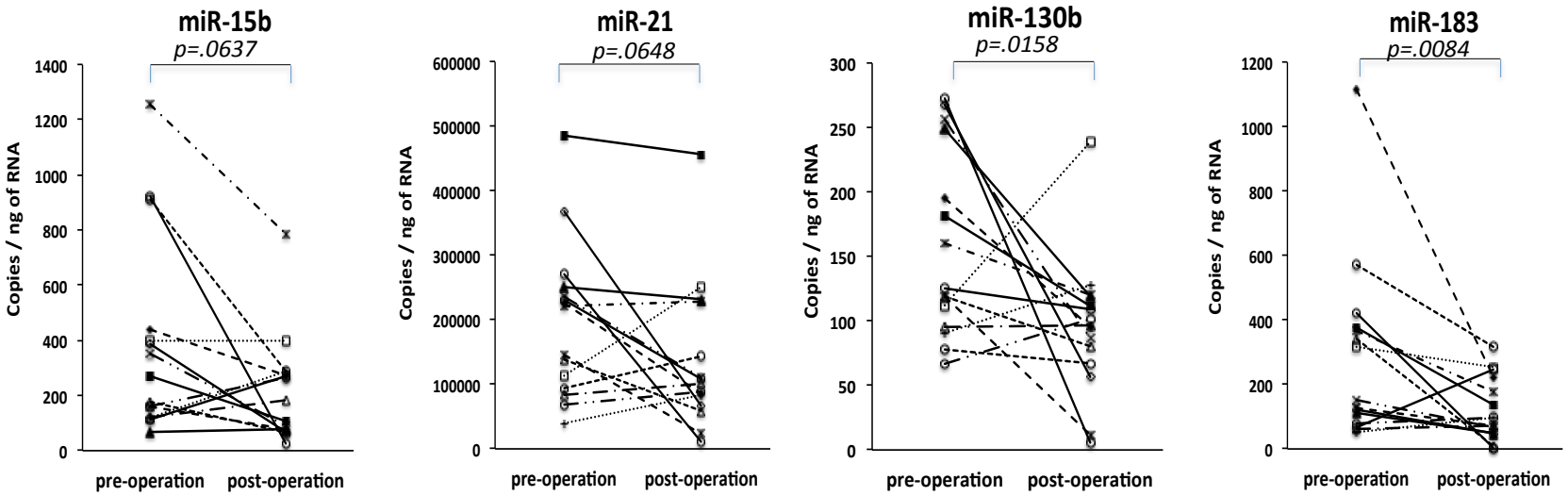


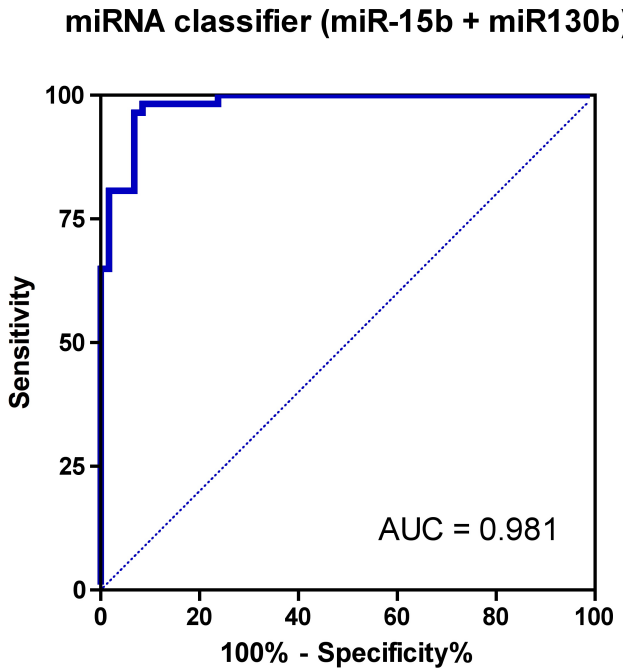
Figure 4



Median levels (copies/ ng RNA)	miR-15b	miR-21	miR-130b	miR-183
pre-operation	177.61	221404.42	124.82	153.27
post-operation	64.13	100140.49	96.18	95.19

Figure 5

A


miRNA classifier (miR-15b + miR130b)
Sensitivity
100
75
50
25
0
0
20
40
60
80
100
100% - Specificity%
AUC = 0.981

B

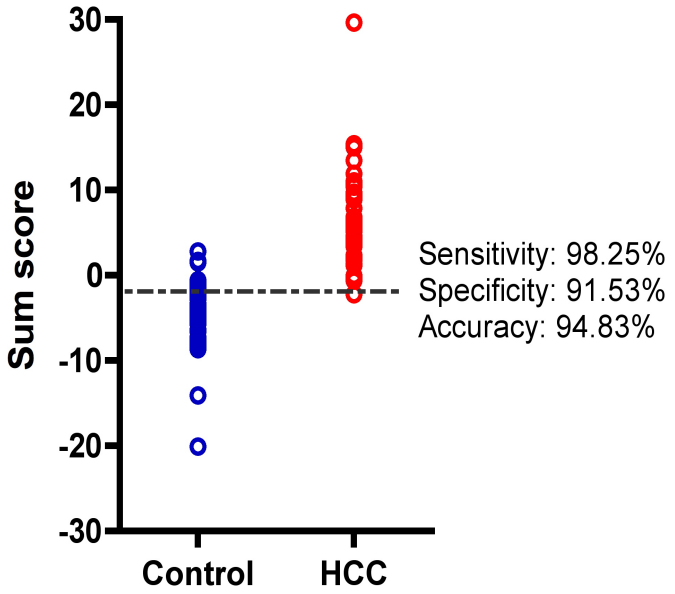
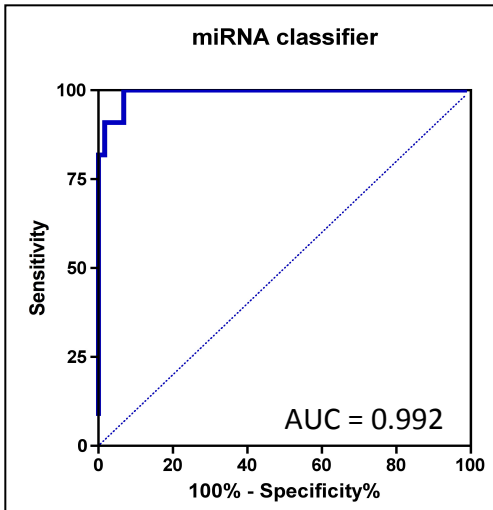
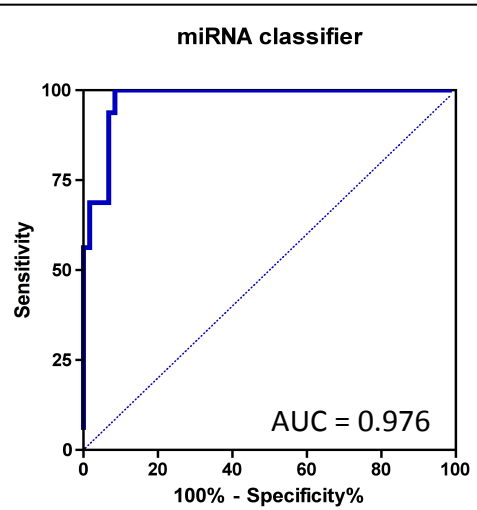
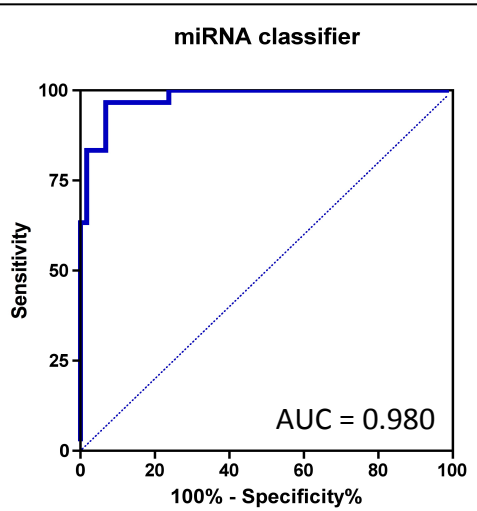

Sum score
30
20
10
0
-10
-20
-30
Control
HCC
Sensitivity: 98.25%
Specificity: 91.53%
Accuracy: 94.83%

Figure 5

C



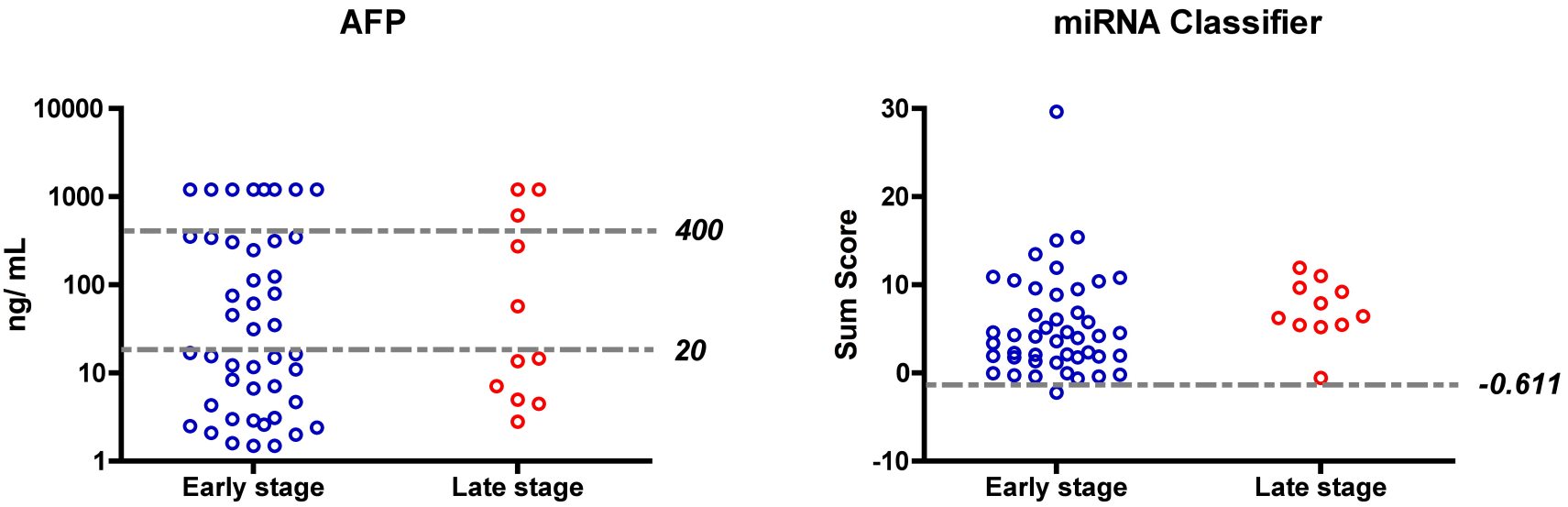




Cut-off at -0.611	Controls (n = 59) vs. HCCs with AFP > 400 (n = 11)	Controls (n = 59) vs. HCCs with AFP 20 - 400 (n = 16)	Controls (n = 59) vs. HCCs with AFP < 20 (n = 30)
Sensitivity	100.00%	100.00%	96.67%
Specificity	91.53%	91.53%	91.53%
False positive rate	8.47%	8.47%	8.47%
False negative rate	0.00%	0.00%	3.33%
Arruracy	92.86%	93.33%	93.26%

Figure 5

D


AFP
10000
1000
100
10
1
ng/ mL
400
20
Early stage
Late stage
miRNA Classifier
30
20
10
0
-10
Sum Score
-0.611
Early stage
Late stage

STARD checklist for reporting of studies of diagnostic accuracy

(version January 2003)

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	2
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	3
METHODS			
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	4
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	4
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	4-5
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	4-5
Test methods	7	The reference standard and its rationale.	4-5
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	5-6
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	6
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	6
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	6
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	6
	13	Methods for calculating test reproducibility, if done.	6
RESULTS			
Participants	14	When study was performed, including beginning and end dates of recruitment.	4-5
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	17
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	19
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	7-9
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	7-9
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	7-9
	20	Any adverse events from performing the index tests or the reference standard.	N/A
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	7-9
	22	How indeterminate results, missing data and outliers of the index tests were handled.	6
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	7-9
	24	Estimates of test reproducibility, if done.	7-9
DISCUSSION	25	Discuss the clinical applicability of the study findings.	9-11