Identification of recurrence-related serum microRNAs in hepatocellular carcinoma following hepatectomy

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Abbreviations: AFP, alphafetoprotein; AUC, area under the curve; CT, computed tomography; DSA, digital subtraction angiography; HCC, Hepatocellular carcinoma; miRNA, microRNA; MRI, magnetic resonance imaging; MVI, microvascular invasion; RFS, relapse free survival; ROC, receiver operating characteristic; US, ultrasonography.

Hepatocellular carcinoma (HCC) is one of the most deadly tumors. Prognosis of patients with HCC is generally poor due to the high recurrence rate. In the present study, TaqMan Real-time PCR microRNA Array was used to identify differentially expressed miRNAs from 10 tumor tissue samples (5 from recurrence group vs. 5 from non-recurrence group) and the matched serum samples. Four differentially expressed miRNAs (miR-486–5p, miR-422a, miR-125b and miR-139–5p) were further quantified in 20 tumor tissues and 116 HCC patients’ serum before they received hepatectomy. Univariate analysis revealed that miR-486–5p, miR-422a and miR-125b were significantly associated with patients’ relapse free survival (RFS). Multivariate analysis demonstrated that miR-486–5p, AFP and microvascular invasion (MVI) were the independent prognostic factors associated with RFS in this cohort (p = 0.000, 0.043, 0.000, respectively). Besides, the expression levels of miR-486–5p were positively correlated in tumor tissues and the paired serum samples, so was miR-422a. The probability of the prognostic accuracy of miR-486–5p in predicting postoperative recurrence of HCC within the first year was 76.79% (65.38% specificity and 81.58% sensitivity), which was almost equal to the classifier established by combination of AFP and MVI (75.98% probability, 63.13% specificity and 85.90% sensitivity). Furthermore, the combination of AFP, MVI and miR-486–5p yielded a ROC curve area of 88.02% (69.20% specificity and 92.10% sensitivity). Our study was the first to identify that serum miR-486–5p could be used to stratify the patients with higher recurrence risk before hepatic resection and potentially guide more effective surveillance strategies for them.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the second most common cause of cancer mortality. An estimated 782,000 new liver cancer cases and 745,000 cancer deaths occurred worldwide. Half of these cases and deaths were estimated to occur in China. Hepatic resection is one of the most effective curative treatments for HCC, but the short-term and long-term relapse free survival (RFS) after operation remains unsatisfactory mainly because of the high recurrence rate. The estimated recurrence rate can exceed 60% at 5 y. Previous studies demonstrated that recurrence within 1 y after primary resection were significant risk factors for poor overall survival and recurrence at >2 years were significantly associated with 10-year survival. Some studies showed that microvascular invasion, poor differentiation, satellites nodules, AFP levels, etc. are parameters that help clinicians to assess the risk of HCC recurrence, but it is necessary to investigate prognostic molecular biomarkers which may provide us with an opportunity to make an early diagnosis and effectively intervene with therapeutic and preventative strategies.
MicroRNAs (miRNAs) are a class of endogenous small regulatory RNA molecules that target mRNA and trigger either translation repression or mRNA degradation. They are responsible for post-transcriptional regulation and participate in nearly all biological processes. The use of miRNA as a cancer biomarker is of particular interest because it could be detected in blood plasma or serum with high stability, though the exact source of circulating miRNAs is a matter of debate. In recent years, there are many reports investigating the possible predictions of clinical outcomes by miRNA in human cancers, including HCC. However, only a few studies have focused on identifying the miRNA-based classifiers and the prognostic value of circulating miRNAs for recurrence of HCC.

In this study, we hypothesized that some miRNAs that are susceptible to fluctuation in serum from tumor tissue may be used to accurately predict recurrence in HCC patients after liver resection. To address this hypothesis, we screened miRNAs that were consistently differentially expressed between recurrence and non-recurrence groups both in tumor tissues and paired serum samples by using microRNA array. We also detected the expression levels of candidate miRNAs in a cohort of 116 serum samples and 20 tumor tissues in order to validate their values of predicting prognosis. Our study suggested that serum miRNAs can be considered as useful biomarkers that could help to assess the risk of recurrence in postoperative HCC patients.

## Results

### Screening phase

To explore whether miRNAs were associated with postoperative recurrence of HCC after hepatectomy, we first compared the expression of miRNAs in 5 tissue samples from recurrence group vs. Five from non-recurrence group and the matched serum samples. Of the 40 miRNAs that differentially expressed (fold2-altered) both in tissue and in serum samples, 15 miRNAs have the same variation tendency. And, 4 of the 15 miRNAs were differentially expressed with a p value below 0.10 between recurrence group and non-recurrence group in tissue samples. Four candidate miRNAs were further validated in 116 independent serum samples and 20 tumor tissues.
value in the 2 pools, (2) showing a 2-fold altered expression. After being analyzed, 159 miRNAs satisfied the criteria.

As shown in Figure 1, we found that 40 miRNAs were differentially expressed both in tissue and in serum samples. As a relatively stable marker, serum expression should be consistent with the expression in cancer tissue. Of these 40 miRNAs, 15 miRNAs have the same variation tendency in tissue and serum samples (Table 2). Among these 15 miRNAs, 4 miRNAs were differentially expressed with a p value below 0.10 between recurrence group and non-recurrence group in tumor tissue samples. As a result, we firstly selected these 4 miRNAs as our potential candidate biomarkers for further research.

The expression of serum miRNAs was associated relapse free survival of postoperative HCC patients

Four miRNAs were analyzed in 116 HCC patients’ serum to identify prognostic factors for recurrence. Clinicopathologic informations of the patients were summarized in Table 3. The expression of individual miRNAs was correlated to RFS with univariate analysis. We divided the 116 patients into 2 groups based on the median value of the expression level of each miRNA. Among the 4 miRNAs, 3 miRNAs (miR-486–5p, miR-422a and miR-125b) were significantly associated with RFS, while the RFS of miR-139–5p groups were not significantly different (p > 0.05). The Kaplan-Meier curves of RFS according miR-486–5p, miR-422a, miR-125b and miR-139–5p were plotted in Figure 2A-D. Comparing each miRNA expression in HCC serum with patient’s RFS time revealed 2 group: those with predominantly higher expression of miR-486–5p, miR-422a, miR-125b and long RFS and those with predominantly lower expression of miR-486–5p, miR-422a, miR-125b and short RFS (p = 0.000, 0.002, 0.003, respectively). The Multivariate Cox proportional hazard regression analysis revealed that miR-486–5p was the independent prognostic factor associated with RFS (Table 4). The scatter diagram of miR-486–5p, miR-422a, miR-125b and miR-139–5p in all patients was showed in Figure S1.

To further understand the significance of miRNAs in the prognosis of postoperative HCC patients, whether the 4 miRNAs expression were significantly associated with the clinicopathologic features were analyzed. As shown in Table 5, univariate analysis showed that the expression of miR-139–5p was considerably associated with preoperative GGT and satellite nodules, miR-125b was associated with microvascular invasion, miR-486–5p was associated with cirrhosis (p < 0.05).

Subsequently, we measured the expression of these 4 miRNAs in 20 HCC tumor tissues samples and performed spearman correlation analysis of the paired serum samples in order

| miRNAs   | In serum | In tissue |
|----------|----------|----------|
|          | Fold change (R/NR)* | Fold change (R/NR)* | P value |
| miR-125b | 0.482    | 0.3917    | 0.0617  |
| miR-139–5p | 0.3856 | 0.2193    | 0.223   |
| miR-422a | 0.0875   | 0.1836    | 0.0874  |
| miR-486–5p | 0.1652 | 0.3516    | 0.0302  |
| miR-142–5p | 0.3234 | 0.4685    | 0.4261  |
| miR-145  | 0.4918   | 0.4854    | 0.3675  |
| miR-199a–3p | 0.362  | 0.3556    | 0.3626  |
| miR-204  | 0.2926   | 0.3893    | 0.2697  |
| miR-212  | 0.1584   | 0.4347    | 0.1676  |
| miR-214  | 0.0768   | 0.4106    | 0.2441  |
| miR-218  | 0.1263   | 0.4308    | 0.2864  |
| miR-449a | 0.3155   | 0.4109    | 0.4856  |
| miR-486–3p | 0.189  | 0.2217    | 0.119   |
| miR-511  | 0.2505   | 0.4982    | 0.2298  |
| miR-627  | 0.2639   | 0.484     | 0.5443  |

R, recurrence; NR, non-recurrence; miR, microRNA.
to identify the 4 candidate microRNAs which were susceptible to fluctuation in serum from tumor tissue. The results revealed that miR-486–5p was positively correlated in tumor tissues and the paired serum samples, the same as miR-422a (Fig. 3).

The classifier of miR-486–5p for predicting postoperative recurrence of HCC

Multivariate analysis revealed that miR-486–5p was the independent prognostic factor associated RFS in HCC patients. Then, the discriminative power of miR-486–5p in predicting the outcome before surgery was verified. According to the RFS, the patients were stratified into 2 subgroups, including a non-recurrence group (≥12 months) and a recurrence group (≤12 months). To evaluate the prognostic value, the ROC curve was used to analyze the sensitivity and specificity. As shown in Figure 2, the ROC curve of miR-486–5p showed an AUC of 76.79% (81.58% sensitivity and 65.38% specificity) (Fig. 4A). In order to further confirming the potential role of miR-486–5p in predicting the recurrence of HCC, the prognostic values of multiple commonly used clinicopathologic features were analyzed with univariate and multivariate analysis. Multivariate Cox proportional hazard regression analysis revealed that AFP and microvascular invasion (MVI) were the independent prognostic factors associated with postoperative recurrence of HCC (Table 5). The ROC curve of AFP and MVI had an AUC of 75.98% (85.90% sensitivity and 63.13% specificity) (Fig. 4B). Furthermore, the combination of AFP, MVI and miR-486–5p, yielded an AUC of 88.02% (92.1% sensitivity and 69.2% specificity) (Fig. 4C). The results demonstrated that the combination of AFP, MVI and miR-486–5p was a much more powerful discrimination tool in predicting postoperative recurrence of HCC.

Discussion

HCC is difficult to manage due to the high recurrence rate after surgery. Therefore, prevention of HCC recurrence after resection has become an issue which is needed to be studied further. miRNAs function as negative regulators of gene expression. In recent years, miRNAs constitute a novel class of non-invasive biomarkers, due to the high stability of endogenous circulating miRNAs. Liver is a rich blood supply organ, easily accessible serum based miRNAs may provide a clue in monitoring of liver diseases. To date, only several studies explored the potential of circulating miRNA in predicting postoperative recurrence of HCC.

In the present study, we screened differentially expressed miRNAs between recurrence group and non-recurrence group of postoperative HCC patients both in tumor tissue and paired serum and identified 4 differentially expressed miRNAs (including miR-486–5p, miR-139–5p, miR-125b and miR-422a) in 116 independent samples. Previous studies have reported that all of these 4 miRNAs were down regulated in liver cancer compared with the matched normal tissues. After further validation, we observed that the levels of miR-486–5p were positively correlated between the paired serum and tumor tissues, so was miR-422a. The results of this study suggested that miR-486–5p and miR-422a were susceptible to fluctuation in serum from tumor tissue, but miR-125b and miR-139–5p were not. The selective release
of specific cellular miRNAs from the tumor cells or from normal cells might explain these results. There may also be other reasons, such as sample size. More importantly, serum miR-486–5p showed a powerful discrimination potential in identifying postoperative recurrence of HCC. We also obtained a relatively high prediction accuracy (AUC 88.02%) by using the combination of serum miR-486–5p, AFP and microvascular invasion (MVI). The concordance of the risk factor (AFP and MVI) in our study with those from previous studies supports the validity of our findings.

Understanding the miRNA targets and the molecular mechanisms by which the miRNAs regulate may promote their clinical application. Among the 4 miRNAs identified in this study, miR-486–5p was first cloned from the fetal liver. Previously, miR-486–5p has been shown to be a direct regulator of several cancer-related genes like PTEN, OLFM4, ARHGAP5, PIM-1, and PIK3R1. The results suggested that miR-486–5p might be a tumor suppressor. Here, we found that miR-486–5p was associated with cirrhosis and was much lower in the patients of recurrence group compared with the patients of non-recurrence group. This is possibly due to the fact that high glucose can increase miR-486–5p expression, and the liver’s capacity to store glycogen is reduced in patients with liver cirrhosis. Nevertheless, the exact mechanism of miR-486–5p dysregulation in HCC remains unknown and additional investigations need to be explored in future study. miR-125b, another miRNA identified in our study, has been extensively investigated in terms of tumor biology and can suppress human liver cancer cell proliferation and metastasis. Several targets of miR-125b have been identified, including LIN28B, placenta growth factor (PIGF), and SIRT7. PIGF is a member of VEGF family and exerts pleiotrophic functions in promoting tumor growth and angiogenesis in normal and malignant hepatic endothelial cells.

In the present study, our data showed that miR-125b was associated with microvascular invasion. It is quite possible that miR-125b might be regulating PIGF as well in our system. Moreover, pioneering study showed that high level of miR-125b in tumor tissue was correlated with good survival of HCC patients. Although our study could not conclude that miR-125b was susceptible to fluctuation in serum from tumor tissue, high level of serum miR-125b was also significantly associated with good RFS. Furthermore, univariate analysis showed that miR-139–5p was associated with satellite nodules. Emerging evidences suggest

| Table 4 | Univariate and Multivariate Cox proportional hazards regression analysis of miRNAs and clinical parameters in relation to postoperative recurrence of HCC |
| --- | --- | --- | --- |
| Overall survival | | | |
| Variable | Univariate analysis | Multivariate analysis |
| HR(95%CI) | p value | HR(95%CI) | p value |
| miR-486–5p | 0.789 (0.698–0.892) | 0.000 | 0.789 (0.698–0.892) | 0.000 |
| miR-422a | 0.689 (0.638–0.736) | 0.003 | 1.105 (1.003–1.217) | 0.043 |
| miR-125b | 0.717 (0.638–0.806) | 0.002 | 0.717 (0.631–0.812) | 0.117 |
| miR-139–5p | 0.837 (0.747–0.932) | 0.000 | 0.837 (0.747–0.932) | 0.000 |
| Microvascular invasion | 1.113 (1.044–1.186) | 0.001 | 1.113 (1.044–1.186) | 0.001 |
| Tumor size | 3.229 (1.55–6.728) | 0.002 | 3.229 (1.55–6.728) | 0.002 |
| Satellit e nodules | 1.150 (1.048–1.261) | 0.003 | 1.150 (1.048–1.261) | 0.003 |
|AFP | 2.577 (1.337–4.970) | 0.005 | 2.577 (1.337–4.970) | 0.005 |
| Differentiation | 1.824 (1.083–3.073) | 0.024 | 1.824 (1.083–3.073) | 0.024 |
| BCLC | 1.884 (1.054–3.367) | 0.033 | 1.884 (1.054–3.367) | 0.033 |
| Hepatitis | 0.685 (0.371–1.262) | 0.225 | 0.685 (0.371–1.262) | 0.225 |
| Cirrhosis | 1.657 (0.652–4.214) | 0.289 | 1.657 (0.652–4.214) | 0.289 |
| Tumor multiplicity | 1.334 (0.565–3.148) | 0.511 | 1.334 (0.565–3.148) | 0.511 |
| Gender | 0.997 (0.482–2.063) | 0.993 | 0.997 (0.482–2.063) | 0.993 |
| Age | 1.000 (0.975–1.026) | 0.996 | 1.000 (0.975–1.026) | 0.996 |

HR, hazard ratio; CI, confidence interval; miR, microRNA.

| Table 5 | Correlation between the expression of 4 miRNAs and clinical parameters of 116 HCC patients underwent hepatectomy |
| --- | --- | --- |
| Parameters | miRNAs | P value |
| Gender | None | 0.290 |
| Age (years) | None | 0.311 |
| Preoperative GGT | miR-139–5p | 0.020 |
| Preoperative AFP | None | 0.083 |
| Viral hepatitis | None | 0.072 |
| Tumor size | None | 0.083 |
| Tumor multiplicity | None | 0.072 |
| Satellite nodules | None | 0.083 |
| Differentiation | None | 0.072 |
| Microvascular invasion | None | 0.083 |
| Envelop invasion | None | 0.072 |
| Cirrhosis | None | 0.083 |
| BCLC | None | 0.072 |
| Recurrence (<12 months) | miR-486–5p | 0.002 |
| miR-125b | 0.002 |
| miR-422a | 0.003 |
that miR-139–5p could suppress metastasis and progression of HCC.\textsuperscript{35-37} As we all know, the present of satellite nodules is a sign of intrahepatic metastasis. A newly-published study showed that miR-422a is negatively correlated with pathological grading, recurrence, and metastasis and overexpression of miR-422a in HCC tumor cells significantly inhibits tumor growth and liver metastasis \textit{in vitro} and \textit{in vivo}.\textsuperscript{19} All these studies may increase the reliability of our findings and provide clue to improve our understanding of the molecular pathogenesis of HCC.

There are some potential limitations of our study. First, long-term follow-up studies are still required to confirm the correlation between serum miRNA levels and patients outcome. Second, the underlying mechanisms of secretion of miR-486–5p have not been demonstrated. In addition, our study lacked an independent, large validation cohort, which must be considered in future investigations to further appreciate the clinical significance of the findings reported in this study.

In summary, our findings highlight that circulating miR-486–5p may serve as a class of non-invasive biomarker with sufficient accuracy in predicting postoperative recurrence of HCC patients. Our work will serve as a helpful tool to stratify the patients with higher recurrence risk and to formulate more effective comprehensive therapy for the high-risk-recurrence patients.

Materials and Methods

Patients and samples
126 patients with HCC were included in this study. All patients were Child-Pugh class A and were treated with curative surgical liver resection. Clinicopathologic informations of the patient were summarized in Table 1 and Table 3. All serum samples were collected before the cancer patients had received surgery at Cancer Institute and Hospital, Chinese Academy of Medical Sciences (CAMS), between Jan 2012 and Oct 2013. Tumor specimens were immediately frozen in liquid nitrogen and stored at $-80\degree$C refrigerator.

This study was approved by ethics committee approval from cancer hospital CAMS, and all the participants signed written informed consent forms.

Clinical evaluation of recurrence
The standard postoperative surveillance program at our study consists of routine follow-up at 3-month intervals for the first 2 y and at 6-month intervals thereafter. Each follow-up visit will include interrogation and physical examination. During which, all patients were screened for the tumor marker alphafetoprotein (AFP), liver function, chest x-ray, abdominal ultrasonography (US) and enhanced CT (CT). If inner-hepatic-recurrence was suspected, the lesion was confirmed by hepatic digital subtraction angiography (DSA) and/or enhanced magnetic resonance imaging (MRI). In addition, enhanced CT scans of thorax or a bone scintigram will be performed as required to investigate possible tumor metastasis. The criteria for diagnosing a recurrence was refer to the “NCCN Guidelines on hepatobiliary cancers” in 2012 and “diagnosis and treatment norms of primary hepatic carcinoma” issued by ministry of health of the PRC in 2011. The recurrence was determined if any of the following was satisfied: (1) at least 2 positive radiographic evidence (US/CT/DSA/MRI) for the same identified new lesion. (2) any radiographic findings of new lesion accompanied with increased serum AFP more than 400ng/mL. (3)

Figure 3. Positive correlations between the expression levels in tumor tissues and the paired serum samples

Figure 4. Receiver operating characteristic curve analysis for predicting prognostic accuracy of postoperative recurrence of HCC patients. (A) ROC curve for miR-486–5p yielded area under the curve (AUC) of 76.79%, the sensitivity of 81.58% and specificity of 65.38% in predicting prognosis. (B) ROC curve for AFP and microvascular invasion yielded AUC of 75.98%, the sensitivity of 85.90% and specificity of 63.13% in predicting prognosis. MVI: microvascular invasion. (C) ROC curve for combination of AFP, MVI and miR-486–5p yielded AUC of 88.02%, the sensitivity of 69.20% and specificity of 92.10% in predicting prognosis.
confirmation by histopathology or cytopathology, but not necessarily the fine needle/needle core aspiration/biopsies were undertaken to assess recurrences. Recurrence time was calculated as the time from the end of surgery to the time of detected recurrence/progression. All of the patients were followed-up until August 2014. Until the last follow-up, 38 patients developed recurrence within the first year after resection. The median recurrence time of the recurrence group was 8 months (n=38). Seventy-eight patients identified as non-recurrence that were followed up at least 18 months. Among them, 9 patients were with tumor recurrence for more than one year.

**TaqMan Real-time PCR microRNA Array**

TaqMan Real-time PCR microRNA Arrays (Card A) (Applied Biosystems, CA) were used to identify differentially expressed miRNAs from 10 tumor tissue samples (5 from recurrence group vs. Five from non-recurrence group) and the matched serum sample. Five serum samples from each group were pooled together, respectively. The Array representing 381 mature miRNAs in Card A. Total RNAs were extracted from the tumor tissues by using a mirVana RNA isolation kit (Ambion) according to the manufacturer’s protocol. Total RNA from pooled serum samples was isolated using mirVana PARIS kit (Ambion) according to the manufacturer’s protocol. Total RNA concentrations were measured using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE USA). Megaplex RT reactions by using 1 μg of total RNA extracted from tumor tissue samples and 100 ng of total RNA extracted from serum samples were performed according to the manufacturer’s protocols (Applied Biosystems, CA USA). Pre-amplification reactions were run for serum samples after Megaplex RT reactions. TaqMan microRNA Arrays were performed on the ABI 7900HT Instrument (Applied Biosystems, CA USA). All reactions were performed according to the standard manufacturer’s protocols. Analysis of the qRT-PCR data was performed by using the SDS 2.0.1 software (settings: automatic baseline, threshold 0.2) and Data Assist v2.0 software (Applied Biosystems, Foster City, CA USA). Let-7d was used as an internal control for normalization. Real-time PCR was performed using the StepOne Plus Real-time system (Applied Biosystems, Foster City, CA USA) and fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. The mean miRNA level from 3 real-time quantitative PCR experiments was calculated for each case.

**Survival analysis**

Univariate Cox proportional hazards regression analysis were done to evaluate the association of each miRNA or clinical parameters to relapse free survival (RFS). The p values were calculated using the Wald test. Multivariate Cox proportional hazards regression analysis were done to evaluate the independent prognostic value of the miRNA signature. The Kaplan-Meier estimator was used to evaluate the median survival time of the RFS that was based on miRNA expression signature. The p value of the Kaplan-Meier analysis was calculated with the log-rank test. Relapse free survival was defined as the time interval from the end of surgery to the time of detected recurrence or censored on the last follow-up.

**Statistical Analysis**

SPSS16.0 software was used for the statistical analysis. Statistical descriptions were used to describe the clinical pathological features, and the t test (Student’s t test) was used to analyze the measurement data. The P value was bilaterally tested, and values less than 0.05 were regarded as statistically significant. Logistic regression analysis was performed to analyze various combinations of clinical parameters and miRNA. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used to determine the feasibility. The Youden’s Index was used to identify the optimal cut-off point. As defined, the corresponding sensitivity and specificity was showed.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

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