Screening of Prognostic Biomarkers for Stereotactic Body Radiation Therapy in Primary Liver Cancer

Zhenzhen Liang, PhD1,4, Chang Xue, MS1, Qing Chen, MS1, Mengke Li, MS1, Guanghui Li, MS1, Hao Feng, MS1, Yi Liu, MS1, Xiaodong Liu, PhD1,2,3, and Shumei Ma, PhD1,2,3

Abstract

Objective: So far there are still no effective immediate-early markers for assessing the efficacy of Stereotactic Body Radiation Therapy (SBRT). To find effective biomarkers for accurate assessment of the efficacy of SBRT in patients with primary liver cancer, we conducted this study including retrospective part and prospective part.

Material and Methods: 589 patients with primary liver cancer were included at Ruikang Hospital affiliated to Guangxi Medical University from January 2012 to December 2018. Follow-up was conducted, clinical information and a total of 17 patients with 51 blood samples (before SBRT, before discharge and 2 months after SBRT) were collected. mRNAs profiles on 2 patients with 6 blood samples were detected by high-throughput sequencing, followed by qPCR verification on 15 patients with 45 blood samples.

Results: The commonly used serum biomarkers such as AFP, CEA, and CA125 shown low prognostic value in distinguishing survival group and death group, indicated by low AUC (less than .7) and Youden indexes (less than .5). Based on high-throughput sequencing of test group and qPCR detection of another verification group, we found 16 up-regulated and 12 downregulated genes after SBRT. Among them, ADIPOR1 and EPB42 showed significantly different between effective and ineffective group after SBRT, ROC suggested that based on the optimal threshold of .5838, ADIPOR1 shown a sensitivity of 100% and a specificity of 83.33% to distinguish effective from ineffective group. And EPB42 had a sensitivity of 75% and a specificity of 100% at the optimal threshold of 1.3817. In addition, GSEA showed that high expression of ADIPOR1 was mainly related to Mismatch repair, Circadian rhythm, Protein processing in endoplasmic reticulum, DNA replication, and Fanconi anemia pathways.

Conclusion: ADIPOR1 in whole blood is a promising candidate to act as prognostic biomarker for predication of SBRT outcomes in primary liver cancer patients.

1 School of Public Health and Management, Wenzhou Medical University, Wenzhou, Zhejiang, China
2 South Zhejiang Institute of Radiation Medicine and Nuclear Technology, Wenzhou, Zhejiang, China
3 Key Laboratory of Watershed Science and Health of Zhejiang Province, Wenzhou Medical University, Wenzhou, Zhejiang, China
4 NHC Key Laboratory of Radiobiology (Jilin University), Changchun, Jilin, China

Received 5 February 2022; accepted 10 April 2022

Corresponding Authors:
Xiaodong Liu, School of Public Health and Management, Wenzhou Medical University, Wenzhou, Zhejiang, China; South Zhejiang Institute of Radiation Medicine and Nuclear Technology, Wenzhou, Zhejiang, China. Email: liuxd2014@126.com

Shumei Ma, School of Public Health and Management, Wenzhou Medical University, Wenzhou, Zhejiang, China; South Zhejiang Institute of Radiation Medicine and Nuclear Technology, Key Laboratory of Watershed Science and Health of Zhejiang Province, Wenzhou Medical University, Wenzhou, Zhejiang, China. Email: shmm2001@126.com

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).
Keywords
sclerotomy body radiation therapy, liver cancer, prognosis, biomarkers

Introduction

Liver cancer is expected to be the seventh most frequently diagnosed cancer and the second leading cause of cancer death around the world in 2020, with 905,777 new cases and 830,180 deaths per year. It is also one of the most common malignant tumors in China, showing No. 5 of morbidity and No. 2 of mortality among malignant tumors in Chinese. Hepatocellular carcinoma (HCC) is the leading pathologic subtype contributing 85–90% of primary liver cancer. The main risk factors for liver cancer are chronic hepatitis B virus HBV or hepatitis C virus HCV, heavy alcohol intake, aflatoxin-contaminated foods, obesity, smoking, etc. Among all cancers in China, liver cancer has the poorest survival except for pancreatic cancer and the age-standardized 5-year relative survival is only 14.1%. In 2022, it is estimated that there will be 431,383 new liver cancer cases and 412,216 new liver cancer deaths in China. Invasion, metastasis and recurrence are the primary factors that affect clinical treatment and prognosis. Besides, the insidious onset of liver cancer is another reason, for the majority of liver cancer patients are diagnosed at a late stage when it is too far advanced to be cured. Being highly malignant with rapid progression, the treatment for advanced liver cancer is difficult.

At present, there are various treatment methods for liver cancer, including surgical treatment, such as radical surgical resection and liver transplantation; non-surgical treatment, such as local ablation therapy, arterial chemoembolization, gene molecular targeted therapy, systemic chemotherapy, and radiotherapy. Surgical resection has proven to be the optimal treatment for patients with unresectable, locally advanced, or recurrent liver cancer. Additionally, NCCN also recommends that SBRT can also be used as an alternative to ablation/Transarterial Chemoembolization (TACE) treatments, options for treatment after ablation/TACE failure, or treatment options for liver cancer patients with ablation/TACE contraindications. Neointerventional therapy is a potential future treatment for liver cancer. However, to date, the evaluation of radiotherapy efficacy relies mainly on imaging data and the calculated local control based on complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD), such long-term index as overall survival (OS), disease-free survival (DFS), progression-free survival (PFS), etc. are also used. In addition, some serum molecules have been used for biomarkers, for example, AFP has long been used for estimation of the liver cancer progression, but even in the advanced stage 15–30% of patients with a normal AFP levels. So far there are still no effective immediate-early markers for assessing the efficacy of radiotherapy. Therefore, finding biomarkers with prognostic value for radiotherapy is still a focus that researchers are paying attention to. Genetics affects the occurrence and development of tumors, whether the change of mRNA profiles in venous blood after radiotherapy could be used as prognostic biomarkers, is our concern. In the current study, we mainly focused on evaluating the prognostic value of serum biomarkers, both the commonly used (AFP, CEA, CA125, CA153, CA199, and CA724) and novel (screened from high-throughput sequencing data) biomarkers, for HCC patients treated by SBRT.

Adiponectin is the most abundant fat specific hormone, which functions by binding to its specific receptors adiponectin receptor protein 1 (ADIPOR1) and adiponectin receptor protein 2 (ADIPOR2). ADIPOR1 is widely expressed in skeletal muscle and liver. The activation of ADIPOR1 plays an important role in regulating glucose metabolism and fatty acid catabolism. High-fat alcoholic diet downregulated the protein levels of hepatic ADIPOR1. In recent years, it has been found that ADIPOR1 also plays an important role in tumorigenesis, invasion, and metastasis. As for ADIPOR1 and liver cancer, it reported that overexpressed miR-221 promoted epithelial-mesenchymal transition (EMT) in hepatocellular carcinoma by targeting ADIPOR1, and low expression of ADIPOR1 was associated with increased risk of recurrence.
and death in patients with liver cancer. In this study, we identified ADIPOR1 as a prognostic marker for SBRT in patients with liver cancer through clinical information, high-throughput sequencing and qPCR analysis.

In short, the main purpose of this study has two points: 1. To evaluate the prognostic value of commonly used tumor markers in patients with primary liver cancer treated with SBRT; 2. Screen for new and effective biomarkers for patients with primary liver cancer treated with SBRT based on sequencing data. This is the first report for screening prognostic biomarkers for liver patients treated with SBRT.

**Material and Methods**

**Patient Selection and Medical Record Collection**

The primary liver cancer patients involved in this study were divided into two parts. In the retrospective study, the clinical records of all primary liver cancer patients who underwent SBRT at Ruikang Hospital affiliated to Guangxi Medical University from January 2012 to December 2018 were retrospectively reviewed and follow-up was conducted in the following years, and the last follow-up date was April 2019. Patient information collected included gender, age, SBRT treatment time, discharge time, reexamination time, tumor marker examination results, and 1–5-year follow-up results. The inclusion criteria for the retrospective study were as follows: (1) First SBRT treatment without previous treatment history; (2) No other combined treatment such as surgery, intervention, chemotherapy, etc. The exclusion criteria were: (1) Patients with previous treatment history; (2) Patients with other malignant tumors. In the prospective study, we collected blood samples from HCC patients who received SBRT from December 2017 to December 2018. Three blood samples for each patient were collected, that is, before SBRT, before discharge and 2 months after SBRT. In addition, the inclusion criteria for the prospective study were as follows: (1) Size less than 10 cm, with Child-Pugh score A or B liver function; (2) First SBRT treatment without previous treatment history, 80 Gy < BED <100 Gy (BED: Biologically Effective Dose); (3) No other comprehensive treatment such as chemotherapy that seriously affects the blood index. The exclusion criteria were: (1) Lost to follow-up and failed to complete three blood collections as scheduled; (2) Received other treatments within two months of SBRT. Patients were all informed of the study and signed a written informed consent form. All patients provided informed written consent and all research and related activities involving human subjects were approved by the Ethics Committee of the first and second hospital affiliated to Jilin University and performed in accordance with guidelines and regulations and the Declaration of Helsinki. Trial registration: ResMan, number: ChiCTR1800015499. Registered 20 April 2018, www.medresman.org.

**Specimen Collection and High-Throughput Sequencing**

A total of six blood samples from two HCC patients were collected for RNA-sequencing. Blood samples were collected before SBRT (first sample), before discharge (second sample) and 2 months after SBRT (third sample). In this study, PAXgene Blood RNA Tubes (PreAnalytiX, Qiagen BD, Valencia, CA) (abbreviated as BRT) were used to collect blood samples for quick protection of the RNA from degradation. PAXgene Blood RNA Kit (PreAnalytiX, Qiagen BD, Valencia, CA) was used for subsequent experimental studies. The whole blood samples (5 mL) were transported to Novogene (Novogene, Beijing) for RNA isolation, quality control, library preparation, and sequencing.

**Quantification of mRNAs by RT-qPCR Analysis**

A total of 45 blood samples from 15 HCC patients were collected for RT-qPCR. Blood sample collection and RNA extraction were all carried as previously described. For the reverse transcription reaction, the RT reaction solution was prepared on ice according to the following components: 2 μL of 5×PrimeScript RT Master Mix (Perfect Real Time), 500 ng of total RNA, followed by RNase Free dH2O up to 10 μL. The reaction mixtures were incubated at 37°C for 15 min, followed by 85°C for 5 sec and saved at 4°C. qRT-PCR was performed using the ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA).

All primer set were designed and synthesized by Wegen Biotechnology Corporation (Shanghai, China). The sense and antisense primers of ADIPOR1 are: TCCGTGCACTAA- CAGGGAAG and GTTGGCGATTACCCGTTTG; the sense and antisense primers of EPB42 are: ACTTTGTGAA CCAGAATGCT CTC and TCCACTTCTC TACCTGCTTG TC; and so on. GAPDH (forward primers: CAATGACCC TTCATTCGCC and reverse primers: GACAAGCTTC TTCATTGACC) was used as the reference control. We used the TB Green™ Premix Ex TaqTM II (Tli RNaseH Plus) (Takara, Code No. RR820 A) as the QPCR test kit following the manufacturer’s recommendations. The 2−ΔΔCt method was performed to calculate the relative levels of mRNAs.

**Statistical Analysis**

Mann–Whitney U test and Student’s t test were used to compare differences between two groups. Area under receiver-operating characteristic (ROC) curve (AUC) was used to determine the optimal cut-off value of blood mRNA levels and their diagnostic ability. P-values (in two-sided tests) ≤0.05 were considered statistically significant. Histograms were plotted by Graphpad Prism 7.0 (San Diego, California). Volcano map, heatmap, box plot, and GSEA plot were drawn by the statistical packages within R package version 4.0.3. Clinical data analysis was performed using SPSS, version 24 (IBM Corp, Armonk,
Table 1. Analysis of the difference in the expression of tumor markers in patients before treatment, 3 months after treatment and 6 months after treatment.

| Tumor Biomarker | 3 months after SBRT vs before Treatment | 6 months after SBRT vs before Treatment |
|-----------------|----------------------------------------|----------------------------------------|
|                 | Median Level in 3 months after SBRT | Median Level before Treatment | Z | P               | Number\(^a\) | Median Level in 6 months after SBRT | Median Level before Treatment | Z | P               |
| AFP             | 367 | 18.00 (5.92,182.20) | 103.10 (7.86,1210.00) | −8.312 .000 | 233 | 11.62 (4.58,128.85) | 52.51 (6.52,1176.00) | −6.141 .000 |
| CEA             | 309 | 2.65 (1.77,4.21)   | 2.73 (1.63, 4.55)    | −.888 .375 | 204 | 2.83 (1.85,3.98)   | 2.86 (1.65,4.31)    | −.363 .716  |
| CA125           | 295 | 20.95 (14.06, 59.95) | 18.91 (10.72, 44.12) | −2.997 .003 | 197 | 21.24 (12.42, 62.92) | 16.56 (9.80, 34.73) | −4.067 .000 |
| CA153           | 281 | 15.00 (10.58,21.68) | 14.57 (10.22,21.29)  | −.540 .589 | 193 | 13.38 (9.33, 20.40) | 13.59 (9.74, 20.00) | −.270 .787  |
| CA199           | 303 | 27.24 (13.17,48.53) | 26.40 (12.01, 50.74) | −.316 .752 | 202 | 21.71 (8.27, 44.24) | 25.93 (9.22, 50.05) | −2.551 .011 |
| CA724           | 285 | 1.42 (1.05, 2.63)   | 1.30 (0.98, 2.89)    | −1.278 .201 | 193 | 1.47 (1.05, 2.69)   | 1.28 (0.99, 2.72)   | −1.204 .229 |

Mann-Whitney U test was used to compare differences between two groups. P-values (in two-sided tests) ≤.05 were considered statistically significant.

\(^a\)means the number of patients with biomarker examination record both in before SBRT and 3 months after SBRT.

\(^b\)means the number of patients with biomarker examination record both in before SBRT and 6 months after SBRT.
Results

Patient Specimens

In the retrospective study, we collected a total of 589 medical records of primary liver cancer patients admitted to hospital for SBRT between January 2012 and December 2018. After excluding 55 patients who were lost for follow-up, a total of 534 patients were involved in the following research. In the prospective study, 51 blood samples from 17 patients were collected, the outcome after SBRT were followed up and recorded, the relationship between markers and prognosis, the ROC and assessment of prognostic value were analyzed.

Table 2. Analysis of the difference in the proportion of tumor markers in the survival group and the death group.

| Follow-up time | Proportion of tumor markers | Survive number | Death number | Proportion of tumor markers in survival group M (P25, P75) | Proportion of tumor markers in death group M (P25, P75) | Z     | P     |
|----------------|-----------------------------|----------------|--------------|----------------------------------------------------------|----------------------------------------------------------|-------|-------|
| One-year       | AFP 3 months vs before      | 161            | 65           | -.318 (−.873, .276)                                      | .000 (−.557, .458)                                       | -1.808 | .071  |
|                | AFP 6 months vs before      | 130            | 26           | -.380 (−.908.0.216)                                      | .000 (−.474, .860)                                       | -2.264 | .024  |
|                | CA125 3 months vs before    | 123            | 49           | .085 (−.325, .580)                                       | .651 (−.217, 2.083)                                      | -3.019 | .003  |
|                | CA125 6 months vs before    | 106            | 21           | .093 (−.358, 0.733)                                      | 2.583 (5.40, 13.327)                                    | -4.056 | .000  |
|                | CA199 6 months vs before    | 108            | 22           | -.063 (−.367, .165)                                      | .333 (−.172.2.472)                                      | -2.791 | .005  |
| Two-year       | AFP 3 months vs before      | 78             | 96           | -.446 (-.909, .272)                                      | .000 (-.593.0.471)                                      | -2.313 | .021  |
|                | AFP 6 months vs before      | 65             | 51           | -.710 (−.928, .268)                                      | .000 (−.571, .716)                                       | -2.934 | .003  |
|                | CA125 3 months vs before    | 59             | 71           | .051 (−.196, .459)                                       | .625 (−.273.1.991)                                      | -2.864 | .004  |
|                | CA125 6 months vs before    | 53             | 39           | -.019 (−.451, .487)                                      | .959 (−.033, 5.315)                                     | -3.504 | .000  |
|                | CA199 6 months vs before    | 56             | 40           | -.063 (−.360.0.164)                                      | .092 (−.131, 1.519)                                     | -2.389 | .017  |
| Three-year     | AFP 3 months vs before      | 33             | 100          | -.349 (-.871, .468)                                      | .000 (-.635, .487)                                      | -1.188 | .235  |
|                | AFP 6 months vs before      | 26             | 59           | -.750 (−.939, .609)                                      | .000 (−.574, .716)                                       | -2.055 | .040  |
|                | CA125 3 months vs before    | 27             | 77           | -.108 (−.316, .489)                                      | .651 (−.252, 1.787)                                     | -2.324 | .020  |
|                | CA125 6 months vs before    | 21             | 46           | .029 (−.348.0.824)                                       | .851 (−.107, 4.753)                                     | -2.365 | .018  |
|                | CA199 6 months vs before    | 22             | 47           | .045 (-.168, .305)                                       | .091 (-.308, .761)                                      | -0.773 | .440  |

NY, USA). In addition, Medcalc software, version 11.5.0.0 (MedCalc Software, Mariakerke, Belgium) was used to evaluate the prognostic value of biomarkers. All authors had access to the study data and had reviewed and approved the final article.

Prognostic Analysis of Commonly Used Serum Tumor Biomarkers

In the retrospective study, we selected patients together with serum biomarker examination before SBRT, 3 months after SBRT, and 6 months after SBRT. The results are shown in Table 1. We found that AFP, CA125, and CA199 were differential expressed before and after SBRT.

Then, we wanted to figure out the relationship between these markers and the overall survival (OS) for 1-year, 2-year, and 3-year (Table 2). From Table 2, significant differences were found between the survival group and the death group, that is, change ratio of AFP in 6 months after SBRT, and CA125 in 3 months and 6 months after SBRT in 1-year, 2-year, and 3-year OS. And no difference was found between the survival group and the death group in the change of CA199 for 3-year OS.

The diagnostic ability of these significant markers was then evaluated. The ROC curve was made and the sensitivity, specificity, AUC (Area Under the ROC Curve), Youden index
Table 3. Evaluation of predictive efficacy in distinguishing survival group and death group of the tumor markers.

|                  | One year |                     |                     | Two-year |                     |                     | Two-year |
|------------------|----------|---------------------|---------------------|----------|---------------------|---------------------|----------|
|                  | Sensitivity | Specificity | Youden index | AUC | Cut-off | Sensitivity | Specificity | Youden index | AUC | Cut-off | Sensitivity | Specificity | Youden index | AUC | Cut-off |
| AFP 3 months vs before | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| AFP 6 months vs before | 40.8 | 92.3 | .33 | .641 | ≤.68 | 52.3 | 84.3 | .37 | .659 | ≤.68 | 53.8 | 81.4 | .35 | .640 | ≤.68 |
| CA125 3 months vs before | 79.7 | 51.0 | .31 | .648 | ≤.63 | 86.4 | 52.1 | .39 | .647 | ≤.55 | 88.9 | 53.2 | .42 | .651 | ≤.54 |
| CA125 6 months vs before | 68.9 | 81.0 | .50 | .781 | ≤.48 | 73.6 | 69.2 | .43 | .715 | ≤.38 | 95.2 | 43.5 | .39 | .681 | ≤1.28 |
| CA199 6 months vs before | 80.6 | 59.1 | .40 | .689 | ≤.23 | 89.3 | 37.5 | .27 | .642 | ≤.35 | — | — | — | — | — |

AUC: Area under receiver-operating characteristic (ROC) curve. Medcalc Software, Version 11.5.0.0 (MedCalc Software, Mariakerke, Belgium) Was Used to Calculate AUC and Cut-Off.
and cut-off values were shown in Table 3. The Youden index of AFP, CA125 and CA199 as prognostic markers for judging the efficacy of SBRT was less than .5. In addition, the AUC values of the change ratio of tumor biomarkers distinguishing survival group and death group were almost less than .70, except for CA125 in 6 months which were .781 and .715 in 1-year group and 2-year group, respectively.

**High-Throughput Sequencing for Gene Profiles**

By the high-throughput sequencing, we obtained gene expression profiles before and after SBRT, the visualization of the heatmap of the HCC samples are shown in Supplementary Figure 1. Then, we compared the changes in gene expression before discharge (group 2) with pre-treatment (group 1), 2 months after SBRT treatment (group 3) with pre-treatment (group 1), and also group 3 vs group 2. Genes with q<.05 (q value is adjusted P value) were used as the differential gene, and the FPKM values of all differential genes in each comparison group were summarized. log$_2$FC>1 (FC: fold change) was considered to be up-regulated, and log$_2$FC <.5 was considered to be downregulated, the visualization of the volcano map of the HCC samples are shown in Supplementary Figure 2. Then, we used log$_2$FC> 2 to define up-regulation/down-regulation, and deleted the transcript with a detection value of 0. By analyzing all the differentially expressed genes of group 2 vs 1, group 3 vs 1 and group 3 vs 2, it was found that a total of 16 differentially expressed genes remained elevated in all HCC patients after SBRT (i.e., up-regulated in group 2 vs 1 and in group 3 vs 1, but no difference in group 3 vs 2) (shown in Supplementary Figure 3(A)). In addition, a total of 12 differentially expressed genes remained downregulation in all HCC after SBRT (i.e. downregulated in group 2 vs 1 and in group 3 vs 1, but no difference in group 3 vs 2) (shown in Supplementary Figure 3(B)).

**Evaluation of the Differential Genes for SBRT Efficacy in HCC**

The expression of the above 28 genes were further verified by qPCR. Nonparametric test was used to determine whether there were differences in gene expression before and after
Table 5. Change ratio of differentially expressed genes in the effective and ineffective groups of liver cancer at 3 months after SBRT.

| Differential gene | Change ratio of the expression before discharge and before SBRT (2vs1) | Change ratio of the expression in 2 months after SBRT and before SBRT (3vs1) |
|-------------------|---------------------------------------------------------------|---------------------------------------------------------------|
|                   | Effective (Z) | Ineffective (Z) | Effective (Z) | Ineffective (Z) |
| ADIPOR1           | .205 (-.112, .571) | 1.020 (.874, 1.484) | -.2304 .021 | .646 (-.098, 1.400) | 1.298 (281, -) | -1.202 .229 |
| ASCC2             | .149 (-.218, .479) | .675 (-.034, 1.591) | -.1455 .146 | .376 (-.080, 1.503) | .315 (.276, -) | -1.277 .782 |
| BCL11 B           | -.514 (-.820, -.127) | -.253 (-.706, -.508) | -.364 .716 | .670 (-.591, 1.578) | -.473 (-.898, -) | -1.387 .166 |
| BCL2L1            | .307 (039, .865) | .196 (-.285, .989) | -.364 .716 | .546 (.005, 1.502) | -.342 (-.619, -) | -1.647 .518 |
| BLK               | -.512 (-.864, .024) | -.496 (-.847, -.325) | -.485 .628 | -.523 (-.842, 1.522) | -.433 (-.926, -) | -1.277 .782 |
| CAT               | -.454 (-.624, .040) | -.176 (-.669, .194) | -.485 .628 | -.369 (-.509, .630) | .310 (-.740, -) | -1.277 .782 |
| CD79 A            | -.817 (-.892, -.507) | -.592 (-.849, -.306) | -.789 .430 | -.564 (-.662, .408) | -.564 (-.680, -) | -1.185 .853 |
| COL19 A           | -.823 (-.918, -.354) | -.384 (-.889, .030) | -.606 .544 | -.608 (-.853, .685) | -.221 (-.933, -) | -1.092 .926 |
| CXC5              | -.628 (-.876, -.357) | -.594 (-.900, -.080) | -.121 .903 | -.495 (-.763, .516) | -.609 (-.912, -) | -1.647 .518 |
| EPB42             | .400 (087, 589) | 2.062 (.747, 2.678) | -.2304 .021 | 1.303 (.537, 1.772) | 1.616 (1.538, -) | -1.757 .079 |
| GOLGA6L9          | -.440 (-.811, -.088) | -.757 (-.869, .526) | -.728 .467 | -.227 (-.656, .093) | -.710 (.860, -) | -1.757 .079 |
| IL7R              | -.721 (-.828, -.514) | -.595 (-.726, -.014) | -.1213 .225 | -.222 (-.672, .955) | -.188 (-.673, .266) | -1.992 .926 |
| KLHL1             | -.846 (-.900, -.566) | -.103 (-.901, 1.542) | -.1455 .146 | -.535 (-.792, -.003) | -.346 (-.955, 1.576) | -1.462 .644 |
| OR2W3             | -.041 (-.150, 1.198) | -.236 (077, 1.156) | -.1091 .275 | .886 (1.156, 1.424) | .316 (-.364, 2.293) | -1.462 .644 |
| PAX5              | -.777 (-.927, 2.233) | -.894 (-.919, -.547) | -.243 .808 | -.561 (-.855, .088) | -.716 (-.888, -.651) | -1.202 .229 |
| FECH              | .117 (1.175, 1.496) | .496 (218, 1.053) | -.970 .322 | .693 (1.146, 1.630) | .313 (-.362, -) | -1.092 .926 |
| SFRP2             | .408 (-.083, 1.400) | .482 (-.03, 3.984) | -.849 .396 | .178 (-.248, 1.267) | .318 (-.629, 1.702) | -1.277 .782 |
| STAP1             | -.737 (-.862, -.539) | -.760 (-.918, -.469) | -.485 .628 | -.344 (-.671, .656) | -.406 (-.919, .075) | -1.647 .518 |
| TMCC2             | .407 (.091, 615) | .961 (.015, 1.467) | -.1334 .182 | 1.441 (1.128, 2.334) | .269 (-.257, 1.577) | -1.832 .405 |
| UBA5              | -.003 (-.281, .679) | .124 (-.067, 1.043) | -.123 .225 | 1.244 (-.096, 1.829) | .307 (0.03, 2.915) | -1.092 .926 |
| UBB               | -.085 (-.238, .578) | .591 (.512, 1.296) | -.1940 .052 | 1.135 (.014, 2.219) | 1.295 (36, 2.302) | -1.462 .644 |

Mann-Whitney U test was used to compare differences between 2 groups. P-values (in two-sided tests) ≤ .05 were considered statistically significant.

Table 6. The evaluation results of the prognostic value of the change ratio of ADIPOR1 and EPB42 (2vs1) for liver cancer patients in 3 months after SBRT.

| Gene     | AUC   | SE    | Sensitivity (%) | Specificity (%) | Yoden Index | Cut-off |
|----------|-------|-------|-----------------|-----------------|-------------|---------|
| ADIPOR1  | .896  | .087  | 100             | 83.33           | .83         | .5838   |
| EPB42    | .896  | .111  | 75.00           | 100.00          | .75         | 1.3817  |

*2vs1, mean a pre-discharge vs before SBRT

SBRT. We found that 21 genes were differently expressed, and the detailed results are shown in Table 4.

Further, we calculated the changes of 21 genes and evaluated whether the change in group 2 vs 1, group 3 vs 1 were different between the effective and ineffective groups after SBRT. According to the imaging data of HCC patients, the therapeutic effects were divided into CR, PR, SD, and PD based on the change of the diameter of HCC, CR and PR were classified into effective group, and SD and PD were classified into ineffective group. The results are shown in Table 5, it can be seen that ADIPOR1 and EPB42 were differentially expressed in pre-discharge vs before SBRT (P<.05), indicating that ADIPOR1 and EPB42 had significant changes in the short term after SBRT treatment. Therefore, in the following study, we mainly focused on the two genes ADIPOR1 and EPB42.

Since there was no significant difference in group 3 vs 1, we only evaluated the prognostic value of the change ratio of group 2 vs 1 in the efficacy of 3-month after SBRT for HCC. The evaluation results of the prognostic value for ADIPOR1 and EPB42 were listed in Table 6 and the ROC curves were shown in Supplementary Figure 3(A) and (B). The results showed that ADIPOR1 had a sensitivity of 100% and a specificity of 83.33%, at the optimal threshold of .5838. And EPB42 had a sensitivity of 75% and a specificity of 100%, at the optimal threshold of 1.3817. Given that the Yoden index of ADIPOR1 is higher than .80, and the Yoden index of EPB42 is lower than .80, ADIPOR1 was considered to be a better
prognostic biomarker than EPB42. Combined Table 5 with Table 6, it can be seen that when the change ratio of ADIPOR1 was lower than .5838 in pre-discharge compared with that before SBRT, patients had a better prognosis.

**Function Analysis Based on TCGA**

In order to further explore the biological functions of the potential prognostic marker ADIPOR1, we used the TCGA database to perform further biometric analysis. By using 371 tumor tissues and 50 paracarcinoma tissues in liver hepatocellular carcinoma (LIHC), we found that ADIPOR1 was significant higher expressed in tumor tissues (shown in Supplementary Figure 5(A)). Further, by using Gene Set Enrichment Analysis (GSEA), we found that high expression of ADIPOR1 is mainly related to the following pathways: mismatch repair, Circadian rhythm, protein processing in endoplasmic reticulum, DNA replication, and Fanconi anemia pathway (top 5), the GSEA plot were shown in Supplementary Figure 5(B).

**Discussion**

Tumor biomarkers, such as AFP, CEA, CA199, CA153, and CA125, are commonly used for estimation of the disease progression. By detecting the levels of these biomarkers, clinician can make a preliminary assessment of the occurrence and progress of tumors. Jung J et al26 reported that liver cancer patients had a better prognosis when AFP levels returned to normal levels 3 months after SBRT, Uemotok et al27 found that the risk of recurrence was associated with the elevated AFP level. Whether those biomarkers are with prognostic value is our concern. Previous study reported that high AFP were associated with worse survival for liver patients who receiving Cyberknife treatment,28 however, all patients they included are in advanced or terminal stage of HCC. In our study, we evaluated the prognostic values of those existing tumor biomarkers, and found that those widely used tumor biomarkers such as AFP, CEA, and CA199, could not precisely predict the outcome of liver cancer patients treated by SBRT due to insufficient sensitivity or specificity, for the Yoden index was less than .5, indicating that these commonly used biomarkers do not provide a good assessment of the therapeutic efficacy of SBRT. Actually, although AFP is a well-known biomarker, the use of AFP as a screening indicator for HCC has been canceled by the 2010 American Association for the Study of Liver Diseases guidelines due to its low sensitivity.29 Therefore, it is necessary to find more effective biomarkers for accurate assessment of the efficacy of SBRT in patients with liver cancer. To this end, we used blood samples from liver cancer patients before and after radiotherapy to discover new and effective biomarkers.

In our research, we explored potential biomarkers in mRNA level for the immediate-early assessment of prognosis after SBRT in patients with primary liver cancer through high-throughput sequencing and PCR. By using strict screening criteria, we finally got 28 differentially expressed mRNAs that were commonly increased or decreased after SBRT. Of all the 28 mRNAs, we found that the change ratio of ADIPOR1 ($Z = -2.304, P=.021$) and EPB42 ($Z = -2.304, P=.021$) in “before discharge” vs “before SBRT” was significantly different between the effective and ineffective groups at 3 months after SBRT.

Previous studies revealed that the expression of ADIPOR1 was significantly lower in liver cancer than non-neoplastic hepatic tissues,30 suggested an inverse relation of ADIPOR1 to malignancy. In our study, ADIPOR1 is elevated after SBRT and had a good sensitivity and specificity in assessing the efficacy of SBRT after 3 months. Similarly, EPB42 was also found to be a good prognostic biomarker for liver cancer patients treated by SBRT. Furthermore, when the change of ADIPOR1 was lower than .5838 in pre-discharge compared with that before SBRT, patients had a better prognosis. And the sensitivity is 100%. That is to say, by detecting the changes of ADIPOR1 before discharge compared with those before SBRT, the efficacy of the patients three months after SBRT could be effectively predicted. In addition, routine blood collection before treatment and before discharge, did not increase burden for patients, it is convenient to monitor the changes of ADIPOR1 and help to estimate whether the patient has a good prognosis, whether the patient needs further radiotherapy, and consequently providing a reliable reference for the determination of the overall clinical treatment plan.

In addition, there are only two studies on ionizing radiation (IR) and ADIPOR1 response, one is on IR and mouse intestine, suggesting that ADIPOR1 in mouse colon tissue has a significant decrease 12 months after 2 Gy irradiation31; one is a recent report by our research group, we found that ADIPOR1 decreased significantly in hepatoma cells after 15 Gy irradiation, and knocking down ADIPOR1 could enhance radiation sensitivity.32 These results suggested that ADIPOR1 was related to the progression of liver cancer, and IR could cause significant changes in ADIPOR1. Targeting ADIPOR1, combined with IR, may improve the efficacy of radiotherapy in patients with liver cancer.

There are still some limitations in this study. First, although ADIPOR1 shown promising prognostic value for SBRT, it still needs more samples and further validation; Secondly, how does ionizing radiation cause changes in ADIPOR1 and then affects the prognosis of patients with liver cancer, and what’s the underlying mechanism, remain to be studied. In addition, the GSEA showed that high expression of ADIPOR1 was mainly related to Mismatch repair, Circadian rhythm, Protein processing in endoplasmic reticulum, DNA replication and Fanconi anemia pathways, perhaps those results may provide some theoretical basis for future mechanism research.
Appendix

Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| SBRT        | Stereotactic body radiotherapy |
| CR           | complete response |
| PR           | partial response |
| SD           | stable disease |
| PD           | progressive disease |
| OS           | overall survival |
| DFS          | disease-free survival |
| PFS          | progression-free survival |
| EBRT         | external beam radiation therapy |
| BED          | Biologically Effective Dose |
| ROC          | receiver-operating characteristic curve |
| AUC          | Area under ROC |

Author Contributions

Zhenzhen Liang: Data analysis, original draft.
Chang Xue and Guanghui Li: Data analysis.
Qing Chen, Mengke Li: Data cleaning.
Hao Feng and Yi Liu: Data collection.
Xiaodong Liu and Shumei Ma: Conceptualization, review & editing.

Information on the authors responsible for statistical analysis

Declaration of Conflicting Interests

The author declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

The author disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Natural Science Foundation of China (81972969, 81872558 and 81773363).

Ethical Approval and Consent to Participate

The study design was approved by the Ethics Committee of the certain hospitals.

Consent for Publication

The authors agree to publication.

Availability of Data and Material

The data used and analyzed in this study are available from the corresponding author upon reasonable request.

ORCID iDs

Zhenzhen Liang  https://orcid.org/0000-0002-4069-2625
Xiaodong Liu  https://orcid.org/0000-0002-6879-990X
Shumei Ma  https://orcid.org/0000-0002-9167-4582

References

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA A Cancer J Clin. 2021;71(3):209-249.
2. Meng Z, Ren Q, Zhong G, et al. Noninvasive detection of hepatocellular carcinoma with circulating tumor DNA features and α-fetoprotein. J Mol Diagn. 2021;23(9):1174-1184.
3. Allemani C, Matsuda T, Di Carlo V, et al. Global surveillance of trends in cancer survival 2000-14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. Lancet (London, England). 2018;391(10125):1023-1075.
4. Xia C, Dong X, Li H, et al. Cancer statistics in China and United States, 2022: Profiles, trends, and determinants. Chinese Med J. 2022;135(5):584-590.
5. Visvader JE. Cells of origin in cancer. Nature. 2011;469(7330):314-322.
6. Zhang H-H, Zhang Y, Cheng Y-N, et al. Metformin in combination with curcumin inhibits the growth, metastasis, and angiogenesis of hepatocellular carcinoma in vitro and in vivo. Mol Carcinog. 2018;57(1):44-56.
7. Kim M, Kay CS, Jang WI, Kim M-S, Lee DS, Jang HS. Prognostic value of tumor volume and radiation dose in moderate-sized hepatocellular carcinoma. Medicine. 2017;96(24):e7202.
8. Klein J, Dawson LA. Hepatocellular carcinoma radiation therapy: Review of evidence and future opportunities. Int J Radiat Oncol Biol Phys. 2013;87(1):22-32.
9. NCCN Clinical Practice Guidelines in Oncology. Hepatobiliary Cancers, Available at: https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1438.
10. Chandra RA, Keane FK, Voncken FEM, Thomas CR Jr. Contemporary radiotherapy: Present and future. Lancet. 2021;398(10295):171-184.
11. Lazarev S, Hardy-Abeloos C, Factor O, Rosenzweig K, Buckstein M. Stereotactic body radiation therapy for centrally located hepatocellular carcinoma: Outcomes and toxicities. J Cancer Res Clin Oncol. 2018;144(10):2077-2083.
12. Murray LJ, Dawson LA. Advances in Stereotactic Body Radiation Therapy for Hepatocellular Carcinoma. Semin Radiat Oncol. 2017;27(3):247-255.
13. Moon DH, Wang AZ, Tepper JE. A prospective study of the safety and efficacy of liver stereotactic body radiotherapy in patients with and without prior liver-directed therapy. Radiother Oncol. 2018;126(3):527-533.
14. Petrelli F, Cornito T, Barni S, Pancra G, Scorsetti M, Ghidini A. Stereotactic body radiotherapy for colorectal cancer liver metastases: A systematic review. Radiother Oncol. 2018;129(3):427-434.
15. Cantaloube M, Castan F, Creoff M, et al. Image-Guided Liver Stereotactic Body Radiotherapy Using VMAT and Real-Time Adaptive Tumor Gating: Evaluation of the Efficacy and Toxicity for Hepatocellular Carcinoma. Cancers. 2021;13(19).
16. Jing W, Gao S, Zhu M, et al. Potential diagnostic value of lncRNA SPRY4-IT1 in hepatocellular carcinoma. *Oncol Rep*. 2016;36(2):1085-1092.

17. Li Q, Tan J-X, He Y, et al. Atractylenolide III ameliorates non-alcoholic fatty liver disease by activating Hepatic adiponectin Receptor 1-Mediated AMPK Pathway. *Int J Biol Sci*. 2022; 18(4):1594-1611.

18. Lewandowski D, Foik AT, Smidak R, et al. Inhibition of ceramide accumulation in AdipoR1-/- mice increases photoreceptor survival and improves vision. *JCI Insight*. 2022; 7(4).

19. Nascimento AF, Ip BC, Luvizotto RA, Seitz HK, Wang XD. Aggravation of nonalcoholic steatohepatitis by moderate alcohol consumption is associated with decreased SIRT1 activity in rats. *Hepatobiliary Surgery and Nutrition*. 2013; 2(5):252-259.

20. Kordafshari M, Nourian M, Mehrvar N, et al. Expression of AdipoR1 and AdipoR2 and serum level of adiponectin in gastric cancer. *Gastrointestinal Tumors*. 2020; 7(4):103-109.

21. Byeon J-S, Jeong J-Y, Kim MJ, et al. Adiponectin and adiponectin receptor in relation to colorectal cancer progression. *Int J Cancer*. 2010; 127(12):2758-2767.

22. Huang Q, Peng L, Sun Y, et al. miR-593-3p Promotes Proliferation and Invasion in Prostate Cancer Cells by Targeting ADIPOR1. *OncoTargets Ther*. 2021; Volume 14: 3729-3737.

23. Sun G, Zhang X, Liu Z, et al. The Adiponectin-AdipoR1 axis mediates tumor progression and tyrosine kinase inhibitor resistance in metastatic renal cell carcinoma. *Neoplasia*. 2019; 21(9):921-931.

24. Li T, Li M, Hu S, et al. MiR-221 mediates the epithelial-mesenchymal transition of hepatocellular carcinoma by targeting AdipoR1. *Int J Biol Macromol*. 2017;103:1054-1061.

25. Shin E, Yu Y-D, Kim D-S, Won NH. Adiponectin receptor expression predicts favorable prognosis in cases of hepatocellular carcinoma. *Pathol Oncol Res*. 2014;20(3): 667-675.

26. Jung J, Yoon SM, Han S, et al. Alpha-fetoprotein normalization as a prognostic surrogate in small hepatocellular carcinoma after stereotactic body radiotherapy: a propensity score matching analysis. *BMC Cancer*. 2015;15:987.

27. Uemoto K, Doi H, Shiomi H, et al. Clinical assessment of microresidual tumors during stereotactic body radiation therapy for hepatocellular carcinoma. *Anticancer research*. 2018;38(2):945-954.

28. Kato H, Yoshida H, Taniguch H, et al. Cyberknife treatment for advanced or terminal stage hepatocellular carcinoma. *World Journal of Gastroenterology*. 2015;21(46): 13101-13112.

29. Brux J, Sherman M. Management of hepatocellular carcinoma: An update. *Hepatology*. 2011;53(3):1020-1022.

30. Hebbard L, Ranscht B. Multifaceted roles of adiponectin in cancer. *Best Practice & Research Clinical Endocrinology & Metabolism*. 2014;28(1):59-69.

31. Suman S, Kallakury BVS, Fornace AJ Jr, Datta K. Protracted upregulation of leptin and IGF1 is associated with activation of PI3K/Akt and JAK2 pathway in mouse intestine after ionizing radiation exposure. *Int J Biol Sci*. 2015;11(3):274-283.

32. Liu Y, Qi M, Liu L, Li M, et al. Blocking Adipor1 enhances radiation sensitivity in Hepatoma Carcinoma Cells. *Arch Biochem Biophys*. 2022;718:109152.