TP53 Mutation in Circulating exoDNA Correlates with Outcomes of Patients with Hepatocellular Carcinoma.

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Abstract

Background: Detection of tumor-specific mutations in exosomal DNA (exoDNA), a promising liquid biopsy material, has been used to assess the prognosis of hepatocellular carcinoma (HCC) patients. This study was the first to use a droplet digital PCR (ddPCR) platform to detect tumor-specific mutations in circulating exoDNA and to evaluate the prognosis of HCC patients.

Methods: Blood samples from 40 HCC patients were obtained between 2018 and 2019, with clinically annotated follow-up until 2020. A ddPCR platform was used to detect an HCC tumor-specific mutation in the TP53 gene. We analyzed the correlation between TP53 mutation detected in circulating exoDNA and patient clinical data. The ratio of mutant droplets/total droplets (MD/TD) was calculated according to ddPCR results.

Results: TP53 mutations in circulating exoDNA were detected in 33 of the 40 patients (82.5%). Patients with high MD/TD (>62.5%) were more likely to show microvascular invasion ($P=0.028$) and high MD/TD predicted a shorter recurrence-free survival time ($P<0.001$).

Conclusions: High MD/TD of TP53 detected in serum was associated with microvascular invasion and might be used to predict the prognosis of HCC patients. The diagnostic performance of detecting exosome-derived tumor DNA will likely improve when more mutations in other tumor-specific genes are combined.

Background

Hepatocellular carcinoma (HCC) is currently the fifth most common malignancy with the third highest mortality rate in the world[1]. Effective treatment and diagnostic strategies for HCC remain difficult problems. Although there are many treatments for HCC, including surgery, transcatheter arterial chemoembolization, and targeted drugs, the recurrence rate of HCC is high[1, 2]. Therefore, timely diagnosis and real-time intervention are required for effective diagnostic and therapeutic measures for HCC. Unfortunately, the diagnostic efficacy of traditional markers, such as AFP, is not ideal[3]. In recent years, multiple studies have used circulating free DNA (cfDNA) from the blood of pancreatic and colorectal cancer patients to identify tumor-specific mutations as cancer markers[4, 5]. However, cfDNA originates from dead cells in damaged tissue or accumulates as a result of physiological cell turnover[6, 7]. This may lead to reduced sensitivity of cfDNA markers and make the identification of cancer-specific mutations more challenging.

Exosomes are extracellular vesicles 40–150 nm in size that are rich in DNA, RNA, and proteins[8]. Although exoDNA shows biological stability and has the potential for clinical application, few studies have examined exoDNA compared with the multiple studies already performed on exosomal RNA. Exosomes are secreted by living and dead cells, as well as cancer cells, which produce high numbers of exosomes[8–10]. Recent studies have shown that circulating exoDNA can be used to identify cancer-specific mutations[9, 11, 12], which is of great significance for the diagnosis and treatment of HCC

patients as well as prognosis assessment. Genetic mutations have been traditionally identified by genome sequencing of biopsy tumor fragments or cfDNA, but this represents only a small fraction of tumor heterogeneity because cfDNA is released by dead tumor cells[6]. ExoDNA is more stable than cfDNA because of the lipid bilayer structure of exosomes[13, 14], and exosomes contain larger DNA fragments, which is conducive to the detection of mutations. Additionally, because exosomes can be released from various cells in tumors, exoDNA in plasma may reflect tumor heterogeneity. Based on these advantages, increasing studies have been reported on tumor-specific mutations in circulating exoDNA, such as *EGFR* mutations in lung adenocarcinoma patients and *KRAS* mutations in pancreatic cancer patients[9, 11, 15]. However, no studies have been reported on the detection of tumor-specific mutations in circulating exoDNA in HCC patients.

In this study, here we report the first investigation of the value and significance of detection of tumor-specific mutations in circulating exoDNA in HCC patients using droplet digital PCR (ddPCR).

Results

Patient characteristics

This study included 40 patients with primary HCC who were enrolled between October 2018 and August 2019 at the Second Affiliated Hospital of Nanchang University. The clinical characteristics of the 40 patients are listed in Table 1, and the preoperative information of the 40 patients is provided in Supplementary Table 1.

Correlation between TP53 mutation in circulating exoDNA and clinicopathologic characteristics

The c.747G>T mutation in TP53 has been identified as one of the hottest mutants in HCC patients[16-18]. And our previous studies have confirmed the presence of TP53 mutations in circulating tumor DNA in HCC patients[16]. Thus, these mutations might be detectable in exoDNA, which also contains abundant tumor cell-derived genetic information. We therefore selected the TP53 gene for analysis and detected TP53 gene mutation status in circulating exoDNA. The copy number of wild-type and mutant TP53 genes in all patients is shown in Supplementary Table 2. TP53 mutations were identified in 33 of 40 patients (sensitivity, 82.5%). The high sensitivity may be due to the wide source and stability of exosomes. We examined the correlation between TP53 mutation status and patient clinical characteristics but found no correlation with clinical characteristics.

The ddPCR analysis results are displayed in Supplementary Figure 1. According to the results of ddPCR, we calculated and analyzed the ratio of mutant droplets/total droplets (MD/TD). Notably, the MD/TD was associated with microvascular invasion (MVI) ($P=0.035$). ROC curve analysis showed that the best cut-off value of MD/TD to predict MVI was $>62.5\%$ (sensitivity 45%, specificity 95%, 95%CI [0.524–0.826], $P=0.024$); the detailed results are shown in Supplementary Figure 2. Multi-factor logistics regression

analysis also demonstrated that high MD/TD was an independent risk factor for MVI ($p=0.028$). Thus, high MD/TD can predict MVI to a certain extent. However, other clinicopathological features such as cirrhosis, cancer thrombin, tumor size, and tumor number were not significantly associated with TP53 mutation status and MD/TD. The detailed results of these analyses are shown in Table 1.

Correlation between TP53 mutation rate in circulating exoDNA and survival

We also analyzed whether TP53 mutation status in exoDNA could predict the prognosis of HCC patients who had undergone surgical treatment. We developed a regular and rigorous follow-up strategy of the 40 HCC patients from the day of operation to the date of tumor recurrence or up to 576 days. We first compared the survival curves of patients with and without TP53 mutation in exoDNA and found no significant difference between groups ($P=0.329$). We analyzed the survival curves of patients with high and low MD/TD and found that the median recurrence-free survival was 61 days (range, 53–202 days) for patients with high MD/TD and 368 days (range, 51–576 days) for patients with low MD/TD ($P<0.001$, log-rank test, Figure 1A). This result showed that patients in the group with high MD/TD were more likely to relapse than those in the group with low MD/TD. We also performed survival analysis for patients with MVI according to MD/TD, and the results also showed that patients with high MD/TD were more likely to relapse than patients with low MD/TD ($P=0.035$, log-rank test, Figure 1B). Thirteen patients did not relapse during follow-up, and all of these patients showed low MD/TD.

Discussion

In this study, TP53 mutation and MD/TD in circulating exoDNA were detected to evaluate their diagnostic and prognostic value in HCC. Exosomes are secreted by a wide range of sources, including tumor cells[10], which is why exosome DNA is easier to detect than cfDNA. This study demonstrated that exosomes and purified DNA can be extracted from serum volumes as low as 250 μ L for the detection of TP53 mutations.

Our results showed that TP53 mutations were detected in 82.5% of patients, which is immensely higher than the previous detection rate for tumor-specific mutations in cfDNA. We previously detected TERT, TP53 and CTNNB1 mutations in cfDNA of HCC patients, with a sensitivity of only 19.5%[16]. Other studies have shown that the different stages of cfDNA at between 30.8% and 57.9%[19]. In addition, a meta-analysis showed that cfDNA lacks robustness in the diagnosis of HCC[20]. This finding may be explained by the single source of cfDNA and the small number of circulating gene fragments produced from tumor tissues, with the total number of circulating DNA fragments $<1.0\%$ [21, 22], and it makes traditional technology difficult to detect. No studies thus far have described the detection of TP53 mutations in circulating exoDNA in patients with HCC. Therefore, we used ddPCR to detect TP53 mutations in circulating exoDNA of HCC patients and explored the possibility of circulating exoDNA as a new non-invasive liquid biopsy method in the diagnosis and prognosis of HCC. Krimmel et al reported

that the detection of TP53 mutations was related to age[23]. However, our study did not find an association between age and TP53 mutation status.

Our results showed that patients with high MD/TD were more likely to show MVI. HCC patients with vascular invasion have a poor prognosis[24], and our study confirmed that patients with high MD/TD were more likely to relapse compared with patients with low MD/TD ($P < 0.001$). Wang et al. reported that circulating tumor DNA correlates with MVI and predicts tumor recurrence of HCC[17]. These results are consistent with our findings. We speculate that the association between MD/TD and vascular invasion may be because MVI makes it easier for nucleic acid fragments from the tumor to enter the circulation. Our findings suggest that HCC patients with high MD/TD are more likely to show MVI and a poor prognosis. Therefore, MD/TD may be a candidate factor to predict the occurrence of MVI in HCC patients and thus to assess patient prognosis.

Like most tumors, HCC tumors show heterogeneity[25, 26]. After the multiple stages of cell division and proliferation during tumor development, tumor cells undergo various genetic changes, resulting in aberrant growth rate, invasion ability, sensitivity to drugs, and other activities[27]. This may also contribute to the limitations of traditional biopsy. Notably, the detection of tumor-specific mutations in circulating exoDNA overcomes obstacles associated with tumor heterogeneity. Detection of mutations in exoDNA is therefore a promising non-invasive liquid biopsy approach for early diagnosis, efficacy assessment, follow-up monitoring, and prognosis assessment in HCC patients.

Targeted sequencing of circulating exoDNA has been applied to a variety of tumors. Allenson et al. detected KRAS mutations in 66.7% (22/33), 80% (12/15) and 85% (17/20) of patients with localized, locally advanced and metastatic pancreatic cancer, respectively[9], with rates similar to our sensitivity detection results of 82.5%. In addition, Yang et al. demonstrated the value of circulating exoDNA as a rapid and low-cost method for identification of cancer-driving mutations in circulating exoDNA such as KRAS and TP53 mutations in pancreatic cancer patients and healthy individuals, respectively[11]. In contrast to these studies, we did not detect TP53 gene mutations in circulating exoDNA in healthy individuals, which also led to the lack of specificity in our experimental results. Uchiyama et al. reported that due to low exosome DNA concentrations, these samples may be in a "gray area" for detecting[28], which may be a potential limitation of circulating exoDNA as a liquid biopsy. Moreover, compared with other reports, we detected the TP53 mutation with a coverage area of only 87 bp, which made the detection of circulating exoDNA less effective as a non-invasive liquid biopsy.

To increase the value of circulating exoDNA detection in HCC application, we are currently planning a prospective study that will cover almost all HCC-specific mutations. We will also improve the exoDNA extraction technology to improve the sensitivity of ddPCR, so as to improve the detection of low exosome DNA concentrations. We anticipate that this approach will provide beneficial information for the molecular evaluation of personalized HCC therapy. The detection of circulating exoDNA may enable further development of precision medical technologies to realize early diagnosis of HCC and to customize personalized treatment strategies.

Conclusions

At present, the diagnosis, treatment and follow-up monitoring of HCC are changing in the world. It is the diversified diagnosis and prognostic monitoring that makes it possible for patients with HCC to receive timely and effective treatment. In our study we proposed for the first time a framework for fluid biopsy of HCC patients by detecting tumor-specific mutations in circulating exoDNA. Our results provided strong evidence that the mutation rate of the TP53 gene in circulating exoDNA was associated with MVI and might be used to predict patients with shorter recurrence-free survival. We also demonstrated that the detection of circulating exoDNA could overcome the limitations caused by tumor heterogeneity. The detection of tumor-specific mutations in circulating exoDNA may be a reliable method for the diagnosis of HCC, and simultaneously detection of multiple tumor-specific genes will improve the diagnostic performance. A future prospective study is required to validate the results of our preliminary study.

Methods

Sample collection

A total of 40 patients with primary HCC at the Second Affiliated Hospital of Nanchang University were enrolled between October 2018 and August 2019. All patients agreed to the analysis of exoDNA mutations in collected preoperative blood. Postoperative pathology revealed HCC. HCC patients were eligible if they agree to have their blood collected for detection of exoDNA before surgery. None of the HCC patients received preoperative cancer-related therapies. Written informed consent was obtained from all patients enrolled in the study. Blood samples were collected in EDTA tubes, and within 1 h, samples were centrifuged at 3000×g for 20 min. Serum samples were stored at -80°C. This study was approved by the Ethics Review committee of the Second Affiliated Hospital of Nanchang University.

Exosome extraction and exoDNA extraction

Exosomes were extracted from serum samples using the ExoQuick™ kit (System Bioscience, CA, USA) following the manufacturer's instructions. We examined the exosomes by electron microscopy (Supplementary Figure 3A) and detection of the exosome-specific protein CD63. Protein expression of CD63 was assessed by western blotting and three experiments were repeated to verify the presence of CD63 (Supplementary Figure 3B). ExoDNA was extracted from exosomes using the ExoDNA™ Extraction kit (BioVision Inc, SF, USA) following the manufacturer's instructions. We used this method to extract exosome DNA and the presence of DNA was confirmed in all samples.

Study design and droplet digital PCR (ddPCR)

DdPCR (also known as single-molecule PCR) generally consists of two steps: PCR amplification and fluorescence signal analysis. Our experimental procedures followed the guidelines proposed for reporting

digital PCR data[29]. The PCR primers and probe sequences were designed using Primer Premier 5.0 software. Each PCR reaction contained 10µl ddPCR Supermix for Probes (Bio-Rad, USA), 3.6µl of primer (Sangon Company, China), 1µl of probe (Sangon Company), 2µl of template DNA from exoDNA and 3.4µl of ddH₂O to give a total volume of 20µl. The PCR conditions were 96°C for 10 min; 40 cycles of 94°C for 30 s and 60°C for 60 s; with a final incubation at 98°C for 10 min.

Preparation and analysis of droplets

We added a new DG8 Cartridge (BioAssay Systems, CA, USA) into the holder and added 20µL of the samples into eight holes in the middle row. Next, 70µL drop-generated Oil (DG Oil) was added into the bottom row of eight cartridges in the same chamber. The gasket was covered and the holes on both sides were firmly hooked. The holder was placed in the QX200 droplet generator (Bio-Rad, CA, USA) for generation of droplets, which generally took about 2 min to complete. Droplets were produced in the top row of cartridges and subsequently transferred into a 96-well plate. The plate was placed on a heat-sealing instrument and covered with a heat-sealing membrane for sealing. The QX200 Droplet analyzer was used for droplet analysis and detection, and the data were uploaded to the computer for final analysis. After the reactions were completed, the threshold line was adjusted to the appropriate position according to the specific reaction conditions for result interpretation. The 5' primer ends of mutant and wild-type genes were labeled with the fluorescent dyes FAM (blue) and VIC (green), respectively, to assess the mutation status of the genotyped PCR product with the QX200 Droplet analyzer. Red fluorescence indicated that FAM and VIC were simultaneously detected. The copy number results of PCR amplification and droplet fluorescence analysis for all patients are shown in Supplementary Table 2 and Supplementary Figure 1, respectively.

Statistical analysis

We used Stata software (version 16.0; Stata Corporation LP; College Station, TX, USA) to perform statistical analyses. Pearson correlation analysis was used to examine the correlation of the ratio of MD/TD with patient MVI. Logistics regression analysis was used to assess the correlation between mutation status and the clinical and pathologic characteristics. Recurrence-free survival (calculated as the time from operation to tumor recurrence) curves were plotted according to Kaplan–Meier method and assessed using the log-rank test. Results were considered statistically significant if the P-value was less than 0.05.

Abbreviations

TP53: Tumor protein p53; ExoDNA: Exosomal DNA; HCC: Hepatocellular carcinoma; DdPCR: Droplet digital Polymerase Chain Reaction (PCR); MD/TD: The ratio of mutant droplets/total droplets; AFP: Alpha-fetoprotein; CfDNA: Circulating free DNA; MVI: Microvascular invasion; RFS: Recurrence-free survival.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Review committee of The Second Affiliated Hospital of Nanchang University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Province, China (20181BBG70010). It contributed to study design and data collection, analysis and interpretation, and it also contributed to authorization of software and paper written.

Availability of data and materials

The clinical data generated during the current study are available in medical system of The Second Affiliated Hospital of Nanchang University. The experimental data of ddPCR are from Sangon Biotech Company (Shanghai, China).

Authors' contributions

All authors read and approved the final manuscript. YL, JW: experiments, conceptualization, formal analysis, software, and writing—original draft. LW, WL: conceptualization, writing—review and editing, project administration. JL, FZ, XY, DH, YM: samples data curation and methodology. All authors had access to the study data and reviewed and approved the final article.

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References

1. Altekruse SF, McGlynn KA, Reichman ME: **Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005.** *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2009, **27**(9):1485-1491.
2. Molla MD, Dessie G, Akalu Y, Ayelign B: **Hepatocellular Expression of SIRT1 and Its Effect on Hepatocellular Carcinoma Progression: A Future Therapeutic Perspective.** *International journal of hepatology* 2020, **2020**:2374615.
3. Benowitz S: **Liver cancer biomarkers struggling to succeed.** *Journal of the National Cancer Institute*, 2007, **99**(8):590-591.
4. Zvereva M, Roberti G, Durand G, Voegelé C, Nguyen MD, Delhomme TM, Chopard P, Fabianova E, Adamcakova Z, Holcatova I *et al*: **Circulating tumour-derived KRAS mutations in pancreatic cancer cases are predominantly carried by very short fragments of cell-free DNA.** *EBioMedicine* 2020, **55**:102462.
5. Lee J, Kim JH, Kang SH, Yoo HM: **Improvement of digital PCR conditions for direct detection of KRAS mutations.** *Journal of clinical laboratory analysis* 2020:e23344.
6. Schwarzenbach H, Hoon DSB, Klaus P: **Cell-free nucleic acids as biomarkers in cancer patients.** *Nature Reviews Cancer*; 2011, **11**(6):426-437.
7. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R: **DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells.** *CANCER RESEARCH* 2001, **61**(4):1659-1665.
8. Sharma A, Johnson A: **Exosome DNA: Critical regulator of tumor immunity and a diagnostic biomarker.** *Journal of cellular physiology* 2020, **235**(3):1921-1932.
9. Allenson K, Castillo J, San Lucas FA, Scelo G, Kim DU, Bernard V, Davis G, Kumar T, Katz M, Overman MJ *et al*: **High prevalence of mutant KRAS in circulating exosome-derived DNA from early-stage pancreatic cancer patients.** *Annals of Oncology* 2017, **28**(4):741-747.
10. Bakhoun SF, Ngo B, Laughney AM, Cavallo JA, Murphy CJ, Ly P, Shah P, Sriram RK, Watkins TBK, Taunk NK *et al*: **Chromosomal instability drives metastasis through a cytosolic DNA response.** *Nature* 2018, **553**(7689):467-472.
11. Yang S, Che SP, Kurywachak P, Tavormina JL, Gansmo LB, Correa de Sampaio P, Tachezy M, Bockhorn M, Gebauer F, Haltom AR *et al*: **Detection of mutant KRAS and TP53 DNA in circulating exosomes from healthy individuals and patients with pancreatic cancer.** *Cancer Biology & Therapy* 2017, **18**(3):158-165.

12. Bernard V, Kim DU, San Lucas FA, Castillo J, Allenson K, Mulu FC, Stephens BM, Huang J, Semaan A, Guerrero PA *et al*: **Circulating Nucleic Acids Are Associated With Outcomes of Patients With Pancreatic Cancer.** *Gastroenterology* 2019, **156**(1):108-118.e104.
13. Liu Y, Wang Y, Lv Q, Li X: **Exosomes: From garbage bins to translational medicine.** *International journal of pharmaceutics* 2020, **583**:119333.
14. Jin Y, Chen K, Wang Z, Wang Y, Liu J, Lin L, Shao Y, Gao L, Yin H, Cui C *et al*: **DNA in serum extracellular vesicles is stable under different storage conditions.** *BMC cancer* 2016, **16**(1):753.
15. Qu X, Li Q, Yang J, Zhao H, Wang F, Zhang F, Zhang S, Zhang H, Wang R, Wang Q *et al*: **Double-Stranded DNA in Exosomes of Malignant Pleural Effusions as a Novel DNA Source for EGFR Mutation Detection in Lung Adenocarcinoma.** *Frontiers in oncology* 2019, **9**:931.
16. Liao W, Yang H, Xu H, Wang Y, Ge P, Ren J, Xu W, Lu X, Sang X, Zhong S *et al*: **Noninvasive detection of tumor-associated mutations from circulating cell-free DNA in hepatocellular carcinoma patients by targeted deep sequencing.** *Oncotarget* 2016, **7**(26):40481-40490.
17. Wang J, Huang A, Wang YP, Yin Y, Fu PY, Zhang X, Zhou J: **Circulating tumor DNA correlates with microvascular invasion and predicts tumor recurrence of hepatocellular carcinoma.** *Annals of translational medicine* 2020, **8**(5):237.
18. Shen T, Li SF, Wang JL, Zhang T, Zhang S, Chen HT, Xiao QY, Ren WH, Liu C, Peng B *et al*: **TP53 R249S mutation detected in circulating tumour DNA is associated with Prognosis of hepatocellular carcinoma patients with or without hepatectomy.** *Liver international* 2020.
19. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM *et al*: **Detection of circulating tumor DNA in early- and late-stage human malignancies.** *Science translational medicine* 2014, **6**(224):224ra224.
20. Liao W, Mao Y, Ge P, Yang H, Xu H, Lu X, Sang X, Zhong S: **Value of quantitative and qualitative analyses of circulating cell-free DNA as diagnostic tools for hepatocellular carcinoma: a meta-analysis.** *Medicine* 2015, **94**(14):e722.
21. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, Thornton K, Agrawal N, Sokoll L, Szabo SA *et al*: **Circulating mutant DNA to assess tumor dynamics.** *Nature medicine* 2008, **14**(9):985-990.
22. Diaz LA, Bardelli A: **Liquid Biopsies: Genotyping Circulating Tumor DNA.** *Journal of Clinical Oncology* 2014, **32**(6):579-586.
23. Krimmel JD, Schmitt MW, Harrell MI, Agnew KJ, Kennedy SR, Emond MJ, Loeb LA, Swisher EM, Risques RA: **Ultra-deep sequencing detects ovarian cancer cells in peritoneal fluid and reveals somatic TP53 mutations in noncancerous tissues.** *Proceedings of the National Academy of Sciences of the United States of America* 2016, **113**(21):6005-6010.
24. Ling YH, Chen JW, Wen SH, Huang CY, Li P, Lu LH, Mei J, Li SH, Wei W, Cai MY *et al*: **Tumor necrosis as a poor prognostic predictor on postoperative survival of patients with solitary small hepatocellular carcinoma.** *BMC cancer* 2020, **20**(1):607.
25. Cabral LKD, Tiribelli C, Sukowati CHC: **Sorafenib Resistance in Hepatocellular Carcinoma: The Relevance of Genetic Heterogeneity.** *Cancers* 2020, **12**(6).

26. Kwa WT, Effendi K, Yamazaki K, Kubota N, Hatano M, Ueno A, Masugi Y, Sakamoto M: **Telomerase reverse transcriptase (TERT) promoter mutation correlated with intratumoral heterogeneity in hepatocellular carcinoma.** *Pathology international* 2020.

27. Zhang J, Fujimoto J, Zhang J, Wedge DC, Song X, Zhang J, Seth S, Chow CW, Cao Y, Gumbs C *et al*: **Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing.** 2014, **346**(6206):256-259.

28. Uchiyama Y, Nakashima M, Watanabe S, Miyajima M, Taguri M, Miyatake S, Miyake N, Saitsu H, Mishima H, Kinoshita A *et al*: **Ultra-sensitive droplet digital PCR for detecting a low-prevalence somatic GNAQ mutation in Sturge-Weber syndrome.** *Scientific reports* 2016, **6**:22985.

29. Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, Hellemans J, Kubista M, Mueller RD, Nolan T *et al*: **The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments.** *Clinical chemistry* 2013, **59**(6):892-902.

Tables

Table 1: Correlation between TP53 mutation and clinicopathological data.

Clinical Characteristics		Patients (n=40)		TP53 mutation	MD/TD>62.5
		No.	%	(n=33)	(n=10)
Age	≤60	28	70	23	9
	>60	12	30	10	1
	P			0.928	0.702
Gender	Female	11	27.5	8	1
	Male	29	72.5	25	9
	P			0.325	0.180
HBV	Yes	39	97.5	32	10
	No	1	2.5	1	0
	P			1.000	1.000
Cirrhosis	Yes	22	55	17	6
	No	18	45	16	4
	P			0.345	0.714
Tumor size	<5cm	23	57.5	18	3
	≥5cm	17	42.5	15	7
	P			0.418	0.051
Tumor thrombus	Yes	35	87.5	29	2
	No	5	12.5	4	8
	P			0.875	0.416
Microvascular invasion	Yes	20	50	18	9
	No	20	50	15	1
	P			0.226	0.028*
Tumor number	Single	36	90	31	10
	Multiple	4	10	2	0
	P			0.100	0.999
Preoperative AFP (ng/ml)	>400	12	30	9	5
	≤400	28	70	24	5
	P			0.419	0.783

Abbreviations: TP53 mutation, tumor protein p53 mutation status in circulating exosomal DNA; MD/TD: The ratio of mutant droplets/total droplets; HBV, hepatitis B virus; AFP, alpha-fetoprotein.

Figures

Figure 1

Recurrence-free survival (RFS) curves for HCC patients included in our study. A. Recurrence-free survival in subjects with detectable high MD/TD in circulating exoDNA. This analysis revealed that patients with detectable high MD/TD in circulating exoDNA were more likely to relapse than those in whom the detection was negative ($P < 0.001$, log-rank test). B. Similar analyses were performed in patients who suffered MVI, revealing a significant association between detectable MD/TD and RFS ($P = 0.035$, log-rank test).

Supplementary Files

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