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ORIGINAL RESEARCH—BASIC

Detection of γ-OHPdG in Circulating Tumor Cells of Patients With Hepatocellular Carcinoma as a Potential Prognostic Biomarker of Recurrence

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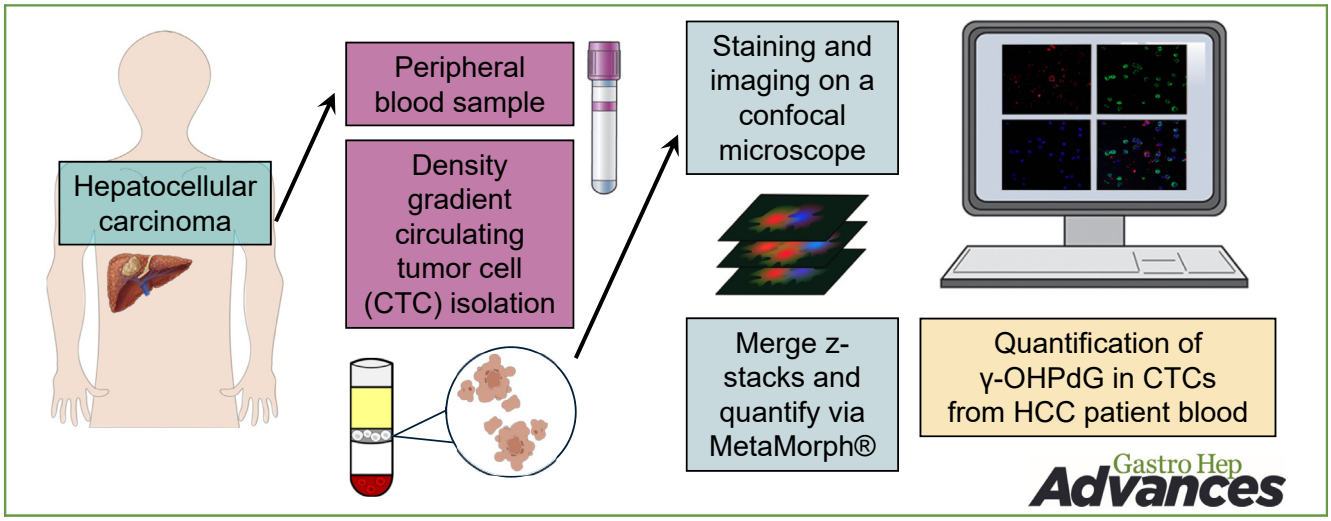

BACKGROUND AND AIMS: Blood-based biomarkers for hepatocellular carcinoma (HCC) and its recurrence are lacking. We previously showed that hepatic γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (γ-OHPdG), an endogenous DNA adduct derived from acrolein by lipid peroxidation, increased during hepatocarcinogenesis. Additionally, higher hepatic γ-OHPdG from HCC patients after surgery were strongly associated with poor survival ($P < .0001$) and recurrence-free survival ($P = .007$) (Fu et al, Hepatology, 2018). These findings suggest that γ-OHPdG is a potential prognostic biomarker for HCC and its recurrence. To attain the goal of using γ-OHPdG as a biomarker in future preventive and therapeutic trials, we developed a blood-based method to detect γ-OHPdG in circulating liver tumor cells from HCC patient blood. **METHODS:** We first established the specificity of anti-γ-OHPdG antibody by determining its dose-response in HepG2 cells treated with acrolein. Then, HepG2 cells in spiked blood of healthy volunteers and circulating tumor cells (CTCs) from 32 HCC patients were isolated using a RosetteSep CD45 Depletion Cocktail and Ficoll Paque. The HCC CTCs identified with anti-asialoglycoprotein receptor 1, a surface protein expressed solely in hepatocytes, were stained with an anti-γ-OHPdG antibody. The number of total HCC CTCs and γ-OHPdG-positive CTCs, as well as the staining intensity, were quantified using MetaMorph software. As an initial effort toward its clinical application, we also evaluated γ-OHPdG in CTCs from these patients along with certain clinical features. **RESULTS:** The γ-OHPdG antibody specificity was demonstrated by an acrolein concentration-dependent increase of γ-OHPdG-positive HepG2 cells and the intensity of γ-OHPdG staining. The recovery of HepG2 cells from spiked blood was ∼50–60%, and the positivity rate of CTCs in blood from 32 patients with advanced HCC was 97%. The MetaMorph analysis

Abbreviations used in this paper: **γ-OHPdG, γ-hydroxy-1,N^2-propano-2′-deoxyguanosine; AFP, α-fetoprotein; ASGR1, asialoglycoprotein receptor 1; CTC, circulating tumor cell; CTP Score, Child-Turcotte-Pugh Score; DAPI, 4′,6-diamidino-2-phenylindole; dG, deoxyguanosine; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HV, healthy volunteer; IF, immunofluorescence; IHC, immunohistochemistry; LPO, lipid peroxidation; NASH, nonalcoholic steatohepatitis; NER, nucleotide excision pathway; PBS, phosphate buffered saline; RFS, recurrence-free survival; RT, room temperature.**

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showed a wide variation among patients in total number of CTCs, γ-OHPdG positivity, and staining intensity. Statistical analysis revealed that γ-OHPdG in CTCs of these patients appears to be associated with multifocality and poor differentiation. **CONCLUSION:** A blood-based method was developed and applied to HCC patients to evaluate the potential of γ-OHPdG in CTCs as a prognostic biomarker.

Keywords: Hepatocellular Carcinoma; Recurrence; Circulating Tumor Cells; DNA Adduct; γ-OHPdG; Biomarker; ASGR1

Introduction

Almost 1 million liver cancer cases are diagnosed worldwide each year.[1] Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related deaths, mainly due to the lack of a suitable biomarker for early detection, inadequate understanding of the molecular features, and resistance to current chemotherapy.[2] Resection, liver transplantation, and ablation remain the only curative treatment modalities for HCC.[3] As surgical procedures and perioperative care have improved, death rates following the resection of HCC have approached zero in experienced treatment centers.[4] However, the risk of recurrence during the first 3 years following resection is estimated at 50%, which increases to between 75% and 100% over 5 years.[5,6] HCC has a poor prognosis with a 5-year survival of only 18% in the United States, and when diagnosed at advanced stages, the median patient survival is around 6–20 months.[7–9] There is a great unmet need to decrease cancer recurrence after surgical resection. Therefore, understanding risk factors for HCC recurrence and developing strategies to address risk factors to improve patient outcomes are important clinical research goals for this deadly disease.

HCC is associated with oxidative stress as a result of chronic inflammation originating from different etiologies, including hepatitis B virus (HBV)/hepatitis C virus (HCV), nonalcoholic fatty liver disease, nonalcoholic steatohepatitis (NASH), and obesity.[10,11] Oxidative stress induces lipid peroxidation (LPO) and has emerged as a major player in the development and progression of liver cancer.[12–14] LPO is associated with increased preneoplastic lesions in hepatocarcinogenesis in animal models.[13] LPO yields a host of reactive aldehydes capable of modifying DNA bases via Michael addition, forming cyclic adducts as endogenous lesions.[15–19] Certain cyclic DNA adducts, including acrolein-derived DNA adducts, are promutagenic and potentially play a role in carcinogenesis and, therefore, may serve as mechanism-based biomarkers to predict cancer risk.[20–24] Currently, α-fetoprotein (AFP) is considered the most widely used biomarker for HCC, despite suffering from poor sensitivity and specificity.[25] Furthermore, at an AFP cutoff of 20 ng/mL, 30% of early-stage HCC patients test negative for HCC.[26] AFP is a US Food and Drug Administration-approved biomarker for risk stratification but not for HCC surveillance. AFP is also not required as part of the recommended HCC surveillance plan by the American Association for the Study of Liver Diseases.[26] A more mechanistically and biologically relevant prognostic biomarker for HCC is urgently needed.

γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (γ-OHPdG) is a background DNA adduct in the cells and tissues of rodents and humans.[15] It is a reaction product of deoxyguanosine (dG) with acrolein.[27] In addition to being a ubiquitous environmental pollutant, acrolein is a major LPO product of polyunsaturated fatty acids.[28] γ-OHPdG is repaired by the nucleotide excision pathway (NER) and is a mutagenic lesion that predominantly induces G to T mutations.[20–23] Intriguingly, ligation-mediated polymerase chain reaction showed that γ-OHPdG forms at sites in the human p53 gene that coincide with p53 mutation hotspots in human lung cancer.[24] These observations support a causative role for γ-OHPdG in somatic mutations in critical tumor suppressor genes, raising the possibility that it may serve as a biomarker for human cancer development. In this context, our recent studies showed strong correlations between hepatic γ-OHPdG levels and liver cancer incidence in a number of animal models, including Xpa−/− mice deficient in NER, diethyl nitrosamine-treated mice, Long-Evans Cinnamon rats, and an obesity model in B-6 mice fed a high-fat diet.[29,30] Consistent with the dominant mutations caused by γ-OHPdG, these studies revealed that GC>TA is a major somatic mutation in liver tumor tissues obtained from Xpa−/− mice and the high-fat diet-fed B-6 mice. Remarkably, these studies showed that Theaphenon E, a standardized formulation of green tea extract, effectively reduced HCC incidence by 86%, 60%, and 100% (a complete blockage of HCC formation) in Xpa−/− mice, diethyl nitrosamine-treated mice, and the high-fat diet-fed B-6 mice, respectively.[29] The tumor reduction in these animals occurred with a concomitant decrease of γ-OHPdG in the liver DNA.[30] The close correlation between γ-OHPdG and hepatocarcinogenesis in these animal studies supports that γ-OHPdG formation in livers may constitute an early event leading to HCC. Reinforcing this notion, using 2 independent sets of liver specimens from 90 and 45 HCC patients, we demonstrated that higher levels of γ-OHPdG scored by immunohistochemistry (IHC) staining are closely associated with low survival ($P < .0001$) and low recurrence-free survival (RFS) ($P = .007$).[29] These data showed that patients with γ-OHPdG-high HCC have an increased risk of recurrence compared to subjects with γ-OHPdG-low HCC independent of stage, differentiation, and other clinical factors. To support future HCC prevention and treatment trials, we strongly believe the development of a blood-based noninvasive method for detecting γ-OHPdG is highly warranted.

In recent years, there has been a surge of interest in using circulating tumor cells (CTC) in HCC because of their great potential in early detection, surveillance, and treatment monitoring, as well as in the identification of biomarkers for targeted therapy, metastatic potential, and disease recurrence.[31] CTCs are cells that detach in small numbers from solid tumors located anywhere in the body

and enter the bloodstream. An HCC CTC method could provide a practical, convenient, and powerful tool for biomarker development for prognosis and assessing therapeutic efficacy.[32,33] Liver biopsy is invasive with risks of tumor seeding. Furthermore, biopsy specimens do not reflect the whole tumor, whereas HCC CTCs are thought to represent tumor heterogeneity better and capture all tumor genetic alterations and other cellular and molecular changes in tumors more accurately. These obvious advantages support the idea of developing a CTC method for detecting and quantifying γ-OHPdG in patients' blood. In this study, we report the development of such a method. As a first step toward the goal of future clinical application, we also evaluated the possible correlation of γ-OHPdG in CTCs with clinical factors in 32 patients with advanced HCC.

Materials and Methods

Identification and Enumeration of Liver Cancer Cells by Immunofluorescence Staining and MetaMorph Analysis

HepG2 cells were seeded in 4-well chamber slides, either untreated or treated with acrolein, washed 3 times with 1× phosphate buffered saline (PBS), and fixed with formalin (10%) at room temperature (RT) for 15 minutes. Fixed cells were washed 3 times with 1× PBS and blocked with 5% goat serum (Sigma) in washing buffer (PBS with 0.1% Tween 20) for 30 minutes at RT. Cells were then washed 3 times with washing buffer and incubated with a primary antibody cocktail (rabbit anti-asialoglycoprotein receptor 1 (anti-ASGR1) antibody, Abcam, and mouse anti-γ-OHPdG antibody, both at 1:500 dilution) in blocking buffer (5% normal goat serum in washing buffer) for 1 hour at RT. After 3 washes with washing buffer, cells were incubated with a secondary antibody cocktail (goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Flour Plus 647, both at 1:500 dilution) (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI) (1 μg/mL final concentration) at RT for 1 hour. Cells were washed 3 times with washing buffer and coated with ibidi mounting medium (ibidi USA Inc), and slides were stored at 4 °C until imaging. Slides were imaged using a Leica SP8 microscope (Leica Microsystems) with a 1.4 numerical aperture oil immersion objective lens. Images were captured as Z stacks using the hybrid detectors and analyzed using the MetaMorph (BioVision)-based journal software (Sunnyvale, CA, USA).

The MetaMorph-based software journal creates a three dimensional image for each image using z-stacks. These three dimensional images were then analyzed for cells that are nucleated based on the DAPI staining. The nucleated cells that were positive for ASGR1 were further quantified for the total number of γ-OHPdG-positive liver cells and the intensity of γ-OHPdG staining as indicators of adduct levels in the cells. The same method was used for staining, imaging, and analysis of HepG2 cells from spiked healthy volunteer (HV) blood samples or CTCs isolated from the HCC patient blood samples.

Spiking Experiments With HV Blood Samples

Peripheral blood was drawn from HVs according to the study protocol approved by the IRB board (Study identifier: MOD00010314). All participants signed informed consent prior to enrollment. Approximately 20 mL of blood samples were collected from HVs in BD ethylenediaminetetraacetic acid vacutainers and processed within 2 hours of collection. HVs did not have any prior or current malignancy or liver problem. A known number of HepG2 cells were spiked into HV blood samples, and samples were processed for circulating HepG2 cell enrichment using RosetteSep Human CD45 depletion cocktail and the Ficoll Paque Plus density solution-based method described below. ASGR1-positive stained cells that also stained positive for γ-OHPdG by immunofluorescence (IF) staining, both in the nucleus and whole cell, were enumerated using MetaMorph analysis as described above. Unspiked blood samples from HVs were used as a negative control.

Blood Samples From HCC Patients

Peripheral blood samples were collected from 32 HCC patients according to the study protocol approved by the IRB board (study identifier MOD00010314). Blood samples (5 mL) were collected from HCC patients in BD ethylenediaminetetraacetic acid vacutainers and processed within 2 hours for CTC enrichment using a RosetteSep Human CD45 depletion cocktail and Ficoll Paque Plus density solution-based method as described below. Isolated CTCs were enumerated for ASGR1-positive stained cells that also stained positive for γ-OHPdG, both in the nucleus and whole cell, by IF staining and MetaMorph analysis as described above.

Protocol for the Isolation of HepG2 Cells From Spiked HV Blood Samples or CTCs From HCC Patient Blood Samples

The HepG2 cells from spiked HV blood samples or CTCs from HCC patient blood samples were isolated following an immunodensity method using the RosetteSep Human CD45 depletion cocktail (StemCell Technologies) combined with Ficoll Paque Plus density gradient solution-based method as per the manufacturer's instruction with some modifications. Briefly, 250 μL (50 μL/mL) of the RosetteSep Human CD45 depletion cocktail was added to the 5 mL of spiked blood or HCC patients' blood samples and incubated for 20 minutes at RT. Blood samples were then diluted with equal volumes of 2% fetal bovine serum (FBS) + 1× PBX and layered carefully onto Ficoll Paque Plus. The samples were then centrifuged at 1200*g* at RT for 20 minutes with brake for separation of enriched cells, which were collected from the interface between Ficoll and plasma. The enriched cells were then washed with 2% FBS + 1× PBS at 1200*g* for 10 minutes RT. Cells were then resuspended in 5 mL of 2% FBS + 1× PBS and centrifuged at 500*g* for 10 minutes. Finally, the cells were resuspended in 800 μL 2% FBS + 1× PBS, and the suspension was strained through a 40 μm pluristrainer by centrifugation at 500*g* for 3 minutes at RT. The cell suspension was then added to the 8-well chamber slide (ibidi USA Inc), and the slides were stored at 4 °C for 72 hours before IF staining and MetaMorph analysis as described above.

Fluorescence-Activated Cell Sorting Analysis of the Cells

A total of 1×10^6 cells from different cancer types, including liver (HepG2), breast (MCF7 and MDA-MB-468), and

lung (NCI- H1975), were stained with rabbit anti-ASGR1 antibody, Abcam (1:500 dilution) for 45 minutes at RT. After a wash with 1× PBS, cells were incubated with a goat anti-rabbit Alexa Fluor 488 at RT for 45 minutes. Cells were washed with 1× PBS and resuspended in 0.5 mL of 1× PBS for flow cytometric analysis using a Becton Dickinson fluorescence-activated cell sorting (FACS) sorter (BD Biosciences, San Jose, CA, USA), and the data were analyzed using the Mod Fit program (Verity Software House, Topsham, ME, USA).

For detecting γ-OHPdG-positive HepG2 cells, 1×10^6 HepG2 cells were fixed with 10% formalin at RT for 15 minutes. Fixed cells were washed with 1× PBS and incubated with a primary antibody cocktail (rabbit anti-ASGR1 and mouse anti-γ-OHPdG, both at 1:500 dilution) in 1× PBS for 45 minutes at RT. After washing with 1× PBS buffer, cells were incubated with a secondary antibody cocktail (goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Flour 647, both at 1:500 dilution) at RT for 45 minutes. Cells were then stained with pacific blue antihuman CD45, brilliant violet 650 antihuman CD42b, and anti-pan-cytokeratin-phycoerythrin, all antibodies at 1:500 dilution, in 1× PBS for 15 minutes at RT and analyzed by flow cytometry as described above. The pacific blue antihuman CD45 and brilliant violet 650 antihuman CD42b antibodies were purchased from Biolegend and anti-pan-cytokeratin-phycoerythrin was from Miltenyi Biotech.

For analyzing HepG2 cells from spiked blood samples of a HV, isolated cells were stained with primary antibody (rabbit anti-ASGR1 at 1:500 dilution) in 1× PBS for 45 minutes at RT. After washing with 1× PBS buffer, cells were incubated with a secondary antibody (goat anti-rabbit Alexa Fluor 488 at 1:500 dilution) at RT for 45 minutes. Cells were then stained with pacific blue antihuman CD45 and brilliant violet 650 antihuman CD42b and were then analyzed by flow cytometry as described above. As a control, blood samples that were not spiked with HepG2 cells were processed similarly and analyzed by flow cytometry.

Statistical Analysis Methods

Data were analyzed by both parametric and nonparametric methods. Group comparisons were made using the *t*-test for continuous variables and Fisher's exact test or chi-squared test for categorical variables. Student's *t*-test and one-way analysis of variance were used to compare patient γ-OHPdG rates/intensities (with log-transform) with various clinical responses. Pearson's product-moment correlation coefficient was used to measure the correlation between patient γ-OHPdG rate/intensity and demographical and clinical features. Linear mixed regression was used to determine the relative expression levels of γ-OHPdG in HCC patients. A P value $<.05$ was considered statistically significant.

Results

Detection of Acrolein Dose-Dependent γ-OHPdG in HepG2 Cells by IF Staining and Flow Cytometry Using an Anti-γ-OHPdG Antibody

Previously, an anti-γ-OHPdG antibody was shown to detect γ-OHPdG in cultured human cells, including colon cancer HT29, bronchial BEAS-2B, and oral cells.[34,35] Here, we detected γ-OHPdG in formalin-fixated HepG2 cells stained with mouse hybridoma anti-γ-OHPdG antibody (Figure 1A). To verify the sensitivity and specificity of γ-OHPdG staining in HepG2 cells, we determined the levels of γ-OHPdG in untreated or acrolein-treated HepG2 cells by IF staining followed by imaging using a Leica SP8 microscope. With IF staining using anti-γ-OHPdG antibody, we detected a concentration-dependent increase in the percentage of positively stained HepG2 cells upon treatment with acrolein (50 or 100 μM) (Figure 1B). In addition to nuclear γ-OHPdG, we detected an increase of cells staining positive for adduct in the whole cell, including the cytoplasm (Figure 1B). Furthermore, acrolein treatment (50 or 100 μM) of HepG2 cells induced a significant increase in staining intensity in both the cell nucleus and cytoplasm compared to the untreated cells (Figure 1C). We also stained HepG2 cells using anti-ASGR1 and anti-γ-OHPdG antibodies without fixation for flow cytometry. ASGR1 is a cell surface protein expressed solely on the surface of hepatocytes and HCC cells but not in other human tissues.[36] Almost all HepG2 cells (99.97%) stained positive for ASGR1, whereas no positive ASGR1 staining was observed for cancer cell lines derived from an extrahepatic origin, including breast (MCF7 and MDA-MB-468) and lung (NCI-H1975) (Figure 1D–G). Furthermore, flow cytometry experiments showed antibody specificity to ASGR1 and γ-OHPdG following indirect detection through Alexa Fluor 488 or 647 conjugated secondary antibodies (Figure 1H–L). 84.57% of the HepG2 cells stained positive for ASGR1 also stained positive for γ-OHPdG (Figure 1M).

Detection and Quantification of γ-OHPdG in HepG2 Cells Spiked in the Blood of a HV

We detected γ-OHPdG in HepG2 cells isolated from spiked blood of a HV. In flow cytometry experiments, we stained HepG2-spiked blood from a HV after negative enrichment of HCC cells through depletion of CD45+ leukocytes. We found 82.68% of cells stained positive for both ASGR1 and γ-OHPdG; cells also stained positive for epithelial cell marker pan-cytokeratin (Figure 2A–C). To further confirm the recovery of HepG2 cells from the spiked HV blood samples, flow cytometry was performed after staining the isolated cells for ASGR1, CD45, and CD42b. HepG2 cells that stained positive only for ASGR1 were isolated and these cells were negative for CD45 and CD42b, suggesting that the recovered cells were HepG2 cells, not hematopoietic cells. In CTC experiment, ASGR1 positively stained liver cancer cells were isolated from 5 mL blood samples of a non-HCC patient spiked with 400 HepG2 cells with a recovery rate of approximately 50% (Figure 2D and E). We detected that, both in the nuclei alone and in the whole cell, approximately 90% of these cells stained positive for γ-OHPdG (Figure 2F). In addition, we observed that the staining intensity was almost 3-fold greater in the whole cell than in the nuclei alone (Figure 2G). As a control, the blood samples from a HV without spiking with HepG2 cells were used. Only 0–3 cells/mL of blood that stained positive for ASGR1 (negative for

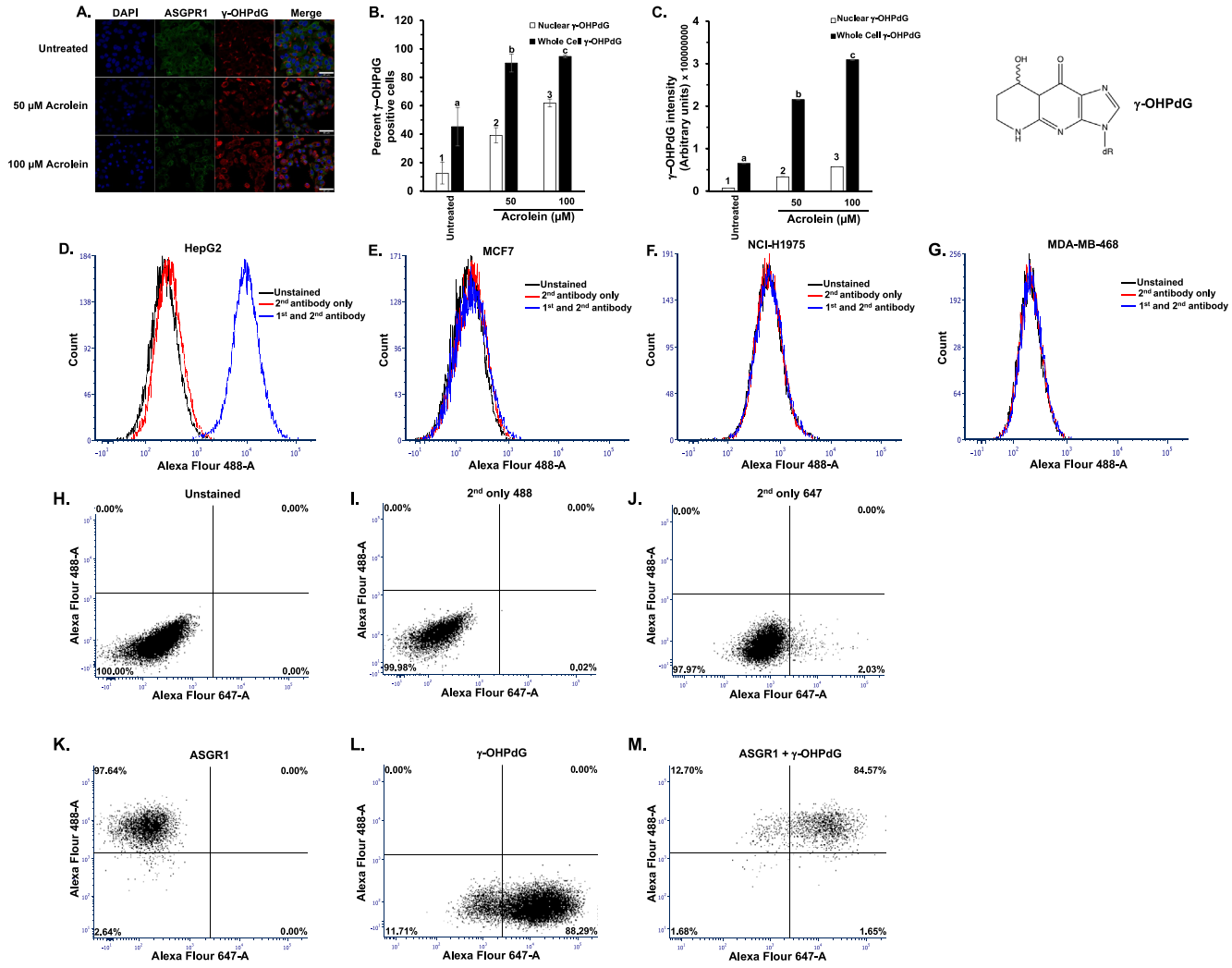

Figure 1. Detection of γ-OHPdG in HepG2 cell cultures by IF and flow cytometry with and without acrolein treatment. HepG2 cells were either untreated or treated with indicated concentrations of acrolein for 5 hours. (A) Representative images of the HepG2 cells stained with γ-OHPdG antibody (red), ASGR1 (green) and DAPI (blue). (B) Percent of cells stained positive for γ–OHPdG in the nucleus only (nuclear) or in nucleus and cytoplasm (whole cell). (C) Intensity of nuclear and whole cell γ–OHPdG. Experiments (A–C) done in duplicate, with error bars for SD and bars bearing different numbers (nucleus) or alphabets (whole cell) statistically different from one another ($P < .05$). In flow cytometry experiments (D–G), (D) HepG2, (E) breast MCF, (F) breast MDA-MB-468, and (G) lung NCI-H1975 cells were stained with or without rabbit anti-ASGR1 primary antibodies and goat-anti-rabbit Alexa Flour 488 secondary antibodies. Lastly, HepG2 cells were further stained for flow cytometry analysis with (H) no antibodies, (I) goat-anti-rabbit Alexa Flour 488 antibodies, (J) goat-anti-mouse Alexa Flour 647, (K) primary rabbit anti-ASGR1 and secondary Alexa Flour 488, (L) primary mouse anti-γ-OHPdG and secondary Alexa Flour 647, or (M) both primary and both secondary antibodies.

CD45 and CD42b) were detected. We verified these results by carrying out a concentration-dependent study to examine whether there is a difference in γ-OHPdG positive cells and the staining intensity in blood samples spiked with HepG2 cells (400 cells/5 mL blood) untreated or treated with different concentrations of acrolein. Analogous to the results shown in Figure 1, we found that γ-OHPdG in HepG2 cells increased upon treatment with increasing concentration of acrolein (Figure 2H–J). These results demonstrated that the HepG2 cells in blood can be isolated with a good recovery rate, allowing the determination of not only the total CTC count but, more importantly, the levels of γ-OHPdG.

Determination of Total CTC Cell Counts, the Number of γ-OHPdG Positive Cells, and Staining Intensities in HCC Patient Blood Samples

Blood samples collected from 32 patients with Barcelona clinic liver cancer stage C HCC with different clinicopathological features were processed and analyzed. We isolated ASGR1-positive CTCs through negative enrichment and

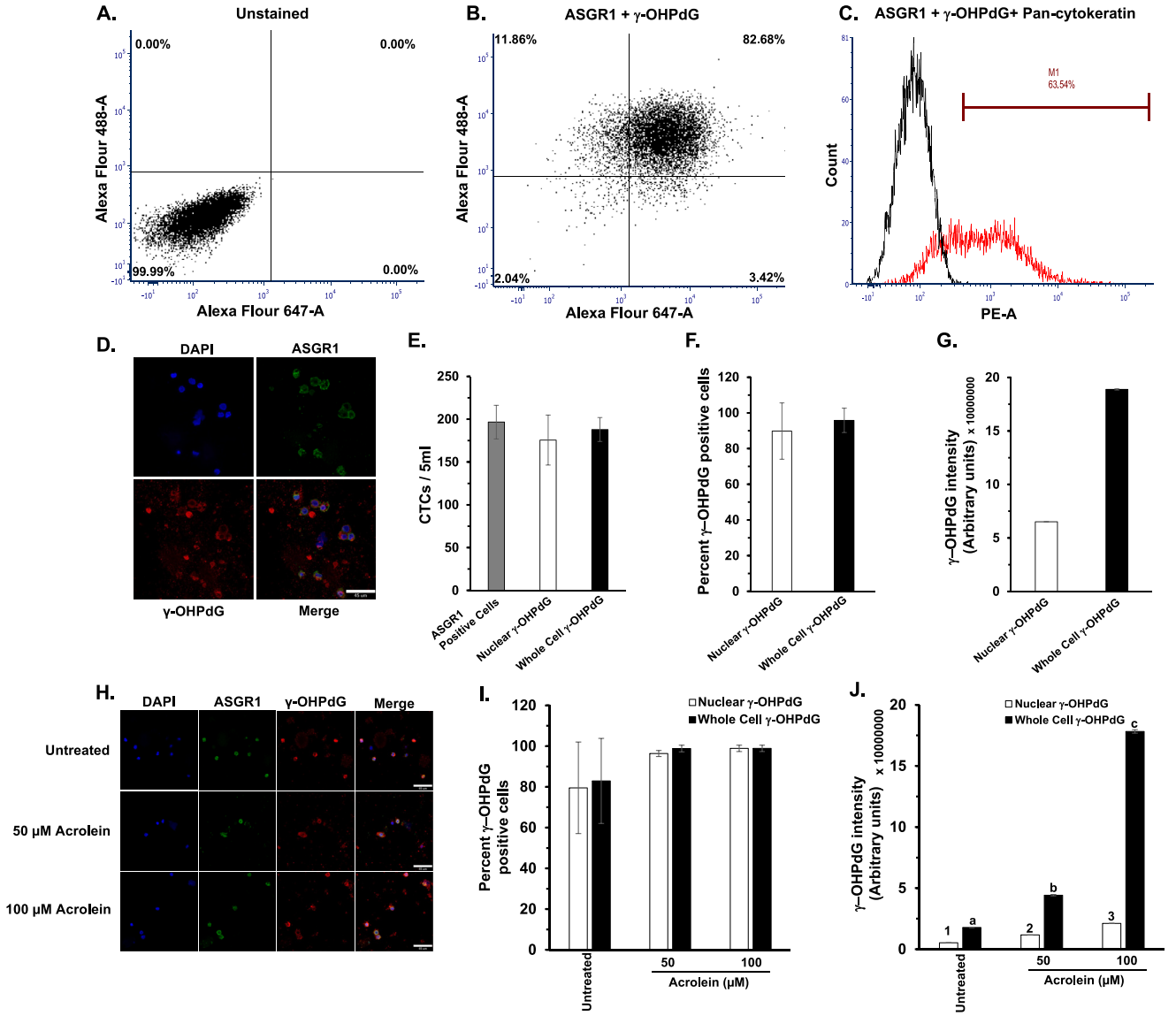

Figure 2. Recovery of HepG2 cells and detection of γ-OHPdG from spiked blood samples of healthy volunteers (HVs). In flow cytometry experiments (A–C), HepG2 cells retrieved from blood were either (A) unstained, (B) stained with primary anti-ASGR1 and anti-γ-OHPdG antibodies before secondary antibodies Alexa Flour 488 and Alexa Flour 647, or (C) stained with anti-ASGR1, anti-γ-OHPdG, and anti-pan-cytokeratin. In immunocytochemistry experiments, 400 HepG2 cells were spiked in 5 mL blood samples from HV. Isolated HepG2 cells were stained by ASGR1 antibody (green), γ-OHPdG antibody (red), and DAPI (blue). HepG2 experiments (D–G) were not treated with acrolein and done in triplicate. (D) Representative images of stained HepG2 cells from spiked blood samples of HV. (E) The total number of HepG2 cells recovered from spiked blood positive for ASGR1, γ-OHPdG in the nucleus only, or γ-OHPdG in the nucleus and cytosol (whole cell). (F) Percent of ASGR1 positive cells also positive for γ–OHPdG in nucleus only or whole cell. (G) Total γ-OHPdG intensity in the nucleus only and whole cell. HepG2 cells were treated with 0, 50, or 100 μM of acrolein for 5 hours before spiking; treatments done in duplicate. (H) Representative images of stained HepG2 cells with and without acrolein retrieved from healthy blood. (I) Percent of cells stained positive for γ-OHPdG in the nucleus only and in the whole cell. (J) Intensity of γ-OHPdG in the nucleus only and the whole cell. Error bars represent standard deviation. Bars within the same graph that bear different numbers (nucleus) or alphabets (whole cell), respectively, are statistically different from one another. $P < .05$.

quantified γ-OHPdG staining in each CTC using the MetaMorph software. Imaging results showed that ASGR1-positive CTCs were successfully isolated from the patient blood samples and costained with the γ-OHPdG antibody (Figure 3A). For each patient, we determined the number of CTCs detected through positive ASGR1 staining, the percentage of positive γ-OHPdG staining in CTCs, and the γ-OHPdG intensity of all CTCs (Figure 3B–D). Because each patient has a different number of CTCs expressing γ-OHPdG and staining intensity, we also determined the average intensity of the γ-OHPdG-positive cells in each patient (Figure 3E).

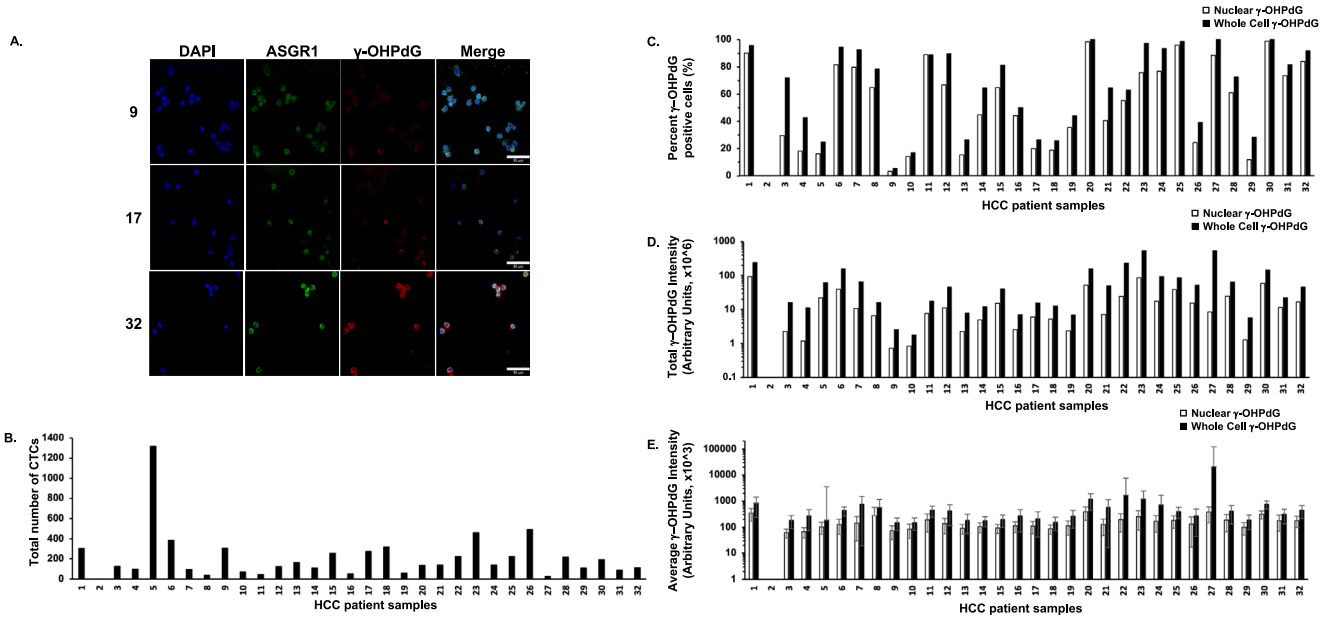

Figure 3. Detection of γ-OHPdG in CTCs isolated from HCC patient blood samples. Five mL of blood from HCC patients was processed by RosetteSep Human CD45 depletion cocktail and Ficoll Paque density solution-based method described in Materials and Methods section. (A) Representative images of CTCs from patients 9, 17, and 32 stained for ASGR1 (green), γ-OHPdG (red), and DAPI (blue) with differing levels of γ-OHPdG. (B) Total count of CTCs detected in each sample. (C) Percent of CTCs stained positive for γ–OHPdG in the nucleus (nuclear) or in the nucleus and cytosol (whole cell). (D) Total intensity of γ–OHPdG detected in the nuclei and whole cells of each sample's CTCs. (E) Average intensity of γ–OHPdG detected in the nuclei and whole cells of a patient's CTCs.

We observed large variations in the number of total CTCs, the percentage of CTCs positive for γ-OHPdG, and the intensity of γ-OHPdG staining (Table A1). We found ASGR1-stained CTCs, ranging from as low as 0 (patient 2) to as high as ∼1300 (patient 5) per sample (Figure 3B). γ-OHPdG was detected in the nucleus and whole cell (nucleus + cytoplasm) of CTCs for quantification. As expected, a parallel relationship was evident in the percentages of γ-OHPdG staining between the nucleus and whole cell (Figure 3C), as well as in the total staining intensity (Figure 3D). We observed considerable patient variability in the percentage of ASGR1-positive CTCs stained for γ-OHPdG. Whole cell positivity ranged from 5.56% (patient 9) to 100% (patient 20, 27, and 30) (Figure 3C). The total γ-OHPdG intensity ranged in magnitudes of 10^5–10^8 arbitrary units (Figure 3D), and the average γ-OHPdG intensity varied between 10^4 and 10^7 (Figure 3E). The total γ-OHPdG intensity represents the levels of adduct in CTCs from each patient, whereas the average γ-OHPdG intensity (a ratio between total intensity and the number of γ-OHPdG positive cells) shows differential adduct levels in each patient. For example, it may distinguish patients who have fewer positive CTCs but with a relatively high γ-OHPdG intensity or, vice versa, patients with more positive CTCs with low γ-OHPdG intensity.

Evaluating Possible Associations of γ-OHPdG Levels in CTCs of 32 Patients With Clinical Manifestations

We have collected demographic information, risk factors of HCC, and characteristics of HCC including size, multifocality, vascular invasion, extrahepatic metastasis, Child-Turcotte-Pugh (CTP) scores, and laboratory findings, including AFP, in these patients (Table 1). The percentage of CTCs staining positive for γ-OHPdG and the total intensity of γ-OHPdG staining are highly correlated (Figure 4A). In our analysis, γ-OHPdG was not related to patient race, gender, age, ethnicity, NASH, CTP score, or HCV or HBV infection. However, patients with multifocal HCC have higher nuclear + cytoplasmic γ-OHPdG intensity scoring than patients without multifocal disease ($P = .037$) (Figure 4B). Among these patients with multifocal HCC, total nuclear γ-OHPdG intensity seemed to be inversely correlated with the largest lesion size ($P = .0143$) (Figure 4C). Among all patients, positivity rates of γ-OHPdG staining in CTCs were not found to be associated with tumor size or total tumor burden as a continuous variable. Rather, those with nuclear γ-OHPdG positivity rates greater than or equal to 75% had smaller largest lesions ($P = .0036$) and lower tumor burdens ($P = .0273$) than those with lower positivity rates (Figure 4D). Total γ-OHPdG intensity in CTCs was not significantly associated with other clinical endpoints (Table 2). Interestingly, average CTC nuclear γ-OHPdG staining intensity was higher in patients with poorly differentiated HCC than those with moderate ($P = .0168$) or moderate/well differentiated HCC ($P = .0078$) (Figure 4E).

Discussion

Early recurrence is one of the leading causes of death in HCC patients after potentially curative resection. Previous

Table 1. Clinical and Demographic Characteristics of 32 HCC Patients Enrolled

Demographic variables	Count	Percentage
Race		
White	16	(50%)
African American	14	(44%)
Asian	2	(2%)
Ethnicity		
Hispanic	3	(9%)
Non-Hispanic	29	(91%)
Sex		
Male	30	(94%)
Female	2	(6%)
Age, range (y)	49–85 (median: 67)	
Clinicopathological variables	**Count**	**Percentage**
CTP score		
Class A	16	(50%)
Class B	16	(50%)
Class C	0	(0%)
Viral hepatitis		
HBV	6	(19%)
HCV	16	(50%)
HBV and HCV	2	(6%)
None	8	(25%)
HCC differentiation		
Well	4	(12.5%)
Moderate	7	(22%)
Poor	4	(12.5%)
Unknown	17	(53%)
Cirrhosis		
Yes	25	(78%)
No	7	(22%)
NASH		
Yes	4	(12.5%)
No	28	(87.5%)
Largest lesion, range (cm)	1–17 (median: 4.8)	
Total tumor burden, range (cm)	1–31 (median: 7.4)	
AFP at time of collection, range (ng/mL)	2.4–110425.2 (median: 73.7)	

studies suggest clinical features, such as vascular invasion, positive margins, and multifocality, are prognostic for early recurrence or early death after surgical resection of HCC. Unfortunately, these factors remain ambiguous until a post-resection review of the final pathology report. These uncertainties present a compelling reason for developing a better biomarker to predict HCC recurrence that can be easily evaluated clinically. The γ-OHPdG levels, based on their distribution (score 0–3 from low to high) and staining intensity (score 0–3), determined by IHC on surgical biopsies from HCC patients, seem to correlate strongly with the risk of recurrence. γ-OHPdG is a mutagenic DNA lesion that primarily induces G to T transversions, a predominant mutation found in HCC.[20–23] While mechanistically the formation of this adduct may be considered as an initiating event in liver carcinogenesis in preclinical models, the prospect of this DNA adduct as a prognostic biomarker in clinical settings needs to be evaluated in a large patient cohort. To meet this goal, we developed a noninvasive method for detecting γ-OHPdG in HCC CTCs isolated from patient blood. Our aim is to apply this low-risk, practical, and economical method, after being fully validated, in future trials toward the treatment and prevention of HCC and its recurrence.

γ-OHPdG is not a specific biomarker of HCC. Instead, as an endogenously formed background DNA lesion found in tissues of animals and humans which induces mutations, its formation is believed to be involved in the hepatocarcinogenic process. Our previous studies demonstrated that hepatic γ-OHPdG levels correlated with liver cancer incidence and multiplicity in various animal models, and it was strongly associated with RFS and overall survival after surgery in a retrospective clinical trial study.[29,30] Therefore, it is a potential small molecule-based risk biomarker of recurrence in HCC patients after surgery. In fact, it was reported earlier that γ-OHPdG is detected in liver biopsies from patients across the liver disease spectrum—from healthy subjects to steatosis to fibrosis to cirrhosis and, finally, HCC.[37] Its levels varied in individual patients and at different disease stages, with a general trend of increasing levels in steatosis, followed by decreasing levels in fibrosis and cirrhosis, and rising in HCC.

While the use of CTCs from liver cancer patients has received much attention in recent years,[31–33,38,39] most CTC studies entail the enumeration of cells and the detection of biomarkers at protein, DNA, or RNA levels. A single study assessed the kinetics of ex vivo-induced platinum guanine-guanine adducts in CTCs from non-small-cell lung cancer as a biomarker for predicting the response and dose for platinum-based chemotherapy.[40] To our knowledge, the method described here is the first to detect a specific DNA adduct in CTCs. An immunomagnetic system that involves magnetic beads coated with an antibody for a tumor cell surface antigen is currently the standard method of CTC detection. Epithelial cell adhesion molecule, expressed widely on the surface of epithelial cells, is used by the CellSearch system to detect CTCs. However, epithelial cell adhesion molecule is expressed in only about 35% of HCC cases, thus limiting its sensitivity as a marker for detecting HCC CTCs.[41–44] We have developed a non-immunomagnetic and biophysical properties-based separation method that uses ASGR1 as a marker to detect CTCs because it is a transmembrane protein expressed exclusively on the surface of hepatocytes. FACS analysis confirmed that the cells isolated from spiked HV blood samples are indeed epithelial HepG2 cells (DAPI+/ASGR1+ and CD45-/CD42b-). However, there are 2 limitations to using the FACS method. First, staining the intracellular markers γ-OHPdG and pan-cytokeratin requires fixation with formalin, leading to very low cell recovery by centrifugation. Second, the assay cannot quantify the intensity of γ-OHPdG in positively stained CTCs. The MetaMorph software program overcomes these limitations as it quantifies the γ-OHPdG intensity in positively stained cells at

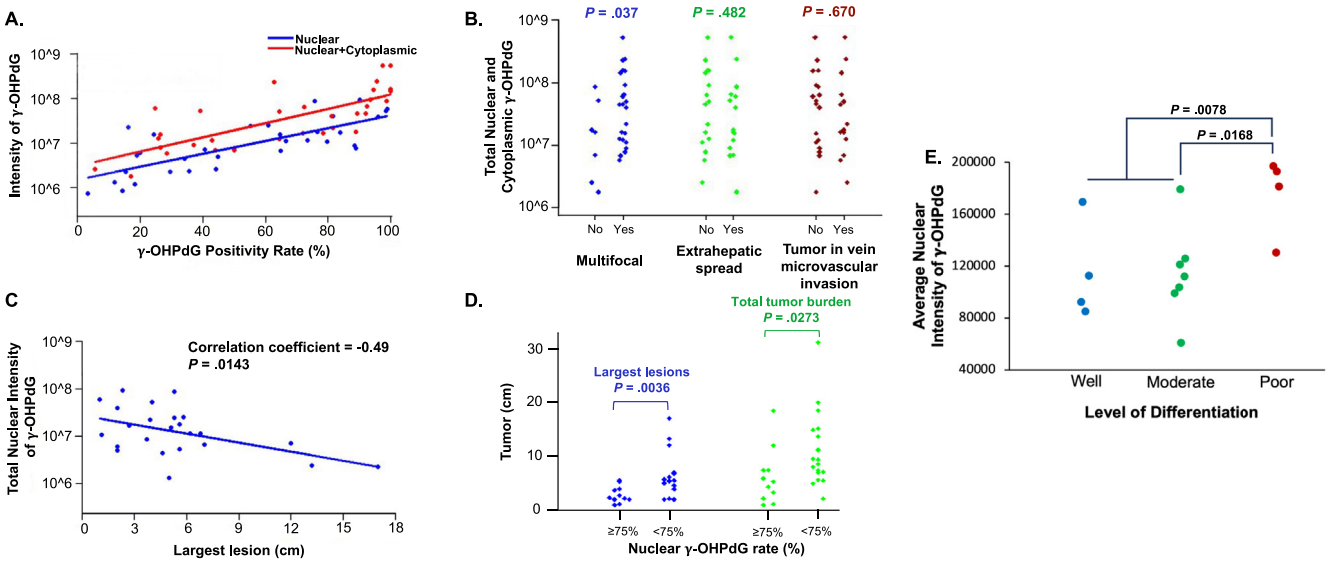

Figure 4. Relationship of γ-OHPdG in CTCs from HCC patients with clinicopathological features. Various clinicopathological features were compared to γ-OHPdG staining intensity in ASGR1-positive CTCs. (A) Associations between total γ-OHPdG intensities to γ-OHPdG positivity rates in CTCs. (B) T-test comparisons of total whole cell γ-OHPdG staining intensity to multifocality, extrahepatic spread, and tumor-in-vein microvascular invasion. (C) Associations between total γ-OHPdG intensities and largest lesion sizes in patients with multifocal HCC (n = 25). (D) T-test comparisons of largest lesion size and total tumor burden in patients with CTCs containing nuclear γ-OHPdG positivity rates ≥75% (n = 11) and <75% (n = 19). (E) Comparisons in average nuclear γ-OHPdG intensity among well (n = 4), moderately (n = 7), or poorly (n = 4) differentiated HCC, excluding patients with unknown differentiation (n = 15).

Table 2. Relationships Between γ-OHPdG and Clinicopathological Features

Clinicopathological features	Nuclear rate	Nuclear + cytosol rate	Total nuclear intensity	Total nuclear + cytosol intensity
Differentiation	0.2182 ($P = .4347$)	0.1681 ($P = .5492$)	0.4002 ($P = .1394$)	0.4076 ($P = .1315$)
NASH	−0.0784 ($P = .6697$)	0.0749 ($P = .6835$)	−0.1171 ($P = .5234$)	−0.0412 ($P = .823$)
Cirrhosis	−0.0065 ($P = .9717$)	0.0316 ($P = .8637$)	0.1351 ($P = .4609$)	0.0901 ($P = .6239$)
HBV or HCV	0.0893 ($P = .6783$)	0.0485 ($P = .8218$)	0.2252 ($P = .29$)	−0.0500 ($P = .8165$)
CTP score	0.1028 ($P = .5754$)	0.1140 ($P = .5346$)	0.0881 ($P = .6316$)	−0.0092 ($P = .9601$)
Largest lesion	−0.3440 ($P = .0627$)	−0.2749 ($P = .1416$)	−0.3479 ($P = .0595$)	−0.2763 ($P = .1394$)
Total tumor burden	−0.1771 ($P = .3491$)	−0.1104 ($P = .5612$)	−0.1565 ($P = .409$)	−0.0992 ($P = .6019$)
AFP	−0.1436 ($P = .433$)	−0.1039 ($P = .5715$)	−0.1484 ($P = .4176$)	−0.0994 ($P = .5881$)
Multifocality	0.1893 ($P = .2995$)	0.2188 ($P = .229$)	0.3266 ($P = .0681$)	**0.3701 ($P = .0370$)**
Portal vein thrombus	0.0921 ($P = .6162$)	0.0652 ($P = .7228$)	−0.1176 ($P = .5214$)	−0.0038 ($P = .9833$)
Extrahepatic spread	0.2182 ($P = .4347$)	0.1681 ($P = .5492$)	0.0551 ($P = .7644$)	−0.1288 ($P = .4822$)
Tumor in vein microvascular invasion	0.1391 ($P = .4478$)	0.1321 ($P = .4709$)	−0.1478 ($P = .4197$)	−0.0783 ($P = .6702$)

Bold indicates $P < .05$.

the individual cellular level. Furthermore, it can assess the cellular localization of γ-OHPdG with high accuracy and efficiency. Thus, using immunocytochemistry plus MetaMorph, detailed information about the percentage of positive cells, the distribution of γ-OHPdG between the nucleus and cytoplasm, and the kinetics of γ-OHPdG over time can be readily obtained. We showed γ-OHPdG is detected in both the nucleus and cytoplasm of HCC CTCs. The cytoplasmic staining could be derived from the unincorporated nucleotide pool and/or damaged mitochondrial DNA. Previous studies have shown that acrolein can cause oxidative damage, leading to membrane disruption and DNA and mitochondrial damage.[45,46] More studies are needed to ascertain whether cytoplasmic γ-OHPdG in CTCs is indeed originated from the acrolein-induced damage to mitochondrial DNA.

We examined peripheral blood samples from 32 HCC patients, all at an advanced stage of disease (Barcelona clinic liver cancer stage C) but of different etiologies, including

NASH, HCV, or HBV, and different levels of tumor burden based on tumor size, number, vascular invasion, or extrahepatic spreading, and other clinical factors including CTP score and AFP. The results showed that CTCs could be detected in 31 of 32 patients with a ~97% positivity rate, a better positivity rate than previously described methods utilizing ASGR1, Hep Par1, pan-cytokeratin, and anti-carbamoyl phosphate synthetase 1 phenotypic markers for CTC sorting with immunomagnetic method for cell enrichment (showing a positivity rate ranging from 81%–89%).[47–49] A wide variation was seen in γ-OHPdG positivity and intensity in patients. These variations could reflect individual differences in DNA damage repair pathways, specifically NER, and/or other conditions that may influence γ-OHPdG formation, including smoking, alcohol consumption, and obesity.

While the main focus of this study is to develop a blood-based method for γ-OHPdG, we made an effort to evaluate the possible relationships of its levels with available clinical factors associated with recurrence and aggressiveness of HCC. Our analyses revealed, based on total staining intensity, that patients with multifocal HCC show higher nuclear + cytoplasmic γ- OHPdG intensity than patients without multifocal disease ($P = .037$). Multifocal HCC can arise synchronously or metachronous either from intrahepatic metastasis (IM-HCC) or multicentric occurrence (MO-HCC). Multifocal HCC is a strong predictor for recurrence after surgery.[50] It is to be determined whether γ-OHPdG contributes to the development of IM or MO, or both γ-OHPdG accumulation seemed not to be correlative with vascular invasion, extrahepatic spreading, or elevated AFP which are known poor prognostic factors for aggressive HCC. It is mechanistically plausible that γ-OHPdG formation is an initiation event giving rise to increased mutations that leads to HCC multifocality. Interestingly, when the staining intensity of only the cells stained positive with adduct was analyzed, an association of higher γ-OHPdG with poorly differentiated HCC was observed ($P < .02$). Studies determining the clonal origin of multifocal HCC have shown that well-differentiated foci of recurrent HCC are more likely to originate from a de novo process (ie, MO-HCC), while poor differentiation and invasive features point to metastatic dissemination (ie, IM-HCC).[51,52] The intriguing adverse relationship between high γ-OHPdG levels (>75% vs <75%) and small tumor size, opposite to that of multifocality or differentiation, is somewhat unexpected. It is tempting to speculate, however, in the context of multistep liver carcinogenesis, γ-OHPdG may be more relevant as an initiating lesion in DNA than a promotion lesion.

Conclusions

We report the development of a noninvasive method to detect and quantify γ-OHPdG in CTCs in HCC patient blood. Our study is unique because this is the first time a method to detect a specific DNA adduct in CTC is reported. The importance of γ-OHPdG as a potential prognostic biomarker lies in its mechanistic relevance with liver carcinogenesis, as well as the observations in HCC patients that the higher levels of γ-OHPdG strongly associated with poor survival ($P < .0001$) and RFS ($P = .007$).[29,30] We applied this method in 32 HCC patients. The data showed a wide individual variability of the adduct levels in HCC CTCs. We analyzed the data against the known clinical features in these patients and found that higher γ - OHPdG scoring in CTCs may be associated with multifocal disease and poor differentiation, but inversely associated with lesion size. The blood-based method for γ -OHPdG described needs to be validated independently, as well as with the IHC method, in prospective trials with large HCC patient cohorts. Nevertheless, we reported a low-risk, convenient, and economical method for γ- OHPdG. This study is of general interest as there is an urgent need for a noninvasive method to detect a mechanism-relevant biomarker for HCC and its recurrence.

Supplementary Materials

Material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.gastha.2024.04.006.

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Monika Aggarwal led this study under supervision and funding acquisition of Fung-Lung Chung. Monika Aggarwal and Fung-Lung Chung contributed to study conceptualization. Monika Aggarwal, Mark Kuo, Zizhao Zhu, Sophie Gould, Bhaskar Kallakury, Karen Creswell, Samira Beheshtian, Aiwu Ruth He, and Alexander Kroemer contributed to data curation and methodology. Peter Johnson, Kevin Zhang, and Susette Mueller also contributed to methodology. Laura Kuhlman supported administration and resources. Hongbin Fang and Zijun Zhao contributed to data analysis and visualization. Monika Aggarwal, Mark Kuo, Aiwu Ruth He, and Fung-Lung Chung contributed to writing and visualization with suggestions by other authors. All authors had access to the data and have reviewed and approved of this manuscript.

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The records of patients were deidentified, and all authors underwent relevant training required by the Georgetown-MedStar Institutional Review Board system. The Georgetown-MedStar IRB system approved of this study (IRB ID: MOD00010314) under investigator A.R.H.

Data Transparency Statement:
The data, analytic methods, and study materials underlying this article may be made available and will be shared by a request to the corresponding authors.

Reporting Guidelines:
Not applicable for this article type.