DNA-PK—A Candidate Driver of Hepatocarcinogenesis and Tissue Biomarker That Predicts Response to Treatment and Survival

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Abstract

Purpose: Therapy resistance and associated liver disease make hepatocellular carcinomas (HCC) difficult to treat with traditional cytotoxic therapies, whereas newer targeted approaches offer only modest survival benefit. We focused on DNA-dependent protein kinase, DNA-PKcs, encoded by PRKDC and central to DNA damage repair by nonhomologous end joining. Our aim was to explore its roles in hepatocarcinogenesis and as a novel therapeutic candidate.

Experimental Design: PRKDC was characterized in liver tissues from of 132 patients [normal liver (n = 10), cirrhotic liver (n = 13), dysplastic nodules (n = 18), HCC (n = 91)] using Affymetrix U133 Plus 2.0 and 500 K Human Mapping SNP arrays (cohort 1). In addition, we studied a case series of 45 patients with HCC undergoing diagnostic biopsy (cohort 2). Histological grading, response to treatment, and survival were correlated with DNA-PKcs quantified immunohistochemically. Parallel in vitro studies determined the impact of DNA-PK on DNA repair and response to cytotoxic therapy.

Results: Increased PRKDC expression in HCC was associated with amplification of its genetic locus in cohort 1. In cohort 2, elevated DNA-PKcs identified patients with treatment-resistant HCC, progressing at a median of 4.5 months compared with 16.9 months, whereas elevation of activated pDNA-PK independently predicted poorer survival. DNA-PKcs was high in HCC cell lines, where its inhibition with NU7441 potentiated irradiation and doxorubicin-induced cytotoxicity, whereas the combination suppressed HCC growth in vitro and in vivo.

Conclusions: These data identify PRKDC/DNA-PKcs as a candidate driver of hepatocarcinogenesis, whose biopsy characterization at diagnosis may impact stratification of current therapies, and whose specific future targeting may overcome resistance. Clin Cancer Res; 21(4): 925–33. ©2014 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer death (1). It arises on a background of chronic liver diseases, such as viral hepatitis, alcoholic liver disease (ALD), and, increasingly, non–alcoholic liver disease (NAFLD; ref. 2). Cirrhosis and advanced HCC stage at presentation in the majority of patients severely restricts both surgical and nonsurgical therapeutic options. Curative treatments are limited to patients with early cancers who are fit enough for resection, liver transplantation, or radiofrequency ablation (3). HCC resistance to conventional palliative cytotoxic agents is compounded by increased toxicity, attributed to hepatic metabolism and their reduced clearance in patients with impaired liver function. Sorafenib represents a major advance in the medical management of these patients (4), but the survival benefit is modest (4). The need to identify key drivers of hepatocarcinogenesis and chemoradioresistance, as well as biomarkers to target therapy effectively, is of paramount importance.

The most widely used palliative treatment for fit patients with intermediate stage HCC [Barcelona Clinic for Liver Cancer (BCLC) stage “B”] is transarterial chemoembolization (TACE). Its overall efficacy, however, is questionable and patient selection is key (3). Tumor-directed radiotherapy is promising (5, 6) but as yet has no proven benefit. Both radiotherapy and the cytotoxic drugs used in TACE cause DNA damage, to which the cell mounts a DNA damage response (DDR). DNA double-strand breaks (DSB) are the most cytotoxic and are repaired by two major pathways, with nonhomologous end joining (NHEJ) being the most active in both replicating and nonreplicating cells alike.
Translational Relevance

Hepatocellular carcinoma (HCC) has few treatment options and is the second most common cause of cancer death globally. This study demonstrates that an increase in DNA-PKcs, a key enzyme in DNA double-strand break repair, drives this deadly cancer. Increased DNA-PKcs copy number and expression were associated with the malignant process and predicted resistance to hepatic transarterial chemoembolization (TACE) therapy. We found that increased DNA-PK activity was an independent indicator of poor survival, and in preclinical studies, inhibition of DNA-PKcs profoundly radiosensitized and chemosensitized HCC.

Our study identifies DNA-PKcs as a candidate biomarker that potentially could be used to stratify patients for TACE therapy and identifies a novel candidate for future therapeutic inhibition to reverse radio- and chemoresistance in HCC. Targeting a ‘driving’ process in hepatocarcinogenesis may ultimately offer significantly improved survival benefit.

Materials and Methods

Patient cohorts

Cohort 1 included tissues from consented consecutive patients undergoing resection or liver transplantation at three university hospitals in the United States (Mount Sinai Hospital, New York, NY) and Europe (Hospital Clinic, Barcelona, Spain, and National Cancer Institute, Milan, Italy) as described previously (13, 14). Patients with extrahepatic spread were excluded. Specific analyses are as described in Fig. 1. Cohort 2 was a case series of 45 patients (Table 1) undergoing pretreatment diagnostic biopsy, either because there was no history/evidence of associated cirrhosis or because of radiological diagnostic doubt, from a total of 632 patients managed in Newcastle between 2000 and 2010 (2). Those who did not consent to the use of their surplus tissues after diagnostic purposes were excluded. Patients were followed until June 30, 2013.

Immunohistochemistry

Using formalin-fixed paraffin-embedded tissues, HCC grading was by two pathologists (15). A detailed immunohistochemistry (IHC) protocol is described in Supplementary Methods. Briefly, antigen retrieval was with an Antigen Access Unit (A. Menarini Diagnostics). Antibodies: anti-DNA-PKcs (rabbit polyclonal, H-163; 1:500; Santa Cruz Biotechnology), anti-phosphorylated Ser2056 DNA-PKcs (rabbit polyclonal, ab20407; 1:500; Abcam), anti-ATM (rabbit polyclonal, MAT3-4G10/8; 1:800; Sigma), anti-phosphorylated Ser1981 ATM (rabbit polyclonal, AF1655; 1:300; R&D Systems). Sections were analyzed using Aperio Image analysis. Hepatocyte nuclei were identified using a modified nuclear algorithm and stained quantified in pixels after background subtraction. The selection of normal versus tumor areas was by a pathologist, whereas the application of the quantification algorithm was by supervised researchers. Both pathologists and researchers were blinded until the study endpoint.

Cell lines and in vitro assays

HCC cell lines SNU-182, SNU-475, HepG2, Hep3B, Huh7 (ATCC), and PLC/PRF/5 (ECACC) were maintained as per suppliers guidelines. All cell lines were authenticated (LGC Standards) and free of Mycoplasma contamination (MycoAlert Assay; Cambrex Bio Science). Mean change in gene expression (±SEM), using Human DNA Repair PCR Profiler Arrays (SA Biosciences; Qiagen), was expressed as ΔΔCt, relative to HPRT1. Western blotting was as described previously (16). Image acquisition/densitometry was performed using a G-box chemiluminescent image analyzer (Syngene). γH2AX and RAD51 foci detection was as previously described (17). Cell survival was assessed by colony formation and automated counting, normalized to untreated control (±SEM; ref. 16). ShRNA-mediated knockdown of DNA-PKcs and subsequent analysis of double-strand break repair (DSBR) activity using the traffic light reporter system (18, 19) are detailed in Supplementary Methods.

Xenograft model

Female nude mice (CD1 nu/nu; Charles River) were maintained as previously described (16). Huh7 cells (1 × 107 in 50 μL culture medium) were implanted subcutaneously. NU7441 (10 mg/kg i.p.) and/or doxorubicin (2 mg/kg i.p.) were administered to tumor-bearing mice daily for 5 days.

Statistical analysis

Data were analyzed using SPSS statistics (version 19.0). A two-way ANOVA was used for the comparison of cell line and treatment means (SAS Institute, Cary, NC). ANOVA was also used to test for significant gene expression changes in TNM stages for Cell lines and in vitro assays. Where appropriate, mean changes were analyzed using Wilcoxon-Mann-Whitney test. Survival analysis was performed using the Kaplan–Meier method and compared using log-rank (Mantel–Cox) and Cox proportional hazards regression. Survival and gene expression data were compared in a log-rank test and Cox proportional hazards regression, respectively. The survival analyses were performed using SPSS statistics (version 19.0; IBM). A P value of 0.05 was used to determine statistical differences.

Results

Amplification of PRKDC in HCC in association with increased mRNA levels

Expression of genes involved in the DDR was evaluated in a cohort of 132 samples (13, 14) of normal, chronically diseased,
and tumor liver tissues (Fig. 1A). PRKDC was upregulated 2.4-fold in HCC relative to noncancerous liver \( (P = 0.0007) \), whereas the mRNA level of ATM (Ataxia Telangiectasia Mutated kinase), central to the DDR involving both homologous recombination repair (HRR) and NHEJ, was unchanged (Fig. 1B). The PRKDC gene locus showed copy-number gains in 55% of HCCs \( (56/101 \text{ samples compared with } 83 \text{ paired cirrhotic hepatitis C virus (HCV)–positive samples; Fig. 1C}) \).

PRKDC copy number correlated significantly with gene expression \( (\text{Spearman rho } = 0.6, P = 10^{-7}) \). There was no correlation between PRKDC mRNA levels and patient outcome. In a small number of supplementary cases from the Newcastle HPB Research Tissue bank, tumor-specific PRKDC locus amplification determined by Multiplex Ligation-dependent Probe Amplification (MPLA) was associated with DNA-PKcs protein overexpression shown in Supplementary Fig. S1.

Increased HCC nuclear DNA-PKcs and treatment resistance

Nuclear DNA-PKcs protein levels assessed by IHC in paired tumor and nontumor liver from an independent cohort of 45 patients (Table 1) were scored as negative or grades one to three based on the positive pixel count (Fig. 2A). Most hepatocyte and HCC nuclei were positive, but the percentage of grade 3 nuclei was higher in tumor tissues (normal hepatocytes 33/65%, versus 50/65% of HCC nuclei; \( P = 0.001 \)) and increased stepwise with the histological grade (Fig. 2B). The HCC DNA-PKcs level, or percentage of grade 3 nuclei, was not associated with overall survival (data not shown). In a subset analysis of patients receiving palliative doxorubicin in the form of TACE as their first-line

Table 1. Clinical features of 45 patients undergoing diagnostic biopsy—cohort two

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NOTE: Continuous data are presented as mean ± SE.

Abbreviations: AIH, autoimmune hepatitis; BMI, body mass index; INR, international normalized ratio; PVT, portal vein thrombosis.

Figure 1.
Increased PRKDC in HCC and amplification at the DNA locus. PRKDC (A) and ATM (B) mRNA expression levels were analyzed in 132 human liver tissues using Affymetrix U133 Plus 2.0 arrays and expressed as fold change relative to normal liver. Tissues included normal liver \( (n = 10) \), cirrhotic liver \( (n = 13) \), low-grade dysplastic nodules (LGDN; \( n = 10) \), high-grade dysplastic nodules (HGDN; \( n = 8) \), and HCV-related HCC \( (n = 9) \). PRKDC was significantly elevated in HCC; \( P = 0.0007) \). Tumor PRKDC locus copy number was determined using the Affymetrix 500 K Human Mapping Array (C). The maximum value of paired cirrhotic samples was used as a cutoff (mean DNA copy number in 0.5 Mb around PRKDC gene locus, cutoff 2.25). D, relationship between PRKDC locus copy number and mRNA levels (Spearman rank correlation \( r = 0.6, P = 10^{-7} \)).
therapy ($n = 26$; Supplementary Table S1), the time to radiological progression (EASL guidelines 2001; ref. 20) after the first treatment was significantly shorter in those with high DNA-PKcs (>48% HCC nuclei DNA-PKcs grade 3) compared with those with lower DNA-PKcs (median, 4.5 months vs. 16.9 months, $P = 0.011$, Kaplan–Meier; Fig. 2C). The high DNA-PKcs association was independent of tumor size, which was the only other factor also predictive of time to radiological progression in this selected subset of patients by univariate analysis ($P = 0.034$; multivariate Cox regression: DNA-PKcs grade 3+ HR 2.5, confidence intervals (CI), 1.0–6.0; $P = 0.041$; tumor size HR 1.12; CI, 0.97–1.23; $P = 0.12$). The BCLC stage (20), combining HCC features, liver function, and patient performance, rather than any single factor, was predictive of survival in this treated group (Fig. 2D and E). There were no differences in ATM levels between HCC and paired nontumor liver tissues.

Elevated HCC DNA-PKcs activity was independently associated with poor survival

DNA-PK activity, assessed by IHC detection of pDNA-PKcs$^{S2056}$, was less prevalent and did not correlate significantly with levels of the native protein (data not shown). However, pDNA-PKcs$^{S2056}$ was elevated in tumor versus nontumor liver (71% of HCC nuclei vs. 29% of nontumor nuclei, $P = 0.003$) and increased with histological tumor grade ($P = 0.011$; Fig. 3A). The median survival of patients with low tumor pDNA-PKcs$^{S2056}$ (<25% 3+ nuclei; $n = 26$) was 35 months compared with 9.9 months in those with >25% 3+ nuclei ($n = 19$; $P = 0.007$, Kaplan–Meier; Fig. 3B). HCC pDNA-PKcs$^{S2056}$ was independently associated with survival ($P = 0.006$; HR, 2.91; CI, 1.37–6.17; Supplementary Table S2). ATM activity (autophosphorylation at serine1981; ref. 21) was unchanged in HCC compared with nontumor liver and was not associated with tumor grade or survival.
DNA-PK expression and activity in a panel of HCC cell lines

Measurement of DDR gene mRNA levels in a panel of 6 HCC cell lines revealed high PRKDC, XRCC6 (Ku70), and XRCC5 (Ku80), with Hep3B and PLC/PRF/5 having the highest and lowest expression, respectively (Fig. 4A). Other genes involved in NHEJ (XRCC4, LIG4, and XRCC6BP1; Supplementary Fig. S2A) and ATM (Fig. 4A) were not as highly expressed. The expression of genes involved in HRR was modest, and there was no indication that nucleotide excision repair, base excision repair, or mismatch repair gene expression was altered across the panel (Supplementary Fig. S2B–S2D). Western blot analysis of DNA-PKcs, Ku70, Ku80 (Fig. 4B and C), XRCC4, Ligase 4, and ATM (Supplementary Fig. S3) revealed abundant DNA-PKcs in all cell lines. DNA-PK function is critical to the DDR and cell survival and treatment with...
IR (10 Gy) induced variable activation of DNA-PK (pDNA-PK<sup>ser2056</sup>) and ATM (pATM<sup>ser1981</sup>; Fig. 4B and C). Irradiation did not affect the levels of Ku70, Ku80, XRCC4, or Ligase 4. To establish a relationship between DNA-PK expression and NHEJ activity, the level of DNA-PKcs was suppressed (shRNA), causing a corresponding reduction in NHEJ repair, assessed using the traffic light reporter system (18), in HuH7 cells (Supplementary Fig. S4 and Supplementary Methods).

Effect of DNA-PK and ATM inhibitors on the repair of DNA DSBs

The effect of DNA-PK and ATM inhibitors, NU7441 and KU55933, on DSB repair was investigated in the cell lines that showed a substantial IR induction of ATM and/or DNA-PK activity. DNA DSB induction and repair by HRR were visualized by γH2AX and RAD51 foci, respectively (Fig. 5). The IR-induced rapid and substantial (5- to 10-fold) increase, then gradual decline, in γH2AX foci was followed by an increase in RAD51 foci, peaking at 24 hours, thereafter gradually declining. NU7441 slightly delayed the time to reach peak γH2AX and hindered resolution of the foci. This was particularly notable in PLC/PRF/5 cells where the foci were still 5x baseline at 24 hours (Fig. 5A). Interestingly, inhibition of NHEJ by NU7441 caused a very substantial increase in RAD51 foci (Fig. 5A, C, and E), a marker of HRR, for which ATM is presumed to be key. Contrary to expectation, the ATM inhibitor KU55933 did not suppress RAD51 foci (Fig. 5B, D, and F).

Doxorubicin sensitivity, radiosensitivity, and the effect of NU7441 and KU55933

Measurement of the survival of HCC cells exposed to IR and doxorubicin (Supplementary Fig. S5) revealed that HepG2 cells, with high levels of DNA-PK and ATM, were highly radio- and chemoresistant. SNU-182 cells were the most chemosensitive, consistent with their low DNA-PK and ATM activity. SNU-182 cells grew slowly, and the more modest induction of DNA-PK autophosphorylation after irradiation may reflect different temporal kinetics of its activation. Otherwise, ATM and DNA-PK expression and activity were not critical determinants of sensitivity to IR or doxorubicin. NU7441 significantly potentiated IR-induced cytotoxicity by 3- to 40-fold and doxorubicin cytotoxicity by 2- to 50-fold (Fig. 6A–D; Supplementary Fig. S6). Inhibition of ATM by KU55933 caused an up to 44-fold radiosensitization and 10-fold chemosensitization (Supplementary Fig. S7).

Although NU7441 or KU55933 alone had only a marginal effect on survival, the two together profoundly inhibited survival, particularly in HepG2 and Huh7 cells (Fig. 6E; Supplementary Fig. S8). The combination of these inhibitors also contributed additional chemo- and radiopotentiation compared with either alone (Supplementary Fig. S9).
The rank order of sensitivity to doxorubicin compared with IR in the cell lines was markedly different, as was sensitization by NU7441 and KU55933. This was not due to effects on doxorubicin efflux as nuclear accumulation was similar in all cells and was not increased by either NU7441 or KU55933. In contrast, verapamil, an established MDR inhibitor, increased the nuclear accumulation of doxorubicin in all cell lines (Supplementary Fig. S10).

In vivo studies

Huh7 cells, which reliably formed subcutaneous xenografts and had high DNA-PK levels and activity, were used to explore chemosensitization by NU7441 in vivo. The poor solubility of KU55933 precluded in vivo evaluation of ATM inhibition. Neither doxorubicin nor NU7441, alone or in combination, caused any significant weight loss in the mice (Supplementary Fig. S11A). Scheduled killing based on tumor burden was 7 days after treatment allocation in the control group versus 12 in the NU7441 + doxorubicin group (Kaplan–Meier, P = 0.043; Supplementary Fig. S11B). In mice treated with NU7441 + doxorubicin, the increasing tumor volume trend was modestly reduced compared with control over the study period (Fig. 6F).

Discussion

Here, we show increased PRKDC mRNA expression in HCC in association with amplification of the PRKDC locus, supporting copy-number gain as a potential mechanism. Having demonstrated tumor-specific overexpression of DNA-PKcs, the protein encoded by PRKDC, in association with MPLA-defined PRKDC locus amplification in resected HCC cases, we demonstrated increased HCC DNA-PKcs in a second cohort of patients undergoing pretreatment diagnostic biopsies. Importantly, increased DNA-PKcs expression was associated with a shorter time to progression in patients receiving cytotoxic TACE therapy. These data support a role for DNA-PK in resistance to HCC cytotoxic therapy. In all patients in the second cohort, DNA-PK activation (pDNA-PKcsE2056) in the diagnostic pretreatment biopsy was independently associated with poorer patient survival. This suggests that activation of DNA-PKcs following gene amplification contributes to tumor progression. Although higher levels of genomic stress associated with inflammation and reactive oxygen species are well recognized in patients with chronic liver disease, and may predispose to carcinogenesis, recent evidence confirms even higher levels of oxidative DNA damage (22, 23) in HCC tissues. Thus, endogenous activation of DNA-PK may reflect high levels of oxidative stress–induced tumor DNA damage in a subgroup of patients with a particularly poor prognosis. DNA-PK stabilization of c-Myc (11), and promotion of genomic instability through competition with high-fidelity HRR (24), are candidate contributory mechanisms. Taken together, these data identify DNA-PK amplification and elevated expression, in the presence of either endogenous or exogenous activation, as a candidate driver of hepatocarcinogenesis or therapy resistance, respectively.

Similarly, HCC cell lines also had high levels of PRKDC mRNA and DNA-PKcs, with lower expression of other NHEJ genes and ATM, supporting a specific role for DNA-PK, rather than a general one for DDR genes, in hepatocarcinogenesis. Variation in the protein levels between the cell lines unrelated to the mRNA expression was in keeping with translation and protein stability.
being dysregulated in cancer (25). Neither DNA-PK nor ATM expression/activity predicted the rate of DSB repair or chemo- or radiosensitivity in the HCC cell lines, reflecting the complexity and multifactorial nature of the DDR. Nevertheless, suppression of DNA-PKcs levels in HuH7 cells reduced repair of I-SceI–induced DSBs by NHEJ. Furthermore, inhibition of DNA-PK activity retarded the resolution of DSBs, sensitizing HCC cells to the effects of doxorubicin and IR.

Both DNA-PK suppression and inhibition conferred a shift toward HRR, consistent with the hypothesis that NHEJ and HRR compete for DNA breaks (26). In contrast with previous reports in other cells (27, 28), KU55933 did not suppress IR-induced RAD51 focus formation, suggesting that ATM kinase activity is not essential for HRR in HCC. Our subsequent demonstration that the combination of NU7441 and KU55933 had a substantial impact on HCC cell survival in the absence of a DNA-damaging agent is indicative of synthetic lethality, as neither alone was significantly cytotoxic. These observations support recent studies indicating that ATM deficiency confers sensitivity to DNA-PK inhibition (29). Furthermore, the ability of NU7441 and KU55933 to chemo- and/or radiosensitize in all the cell lines was encouraging for their potential as anticancer therapeutics. Although extending these studies to the in vivo setting was hampered by the rapid growth of the tumor xenografts, the combination of NU7441 with doxorubicin did suppress the rate of xenograft growth.

These data are novel and suggest that (i) the combination of inhibitors of DNA repair pathway inhibitors may induce HCC cell death and (ii) the potentiation of tumor damage by cytotoxic agents may facilitate their use at doses much less toxic to non-tumor liver, warranting further exploration. Of more immediate clinical relevance is the evidence that HCC DNA-PKcs expression by IHC in pretreatment diagnostic biopsy material predicts responsiveness to TACE. TACE treatment has been controversial, clinical relevance is the evidence that HCC DNA-PKcs expression

Disclosure of Potential Conflicts of Interest
A. Villanueva is a consultant/advisory board member for Bayer Pharmaceuticals. D. Newell reports receiving a commercial research grant from and is a consultant/advisory board member for Astex Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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