This is a post-peer-review, pre-copyedit version of an article published in Journal of Gastroenterology. The final authenticated version is available online at: https://doi.org/10.1007/s00535-020-01705-8

#### Synergistic anti-tumor activity of miriplatin and radiation through PUMA- $\frac{2}{3}$  mediated apoptosis in hepatocellular carcinoma  $3 \t\t\t\t\t...$

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**Short title:** Chemoradiotherapy with miriplatin 

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 $\frac{52}{53}$  5996 words  $9990$  MOIDS

#### Abstract

 $\frac{2}{3}$  **Background:** The prognosis for patients with unresectable advanced hepatocellular  $\frac{4}{5}$  carcinoma (HCC) is poor. Miriplatin is a hydrophobic platinum compound that has a  $\frac{7}{2}$  long retention time in lesions after transarterial chemoembolization (TACE). We  $\frac{9}{10}$  investigated anti-tumor activity of miriplatin combined with irradiation on HCC cells, and its underlying mechanism of apoptosis. We also analyzed the effectiveness of  $\frac{14}{15}$  miriplatin-TACE and radiotherapy for locally advanced HCC.  $3 - 2$  and  $3 - 3$  carcinoma (HCC) is poor. Mirip  $6\overline{6}$  and  $\sim$  and  $\sim$  8 mesugated anti-tumbre activity 13 and the contract of the con minimum role and radiomera

**Methods:** Human HCC cell lines HepG2 and HuH-7 were treated with DPC (active  $\frac{19}{20}$  form of miriplatin) and radiation, and synergy was evaluated using a combination index  $\frac{21}{22}$  (CI). Apoptosis-related proteins and cell cycles were analyzed by western blotting and  $^{24}_{25}$  flowcytometry. We retrospectively analyzed treatment outcomes in 10 unresectable  $\frac{26}{27}$  HCC patients with vascular/bile duct invasion treated with miriplatin-TACE and 29 radiotherapy. **Company and reduced**  $_{22}$  (CI). Apoptosis-related proteins  $_{27}$   $_{\text{NU}}$  patients with vascular) 

 $\frac{31}{32}$  Results: DPC or X-ray irradiation decreased cell viability dose-dependently. DPC plus irradiation decreased cell viability synergistically in both cell lines (CI<1 respectively).  $\frac{36}{27}$  Cleaved PARP expression was induced much more strongly by DPC plus irradiation than by each treatment alone. Expression of p53 up-regulated modulator of apoptosis  $^{41}$  (PUMA) was significantly induced by the combination, and knockdown of PUMA with  $\frac{43}{44}$  siRNA significantly decreased apoptosis in both cell lines. DPC plus irradiation caused <sup>46</sup> sub-G1, G2/M, and S phase cell arrest in those cells. The combination of miriplatin- $^{48}_{49}$  TACE and radiotherapy showed a high response rate for patients with locally **advanced HCC** despite small number of patients. Nesults. Dr  $\sigma$  or  $\lambda$ -ray irradiation Ocaved T and expression was **than by each treatment alone.**  44 SIRINA SIGNIFICANTLY decreased TACE and Tagonierapy show 

 $\frac{53}{54}$  Conclusions: Miriplatin plus irradiation had synergistic anti-tumor activity on HCC cells through PUMA-mediated apoptosis and cell cycle arrest. This combination may possibly be effective in treating locally advanced HCC. **Concresions.** Interpretative pros **cells through PUMA-mediated**  Possibly be shootive in treating

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Key words: hepatocellular carcinoma, miriplatin, radiation, synergistic effect,

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 $\frac{2}{3}$  apoptosis approximation of  $\frac{1}{2}$ 

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#### Introduction

 $\frac{2}{3}$  Hepatocellular carcinoma (HCC) is the fourth-leading cause of cancer-related  $\frac{4}{5}$  death worldwide [1]. Most patients with HCC are diagnosed at an advanced stage,  $\frac{7}{2}$  when curative therapies, such as surgical resection and percutaneous ablation, are of  $\frac{9}{10}$  limited utility. A majority of HCCs exhibit intrinsic resistance to many cytotoxic anticancer agents and, therefore, interventional treatments such as transarterial  $\frac{14}{15}$  chemotherapy have been applied for advanced HCC [2, 3]. Recently, the therapeutic efficacy of systemic treatment with sorafenib, a tyrosine kinase inhibitor, on  $^{19}_{22}$  unresectable HCC was reported; sorafenib significantly improved overall survival (OS)  $\frac{21}{22}$  in patients with unresectable advanced HCC in randomized controlled phase III trials  $^{24}$  [4]. More recently, other tyrosine kinase inhibitors such as regorafenib and lenvatinib  $\frac{26}{27}$  were reported to be effective for the treatment of advanced HCC [5, 6]. However, a serious limitation of these multi-kinase inhibitors is that they show poor efficacy for  $\frac{31}{32}$  treating HCCs that invade the major portal vein, hepatic vein, or bile duct. Therefore, the prognosis for these locally advanced HCC is extremely poor [7, 8]. For example,  $\frac{36}{27}$  Cheng and associates reported that the median survival time for cases of HCC with major vascular invasion was only 5.6 months despite treatment with sorafenib [9]. aeath worldwide [1]. Most patients  $6\overline{6}$   $_{10}$  minied dully. A majority of Fig. Crientotricially have been appl 20 am coolding not had reported  $_{22}$  in patients with unresectable a **c**  $_{27}$  were reported to be effective areally rives that invade the Oneng and associates reports **major vascular invasion was o** 

 $\frac{41}{42}$  With recent advances in three-dimensional radiation techniques, radiotherapy has  $\frac{43}{44}$  become a treatment option for unresectable HCC. In particular, radiotherapy has been <sup>46</sup> reported to be effective for treatment of portal vein and/or inferior vena cava tumor  $\frac{48}{49}$  thrombosis in HCC [10, 11]. Moreover, transarterial chemoembolization (TACE) combined with radiotherapy was reported to be effective in treating HCC with portal  $\frac{53}{54}$  vein tumor thrombus [12]. Notably, Yoon and associates reported that TACE with cisplatin (CDDP) combined with radiotherapy was very effective for the treatment of  $^{58}_{58}$  unresectable HCC invading the portal vein; the response rate (RR) and median overall **Andrew Communication**  Decome a treatment option for and an and  $10$ ,  $11$ . vein turnor unombus  $[12]$ . No 56 cisplatin (CDDP) combined wi am cooled by  $100$  meaning and

survival (OS) were 39.6% and 10.6 months, respectively [13]. In a randomized clinical  $\frac{2}{3}$  trial, the same group showed that TACE with CDDP combined with radiotherapy was  $\frac{4}{5}$  superior to sorafenib for the treatment of HCC with macroscopic vascular invasion [14].  $\frac{7}{2}$  However, these outcomes with respect to RR and OS of treatment with TACE with  $\frac{9}{10}$  CDDP and radiotherapy remain unsatisfactory. In addition, CDDP has various adverse effects such as renal toxicity, vascular damage, and GI toxicity, and it shows poor 12  $\frac{14}{15}$  tumor retention when administered as TACE [15, 16]. 1  $3<sup>2</sup>$  $5^5$  superior to soratenip for the trea  $6\overline{6}$  8  $_{10}$  CDDF and radiomerapy remain 11 13  $15$  and the returnal writer durinos

17 Miriplatin (cis-[1R,2R]-1,2-cyclohexanediamine-N,N']bis[myristate])-platinum(II)  $\frac{19}{20}$  monohydrate; Sumitomo Dainippon Pharma Co, Ltd. Osaka, Japan) is a novel  $\frac{21}{22}$  lipophilic anti-cancer drug that can be suspended in lipiodol, a lipid lymphographic  $^{24}_{\sim}$  agent for TACE [16], and it is less toxic and has a much longer retention time in tumor  $\frac{26}{27}$  tissues compared with CDDP. A phase II study of TACE with miriplatin and iodized oil showed promising efficacy for unresectable HCC with a mild toxicity profile [17]. 29  $\frac{31}{32}$  Subsequent studies of TACE with miriplatin also revealed better efficacy and much lower toxicity for the treatment of unresectable HCC compared with other anti-cancer 34  $\frac{36}{27}$  agents, despite a few contradictory reports [18, 19]. However, combination therapy with miriplatin and radiotherapy for HCC has not been studied to date. Therefore, in  $\frac{41}{42}$  this study, we first investigated the anti-cancer activity and synergistic effect of  $\frac{43}{44}$  miriplatin plus X-ray combination therapy using HCC cell lines. Since we ultimately 46 betweed synergistic anti-tumor activity with this combination, we next examined the  $\frac{48}{49}$  underlying mechanism of synergistic activity, including apoptosis, using HCC cell lines. We also evaluated the anti-tumor activity of TACE with miriplatin combined with 51  $\frac{53}{54}$  radiotherapy in HCC patients with vascular and/or bile duct invasion. 18 20 mononyarato, cannonio Ban  $_{22}$   $\qquad$  **IIpophilic anti-cancer drug tha** 23 25  $_{27}$  ussues compared with CDDP. 28 30  $32$  OUDSEQUETIC SIGNES OF TAGE 33 35  $37$  agonto, acoptic a low contrade 38 39 **With miriplatin and radiotherap** 40 42 and start, no met missinger 44 minplatin plus x-ray combinat 45 47  $49$  underlying meditams in or syne 50 52  $_{54}$  radiomerapy in rice patients  $\sqrt{ }$ 

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#### 58 MATERIALS AND METHODS 59 **MARIAMED AND METHODS**

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 $\frac{2}{3}$  The human HCC cell lines HepG2 and HuH-7 were purchased from American  $\frac{4}{5}$  Tissue Culture Collection. Cells were maintained in DMEM supplemented with 10%  $\frac{7}{1}$  fetal bovine serum (FBS). I ISSUE CUITURE COILECTION. CEILS  $6\overline{6}$ 8 and 2012 and 2012

#### **Drugs and reagents**

 $\frac{14}{15}$  Miriplatin and dichloro [(1R, 2R)-1,2-cyclohexanediamine-N, N'] platinum (DPC), an active form of miriplatin, were provided by Sumitomo Dainippon Pharma Co, Ltd.  $^{19}_{22}$  DPC was dissolved in N,N-dimethylformamide (DMF) and CDDP (Sigma-Aldrich, St  $\frac{21}{22}$  Louis, MO) was dissolved in saline. minplatin and digition  $\Gamma$   $_{22}$  Louis, MO) was dissolved in sa

#### $\frac{26}{27}$  Radiation exposure  $_{27}$  Radiation exposure

Cells were irradiated in the medium at room temperature with an X-ray generator  $\frac{31}{32}$  (MBR-1520R-3, Hitachi, Japan) operating at 150 kV–20 mA with a filter of 0.1 mm Cu and 0.5 mm Al at a dose rate of 1.0 Gy/min. (MDIN-TOZUN-O, FiliaCHI, Japan 

#### Cell viability assay 38 and <u>the second contract of the sec</u> **Cell viability assay**

 $^{41}_{42}$  Cell viability was examined by WST-8 assay, according to manufacturer's **instructions**. **Community** that external 

#### $^{48}_{49}$  Calculation of Combination index (CI) Calculation of Compilation

 DPC or CDDP was added to the cells at a concentration of 0 – 10 µM, and the cells  $^{53}_{54}$  were irradiated with X-rays at 0 – 10 Gy. They were subsequently incubated at 37°C for 120 h and cell viability was determined by WST-8 assay to generate a new cell  $\frac{58}{58}$  viability curve. Based on these data, a combination index (CI) was calculated were inducted with  $\lambda$ -rays at **for 120 h and cell viability wa**  Maximity barrot babba on the

according to the method of Chou and Talalay using CompuSyn software (ComboSyn,  $\frac{2}{3}$  Paramus, NJ) [20, 21]. The CI value was plotted against the fraction of affected cells  $\frac{4}{5}$  (Fa), which represents the percentage of growth inhibition (CI-Fa plot). Three  $\frac{7}{2}$  independent experiments consisting of quintuplicate assays for each condition were  $\frac{9}{10}$  performed to generate CI-Fa plots. A CI value < 1.0 indicates synergism of the 12 combination. (Fa), which represents the p  $6\overline{6}$ 8 and 10 and  $_{10}$  performed to generate GP-ra 

#### Western blot analysis

 $^{19}$  Expression of apoptosis-related proteins was analyzed by western blot analysis,  $\frac{21}{22}$  as described previously [22]. The primary antibodies used are listed in Supplementary Table 1. 20 = Presence of approaches  $_{22}$  as described previously [22]. T 25 and 26 an

#### Cell cycle analysis

 $\frac{31}{32}$  Cell cycle was analyzed using a Cell Cycle Phase Determination Kit (Cayman **chemical, Ann Arbor, MI) according to manufacturer's instruction. Briefly, cells were**  $\frac{36}{27}$  seeded in 10-cm dishes and the culture medium was changed to serum-free medium  $\frac{38}{39}$  for 24 h to facilitate cell cycle synchronization. DPC was added to the wells with or  $^{41}_{12}$  without irradiation. After incubation for 24 h, the DNA content of propidium iodide (PI)- $\frac{43}{44}$  stained cell nuclei was determined using a BD FACSVerse flow cytometer (BD Biosciences, San Jose, CA). For analysis of DNA synthetic activity, BrdU assay was  $^{48}_{49}$  performed using an FITC BrdU Flow Kit (BD Biosciences), according to 51 manufacturer's instructions. Cell cycle was allalyzed u **Coded in the city district dividend Tor 24 h to facilitate cell cycle**  stairied cell nuclei was deter performed doing an inc 

Retrospective study of TACE with miriplatin combined with radiotherapy for unresectable HCC with portal/hepatic and/or bile duct invasion **Retrospective study of TAC CALL COOPERATION** 1.000 Main points

We retrospectively analyzed the anti-tumor effect of TACE with miriplatin or CDDP  $\frac{2}{3}$  combined with radiotherapy for patients with HCC between February 2012 and April  $\frac{4}{5}$  2019 at Tokushima University Hospital. The criteria for enrollment were as follows; (i)  $\frac{7}{2}$  unresectable HCC that macroscopically invaded the major branch of the portal vein  $\frac{9}{10}$  (Vp1 - 3), hepatic vein (Vv1 - 3) or bile ducts (B1 - 4) in contrast-enhanced CT; and (ii) 12 Child–Pugh classification A or B. Patients with extrahepatic metastasis or with  $\frac{14}{15}$  invasion to the main trunk of the portal vein were excluded. A total of 10 patients in the miriplatin group and 15 patients in the CDDP group who satisfied the eligibility  $\frac{19}{20}$  criteria were evaluated. This study was approved by the institutional review board of our university. 2019 at Tokushima University F  $6\overline{6}$ 8 and 2010  $_{10}$  (vp i - 3), riepalic velli (vv i - 3) **IIIVASION** to the main trunk of t **Chrona More Sydnamical** This S 

 We evaluated primarily the main tumor with vascular/bile duct invasion inside the  $\frac{26}{27}$  radiation field by serial CT scans performed 3 months after the completion of TACE and radiotherapy, according to criteria described previously [23], as follows: complete  $\frac{31}{32}$  response (CR), complete disappearance of tumor; partial response (PR), >30% reduction of tumor in the largest diameter without complete disappearance of tumor;  $\frac{36}{27}$  progressive disease (PD), >20% extension of tumor in the largest diameter; stable disease (SD), neither sufficient regression to qualify for PR nor sufficient growth to  $\frac{41}{42}$  qualify for PD. We also evaluated secondarily intrahepatic nodules outside the  $\frac{43}{44}$  radiation field according to modified RECIST criteria [24]. OS was measured from the 46 date of initial treatment to the date of death or last follow-up and assessed by the  $\frac{48}{49}$  Kaplan–Meier method.  $_{27}$  radiation field by serial CT sca 30 and the set of the <br>Set of the set of the s response (Cry), complete als progressive disease ( $\overline{p}$ ),  $\overline{p}$  **disease (SD), neither sufficier**  44 radiation field according to mo Napidi-Meler metricu.

Detailed information on the method for clonogenic cell survival assay, apoptosis  $\frac{53}{54}$  assay, siRNA transfection, and TACE with radiotherapy is provided in Supplementary Methods and Results. assay, sirved databased and 

#### **Results**

#### $\frac{2}{3}$  Anti-tumor activity of DPC, CDDP and X-ray irradiation on hepatocellular  $\frac{4}{5}$  carcinoma cell lines 3  $5$  carcinoma cell lines

 $\frac{7}{2}$  We first analyzed the anti-tumor effect of DPC, an active form of miriplatin, and  $^{9}_{10}$   $\,$  CDDP on HepG2 and HuH-7 cells by cell viability assay. The viability of HepG2 cells treated with DPC or CDDP decreased in a dose-dependent manner. The IC50 values 12  $^{14}_{15}$  for DPC and CDDP were 3.66 ± 1.45 and 6.30 ± 1.66 µM respectively; i.e., the IC50 of DPC was significantly lower than that of CDDP ( $\rho$ <0.03 by Student's  $t$  test; Fig.1A).  $^{19}_{20}$  Similarly, the IC50 of DPC in HuH-7 cells (0.90 ± 0.34 µM) was significantly lower than  $\frac{21}{22}$  that of CDDP (5.84 ± 1.07µM, *p*<0.01). Thus, DPC exhibited greater anti-tumor activity  $\frac{24}{25}$  than CDDP against both HCC cell lines. 8 and 2 <br>But the contract of the contrac  $_{10}$  CDDF on HepGZ and Hum-7  $\sigma$ 11 13  $15$  TOT DT C and CDDT Were 0.00 16 17 of DPC was significantly lower 18  $20$  cannot be considered by  $20$  $_{22}$  that of CDDP (5.84 ± 1.07 µM,  $\mu$ 23 25

 $\frac{26}{27}$  When HepG2 or HuH-7 cells were irradiated with X-rays at 0 – 10 Gy, cell viability decreased in a dose-dependent manner, with ED50s of 5.6 Gy and 5.2 Gy, 29  $\frac{31}{32}$  respectively (Supplementary Fig.1A).  $27$  vien HepGZ or Hum-7 cen 28 30  $32$  respectively (supplementary r

#### $\frac{36}{27}$  Synergistic effect of DPC and X-ray irradiation on HCC cells  $37$  by order the contract of  $\mathbf{D}$  by  $\mathbf{D}$

To evaluate the synergistic effect of anti-tumor drug treatment and X-ray irradiation,  $^{41}_{42}$  we examined the viability of HepG2 and HuH-7 cells using the combination index  $\frac{43}{44}$  method after treatment with various concentrations of DPC or CDDP in combination  $^{46}$  with 0 – 10 Gy X-ray irradiation. HepG2 and HuH-7 cell viability decreased in a dose- $\frac{48}{49}$  dependent manner following treatment with DPC in each X-ray dose group from 0 – 10 Gy (Fig.1B,C). Moreover, the viability curve for each X-ray exposure group 51  $\frac{53}{54}$  gradually decreased with increasing X-ray doses. The Chou and Talalay (CI – Fa) plot revealed that all the CI values were less than 1.0 in the range of Fa ≥0.2 in HepG2  $^{58}$  and HuH-7 cells, clearly showing a downward trend of CI values with increasing Fa. 39 **The Valuate the synergistic** 40 42 44 method after treatment with va 45 47 49 dependent manner following the 50 52  $54$  gradually decreased with incre 55 56 **revealed that all the CI values** 57  $59$  and that the solid, did in  $\frac{1}{2}$ 

Since it is well known that synergism (CI value) at higher Fa levels is more relevant to  $\frac{2}{3}$  the anti-cancer therapeutic effect than that at lower Fa levels [20, 25], DPC and X-ray  $\frac{4}{5}$  irradiation were judged to have synergistic effects in both cell lines. In contrast, for  $\frac{7}{2}$  CDDP plus X-ray irradiation only one CI value was less than 1.0 (around 0.6 of Fa)  $\frac{9}{10}$  but the other 3 CI values (including the one at the highest Fa level) were greater than  $12 \hspace{25pt}$  1.0, failing to show a downward trend of CI values with increasing Fa in both cell lines  $^{14}_{15}$  (Fig.1D,E). Thus, CDDP plus X-ray irradiaition was judged to have little synergy or a very weak synergistic effect. rradiation were judged to have  $6\overline{6}$ 8 and 1 Dut the other 3 GT values (filter (rig.  $10, L$ ). Thus, ODDT plus 

 $\frac{19}{20}$  To evaluate the long-term combination effect of DPC and X-ray irradiation, we  $\frac{21}{22}$  performed colony forming assays in HepG2 and HuH-7 cells. Representative images  $\frac{24}{35}$  of colonies in each HepG2 treatment group are shown in Fig.2A. The number of  $\frac{26}{27}$  colonies of HCC cells formed was lowest in cells treated with DPC plus X-ray irradiation as compared with all other treatment groups, although the number of  $\frac{31}{32}$  colonies formed in cells treated with DPC or X-ray irradiation alone was also lower compared with control cells. Mean clonogenic survival (percentage of control cells) in  $\frac{36}{27}$  the DPC plus X-ray irradiation group (38.3 ± 2.1) was significantly lower than that in the DPC alone (51.7  $\pm$  3.0) and X-ray irradiation alone groups (86.8  $\pm$  3.7) (p<0.05 <sup>41</sup> respectively, by Turkey's test; Fig.2B). Mean clonogenic survival in the latter 2 groups  $\frac{43}{44}$  was significantly lower compared with control cells (*p*<0.05 respectively). Similar <sup>46</sup> results were obtained in the colony forming assay with HuH-7 cells (Fig.2C,D;  $p$ <0.05  $\frac{48}{49}$  respectively). 20 Protocolated the long term  $_{22}$  performed colony forming ass. colonies of  $HUC$  cells forme colonies formed in cells treate and  $21$  o plus x ray irrediction the DPC alone  $(51.7 \pm 3.0)$  are **was significantly lower comp**  respectively).

#### $\frac{53}{54}$  Apoptosis induction with DPC plus X-ray irradiation  $_{54}$   $_{\text{A}}$   $_{\text{A}}$   $_{\text{B}}$   $_{\text{B}}$

To investigate the mechanism of the synergistic anti-tumor effect of the  $^{58}$  combination treatment on HCC cells, apoptosis was examined by western blotting and **To investigate the mech Completed Computer** 

flow cytometry in HCC cells treated with DPC and/or X-ray irradiation, in comparison  $\frac{2}{3}$  with CDDP and/or X-ray irradiation. When HepG2 and HuH-7 cells were treated with  $\frac{4}{5}$  various concentrations of DPC or CDDP, the expression of cleaved PARP and  $\frac{7}{2}$  caspase-3 was induced in a dose-dependent manner at 0 – 20  $\mu$ M (including low  $\frac{9}{10}$  concentrations of 0 – 5 µM) in both cell lines (Supplementary Fig.2A,B). Moreover, cleavage of PARP and caspase-3 was also induced in both cell lines in a dose-  $\frac{14}{15}$  dependent manner after treatment with X-ray irradiation alone (Supplementary Fig.1B). When HepG2 and HuH-7 cells were treated with DPC plus X-ray irradiation, the  $\frac{19}{20}$  induction of cleaved PARP and caspase-3 expression was much stronger than with  $\frac{21}{22}$  each treatment, and with CDDP plus X-ray irradiation**, suggesting that the synergistic**  $^{24}_{\sim}$  anti-tumor effect of DPC plus X-ray irradiation is caused by apoptosis in both cell lines (Fig.3A).  $3 \cdot 3$  various concentrations of DPC  $6\overline{6}$ 8 and 10 and  $_{10}$  concentrations or  $v - 3$  plw) if advertisement manner after treatment 17 When HepG2 and HuH-7 cel  $\ldots$   $\ldots$   $\ldots$   $\ldots$   $\ldots$   $\ldots$ **each treatment**, and with CDD  $(\mathbf{F}^{\prime}, \mathbf{Q}\mathbf{A})$ 

**In the flow cytometry analysis, the percentage of annexin V (+) in HepG2 cells**  $\frac{31}{32}$  treated with DPC plus X-ray irradiation (53.5 ± 1.7%) was significantly higher 34 compared with control cells treated with vehicle alone (11.4  $\pm$  0.4%;  $p$ <0.01 by  $\frac{36}{27}$  Turkey's test) or CDDP plus X-ray irradiation (33.1 ± 2.4%; p<0.01; Fig.3B). Similarly, the percentage of annexin V (+) in HuH-7 cells treated with DPC plus X-ray irradiation  $^{41}_{42}$  (50.8 ± 1.0%) was significantly higher than that with vehicle treatment alone (13.2 ± 1.6%, **p<0.01)** or CDDP plus X-ray irradiation (41.5 ± 1.5%; p<0.01; Fig.3C). arealed with DFC plus  $\lambda$ -ray and  $\sqrt{9}$  decay of ODD, place  $\sqrt{2}$  **the percentage of annexin V (+**  1.0%,  $p$ <0.01) or CDDP plus  $\lambda$ 

#### $\frac{48}{49}$  Mechanism of synergistic effect of DPC and X-ray irradiation mechanism of synergistic entry

To further investigate the mechanism of apoptosis induction by DPC and X-ray  $\frac{53}{54}$  irradiation, we examined the expression of major apoptosis-related proteins (Bim, Bik, p53 up-regulated modulator of apoptosis [PUMA], Noxa, Bcl-2, Mcl-1, and Bcl-xl) in  $^{58}$  HepG2 and HuH-7 cells by western blot analysis. When HepG2 cells were treated with  $\cdots$   $\cdots$   $\cdots$   $\cdots$   $\cdots$   $\cdots$  **p53 up-regulated modulator o**   $\ldots$   $\ldots$   $\ldots$   $\ldots$   $\ldots$   $\ldots$ 

DPC alone, PUMA expression was significantly increased in a dose-dependent  $\frac{2}{3}$  manner at 0 – 20 µM (including low concentrations of 0 – 5 µM), whereas expression  $\frac{4}{5}$  of Bim, Bik, Noxa, Bcl-2, Mcl-1, and Bcl-xl did not change significantly (Supplementary Fig.2A). Conversely, PUMA expression was not changed by treatment with CDDP,  $\frac{9}{10}$  irrespective of the concentration, suggesting that the mechanism of apoptosis induction by CDDP differs from that of DPC. Moreover, when HepG2 cells were treated 12  $\frac{14}{15}$  with DPC plus X-ray irradiation, PUMA expression was much stronger than that of each treatment individually, whereas the expression of other apoptosis-related 17  $\frac{19}{20}$  proteins did not change significantly (Fig.4A and Supplementary Fig.3). Quantitative  $\frac{21}{22}$  imaging analysis showed that the PUMA expression level (PUMA/β-actin ratio) of  $^{24}_{\sim}$  HepG2 cells treated with DPC plus X-ray irradiation was significantly higher than that  $\frac{26}{27}$  with treatment with DPC, X-ray irradiation alone, or CDDP plus X-ray irradiation, as well as control cells (Fig.4B). Similarly, PUMA expression was significantly increased 29  $\frac{31}{32}$  in a dose-dependent manner in HuH-7 cells after treatment with DPC, whereas it did not change after treatment with CDDP (Supplementary Fig.2B). Moreover, PUMA 34  $\frac{36}{27}$  expression of HuH-7 cells treated with DPC plus X-ray irradiation was significantly higher than with treatment with DPC, X-ray irradiation alone, or CDDP plus X-ray  $^{41}_{42}$  irradiation, as well as control cells (Fig.4C,D). These results indicate that PUMA plays  $\frac{43}{44}$  a critical role in the induction of apoptosis by DPC and X-ray irradiation in combination. 46 To investigate the mechanism of PUMA induction, we examined the expression of  $^{48}_{49}$  JNK, c-Jun, and CHOP expression in HepG2 and HuH-7 cells by western blotting. Expression of p-JNK and p-c-Jun in HepG2 cells after treatment with DPC plus X-ray 51  $\frac{53}{54}$  irradiation was significantly higher compared with control cells and cells after treatment with CDDP plus X-ray irradiation (Fig.4E). However, expression of ER stress-related 58 protein (CHOP) did not change after treatment with DPC plus X-ray irradiation or 1  $3 \frac{1}{2}$  $5$  of BIM, BIK, NOXA, BCI-Z, MCI-1,  $6\overline{6}$  8  $10$  inespective of the concentral 11 13  $15$  with Dr C plus X-lay induiction 16 18 20 Protonio did not ondrigo olgrim <sub>22</sub> **Imaging analysis showed that** 23 25  $_{27}$  with treatment with DPC,  $\lambda$ -ra 28 30  $32$   $\cdots$   $\cdots$   $\cdots$   $\cdots$   $\cdots$   $\cdots$   $\cdots$   $\cdots$   $\cdots$ 33 35  $37$  CAPICOMOTION TRITT CONSTRUCT 38 39 **higher than with treatment wi** 40 42 measurely, as from as seriously  $44$  a critical role in the induction of 45 47 49 JIVN, G-JUII, and UNUF Exple 50 52  $54$   $\ldots$   $\ldots$   $\ldots$   $\ldots$ 55 56 with CDDP plus X-ray irradiati 57  $59$  Proton (Orior) and not one.

CDDP plus X-ray irradiation. Similar results were obtained in HuH-7 cells after  $\frac{2}{3}$  treatment with DPC plus X-ray irradiation (Fig.4F). These results suggest that the  $\frac{4}{5}$  combination of DPC plus X-ray irradiation induced PUMA expression dependent on  $\frac{7}{2}$  the JNK signaling pathway. 1  $3 \cdot 3$  $5$  **compination** of DPC plus X-ray  $6\overline{6}$ 8

 $\frac{9}{10}$  To confirm the role of PUMA in the synergistic effect of DPC and X-ray irradiation, 12 blow we knocked down the PUMA gene in HepG2 and HuH-7 cells using siRNA and  $\frac{14}{15}$  examined changes of apoptosis. The relative mRNA levels of PUMA in the knocked-17 down cells were suppressed to 20% or less at 24 – 96 h after transfection with siRNA  $\frac{19}{20}$  as compared with that of control cells (Fig.5A). The apoptotic fraction of HepG2 cells  $\frac{21}{22}$  transfected with PUMA siRNA was 22.7 ± 3.5%, which was significantly lower than <sup>24</sup> that of control cells (46.5 ± 3.6%) (p<0.01 by Student's t test; Fig.5B). Similarly, the  $\frac{26}{27}$  apoptotic fraction of HuH-7 cells transfected with PUMA siRNA (37.0 ± 2.6%) was <sup>29</sup> significantly lower than that of control siRNA (45.9  $\pm$  2.6%) (*p*<0.01; Fig.5C). Thus,  $\frac{31}{32}$  knockdown of PUMA resulted in escape from apoptosis by the combination of DPC and X-ray irradiation in both HepG2 and HuH-7 cells. In addition, knockdown of PUMA 34  $\frac{36}{27}$  decreased most strongly the cytotoxic activity of DPC plus X-ray irradiation among the treatments (vehicle, DPC, X-ray alone and DPC/X-ray combination), in both cell lines,  $\frac{41}{42}$  suggesting that PUMA may play an important role in the synergistic effect of this  $\frac{43}{44}$  combination as well as in apoptosis induction (Supplementary Table 2).  $10$  TO COMMANDER TO LOT POINT 11 13  $15$  examined changes of apoptos 16 18 20 ab compared with that of comp 22 **Iransfected With PUMA SIRNA** 23 25  $_{27}$  apoptone iraction of HuH-7 ce 28 30 32 MIUCROUWII UI FUIVIA IESUIIEU 33 35  $37$  accreased most strongly the  $\sigma$ 38 39 **treatments (vehicle, DPC, X-ra** 40  $42$  $44$  compination as well as in apop

#### $^{48}_{49}$  Cell cycle arrest with DPC or CDDP plus X-ray irradiation  $49$  Cell cycle arrest with DFC of

To investigate the effects of DPC or CDDP plus X-ray irradiation on the cell cycle, 51  $^{53}_{54}$  we quantified cell subpopulations in sub-G1, G0/1, S, and G2/M phases after treatment with DPC/CDDP and/or X-ray by flow cytometry. DPC treatment significantly  $^{58}$  increased the sub-G1 population in HepG2 cells (65.6 ± 1.5%) compared with control 52  $54$  we quantified cell suppopula 55 56 **treatment with DPC/CDDP and** 57 59 more does a horse or popular

cells  $(26.8 \pm 1.7\%; p<0.01$  by Dunnett's test; Fig.6A), indicative of apoptosis induction.  $\frac{2}{3}$  In contrast, X-ray irradiation significantly increased the G2/M phase population in  $^{4}_{5}$  HepG2 cells (30.7  $\pm$  1.1%) compared with control cells (25.7  $\pm$  0.7%; *p*<0.01), suggesting G2/M arrest. DPC plus X-ray irradiation significantly increased the 7  $\frac{9}{10}$  apoptotic sub-G1 population (70.2 ± 0.6%; *p*<0.01). Similar patterns were observed in HepG2 cells treated with CDDP, and with CDDP plus X-ray irradiation. In contrast, 12  $^{14}_{15}$  experiments with HuH-7 cells showed that DPC significantly increased the S phase population (44.5 ± 5.7% vs 11.1 ± 0.6% for control; p<0.01; Fig.6B) as well as sub-G1  $^{19}_{20}$  population (6.7 ± 0.4% vs 4.3 ± 0.6% for control; p<0.01), and X-ray irradiation induced  $\frac{21}{22}$  sub-G1 (8.1 ± 0.9%; *p*<0.01) and G2/M arrest (37.3 ± 0.5% vs 25.3 ± 0.8% for control;  $p$ <0.01). DPC plus X-ray irradiation significantly induced the S phase (29.1  $\pm$  3.7%;  $^{26}_{27}$  p<0.01), sub-G1 (8.2 ± 0.6%; p<0.01), and G2/M phase populations (34.3 ± 1.0%; <sup>29</sup>  $p<sub>0.01</sub>$  in HuH-7 cells. While CDDP increased the sub-G1 (9.7  $\pm$  0.4%;  $p<sub>0.01</sub>$ ) and  $\frac{31}{32}$  G0/G1 population (72.2 ± 3.0% vs 59.4 ± 0.4% for control; p<0.01), possibly leading to apoptosis. CDDP plus X-ray irradiation increased only the apoptotic sub-G1 34  $\frac{36}{27}$  population (7.8 ± 0.8%; p<0.01). Thus, the effect of DPC on the cell cycle in HuH-7 cells differed from that of CDDP. 1 3  $5$  HepGZ cells (30.7 ± 1.1%) C  $6\overline{6}$  8  $_{10}$  apoptotic sub-G i population (*f* 11 13  $15$  CAPCHINGIUS WILL FIGHT-T CCIS 16 17 population  $(44.5 \pm 5.7\% \text{ vs } 11.$ 18 20 Population (6.1  $\pm$  6.170 to 1.0  $\pm$ 22 SUD-G1 (8.1 ± 0.9%;  $p$ <0.01) a 23 25  $\sqrt{25}$ 27  $p$ SUUT), SUD-GT (6.2 ± 0.0%) 28  $30 \qquad \qquad$  $32$  BUG Population (12.2 ± 3.0) 33 35  $37$  Population (1.0  $\pm$  0.070, p  $\cdot$ 0.0 38 39 **Cells differed from that of CDD** 

 $^{41}_{42}$  Subsequently a BrdU incorporation assay was performed to determine DNA  $\frac{43}{44}$  synthetic activity in HepG2 and HuH-7 cells treated with DPC, X-ray irradiation, and both in combination. Representative data are shown in Fig.6C. Both DPC and X-ray 46  $\frac{48}{49}$  irradiation significantly decreased the number of BrdU-incorporated cells in both cell lines. The combination of DPC plus X-ray irradiation decreased DNA synthetic activity 51  $^{53}_{54}$  most strongly (up to 0.5 - 1.0%) in both cell lines. It is expected that not only HepG2 cells but also HuH-7 cells, which showed S phase arrest, would undergo subsequent  $^{58}$  apoptosis as described previously [26].  $42$  $_{44}$  synthetic activity in HepGZ an 45 47  $49$   $\blacksquare$  induction significative decrease 50 52  $54$  most strongly (up to 0.0 - 1.07) 55 56 **cells but also HuH-7 cells, whi** 57 59 apoptosis as assembla provisi

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To examine the expression of cell cycle regulators, we performed western blotting  $\frac{2}{3}$  for cyclins A, B1, D1, and E (Fig.6D). DPC or DPC plus X-ray irradiation decreased  $\frac{4}{5}$  cyclin D1 expression strongly in HepG2 cells, which may be associated with an  $\frac{7}{2}$  increased sub-G1 population [27]. X-ray irradiation caused cyclin B1 accumulation in  $\frac{9}{10}$  both cell types, consistent with G2/M arrest in those cells [28]. Moreover, DPC or DPC plus X-ray irradiation decreased cyclins A and E in p53 wild-type HepG2 cells, also  $\frac{14}{15}$  consistent with previous reports [29, 30]. However, considerably weaker effects of CDDP plus X-ray irradiation on those cyclins were observed compared with DPC plus  $\frac{19}{20}$  X-ray irradiation.  $3^{3}$  and  $3^{3}$  cyclin DT expression strongly  $6\overline{6}$ 8 and 1.1 and 1 DOLLET Lypes, consistent with consistent with previous report 20 Array medicines.

#### $^{24}_{\sim}$  Effect of miriplatin plus X-ray irradiation on locally advanced HCC

 $\frac{26}{27}$  To evaluate the therapeutic efficacy of miriplatin plus radiotherapy on locally advanced HCC, we retrospectively compared the therapeutic efficacy of miriplatin-  $\frac{31}{32}$  TACE plus radiotherapy versus CDDP-TACE plus radiotherapy for locally advanced HCC patients with macroscopic vascular and/or bile duct invasion. The baseline  $\frac{36}{27}$  characteristics of the patients are summarized in Supplementary Table 3, and more detailed information is provided in Supplementary Table 4. No significant differences  $\frac{41}{42}$  in characteristics including age; sex; etiology; laboratory data; liver functional reserve;  $\frac{43}{44}$  and tumor size, number, degree of vascular/bile duct invasion, and stage were <sup>46</sup> observed between the miriplatin and CDDP groups. Representative CT images of CR,  $^{48}_{49}$  PR, SD and PD for primary evaluation of the main tumor with vascular/bile duct invasion inside the radiation field are shown in Fig.7A-D. The HCC tumor invading the  $^{53}_{54}$  portal vein completely disappeared in case 9 (CR), and the tumor invading the portal vein shrank but still remained in case 4 (PR) after treatment with miriplatin-TACE and  $^{58}$  radiotherapy. However, the tumor invading the portal vein showed little change in case  $_{27}$  To evaluate the therapeur TAGE plus radiotricially versu 37 Conditionships of the patients **detailed information is provide**  and tumor size, number, deg rn,  $3D$  and rD for primary  $_{54}$  portal velli completely disapper 56 vein shrank but still remained i reductionary: However, the tan

21 (SD), and the tumor invading the portal vein progressed in case 24 (PD) after  $\frac{2}{3}$  treatment with CDDP-TACE and radiotherapy. In total, the overall RR in the miriplatin  $\frac{4}{5}$  group (100% [10/10]; 8 CR and 2 PR) was significantly higher than in the CDDP group  $\frac{7}{2}$  (53.3% [8/15]); 6 CR, 2 PR, 5 SD, 2 PD;  $p$ <0.05; Fig.7E), despite the small number of  $\frac{9}{10}$  patients. We also evaluated secondarily intrahepatic nodules outside the radiation field based on modified RECIST criteria; the RR in the miriplatin group was 30% (3/10; 2  $^{14}_{15}$  CR, 1 PR, 2 SD, 5 PD), and was similar to that in the CDDP group (40% [6/15]; 5 CR, 17 1 PR, 6 SD, 3 PD; Supplementary Table 5). OS in the miriplatin group (23.6 months;  $\frac{19}{20}$  95% CI 6.9 – 33.3) was fairly higher than in the CDDP group (10.4 months; 95% CI  $\frac{21}{22}$   $\,$   $\,$   $\,$  4.4 – 21.6), although the difference was not statistically significant (p=0.16 by log rank  $\,$  $^{24}_{\sim}$  test; Fig.7F). These results may suggest that the tumor with vascular/bile duct invasion  $\frac{26}{27}$  determined the prognosis (OS) in locally advanced HCC, as described previously [7]. Thus, our data suggest that combination treatment with miriplatin and X-ray irradiation  $\frac{31}{32}$  may possibly be more effective against locally advanced HCC compared with CDDP-TACE plus radiotherapy. group (100% [10/10]; 8 CR and  $6\overline{6}$  $8 \qquad \qquad 8 \qquad \qquad 8$  patients. We also evaluated set UI, IIII, Z OD, JT DJ, and We  $_{22}$  4.4 – 21.6), although the differe  $_{27}$  determined the prognosis ( $\sim$   $\cdots$   $\cdots$   $32$ 

## **Discussion**<br>40

 $\frac{41}{42}$  In this study, we demonstrated that miriplatin and X-ray irradiation in combination  $\frac{43}{44}$  has a synergistic effect on HCC cells. This is the first study to have investigated the anti-tumor activity of miriplatin plus X-ray irradiation in vitro and in vivo. We also  $\frac{48}{49}$  showed that this combination caused strong apoptosis mediated via PUMA expression. Synergistic induction of PUMA in HCC cells after treatment with the combination, and  $\frac{53}{54}$  inhibition of apoptosis by knocking down the PUMA gene in HCC cells, indicate a pivotal role of PUMA in apoptosis caused by the combined treatment. Moreover, TACE  $^{58}$  with miriplatin concurrently combined with radiotherapy showed a high response rate **All State**, *All State*, *All States, <i>All*  $_{44}$  and  $_{44}$  and SHOWED that this compliance to **IMMOND OF apoptosis by KID pivotal role of PUMA in apoptos Martimipidan concentently con** 

in patients with unresectable HCC invading macroscopically portal/hepatic veins  $\frac{2}{3}$  and/or bile ducts, despite a limited number of patients studied. Conversely, CDDP  $\frac{4}{5}$  showed little synergistic effect on HCC cell lines nor did it induce PUMA to trigger <sup>7</sup> apoptosis. Showed little synergistic effect  $6\overline{6}$  $8 \cdot 1 \cdot 1$ 

 $^{10}_{11}$  PUMA is a key molecule for apoptosis induction by anti-tumor drugs and by 13 radiotherapy. The expression of PUMA is reportedly elevated in response to DNA- $\frac{15}{16}$  damaging stimuli through p53-dependent or p53-independent transcription. It has  $^{17}_{18}\,$  been reported that X-ray irradiation enhances PUMA expression mainly through p53- $^{20}$  dependent transcription activation, leading to apoptosis in oral cancer,  $\frac{22}{23}$  masopharyngeal cancer, and breast cancer [31]. However, no published studies have investigated the mechanism of apoptosis induced by miriplatin. Notably, oxaliplatin,  $\frac{27}{28}$  whose active form, DPC, is the same as miriplatin, enhances PUMA expression in colorectal cancer [32]. Although it is unclear how radiation and DPC exert synergistic  $\frac{32}{22}$  anti-tumor activity in detail, it is plausible that the combination of miriplatin and X-ray irradiation synergistically enhances PUMA expression, leading to strong induction of  $\frac{37}{20}$  apoptosis in HCC cells. In addition, we used the 2 cell lines, HepG2 with p53 wild and  $_{\rm 40}^{\rm 39}$  HuH-7 with p53 mutant, and both lines showed similarly enhanced PUMA expression after treatment with DPC plus X-ray irradiation, possibly leading to induction of  $\frac{44}{45}$  apoptosis.  $10$ <sub>NN</sub> is a Key molecule admaging burnan unbagn pot been reported that X-ray irradi **Experimental Exercise Section**  $_{23}$  rasopharyngeal cancer, and b 26 and 20 an WIIOSE ACTIVE IOIIII, DFC, IS II and tamor address in additional  $33$  **Tradiation synergistically enha**  38 September 2016 11:12 September 2016 HuH-7 with p53 mutant, and be apoptosis.

Eventually, DPC increased PUMA and cleaved PARP expression levels similarly  $\frac{50}{51}$  in a dose-dependent manner in HepG2 and HuH-7 cells. Notably, their expression  $^{52}_{53}$  levels started to increase at lower DPC concentrations (0 – 1.25 µM) in HuH-7 cells  $^{55}_{20}$  than in HepG2 cells (2.5 – 5.0 µM; Suppler 51 magazine appointment **Ievels started to increase at lo**  

with the difference in IC50 of these cells for DPC, and also suggest a close association  $\frac{2}{3}$  of PUMA with apoptosis in both cells.  $3 \,$   $\ldots$   $\ldots$   $\ldots$   $\ldots$   $\ldots$ 

 $\frac{5}{6}$  Regarding the mechanism of PUMA induction, we found enhanced p-JNK and p-8 C-JUN expression but not ER stress-related protein (CHOP) expression in both cell  $\frac{10}{11}$  lines after treatment with DPC plus X-ray irradiation (Fig.4E,F). These results suggest that DPC plus X-ray irradiation induced PUMA expression via the JNK signaling  $^{15}_{16}$  pathway, consistent with a previous report on JNK-dependent PUMA induction [33]. It  $^{\rm 17}_{\rm 18}$   $\qquad \qquad$  is also assumed that induced PUMA activates Bax and Bak, leading to activation of  $\frac{20}{3}$  the caspase cascade and ultimately to cell apoptosis. Thus, the combination of  $\frac{22}{23}$  miriplatin and X-ray irradiation would be effective on HCC cells regardless of the status  $^{25}$  of p53 mutation. In addition, the PUMA mRNA expression level was significantly lower  $\frac{27}{28}$  in primary normal hepatocytes compared with HCC cells, and the difference in expression was enhanced roughly tenfold after treatment with DPC and X-ray  $\frac{32}{22}$  irradiation (Supplementary Fig.4). These data suggest that DPC plus X-ray irradiation would be selectively effective against HCC cells without affecting mostly normal  $\frac{37}{28}$  hepatocytes. Regarding the mechanism of  $6$   $\ldots$   $\ldots$   $\ldots$  and  $\ldots$   $\ldots$   $\ldots$   $\ldots$  pairway, concident with a pro-**IS also assumed that induced**  21 and the part of the most sense of the  $_{23}$  minplatin and X-ray irradiation in primary normal nepatocyte magnetion (supplementary rig **Would be selectively effective**  

 $\frac{40}{41}$  Cisplatin, a hydrophilic agent like most other chemotherapeutic agents, is <sup>43</sup> commonly used for TACE with or without radiation therapy in patients with HCC [34].  $^{45}_{46}$  A retrospective analysis by Oguro and associates reported that TACE with miriplatin demonstrated a significantly lower response rate but lower rates of adverse events  $^{50}_{51}$  than TACE with CDDP [35]. In their report, however, the mean dose of miriplatin  $^{52}$  administered (50.4 mg, 64.4 µmol) was lower than that of CDDP (55.9 mg, 186.3 µmol).  $\frac{55}{2}$  In our study, the IC50 value of DPC (miriplatin) in HCC cell lines was significantly lower  $\frac{57}{58}$  than that of CDDP. Therefore, it is plausible that miriplatin has stronger anti-tumor Cispiauri, a riyurophinc a and those was obtharpoon.  $_{53}$  administered (50.4 mg, 64.4  $\mu$ r  $_{58}$   $\ldots$  man mat or GDDP. Therefore

activity than CDDP against HCC at quimolar doses. It remains controversial as to whether or not the combination of CDDP and radiation has a synergistic effect [36,37].  $\frac{4}{5}$  However, our results indicated that CDDP plus X-ray showed little synergy. This may  $\frac{7}{2}$  be related to the fact that CDDP did not induce PUMA expression in cancer cells as  $\frac{9}{10}$  revealed by our group (Supplementary Fig.2) and other investigators [39]. However, our results indicated to  $6\overline{6}$ 8 and 2010  $_{10}$  revealed by our group (Supple

**In addition to analysis of apoptosis to determine the mechanism underlying the**  $^{15}_{16}$  effect of miriplatin in combination with radiation, we also analyzed the effects of DPC  $^{17}_{18}$  (miriplatin) plus radiation on cell cycle arrest by flow cytometry (Fig.6). DPC alone  $^{20}$  induced sub-G1 arrest in HepG2 cells with wild-type p53, probably leading to p53  $\frac{22}{23}$  dependent apoptosis. In contrast, DPC alone mainly induced S phase arrest as well as sub-G1 arrest in HuH-7 cells with mutant p53, which is consistent with previous  $\frac{27}{28}$  studies showing that several anti-cancer drugs induce S phase arrest in cancer cells, subsequently leading to apoptosis [26]. Interestingly, our results are also compatible  $\frac{32}{22}$  with a previous report showing that zolendronic acid induced PUMA and S phase arrest in cancer cells [39]. On the other hand, X-ray irradiation alone induced G2/M  $\frac{37}{20}$  arrest in these cell lines, consistent with previous reports [28]. The combination of DPC  $_{\rm 40}^{\rm 39}$  plus irradiation induced both sub-G1 and S phase arrest and a much greater degree of G2/M arrest in HCC cells. In our study, DPC alone decreased cyclin D1 expression,  $\frac{44}{45}$  while X-ray irradiation caused cyclin B1 accumulation, consistent with previous reports [28, 40]. Thus, cell cycle arrest might be associated with the synergistic effect of DPC  $^{49}_{50}$  and X-ray irradiation. on our or immediate in complete. 18 (miriplatin) plus radiation on d  $_{23}$  aependent apoptosis. In contra Studies Showing that several a with a provided report shown **arrest in cancer cells [39]. On plus irradiation induced both s**  write  $\lambda$ -ray irragiation caused of and  $\lambda$ -ray madiation.

 $\frac{52}{53}$  In preliminary experiments, we investigated invasion capability in 5 HCC cell lines 55 (HepG2, HuH-7, PLC/PRF5, HuH-1, and Hep3B) using a transwell invasive assay.  $^{57}_{58}$  HepG2 and HuH-7 cells showed the highest invasion activities, followed by HuH-1, <sub>53</sub> In preliminary experiments,  $_{58}$   $\phantom{00}$   $\phantom{0}$   $\phantom{0$ 

PLC/PRF5, and Hep3B cells (Supplementary Fig.5A,B). Moreover, HepG2 and HuH- $\frac{2}{3}$  7 cells were p53 wild and mutant, respectively, and thus it appears that HepG2 and  $_5^{4}$  HuH-7 cells were suitable for the current experiments. In addition, when HepG2 and <sup>7</sup> HuH-7 cells were treated with DPC plus X-ray irradiation, invasion activity was  $\frac{9}{10}$  significantly decreased compared with control cells after correction for viable cell 12 numbers (p<0.05; Supplementary Fig.5C-E). HuH-*I* cells were suitable for tr  $6\overline{6}$ 8 and 2010  $_{10}$  significatity decreased compa 

 $\frac{15}{16}$  Our retrospective analyses of miriplatin plus radiotherapy showed a higher  $\frac{17}{18}$  response rates of the main tumor with vascular/bile duct invasion and longer OS times  $\frac{20}{3}$  in locally advanced HCC than those of CDDP plus radiotherapy, despite the small  $\frac{22}{23}$  number of patients. These values were also greater than those previously reported for CDDP plus radiotherapy [13, 14]. The total radiation doses in previous reports of  $\frac{27}{28}$  CDDP plus radiotherapy ranged from 37.5 – 45 Gy, which is lower than the dose used in our study (50 Gy). The dose of CDDP in previous reports was 2 mg/kg (6.7  $\frac{32}{22}$  whereas the dose of miriplatin in our study was 2 mg/kg (2.6 µmol/kg). Consequently, it appears that the synergistic effect of miriplatin and radiotherapy in this study might  $\frac{37}{20}$  be attributable to the high RR of the main tumor. In addition, no grade 3/4 adverse  $\frac{39}{40}$  event was observed in the miriplatin group, although grade 3/4 adverse events were observed at a rate of 53.3% (8/15) in the CDDP group (Supplementary Table 6),  $\frac{44}{45}$  consistent with previous reports showing that miriplatin is much less toxic than cisplatin Car Tomoopoom analyse response rates of the main tum  $_{23}$  mumber of patients. These value 26 and the contract of the con  $_{28}$  CDDF plus radiomerapy range **MICRO GIC GOOD OF IMPROVER It appears that the synergistic 38**  event was observed in the mir consistent with previous report. 

[41].

50 A limitation of this study is that the human data on miriplatin-TACE and CDDP-TACE with radiotherapy are small sample-sized and retrospective analysis. Therefore, 55 a prospective large-scale study to compare those therapies should be performed in  $\frac{57}{58}$  the future. 51 Million of the staat to **I ACE with radiotherapy are sm**  58 The future.

In conclusion, our data suggest that combination treatment with miriplatin and X- $\frac{2}{3}$  ray irradiation has a synergistic effect on HCC cells, and that this combination induces  $^{4}_{5}$  PUMA-mediated apoptosis as well as cell cycle arrest in HCC cells, regardless of the  $\frac{7}{2}$  status of p53 mutation. Our results suggest that TACE with miriplatin combined with  $\frac{9}{10}$  radiotherapy may be possibly effective for locally advanced HCC.  $3 \t\times 3$  PUMA-mediated apoptosis as v  $6\overline{6}$ 8 and 1  $_{10}$  adjoint apy may be possibly  $\epsilon$ 

#### $^{15}_{16}$  Acknowledgements

 $^{17}_{18}$  We are grateful to Misato Hirata and Masahiro Bando for their technical assistance. We are grateful to Misato Hi

 $\frac{22}{23}$  Competing interests: Tetsuji Takayama received DPC from Sumitomo Dainippon Pharma Co, Ltd. (Osaka Japan) in this study.  $_{23}$  competing interests: retsuji 

**Funding:** This work was partly supported by a Grant-in-Aid for Scientific Research  $\frac{32}{22}$  from the Japan Society for the Promotion of Science (JSPS; grant number 17K15950).  $\ldots$   $\ldots$   $\ldots$   $\ldots$ 

#### References

1

2<br>
1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of  $\frac{3}{3}$  incidence and mortality worldwide for  $\frac{4}{1000}$  and mortally worldwide to  $\frac{424}{1}$ 

 $6$  2. Llovet JM, Real MI, Montaña X, et symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised<br>8 controlled trial Jancet 2002:359:1734-9  $\frac{8}{9}$  controlled trial. Lancet. 2002;359:1734-9.

3. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular  $10$ 10 St. Elovetski, Brans, Systematic Tex 11 Carcinoma. Chemoembolization imp

12  $\,$  4. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J<br>13 Med. 2008;359:378-90. 13 Med. 2008;359:378-90.<br>14 5 Kudo M, Finn RS, Oi

5. Kudo M, Finn RS, Qin S, et al. Lenvatinib versus sorafenib in first-line treatment of patients with  $\frac{15}{15}$  unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet.  $\frac{16}{17}$  2018;391:1163-73.

 $17$  2010, 331.1103-73.  $18$  b. Bruix J, Qin S, Merie P, et al. Reg 19 progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, 20 phase 3 trial. Lancet. 2017;389:56-66.

21 7. Cabibbo G, Enea M, Attanasio M, et al. A meta-analysis of survival rates of untreated patients in<br>22 magdanized divind this of hands all the services Henrich and 2010 F1:1274.93  $\frac{22}{23}$  randomized clinical trials of hepatocellular carcinoma. Hepatology. 2010;51:1274-83.

23 manusimized entired that of hepatoc  $24$   $\alpha$ , Masuda K, Uno A, Alkata H, et al 25 of extreme poor prognosis for advanced hepatocellular carcinoma treated with sorafenib and 26 hepatic arterial infusion chemotherapy. J Gastroenterol. 2018;53:107-18.<br>27 9 Cheng Al Guan 7 Chen 7 et al Efficacy and safety of sorafenih in pat

27 and 9. Cheng AL, Guan Z, Chen Z, et al. Efficacy and safety of sorafenib in patients with advanced 28 beneficially consiners according to begaling at two subsets and we observe the phase III Cambo 28 29 **State Project Line Communication** 

 $30$  Asia-Facilic trial. Eur J Callcel. 2012,  $_{31}$  and 20. Zeng ZC, Fan J, Tang ZY, et al. A comparison of treatment combinations with and without 32 radiotherapy for hepatocellular carcinoma with portal vein and/or inferior vena cava tumor 33 thrombus. Int J Radiat Oncol Biol Phys. 2005;61:432-43.

 $\frac{34}{11}$  11. Huang YJ, Hsu HC, Wang CY, et al. The treatment responses in cases of radiation therapy to 35 **Samuel Library Strategiers** and the set of  $36$  portal vehiclinomisosis in advanced  $\frac{37}{37}$  2009;73:1155-63.

38 12. Yamada K, Izaki K, Sugimoto K, et al. Prospective trial of combined transcatheter arterial 39 chemoembolization and three-dimensional conformal radiotherapy for portal vein tumor thrombus <sup>40</sup> in patients with unresectable hepatocellular carcinoma. Int J Radiat Oncol Biol Phys. 2003;57:113-9. 41<br>42 **13. Yoon SM, Lim YS, Won HJ, et al. Radiotherapy plus transarterial chemoembolization for** 

42 **13. 1880 Strip Emilio, woming, et al.** 43 **Superior Contract Calculorii de la constituto de la constitución de la constitución de la constitución de l** 44 Biol Phys. 2012;82:2004-11.<br>45 14. Yoon SM, Ryoo BY, Lee S

14. Yoon SM, Ryoo BY, Lee SJ, et al. Efficacy and Safety of Transarterial Chemoembolization Plus<br>46 Sternal Beam Radiotherany vs Sorafenih in Henatocellular Carcinoma With Macrosconic Vascul 46 External Beam Radiotherapy vs Sorafenib in Hepatocellular Carcinoma With Macroscopic Vascular<br>47 September 10 Reprinsived Clinical Trial 1984 Creat 2018 4:661 0 Invasion: A Randomized Clinical Trial. JAMA Oncol. 2018;4:661-9.

48 The Deble N. Deng 7. Cicelatin nephr  $49$  **10.** Fabia IV, DOIIg Z. Cispia III Hepin  $\frac{1}{50}$  2008;73:994-1007.<br>51 16. Hanada M, Bab

51 16. Hanada M, Baba A, Tsutsumishita Y, et al. Intra-hepatic arterial administration with miriplatin<br>52 suspended in an oily lymphographic agent inhibits the growth of tumors implanted in rat livers by  $52$  suspended in an oily lymphographic agent inhibits the growth of tumors implanted in rat livers by<br> $53$  inducing platinum DNA adducts to form and massive apoptosis. Cancer Chamother Pharmacol inducing platinum-DNA adducts to form and massive apoptosis. Cancer Chemother Pharmacol.  $\frac{54}{15}$  2009;64:473-83.

 $55$  2009,04.479 09.  $56$  17. Okusaka 1, Okada 5, Nakanisni 1 57 lipophilic platinum derivative (SM-11355) in patients with hepatocellular carcinoma. Invest New<br>58 Drugs. 2004;22:169-76. Drugs. 2004;22:169-76.

22

- 59 60
- 61 62
- 64 65

- 18. Imai Y, Chikayama T, Nakazawa M, et al. Usefulness of miriplatin as an anticancer agent for 1 transcatheter arterial chemoembolization in patients with unresectable hepatocellular carcinoma. J 2 Gastroenterol. 2012;47:179-86.  $\frac{3}{4}$  19. Ikeda M, Kudo M, Aikata H, et al. Transarterial chemoembolization with miriplatin vs. epirubicin  $\frac{4}{\epsilon}$  c c  $\frac{1}{\epsilon}$   $5$  for unresectable neparocentrial carcing  $\frac{6}{7}$  2018;53:281-90.<br>7 20. Chou TC. Dru 20. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay<br>8 method Cancer Res. 2010:70:440-6  $\frac{8}{9}$  method. Cancer Res. 2010;70:440-6. 21. Liao B, Zhang Y, Sun Q, et al. Vorinostat enhances the anticancer effect of oxaliplatin on 10 **Explores Exception** Continues and Co  $11$  reparticular calculofiid cens. Cand 12 the Antitumor Effect in Colorectal Cancer. Mol Cancer Res. 2017;15:1445-54.<br>14 23. Onishi H. Nouso K. Nakamura S. et al. Efficacy of henatic arterial infusion 23. Onishi H, Nouso K, Nakamura S, et al. Efficacy of hepatic arterial infusion chemotherapy in  $15$  combination with irradiation for advanced hepatocellular carcinoma with portal vein invasion.  $16$ <br>Hepatol Int. 2015;9:105-12. 17  $18$  24. Elsenhauer EA, Therasse P, Boga 19 revised RECIST guideline (version 1.1). Eur J Cancer. 2009;45:228-47. 20 25. Shen D, Wang H, Zheng Q, et al. Synergistic effect of a retinoid X receptor-selective ligand 21 bexarotene and docetaxel in prostate cancer. Onco Targets Ther. 2019;12:7877-86.<br>22 22 26 Sharing Cl. Harnor IW, Antiopage drug terrate: call augh and sharknesist centre 22 26 Shaniro GL Harner IW Anticance 23 **2000-104-164F** F2  $24$  1999;104:1645-53. 25 27. Kranenburg O, van der Eb AJ, Zantema A. Cyclin D1 is an essential mediator of apoptotic 26 meuronal cell death. EMBO J. 1996;15:46-54.<br>27 28 Lin 77 Chou CH Cheng AL et al Badioser 27 28. Lin ZZ, Chou CH, Cheng AL, et al. Radiosensitization by combining an aurora kinase inhibitor with  $28$ <br>28 and interface in happines like arcinoma through sell ovels interruption. Int LCancer, 2014;125:402 28 radiotherany in henatocellular carcin  $29$   $29$  $30$   $301$ .  $31$  29. Voland C, Bord A, Peleraux A, et 32 cisplatin in human colon cancer cells. Mol Cancer Ther. 2006;5:2149-57. 33 30. Jiang T, Zhang H, Liu X, et al. Effect of oxaliplatin combined with polyenephosphatidylcholine on <sup>34</sup> the proliferation of human gastric cancer SGC-7901 cells. Oncol Lett. 2016;12:4538-46.  $\frac{35}{36}$  31. Yu J, Zhang L. PUMA, a potent killer with or without p53. Oncogene. 2008;27 Suppl 1:S71-83.  $36$  32.  $10^{3}$ ,  $20^{11}$ ,  $30$  $37$  32. Wang  $\lambda$ , Li Wi, Wang J, et al. The 38 apoptosis in colon cancer cells. Biochem Pharmacol. 2006;71:1540-50. 39 33. Cazanave SC, Mott JL, Elmi NA, et al. JNK1-dependent PUMA expression contributes to 40 hepatocyte lipoapoptosis. J Biol Chem. 2009;284:26591-602. 41 34. Chung SR, Kim JH, Yoon HK, et al. Combined Cisplatin-Based Chemoembolization and Radiation 42 **Theren: for Heretecollish Corpinan** 43 Therapy for Hepatocentrial Carcinon  $\frac{44}{45}$  2015;26:1130-8.<br>45 35. Oguro S, Has 45 46 chemoembolization using miriplatin-lipiodol suspension for hepatocellular carcinoma. Jpn J Radiol.<br>47 2012:20:725.42 2012;30:735-42.  $\frac{48}{40}$  36. Chenoufi N, Raoul JL, Lescoat G, et al. In vitro demonstration of synergy between radionuclide 49 **So.** Chenoam W, Rabar SE, Eesebat G,  $50$  and chemotherapy. J Nucli Med. 199 51 37. Kitabayashi H, Shimada H, Yamada S, et al. Synergistic growth suppression induced in esophageal<br>52 squamous cell carcinoma cells by combined treatment with docetaxel and heavy carbon-ion beam 52 squamous cell carcinoma cells by combined treatment with docetaxel and heavy carbon-ion beam<br>53 stradiation Oncol Ben 2006:15:913-8  $\frac{53}{54}$  irradiation. Oncol Rep. 2006;15:913-8.  $54$  38 Wang L 7hou IV Wu GS Rim pro  $55$  30.  $\frac{3041}{20622220402}$  $\frac{56}{56}$  2011;286:22384-92.<br>57 39. Liu SS, Wang XP, 57 39. Liu SS, Wang XP, Li XB, et al. Zoledronic acid exerts antitumor effects in NB4 acute promyelocytic<br>58 leukemia cells by inducing apoptosis and S phase arrest. Biomed Pharmacother. 2014;68:1031-6. leukemia cells by inducing apoptosis and S phase arrest. Biomed Pharmacother. 2014;68:1031-6. 59 60 23 61 62 63 64
- 
- 65

40. Qu K, Xu X, Liu C, et al. Negative regulation of transcription factor FoxM1 by p53 enhances oxaliplatin-induced senescence in hepatocellular carcinoma. Cancer Lett. 2013;331:105-14.<br>2 1 Otsuii K Takai K Nishigaki Y et al Efficacy and safety of cisplatin versus miriplatin in <sup>2</sup> 41. Otsuji K, Takai K, Nishigaki Y, et al. Efficacy and safety of cisplatin versus miriplatin in<br><sup>2</sup> transacthotor arterial shomoombolization and transactorial infusion shomothorony for  $\frac{3}{4}$  transcatheter arterial chemoembolization and transarterial infusion chemotherapy for  $\frac{4}{1}$   $\frac{1}{1}$   $\frac{1}{1}$ reparacement cardinalities. A randomis

Figure legends

#### $\frac{2}{3}$  Figure 1.

**Figure 1**.<br>Viability of HCC cells treated with DPC and CDDP. A. HepG2 and HuH-7 cells were  $\frac{7}{10}$  treated with DPC or CDDP for 72 h. Cell viability was then determined by WST-8 assay.  $\frac{9}{10}$  IC50 values were calculated by non-linear regression analysis. Combination index analysis of DPC or CDDP combined with X-ray irradiation in HCC cells. HepG2 or  $^{14}_{15}$  HuH-7 cells were incubated with various concentrations of DPC (B, C) or CDDP (D, **E**) combined with X-ray exposure (0 – 10Gy) at a fixed ratio. After 120 h, cell viability  $\frac{19}{20}$  was determined by WST-8 assay. The CI was calculated as described in Materials  $\frac{21}{22}$  and Methods. A CI value <1 and >1 indicates synergistic and antagonistic effects, respectively.  $\cdots$   $\cdots$   $\cdots$  viability of HCC cells treated w  $6\overline{6}$ 8 and 2010 ICOU values were calculated TRIP CERS WEIGHT CONDECT IN **mac accommod** by **not** b ac  $_{22}$  and Methods. A CI value  $\leq$ 1 

### **Figure 2.**

 $\frac{29}{30}$  Figure 2.<br> $\frac{31}{32}$  Colony formation in HCC cells after exposure to DPC and X-ray irradiation. A, C. Representative images of colony formation in HepG2 (A) and HuH-7 cells (C). Cells  $\frac{36}{27}$  were treated with DPC (250 nmol/L) with or without X-ray irradiation (1 Gy), followed by incubation for 14 days. They were then fixed with methanol and stained with 0.4% <sup>41</sup> crystal violet. B, D. The number of colonies in HepG2 (B) and HuH-7 cells (D) with  $\frac{43}{44}$  greater than 50 cells was determined and expressed as a percentage of the number 46 of control cells. Data represent mean ± standard deviation (SD) of triplicated  $^{48}_{49}$  experiments. \*  $p$  < 0.05 by Turkey's test. COION FORMATION IN TIGG CENT were abated with Dr  $\sigma$  (200 m 39 by incubation for 14 days. The greater than 50 cells was dete experiments.  $p > 0.03$  by Fur

#### $\frac{53}{54}$  Figure 3. rigults J.

**Figure 3**.<br>Apoptosis induction in HCC cells after treatment with DPC (5 µM) or CDDP (10 µM)  $^{58}$  and/or X-ray irradiation (5 Gy). A. Cells were treated with vehicle, DPC, CDDP or X- **Apoptosis induction in HCC ce**  and/or  $\lambda$  ray measurem ( $\sigma$   $\sigma$ )

ray irradiation alone, or DPC/CDDP + X-ray, then solubilized and subjected to western  $\frac{2}{3}$  blot analysis for cleaved PARP and caspase-3. β-actin was used as a loading control.  $\frac{4}{5}$  B, C. Flow cytometric analysis of HepG2 (B) and HuH-7 cells (C) for annexin V-FITC  $\frac{7}{2}$  and propidium iodide (PI) staining of cells treated with vehicle or DPC/CDDP + X-ray.  $\frac{9}{10}$  Data represent mean ± SD of triplicate experiments. \*  $p$  < 0.01 by Turkey's test. 1  $3$  $5$  B, C. Flow cytometric analysis of  $6\overline{6}$  $8 \hspace{1.5cm} \cdot \cdot \cdot$  $_{10}$  Data represent mean ± 3D on

#### $14$   $\blacksquare$  $15$  riguit  $\frac{1}{2}$ .

 $^{\texttt{14}}_{\texttt{15}}$  **Figure 4.**<br><sup>16</sup> Expression of apoptosis-related protein, JNK signaling, and ER stress-related protein **Expression of apoptosis-related prote**in  $\frac{19}{20}$  in HCC cells treated with DPC, CDDP, and X-ray irradiation. A, C. HepG2 (A) or HuH-7 cells (C) were treated with vehicle, DPC (5  $\mu$ M), CDDP (5  $\mu$ M), or X-ray (5 Gy) alone,  $^{24}_{25}$  or DPC/CDDP + X-ray, then solubilized and subjected to western blot analysis for BH3  $_{27}$  only protein and Bcl-2 family protein. β-actin was used as a loading control. B, D. Each  $\,$  $^{29}_{22}$  of the PUMA protein bands in HepG2 (B) and HuH-7 cells (D) was specifically  $\frac{31}{32}$  quantified and normalized to β-actin using the Image J program (National Institutes of <sup>34</sup> Health, Bethesda, MD). \*\*p < 0.05 vs other treatment groups by Turkey test. E, F.  $\frac{36}{37}$  Western blot analysis was performed for JNK, p-JNK, c-Jun, p-c-Jun, and CHOP protein in HepG2 (E) and HuH-7 cells (F) after treatment with DPC/CDDP plus X-ray 39  $\frac{41}{42}$  irradiation. 18  $20$   $\ldots$   $\ldots$ 21 22 7 cells (C) were treated with ve 23  $25$  or Brossberry, aloned  $_{27}$  only protein and BcI-2 family pr 28 30 **and the complete of the state of the**  $32$  quantified and normalized to  $\beta$ 33  $35$ 37 **WESTELL DIOL ALIALYSIS WAS PE** 38 40 42 magazines.

#### $46 \over 47$  Figure 5.  $47$  and  $\bullet$ .

**Figure 5**.<br>Knockdown of the PUMA gene resulted in escape from apoptosis by DPC and X-ray  $^{51}_{-2}$  irradiation in HCC cells. A. The PUMA gene in HepG2 and HuH-7 cells was knocked  $^{53}_{54}$  down by siRNA. The relative mRNA levels at 24, 48, 72 and 96 h were determined by 56 Taqman PCR.  $* p < 0.01$ . B, C. HepG2 (B) or HuH-7 cells (C) were transfected with  $^{58}_{58}$  siRNA against PUMA or control siRNA and treated with DPC (5 µM) plus X-ray 48 49 KNOCKOOWN OT The PUMA gene 50 52  $_{54}$  about by SIRNA. The relative from 55 57  $\frac{59}{2}$  SINNA ayalılar FUMA VI CON

irradiation (5 Gy). Flow cytometric analysis for annexin V-FITC as well as for propidium  $\frac{2}{3}$  iodide (PI) was performed. The percentages of annexin V-positive cells are presented  $\frac{4}{5}$  as mean ± SD of triplicate experiments. \*\* $p$  < 0.01 by Student's *t* test. as mean  $\pm$  SD or triplicate expe

#### $_{10}$  rigule o.

 $\frac{10}{10}$  Figure 6.<br>11 Cell cycle analysis in HCC cells after exposure to DPC or CDDP and X-ray irradiation. X-ray irradiation (5 Gy). HepG2 (A) and HuH-7 cells (B) were treated with vehicle,  $^{19}_{22}$  DPC, CDDP, X-ray irradiation alone, or DPC or CDDP plus X-ray irradiation, and cell  $\frac{21}{22}$  cycle profile was analyzed after 24 h by flow cytometry. \*\*;  $\bm{p}$  < 0.01 by Dunnett's test.  $^{24}$  C. HepG2 and HuH-7 cells were treated with vehicle, DPC or X-ray irradiation alone,  $\frac{26}{27}$  or DPC plus X-ray irradiation, and BrdU incorporation was analyzed after 24 h by flow cytometry. D. HepG2 and HuH-7 cells were treated with vehicle, DPC, CDDP, X-ray  $\frac{31}{32}$  irradiation alone, DPC plus X-ray irradiation, or CDDP plus X-ray irradiation, and cyclin expression was analyzed after 24 h by western blotting.  $14 \rightarrow$   $\sim$  P Coll oveloperatile of HCC  $20$  $_{22}$  cycle profile was analyzed after **Exercise 25**  $_{27}$  or DPC plus X-ray irradiation, a  $30 \qquad \qquad$  in duration divite, Dr  $\vee$  plus  $\wedge$ - $\vee$ 

#### **Figure 7.**

Figure 7. Response and survival of HCC with vascular/bile duct invasion to transarterial  $\frac{43}{44}$  chemoembolization (TACE) with miriplatin or TACE with CDDP combined with <sup>46</sup> radiotherapy. Representative CT images of CR, PR, SD, and PD before and after  $^{48}_{49}$  treatment. A. An abdominal CT scan during the portal-venous phase in case 9 with HCC invading the portal vein. The tumor and portal invasion completely disappeared  $^{53}_{54}$  (CR) after miriplatin-TACE with radiotherapy. B. An abdominal CT scan during the portal-venous phase in case 4 with tumor invading the portal vein. The tumor invading  $^{58}$  the portal vein shrank but still remained (PR) after miriplatin-TACE with radiotherapy. 42 response and cannot control 42  $_{44}$  chemoembolization (TACE) \ u datument. A. An abdominar G (Civ) and imipiduit role with **portal-venous phase in case 4**  are portal voir official backups  $59$ 

C. The tumor invading portal vein showed little change (SD) in case 21 after CDDP- $\frac{2}{3}$  TACE with radiotherapy. D. The size of tumor invading portal vein enlarged (PD) in  $\frac{4}{5}$  case 24 after CDDP-TACE with radiotherapy. E. Summary of response in miriplatin <sup>7</sup> and CDDP groups. F. Overall survival of patients in the miriplatin and CDDP groups  $\frac{9}{10}$  was estimated by Kaplan-Meier analysis and differences were evaluated by log rank 12 test.  $3 \cdot 3$  case 24 after CDDP-TACE with  $6\overline{6}$  10 Was estimated by Rapidit-Men 

#### $^{19}$  Supplamentary Figure 1 **Suppromontary rights**  $\cdot$

**Supplementary Figure 1**.<br>Anti-tumor effect of X-ray irradiation on HCC cells. A. HepG2 and HuH-7 cells were  $^{24}_{25}$  irradiated with X-rays at 0, 2.5, 5, 7.5 and 10 Gy. After 5 days, cell viability was  $\frac{26}{27}$  analyzed by WST-8 assay. ED50 values were calculated by non-linear regression analysis. B. HCC cells were irradiated with X-rays at each dose, then solubilized after  $\frac{31}{32}$  5 days and subjected to western blot analysis for PARP. **Anti-tumor effect of X-ray irrac**   $_{27}$  analyzed by  $\text{two}$  -6 assay.  $\text{E}$  U days and subjected to wester

#### Cupplamentary Figure 2 **Supplementary rights 2.**

**Supplementary Figure 2**.<br>Expression of apoptosis-related protein in HCC cells after treatment with DPC or  $^{41}_{42}$  CDDP. HepG2 (A) and HuH-7cells (B) were treated with DPC or CDDP at 0 - 20 µM  $\frac{43}{44}$  for 48 h, then solubilized and subjected to western blot analysis for BH3 only protein  $^{46}$  and Bcl-2 family proteins.  $\beta$ -actin was used as a loading control. **Expression of apoptosis-relate**  Tor 48 n, then solubilized and  $\frac{4}{3}$ 

## 51 **Supplementary Figure 3.**<br>52

Supplementary Figure 3.<br>52<br>53 Quantitation of apoptosis-related protein expression in HepG2 and HuH-7 cells. The expression levels of Bim, Blk, Noxa, Bcl-2, Mcl-1 and Bcl-xl protein in HepG2 (A) and Quantitation of apoptosis-relation **expression levels of Bim, Blk,** I

HuH-7 cells (B) detected by western blot analysis were specifically quantitated and  $\frac{2}{3}$  normalized to  $\beta$ -actin using the Image J program.  $3 \cdot 3$ 

#### $\frac{7}{8}$  Supplementary Figure 4. and  $\sim$   $\sim$   $\sim$

 $\frac{7}{8}$  Supplementary Figure 4.<br> $\frac{9}{10}$  The expression of PUMA mRNA in normal hepatocytes and HepG2 cells after T treatment with DPC, X-ray irradiation, and DPC plus X-ray irradiation. Normal  $^{14}_{15}$  hepatocytes or HepG2 cells were treated with DPC plus X-ray irradiation, and PUMA mRNA expression levels were determined by Taqman PCR. N.S., not significant. \*p <  $\frac{19}{20}$  0.01 by Turkey's test. \*\*The relative mRNA level of PUMA in normal hepatocytes was  $\frac{21}{22}$  significantly lower than in HepG2 cells.  $\bm{p}$  < 0.01 by Student's  $\bm{t}$  test. The expression of FOMA III reparcious or repoz cens we 17 mRNA expression levels were  $0.01$  by Tamby 0 tool. The To  $_{22}$  significantly lower than in Hep

#### $\frac{26}{27}$  Supplementary Figure 5.  $_{27}$  Supplementary Figure 5.

 Invasion capability of HCC cells treated with DPC and X-ray irradiation. A. The  $\frac{31}{32}$  invasion capability of 5 HCC cell lines was determined by transwell assay. A representative image of each cell line is shown. B. Five randomly selected fields were  $\frac{36}{27}$  imaged and the number of invading cells was counted as described in Supplementary information. \*\* $p$  < 0.01 by Turkey's test. C. HepG2 and HuH-7 cells were treated with  $^{41}_{42}$  vehicle alone or DPC plus X-ray irradiation, cultured for 48 h, and invasion assay was  $\frac{43}{44}$  performed. Representative images of HepG2 and HuH-7 cells are shown. D,E. The <sup>46</sup> mean numbers of invading cells in HepG2 and HuH-7 cells are shown.  $* p < 0.01$  by  $\frac{48}{49}$  Student's *t* test. IIIVASION CAPADING OF J TICK magda and the number of five 39 Information.  $\sim p < 0.01$  by Turk 44 performed. Representative im- Students thest.







Figure 2



B





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Figure 3



B



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Figure 5



Figure 6

