CONCLUSION

In summary, HCC, pancreatic cancers, and their surrounding tissues express AR at various levels (Figure 1). AR, but not androgen, might be involved in the carcinogenesis and cancer development of HCC or pancreatic cancer. In HBV-related hepatocarcinogenesis, the interaction between the CAG repeats in the AR gene as was reported from Taiwan[37-41]. AR also promotes the transcription of HBV, which leads to a higher HBV titer in male HBV carriers and an increased risk of HCC[47-49,93]. Currently, the effect of AR on HCV replication is unclear, however, HCV increases AR-mediated transcriptional activity especially in the presence of AR[58]. AR might play an important role in pancreatic carcinogenesis and the development of pancreatic cancer[72]. AHR is involved in both HCC[80-82] and pancreatic cancer[83,84]. AR could be in involved the carcinogenesis of HCC and pancreatic cancer through MICA/B[87-90]. Future directions in treatment development should specifically target AR in these cancers.

REFERENCES

1 **Zaret KS**, Grompe M. Generation and regeneration of cells of the liver and pancreas. *Science* 2008; **322**: 1490-1494 [PMID: 19056973 DOI: 10.1126/science.1161431]
2 **Chalmers AD**, Slack JM. The Xenopus tadpole gut: fate maps and morphogenetic movements. *Development* 2000; **127**: 381-392 [PMID: 10603354]
3 **Tremblay KD**, Zaret KS. Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. *Dev Biol* 2005; **280**: 87-99 [PMID: 15766750]
4 **Field HA**, Dong PD, Beis D, Stainier DY. Formation of the digestive system in zebrafish. II. Pancreas morphogenesis. *Dev Biol* 2003; **261**: 197-208 [PMID: 12941629]
5 **Slack JM**. Developmental biology of the pancreas. *Development* 1995; **121**: 1569-1580 [PMID: 7600975]
6 **Shen CN**, Slack JM, Tosh D. Molecular basis of transdifferentiation of pancreas to liver. *Nat Cell Biol* 2000; **2**: 879-887 [PMID: 11146651]
7 **Tanaka H**, Imai Y, Hiramatsu N, Ito Y, Imanaka K, Oshita M, Hijioka T, Katayama K, Yabuuchi I, Yoshihara H, Inoue A, Kato M, Takehara T, Tamura S, Kasahara A, Hayashi N, Tsukuma H. Declining incidence of hepatocellular carcinoma in Osaka, Japan, from 1990 to 2003. *Ann Intern Med* 2008; **148**: 820-826 [PMID: 18519928]
8 **Mittal S**, El-Serag HB. Epidemiology of hepatocellular carcinoma: consider the population. *J Clin Gastroenterol* 2013; **47** Suppl: S2-S6 [PMID: 23632345 DOI: 10.1097/MCG.0b013e3182872f29]
9 **Beasley RP**, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. *Lancet* 1981; **2**: 1129-1133 [PMID: 6118576]
10 **Hayashi PH**, Di Bisceglie AM. The progression of hepatitis B- and C-infections to chronic liver disease and hepatocellular carcinoma: epidemiology and pathogenesis. *Med Clin North Am* 2005; **89**: 371-389 [PMID: 15656931]
11 **Akita T**, Ohisa M, Kimura Y, Fujimoto M, Miyakawa Y, Tanaka J. Validation and limitation of age-period-cohort model in simulating mortality due to hepatocellular carcinoma from 1940 to 2010 in Japan. *Hepatol Res* 2013; Epub ahead of print [PMID: 23730747 DOI: 10.1111/hepr.12177]
12 **Ikai I**, Arii S, Kojiro M, Ichida T, Makuuchi M, Matsuyama Y, Nakanuma Y, Okita K, Omata M, Takayasu K, Yamaoka Y. Reevaluation of prognostic factors for survival after liver resection in patients with hepatocellular carcinoma in a Japanese nationwide survey. *Cancer* 2004; **101**: 796-802 [PMID: 15305412]
13 **Shiina S**, Tateishi R, Arano T, Uchino K, Enooku K, Nakagawa H, Asaoka Y, Sato T, Masuzaki R, Kondo Y, Goto T, Yoshida H, Omata M, Koike K. Radiofrequency ablation for hepatocellular carcinoma: 10-year outcome and prognostic factors. *Am J Gastroenterol* 2012; **107**: 569-77; quiz 578 [PMID: 22158026 DOI: 10.1038/ajg.2011.425]
14 **Yoshikawa M**, Ono N, Yodono H, Ichida T, Nakamura H. Phase II study of hepatic arterial infusion of a fine-powder formulation of cisplatin for advanced hepatocellular carcinoma. *Hepatol Res* 2008; **38**: 474-483 [PMID: 18430093 DOI: 10.1111/j.1872-034X.2008.00338.x]
15 **Shao YY**, Hsu CH, Cheng AL. Predictive biomarkers of anti-angiogenic therapy for advanced hepatocellular carcinoma: where are we? *Liver Cancer* 2013; **2**: 93-107 [PMID: 24159601]
16 **Raimondi S**, Maisonneuve P, Lowenfels AB. Epidemiology of pancreatic cancer: an overview. *Nat Rev Gastroenterol Hepatol* 2009; **6**: 699-708 [PMID: 19806144 DOI: 10.1038/nrgastro.2009.177]
17 **Yadav D**, Lowenfels AB. The epidemiology of pancreatitis and pancreatic cancer. *Gastroenterology* 2013; **144**: 1252-1261 [PMID: 23622135 DOI: 10.1053/j.gastro.2013.01.068]
18 **Conlon KC**, Klimstra DS, Brennan MF. Long-term survival after curative resection for pancreatic ductal adenocarcinoma. Clinicopathologic analysis of 5-year survivors. *Ann Surg* 1996; **223**: 273-279 [PMID: 8604907]
19 **Egawa S**, Toma H, Ohigashi H, Okusaka T, Nakao A, Hatori T, Maguchi H, Yanagisawa A, Tanaka M. Japan Pancreatic Cancer Registry; 30th year anniversary: Japan Pancreas Society. *Pancreas* 2012; **41**: 985-992 [PMID: 22750974]
20 **Chang C**, Lee SO, Yeh S, Chang TM. Androgen receptor (AR) differential roles in hormone-related tumors including prostate, bladder, kidney, lung, breast and liver. *Oncogene* 2014; **33**: 3225-3234 [PMID: 23873027 DOI: 10.1038/onc.2013.274]
21 **Gelmann EP**. Molecular biology of the androgen receptor. *J Clin Oncol* 2002; **20**: 3001-3015 [PMID: 12089231]
22 **Heinlein CA**, Chang C. Androgen receptor in prostate cancer. *Endocr Rev* 2004; **25**: 276-308 [PMID: 15082523]
23 **Yoon G**, Kim JY, Choi YK, Won YS, Lim IK. Direct activation of TGF-beta1 transcription by androgen and androgen receptor complex in Huh7 human hepatoma cells and its tumor in nude mice. *J Cell Biochem* 2006; **97**: 393-411 [PMID: 16187311]
24 **Bolton EC**, So AY, Chaivorapol C, Haqq CM, Li H, Yamamoto KR. Cell- and gene-specific regulation of primary target genes by the androgen receptor. *Genes Dev* 2007; **21**: 2005-2017 [PMID: 17699749]
25 **Jiang X**, Kanda T, Nakamoto S, Miyamura T, Wu S, Yokosuka O. Involvement of androgen receptor and glucose-regulated protein 78 kDa in human hepatocarcinogenesis. *Exp Cell Res* 2014; **323**: 326-336 [PMID: 24583399 DOI: 10.1016/j.yexcr.2014.02.017]
26 **Nagasue N**, Ito A, Yukaya H, Ogawa Y. Androgen receptors in hepatocellular carcinoma and surrounding parenchyma. *Gastroenterology* 1985; **89**: 643-647 [PMID: 2991072]
27 **Negro F**, Papotti M, Pacchioni D, Galimi F, Bonino F, Bussolati G. Detection of human androgen receptor mRNA in hepatocellular carcinoma by in situ hybridisation. *Liver* 1994; **14**: 213-219 [PMID: 7968281]
28 **Vizoso FJ**, Rodriguez M, Altadill A, González-Diéguez ML, Linares A, González LO, Junquera S, Fresno-Forcelledo F, Corte MD, Rodrigo L. Liver expression of steroid hormones and Apolipoprotein D receptors in hepatocellular carcinoma. *World J Gastroenterol* 2007; **13**: 3221-3227 [PMID: 17589901]
29 **Nagasue N**, Yu L, Yukaya H, Kohno H, Nakamura T. Androgen and oestrogen receptors in hepatocellular carcinoma and surrounding liver parenchyma: impact on intrahepatic

recurrence after hepatic resection. *Br J Surg* 1995; **82**: 542-547 [PMID: 7613907]
30 **Boix L**, Castells A, Bruix J, Solé M, Brú C, Fuster J, Rivera F, Rodés J. Androgen receptors in hepatocellular carcinoma and surrounding liver: relationship with tumor size and recurrence rate after surgical resection. *J Hepatol* 1995; **22**: 616-622 [PMID: 7560855]
31 **Zhang X**, He L, Lu Y, Liu M, Huang X. Androgen receptor in primary hepatocellular carcinoma and its clinical significance. *Chin Med J* (Engl) 1998; **111**: 1083-1086 [PMID: 11263369]
32 **Kalra M**, Mayes J, Assefa S, Kaul AK, Kaul R. Role of sex steroid receptors in pathobiology of hepatocellular carcinoma. *World J Gastroenterol* 2008; **14**: 5945-5961 [PMID: 18932272]
33 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539 [PMID: 17256718]
34 **Di Bisceglie AM**. Hepatitis B and hepatocellular carcinoma. *Hepatology* 2009; **49**: S56-S60 [PMID: 19399807 DOI: 10.1002/hep.22962]
35 **Wu S**, Kanda T, Imazeki F, Nakamoto S, Shirasawa H, Yokosuka O. Nuclear receptor mRNA expression by HBV in human hepatoblastoma cell lines. *Cancer Lett* 2011; **312**: 33-42 [PMID: 21903321 DOI: 10.1016/j.canlet.2011.07.025]
36 **Yu MW**, Yang YC, Yang SY, Cheng SW, Liaw YF, Lin SM, Chen CJ. Hormonal markers and hepatitis B virus-related hepatocellular carcinoma risk: a nested case-control study among men. *J Natl Cancer Inst* 2001; **93**: 1644-1651 [PMID: 11698569]
37 **Yu MW**, Cheng SW, Lin MW, Yang SY, Liaw YF, Chang HC, Hsiao TJ, Lin SM, Lee SD, Chen PJ, Liu CJ, Chen CJ. Androgen-receptor gene CAG repeats, plasma testosterone levels, and risk of hepatitis B-related hepatocellular carcinoma. *J Natl Cancer Inst* 2000; **92**: 2023-2028 [PMID: 11121465]
38 **Yu MW**, Yang YC, Yang SY, Chang HC, Liaw YF, Lin SM, Liu CJ, Lee SD, Lin CL, Chen PJ, Lin SC, Chen CJ. Androgen receptor exon 1 CAG repeat length and risk of hepatocellular carcinoma in women. *Hepatology* 2002; **36**: 156-163 [PMID: 12085360]
39 **Yeh SH**, Chang CF, Shau WY, Chen YW, Hsu HC, Lee PH, Chen DS, Chen PJ. Dominance of functional androgen receptor allele with longer CAG repeat in hepatitis B virus-related female hepatocarcinogenesis. *Cancer Res* 2002; **62**: 4346-4351 [PMID: 12154039]
40 **Yeh SH**, Chiu CM, Chen CL, Lu SF, Hsu HC, Chen DS, Chen PJ. Somatic mutations at the trinucleotide repeats of androgen receptor gene in male hepatocellular carcinoma. *Int J Cancer* 2007; **120**: 1610-1617 [PMID: 17230529]
41 **Chan HL**, Sung JJ. Hepatocellular carcinoma and hepatitis B virus. *Semin Liver Dis* 2006; **26**: 153-161 [PMID: 16673293]
42 **Kim CM**, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature* 1991; **351**: 317-320 [PMID: 2034275]
43 **Kanda T**, Yokosuka O, Imazeki F, Yamada Y, Imamura T, Fukai K, Nagao K, Saisho H. Hepatitis B virus X protein (HBx)-induced apoptosis in HuH-7 cells: influence of HBV genotype and basal core promoter mutations. *Scand J Gastroenterol* 2004; **39**: 478-485 [PMID: 15180187]
44 **Chiu CM**, Yeh SH, Chen PJ, Kuo TJ, Chang CJ, Chen PJ, Yang WJ, Chen DS. Hepatitis B virus X protein enhances androgen receptor-responsive gene expression depending on androgen level. *Proc Natl Acad Sci USA* 2007; **104**: 2571-2578 [PMID: 17259306]
45 **Zheng Y**, Chen WL, Ma WL, Chang C, Ou JH. Enhancement of gene transactivation activity of androgen receptor by hepatitis B virus X protein. *Virology* 2007; **363**: 454-461 [PMID: 17335866]
46 **Yang WJ**, Chang CJ, Yeh SH, Lin WH, Wang SH, Tsai TF, Chen DS, Chen PJ. Hepatitis B virus X protein enhances the transcriptional activity of the androgen receptor through c-Src and glycogen synthase kinase-3beta kinase pathways. *Hepatology* 2009; **49**: 1515-1524 [PMID: 19205031 DOI: 10.1002/hep.22833]
47 **Wang SH**, Yeh SH, Lin WH, Wang HY, Chen DS, Chen PJ. Identification of androgen response elements in the enhancer I of hepatitis B virus: a mechanism for sex disparity in chronic hepatitis B. *Hepatology* 2009; **50**: 1392-1402 [PMID: 19670412 DOI: 10.1002/hep.23163]
48 **Wu MH**, Ma WL, Hsu CL, Chen YL, Ou JH, Ryan CK, Hung YC, Yeh S, Chang C. Androgen receptor promotes hepatitis B virus-induced hepatocarcinogenesis through modulation of hepatitis B virus RNA transcription. *Sci Transl Med* 2010; **2**: 32ra35 [PMID: 20484730 DOI: 10.1126/scitranslmed.3001143]
49 **Tian Y**, Kuo CF, Chen WL, Ou JH. Enhancement of hepatitis B virus replication by androgen and its receptor in mice. *J Virol* 2012; **86**: 1904-1910 [PMID: 22156518 DOI: 10.1128/JVI.06707-11]
50 **Lee WM**, Dienstag JL, Lindsay KL, Lok AS, Bonkovsky HL, Shiffman ML, Everson GT, Di Bisceglie AM, Morgan TR, Ghany MG, Morishima C, Wright EC, Everhart JE. Evolution of the HALT-C Trial: pegylated interferon as maintenance therapy for chronic hepatitis C in previous interferon nonresponders. *Control Clin Trials* 2004; **25**: 472-492 [PMID: 15465617]
51 **Saito I**, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990; **87**: 6547-6549 [PMID: 2168552]
52 **Ray RB**, Lagging LM, Meyer K, Steele R, Ray R. Transcriptional regulation of cellular and viral promoters by the hepatitis C virus core protein. *Virus Res* 1995; **37**: 209-220 [PMID: 8533458]
53 **Ray RB**, Lagging LM, Meyer K, Ray R. Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype. *J Virol* 1996; **70**: 4438-4443 [PMID: 8676467]
54 **Moriya K**, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4**: 1065-1067 [PMID: 9734402]
55 **Ray RB**, Meyer K, Ray R. Hepatitis C virus core protein promotes immortalization of primary human hepatocytes. *Virology* 2000; **271**: 197-204 [PMID: 10814584]
56 **Basu A**, Meyer K, Lai KK, Saito K, Di Bisceglie AM, Grosso LE, Ray RB, Ray R. Microarray analyses and molecular profiling of Stat3 signaling pathway induced by hepatitis C virus core protein in human hepatocytes. *Virology* 2006; **349**: 347-358 [PMID: 16545852]
57 **Saito K**, Meyer K, Warner R, Basu A, Ray RB, Ray R. Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein. *J Virol* 2006; **80**: 4372-4379 [PMID: 16611896]
58 **Kanda T**, Steele R, Ray R, Ray RB. Hepatitis C virus core protein augments androgen receptor-mediated signaling. *J Virol* 2008; **82**: 11066-11072 [PMID: 18768969 DOI: 10.1128/JVI.01300-08]
59 **Ueda T**, Mawji NR, Bruchovsky N, Sadar MD. Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. *J Biol Chem* 2002; **277**: 38087-38094 [PMID: 12163482]
60 **White DL**, Tavakoli-Tabasi S, Kuzniarek J, Pascua R, Ramsey DJ, El-Serag HB. Higher serum testosterone is associated with increased risk of advanced hepatitis C-related liver disease in males. *Hepatology* 2012; **55**: 759-768 [PMID: 21858849 DOI: 10.1002/hep.24618]
61 **Ma WL**, Hsu CL, Wu MH, Wu CT, Wu CC, Lai JJ, Jou YS,

Chen CW, Yeh S, Chang C. Androgen receptor is a new potential therapeutic target for the treatment of hepatocellular carcinoma. *Gastroenterology* 2008; **135**: 947-55, 955.e1-5 [PMID: 18639551 DOI: 10.1053/j.gastro.2008.05.046]

62 **Ma WL**, Hsu CL, Yeh CC, Wu MH, Huang CK, Jeng LB, Hung YC, Lin TY, Yeh S, Chang C. Hepatic androgen receptor suppresses hepatocellular carcinoma metastasis through modulation of cell migration and anoikis. *Hepatology* 2012; **56**: 176-185 [PMID: 22318717 DOI: 10.1002/hep.25644]

63 **Ao J**, Meng J, Zhu L, Nie H, Yang C, Li J, Gu J, Lin Q, Long W, Dong X, Li C. Activation of androgen receptor induces ID1 and promotes hepatocellular carcinoma cell migration and invasion. *Mol Oncol* 2012; **6**: 507-515 [PMID: 22819717 DOI: 10.1016/j.molonc.2012.06.005]

64 **Li Z**, Tuteja G, Schug J, Kaestner KH. Foxa1 and Foxa2 are essential for sexual dimorphism in liver cancer. *Cell* 2012; **148**: 72-83 [PMID: 22265403 DOI: 10.1016/j.cell.2011.11.026]

65 **DePrimo SE**, Diehn M, Nelson JB, Reiter RE, Matese J, Fero M, Tibshirani R, Brown PO, Brooks JD. Transcriptional programs activated by exposure of human prostate cancer cells to androgen. *Genome Biol* 2002; **3**: RESEARCH0032 [PMID: 12184806]

66 **Nelson PS**, Clegg N, Arnold H, Ferguson C, Bonham M, White J, Hood L, Lin B. The program of androgen-responsive genes in neoplastic prostate epithelium. *Proc Natl Acad Sci USA* 2002; **99**: 11890-11895 [PMID: 12185249]

67 **Corbishley TP**, Iqbal MJ, Wilkinson ML, Williams R. Androgen receptor in human normal and malignant pancreatic tissue and cell lines. *Cancer* 1986; **57**: 1992-1995 [PMID: 3955505]

68 **Noh KW**, Pungpapong S, Wallace MB, Woodward TA, Raimondo M. Do cytokine concentrations in pancreatic juice predict the presence of pancreatic diseases? *Clin Gastroenterol Hepatol* 2006; **4**: 782-789 [PMID: 16713745]

69 **Ueda T**, Bruchovsky N, Sadar MD. Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. *J Biol Chem* 2002; **277**: 7076-7085 [PMID: 11751884]

70 **Furukawa T**. Molecular targeting therapy for pancreatic cancer: current knowledge and perspectives from bench to bedside. *J Gastroenterol* 2008; **43**: 905-911 [PMID: 19107333 DOI: 10.1007/s00535-008-2226-1]

71 **Yang L**, Wang L, Lin HK, Kan PY, Xie S, Tsai MY, Wang PH, Chen YT, Chang C. Interleukin-6 differentially regulates androgen receptor transactivation via PI3K-Akt, STAT3, and MAPK, three distinct signal pathways in prostate cancer cells. *Biochem Biophys Res Commun* 2003; **305**: 462-469 [PMID: 12763015]

72 **Okitsu K**, Kanda T, Imazeki F, Yonemitsu Y, Ray RB, Chang C, Yokosuka O. Involvement of interleukin-6 and androgen receptor signaling in pancreatic cancer. *Genes Cancer* 2010; **1**: 859-867 [PMID: 21779469 DOI: 10.1177/1947601910383417]

73 **Konduri S**, Schwarz MA, Cafasso D, Schwarz RE. Androgen receptor blockade in experimental combination therapy of pancreatic cancer. *J Surg Res* 2007; **142**: 378-386 [PMID: 17559882]

74 **Kollara A**, Brown TJ. Four and a half LIM domain 2 alters the impact of aryl hydrocarbon receptor on androgen receptor transcriptional activity. *J Steroid Biochem Mol Biol* 2010; **118**: 51-58 [PMID: 19815066 DOI: 10.1016/j.jsbmb.2009.09.017]

75 **Wu Y**, Baumgarten SC, Zhou P, Stocco C. Testosterone-dependent interaction between androgen receptor and aryl hydrocarbon receptor induces liver receptor homolog 1 expression in rat granulosa cells. *Mol Cell Biol* 2013; **33**: 2817-2828 [PMID: 23689136 DOI: 10.1128/MCB.00011-13]

76 **Gradin K**, McGuire J, Wenger RH, Kvietikova I, fhitelaw ML, Toftgård R, Tora L, Gassmann M, Poellinger L. Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. *Mol Cell Biol* 1996; **16**: 5221-5231 [PMID: 8816435]

77 **Rowlands JC**, McEwan IJ, Gustafsson JA. Trans-activation by the human aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator proteins: direct interactions with basal transcription factors. *Mol Pharmacol* 1996; **50**: 538-548 [PMID: 8794892]

78 **Fernandez-Salguero PM**, Hilbert DM, Rudikoff S, Ward JM, Gonzalez FJ. Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity. *Toxicol Appl Pharmacol* 1996; **140**: 173-179 [PMID: 8806883]

79 **Anderson G**, Beischlag TV, Vinciguerra M, Mazzoccoli G. The circadian clock circuitry and the AHR signaling pathway in physiology and pathology. *Biochem Pharmacol* 2013; **85**: 1405-1416 [PMID: 23438471 DOI: 10.1016/j.bcp.2013.02.022]

80 **Buzzelli G**, Dattolo P, Pinzani M, Brocchi A, Romano S, Gentilini P. Circulating growth hormone and insulin-like growth factor-I in nonalcoholic liver cirrhosis with or without superimposed hepatocarcinoma: evidence of an altered circadian rhythm. *Am J Gastroenterol* 1993; **88**: 1744-1748 [PMID: 8213718]

81 **Zhao B**, Lu J, Yin J, Liu H, Guo X, Yang Y, Ge N, Zhu Y, Zhang H, Xing J. A functional polymorphism in PER3 gene is associated with prognosis in hepatocellular carcinoma. *Liver Int* 2012; **32**: 1451-1459 [PMID: 22809120 DOI: 10.1111/j.1478-3231.2012.02849.x]

82 **Naik A**, Košir R, Rozman D. Genomic aspects of NAFLD pathogenesis. *Genomics* 2013; **102**: 84-95 [PMID: 23545492 DOI: 10.1016/j.ygeno.2013.03.007]

83 **Sato F**, Nagata C, Liu Y, Suzuki T, Kondo J, Morohashi S, Imaizumi T, Kato Y, Kijima H. PERIOD1 is an anti-apoptotic factor in human pancreatic and hepatic cancer cells. *J Biochem* 2009; **146**: 833-838 [PMID: 19675098 DOI: 10.1093/jb/mvp126]

84 **Relles D**, Sendecki J, Chipitsyna G, Hyslop T, Yeo CJ, Arafat HA. Circadian gene expression and clinicopathologic correlates in pancreatic cancer. *J Gastrointest Surg* 2013; **17**: 443-450 [PMID: 23254314 DOI: 10.1007/s11605-012-2112-2]

85 **Monteleone I**, MacDonald TT, Pallone F, Monteleone G. The aryl hydrocarbon receptor in inflammatory bowel disease: linking the environment to disease pathogenesis. *Curr Opin Gastroenterol* 2012; **28**: 310-313 [PMID: 22450895 DOI: 10.1097/MOG.0b013e328352ad69]

86 **Kung T**, Murphy KA, White LA. The aryl hydrocarbon receptor (AhR) pathway as a regulatory pathway for cell adhesion and matrix metabolism. *Biochem Pharmacol* 2009; **77**: 536-546 [PMID: 18940186 DOI: 10.1016/j.bcp.2008.09.031]

87 **Kumar V**, Kato N, Urabe Y, Takahashi A, Muroyama R, Hosono N, Otsuka M, Tateishi R, Omata M, Nakagawa H, Koike K, Kamatani N, Kubo M, Nakamura Y, Matsuda K. Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. *Nat Genet* 2011; **43**: 455-458 [PMID: 21499248 DOI: 10.1038/ng.809]

88 **Kumar V**, Yi Lo PH, Sawai H, Kato N, Takahashi A, Deng Z, Urabe Y, Mbarek H, Tokunaga K, Tanaka Y, Sugiyama M, Mizokami M, Muroyama R, Tateishi R, Omata M, Koike K, Tanikawa C, Kamatani N, Kubo M, Nakamura Y, Matsuda K. Soluble MICA and a MICA variation as possible prognostic biomarkers for HBV-induced hepatocellular carcinoma. *PLoS One* 2012; **7**: e44743 [PMID: 23024757 DOI: 10.1371/journal.pone.0044743]

89 **Jinushi M**, Takehara T, Tatsumi T, Kanto T, Groh V, Spies T, Kimura R, Miyagi T, Mochizuki K, Sasaki Y, Hayashi N. Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid. *Int J Cancer* 2003; **104**: 354-361 [PMID: 12569559]

90 **Kim H**, Bose SK, Meyer K, Ray R. Hepatitis C virus impairs natural killer cell-mediated augmentation of complement synthesis. *J Virol* 2014; **88**: 2564-2571 [PMID: 24352446 DOI:

10.1128/JVI.02988-13]
91 **Friedl P**, Sahai E, Weiss S, Yamada KM. New dimensions in cell migration. *Nat Rev Mol Cell Biol* 2012; **13**: 743-747 [PMID: 23072889 DOI: 10.1038/nrm3459]
92 **Castoria G**, Giovannelli P, Di Donato M, Hayashi R, Arra C, Appella E, Auricchio F, Migliaccio A. Targeting androgen receptor/Src complex impairs the aggressive phenotype of human fibrosarcoma cells. *PLoS One* 2013; **8**: e76899 [PMID: 24130806 DOI: 10.1371/journal.pone.0076899]
93 **Kanda T**, Yokosuka O, Omata M. Androgen Receptor and Hepatocellular Carcinoma. *J Gastroint Dig Syst* 2013; **S12**: 012 [DOI: 10.4172/2161-069X.S12-012]

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Knockdown of glucose-regulated protein 78 enhances poly(ADP-ribose) polymerase cleavage in human pancreatic cancer cells exposed to endoplasmic reticulum stress

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Abstract. The present study examined the expression of glucose-regulated protein 78 (GRP78/Bip) in human pancreatic cancer cell lines and the effect of knockdown of GRP78 on the cleavage of poly(ADP-ribose) polymerase (PARP). Human pancreatic cancer cell lines (KP-2, MIAPaCa-2, Panc-1 and SUIT-2), constitutively expressed GRP78. We also demonstrated that ER stress induced by thapsigargin upregulated protein levels of GRP78. In the presence of thapsigargin, knockdown of GRP78 enhanced the PARP cleavage in the human pancreatic cancer cells. These results provide evidence that GRP78 is a potential therapeutic target for 'difficult-to-treat' pancreatic cancer, in which ER stress signaling in part falls into disorder.

Introduction

Pancreatic cancer is almost the deadliest of all malignancies (1). In Japan, pancreatic cancer is currently the fifth leading cause of cancer-related death among individuals of both genders (2,3). Resection surgery is still the only potentially curative treatment for pancreatic cancer, and recent improvements in operative technique have been reported (4). Although advances in adjuvant treatment have been observed (5), in general, the prognosis of patients with pancreatic cancer is still poor. Further studies of the mechanisms of pancreatic carcinogenesis and cancer development are needed, and new therapeutic options are highly desirable.

Endoplasmic reticulum (ER) stress response in tumor cells is critical for tumor cell growth and cancer progression (6). The ER stress response is mediated by at least three sensor molecules: inositol-requiring enzyme 1α (IRE1α), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6), which are usually associated with glucose-regulated protein 78 (GRP78/Bip) (7). ER stress, which is associated with the accumulation of unfolded proteins, induces unfolded protein response (UPR), yet if ER stress is overloaded, cells could face death such as by apoptosis and autophagy. Downstream of IRE1α and PERK, the effector molecules, X-box-binding protein 1 (XBP1) and C/EBP homologous protein (CHOP), and growth arrest and DNA damage gene 34 (GADD34) all exist, and they are activated by ER stress. ER stress also leads to the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) (8). For example, p90ATF6 is converted to the activated form p50ATF6, and p50ATF6 translocates to the nucleus (9). Basic leucine-zipper family factors p50ATF6 and XBP1 could induce expression of a subset of UPR-related genes, which include ER stress elements, and are involved in efficient protein folding, maturation and degradation in the ER (6).

The association between ER stress response and tumor growth and progression has been reported (10). We and others have reported that GRP78 is involved in cancer development and innate immune response in the liver (11-14). Liver and pancreas progenitors commonly develop from endoderm cells in the embryonic foregut (15). Pancreatic epithelial cells have a highly developed ER due to a strong engagement in digestive enzyme secretion (16). GRP78 is the main target of UPR signaling that promotes pancreatic cancer cell survival (17). GRP78 is involved in cancer progression as well as drug resistance (18,19). Hence, to decrease the ability of pancreatic cancer cells to survive and proliferate, it may be necessary to block GRP78 expression (17).

We previously demonstrated that blocking of the induction of UPR, as well as inhibition of GRP78 expression is associated with the cleavage of poly(ADP-ribose) polymerase (PARP) (13). In the present study, we examined the expression of ER stress-related molecules in human pancreatic cancer cell lines in the presence or absence of thapsigargin, one of the ER stress-inducers. We also investigated whether knockdown of GRP78 by small interfering RNA (siRNA) enhances the PARP cleavage in human pancreatic cancer cell lines exposed to ER stress.

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Key words: apoptosis, ER stress, GRP78/Bip, pancreatic cancer, resistance, UPR

Materials and methods

Cell culture. Human pancreatic cancer cell lines (KP-2, MIAPaCa-2, Panc-1 and SUIT-2) were grown in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. Inhibitor of sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+} ATPases (SERCA), thapsigargin, control siRNA (si-control) and siRNA for GRP78 (si-GRP78) were purchased from BioVision (Milpitas, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

Western blotting. Twenty-four hours after thapsigargin (1 μM) treatment, cells were lysed in sodium dodecyl sulfate sample buffer, and after sonication, lysates were processed for western blot analysis (11). Briefly, protein samples were subjected to electrophoresis on 5-20% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (ATTO, Tokyo, Japan). Membranes were probed with antibodies specific for ATF4, ATF6 and tubulin (Abcam, Cambridge, UK); GADD34, gyceraldehyde-3-phosphate dehydrogenase (GAPDH) and XBP1 (Santa Cruz); eIF2α, phospho-eIF2α (Ser51), GRP78/Bip and PARP (Cell Signaling Technology, Tokyo, Japan). After washing with PBS-T, the membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Signals were detected by means of enhanced chemiluminescence (GE Healthcare, Tokyo, Japan) and scanned by image analyzer LAS-4000 and Image Gauge (version 3.1) (Fuji Film, Tokyo, Japan) and ImageJ software (NIH, Bethesda, MD, USA).

Transfection of siRNA. To confirm the effects of GRP78 knockdown on apoptosis, we examined GRP78 knockdown by small-interfering RNA (siRNA). Cells were transfected with 50 nM si-GRP78 or si-control, using Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol (20). After 24 h of transfection, cells were treated with 1 μM thapsigargin for 24 h.

Statistical analysis. Results are expressed as means ± standard deviation (SD). Statistical analysis was performed using the Student's t-test. A P-value <0.05 was considered to indicate a statistically significant result.

Results

Human pancreatic cancer cell lines express GRP78. First, we examined the GRP78 expression in the human pancreatic cancer cell lines SUIT-2, MIAPaCa-2, Panc-1 and KP-2 (3). Protein samples were collected from the four pancreatic cancer cell lines, and protein levels of GRP78 were investigated by western blotting with a specific antibody for GRP78 (Fig. 1). We confirmed that all four pancreatic cancer cell lines variably expressed GRP78.

Thapsigargin upregulates the protein levels of GRP78 in the human pancreatic cancer cell lines. Next, we examined the effect of thapsigargin, one of the ER stress-inducers, on GRP78 expression in the human pancreatic cancer cell lines (Fig. 2).

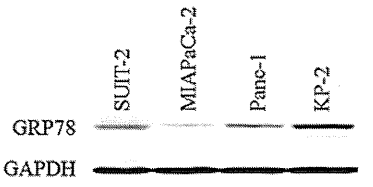

Figure 1. Glucose-regulated protein 78 (GRP78/Bip) is expressed at various levels in the human pancreatic cancer cell lines. Western blot analyses of GRP78 and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) in SUIT-2, MIAPaCa-2, Panc-1 and KP-2 cells.

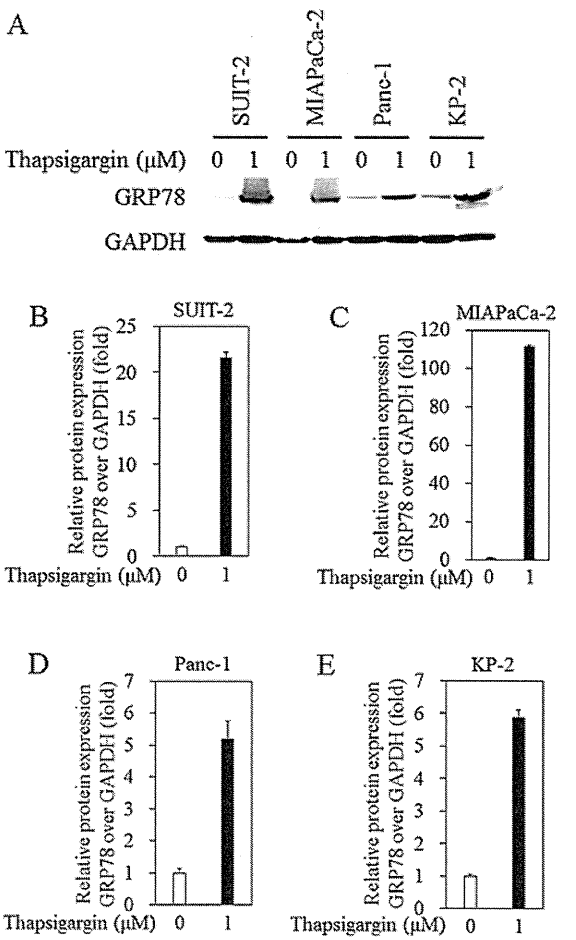

Figure 2. Effects of thapsigargin on glucose-regulated protein 78 (GRP78/Bip) expression in human pancreatic cancer cell lines. (A) Western blot analyses of GRP78 and GAPDH in SUIT-2, MIAPaCa-2, Panc-1 and KP-2 cells treated with or without 1 μM thapsigargin for 24 h. GRP78/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ratios from 3 independent experiments were measured using ImageJ software in (B) SUIT-2, (C) MIAPaCa-2, (D) Panc-1 and (E) KP-2 cells, respectively.

Treatment of 1 μM thapsigargin for 24 h led to the upregulation of GRP78 expression at the protein level [21.5±0.7 vs. 1±0.1 (in untreated control), n=3, p=0.00015; 111.5±1.0 vs. 1±0.12, n=3, p=0.000010; 5.2±0.57 vs. 1±0.1, n=3, p=0.0023; and 5.9±0.2 vs. 1±0.1, n=3, p=0.00013, respectively, in the SUIT-2, MIAPaCa-2, Panc-1 and KP-2 cells]. In the MIAPaCa-2, cells GRP78 expression was more strongly induced than in the other three cell lines.

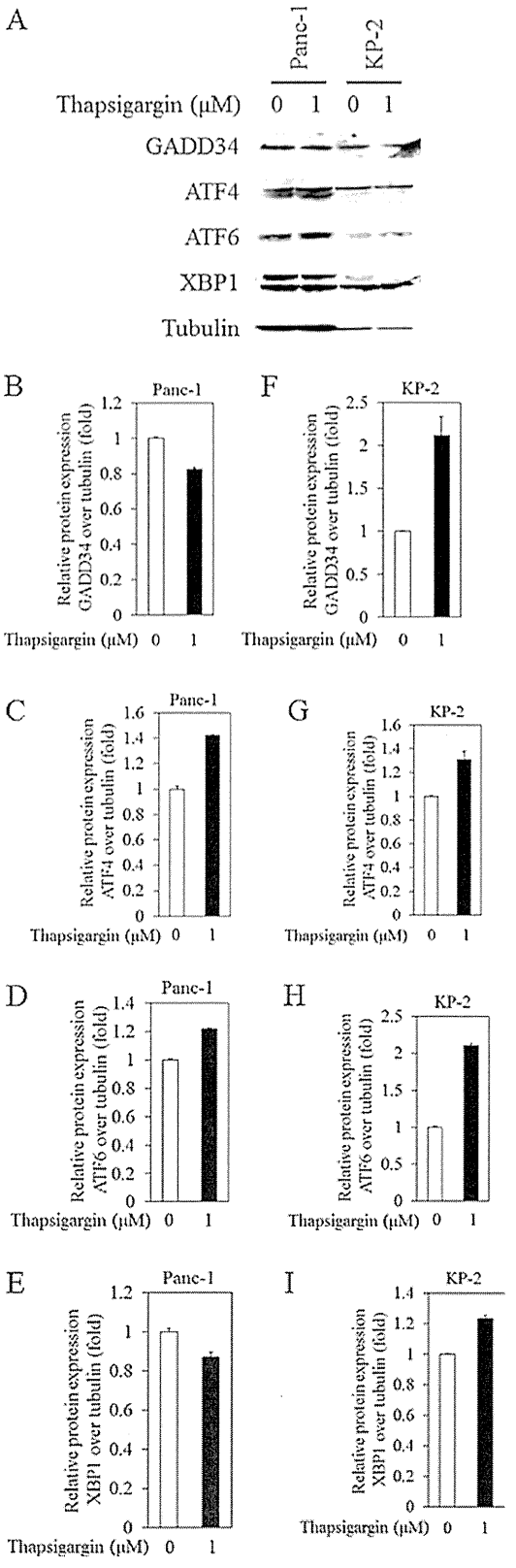

Figure 3. Effects of thapsigargin on growth arrest and DNA damage gene 34 (GADD34), activating transcription factor 4 (ATF4), ATF6 and X-box-binding protein 1 (XBP1) expression in human pancreatic cancer cell lines Panc-1 and KP-2. (A) Western blot analyses of GADD34, ATF4, ATF6, XBP1 and tubulin in Panc-1 and KP-2 cells treated with or without 1 μM thapsigargin for 24 h. (B) The ratios of GADD34 over tubulin, (C) ATF4 over tubulin, (D) ATF6 over tubulin and (E) XBP1 over tubulin in Panc-1 cells were measured using ImageJ software. (F) The ratios of GADD34 over tubulin, (G) ATF4 over tubulin, (H) ATF6 over tubulin and (I) XBP1 over tubulin in KP-2 cells were also measured using ImageJ software.

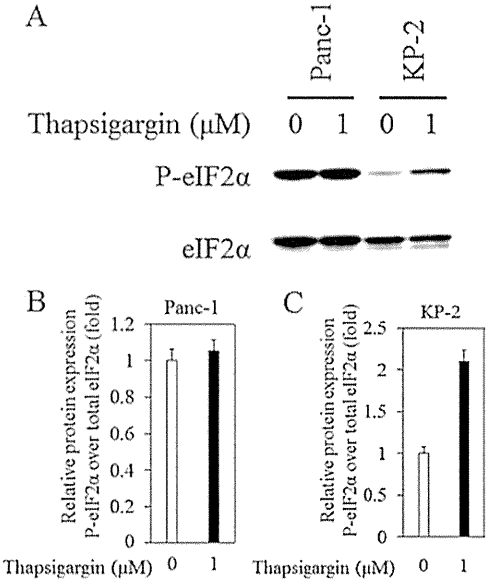

Figure 4. Effects of thapsigargin on the phosphorylation of Ser51-eIF2α (p-eIF2α) in human pancreatic cancer cell lines Panc-1 and KP-2. (A) Western blot analyses of p-eIF2α and total eIF2α in Panc-1 and KP-2 cells treated with or without 1 μM thapsigargin for 24 h. The ratios of p-eIF2α over total eIF2α were measured using ImageJ software in (B) Panc-1 and (C) KP-2 cells.

Effects of thapsigargin on GADD34, ATF4, ATF6 and XBP1 protein expression levels in the human pancreatic cancer cell lines. We examined the protein expression of ER stress signaling-associated molecules in the human pancreatic cell lines treated with or without thapsigargin. The results for the Panc-1 and KP-2 cells are shown in Fig. 3. In the Panc-1 cells, ATF4 and ATF6 expression was upregulated in the presence of 1 μM thapsigargin [1.4±0.010 vs. 1±0.023 (in untreated control), n=3, p=0.000089; and 1.2±0.0027 vs. 1±0.010, n=3, p=0.00019, respectively] (Fig. 3A, C and D. In the Panc-1 cells, GADD34 and XBP1 expression at the protein level was down-regulated in the presence of 1 μM thapsigargin [0.82±0.012 vs. 1±0.0076 (in untreated control), n=3, p=0.0000414; and 0.87±0.024 vs. 1±0.019, n=3, p=0.0012, respectively] (Fig. 3A, B and E).

On the other hand, in KP-2 cells, the protein expression levels of GADD34, ATF4, ATF6 and XBP1 were upregulated in the presence of 1 μM thapsigargin [2.1±0.22 vs. 1±0.012 (in untreated control), n=3, p=0.0063; 1.3±0.073 vs. 1±0.0062, n=3, p=0.0088; 2.1±0.022 vs. 1±0.014, n=3, p=0.0000008; and 1.2±0.019 vs. 1±0.0063, n=3, p=0.00043, respectively] (Fig. 3A and F-I).

XBP1 was also upregulated in the presence of 1 μM thapsigargin in both SUIT-2 and MIAPaCa-2 cells, yet we did not observe any enhancement of GADD34, ATF4 or ATF6 by thapsigargin (data not shown).

Effects of thapsigargin on the phosphorylation of eIF2α in the human pancreatic cancer cell lines. We also examined the phosphorylation status of eIF2α to understand how thapsigargin affects ER stress signaling in Panc-1 and KP-2 cells (Fig. 4A). In Panc-1 cells, phosphorylation of Ser51-eIF2α in the presence of thapsigargin tended to increase, compared with that in the absence of thapsigargin (Fig. 4B; 1.1±0.059

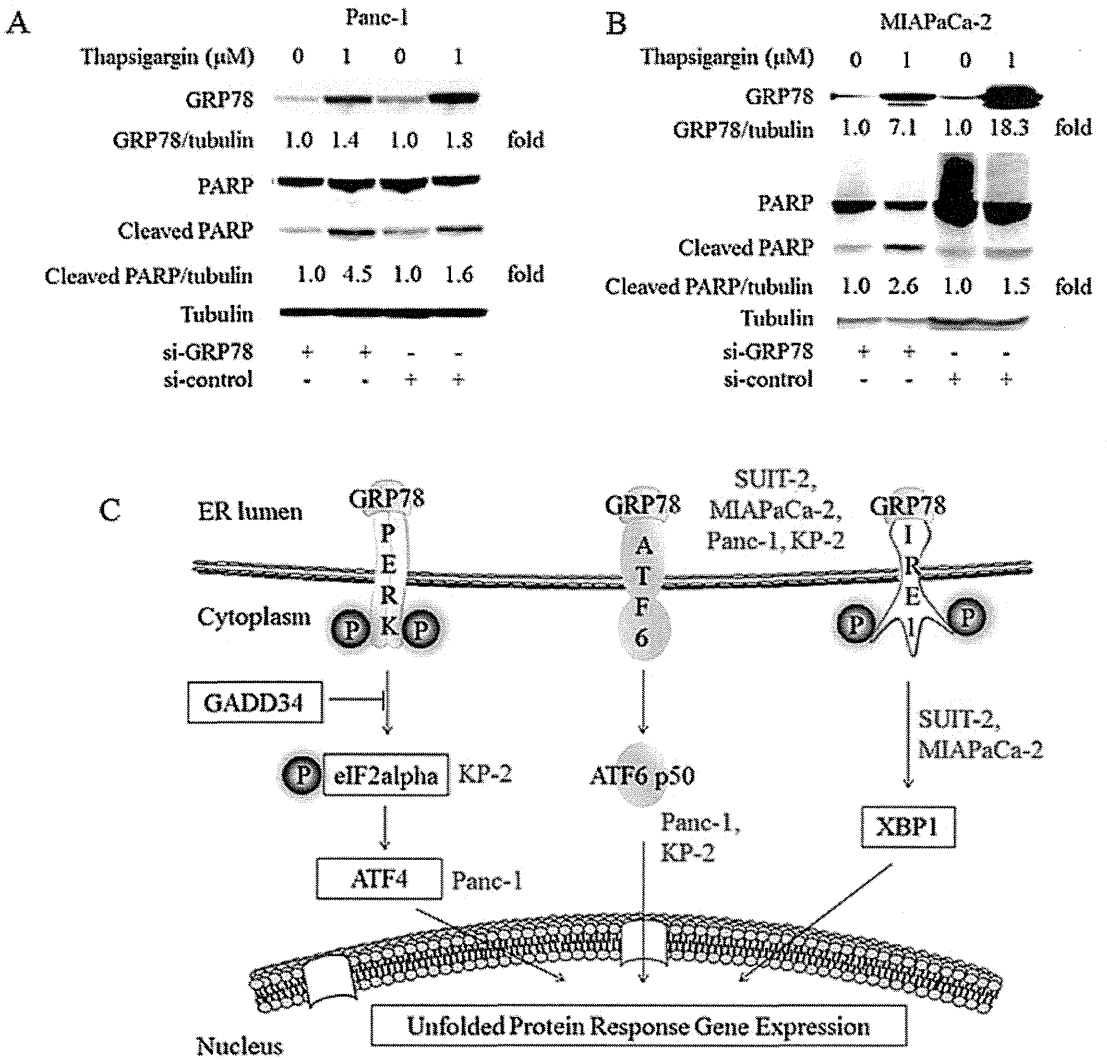

Figure 5. Knockdown of endogenous glucose-regulated protein 78 (GRP78/Bip) by siRNA enhances poly(ADP-ribose) polymerase (PARP) cleavage in pancreatic cancer cells. Western blot analyses of GRP78 and tubulin in (A) Panc-1 and (B) MIAPaCa-2 cells treated with or without 1 μM thapsigargin for 24 h. Cell lysates were analyzed for GRP78, PARP and tubulin expression using specific antibodies. Bands were analyzed using ImageJ software. (C) Schematic presentation of endoplasmic reticulum stress (ER) pathways in human pancreatic cancer cell lines.

vs. 1±0.064, n=3, p=0.17). In the KP-2 cells, significant phosphorylation of Ser51-eIF2α in the presence of thapsigargin was observed when compared with that in the absence of thapsigargin (Fig. 4C; 2.1±0.14 vs. 1±0.075, n=3, p=0.00050).

Knockdown of endogenous GRP78 enhances PARP cleavage in the pancreatic cancer cells. We confirmed that the expression of GRP78 at the protein level was upregulated in all four human pancreatic cancer cell lines tested, yet other molecules downstream of GRP78 reported to be involved in ER stress were expressed at variable levels depending on the individual cell line. Thus, we focused our examination on GRP78. Our previous study (13) demonstrated that blocking of GRP78 induction led to PARP cleavage in hepatocyte apoptosis. We investigated the effect of knockdown of GRP78 by siRNA on PARP cleavage in pancreatic cancer cells treated with thapsigargin (Fig. 5A and B).

GRP78 expression was significantly inhibited by transfection with si-GRP78 in the presence of thapsigargin, compared with that with si-control [1.4±0.040 vs. 1.8±0.040, n=3, p=0.00014; and 7.1±0.24 vs. 18.3±0.37, n=3, p=0.0000038, respectively, in Panc-1 (Fig. 5A) and MIAPaCa-2 cells (Fig. 5B)].

PARP cleavage was significantly enhanced by transfection with si-GRP78 in the presence of thapsigargin, compared with that with si-control [4.5±0.045 vs. 1.6±0.085, n=3, p=0.00000080; and 2.6±0.13 vs. 1.5±0.047, n=3, p=0.00016, respectively, in Panc-1 (Fig. 5A) and MIAPaCa-2 cells (Fig. 5B)].

Discussion

In the present study, we demonstrated that i) human pancreatic cancer cell lines expressed GRP78; ii) ER stress induced by thapsigargin upregulated protein levels of GRP78 in human pancreatic cancer cell lines; iii) ER stress-related molecules downstream of GRP78 were expressed at various levels according to the respective human pancreatic cancer

cell lines; and iv) finally, knockdown of GRP78 by siRNA enhanced PARP cleavage in the human pancreatic cancer cell lines. To our knowledge, this is the first report to show the association between GRP78 and PARP cleavage in pancreatic cancer cell lines treated with thapsigargin.

Our results that human pancreatic cancer cell lines express GRP78 supported a previous study (21) showing that the heat shock proteins HSP90 and GRP78 are constitutively expressed in gastrointestinal cancers including human pancreatic cancer. We also observed that ER stress induced by thapsigargin upregulated protein levels of GRP78 in human pancreatic cancer cell lines. However, ER stress-related molecules downstream of GRP78, such as GADD34, ATF4, ATF6, XBP1 and phospho-eIF2α were not constitutively increased by thapsigargin, but rather were dependent on individual cell lines (Figs. 2-4). These results suggest that GRP78 may have an impact on many different cellular processes and survival of pancreatic cancer and that ER stress signaling downstream of GRP78 can be expected to be disturbed in pancreatic cancer.

It was reported that an increase in GRP78 expression in pancreatic cancer cells may enhance and account for the altered sensitivity of pancreatic cancer to chemotherapeutic agents (21). UPR regulator GRP78 is an anti-apoptotic protein that is usually upregulated in cancer and plays a critical role in chemoresistance in various types of cancers (22). Recently it was also reported that UPR induction in tumor endothelial cells under an acidic pH condition is related to chemoresistance and may contribute to therapeutic failure in response to chemotherapy (23). It was also reported that GRP78 is overexpressed in malignant cells resistant to therapy (24).

PARP is one of the proteins processed by post-translational modification and plays a crucial role in many processes, including DNA repair and cell death (25). During apoptosis, caspases cause PARP cleavage and inactivation, in which PARP proteolysis produces an 89-kDa C-terminal fragment and a 24-kDa N-terminal (25). We observed that in the presence of thapsigargin, knockdown of GRP78 enhanced PARP cleavage in human pancreatic cancer cells Panc-1 as well as MIAPaCa-2. Wang *et al* reported that suppression of GRP78 by taxol and vinblastine potentiated the activation of JNK phosphorylation, caspase-7 and PARP cleavage in the human breast cancer cell line MCF-7 (26). The Hsp90 inhibitor SNX-2112 also induced PARP cleavage as well as the reduction in GRP78 expression in the multidrug-resistant human chronic myeloid leukemia K562/ADR cell line (27).

Collectively, our results suggest that both GRP78 and PARP may have key roles in the chemoresistance of pancreatic cancer (28) and that GRP78 may be one of the valid targets against chemoresistance (24). In conclusion, GRP78 is a potential therapeutic target for 'difficult-to-treat' pancreatic cancer, in which ER stress signaling in part falls into disorder.

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References

1. Lennon AM, Wolfgang CL, Canto MI, *et al*: TThe early detection of pancreatic cancer: what will it take to diagnose and treat curable pancreatic neoplasia? Cancer Res 74: 3381-3389, 2014.
2. Kuroda T, Kumagi T, Yokota T, *et al*: Improvement of long-term outcomes in pancreatic cancer and its associated factors within the gemcitabine era: a collaborative retrospective multicenter clinical review of 1,082 patients. BMC Gastroenterol 13: 134, 2013.
3. Okitsu K, Kanda T, Imazeki F, Yonemitsu Y, Ray RB, Chang C and Yokosuka O: Involvement of interleukin-6 and androgen receptor signaling in pancreatic cancer. Genes Cancer 1: 859-867, 2010.
4. Miyazaki M, Yoshitomi H, Shimizu H, *et al*: Repeat pancreatectomy for pancreatic ductal cancer recurrence in the remnant pancreas after initial pancreatectomy: is it worthwhile? Surgery 155: 58-66, 2014.
5. Sudo K, Ishihara T, Hirata N, *et al*: Randomized controlled study of gemcitabine plus S-1 combination chemotherapy versus gemcitabine for unresectable pancreatic cancer. Cancer Chemother Pharmacol 73: 389-396, 2014.
6. Mahadevan NR, Rodvold J, Sepulveda H, Rossi S, Drew AF and Zanetti M: Transmission of endoplasmic reticulum stress and pro-inflammation from tumor cells to myeloid cells. Proc Natl Acad Sci USA 108: 6561-6566, 2011.
7. Schroder M and Kaufman RJ: ER stress and the unfolded protein response. Mutat Res 569: 29-63, 2005.
8. Hamanaka RB, Bennett BS, Cullinan SB and Diehl JA: PERK and GCN2 contribute to eIF2α phosphorylation and cell cycle arrest after activation of the unfolded protein response pathway. Mol Biol Cell 16: 5493-5501, 2005.
9. Xu W, Liu L, Charles IG and Moncada S: Nitric oxide induces coupling of mitochondrial signalling with the endoplasmic reticulum stress response. Nat Cell Biol 6: 1129-1134, 2004.
10. Ma Y and Hendershot LM: The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 4: 966-977, 2004.
11. Jiang X, Kanda T, Nakamoto S, Miyamura T, Wu S and Yokosuka O: Involvement of androgen receptor and glucose-regulated protein 78 kDa in human hepatocarcinogenesis. Exp Cell Res 323: 326-336, 2014.
12. Shuda M, Kondoh N, Imazeki N, *et al*: Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. J Hepatol 38: 605-614, 2003.
13. Jiang X, Kanda T, Tanaka T, Wu S, Nakamoto S, Imazeki F and Yokosuka O: Lipopolysaccharide blocks induction of unfolded protein response in human hepatoma cell lines. Immunol Lett 152: 8-15, 2013.
14. Martinon F and Glimcher LH: Regulation of innate immunity by signaling pathways emerging from the endoplasmic reticulum. Curr Opin Immunol 23: 35-40, 2011.
15. Kanda T, Jiang X and Yokosuka O: Androgen receptor signaling in hepatocellular carcinoma and pancreatic cancers. World J Gastroenterol 20: 9229-9236, 2014.
16. Nawrocki ST, Carew JS, Dunner K Jr, *et al*: Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. Cancer Res 65: 11510-11519, 2005.
17. Mujumdar N, Banerjee S, Chen Z, *et al*: Triptolide activates unfolded protein response leading to chronic ER stress in pancreatic cancer cells. Am J Physiol Gastrointest Liver Physiol 306: G1011-G1020, 2014.
18. Fu Y and Lee AS: Glucose regulated proteins in cancer progression, drug resistance and immunotherapy. Cancer Biol Ther 5: 741-744, 2006.
19. Lee E, Nichols P, Spicer D, Groshen S, Yu MC and Lee AS: GRP78 as a novel predictor of responsiveness to chemotherapy in breast cancer. Cancer Res 66: 7849-7853, 2006.
20. Kanda T, Yokosuka O, Imazeki F, Arai M and Saisho H: Enhanced sensitivity of human hepatoma cells to 5-fluorouracil by small interfering RNA targeting *Bcl-2*. DNA Cell Biol 24: 805-809, 2005.
21. Ehrenfried JA, Herron BE, Townsend CM Jr and Evers BM: Heat shock proteins are differentially expressed in human gastrointestinal cancers. Surg Oncol 4: 197-203, 1995.
22. Tsai HY, Yang YF, Wu AT, *et al*: Endoplasmic reticulum ribosome-binding protein 1 (RRBP1) overexpression is frequently found in lung cancer patients and alleviates intracellular stress-induced apoptosis through the enhancement of GRP78. Oncogene 32: 4921-4931, 2013.

23. Visioli F, Wang Y, Alam GN, Ning Y, Rados PV, Nör JE and Polverini PJ: Glucose-regulated protein 78 (Grp78) confers chemoresistance to tumor endothelial cells under acidic stress. PLoS One 9: e101053, 2014.
24. Roller C and Maddalo D: The molecular chaperone GRP78/BiP in the development of chemoresistance: mechanism and possible treatment. Front Pharmacol 4: 10, 2013.
25. Soldani C and Scovassi AI: Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis 7: 321-328, 2002.
26. Wang J, Yin Y, Hua H, *et al*: Blockade of GRP78 sensitizes breast cancer cells to microtubules-interfering agents that induce the unfolded protein response. J Cell Mol Med 13: 3888-3897, 2009.
27. Wang R, Shao F, Liu Z, *et al*: The Hsp90 inhibitor SNX-2112, induces apoptosis in multidrug resistant K562/ADR cells through suppression of Akt/NF-κB and disruption of mitochondria-dependent pathways. Chem Biol Interact 205: 1-10, 2013.
28. Lei Y, Henderson BR, Emmanuel C, Harnett PR and Defazio A: Inhibition of ANKRD1 sensitizes human ovarian cancer cells to endoplasmic reticulum stress-induced apoptosis. Oncogene: Feb 17, 2014 (Epub ahead of print).

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Research Article

Involvement of androgen receptor and glucose-regulated protein 78 kDa in human hepatocarcinogenesis

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ABSTRACT

Previous studies demonstrated that androgen receptor (AR) is expressed in human hepatocellular carcinoma (HCC), one of the male-dominant diseases. Glucose-regulated protein 78 kDa (GRP78/Bip), which has a role in cancer development, is one of the androgen response genes in prostate cell lines. The aim of this study was to investigate the impact of AR on endoplasmic reticulum (ER)-stress signaling in human hepatoma. AR and GRP78 expressions were examined in human liver tissue panels. Human hepatoma cells stably expressing short hairpin RNA targeting AR and cells over-expressing AR were generated. The expressions of ER-stress molecules and AR were measured by real-time RT-PCR and Western blotting. The effect of AR on ER-stress responsive gene expression was examined by reporter assay. Strong positive correlation between AR mRNA and GRP78 mRNA was observed in stage I/II-HCCs. AR enhanced ER-stress responsive element activities and GRP78 expression, and regulated ER-stress response in hepatocytes. Sorafenib strongly induced significant apoptosis in HepG2 cells by the inhibition of AR and inhibition of the downstream GRP78. AR seems a co-regulator of GRP78 especially in earlier-stage HCC. AR plays a critical role in controlling ER-stress, providing new therapeutic options against HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer, which is the fifth and seventh most common cancer in men and women, respectively, worldwide [1]. Most cases of HCC are associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections [1,2]. Liver resection, transplantation, radiofrequency ablation and transcatheter arterial chemoembolization are possible modalities for improvement of the prognosis of HCC patients [3]. One-third of HCC patients undergoing surgical resection reportedly have recurrence [4]. Modalities other than repeated hepatectomy, liver transplantation and systemic chemotherapy using molecular target agents such as sorafenib, an oral multiple kinase inhibitor of vascular endothelial growth factor receptor (VEGF-R), platelet-derived growth factor receptor and Raf-kinase (effector of Ras) are needed for recurrent HCC [5]. Understanding

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the molecular pathways of cell growth and death accompanying human HCC would help to improve diagnosis and prognosis, and lead to new therapeutic options.

Gender difference has long been considered a unique feature of human HCC, a male-dominant disease [6]. In fact, virtually all epidemiological studies have shown that males are dominant in hepatocarcinogenesis [7]. Androgen receptor (AR) is a ligand-dependent transcription factor belonging to the nuclear receptor superfamily [8,9]. AR pathways are involved in sexual development and in the development of several cancers [10,11]. Androgen binds to AR, a member of the intracellular receptor family of transcriptional regulators, which in turn associates with genomic androgen response elements (AREs) [12,13]. AR responsive genes (ARGs) include VEGF, transforming growth factor-β1 (TGF-β1) and glucose-regulated protein 78 kDa (GRP78/Bip), all with roles in cancer development [13–15]. ARs exist in normal liver tissues as well as in human HCC tissues [16]. Further, we and others have also demonstrated that AR-mediated signaling is associated with HBV- and HCV-related hepatocarcinogenesis [17,18].

Endoplasmic reticulum (ER)-stress pathway could be involved in hepatocarcinogenesis [19]. GRP78 is a key component of the unfolded protein response (UPR) and promotes cell survival under ER-stress [20,21]. GRP78 functions as an ER chaperone and induces proper folding and assembly of other polypeptides, leading to the formation of functional proteins, retention of unassembled precursors to ER, targeting of misfolded protein for degradation, ER Ca^{2+} binding, and the regulation of transmembrane ER-stress inducers [22–24]. Three distinct signaling pathways comprise the mammalian UPR and are initiated by the ER transmembrane sensors inositol-requiring enzyme 1 (IRE1), protein kinase RNA activated-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [25], which are associated with the status of GRP78 expression. Two transmembrane ER kinases, IRE1α and IRE1β, target the transcription factor X-box-binding protein 1 (XBP1) [26,27]. IRE1 catalyzes a unique splicing event that removes 26 nucleotides from XBP1 mRNA, and this is followed by a relegation that alters the XBP1 open reading frame [27,28]. Activated PERK phosphorylates the alpha subunit of eukaryotic initiation factor-2 (eIF2α), effectively down-regulating protein synthesis [29]. Proteolytic processing of ATF6 yields an active transcription factor [30,31] that up-regulates the expression of ER resident proteins, including chaperones and ER-associated degradation (ERAD) components [32]. ATF6 and XBP1 are both implicated in the UPR-target gene expression.

In the present study, we examined the effects of the knockdown of AR on ER-stress pathways and on apoptotic cell death in human hepatoma cells. We found that AR knockdown suppressed ER-stress proteins located down-stream of GRP78, as well as GRP78. We also found that GRP78 expression was dependent on AR expression in the early clinical stage of human HCC and that knockdown of AR and GRP78 enhanced apoptosis in human hepatoma cell lines. Although AR and ER-stress pathways are involved in hepatic cell apoptosis [33,34], their interaction is believed to play an important role in human HCC development.

Materials and methods

Cell culture and reagents

The human hepatoma cell lines HepG2 and Huh7 and immortalized human hepatocyte (IHH) [18] were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL of penicillin G and 100 μg/mL of streptomycin at 37 °C in a 5%–CO_2 incubator. Plasmid expressing human AR protein (pSG5-AR) [35] and AR-response element-directed luciferase reporter plasmid (pARE4-luc) were kindly provided by Prof. Chawnshang Chang (University of Rochester, NY, USA) [12], and ER-stress response element (ERSE)-directed luciferase reporter plasmid (pERSE-luc) was purchased from Qiagen (Hilden, Germany). Plasmids AR-shRNA (shAR), STAT3-shRNA (shSTAT3) and control-shRNA (shC) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), corresponding to their respective targets, human AR, human STAT3, and an unrelated gene as negative control, respectively. 5α-androstan-17β-ol-3-one (DHT) and 5-aza-2′-deoxycytidine (5-aza-dC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorafenib and lipopolysaccharide (LPS) were purchased from Cayman (Ann Arbor, MI, USA) and Imgenex (San Diego, CA, USA), respectively. The other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Clinical HCC specimens and quantitative RT-PCR

A total of 23 primary HCC and 8 non-HCC (chronic hepatitis or cirrhosis) cDNA samples were purchased from OriGene (Rockville, MD, USA). All samples were collected under IRB-approved protocols by OriGene: http://www.origene.com/tissue/tissue_qc.aspx. OriGene declares that the proper collection and use of human biospecimens begins with complete protection for the rights and privacy of the individual. All human subjects are fully informed and are explicitly asked for their consent to future research use of their samples, even in cases where such uses are unknown at the time. The samples considered non-HCC were free of cancer cells on the basis of pathologic examination. Information of these samples was available from OriGene: http://www.origene.com/qPCR/Tissue-qPCR-Arrays.aspx. Quantitative RT-PCR (qRT-PCR) was performed using primers specific for AR, GRP78, and β-actin [sense primer (5′–CAGCC-ATGTACGTTGCTATCCAGG-3′) and antisense primer (5′–AGGTCCAGA-CGCAGGATGGCATG-3′)] with Power SYBR Green PCR Master Mix (Applied Biosystems, Forester City, CA, USA). Quantitative expression data were acquired using the ABI Prism 7300 system (Applied Biosystems), and results were analyzed by the ddCt method [33].

Transfection of shRNA and siRNA

To stably establish AR-knockdown cells (HepG2-shAR or Huh7-shAR), STAT3-knockdown cells (HepG2-shSTAT3) and control cells (HepG2-shC or Huh7-shC), plasmids AR-shRNA (shAR), STAT3-shRNA (shSTAT3) and control-shRNA (shC), respectively, were used. Approximately 0.5×10^5 HepG2 cells were placed in 35 mm-plates (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection [36]. Cells were transfected with 0.3 μg of each plasmid using Effectene Transfection Reagent (Qiagen) following the manufacturer's protocol. After 48-h transfection, cells were split and treated with 1 μg/mL puromycin for selection of antibiotic-resistant colonies over a 2-week period. We then examined for the expression of endogenous AR or STAT3. Similar to HepG2 cells, we also used Huh7-shAR and Huh7-shC.

To further validate the effects of AR-knockdown on apoptosis, we also examined AR- and GRP78-knockdown by small-interfering RNA (siRNA). Cells were transfected with 50 nM each

of AR siRNA (si-AR), GRP78 siRNA (si-GRP78), or control siRNA (si-control), using Effectene Transfection Reagent (Qiagen) according to the manufacturer's protocol [36]. After 24 h of transfection, cells were treated with 5 μM sorafenib and/or 0.5 μM 5-aza-dC for 24 h.

RNA extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen), and then 5 μg of RNA was reverse-transcribed using PrimeScript RT reagent (Perfect Real Time; Takara, Otsu, Japan). PCR amplification was performed on cDNA templates using primers specific for GRP78 [sense primer (5′- GCCTGTATTTCTAGACCTGCC -3′) and antisense primer (5′–TTCATCTTGCCAGCCAGTTG-3′)], CHOP [sense primer (5′–TTAAGTCTAAGGCACTGAGCGTATC-3′) and antisense primer (5′–TGCTTTCAGGTGTGGTGATG-3′)], GADD34 [sense primer (5′–ATGTATGGTGAGCGAGAGGC-3′) and antisense primer (5′–GCAGTGTCCTTATCAGAAGGC-3′)], and spliced XBP1 mRNA (XBP1s) [sense primer (5′–AATGAAGTGAGGCCAGTGG-3′) and antisense primer (5′–TCAATACCGCCAGAATCCATG-3′)]. The primers for AR and GAPDH were chosen as described previously [18]. For RNA quantification, real-time PCR was performed using SYBR Green I in the StepOne real-time PCR system (Applied Biosystems). Data analysis was based on the ddCt method. Expressions of the genes of interest were normalized to the expression of GAPDH.

Western blot analysis

Cells were lysed in sodium dodecyl sulfate sample buffer and processed for Western blot analysis as previously described [18]. Briefly, proteins were subjected to electrophoresis on 5–20% polyacrylamide gels and transferred onto polyvinylidene difluoride membrane (ATTO, Tokyo, Japan). The membrane was probed with antibodies specific to GRP78, PERK, phospho-eIF2-α (Ser51), IRE1α, or eIF2-α (Cell Signaling, Boston, MA, USA), and XBP1, ATF6, AR and β-tubulin (Santa Cruz Biotechnology). After washing, the membrane was incubated with secondary horseradish peroxidase-conjugated antibodies. Signals were detected by enhanced chemiluminescence (GE Healthcare, Tokyo, Japan) and scanned by image analyzer [LAS-4000, Fuji Film, Tokyo, Japan, and Scion Image (http://scion-image.software.informer.com/)].

Luciferase assay

Cells in six-well plates were transfected with 0.2 μg of reporter plasmids using Effectene (Qiagen) following the manufacturer's instructions. Cells were lysed with reporter lysis buffer (Promega, Madison, WI, USA), and luciferase activity was determined by luminometer (Luminescencer-JNR II AB-2300; ATTO, Tokyo, Japan).

Detection of apoptotic cells

APOPercentage Apoptosis Assay (Biocolor, Belfast, Northern Ireland) was used to quantify apoptosis following the manufacturer's instructions. Transfer and exposure of phosphatidylserine to the exterior surface of the membrane have been linked to the onset of apoptosis. Phosphatidylserine transmembrane movement results in uptake of APOPercentage dye by apoptosis-committed cells. Purple-red stained cells were identified as apoptotic cells by light microscopy. Purple-red cells/300 cells were counted as previously described [33].

Statistical analysis

All experiments were repeated at least three times independently, and statistical analysis was performed using Student's t test with Microsoft Office software (Excel 2010; Microsoft, Redmond, WA, USA).

Results

AR and GRP78 mRNA expressions in clinical HCC specimens

First, we evaluated AR and GRP78 mRNA expressions from the 23 HCC tumor and 8 non-HCC samples. The expression levels of AR mRNA or GRP78 mRNA did not differ significantly between HCC and non-HCC (data not shown). Spearman's rank test showed positive correlation between the expressions of AR mRNA and GRP78 mRNA in total specimens ($P=0.00031$, Fig. 1A). Positive correlations between the expressions of AR mRNA and GRP78 mRNA in total specimens were observed in the 8 non-HCC and 23 HCC samples ($P=0.047$, and $P=0.032$, respectively, Fig. 1B and C). Thirteen stage I/II HCC samples showed strong positive correlation between the expressions of AR mRNA and GRP78 mRNA ($P=0.0044$, Fig. 1D), but 10 stage III/IV HCC samples showed no correlation between these expressions ($P=0.21$, Fig. 1E). These results suggested that the activation of GRP78 gene through AR could play a critical role in hepatocarcinogenesis in certain early-stage HCC patients.

Knockdown of endogenous AR decreased GRP78 expression in human hepatoma cells

To investigate the role of endogenous AR in ER-stress response, we knocked down endogenous AR in the human hepatoma cell line HepG2 using RNA interference. For the generation of stable cell lines, HepG2 cells were transfected with the plasmid DNA shAR, shSTAT3 or control shC, and puromycin-resistant cells were selected. Cell lysates were prepared for Western blot analysis to confirm the knockdown of each targeting protein using specific antibodies. We observed 95% inhibition of AR in HepG2-shAR as compared with HepG2-shC ($P<0.05$; $n=3$) (Fig. 2A) and 90% inhibition of STAT3 in HepG2-shSTAT3 as compared with HepG2-shC ($P<0.05$; $n=3$) (Fig. 2B).

We examined whether knockdown of AR modulates GRP78 expression at the mRNA level in HepG2 cells by real-time RT-PCR, and found 97% inhibition of the GRP78 mRNA level in HepG2-shAR as compared with HepG2-shC ($P<0.05$; $n=3$) (Fig. 2C). However, the GRP78 mRNA level in HepG2-shSTAT3 showed no inhibition as compared with HepG2-shC [*not significant* (*N.S.*); $n=3$] (Fig. 2C).

Next, we examined whether knockdown of AR modulates GRP78 expression at the protein level in HepG2 cells by Western blotting, and we observed 80% inhibition of GRP78 protein in HepG2-shAR as compared with HepG2-shC ($P<0.05$; $n=3$) (Fig. 2D). However, the GRP78 protein level in HepG2-shSTAT3

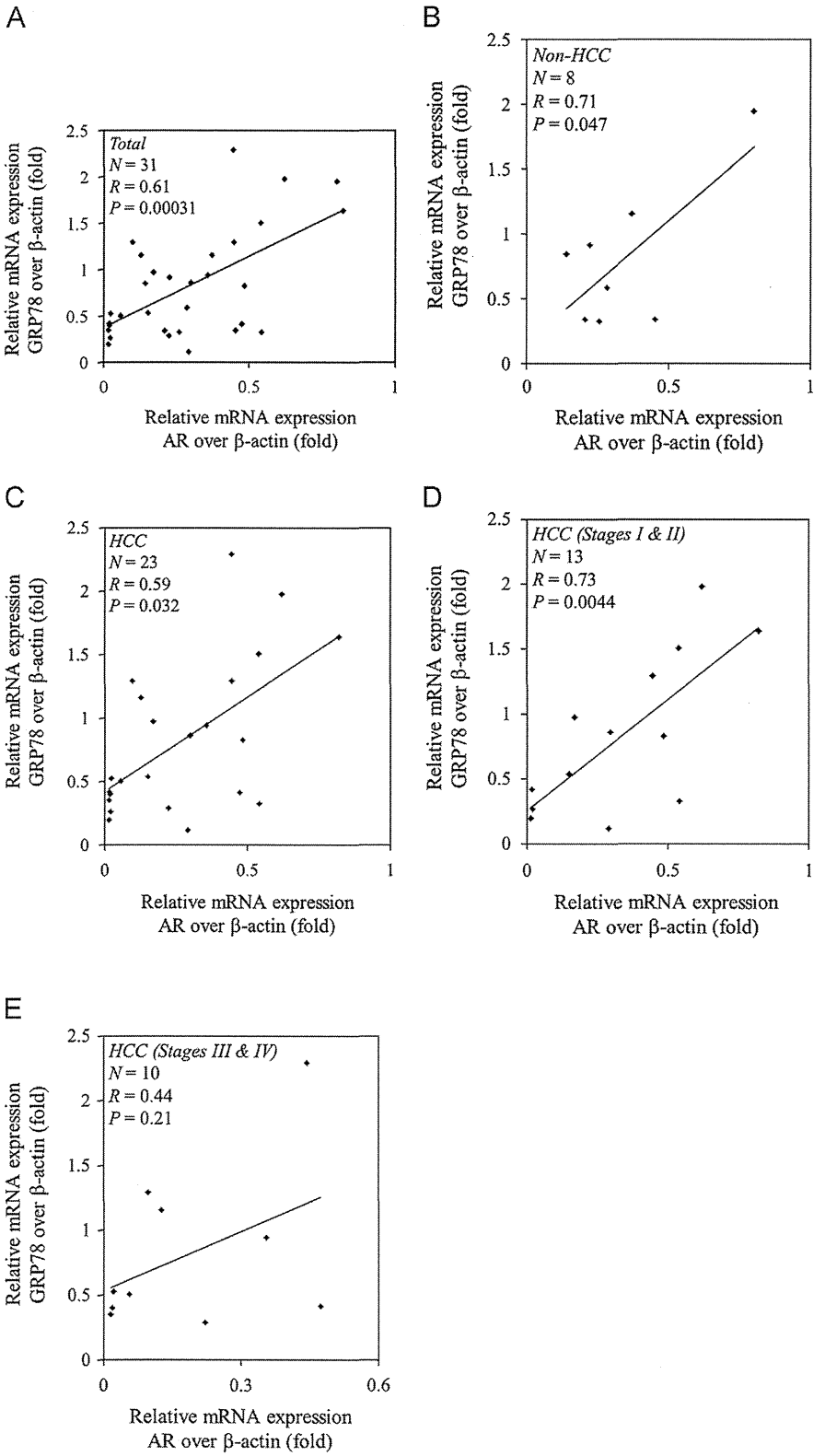

Fig. 1 – Expression levels of androgen receptor (AR) and glucose-regulated protein 78 kDa (GRP78) mRNA in hepatocellular carcinoma (HCC). (A) Total samples ($n=31$). (B) Non-HCC samples ($n=8$). (C) HCC samples ($n=23$). (D) Stage I/II HCC samples ($n=13$). (E) Stage III/IV HCC samples ($n=10$). Expression levels of AR and GRP78 mRNA from the clinical samples were measured by real-time PCR. β-actin was used for normalization.

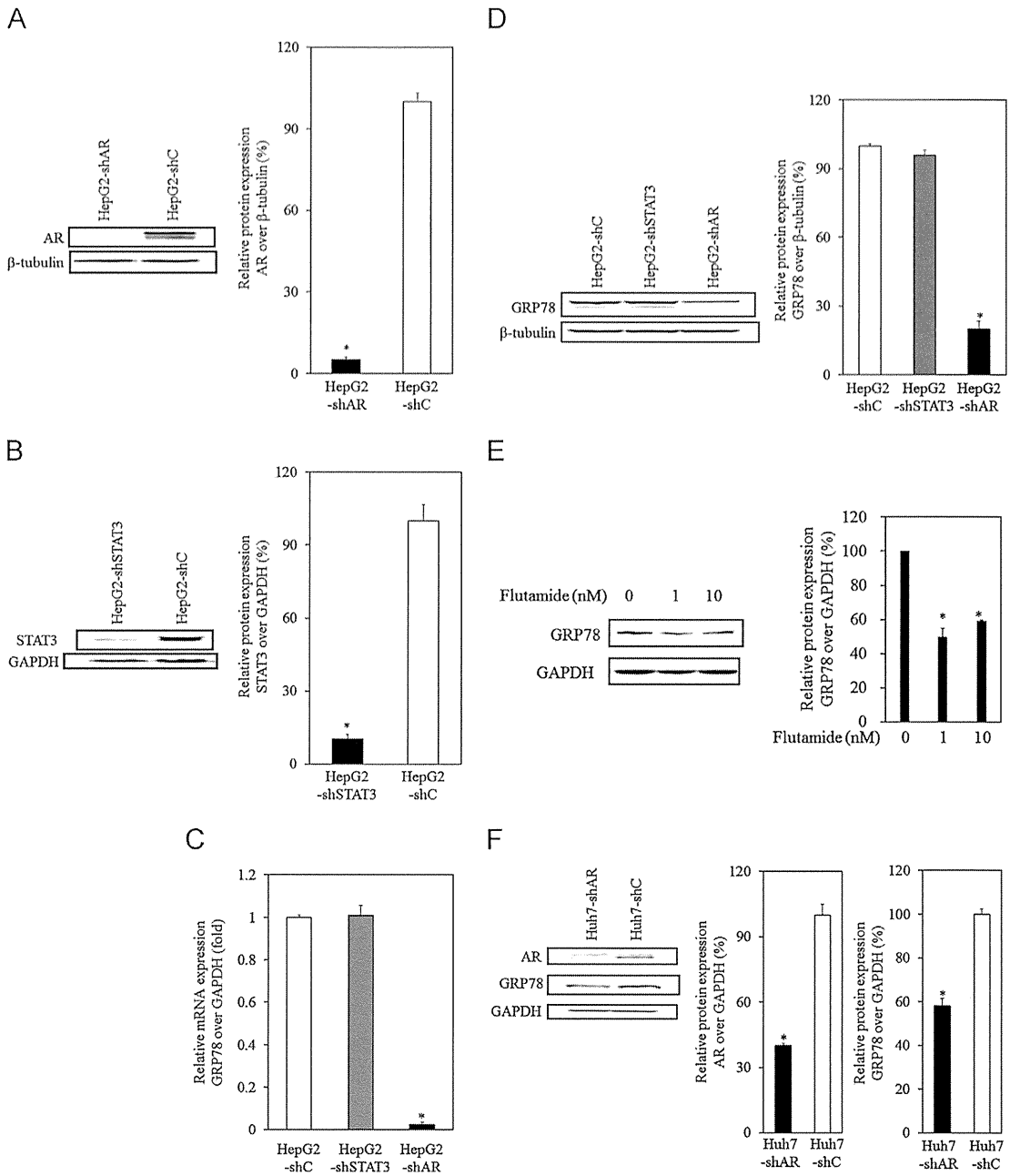

Fig. 2 – Knockdown of endogenous androgen receptor (AR) decreased glucose-regulated protein 78 kDa (GRP78) expression in human hepatoma cells. (A) Western blot analyses of AR and β-tubulin expression in HepG2-shAR and HepG2-shC cells. Densitometric analyses were performed using Scion Image (Scion). (B) Western blot analyses of STAT3 and GAPDH expression in HepG2-shSTAT3 and HepG2-shC cells. (C) Real-time polymerase chain reaction (PCR) analyses of GRP78 mRNA expression in HepG2-shC, HepG2-shSTAT3, and HepG2-shAR cells. GRP78 mRNA levels were normalized to GAPDH levels. (D) Western blot analyses of GRP78 expression in HepG2-shC, HepG2-shSTAT3, and HepG2-shAR cells. *$P<0.05$, compared with that in HepG2-shC. (E) Western blot analyses of GRP78 expression in HepG2 treated with flutamide (0, 1 or 10 nM) for 24 h. *$P<0.05$, compared with untreated control. (F) Western blot analyses of AR, GRP78 and GAPDH expression in Huh7-shAR and Huh7-shC cells. *$P<0.05$, compared with Huh7-shC. Data are mean values ± SD of three independent experiments.

showed no inhibition as compared with HepG2-shC (*N.S.*; $n=3$) (Fig. 2D).

We also examined whether flutamide, a non-steroidal anti-androgen drug, modulates GRP78 expression at the protein level in HepG2 cells by Western blotting. We observed 50 and 41% inhibition of the GRP78 protein level treated with 1 nM and 10 nM flutamide, respectively, as compared with untreated control (Fig. 2E).

Similarly, we also established Huh7-shAR and Huh7-shC. We observed 60% inhibition of AR in Huh7-shAR as compared with Huh7-shC ($P<0.05$; $n=3$) (Fig. 2F). Similar to HepG2, we also observed that knockdown of endogenous AR led to 42% inhibition

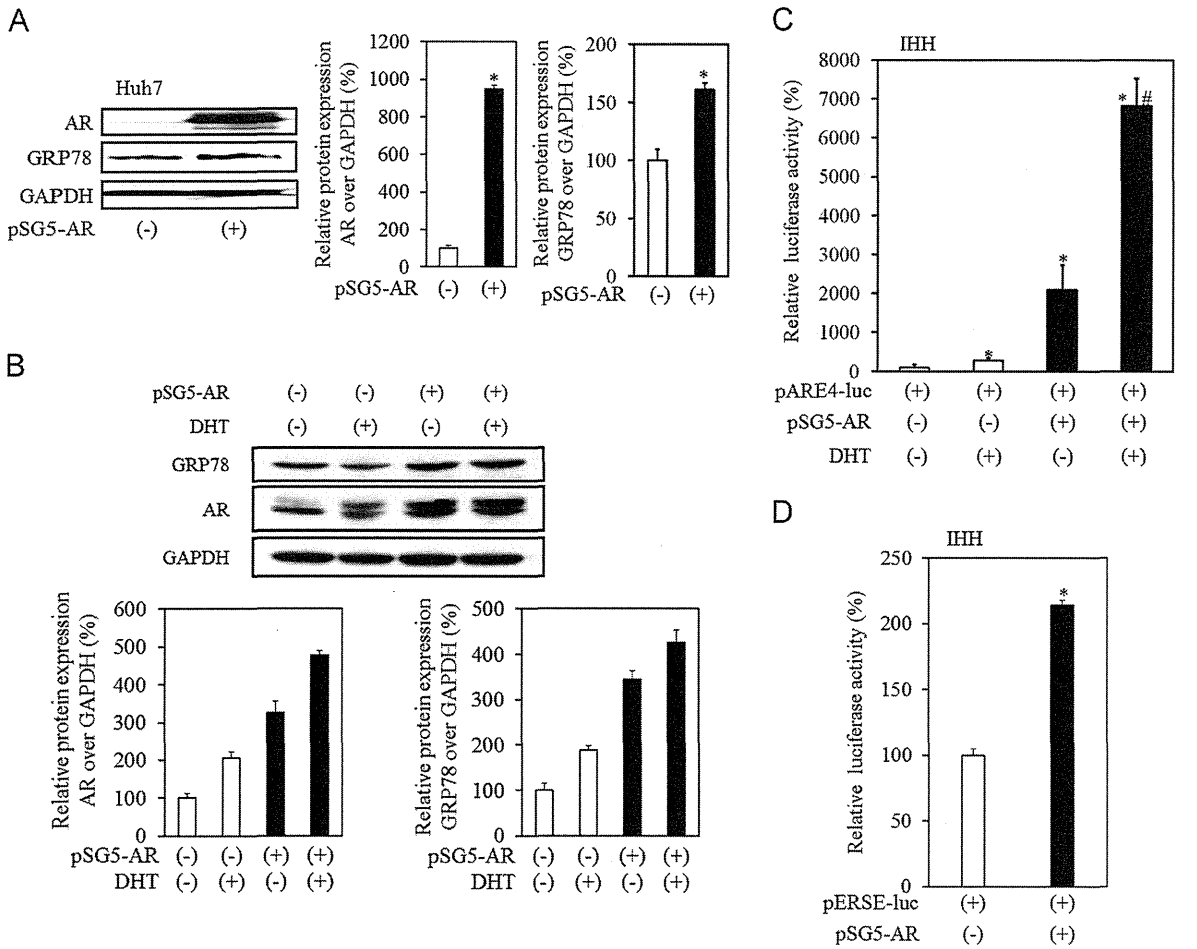

Fig. 3 – Overexpression of androgen receptor (AR) increased GRP78 expression and endoplasmic reticulum (ER)-stress responsive gene expressions in human hepatocytes. (A) Western blot analyses of AR, GRP78 and GAPDH expressions in Huh7 transiently transfected with pSG5-AR or vector-control. *$P<0.05$, compared with Huh7 transfected with vector-control. (B) Western blot analyses of AR, GRP78 and GAPDH expression in IHH transiently transfected with pSG5-AR or vector-control. (C) Overexpression of AR enhances androgen response element (ARE) activities in human hepatocyte IHH with or without 10 nM DHT. The relative luciferase activity of control in the absence of DHT was arbitrarily set at 100% for comparison. *$P<0.05$, compared with IHH transfected with vector-control and treated without DHT; #$P<0.05$, compared with IHH transfected with vector-control and treated with 10 nM DHT. (D) Overexpression of AR enhances ER-stress responsive element (ERSE) activities in human hepatocyte IHH. The relative luciferase activity of control in the absence of pSG5-AR was arbitrarily set at 100% for comparison. *$P<0.05$, compared with IHH transfected with vector-control. Luciferase activities are presented as an average from three independent experiments.

of GRP78 protein in Huh7-shAR as compared with Huh7-shC ($P<0.05$; $n=3$) (Fig. 2F). Together, knockdown of AR resulted in inhibition of GRP78 expression in human hepatoma cell lines.

Overexpression of AR increased ER-stress responsive gene expression in human hepatocytes

We also overexpressed AR in Huh7 and IHH cells, in which AR expressions were relatively lower than that of HepG2, to more conclusively validate the effect of AR on regulating GRP78 expression. In Huh7 transiently transfected with pSG5-AR, the expression of GRP78 was increased about 1.6-fold ($P<0.05$; $n=3$) (Fig. 3A). Similarly, in IHH transfected with pSG5-AR, the expression of GRP78 was increased about 1.9-fold ($P<0.05$; $n=3$) (Fig. 3B). Thus, overexpression of AR led to GRP78 induction in hepatocytes, supporting the previous observation in prostate cancer cells [15].

We observed that AR enhanced AR-responsive gene expression in IHH cells by luciferase assay (Fig. 3C) as well as GRP78 expression by Western blot analysis with or without DHT ($P<0.05$; $n=3$) (Fig. 3B). We also confirmed that overexpression of AR enhanced ERSE activities in IHH cells by luciferase assay (Fig. 3D). Moreover, the promoter region of GRP78 genes (MN_005347) contains at least five AR-binding sites (TGTACA, TCAACA, GGGACA, TGTTCT and AGAACA) (data not shown). These results suggested that AR could regulate ER-stress responsive gene expression as well as GRP78 expression in hepatocytes.

Effects of AR-knockdown on ER-stress pathway in HepG2 cells

UPR is an intracellular signaling pathway that relays signals from the ER lumen to activate target genes in the nucleus [27].

Activation of UPR signaling could be triggered by the dissociation of GRP78 from a class of novel ER transmembrane receptors: PERK, IRE1, and ATF6. To confirm the effects of the knockdown of AR on ER-stress responsive gene expression, we examined whether these three canonical branches of UPR were down-regulated in HepG2 after knockdown of AR by Western blot analysis. We observed that PERK, IRE1α and ATF6 expressions were significantly down-regulated (54%, 63% and 27% inhibition, respectively) in HepG2-shAR as compared to HepG2-shC ($P<0.05$; $n=3$) (Fig. 4A). We also observed that PERK, IRE1α and ATF6 expressions were significantly down-regulated (31%, 37% and 20% inhibition, respectively) in HepG2-shSTAT3 as compared to HepG2-shC ($P<0.05$; $n=3$) (Fig. 4A). However, the effect of AR knockdown on UPR was stronger than that of STAT3.

To unravel the effect of the down-regulation of IRE1 induced by knockdown of AR, we examined the expression of IRE1 downstream transcription factor XBP1, a component of the integrated stress response. Activation of the IRE1 branch can specifically be observed by splicing a 26-nucleotide intron from inactivated XBP1 mRNA (XBP1u), which results in generation of spliced XBP1 mRNA (XBP1s) that encodes the active transcription factor [26]. At the protein level, spliced XBP1 expression was also significantly down-regulated (85% inhibition) in HepG2-shAR as compared to HepG2-shC ($P<0.05$; $n=3$) (Fig. 4B). XBP1s mRNA was significantly down-regulated by about 93% in HepG2-shAR as compared to HepG2-shC ($P<0.05$; $n=3$) (Fig. 4C). A change of XBP1 expression in HepG2-shSTAT3 in comparison to HepG2-shC was not observed (*N.S.*; $n=3$) (Fig. 4B and C).

Phosphorylation of eIF2α involves the ER-resident kinase PERK. Next, we tested the expression of total eIF2α and phospho-eIF2α (Ser51). We found that the phosphorylated form of eIF2α was also slightly down-regulated in HepG2-shAR as compared to HepG2-shC or HepG2-shSTAT3 (Fig. 4B). GADD34 is induced by the PERK branch and serves as negative feedback mechanism that dephosphorylates eIF2α and restores protein translation [37]. GADD34 expression at protein levels was significantly down-regulated (65% inhibition) in HepG2-shAR as compared to HepG2-shC (Fig. 4B). We also confirmed that GADD34 mRNA was significantly down-regulated (75% inhibition) in HepG2-shAR as compared to HepG2-shC (Fig. 4D). Together, these results showed that AR signaling regulated the ER-stress response.

Knockdown of endogenous AR potentiated HepG2 cells susceptible to LPS- and sorafenib-induced apoptosis

We have also reported that GRP78 plays an important role in hepatic cell apoptosis associated with innate immune response and that LPS administration appeared to impair UPR in human hepatoma cells [33]. Next, we examined whether knockdown of endogenous AR made HepG2 susceptible to apoptosis induced by LPS. We observed an increase of LPS-induced apoptosis (67%) in HepG2-shAR, compared to that (47%) in HepG2-shC ($P<0.05$; $n=3$) after these cells were treated with 5 μg/mL LPS for 24 h (Fig. 5A).

Next, we examined the effects of sorafenib, a useful agent for inoperable HCC [5], with or without 5-aza-dC, which could also suppress cancer cell proliferation, on apoptosis of HepG2 cells transfected with si-AR and/or si-GRP78 or si-control (Fig. 5B). We observed that sorafenib with or without 5-aza-dC induced significant apoptosis in HepG2 cells transfected with the combination of si-AR and si-GRP78, compared with HepG2 transfected with si-control, si-AR, or si-GRP78. These siRNAs were validated by Western blotting (Fig. 5C and D).

Discussion

The present study showed that knockdown of AR led to the down-regulation of ER-stress markers in hepatocytes, supporting previous reports that GRP78 is one of the target genes of AR [15,38–40]. UPR is activated in human cancer including HCC [19], and HCC is a male-dominant disease [6,7]. AR mediates the pathophysiological effects of androgens, including sexual differentiation, prostate development, and cancer progression, by binding to genomic androgen response elements (AREs), which influence the transcription of AR target genes [13]. Our results indicated that cross-talk between AR and ER-stress response might play a critical role in certain types of HCC.

In prostate cancer, significant co-expression of GRP78, phosphorylated serine/threonine protein kinase Akt (pAKT), v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (HER2), HER3 and AR was observed [15]. It was also reported that up-regulated expression of AR and GRP78 expression in untreated prostate cancer predicts a less favorable outcome [15]. Bennett et al. [38] reported that AR signaling promotes temporary adaptation to ER-stress, and which in turn may contribute to evasion of prostate tumor cell death. AR could be related to the increased expression of prostate cancer-related survival markers such as heat shock protein 27 (HSP27), GRP78, clusterin (CLU), and cellular FLICE-like inhibitory protein (c-FLIP) in late-stage prostate cancer. Also, AR gene inhibition could be a therapeutic target in late-stage prostate cancer [39], although our present study showed that AR was associated with increased GRP78 in early-stage HCC. AR inclusions acquire GRP78 to ameliorate androgen-induced protein misfolding stress in embryonic stem cells [40]. The above studies indicated the possible association between AR and GRP78 in carcinogenesis and cancer development.

The STAT3 signaling pathway could participate in HCC genesis and development [41]. In the present study, knockdown of STAT3 did not inhibit GRP78 expression (Fig. 2D), but inhibited the expressions of PERK, IRE1α, and ATF6α weakly (Fig. 3). It was reported that STAT3 cross-talked with AR-signaling in hepatocytes [18]. Further studies will be needed regarding this point.

In the present study, we observed that knockdown of AR led to down-regulation of GRP78 and also potentiated the susceptibility of hepatoma cells to apoptosis induced by LPS. The transcription factor CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP; also known as DDIT3/GADD153) mRNA expression in HepG2-shAR was similar to that of HepG2-shC (data not shown), although CHOP was reported to be involved in apoptosis of the liver [42]. The inherent roles and anti-apoptotic capabilities of GRP78 indicate a potential role in cancer progression. Suppression of the GRP78 level results in inhibition of tumor growth in fibrosarcoma [43] and in apoptosis in hepatoma cell lines [44]. Elevation of GRP78 in the microenvironment of tumors due to nutrient deprivation or hypoxia confers a survival advantage to cancer cells and leads to resistance to therapeutics [44]. Further, positive correlation between GRP78 expression and prognostic factors of HCC has been reported [19,45].

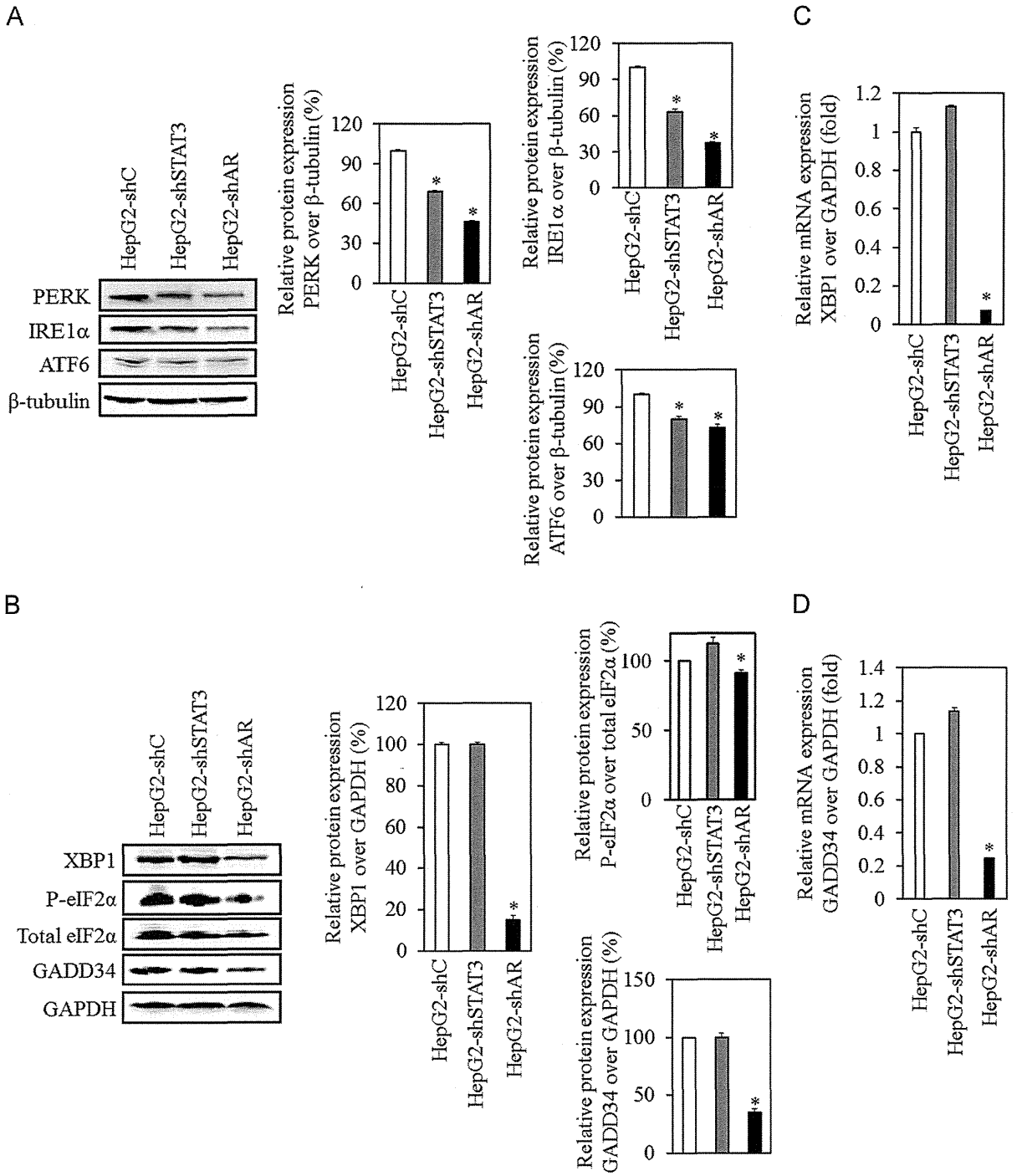

Fig. 4 – Knockdown of endogenous AR decreased unfolded protein response in human hepatoma cells. (A) Western blot analyses of PERK, IRE1α, ATF6 and β-tubulin expressions in HepG2-shC, HepG2-shSTAT3, and HepG2-shAR. Densitometric analyses were performed using Scion Image (Scion). *$P<0.05$, compared with HepG2-shC. (B) Western blot analyses of XBP1, phosphorylated-eIF2α (P-eIF2 α), eIF2α (total eIF2 α), GADD34 and GAPDH expressions in HepG2-shC, HepG2-shSTAT3, and HepG2-shAR. *$P<0.05$, compared with HepG2-shC. (C) Real-time PCR analysis of XBP1 expression in HepG2-shC, HepG2-shSTAT3, and HepG2-shAR cells. XBP1 mRNA levels were normalized to GAPDH levels. *$P<0.05$, compared with HepG2-shC. (D) Real-time PCR analysis of GADD34 expression in HepG2-shC, HepG2-shSTAT3, and HepG2-shAR cells. GADD34 mRNA levels were normalized to GAPDH levels. *$P<0.05$, compared with HepG2-shC.

Sorafenib was the first-approved drug for the systemic chemotherapy of HCC, and improved survival of HCC patients was documented [5]. In the present study, combination of si-AR with sorafenib without si-GRP78 did not enhance apoptosis in HepG2 cell, although VEGF is one of the AR-responsive genes [13,18] and sorafenib is reported to be an inhibitor of angiogenesis [5].

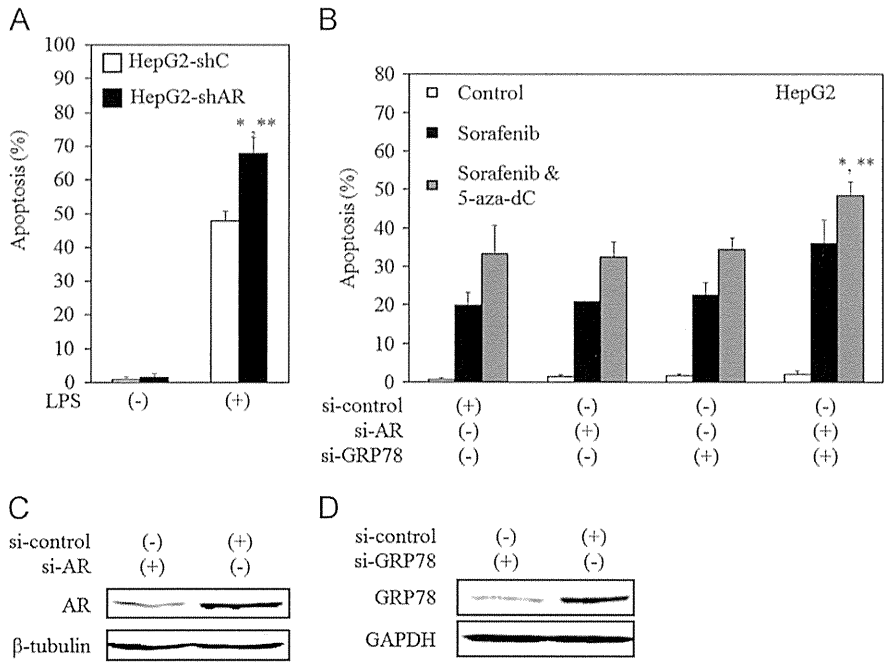

Fig. 5 – Effects of knockdown of endogenous androgen receptor (AR) and/or glucose-regulated protein 78 kDa (GRP78) on apoptosis of human hepatoma cell lines. (A) HepG2 expressing shAR became susceptible to apoptosis induced by lipopolysaccaride (LPS). Twenty-four hours after 1×10^5 cells were split, cells were treated with 5 μg/mL LPS for 24 h. Apoptosis was evaluated by Apopercentage Apoptosis Assay. White column, HepG2-shC; black column, HepG2-shAR. $^*P<0.05$, compared with HepG2-shC untreated with LPS; $^{**}P<0.05$, compared with HepG2-shC treated with LPS. (B) Transfection of AR siRNA (si-AR) and GRP78 siRNA (si-GRP78) into HepG2 enhanced apoptosis induced by sorafenib with or without 5-aza-dC. HepG2 cells (1×10^5 cells) were transfected with 50 nM (si-control, siAR, siGRP78, or siAR and siGRP78) and after 24 h, cells were treated with 5 μM sorafenib with or without 0.5 μM 5-aza-dC. Apoptosis was evaluated by Apopercentage Apoptosis Assay. $^*P<0.05$, compared with others; $^{**}P<0.05$, compared with HepG2-shC treated with 5 μM sorafenib only. (C) Knockdown of AR expression and (D) knockdown of GRP78 expression in HepG2 cells. At 48 h after transfection of control siRNA (si-control), siRNA targeting AR (si-AR), or siRNA targeting GRP78 (si-GRP78), lysates were collected from HepG2 cells and immunoblotted with specific antibodies.

Further studies may be needed to investigate the effect of the combination knockdown of AR with sorafenib on angiogenesis in HCC development. Recent studies suggested that Raf/MEK inhibitors, and inhibition of the downstream kinase ERK, might be needed for durable control of B-Raf-mutant melanoma [46,47]. Sorafenib induced significant apoptosis in HepG2 cells transfected with a combination of AR-siRNA and GRP78-siRNA, suggesting that more/potent inhibitors of AR, and/or inhibition of the downstream of GRP78 may be needed for effective control of sorafenib-resistant HCC.

At present, the early steps of HCC development are not fully understood pathologically. In the current study, we found that AR is a co-regulator of GRP78 especially in the earlier stage of HCC, and we have uncovered an important role for AR in controlling ER-stress response in human hepatoma cell lines. AR might have distinct functional roles in HCC development through the ER-stress pathway and possibly provide new therapeutic options against HCC.

Author contributions

X.J., T.K. conceived and designed the experiments, analyzed the data, and drafted the manuscript. X.J., T.K., S.N., T.M., S.W. performed the experiments. X.J., T.K., S.N., T.M., S.W., O.Y. wrote the manuscript.

Conflicts of interest

Prof. Yokosuka reports receiving grant support from Chugai Pharmaceutical, Bayer, MSD, Daiichi-Sankyo, Mitsubishi Tanabe Pharma, and Bristol-Myers Squibb. These do not alter the authors' adherence to all the Experimental Cell Resarch policies on sharing data and materials.

Declaration of funding source

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