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Inhibition of P2RX7 contributes to cytotoxicity by suppression of glycolysis and AKT activation in human hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer. HCC occurs people with chronic liver diseases. The purinergic receptor P2X 7 (P2RX7) is involved in tumor proliferation and growth. Also, P2RX7 is associated with tumor invasion and metastatic dissemination. High glucose utilization is important for the survival of various types of tumors. However, the role of P2RX7 in glucose metabolism and cellular survival of HCC remains unclear. Here, our results show that the gene and protein levels of P2RX7 were elevated in tumor cells of patients with HCC. The pharmacological inhibition of P2RX7 by A-804598, a selective P2RX7 antagonist, and genetic inhibition by P2RX7 knockdown suppressed the glycolytic activity by reduction of hexokinase 2 (HK2), a key enzyme of the glycolysis pathway, in human HCC cells. Also, both A-804598 treatment and P2RX7 knockdown induced cytotoxicity via inhibition of AKT activation which is critical for tumor cell survival in human HCC cells. Moreover, A-804598 treatment and P2RX7 knockdown increased cytotoxicity and caspase-3 activation in human HCC cells. These results suggest that inhibition of P2RX7 contributes to cytotoxicity by suppression of glycolysis and AKT activation in human HCC. [BMB Reports 2024; 57(10): 459-464]

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most diagnosed cancer (1), and a leading cause of death via cancer (1, 2). The main risk factor of HCC is chronic liver diseases, including hepatitis viral infection, nonalcoholic fatty liver disease (NAFLD), chronic alcohol consumption, and all causes of cirrhosis-inducing conditions (3, 4).

High glucose utilization is critical for cellular proliferation, growth, and homeostasis in various types of tumors (5, 6). High glucose consumption is associated with tumorigenicity, tumor aggressiveness, and tumor growth, and is linked to multiple branched pathways through the glycolytic pathway for the synthesis of biomacromolecules in HCC cells (7, 8). Hexokinase 2 (HK2) is the major isoform of hexokinase in cancers (9). HK2 promotes the cellular uptake and utilization of glucose and is expressed in various cancer cells (10). During glucose utilization, HK2 is activated during hepatocarcinogenesis, and it correlates with poor overall survival in HCC (7, 8, 11, 12).

The purinergic receptor P2X 7 (P2RX7) is a ligand-gated cation channel and is stimulated in response to extracellular adenosine 5'−triphosphate (ATP) binding (13). P2RX7 regulates various downstream events in a cell-dependent manner, such as inflammatory response, cell proliferation, cell death, cellular metabolism, and phagocytosis (14-17). The role of P2RX7 has been identified in the context of neoplastic transformation and the progression of various solid tumors that include breast cancer, colorectal cancer, non-small cell lung cancer (NSCLC), prostate cancer, and renal cancer (18). In HCC, P2RX7 is associated with the invasion and migration of HCC cells (19). Also, P2RX7 is linked to overall survival and recurrence-free survival and inflammation in HCC patients (20, 21). However, the role of P2RX7 in glucose utilization and the survival of HCC during the progression of HCC remains unclear.

Here, our findings show that P2RX7 gene levels were increased in the tumor tissues of patients with HCC. Also, the protein levels of P2RX7 were increased in the tumor cells of patients with HCC. Pharmacological inhibition of P2RX7 by A−804598, a selective P2RX7 antagonist, suppressed the

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glycolytic activity by the reduction of HK2 protein levels in human HCC cells. A−804598 induced cytotoxicity via AKT inhibition and caspase-3 activation in human HCC cells. Consistent with the pharmacologic inhibition of P2RX7, genetic inhibition by P2RX7 knockdown decreased the glycolytic activity by the reduction of HK2 in human HCC cells. Moreover, P2RX7 knockdown induced cytotoxicity via AKT inhibition and the activation of caspase-3 in human HCC cells. Our findings suggest that the inhibition of P2RX7 contributes to cytotoxicity by the suppression of glycolysis and AKT activation in human HCC.

RESULTS

P2RX7 levels are increased in the tumor cells of patients with HCC

We investigated the role of P2RX7 in tumor cells during the pathogenesis of human HCC. First, we examined whether P2RX7 gene levels are increased in the tumor cells of patients with HCC. We measured the levels of P2RX7 gene in tumor tissues from patients with HCC and non-tumor tissues using the nCounter gene expression panel and analysis system (Fig. 1A, and Table S1, S2 of the Supplementary Information [SI]). The levels of P2RX7 mRNA were significantly elevated in the tumor tissues of HCC patients (HCC) compared to those in the non-tumor tissues of patients (Normal) (Fig. 1B). Next, we analyzed P2RX7 protein levels in alpha-fetoprotein (AFP), which is one of the diagnostic marker proteins that is correlated to tumor aggressiveness in HCC, and the positive tumor cells of tumor tissues from patients with HCC. Immunofluorescence staining showed the intensity of P2RX7-positive staining was significantly increased in the tumor tissues from HCC patients (HCC), compared to that of the non-tumor tissues of patients (Normal) (Fig. 1C, D, and Fig. S1 of the Supplementary Information [SI]). Moreover, the intensity of AFP-positive staining was significantly higher in the tumor tissues of HCC patients (HCC) than that in the non-tumor tissues of patients (Normal) (Fig. 1C, E). Notably, the number of cells with P2RX7 and AFP double-positive staining was significantly increased in patients with HCC (HCC), compared to that in the non-tumor tissues of patients (Normal) (Fig. 1C, F). These results suggest that both P2RX7 gene and protein levels were increased in the tumor cells of patients with HCC.

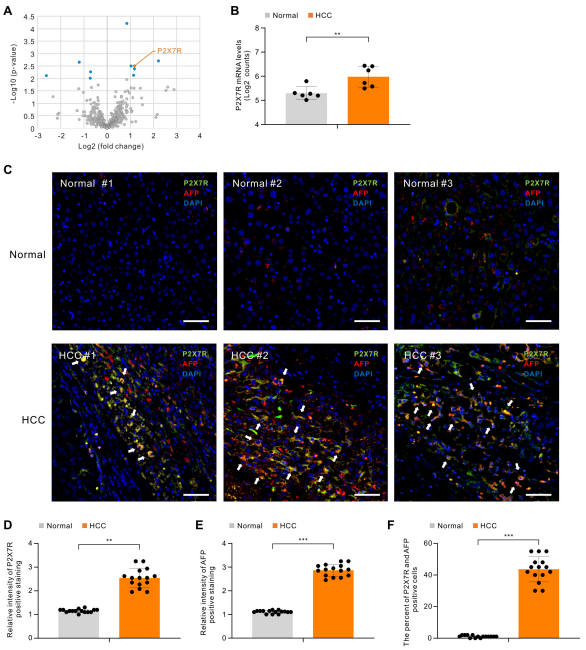

Fig. 1. The levels of P2RX7 are elevated in tumor cells of patients with HCC. (A) Volcano plots from nanostring nCounter gene expression analysis of tumor tissues from patients with HCC (HCC) (n = 3) and non-tumor (Normal) (n = 3). Volcano plots display log2 fold change (x-axis) and -log10 P-values (y-axis) and indicate genes comparing tumor tissues from patients with HCC (HCC) and non-tumor (Normal). Orange dot represents P2RX7 gene. Blue dots represent differentially expressed genes with P-value < 0.01. Gray dots represent differentially expressed genes with P-value ≥ 0.05. (B) P2RX7 mRNA levels (log2 counts) in patients with HCC (HCC) and non-tumor (Normal) by Nanostring nCounter gene expression analysis (n = 6 from duplication of individual subject). (C) Representative immunofluorescence images of P2RX7 and AFP staining in tumor cells (HCC #1, HCC #2, and HCC #3) or non-tumor cells (Normal #1, Normal #2, and Normal #3) of patients with HCC showing P2RX7 (green), AFP (red), and DAPI-stained nucleus (blue) (n = 15). Scale bars, 200 μm. White arrows indicate P2RX7 and AFP-positive tumor cells. (D) Quantification for intensity for P2RX7-positive staining in tumor cells, (E) quantification for intensity for AFP-positive staining in tumor cells, and (F) quantification of the number of P2RX7 and AFP double-positive tumor cells from immunofluorescence images of C (n = 15). Data are presented as mean ± SD. **P < 0.01, ***P < 0.001 by Student's two-tailed t-test.

Pharmacological inhibition of P2RX7 reduces glycolytic activity and HK2 expression, and induces cytotoxicity via AKT inhibition and caspase-3 activation in human HCC cells

To find the role of P2RX7 in the regulation of glycolysis, which contributes to the tumor survival of HCC, we examined whether the pharmacological inhibition of P2RX7 could suppress the activation of glycolysis in human HCC cells. To examine the effects of pharmacological inhibition of P2RX7 by A−804598, a selective P2RX7 antagonist, on the activity of glycolysis in human HCC cells, we measured the levels of extracellular acidification rate (ECAR) as a parameter of glycolysis activity (Fig. 2A). The glycolytic activity was monitored and measured by the following order, such as basal, glucose treatment, oligomycin, a mitochondrial respiration selective inhibitor, treatment, and 2-deoxyglucose (2−DG), a selective glycolysis inhibitor, treatment (Fig. 2A). A−804598 significantly decreased the ECAR levels in response to glucose or oligomycin, compared to that in cells treated with control (Fig. 2A, B). Consistently, the glycolytic capacity, the maximum capacity of glycolysis to generate ATP, was also significantly decreased by A−804598, compared to that in cells treated with control (Fig. 2C). Next, we analyzed the molecular target of P2RX7 inhibi-

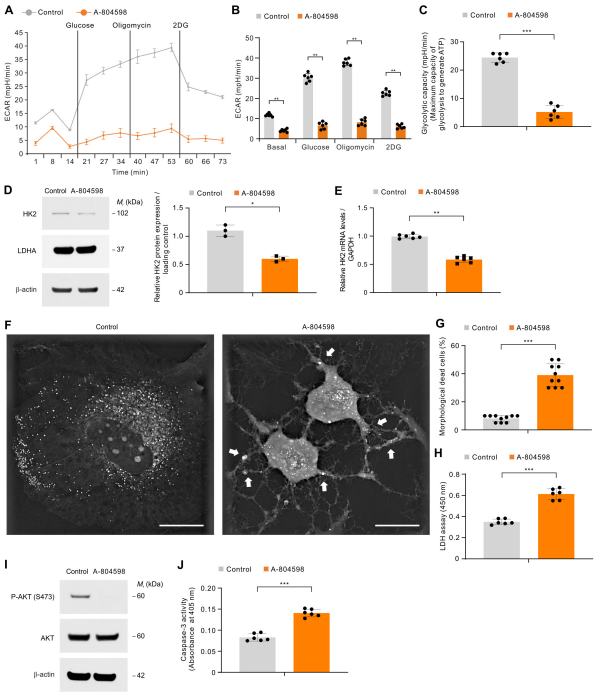

Fig. 2. Pharmacological inhibition of P2RX7 reduces glycolytic activity and HK2 expression and induces cytotoxicity via AKT inhibition and caspase-3 activation in human HCC cells. (A) Representative changes of ECAR levels in response to glucose, oligomycin, and 2DG, (B) quantification of ECAR levels, and (C) glycolytic capacity in human Huh7 cells treated with A-804598 or control (n = 6). Data are mean ± SEM. **P < 0.01, ***P < 0.001 by Student's two-tailed t-test. (D) Representative immunoblot images (left) and densitometry analysis (right) for HK2 and LDH-A protein levels in human Huh7 cells treated with A-804598 or control (n = 3). In immunoblots, the levels of β-actin were used as loading control. Data are presented as mean ± SD. *P < 0.05 by Student's two-tailed t-test. (E) Relative HK2 mRNA levels in human Huh7 cells treated with A-804598 or control (n = 6). (F) Representative 3D images of human Huh7 cells treated with A-804598 or control. White arrows indicate morphological features of cytotoxicity. Scale bars, 20 μm. (G) Quantification for morphological dead cells in human Huh7 cells treated with A-804598 and control (n = 10). (H) Cytotoxicity assay in human Huh7 cells treated with A-804598 or control was determined by lactate dehydrogenase (LDH) levels (n = 6). (I) Representative immunoblot images for phospho-AKT at Ser473 protein levels in human Huh7 cells treated with A-804598 or control (n = 3). In immunoblots, the levels of total AKT were used as loading control. (J) Quantification for caspase-3 activity in human Huh7 cells treated with A-804598 or control (n = 6). Data are presented as mean ± SD. *P < 0.05, ***P < 0.001 by Student's two-tailed t-test.

tion in the regulation of glycolysis in human HCC cells. We examined whether A−804598 could suppress the levels of HK2, a key enzyme in the glycolysis pathway in tumor cells, in human HCC cells. Notably, A−804598 decreased the HK2 protein levels, compared to that in control (Fig. 2D). The lactate dehydrogenase A (LDHA) protein levels, another glycolytic enzyme in glycolysis pathway, were not changed (Fig. 2D). Consistent with the protein levels, the HK2 mRNA levels were decreased by A−804598, relative to that in control (Fig. 2E). These results suggest that the pharmacological inhibition of P2RX7 suppresses glycolytic activity and HK2 expression in human HCC cells. Since high glycolytic activity contributes to the survival of HCC cells, we examined whether the pharmacological inhibition of P2RX7 could induce cytotoxicity in human HCC cells. First, we investigated the cytotoxic effects of A−804598 by morphological changes in human HCC cells. We analyzed the morphological changes by cell death by A−804598 in human HCC cells using 3D image analyzer (Fig. 2F). A−804598 induced the features of cytotoxic morphology, such as cytoplasmic shrinkage and membrane blebbing compared to control (Fig. 2F). Consistent with the morphological features, A−804598 significantly increased the number of morphological dead cells, relative to control (Fig. 2G). Moreover, A−804598 significantly induced the increase of cytotoxicity, relative to control (Fig. 2H). Since the AKT activation in PI3K/AKT signaling pathway is critical for tumor survival, we examined whether A−804598 could inhibit the activation of AKT in human HCC cells (Fig. 2I). A−804598 decreased AKT activation by the reduction of AKT phosphorylation at Ser473, relative to control (Fig. 2I). Since the suppression of AKT signaling is associated with apoptotic cell death, we investigated whether the pharmacological inhibition of P2RX7 by A−804598 could induce caspase-3 activation, which is linked to apoptotic cell death by extrinsic and intrinsic pathways, in human HCC cells (Fig. 2J). A−804598 significantly increased the caspase-3 activity, relative to control, in human HCC cells (Fig. 2J). These results suggest that pharmacological inhibition of P2RX7 induces cytotoxicity via AKT inhibition and caspase-3 activation in human HCC cells.

Genetic inhibition of P2RX7 suppresses glycolytic activity and HK2 expression and induces cytotoxicity via AKT inhibition and caspase-3 activation in human HCC cells

To investigate whether the genetic inhibition of P2RX7 could suppress the activation of glycolysis in human HCC cells, we examined the effects of P2RX7 knockdown on the glycolytic activity in human HCC cells by the ECAR measurement (Fig. 3A). Knockdown of P2RX7 (P2RX7 siRNA) significantly decreased the ECAR levels in response to glucose or oligomycin, compared to that in cells treated with control (Control siRNA) (Fig. 3A, B). Consistently, the glycolytic capacity was also significantly reduced by P2RX7 knockdown, compared to that in cells treated with control (Fig. 3C). Next, we examined whether P2RX7 knockdown could reduce the HK2 expression levels in human HCC cells (Fig. 3D). Notably, P2RX7 knockdown decreased the HK2 protein levels, relative to that in control (Fig. 3D). The protein levels of LDHA were not changed (Fig. 3D). Consistent with the protein levels, the HK2 mRNA levels were decreased by P2RX7 knockdown, relative to those in control (Fig. 3E). These results suggest that the genetic inhibition of

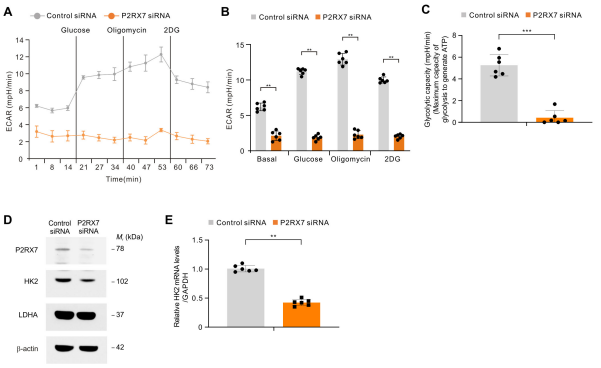

Fig. 3. Genetic inhibition of P2RX7 suppresses glycolytic activity and HK2 expression in human HCC cells. (A) Representative changes of ECAR levels in response to glucose, oligomycin, and 2DG, (B) quantification of ECAR levels, and (C) glycolytic capacity in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells (n = 6). Data are mean ± SEM. **P < 0.01 by Student's two-tailed t-test. (D) Representative immunoblot images for P2RX7, HK2, and LDH-A protein levels in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells (n = 3). In immunoblots, the levels of β-actin were used as loading control. (E) Relative HK2 mRNA levels in in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells (n = 3). Data are presented as mean ± SD. **P < 0.01, ***P < 0.001 by Student's two-tailed t-test.

P2RX7 suppresses glycolytic activity and HK2 expression levels in human HCC cells.

Next, we examined whether P2RX7 knockdown could induce cytotoxicity in human HCC cells. We measured the morphological changes by the cytotoxic effects of P2RX7 knockdown in human HCC cells using 3D image analyzer (Fig. 4A). P2RX7 knockdown increased cytoplasmic shrinkage and membrane blebbing related to cytotoxic features, relative to control (Fig. 4A). Consistently, P2RX7 knockdown significantly increased the number of morphological dead cells, compared to control (Fig. 4B). Moreover, P2RX7 knockdown significantly induced the cytotoxicity, relative to control (Fig. 4C). Next, we examined whether P2RX7 knockdown could inhibit the AKT activation in human HCC cells (Fig. 4D). P2RX7 knockdown decreased AKT activation by the reduction of AKT phosphorylation at Ser473, relative to control (Fig. 4D). Next, we investigated whether P2RX7 knockdown could induce the activation of caspase−3 in human HCC cells. We measured the effects of P2RX7 knockdown on the intracellular activation of cleaved-caspase−3, an active cleaved form of caspase−3, by immunofluorescence staining in human HCC cells (Fig. 4E). Notably, P2RX7 knockdown increased the intensity of positive staining for cleaved-caspase−3, compared to control (Fig. 4E, F). The number of subcellular co-localization cells with double positive staining for both cleaved-caspase−3 and AFP in was significantly elevated by P2RX7 knockdown, relative to control (Fig. 4G). Consistently, P2RX7 knockdown significantly increased the activity of caspase−3 in human HCC cells, compared to control (Fig. 4H). These results suggest that the genetic inhibition of P2RX7 induces

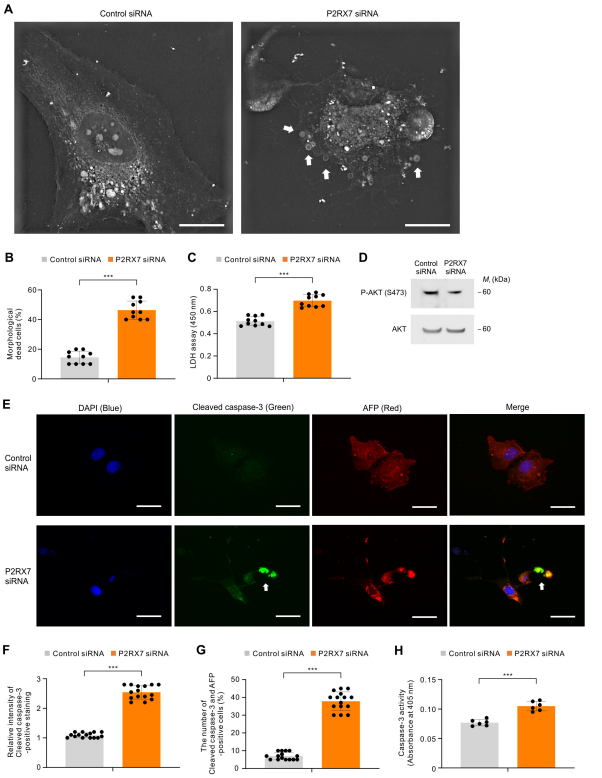

Fig. 4. Genetic inhibition of P2RX7 induces cytotoxicity via AKT inhibition and caspase-3 activation in human HCC cells. (A) Representative 3D images of P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells. White arrows indicate morphological features of cytotoxicity. Scale bars, 20 μm. (B) Quantification for morphological dead cells in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells (n = 10). (C) Cytotoxicity assay in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells was determined by lactate dehydrogenase (LDH) levels (n = 10). (D) Representative immunoblot images for phospho-AKT at Ser473 protein levels in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells (n = 3). In immunoblots, the levels of total AKT were used as loading control. (E) Representative immunofluorescence images of cleaved caspase-3-positive cells in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells showing cleaved caspase-3 (green), AFP (red), and DAPI-stained nucleus (blue). Scale bars, 50 μm. (F) Quantification of intensity for P2RX7-positive staining in AFP-positive cells in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells (n = 15). (G) Quantification for cleaved caspase-3-positive cells in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells (n = 15). (H) Quantification for caspase-3 activity in P2RX7 knockdown (P2RX7 siRNA) and control (Control siRNA) human Huh7 cells (n = 6). Data are presented as mean ± SD. ***P < 0.001 by Student's two-tailed t-test.

cytotoxicity via AKT inhibition and activation of caspase−3 in human HCC cells.

DISCUSSION

Here, we found that the inhibition of P2RX7 contributes to the suppression of glycolysis and AKT activation for survival in human HCC cells. Our findings showed that P2RX7 levels are increased in the AFP–positive tumor cells of patients with HCC. Also, our findings indicate that pharmacological and genetic P2RX7 inhibition reduces glycolytic activity and AKT activation in human HCC cells. P2RX7 inhibition induces cytotoxicity and caspase–3 activation. Our findings suggest that P2RX7 might play an important role in tumor survival in human HCC.

High levels of HK2 are correlated with poor overall survival in human HCC (11, 12). HK4 is a typical isoform in liver and pancreas (22, 23). During liver tumorigenesis, HK2 levels are highly increased, while HK4 levels are decreased (12). In our study, we show that P2RX7 inhibition reduced HK2 gene and protein expression in human HCC cells. Our results also show that P2RX7 inhibition suppressed the AKT activation in human HCC cells. In the PI3K/AKT pathway, AKT signal is linked to the activation of aerobic glycolysis in HCC (24). The activation of PI3K/AKT pathway can directly promote HK2 activity (25). Consistent with previous reports, our results indicate that P2RX7 inhibition could suppress the glycolytic activity through the reduction of AKT activation. Our findings suggest that P2RX7 might be a pharmacological target of aerobic glycolysis inhibition in human HCC cells.

Glutamine metabolic reprogramming plays an important for the progression and proliferation of HCC (26). Increased glutamine uptake and utilization are linked to the regulation of mitochondrial metabolism and mitochondria-mediated apoptosis in HCC (27). Although our results showed the role of P2RX7 inhibition in the impairment of aerobic glycolysis in human HCC cells, P2RX7 inhibition might affect the suppression of glutamine metabolism, and disrupt mitochondrial function under aerobic glycolysis. Further studies are needed to understand the role of P2RX7 inhibition in the regulation mechanism of glutamine metabolism and mitochondria-mediated apoptosis in human HCC cells.

The approach of systemic chemotherapy is limited with few multi-kinase inhibitors, such as sorafenib and lenvatinib, in patients with advanced HCC (28, 29). Recently, immunotherapy with checkpoint inhibitors, including the drug combination of bevacizumab, a VEGF–neutralizing antibody and atezolizumab, an anti-PDL1 antibody, has emerged as first-line treatment for HCC (30). Additionally, the FDA approval for combination therapy with durvalumab, anti-programmed cell death ligand–1 and tremelimumab, anti-cytotoxic T lymphocyte–associated antigen 4 (CTLA-4), indicates a paradigm change in the chemotherapy trial of HCC (31). In our study, we showed that P2RX7 inhibition induced cytotoxicity and the activation of caspase–3 in human HCC cells. These results suggest that the inhibition of P2RX7 might be a therapeutic approach to inhibit the survival of HCC.

P2RX7 plays various roles, such as a pattern recognition receptor in response to extracellular ATP during inflammation, and as a regulator of receptor trafficking (15, 32-34). The P2RX7 receptor signaling regulates the secretion and activation of TNF–α, IL−1β, and IL−6 as the pro-inflammatory cytokines (35, 36). Also, P2RX7 receptor signaling is linked to the production of pro-inflammatory cytokines, including chemokine (C−X−C motif) ligand 2 (CXCL2) and chemokine (C−C motif) ligand 3 (CCL3) (37). Although we showed the role of P2RX7 as a regulator of survival in human HCC cells, further studies are needed to investigate the function of P2RX7 in the inflammatory response of HCC during the progression of HCC.

In summary, our findings suggest that P2RX7 inhibition contributes to cytotoxicity by the suppression of glycolysis and AKT activation in human HCC.

MATERIALS AND METHODS

Materials and methods are available in the Supplemental information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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