HEATR1 Negatively Regulates Akt to Help Sensitize Pancreatic Cancer Cells to Chemotherapy

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Abstract

Elucidating mechanisms of chemoresistance is critical to improve cancer therapy, especially for the treatment of pancreatic ductal adenocarcinoma (PDAC). Genome-wide association studies have suggested the less studied gene HEAT repeat-containing protein 1 (HEATR1) as a possible determinant of cellular sensitivity to different chemotherapeutic drugs. In this study, we assessed this hypothesized link in PDAC, where HEATR1 expression is downregulated significantly. HEATR1 silencing in PDAC cells increases resistance to gemcitabine and other chemotherapeutics, where this effect was associated with increased Akt kinase phosphorylation at the Thr308 regulatory site. Mechanistically, HEATR1 enhanced cell responsiveness to gemcitabine by acting as a scaffold to facilitate interactions between AKT and the protein phosphatase PP2A, thereby promoting Thr308 dephosphorylation. Consistent with these findings, treatment with the Akt inhibitor triciribine sensitized HEATR1-depleted PDAC cells to gemcitabine, suggesting that this therapeutic combination may overcome gemcitabine resistance in patients with low HEATR1 expression. Clinically, we found that HEATR1 downregulation in PDAC patients was associated with increased Akt phosphorylation, poor response to tumor resection plus gemcitabine standard-of-care treatment, and shorter overall survival. Collectively, our findings establish HEATR1 as a novel regulator of Akt and a candidate predictive and prognostic indicator of drug responsiveness and outcome in PDAC patients. Cancer Res; 76(3): 572–81. © 2015 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains a lethal malignancy. The prognosis of patients with PDAC is dismal with a 5-year survival rate of less than 5%. Antitumor drugs and radiotherapy are current treatment options for PDAC; however, drug resistance frequently occurs. Thus, understanding molecular mechanisms contributing to the resistance of PDAC to chemotherapy will provide clues for new targeted therapies.

Akt is a central module to regulate cell proliferation and survival, angiogenesis, and glucose metabolism (1, 2). Akt controls these cellular functions through phosphorylating substrates. Akt directly phosphorylates BAD, preventing it from inhibiting prosurvival Bcl-2 family members (5, 6). Akt regulates glucose metabolism through phosphorylating and inactivating GSK3 (7). In addition, Akt negatively regulates FOXO and p53 and blocks the transcription of BIM, Puma, and Noxa (8, 9). Furthermore, Akt promotes protein synthesis and cell growth through activation of mTOR (10).

Akt activity is tightly controlled at multiple levels. PI3K, a critical upstream kinase of Akt signaling, is activated by growth factors, cytokine, and others (2) and converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 recruits Akt to plasma membrane, where Akt is phosphorylated at Thr308 (11). Ubiquitination of Akt by TRAF6 and Skp2-SCF E3 ligase is required for the recruitment of Akt to plasma membrane (12, 13). Full Akt activity requires phosphorylation of both Thr308 and Ser473 mediated by phosphoinositide-dependent kinase 1 (PDK1; ref. 14) and mTOR complex 2 (mTORC2; ref. 15), respectively. On the other hand, protein phosphatase 2A (PP2A; refs. 16–18) and PH domain leucine-rich repeat protein phosphatase (PHLP; refs. 19, 20) dephosphorylate AktThr308 and Ser473, respectively. FKBPS11 promotes dephosphorylation of Akt Ser473 through acting as a scaffolding protein for Akt and PHLP (21). However, how Akt is targeted to PP2A is not clear.

HEAT repeat-containing protein 1 (HEATR1) contains HEAT repeats, which was initially found in a diverse family of proteins including huntingtin, elongation factor-3, and the PR65/A subunit of protein phosphatase 2A (22, 23). Except for a couple of reports suggesting that HEATR1 may regulate rRNA synthesis and CTLs in
patients with glioma (23, 24), the cellular function of HEATR1 remains largely unknown. Here, we report that HEATR1 regulates cancer cell response to multiple classes of chemotherapeutic drugs. Mechanistically, HEATR1 affects survival of pancreatic cancer cells to chemotherapy by affecting Akt activity. We demonstrate that HEATR1 functions as a scaffold protein to regulate Akt phosphorylation by PP2A. Furthermore, our study identifies HEATR1 as a potential prognostic marker of pancreatic cancers.

Materials and Methods

Cell culture and plasmids

Human pancreatic cancer cell lines SU86.86, ASPC-1, and PANC-1 were purchased from ATCC in 2014 and the identities of all cell lines were confirmed by the medical genome facility at Mayo Clinic Center (Rochester, MN) using short tandem repeat profiling upon receipt. The cell lines were maintained in RPMI 1640 with 10% FBS. HEATR1 cDNA was purchased from Mayo Clinic Center (Rochester, MN) using short tandem repeat fingerprinting upon receipt. The cell lines were maintained in RPMI 1640 with 10% FBS. HEATR1 cDNA was purchased from Mayo Clinic Center (Rochester, MN). PANC-1 cells stably expressing Ctrl and HEATR1 siRNA or shRNA were transfected, and the growth-inhibitory effect was performed as described previously (21).

MTS assay

Gemcitabine was from Eli Lilly and Company. Paclitaxel, SN-38, mitomycin C (MMC), oxaliplatin, and etoposide were from Sigma-Aldrich. Triciribine (TCN) was from EMD Biosciences. Ctrl or HEATR1 siRNA or shRNA were transfected, and the growth-inhibitory effect was performed as described previously (21).

Tumor xenograft study

Female athymic nu/nu mice were obtained from the NCI (Bethesda, MD). Experiments were performed under the approval of the Institutional Animal Care and Use Committee at Mayo Clinic (Rochester, MN). PANC-1 cells stably expressing Ctrl and HEATR1 shRNA were injected subcutaneously in mouse flanks (10^6 cells/mouse flank). Tumor volumes were measured every 3 days. When tumors reached 100 mm³, mice were randomized to four groups (n = 8): vehicle; TCN (0.5 mg/kg/day), administered intraperitoneally once daily; gemcitabine (50 mg/kg), administered intraperitoneally every 3 days; and a combination of two drugs. Mechanistically, HEATR1 affects survival of pancreatic cancer cell response to multiple classes of chemotherapeutic drugs. These results establish an important role of HEATR1 in regulating chemotherapeutic response to different antitumor agents.

Results

HEATR regulates response of pancreatic cancer cells to chemotherapy

We have performed several genome-wide association studies to identify genes whose expression associates with sensitivity to chemotherapy. Interestingly, we found that HEATR1 gene, although not among the top hits in these studies, associates with response to several drugs, including gemcitabine and 1-beta-d-arabinofuranosylcytosine (26). We depleted HEATR1 with siRNA in cells and treated cells with gemcitabine. Downregulation of HEATR1 resulted in increased resistance to gemcitabine (Fig. 1A). Similar results were obtained with two HEATR1-specific shRNAs (Fig. 1B and Supplementary Fig. S1A). Next, multiple classes of chemotherapeutic drugs including oxaliplatin, SN-38, MMC, paclitaxel, camptothecin, and etoposide were used to treat cells. Similarly, cells with HEATR1 knockdown were significantly resistant to these drugs (Fig. 1C and Supplementary Fig. S1B). These results establish an important role of HEATR1 in regulating chemotherapeutic response to different antitumor agents.

HEATR1 regulates Akt phosphorylation at Thr308 by promoting Akt–PP2Aβ56β interaction

As HEATR1 affects cellular response to a broad range of chemotherapeutic agents, it is likely that HEATR1 affects a common pathway such as apoptosis. Gemcitabine induced apparently stronger apoptosis in control cells than in cells transfected with HEATR1 shRNA (Supplementary Fig. S2A). Next, we investigated how HEATR1 affects chemotherapeutic response by probing several pathways regulating cell death. We found that downregulation of HEATR1 led to an increased phosphorylation of Akt at Thr308, but not at Ser473 (Fig. 2A and B). These results suggest that depletion of HEATR1 affects steady-state Akt phosphorylation, which is consistent with an increase in cell growth.
HEATR1 regulates cancer cell response to chemotherapy. A, cells were transfected with indicated siRNA and treated with gemcitabine. Cell survival was determined. The data presented are mean ± SD (n = 6). **, P < 0.01; ***, P < 0.001 (Ctrl vs. HEATR1 siRNA). B, SU86.86 cells stably expressing indicated shRNA were treated with gemcitabine. Cell survival was determined. +++, P < 0.01 (Ctrl vs. HEATR1 shRNA #1 or #2, respectively). C, SU86.86 cells were transfected with indicated siRNA and treated. Cell survival was determined.

As HEATR1 specifically regulates Thr308, it is likely that HEATR1 regulates signaling events that directly control Akt phosphorylation at Thr308. Thr308 of Akt is phosphorylated by PDK1 and dephosphorylated by PP2A (16, 18, 27). No apparent difference of PDK1 phosphorylation was observed in control and HEATR1-depleted cells (Fig. 2C). HEATR1 neither interacted with PDK1 nor affected the interaction between Akt and PDK1 (Supplementary Fig. S3B and S3C), indicating that HEATR1 regulates Akt activity independent of PDK1. Next, we examined whether HEATR1 regulates Thr308 phosphorylation through PP2A. Treatment of PP2A inhibitor okadaic acid significantly increased Akt phosphorylation at Thr308, and HEATR1 knockdown did not further increase Thr308 phosphorylation (Supplementary Fig. S3D). As shown in Fig. 2G, HEATR1 interacted with PP2A scaffold subunit Act, catalytic subunit Cα, and regulatory subunits B55α and B56β, which have been reported to specifically target Akt Thr308 dephosphorylation (16–18, 27). Furthermore,
HEATR1 only interacted with recombinant GST-B56β, suggesting a direct interaction between HEATR1 and B56β (Fig. 2H). Because HEATR1 interacts with both Akt and B56β and regulates Akt phosphorylation, we hypothesized that HEATR1 may function as a scaffolding protein to facilitate Akt dephosphorylation by PP2A. Downregulation of HEATR1 decreased the...
interaction between B56β and Akt (Fig. 2I), while HEATR1 over-expression increased it (Fig. 2I). Furthermore, purified FLAG-HEATR1 increased the interaction between recombinant His-Akt1 and GST-B56β (Fig. 2K). These results suggest that HEATR1 promotes the interaction between Akt and PP2A, facilitating the dephosphorylation of Akt at Thr308.

HEATR1 regulates Akt phosphorylation and cell response to gemcitabine through its scaffolding function

To investigate specific regions of HEATR1 for Akt and B56β interaction, we generated deletion mutants of HEATR1 (Fig. 3A). N-terminal region (aa 1–420) was essential for the binding of HEATR1 with B56β, whereas the middle region (420–1420) was responsible for the binding of HEATR1 with Akt (Fig. 3B and C). These results suggest that HEATR1 binds Akt and B56β using different regions. To further confirm that the scaffolding function of HEATR1 is important for the regulation of Akt phosphorylation, we overexpressed wild-type (WT) and mutants in cells (Fig. 3D). WT decreased Akt phosphorylation at Thr308 and cellular sensitivity to gemcitabine, while HEATR1 mutants that abolish Akt or B56β interaction failed to do so (Fig. 3D and E). These results suggest that HEATR1 regulates Akt phosphorylation and cell response to gemcitabine through its scaffolding function.

Akt inhibitor sensitizes pancreatic cells with HEATR1 knockdown to gemcitabine

The results described above suggest that HEATR1 regulates gemcitabine sensitivity at least partly through regulating Akt, and hyperactivation of Akt in cells with low HEATR1 level might be responsible for increased chemoresistance. If this was the case,

\[
\begin{align*}
\text{Akt} & \quad \text{B56β} \\
\text{WT} & \quad + \\
\text{M1} & \quad - \\
\text{M2} & \quad + \\
\text{M3} & \quad - \\
\text{M4} & \quad + \\
\end{align*}
\]

Figure 3. HEATR1 scaffolding function regulates Akt phosphorylation and cell survival. A, schematic diagram of WT and deletion mutants of HEATR1 used in the study. B and C, cells were transfected with indicated constructs. Precipitation reactions were conducted with S-protein agarose beads and subjected to Western blotting. D, cells were transfected with indicated constructs. Western blotting was performed. E, cells were transfected as in D and gemcitabine sensitivity was examined. The data presented are mean ± SD (n = 6). +, P < 0.05; ++, P < 0.01 (Ctrl vs. HEATR1 KD).
treating cells with an Akt inhibitor should reverse chemoresistance in cells depleted of HEATR1. TCN-P, the active metabolite of Akt inhibitor TCN, binds to PH domain of Akt and prevents its recruitment to cell membrane, where Akt is phosphorylated by PDK1 at Thr308 (28). When TCN was used alone, no significant difference of cytotoxicity and apoptosis was observed in different cell lines transfected with control and HEATR1 siRNA (Supplementary Fig. S4A–S4E). In addition, TCN did not significantly affect gemcitabine sensitivity and apoptosis in control cells. However, TCN sensitized cells depleted of HEATR1 to gemcitabine (Fig. 4A and B, Supplementary Fig. S4B–S4E). We next tested whether the addition of TCN would reverse chemoresistance of HEATR1 Negatively Regulates Akt

Figure 4.
Akt inhibitor sensitizes pancreatic tumors with HEATR1 knockdown to gemcitabine. A and B, PANC-1 (A) or ASPC-1 (B) cells were transfected with indicated siRNA. Gemcitabine (Gem) sensitivity was examined in the presence of vehicle or 10 μmol/L TCN. Data presented are mean ± SD (n = 6). ++, P < 0.01 (control vs. HEATR1 siRNA); ***, P < 0.01 (HEATR1 siRNA vs. HEATR1 siRNA + TCN). C–E, mice with subcutaneously established tumors from PANC-1 cells stably expressing indicated shRNA were treated with PBS, TCN (0.5 mg/kg), gemcitabine (50 mg/kg), or combination. Xenograft tumors were dissected and tumor weights were measured after mice were sacrificed (C). ANOVA was performed (Mann-Whitney test) ++, P < 0.05; +++, P < 0.01 (Ctrl vs. KD). Tumor volumes were measured every 3 days (D). ++, P < 0.01 (vehicle vs. gemcitabine); ***, P < 0.01 (vehicle vs. TCN); and ##, P < 0.01 (vehicle vs. gemcitabine plus TCN). Representative photographs of indicated xenograft tumors are shown in E. KD, knockdown.
tumors with low HEATR1 level in vivo. Xenograft experiments showed that downregulation of HEATR1 promoted tumor growth and resistance to gemcitabine (Fig. 4C–E and Supplementary Fig. S4F). TCN treatment resensitized response of these cells to gemcitabine. This is consistent with in vitro results using pancreatic cancer cell lines and indicates that HEATR1 affects chemosensitivity through regulating Akt activity in vivo.

Association of HEATR1 expression in pancreatic cancer patients with survival and response to chemotherapy

We next used clinical samples to examine the role of HEATR1 in clinical response. Among 100 patients with PDAC, 76 patients died and 24 patients survived with the median survival time of 18 months. Pathologic analysis using PDAC and peritumoral tissues from patients was performed. Statistical analysis revealed that the expression of HEATR1 was significantly lower in pancreatic tumor tissue than in normal pancreatic tissues (Fig. 5A). The staining of Akt phosphorylation at Thr308 was significantly higher in pancreatic tumor tissue than in normal pancreatic tissue (Fig. 5A). Furthermore, low HEATR1 protein levels correlate with increased Akt phosphorylation at Thr308 in representative pancreatic cancer samples (Fig. 5B). We next evaluated whether HEATR1 expression was associated with the response of patients to standardized gemcitabine chemotherapy. As shown in Fig. 5C and D, Supplementary Fig. S5, and Supplementary Table S1, PDAC patients with high HEATR1 or low AktT308 expression in tumors had a significant improvement in OS. Thus, determination of HEATR1 expression in PDAC tissues may be useful as an independent predictor for gemcitabine response. On the basis of IOD value, we observed a negative correlation between HEATR1 expression and AktT308 phosphorylation and TNM staging, and lymph node metastasis, but we did not find the correlation between HEATR1 expression and clinical pathologic feature (Supplementary Table S2). From multivariate survival analysis, HEATR1 expression and AktT308 phosphorylation might be independent prognostic factors among these variables (Supplementary Table S3). Overall, our results
showed that HEATR1 negatively regulates Akt activation, and upregulation of Akt activity by the loss of HEATR1 in pancreatic cancers might give rise to the resistance to chemotherapy (Fig. 6).

Discussion

Despite continuous efforts, effective early detection markers of PDAC are currently not available and approximately 80% of patients are diagnosed at locally advanced or metastatic stages (29). In these patients at advanced stage, limited response to current treatments results in an extremely poor prognosis (30). Thus, improvements in diagnosis and predictability of patient response to existing therapies are urgently needed for PDAC. Cancer treatment by chemotherapy mainly induces apoptosis to kill cancer cells. However, failure to activate apoptosis causes resistance to therapy, which is an important clinical problem in the treatment of cancers.

The Akt pathway, one of the most frequently hyperactivated signaling pathways in human cancers, plays an important role in both tumorigenesis and chemoresistance. Half of PDAC tumors demonstrated activation of Akt by immunohistochemical staining of pAkt (31). Significant correlation between activation of Akt and poor survival suggests an important role of Akt activation in pancreatic cancer (32). Thus, targeted inhibition of Akt shows promise in the treatment of pancreatic cancers given its role in tumorigenesis and chemoresistance. Currently, several small-molecule inhibitors of Akt have been developed and are in various stages of clinical testing (33). For instance, in combination with a cyclin-dependent kinase inhibitor (dinaciclib), the oral pan-AKT inhibitor MK-2206 can dramatically block pancreatic tumor growth and metastases in patient-derived xenograft models (34). Phase I trial results of MK-2206 in patients with advanced solid tumors indicate that MK-2206 was well tolerated, with evidence of Akt signaling blockade (35). RX-0201, an antisense oligonucleotide to mRNA encoding Akt1, in combination with gemcitabine were investigated in a phase II study and the safety and efficacy results are awaited (36). Thus, these Akt inhibitors alone or in combination with other clinically approved anticancer agents should be further explored in clinical studies as potential novel therapeutic agents.

Here we found that depletion of HEATR1 in pancreatic cancer cells causes resistance to gemcitabine and other chemotherapeutic agents (Fig. 1 and Supplementary Fig. S1). We propose that HEATR1 functions in gemcitabine resistance through inhibiting Akt activity. The evidence we observed are as follows. First, the interaction between Akt and B56β was decreased when HEATR1 was depleted, indicating that HEATR1 acts as a scaffolding protein for Akt and B56β, thereby enhancing the phosphatase activity of B56β toward Thr308 (Fig. 2). Second, HEATR1 mutants that abolish Akt or B56β interaction failed to affect Akt Thr308 phosphorylation and chemosensitivity (Fig. 3). Third, addition of TCN sensitizes gemcitabine treatment in cells with low HEATR1 level both in vitro (Fig. 4A) and in animal models (Fig. 3B–D). Finally, we demonstrated that the expression of HEATR1 is positively correlated with OS in patients with pancreatic cancer (Fig. 5).

Identification of diagnostic and prognostic biomarkers for PDAC patients has attracted much attention (29). However, existing biomarkers such as CA19-9 are not adequate as early detection markers of pancreatic cancer and predictor to treatment response because of low sensitivity and specificity (37). The biologic function of HEATR1 in sensitizing pancreatic cancer cell to gemcitabine is potentially important. We report here that HEATR1 expression level in PDAC significantly correlates with patient survival (Fig. 5), suggesting a potentially prognostic value. Because gemcitabine represents the standard-of-care for PDAC, therapeutic interventions targeting HEATR1 may improve outcome in combination with existing therapies. HEATR1 contains
whether HEATR1 is potentially implicated in cancer development should be further tested. It would also be interesting to investigate of the combination of therapies targeting Akt plus gemcitabine resistance in PDAC.

Akt inhibitor and gemcitabine was able to overcome gemcitabine cancers. As an alternative strategy, combination therapy using an Akt inhibitor or gemcitabine was able to overcome gemcitabine resistance in PDAC. HEATR1 affects the response to other chemotherapeutic agents and HEATR1 mRNA to be downregulated in pancreatic cancer cases (3.4%; TCGA); however, this could not account for HEATR1 regulation of HEATR1 is deregulated in PDAC is unclear. From public datasets, we did not find HEATR1 mRNA to be downregulated in PDAC [The Cancer Genome Atlas (TCGA) and Oncomine]. The HEATR1 gene is mutated in a small percentage of pancreatic cancer cases (3.4%; TCGA); however, this could not account for all pancreatic cancers showing weak immunohistochemical staining. We speculate that HEATR1 is downregulated in pancreatic cancer at posttranscriptional level. Possible mechanisms include decreased translation or protein turnover. Thus, further investigation is essential to understand how HEATR1 is dysregulated in cancers. As an alternative strategy, combination therapy using an Akt inhibitor and gemcitabine was able to overcome gemcitabine resistance in PDAC.

Our study provides evidence of HEATR1 as a potential biomarker for predicting chemoresistance, although clinical outcomes of the combination of therapies targeting Akt plus gemcitabine should be further tested. It would also be interesting to investigate whether HEATR1 is potentially implicated in cancer development considering the role of Akt in cancers. Furthermore, although our study mostly focused on gemcitabine and PDAC, our findings that HEATR1 affects the response to other chemotherapeutic agents would have broader impact for the treatment of other cancers and should be validated in the future. In summary, we identify HEATR1 as a negative regulator of the Akt pathway in PDAC and a potential biomarker for predicting chemoresistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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