**SUPPLEMENTAl Materials**

HEATR1 Affects Pancreatic Cancer Cell Response to Chemotherapy by Negatively Regulating Akt

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**Conflict of Interest statement** No author has conflict of interest with the contents of this article.

**SUPPLEMENTAL Methods**

**Antibodies**

Antibodies against Akt (9272), phospho-Akt(Thr308) (9275), phospho-Akt(Ser473) (9271), GSK3β (9315), phospho-GSK3β (9336), PDK1 (3062), phospho-PDK1 (3061), PP2A Aα (2039), B55α (4953), Cα (2038) and cleaved PARP1 (5625) were purchased from Cell Signaling. Anti-HEATR1 antibody was generated with GST fusion protein of HEATR1 (1894-2144). Anti-FLAG (F3165), anti-HA (H3663) and anti-β-actin (A2228) antibodies were purchased from Sigma. Mouse anti-Akt (2920) used for immunofluorescence staining were purchased from Cell Signaling. Anti-HEATR1 (HPA046917) and anti-phospho Akt(Thr308) (SAB4504332) antibodies used for immunohistochemical staining were obtained from Sigma.

**Immunoprecipitation, immunoblotting, and *In vitro* pull-down assay**

We prepared cell lysates, performed immunoprecipitation, and immunoblotting as previously described. In brief, cells were lysed with NETN buffer (20mM Tris-HCl, pH 8.0, 100mM NaCl, 1mM EDTA, 0.5% Nonidet P-40) containing 50mM b-glycerophosphate, 10mM NaF, and 1 mg/ml each of pepstatin A and aprotinin. Whole cell lysates were pulse-sonicated and obtained by centrifugation. Whole cell lysates were incubated with 2 µg of antibody and protein A or protein G Sepharose beads (Amersham Biosciences) for 2 hr or overnight at 4°C. Theimmunocomplexes were then washed with NETN buffer for three times and separated by SDS–PAGE. Immunoblotting was performed following standard procedures. GST fusion proteins were bound to glutathione-Sepharose overnight at 4 °C. The beads were washed with PBS four times and incubated with cell lysates for 1 h at 4 °C. After washing with NETN for three times, the bound proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies.

**Immunofluorescence staining**

Cells grown on coverslips were fixed in 4% paraformaldehyde for 15 min at RT. Slides were washed in phosphate-buffered saline and blocked with 5% goat serum for 1hr at room temperature, then incubated with primary antibody mouse anti-Akt (Cell signaling ) and rabbit anti-HEATR1 at 37 °C for 30 min. After washing with PBS twice, cells were incubated with FITC or rhodamine-conjugated secondary antibodies at 37 °C for 30 min. Nuclei were counterstained with 4'6-diamidino-2-phenylindole (DAPI). After a final wash with PBS, coverslips were mounted with glycerin containing paraphenylenediamine and examined using a confocal microscopy.

**SUPPLEMENTAry TABLES**

**Supplementary Table 1 Total survival of pancreatic cancer patients**

|  |
| --- |
| HEATR1 Patient number Patients of Events Censored  Death Alive Percent  |
| Strong 28 Weak 72 Overall 100 | 14 6276 |  14 13 1024 |  | 50.0%13.8%24.0% |
|  |  |  |  |  |

**Supplementary Table 2 Relationship between expression of tumoral HEATR1 & pAkt T308 and clinicopathological features**

|  |  |  |  |
| --- | --- | --- | --- |
| Clinicopathological Features | Tumoral expression of HEATR1 |  | Tumoral expression of pAkt T308 |
| Low. | High. | *P* |  | Low.. | High | *P* |
| Age, years |  |  | 0.229 |  |  |  | 0.422 |
| <60 | 20 | 26 |  |  | 25 | 21 |  |
| >=60 | 30 | 24 |  |  | 25 | 29 |  |
| Gender |  |  | 0.84 |  |  |  | 0.069 |
| Male | 29 | 28 |  |  | 33 | 24 |  |
| Female | 21 | 22 |  |  | 17 | 26 |  |
| Tumor location |  |  | 0.476 |  |  |  | 0.812 |
| Head | 10 | 13 |  |  | 12 | 11 |  |
| distal | 40 | 37 |  |  | 38 | 39 |  |
| Differentiation level |  |  | 0.579 |  |  |  | 0.423 |
| low | 24 | 26 |  |  | 26 | 22 |  |
| high | 24 | 26 |  |  | 24 | 28 |  |
| Lymph node metastasis |  |  | 0.688 |  |  |  | 0.005 |
| no | 26 | 28 |  |  | 34 | 20 |  |
| yes | 24 | 22 |  |  | 16 | 30 |  |
| Neural invasion |  |  | 0.592 |  |  |  | 0.061 |
| no | 12 | 12 |  |  | 16 | 8 |  |
| yes | 38 | 38 |  |  | 34 | 42 |  |
| Vascular metastasis |  |  | 0.505 |  |  |  | 0.505 |
| no | 46 | 44 |  |  | 46 | 44 |  |
| yes | 4 | 6 |  |  | 4 | 6 |  |
| TNM staging |  |  | 0.583 |  |  |  | 0.01 |
| I | 8 | 12 |  |  | 12 | 8 |  |
| IIA | 19 | 16 |  |  | 23 | 12 |  |
| IIB | 23 | 22 |  |  | 15 | 30 |  |

**Supplementary Table 3 Univariate and multivariate analyses of factors associated with survival**

|  |  |
| --- | --- |
| Factor | OS |
| Univariate*P* | Multivariate |
| HazardRatio | 95% CI | *P* |
| Age, y (>=60 vs <60) | 0.301 |  |  | NA |
| Gender (female vs male) | 0.353 |  |  | NA |
| Tumor location(head vs distal) | 0.147 |  |  | NA |
| Differentiaion level(high vs low) | 0.162 |  |  | NA |
| Lymph node metastasis (yes vs no) | 0.001 |  |  | NS |
| Neural invasion (yes vs no) | 0.011 |  |  | NS |
| Vascular metastasis (yes vs no) | 0.369 |  |  | NA |
| TNM staging (I, IIA,IIB) | 0.006 |  |  | NS |
| HEATR1 expression in tumor | 0.002 | 0.549 | 0.339-0.887 | 0.014 |
| pAkt T308 expression in tumor | 0.000 | 6.379 | 3.605-11.288 | 0.000 |

（OS, overall survival; NA, not adopted; NS, not significant）

**sUPPLEMENTAry FIGURE LEGENDS**

**Supplemental Fig.S1.** HEATR1 regulates cancer cell response to chemotherapy

(A) PANC-1 and ASPC-1 cells stably expressing control or HEATR1 shRNA treated with gemcitabine. Cell survival was determined as described in Figure 1A. The data presented are mean ± SD for six independent experiments. ANOVA analysis was performed. +P<0.05, ++ P<0.01 (Ctrl vs HEATR1 shRNA #1) \*P<0.05, \*\* P<0.01 (Ctrl vs HEATR1 shRNA #2). (B) PANC-1 and ASPC-1 cells were transfected with control or HEATR1 siRNA and then treated with the indicated drugs. Cell survival was determined as described in A. The data presented are mean ± SD for six independent experiments. ANOVA analysis was performed. +P<0.05, ++ P<0.01 (Ctrl vs HEATR1 KD)

**Supplemental Fig.S2.** HEATR1 regulates cancer cell response to chemotherapy and cell growth

(A) SU86.86 cells stably expressing control or HEATR1 shRNA treated with gemcitabine (10µM). Cell lysate were collect at 48 hours and western blotting was performed with indicated antibodies. (B) Cells stably expressing control or HEATR1 shRNA were seeded in each well (5x104/well) and cell numbers were counted every 24 hour. Data are represented as the mean ± SD of four independent experiments.

**Supplemental Fig.S3.** HEATR1 colocalized with Akt and regulates Akt phosphorylation independent of PDK1

(A) Intracellular co-localization of HEATR1 and AKT were observed by using confocal microscopy in Su86.86, PANC-1 and ASPC-1 cells. (B) Cell lysates were subjected to immunoprecipitation with control IgG or anti-HEATR1 antibodies. The immunoprecipitates were then blotted with indicated antibodies. (C) Cells were transfected with control or HEATR1 siRNA, and the interaction between Akt and PDK1 was examined. (D) SU86.86 Cells transfected with control or HEATR1 siRNA were serum starved for 16 hours (0.1% serum), pretreated with DMSO or okadaic acid (5nM) and then serum was added. Whole-cell lysates were harvested at indicated time and western blotting was performed with indicated antibodies.

**Supplemental Fig.S4.** Akt inhibitor sensitizes pancreatic tumors with HEATR1 knockdown to gemcitabine.

(A) SU86.86 cells were transfected with control or HEATR1 siRNA and then treated with AKT inhibitor triciribine (TCN). Cell survival was determined as described in the Methods. (B-E) PANC-1 (B-C) and SU86.86 (D-E) cells were transfected with control or HEATR1 siRNA and then treated with vehicle (N), 10µM gemcitabine (G), 10µM TCN (T) or gemcitabine plus TCN (G+T). Cell lysate were collect at 48 hours and western blotting was performed with indicated antibodies (B,D). Percentage of Sub-G1 cells were measured by flow cytometry (C,E). ANOVA analysis was performed. ++ P<0.01 (Ctrl vs HEATR1 siRNA); \*\* P<0.01 (G vs G+T) (F) Mice with subcutaneously established tumors from PANC-1 cell stably expressing control or HEATR1 shRNA were treated as in Figure 4C. Xenograft tumors were collected and western blotting was performed with indicated antibodies.

**Supplemental Fig.S5.** Representative strong and weak staining of HEATR1 in pancreatic ductal adenocarcinoma. Analysis of HEATR1 staining in pancreatic ductal adenocarcinoma in was shown in Supplementary Table 1.