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MASTL Regulates EGFR Signaling to Impact Pancreatic Cancer Progression

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Abstract

Pancreatic cancer (PC) remains a major cause of cancer-related deaths primarily due to its inherent potential of therapy resistance. Checkpoint inhibitors have emerged as promising anti-cancer agents when used in combination with conventional anti-cancer therapies. Recent studies have highlighted a critical role of the Greatwall kinase (MASTL; Microtubule-associated serine/threonine-protein kinase-like) in promoting oncogenic malignancy and resistance to anti-cancer therapies; however, its role in PC remains unknown. Based on a comprehensive investigation involving PC patient samples, murine models of PC progression (*Kras;PdxCre-KC* and *Kras;p53;PdxCre-KPC*), and loss and gain of function studies, we report a previously undescribed critical role of MASTL in promoting cancer malignancy and therapy resistance. Mechanistically, MASTL promotes PC by modulating the epidermal growth factor receptor (EGFR) protein stability and, thereupon, kinase signaling. We further demonstrate that combinatorial therapy targeting MASTL promotes the efficacy of the cell-killing effects of Gemcitabine using both genetic and pharmacological inhibitions. Taken together, this study identifies a key role of MASTL in promoting PC progression and its utility as a novel target in promoting sensitivity to the anti-pancreatic cancer therapies.

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Keywords

MASTL; Pancreatic Cancer; EGFR; chemoresistance

Introduction

Pancreatic cancer (PC) is the third leading cause of cancer-related deaths, with a 5-year survival rate of ~10% [1] and a strong ability to rapidly metastasize [2] . PC is projected to become the second leading cause of cancer-related death by 2030 if its clinical management is not improved [3]. Thus, it is important to identify novel biomarkers that can accurately predict disease prognosis as well as help improve molecular understanding of the disease for the development of novel therapies. MASTL/Greatwall kinase has recently emerged as an oncogene potentially upregulated in certain cancers [4–8]. MASTL expression has also been associated with poor patient prognosis and therapy resistance as MASTL overexpression is associated with a higher grade and a more advanced cancer stage [7, 9–12]. These studies have implicated a role of MASTL in multiple cellular processes, including cell cycle progression[13], re-entry in response to DNA damage[14, 15] and cellular transformation in manners dependent on the Wnt/β-catenin and Akt-signaling [4, 6, 12]. However, the status of MASTL and its potential causal role in PC progression and therapy resistance remain unknown.

The epidermal growth factor receptor (EGFR) activation is involved in both the PC initiation and its progression to metastatic cancer [16–18]. The auto-phosphorylation of the tyrosine kinase domains amplifies downstream cancer-promoting signaling pathways, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), leading to oncogenic growth, angiogenesis, and metastasis [19, 20]. Although drugs targeted against EGFR have been developed for the treatment of PC, the patients, in general, develop resistance to these therapies, posing a challenge in the effective clinical management of the disease and patient survival [21, 22].

In the current study, we demonstrate for the first time that MASTL is a novel regulator of PC progression. Using analysis of the public databases and independent cohort of PC patients, we also demonstrate that MASTL expression associates with PC progression and a low probability of patient survival. Using loss and gain of function approaches and a novel pharmacological inhibitor, we further demonstrate a causal role of MASTL in PC progression and therapy resistance. We also provide mechanistic data that MASTL regulates EGFR protein stability and dependent signaling for its oncogenic effects in PC progression. Overall, our results establish MASTL as a promising therapeutic target in curbing cancer malignancy in PC and patient survival.

Results

MASTL expression is highly upregulated in PC:

To characterize a role for MASTL, we first determined the status of MASTL expression in PC. Immunoblotting using total cell lysates from a panel of PC cell lines {Capan-1, BxPC3,

MiaPaCa, CD18/HPAF and transformed (tHPNE) cells} demonstrated an upregulated MASTL expression compared to the non-transformed pancreatic cell line (HPNE) (Fig 1A). We further examined MASTL expression in a murine model of PC progression using immunoblotting and immunohistochemical (IHC) analysis. In this regard, we used the primary tumor-derived murine PC cell lines and tumor sections from KC ($LSLKras^{G12D}$; Pdx1-Cre) and KPC ($LSLKras^{G12D}$; $Trp53^{R172H}$; Pdx1-Cre) mouse models [23]. The expression of MASTL was sharply upregulated in a murine PC cell line derived from primary tumors of KPC mice as compared to the KC mice derived cell lines (Fig 1Bi). Next, we observed that expression of MASTL also increased in10 and 25 weeks old tamoxifen induced KPC mouse PC tissues (Fig 1Bii).

To determine the clinical relevance of the above data, we determined the MASTL expression in matched primary and metastatic pancreatic cancer patient samples using a custom in-home array (14 samples). IHC analysis showed that MASTL expression was highly upregulated in malignant pancreatic cancer tissues and metastatic livers as compared to the normal tissue counterparts (Fig 1Ci). Next, we evaluated the correlation of MASTL expression with patient survival using the TCGA (The Cancer Genome) database. Kaplan-Meier analysis exhibited that higher MASTL expression was associated significantly with the lower overall patients' survival *(177 patients, $p = 0.0024$)* (Fig 1Cii). All these observations supported a positive correlation of MASTL expression with (PC) progression.

MASTL knockdown (MKD) in PC cells inhibits tumorigenic growth and invasive ability:

To elucidate a functional role for MASTL in PC progression, we knocked down MASTL expression using a gene-specific shRNA construct. Capan-1 and transformed HPNE (tHPNE) cell lines with high endogenous MASTL expression, as shown in Fig 1A, we confirmed MASTL knockdown in these cell lines by immunoblotting, qRT-PCR and immunofluorescence analysis (Fig 2Ai, Suppl. Fig 2Ai & 2Aii). Inhibition of MASTL induced a significant decrease in cell survival in both, Capan-1^{MKD} and $tHPNE^{MKD}$ cells by MTT-assays as compared to the respective controls (Fig 2Aii). Next, we determined if MASTL knockdown affects cell survival by affecting cell cycle progression and/or cell death. Effect of MKD was determined on cell cycle distribution as described in Materials and Methods and as described previously [6, 24]. In brief, cells were synchronized by Nocodazole (0.5 μM) treatment for 16 hours and then released into the normal cell cycle by exposure to serum containing fresh medium for 2 h, and flow cytometry analysis was done. As shown in Fig 2Bi, the Capan-1^{MKD} or $tHPNE^{MKD}$ cells failed to enter into the mitosis when compared to the control cells, which proceeded to the G0/G1 phase, as expected (Fig 2Bi, Suppl. Fig 2Bi & Bii). Interfering in G2/M phase commonly results in apoptosis[25–27]. Accordingly, Annexin-V uptake assay showed significant increases in both early and late apoptosis in these cells compared to their respective controls *($p < 0.05$*; Fig 2Bii & Suppl. Fig 2Ci & Cii). Further determinations showed that the loss of MASTL expression also inhibited cell invasion *($P < 0.01$)* and anchorage-independent growth (colony formation in soft agar; 65–70%) as compared to the control cells (Fig 2Ci & Cii).

MASTL knockdown inhibits xenograft tumor formation:

To further determine the role of MASTL in tumorigenesis *in vivo*, we performed a subcutaneous xenograft tumor generation using tHPNE^{MKD} and control cells. 1X10^6 control or MKD cells were implanted on the opposite flank of the athymic nude mice (n = 5/group). Mice receiving control tHPNE (transformed) cells displayed tumor progression as early as two weeks post-implantation of cancer cells *($p < 0.01$)*, and average tumor volume increased to 200 ±25.2 mm^3 at 6-weeks. In contrast, tumors resultant from the implantation of tHPNE^{MKD} cells were noticeably smaller and significantly lower in volume and weight *($p<0.0001$)* at 6-weeks post-implantation compare to control (Fig 3Ai & Aii, Suppl. Fig 3A & B). Immunoblotting using the total tumor lysate and IHC of respective tumor section confirmed the inhibition of MASTL expression in tHPNE^{MKD} cells (Fig 3Bi & Bii). To further determine the rate of proliferation/apoptosis, we determined the expression of Ki67 and cleaved caspase-3. A decrease in proliferation (Ki67) while an increase in apoptosis (cleaved caspase-3), was observed in the tumors originating from tHPNE^{MKD} cells versus control cells (Fig 3C).

Pharmacological inhibition of MASTL inhibits cell survival:

Greatwall Kinase inhibitor (GKI-1) is a novel ‘first-generation inhibitor’ of human MASTL [28, 29]. Therefore, we further determined if inhibiting MASTL using the GKI-1 could provide effects similar to the effects of its genetic inhibition. The GKI-1 treatment of PC cells at 25 and 50μM inhibited MASTL expression; however, it did not affect the expression of Aurora kinase, and thus confirmed specificity at these doses (Fig 4A). We then treated Capan-1 and tHPNE cells with increasing concentration of GKI-1 (10, 25, 50 and 100 μM), which demonstrated a dose-dependent decrease in cell viability in GKI-1 treated versus control cells (Fig 4B). Using Annexin V FITC analysis, we further observed that GKI-1 induces early and late apoptosis in dose-dependent manner in both, Capan-1 and tHPNE cells (Fig 4Ci & Cii).

Pharmacological inhibition of MASTL inhibits tumor formation *in vivo*:

We further examined the therapeutic efficacy of GKI-1 in *in vivo* using xenograft tumor transplantation model. Capan-1 and tHPNE cells were implanted subcutaneously in athymic nude mice. After confirming palpable tumor growth (8–12 days after the initial transplantation), the tumors were either left untreated or treated with GKI-1 (10 mg/kg; IP, once/week; n = 5 mice/group). Mice were sacrificed ~5-weeks post-treatment. GKI-1 administration remarkably decreased tumor volume (mm^3) as early as day 16- or day 28 post-transplantation in Capan-1 *($p < 0.001$)* and tHPNE cells *($p < 0.01$)* cells y, and a reduced tumor volume was maintained in the GKI-1-treated mice throughout the study (45 days post-transplantation) (Fig 5Ai, Aii & Suppl Fig 4). The resulting tumors were evaluated for MASTL expression using immunoblotting and IHC to validate the efficacy of the GKI-1. We observed a significant downregulation of MASTL upon GKI-I treatment compared to vehicle control (Fig 5Bi & Bii). Analysis of the Ki67 and cleaved caspase-3 expressions further supported decreased cell proliferation and increased apoptosis in GKI-1-treated tumors (Fig 5Ci & Cii).

MASTL promotes PC progression by modulating EGFR-signaling:

Next, to elucidate further how MASTL-mediates PC progression, we probed a commercial and well-controlled oncogenic array (R&D) with total cell lysate from the tHPNE^{MKD} and control cells (Suppl Fig 5Ai). Interestingly, we found sharp inhibition of the phosphorylated expression of the EGFR family members {EGFR (Her1), ErbB2 (Her2), ErbB3 (Her3), and ErbB4 (Her4)} in MKD cells compared to the control cells (Suppl Fig 5Aii). In addition, Bcl-xL, Survivin, and Galectin-1 were differentially expressed in the MKD cells versus control cells (Suppl Fig 5Ai). Immunoblotting using the total cell lysate from Capan-1^{MKD} and tHPNE^{MKD} cells supported these findings, however the most remarkable changes were observed in the pEGFR, EGFR, Bcl-xL, Survivin, and Galectin-1 expression in the MKD versus control cells (Fig 6Ai & Aii). Galectin-3 expression in same samples did not change thus suggesting specificity for inhibition of Galectin-1. Further determination demonstrated a sharp downregulation of pAkt, a downstream target of the EGFR signaling in MASTL-inhibited PC cell lines as compared to respective controls (Fig 6Ai & Aii). Similar changes were observed in the EGFR, pAkt, Bcl-xL, Survivin, and Galectin-1 expressions in the xenograft mouse tumor lysates and tumor sections from tHPNE^{MKD} cells (Fig 6Bi & Suppl Fig 5Aiii) as well as the GKI-1 treated tumor samples from the Capan-1 and tHPNE cells (Fig 6Bii, Biii, Suppl Fig 5B & C). A decrease in the expression of EGFR protein upon inhibiting MASTL expression suggested that MASTL might regulate EGFR-activation by regulating its expression. To confirm, full-length MASTL plasmid was overexpressed in non-transformed human pancreatic duct cell line HPNE, with minimal endogenous expression of MASTL protein. Our results demonstrated MASTL overexpression not only increased the cell proliferation *($p < 0.001$,* Suppl Fig 6A) but also induced expression of EGFR, Bcl-xL, Survivin, and Galectin-1 as compared to control cells (Suppl Fig 6B).

To examine if MASTL modulation affects EGFR transcription, we examined the *EGFR* mRNA levels in Capan-1^{MKD} versus control cells. As shown in Suppl Fig 6C, suppression of MASTL expression did not significantly affect *EGFR* mRNA expression. We further determined if MASTL regulates the stability of EGFR protein or mRNA to regulate its expression. To examine, we treated the Capan-1^{MKD} cells and control cells with Cycloheximide or Actinomycin D in a time dependent manner over a period of 24-hrs. We found a significant decrease in EGFR protein stability but not the mRNA stability in MKD cells versus control cells (Fig 6C), suggesting that MASTL regulates EGFR protein stability to regulate its expression and dependent signaling.

MASTL and EGFR expression positively associate with PC progression:

To evaluate the clinical relevance of the novel association of MASTL with EGFR expression, we performed *in silico* analysis in the TCGA (The Cancer Genome Atlas) database using an online publicly available software, UALCAN (ualcan.path.uab.edu/home). Scatter Plot demonstrated a Pearson's correlation coefficient=0.31, suggesting a positive association between EGFR and MASTL expression (Fig 7A). To further validate, we determined MASTL and EGFR expression using a human commercial PC samples array from different stages of the PC (normal; 30 samples, inflammation (Pancreatitis); 11 samples, malignant (ductal adenocarcinoma); 46 samples) by IHC analysis. We observed an increase in Pancreatitis and ductal adenocarcinoma samples as compared to normal

controls (Fig 7Bi). To determine the correlation MASTL and EGFR in this dataset, we used Scatter Plot analysis, which demonstrated a significant correlation with Spearman correlation rho=0.34 and p=0.0014 (Fig 7Bii). Further, quantification of IHC-intensity scores of MASTL and EGFR expression showed a higher level in pancreatitis, however a significant increase in ductal adenocarcinoma samples as compared to normal controls (Fig 7Biii). We also determined the expression of MASTL and EGFR in matched primary and metastatic samples from 12 PC patients (custom in-home array). A high MASTL and EGFR expression were observed in the primary and metastatic patient samples (Fig 7C & Suppl Fig 7A). In parallel studies, we determined the expression of EGFR in PC mouse model. EGFR expression increased at 20 and 25 weeks in tumors from KPC mice, which was also associated with MASTL expression (Fig 1B & Suppl Fig 7B). These observations strongly supported a positive association between MASTL and EGFR expression in PC progression.

EGFR expression is integral to the oncogenic effects of MASTL expression:

Above data suggested that MASTL may regulate EGFR-expression and the downstream signaling for its oncogenic functions. To test this postulation, we ectopically expressed a full-length EGFR construct in the Capan-1^{MKD} or tHPNE^{MKD} cells to determine whether EGFR overexpression compensates for the loss of MASTL expression. EGFR overexpression promoted cell viability despite the persistent knockdown of MASTL protein in both cells (Fig 8Ai & Bi). Conversely, inhibiting EGFR-signaling using PD153035, a potent inhibitor of the EGFR tyrosine kinase activation, significantly reduced the cell viability in MASTL overexpressing HPNE cells (Fig 8Ci). To determine the effects of EGFR modulation in MASTL manipulated cells on downstream signaling, we also determined the effect on the expression of Bcl-xl, Survivin, and Galectin-1. EGFR overexpression increased Bcl-xl, Survivin, and Galectin-1 expression in Capan-1^{MKD} or tHPNE^{MKD} cells (Fig 8Aii & Bii), while inhibition of EGFR by PD153035 treatment in MASTL overexpressing cells inhibited these signaling pathways (Fig 8Cii). Overall, the above data suggested a key role for EGFR-signaling in MASTL mediated oncogenic function potentially by modulating the EGFR/Galectin-1/Bcl-xl/Survivin signaling.

MASTL promotes resistance to PC therapy:

Up-regulation of MASTL expression promotes tumor recurrence after initial cancer therapy in some cancers [6, 11]. Hence, in light of our data that MASTL expression is directly proportional to the PC progression, we wondered if increased MASTL expression also promotes resistance against PC therapy. To test, Capan-1 and tHPNE cells were treated with Gemcitabine (Gem) (1, 5, 10μM) [30], the common drug used for treating PC. Interestingly, immunoblotting of the total cell lysate from the above study showed a sharp increase in MASTL expression in the Gem-treated cells compared to the vehicle-treated cells (Fig 9Ai). The MASTL expression was also accompanied by an increase in Survivin expression, supporting a potential role of MASTL in resistance to the Gem therapy (Fig 9Ai). Further analysis showed that the CD18/HPAF cells, a PC cell line selected to be resistant to Gem (CD18Gem), also express high levels of MASTL (Fig 9Aii). We then subjected Capan-1 and tHPNE cells to Gem alone or in combination with GKI-1 to further assess the role of MASTL in resistance to chemotherapy. As shown in Fig 9Bi, GKI-1 was effective only at ≥25μM when used alone, but in combination with Gem, GKI-1 had a much higher

potential to interfere with cell growth, even at a concentration of 5μM, demonstrating that combination therapy was more effective in regulating cell viability as compared to Gem alone ($p<0.001$ for Capan-1; $p<0.01$ for tHPNE). Furthermore, the expression of Survivin was induced by Gem alone but markedly reduced in the cells receiving combinatorial treatment (Fig 9Bii). We found a similar effect of MASTL expression on treatment with 5-FU (5-Florouracil) treatment as 5-FU mediated cell killing effects was significantly increased in Capan-1^{MKD} and $tHPNE^{MKD}$ cells as compared to the respective controls (Suppl Fig 8A & B). Overall, these studies supported the role of MASTL in promoting resistance against conventional anti-pancreatic therapy using Gem or 5-FU.

To further validate, we used tumor-organoids derived from KPC mice, which were then subjected to Gem (1μM) or GKI-1 treatment at 1, 100 μM, and 150 μM concentrations for three days. Thereafter, organoids in each group were carefully followed for morphological or structural variations. GKI-1 treatment showed decreased tumor organoid size with the greatest reductions at 150μM dose (>28% at 100μM, $p<0.05$ and >45% at 150μM, $p<0.01$). We also observed an increased number of dead organoids when treated with GKI-1 compared to untreated organoids (Fig 9Ci, Cii and Suppl Fig 8C). Gem alone did not decrease the size of the organoid during this time course. These *ex-vivo* experiments further supported the role of MASTL as an important therapeutic target in treating PC.

Discussion

Pancreatic cancer (PC) is one of the most aggressive cancer with an extremely poor prognosis [31]. Thus, thorough knowledge underlying malignant PC features has become increasingly critical to overcome the poor clinical outcome of the patients with this lethal disease [32, 33]. Our data demonstrates a novel role of MASTL in PC progression and patient survival. Notably, this conclusion is based upon an extensive analysis using multiple cancer cell lines, mouse models, and samples from different stages of human PC. Our data shows that MASTL upregulates Galectin-1/EGFR/Survivin/Bcl-xl signaling to mediate its oncogenic effect in PC, yet another important finding especially considering the known implication of EGFR-signaling in PC progression and therapy resistance [22]. Overall, our data support the efficacy of MASTL as a novel biomarker of PC progression and therapeutic target.

A number of previous studies have shown that MASTL kinase serves a critical role in regulating mitosis [34–37]. Activated MASTL during the G2/M transition promotes inhibition of tumor suppressor PP2A-B55 through phosphorylation of its substrates, Arpp19 and ENSA [38]. However, recent studies have suggested that the MASTL upregulation in cancer is associated with mitotic activity and the oncogenic potential of MASTL [5, 10–12, 39]. Mechanistically, it has also been shown that MASTL promotes oncogenic cell transformation and associates with tumor progression through hyperactivation of Akt or Wnt/ β-catenin signaling cascade [6, 12, 40]. Finally, we show that MASTL levels modulate tumor growth *in vivo*. Our findings illustrate a regulatory role of MASTL in PC not only by regulating the G2/M transition but also by regulating the oncogenic and invasive properties, as evidenced by the soft agar and invasion assay *in vitro* and tumor formation *in vivo*.

Recently, two groups have reported putative MASTL inhibitors [28, 29]. Ocasio et al. [28] demonstrated inhibition of the kinase activity of MASTL *in vitro* using the GKI-1 inhibitor. Ammarah et al. [29] also reported two potential MASTL inhibitors by *in silico* analysis, which were predicted to interact with the kinase domain of MASTL. Notably, GKI-1 binds to the active site of the protein and shows cellular efficacy in inhibiting MASTL expression, however, potential anti-cancer effects of the GKI-1 remain unknown [28, 29]. Our findings not only validate the efficacy of this inhibitor in inhibiting MASTL expression but also for the first time demonstrate its anti-cancer effects. Of interest, profound inhibition of the PC growth was achieved *in vivo* with GKI-1 at well-tolerated doses and without notable signs of toxicity. Taken together, it is possible that the selection of patients based on the MASTL as a biomarker could be useful for decisions on MASTL targeted therapy though additional studies are needed in that direction and are underway. Another significant finding in our study is the positive association between MASTL and EGFR. Of note, EGFR overexpression has been implicated in the pathogenesis of several epithelial cancers [41]. Overexpression of EGFR contributes to tumor aggressiveness and chemoresistance in PC [42]. Although many patients are sensitive to EGFR inhibitors in the initial treatment, they often develop resistance to these therapies, potentially due to secondary mutations in the EGFR gene. Therefore, developing novel therapeutic targets to inhibit EGFR signaling are critical. We found a significant correlation between MASTL and EGFR expression in human PC samples, TCGA database as well as KPC mouse model. Our data further demonstrate a novel role of MASTL in the regulation of EGFR expression to regulate PC cells. It is noteworthy that the protein level of EGFR, but not the mRNA stability, is regulated by silencing MASTL expression, indicating that MASTL affects EGFR post-transcriptional processes. Notably, inhibition of MASTL inhibited Galectin-1 expression in addition to EGFR, p-Akt, Bcl-xl, and survivin downregulation. Galectin-1 has an important role in PC progression by promoting pro-tumorigenic activity and suppressing T cell function, which affects the tumor microenvironment [43, 44]. In addition, in a mouse model of PDAC (EIa-myc mice), genetic ablation of Galectin-1 reduced tumor progression by inhibiting cell proliferation, angiogenesis, desmoplastic reaction, and enhancing tumor-associated immune response[45]. At the cell surface, galectins regulate spatial distribution, trafficking, and function of glycoproteins, including three RTK members: EGFR, VEGFR, and IR[46–49]. Notably, Galectin-3 is associated with EGFR to regulate pancreatic and lung cancer cells and without Galectin-3 expression, the surface levels of EGFR are dramatically reduced and the receptor accumulates diffusely in the cytoplasm [50–52]. This function of galectin-3 might be mediated through interaction with its binding partner Alix, which is a protein component of the endosomal sorting complex required for transport (ESCRT) machinery[52]. Galectin-8 also regulates epithelial-mesenchymal transition through focal adhesion kinase (FAK) mediated EGFR trans-activation[53]. We postulate that Alix/Galectin-1 may associate with EGFR and play a similar role in MASTL mediated regulation of EGFR expression. Our ongoing studies are focused on understanding details of MASTL mediated regulation of EGFR expression and trafficking. Galectin can also promote Sox2 (CSC marker) expression in an EGFR activation-dependent manner in lung cancer cells and forced expression of Sox2 blunted the effect of galectin-3 knockdown on lung cancer sphere formation ability and thus chemoresistance [50]. These results suggest that MASTL might mediate its effect on chemoresistance by regulating Galectin-1 to promote EGFR activation

leading to the upregulation of CSCs properties and thus chemoresistance. Future studies are necessary to better characterize the mechanistic role of MASTL in PC progression and recurrence, as well as to investigate the clinical potential of MASTL inhibition for cancer therapy application.

Furthermore, inhibition of MASTL genetically or pharmacologically sensitized the mouse or human PC cells to chemotherapy [6, 11]. Our additional data demonstrated that inhibition of MASTL expression significantly promoted efficacy of the conventional anti-pancreatic cancer drug Gem, which further highlights its efficacy in effective clinical management for PC. Similar role of MASTL in tumor resistance has been observed in other cancers including colon, head and neck cancer patients [6, 11]. Also, MASTL depletion in recurrent tumor cells re-sensitize their response to cancer therapy[10, 11]. Most importantly, MASTL targeting precisely induced apoptosis in cancer cells, while not in normal cells [14], showing that MASTL upregulation promotes cancer progression and tumor recurrence after initial cancer therapy. Similarly, in PC, MASTL inhibition enhanced sensitivity to apoptosis that has been induced by Gem or 5FU-treatment by inhibiting EGFR signaling cascade. This suggested MASTL might be a potential therapeutic target for chemoresistant PC.

In summary, the results of the present study indicate that MASTL plays a pivotal role in human pancreatic malignancy based on *in silico*, *in vitro*, *and in vivo*-clinical evidence. We have identified a critical role for EGFR in MASTL mediated effects on pancreatic tumorigenesis. Targeting MASTL might be a promising strategy to inhibit malignant PC by ablating the EGFR signaling pathway to restore homeostasis and thereby reduce the chance of tumorigenesis.

Material and Methods:

Cell Culture, Cell Synchronization and Proliferation Assay:

Human PC cell lines were either purchased from ATCC or derived from KC and KPC mice described in details in Supplementary materials [54]. Synchronization of cell lines at G1, for cell cycle progression analysis, were described elsewhere [6, 24]. GKI-1 was purchased from (#GLXC-09247) Glixx Laboratories, Hopkinton, MA, USA. ActinomycinD and Cycloheximide compounds were purchased from Sigma-Aldrich, St. Louis, MO, USA. Gemcitabine (Gem) and 5-fluorouracil (5FU) were procured from the University of Nebraska Medical Center (UNMC), Omaha, USA pharmacy. Cells were transfected using Turbo DNAfectin™ 300 reagents (#TDNAF-1000 MidSci), according to the manufacturer's protocol.

Immunohistochemistry and immunofluorescence analysis:

These analyses were performed using the standard protocols as described before [55].

Oncogenic Array:

Oncogenic array analysis was performed in tHPNE control and MKD cells using proteome profiler human XL oncology array kit (#ARY026, R&D Systems, Minneapolis, MN) as per manufacturer's instructions.

RNA and Real-Time PCR:

Isolation of total RNA was performed using RNeasy Plus Mini Kit (QIAGEN) according to manufacturer's protocol and as described before [56]. Primer pairs for each gene are provided in Additional File2: Supplemental Table S1.

Cell extraction and Western blotting:

Transfected or treated cells were lysed and immunoblotted as previously described [57] and antibodies used are described in Supplementary materials.

Soft Agar Assay:

Anchorage-independence growth assays or soft agar was used to determine the growth potential of control and MKD cells as described before [56]. After 3 weeks, viable colony numbers and sizes were counted and measured.

Transwell migration assay:

An invasion assay was carried out using 24-well plate (#07–200-169, Corning). A polyvinyl-pyrrolidone-free polycarbonate filter (8 μm pore size) was coated with matrigel (#356237, Corning) as described before [56]. Cells were observed and counted at five selected randomly fields using a light microscope (Olympus).

Annexin V-fluorescein isothiocyanate/ propidium iodide staining:

Annexin V-fluorescein isothiocyanate (FITC)/ propidium iodide (PI) triple staining detection system was used to assess cell apoptosis. Annexin V-FITC and PI solutions were added to stain cells in the dark at room temperature for 15 minutes and were further carried for FACS analysis.

Cell cycle analysis:

PC cell lines tHPNE and Capan-1 were treated for GKI-1 at 10 μM, 25 μM and 50 μM concentration for 48h. After this, cells were treated with Nocodazole (#M-1404, Sigma, St. Louis, MO), (arrests cell cycle at G_2/M phase) for 16 h. The percentage of cells in G0/G1, S, and G2/M phases of the cell cycle was determined using flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA) after PI staining.

Organoid Culture:

Organoid cultures were performed as previously described [58]. PDX tumors were minced into 1–2 mm fragments then digested with 1mg/ml collagenase/dispase (#10269638001, Sigma, St. Louis, MO) for 30– 40 minutes. The digestion was stopped by adding an equal volume of 1%BSA in DMEM, then centrifuged at 1500 rpm x 5min. Pellets were further digested with Accutase for 30 minutes then collected by centrifugation at 1500 rpm x 5minand were grown in organoid growth medium containing Y-27632, 5% matrigel, and growth factors such insulin, FGF2 on pre-coated with matrigel.

Xenograft-tumor studies:

All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of UNMC. The tumorigenicity of cells under study was assessed using subcutaneous flank inoculation of 1×10^6 cells in 6-week-old athymic nude mice. Animals were observed for 7–8 weeks after the inoculation for tumor incidence/growth and then were sacrificed. Tumor volume was measured using the formula Tumor volume = $1/2(\text{length} \times \text{width}^2)/2$ as previously described [59, 60].

Statistical methods:

For all the experiments, statistical analysis was performed using Graphpad Prism software, including students' t-test and one-way ANOVA. All graphs were generated using Microsoft Excel and Graphpad Prism software. The error bars were calculated and represented in terms of mean±SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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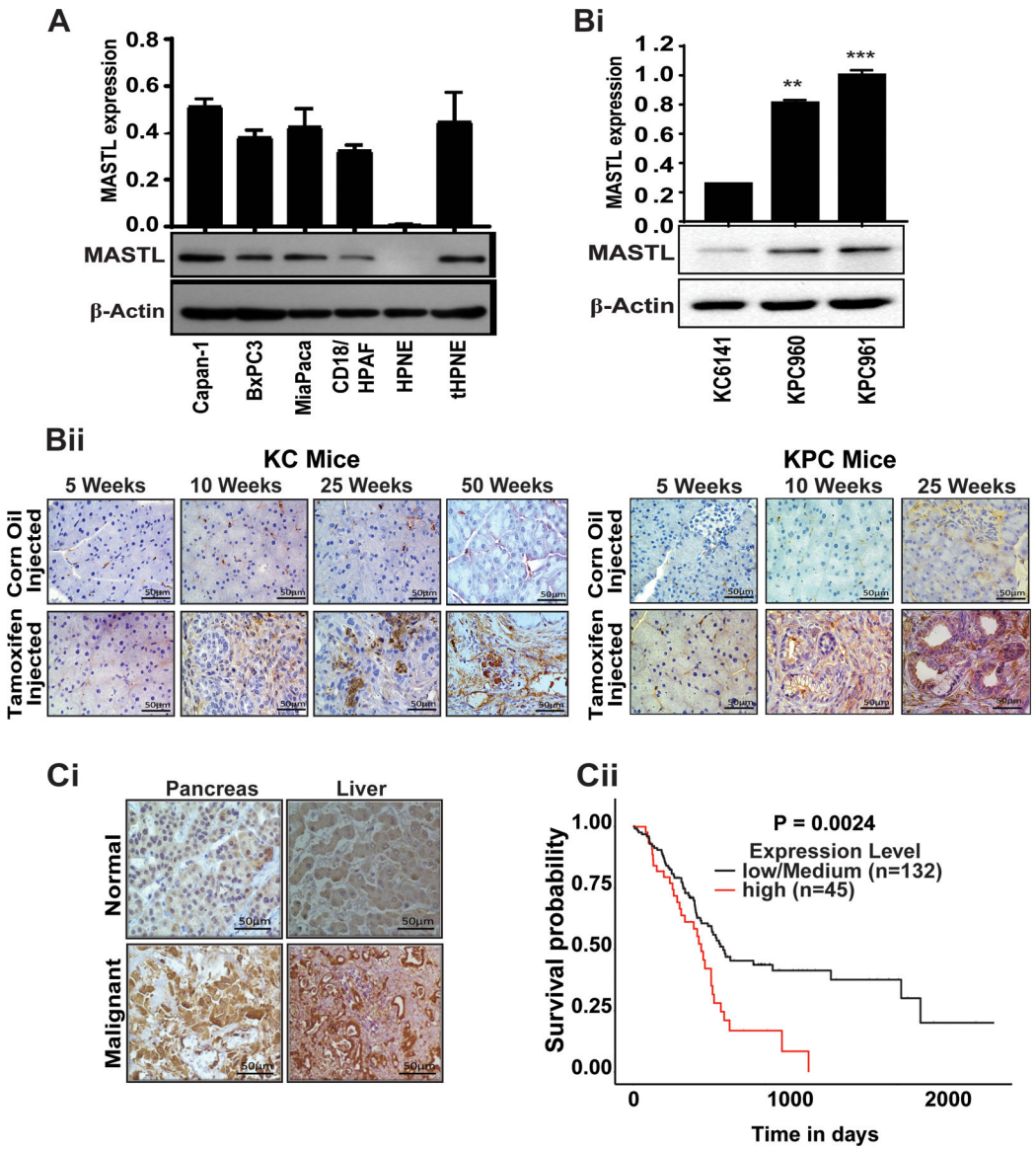

Figure 1. MASTL expression correlates with Pancreatic cancer

(Ai) MASTL expression was determined in different Pancreatic cancer cells by immunoblotting; (Bi) Detection of MASTL expression in cell lines derived from KC (KC-6141) and KPC mice (KPC-960, and KPC-961) PDAC cell lines; (Bii) MASTL expression in the different stages of tumors in corn oil and tamoxifen injected KC and KPC mice using IHC analysis; (Ci) MASTL expression in the tissue sections of normal and PC patients (scale equals 50μm); (Cii) Kaplan-Meier analysis in 177 pancreatic cancer patients to determine survival probability in correlation with MASTL expression. High MASTL expression (red line) correlated with considerably worse overall survival (*P = 0.0024*, n = 45–132) as compared to low MASTL expression (black), which correlated with better survival. For graphs, data represent mean ± SD; ***, P < 0.01; ***, P < 0.001;* vs KC6141 cells.

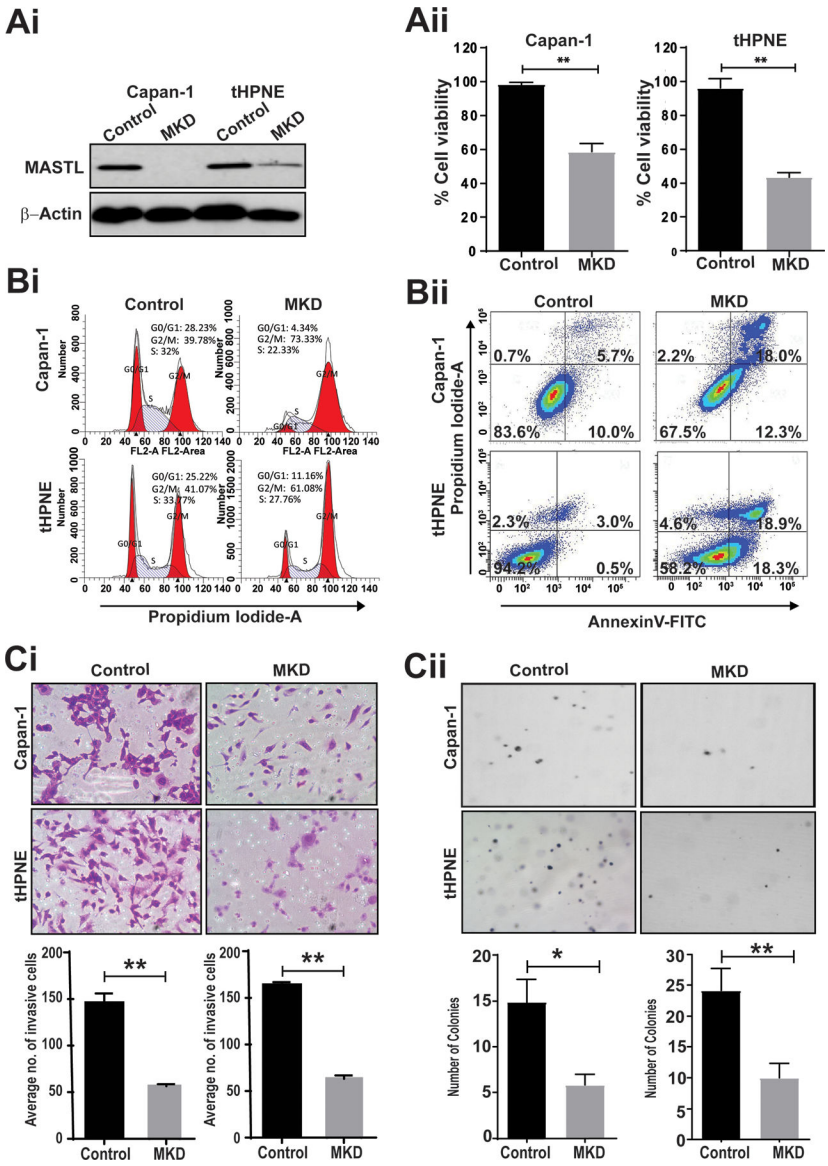

Figure 2. MASTL knockdown alters cellular functions to regulate pancreatic cancer cells.

(Ai) MASTL knockdown acquired by shRNA depletion of MASTL in Capan-1 and tHPNE cells was confirmed by immunoblotting; (Aii) Cell viability was determined in Capan-1^{MKD} and tHPNE^{MKD} cells as compared to respective controls *(P<0.01)*; (Bi) Capan-1^{MKD} and tHPNE^{MKD} cells were unable to overcome the blockage of G2/M phase as compared to control cells; (Bii) FITC Annexin V staining analysis via FACS at both early and late phases in Capan-1^{MKD} and tHPNE^{MKD} cells compared to control cells; (Ci & Cii) Invasive or tumorigenic potential of MASTL were assessed by the ability to invade in boyden chamber or form colonies in soft agar assay in Capan-1^{MKD} and tHPNE^{MKD} cells as compared to their respective controls. For graphs, data represent mean ± SD; **, P < 0.05; **, P < 0.01;* vs control.

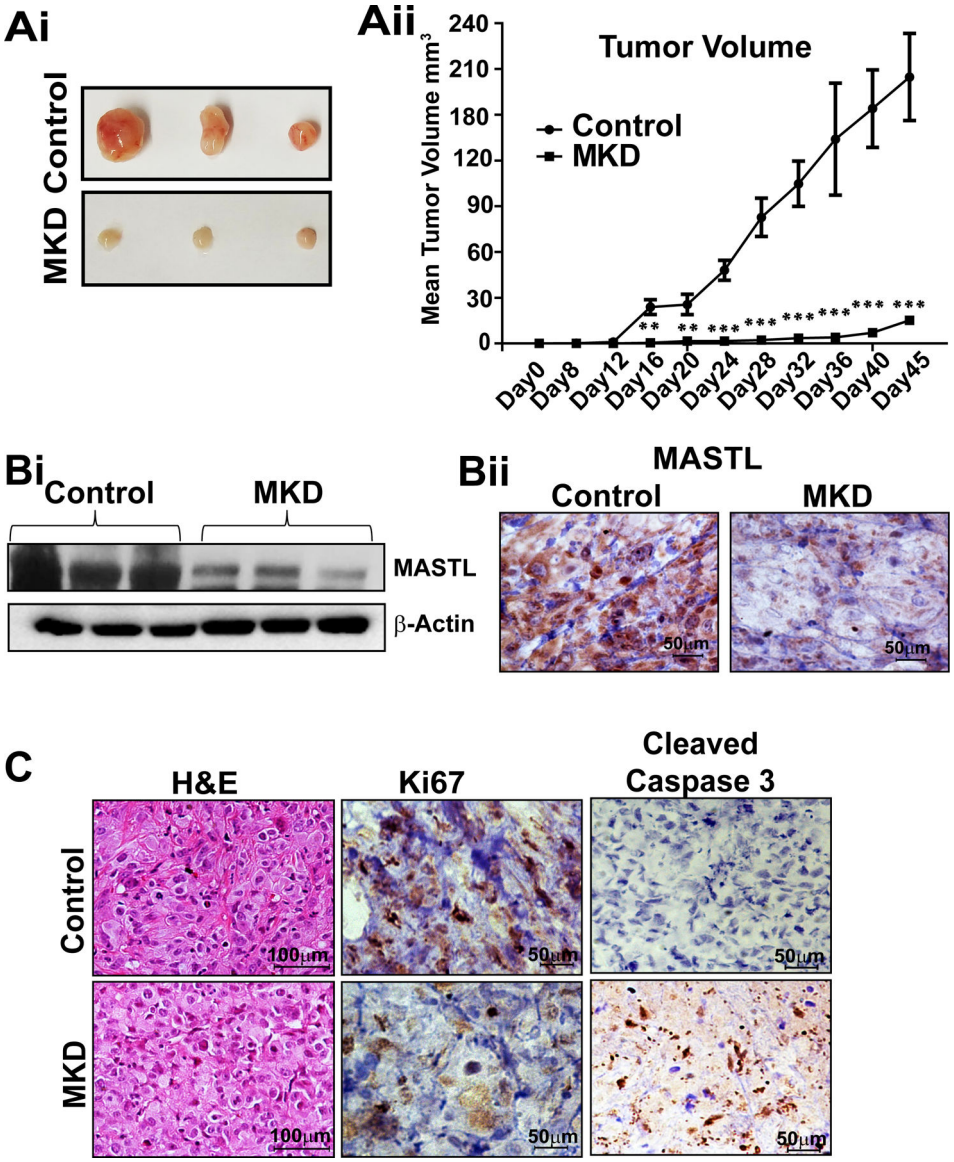

Figure 3. MASTL inhibition significantly inhibits tumor formation in xenograft model *in-vivo*.

(Ai) Tumor picture of tHPNE^{MKD} cells as compared to respective controls after 6 weeks of inoculation in athymic nude mice; (Aii) Tumor volume (mm^3) development, after subcutaneous injection (n = 5 mice per group) in Control and tHPNE^{MKD} cells; (Bi) MASTL expression was analyzed in tumor lysates by immunoblotting from 3 representative mice from each group; (Bii) IHC analysis of MASTL (scale equals 50μm) were observed in Control and MKD tumor sections (C) H & E (scale equals 100μm) and IHC analysis of proliferation marker (Ki67) and apoptotic marker (cleaved caspase-3) (scale equals 50μm) were observed in tumor sections. For graphs, data represent mean ± SD; **, P < 0.05;* vs control.

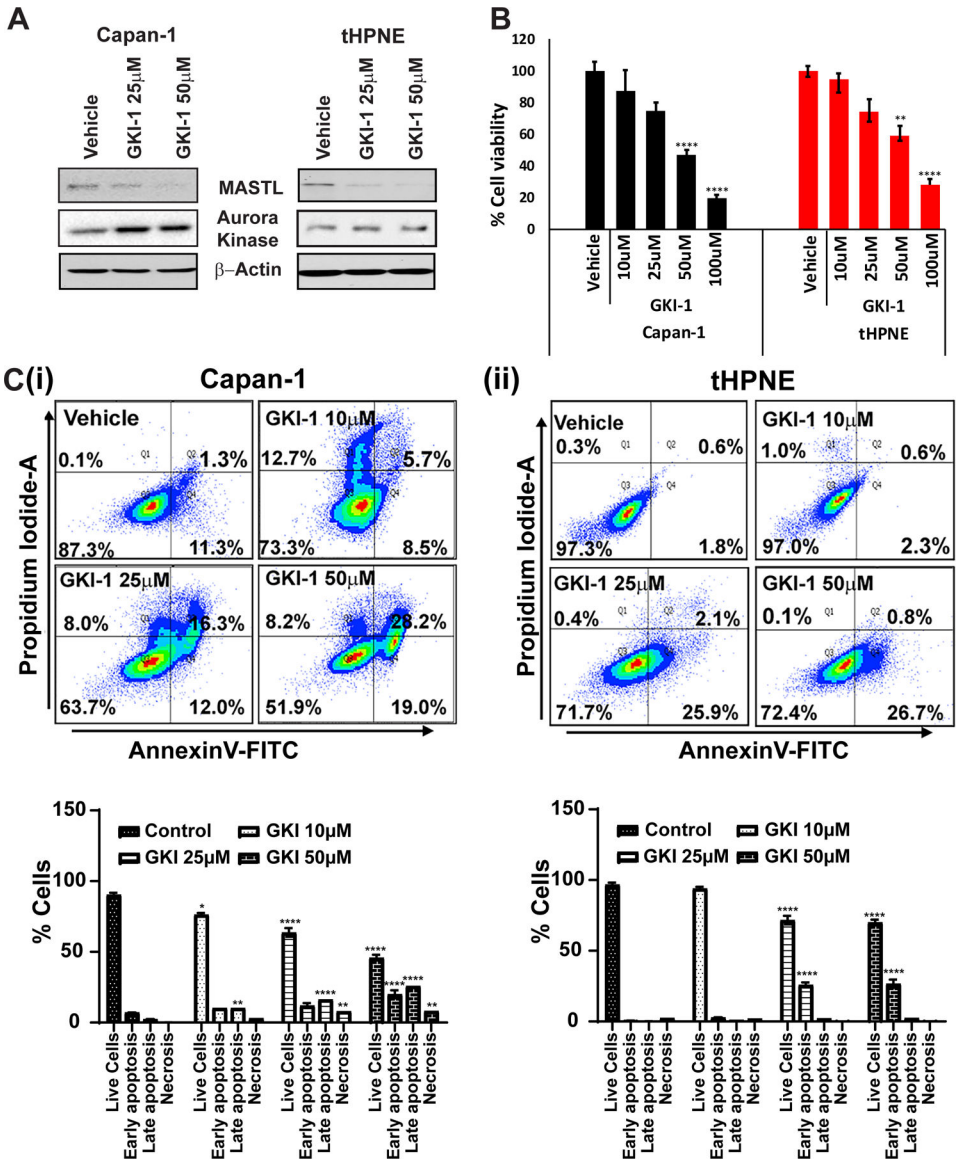

Figure 4. Greatwall Kinase Inhibitor (GKI-1) inhibited MASTL expression and altered functional characteristics in PC cell lines *in-vitro*.

(A) MASTL and Aurora kinase expression was determined in Capan-1 and tHPNE cells upon GKI-1 treatment (25 and 50μM) by immunoblotting; (B) GKI-1 treatment (10–100μM) significantly reduced cell viability in a dose dependent manner in both Capan-1 and tHPNE cells, compared with those in the control group; (Ci & ii) Cell apoptosis on treatment with GKI-1 (10, 25, 50μM) in Capan-1 and tHPNE cells. Cells were synchronized and FITC Annexin V staining analysis via FACS to determine apoptosis at both early and late phases. For graphs, data represent mean ± SD; ***, P < 0.01; ***, P < 0.001; ****, P < 0.0001* vs control.

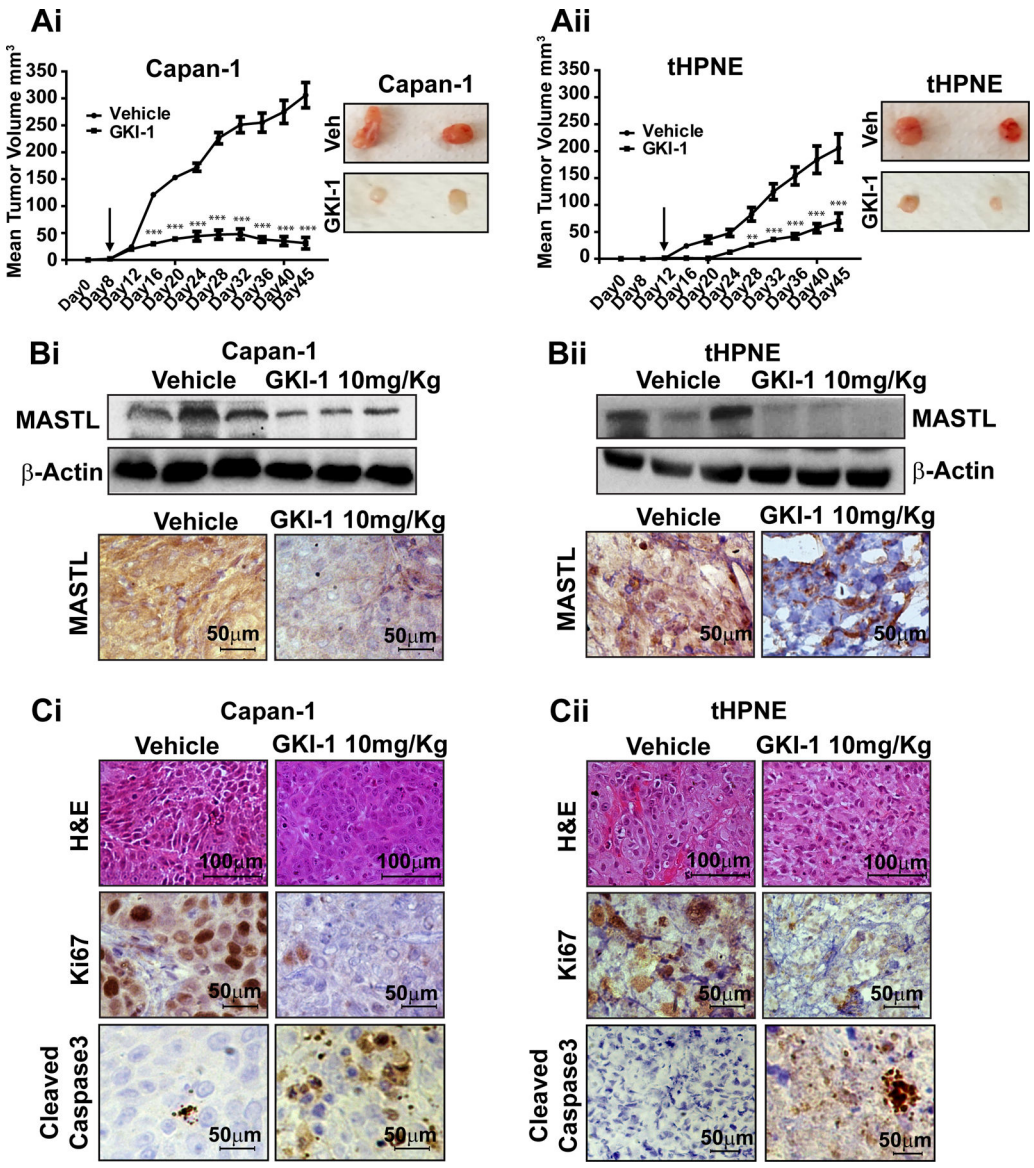

Fig. 5. Anti-tumorigenic potential of GKI-1 was analyzed on tumor xenograft in *in-vivo*.

(A) Tumor picture of vehicle and GKI-1 (10mg/kg; IP, intraperitoneal injection) treated athymic nude mice after 6 weeks of inoculation of (Ai) Capan-1 and (Aii) tHPNE cells. Flank xenograft tumor development, after subcutaneous injection (n = 5 mice per group), were compared between Control and GKI-1 (10mg/kg) treatment in Capan-1 and tHPNE cells injected nude mice; (Bi & ii) MASTL expression was measured upon GKI-1 (10mg/kg) treatment in tumor lysates by Immunoblotting from 3 representative mice from each group and by IHC analysis; (Ci & ii) H & E (scale equals 100μm) and IHC analysis of proliferation marker (Ki67) and apoptotic marker (cleaved caspase3) (scale equals 50μm) were observed in respective tumor sections. For graphs, data represent mean ± SD; ***, P < 0.01; ***, P < 0.001;* vs vehicle treated control group.

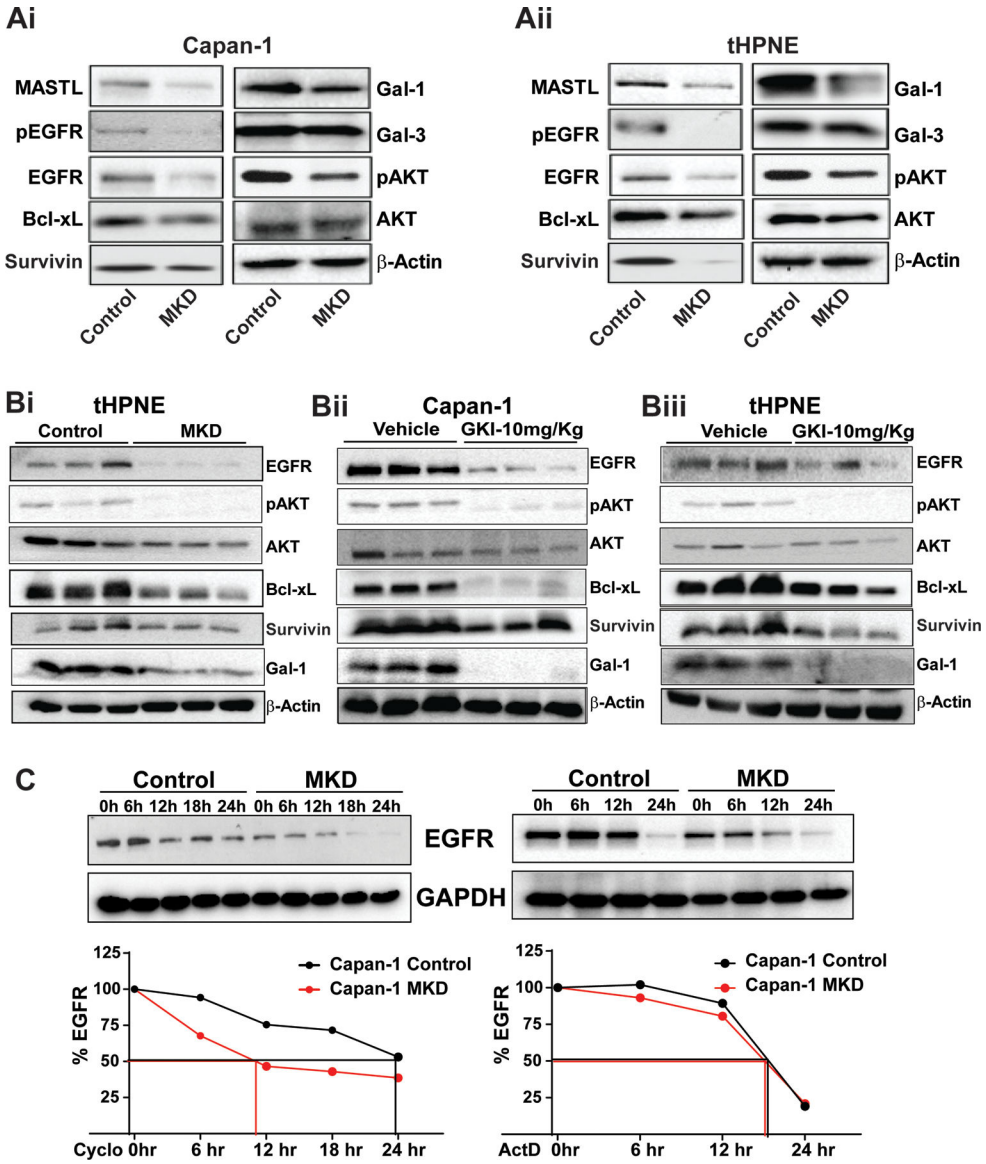

Fig. 6. MASTL mediates its effect through the regulation of EGFR/Galectin-1/Survivin/Bcl-xl signaling.

(Ai & ii) Immunoblotting analysis of MASTL, pEGFR, EGFR, Bcl-xl, Survivin, Galectin-1, Galectin-3, pAkt and Akt expression were observed in Capan-1^{con} and Capan-1^{MKD} cells; tHPNE^{con} and tHPNE^{MKD} cells; (Bi) EGFR, pAkt, Akt, Bcl-xl, Survivin and Galectin-1 expression were measured in tumor lysates of tHPNE^{con} and tHPNE^{MKD} by immunoblotting from 3 representative mice from each group; EGFR, pAkt, Akt, Bcl-xl, Survivin and Galectin-1 expression were measured in tumor lysates by immunoblotting from 3 representative mice from each group of (Bii) Capan-1 cells untreated or treated with GKI-1 (10mg/kg); (Biii) tHPNE cells untreated and treated with GKI-1 (10mg/kg) in athymic nude mice; (C) Capan-1^{con} and Capan-1^{MKD} were treated with Cyclohexamide or Actinomycin D at different time points (6h, 12h, 18h and 24h) and effects on MASTL protein or mRNA stability was determined.

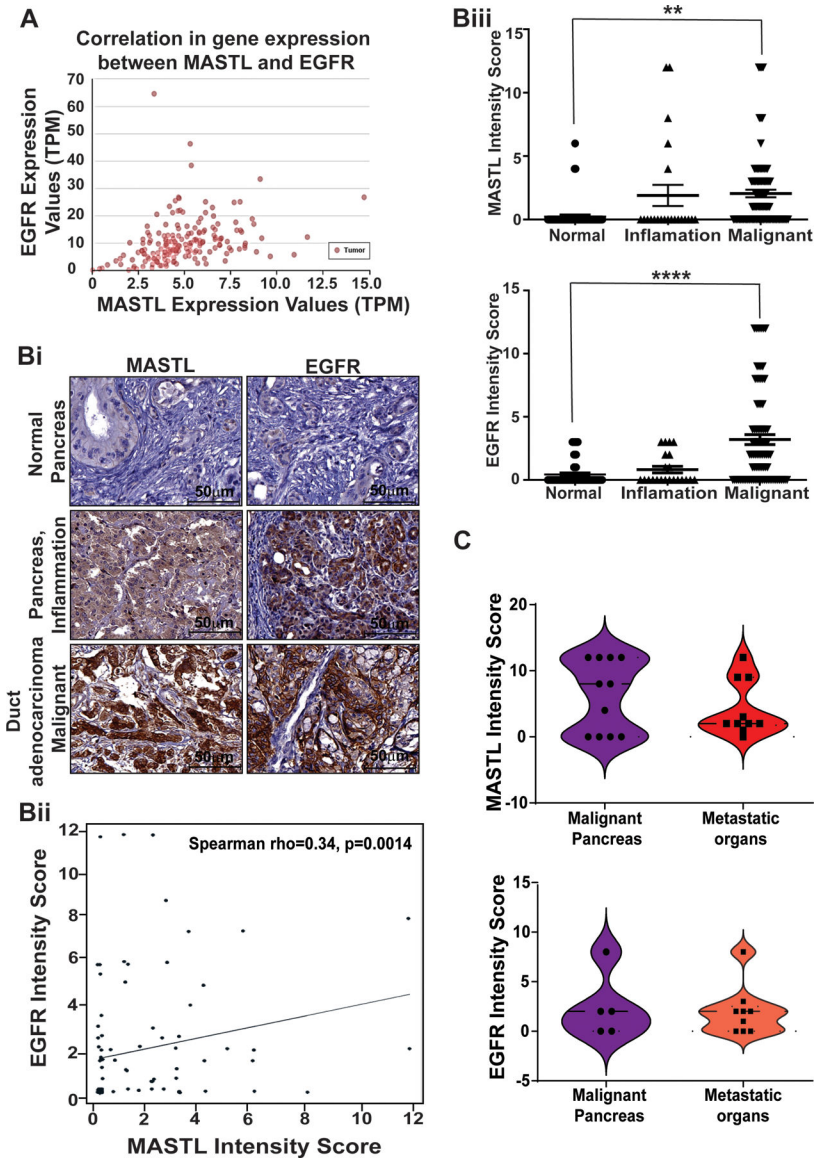

Fig. 7. MASTL expression correlates directly with EGFR expression in pancreatic cancer.

(A) TCGA data analysis to determine the correlation in gene expression between MASTL and EGFR using Scatter Plot analysis (Pearson's Correlation coefficient=0.31); (Bi) IHC analysis for MASTL and EGFR in the tissue sections from a commercially available human tissue microarray (scale equals 50μm) in normal, pancreatitis and ductal adenocarcinoma; (Bii) Scatter Plot analysis for above described patient samples demonstrated a Spearman correlation of rho=0.34, p=0.0014 for the correlation between EGFR & MASTL; (Biii) The intensity score of MASTL and EGFR was quantitated based on pathologic scoring and was plotted using dot plot; (C) Violin plot to determine intensity of MASTL and EGFR in matched primary and metastatic patient samples (12 samples) from in home array. For graphs, data represent mean ± SD; ***, P < 0.01; ****, P < 0.0001.*

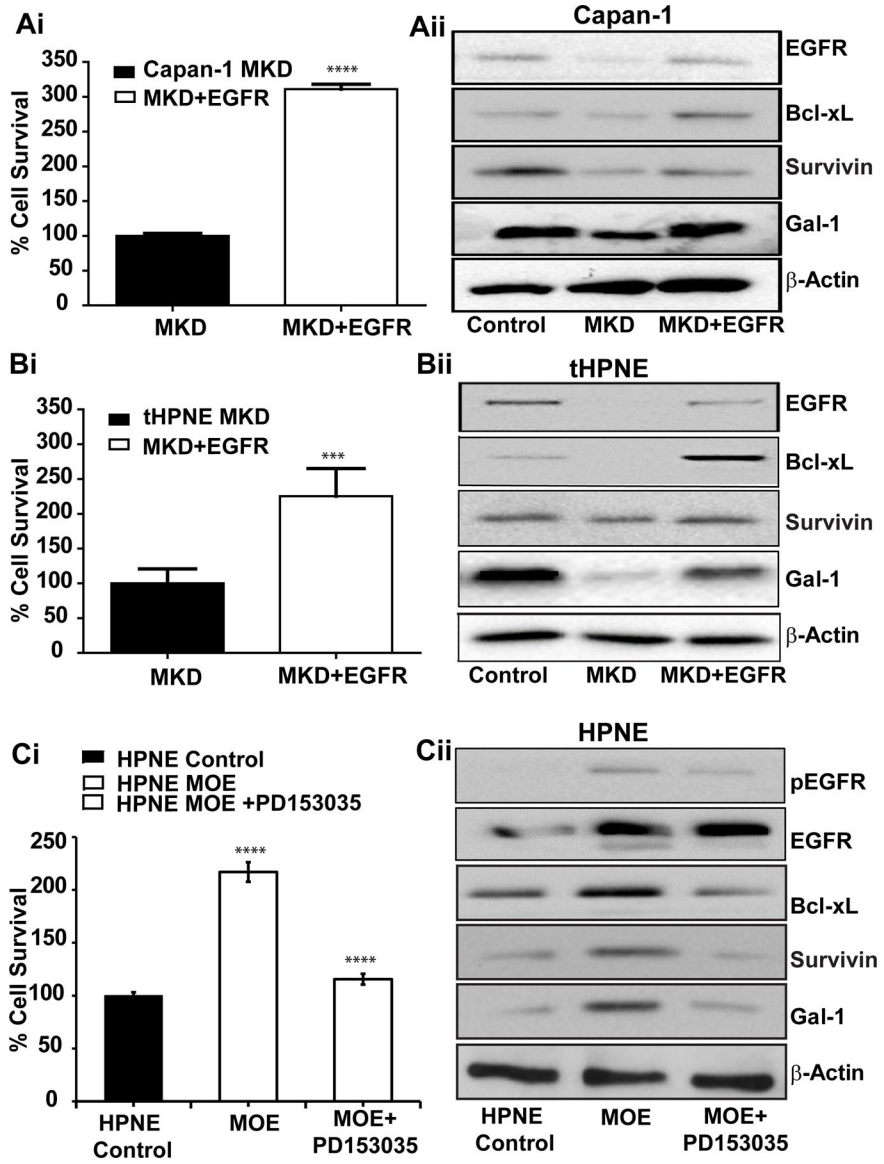

Fig. 8. EGFR overexpression in tHPNE^{MKD} and Capan-1^{MKD} cells abrogated the effects of MASTL inhibition on cell survival and downstream signaling.

(Ai & Bi) Cell survival was determined in tHPNE^{MKD} and Capan-1^{MKD} cells after EGFR overexpression; (Ci) HPNE cells overexpressing MASTL (MOE) were treated with EGFR-inhibitor PD153035 and effects on cell viability were determined; (Aii & Bii) Effect on EGFR, Bcl-xl, Survivin and Galectin-1 expression were determined by immunoblotting in Capan-1^{MKD} and tHPNE^{MKD} cells with overexpression of EGFR; (Cii) Effect on pEGFR, EGFR, Bcl-xl, Survivin and Galectin-1 expression were observed by immunoblotting in MASTL overexpressing HPNE cells (MOE) on treatment with EGFR inhibitor PD153035 (10μM). For graphs, data represent mean ± SD; **, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.*

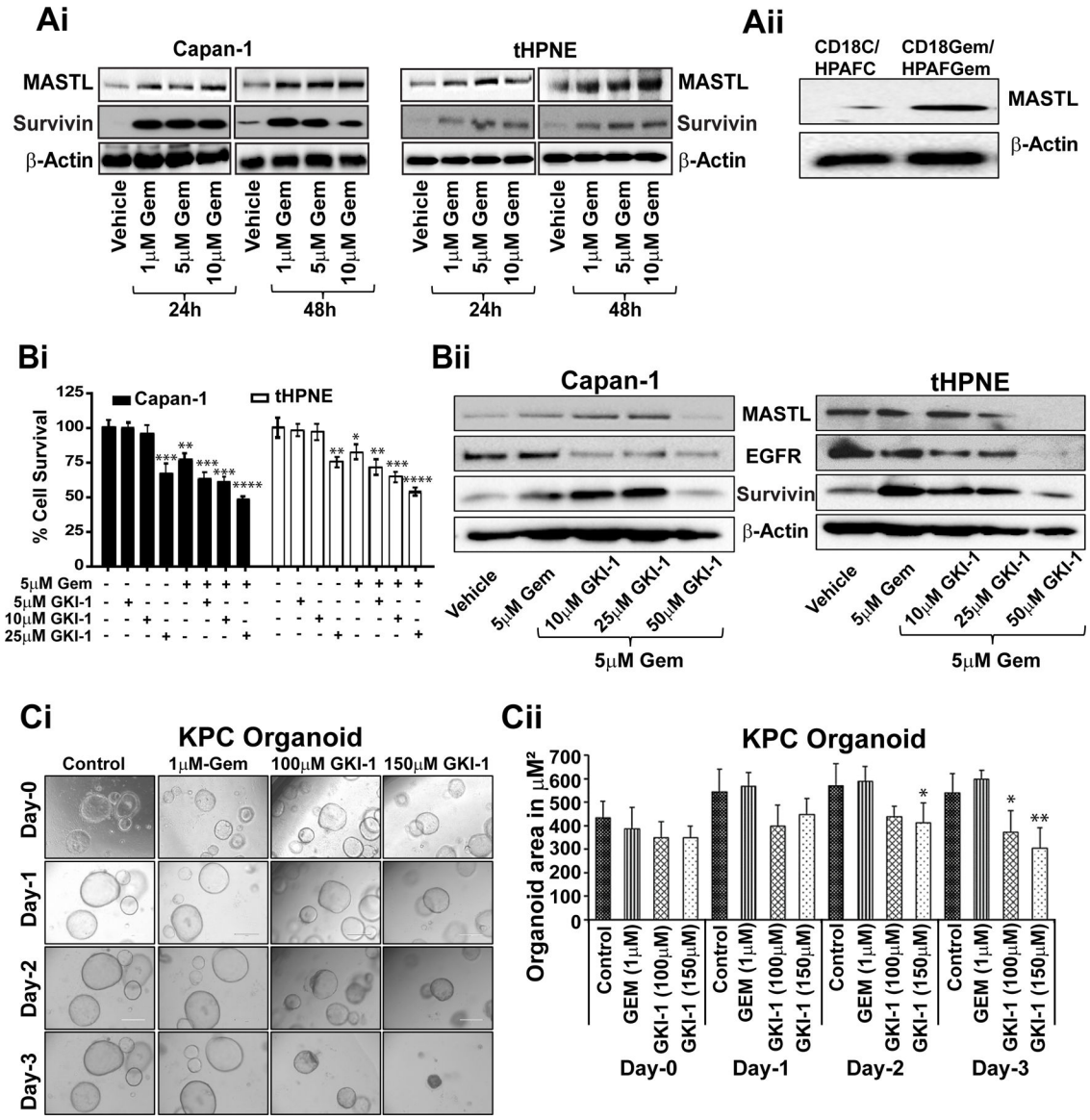

Fig. 9. MASTL expression enhances chemoresistance through EGFR/Survivin pathway.

(Ai) Capan-1 and tHPNE cells were treated with different concentrations of gemcitabine (Gem) (1, 5, 10μM) for 24h & 48h and effects on expression of MASTL and Survivin was determined by immunoblotting; (Aii) MASTL expression was determined in Control and Gem resistant CD18/HPAF cells; (Bi) Cell survival was determined in Capan-1 and tHPNE cells on treatment with Gem (5μM) alone or in combination with MASTL inhibitor-GKI-1 (5, 10 and 25μM); (Bii) Effects on MASTL, EGFR and Survivin expression in Capan-1 and tHPNE treated with Gem (5μM) alone or in combination with GKI-1 (10, 25, 50μM); (Ci & Cii) Representative bright-field images and bar graph of size of tissue organoids from KPC mouse which were treated with different concentration of GKI-1 (1,100 and 150μM). For graphs, data represent mean ± SD; **, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 vs* control.