ANGIOTENSIN II TYPE 2 RECEPTOR: A NOVEL MODULATOR OF INFLAMMATION IN PANCREATIC DUCTAL ADENOCARCINOMA THROUGH REGULATION OF NF-KB ACTIVITY.

Ankit V. Gandhi[1],[*]Galina Chipitsyna[2],Daniel Relles[1], Charles J. Yeo[1],Hwyda A. Arafat[2].

Authors affiliations:

[1]Departments of Surgery, Jefferson Pancreatic, Biliary and Related Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA, 19107, [2]Department of Biomedical Sciences, University of New England, Biddeford, ME, USA, 04005.
*galina727@gmail.com

ABSTRACT:

Aims: The aim of this study was to explore potential pro-inflammatory, pro-migratory and pro-invasive roles of angiotensin II type 2 receptor (AT2R) in pancreatic ductal adenocarcinoma (PDAC) cells.

Study design: AT2R-specific agonist (CGP42112A, CGP), blocking antibody, or siRNA were interchangeably used to analyze functional impact of AT2R on PDAC cell proliferation, migration, and invasion.

Results: This study revealed that AT2R blockade reduced proliferation, migration and invasion of PDAC cells. Blocking AT2R significantly lowed the expression of oxidative-stress genes Nox1, Nox3, Nox4, and Nox5, and pro-invasive genes MMP-2, MMP-9, OPN, and α5-integrin. AT2R siRNA significantly downregulated the expression levels of pro-inflammatory IL-1beta, IFN-γ, iNOS, IL-6, IL-8, and IL-15 mRNA. Blocking AT2R also inhibited the intrinsic and angiotensin II (AngII) or CGP-mediated activation of NF-κB.

Conclusion: These findings demonstrate previously unknown pro-inflammatory, pro-migratory and pro-invasive effects of AT2R in PDAC cells. Our data suggest that one mechanism by which AT2R promote inflammation is through activation of constitutive and AngII-mediated NF-κB. Thus, AT2R blockade could be a novel therapeutic strategy to target multiple pathways that mediate PDAC carcinogenesis.

INTRODUCTION:

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of adult cancer mortality in the United States (1). Associated with poor prognosis and a 5-year survival rate of 5%, this aggressive and chemo-resistant cancer metastasizes early to distant organs prior to diagnosis. Symptoms are usually nonspecific until cancer has reached an advanced stage (2). As a result, elucidating PDAC's carcinogenic mechanism would significantly aid in the development of novel and effective therapies.

The renin-angiotensin system (RAS) is a major regulator of blood pressure and cardiovascular homeostasis. The

effects of angiotensin II (AngII) are mediated via two G-protein-coupled receptors, angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors. Most physiological effects of AngII have been attributed to stimulation of the AT1R (3), whereas AT2R often functions as a counter-regulatory receptor (4).

There is increasing evidence that angiotensin II (AngII) is involved in regulation of cell proliferation, angiogenesis, inflammation and tissue remodeling, and associated with cancer (5-7). AngII has been shown to stimulate VEGF in various types of cancer cells (8). Importantly, blocking AngII has been reported to decrease preneoplastic lesions, cancer cell growth, angiogenesis, and VEGF levels in many experimental models of cancer (9-19). Epidemiologic studies involving hypertensive patients have suggested that blocking AngII may decrease cancer risk (20-22). AT1R overexpression correlates with tumor invasiveness. These data suggest that blockade of the AT1R could be an effective anticancer strategy (11-19). Thus, AT1R antagonists that are already used clinically as potent antihypertensive drugs with mild side effects can be considered as potential tools in future anticancer strategies. However, if AT1R blockers are to be used in anticancer therapy, it is important to assess the role of the AT2R in tumor progression and angiogenesis. Indeed, long-term blockade of the AT1R subtype leaves AT2R fully activated. Studies have shown AT2R can be proangiogenic and to work in concert with the AT1R subtype to increase VEGF levels and blood vessel formation (23-25), thus leaving open the question of whether AT2R activation has beneficial or deleterious effects on tumor progression.

Several studies have identified a local renin angiotensin system in the pancreas and described its role in promoting the progression of pancreatic ductal adenocarcinoma (PDAC) (26-27). We and others have described the role of angiotensin II on the type 1 receptor in promoting PDAC inflammation and angiogenesis (28-30). However, the role of type 2 receptor (AT2R) in PDAC progression has not been identified.

We showed recently that in PDAC cells, AT2R plays a role in tumor metabolism through mediating the induction of fatty acid synthase by AngII and through down-regulating the activity of AMP-activated protein kinase (AMPK) (31). In this study, we explored potential pro-inflammatory, pro-migratory and pro-invasive roles of AT2R in PDAC cells.

METHODS:

Immunohistochemistry. To localize AT2R, formalin fixed, paraffin embedded tissue blocks from PDAC tissue, matching controls and intraductal papillary mucinous neoplasms (IPMN) were prepared. 5 μm-thick serial sections were incubated for 3 h at 4°C with a rabbit polyclonal antibody against human AT2R (1:200) (Santa Cruz Biotechnology). A vectastain universal elite ABC kit and 3,3'-diaminobenzidine tetrahydrochloride chromogenic substrate (Vector Laboratories Inc.) were used according to the manufacturer's protocol to visualize the tissue

reaction. Antibody specificities were validated with nonimmune isotype serum.

Cell culture and treatment. MiaPaCa-2and AsPC-1 cells, originally established from a primary PDAC, were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum in a humid atmosphere of 5% CO_2. MiaPaCa-2 cells were transfected with scrambled or AT2R siRNA, treated with and without the AT2R agonist, CGP42112A (10^{-5} M), for 24h, collected and examined for a panel of proinflammatory and metastatic genes, and genes involved in ROS expression. To evaluate NFκB activity, MiaPaCa-2 cells were transfected with scrambled or AT2R siRNA, treated with and without AngII (10^{-7} M) and CGP42112A (10^{-5} M) for one hour, and analyzed for NFκB activity via ELISA (ENZO LIFE SCIENCES INT'L, INC.). Angiotensin II was purchased from AnaSpec Inc., CGP42112A was purchased from Sigma Chemical Co.

MTT assay. To examine the effect of blocking antibody for AT2R on in vitro cell proliferation, MiaPaCa-2 and AsPC-1 cells were plated in 48-well plates and incubated in growth medium at 37°C and 5% CO_2. The cells were treated with or without AT2R antibody. MTT conversion assay was used to measure cell viability. MTT (Sigma Chemical Co.) was added (500 µg /ml) for 4h after 24, 48, and 72 h of incubation. The resulting formazan crystals were solubilized with DMSO, and the optical density was measured at 570 nm. Optical density correlated with extent of cell viability and proliferation.

Cell migration assay. 2×10^5 MiaPaCa-2 or AsPC-1 cells were plated in 6-well plates (Falcon Becton Dickinson), and incubated in growth medium at 37°C and 5% CO_2.A scratch was made through the bottom of the well using 1 ml sterile pipette tip 24 h later. The scratch width was measured at three marked locations per well. Cells were treated with or without blocking AT2R antibody (0.4µg/ml), incubated for 24-72 h, and scratch width was measured at the same marked locations. Migration distance was estimated using ImageJ software. Closure was calculated as a percent of initial scratch width.

Transwell invasion assay. The BD24-well invasion chambers were used in accordance with manufacturer's protocol (BD Biosciences). 1×10^5MiaPaCa-2 or AsPC-1 cells were seeded onto 8µM pore polycarbonate membrane of the upper chamber in serum-free medium, lower chamber contained full growth medium with or without AT2R blocking antibody (0.4µg/ml). After 48-72 h of incubation cells were removed from the upper chamber, and cells that migrated through the membrane to the lower surface were treated with MTT solution for 4h. The resulting formazan crystals were solubilized with DMSO, and the optical density was measured at 570 nm. Optical density correlated with the number of cells migrated through transwell membrane.

RNA extraction and reverse transcriptase PCR. Total RNA was isolated using Tri-Reagent (Life Technologies). RNAs were quantified and cDNAs were synthesized using ImProm-II™ Reverse Transcription System (Promega).

TaqMan gene expression assays for pro-inflammatory genes (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-15, IFN-gamma, TNF-alpha, iNOSand MCP-1), metastatic genes (MMP-2, MMP-9, VEGF, OPN, α5- and α7- integrins),reactive oxygen species(ROS) genes (Nox1, Nox2, Nox3, Nox4, and Nox5), and GAPDH (internal control) were purchased from Applied Biosystems.cDNAs were subjected to real-time qPCR using HotStart-ITor VeriQuest Probe qPCR Master Mixes (Affymetrix) and TaqMan technology(7500 Sequence Detector, Applied Biosystems). The relative mRNA levels were quantified using Applied Biosystems software.

NF-κB ELISA. Cell lysates were obtained from MiaPaca-2 cells transfected with scrambled or AT2R siRNA, treated with and without AngII (10^{-7} M) and CGP42112A (10^{-5} M). Elisa kit (ENZO LIFE SCIENCES INT'L, INC.) was used to quantify the active form of p65 NF-κB subunit according to the protocol. Chemiluminescence activity was measured using Veritas Microplate Luminometer (Turner Designs).

Statistical analyses. All experiments were performed 3 to 5 times. Data were analyzed for statistical significance by ANOVA with post-hoc Student's *t*-test analysis. Data are presented as mean ± SEM. Continuous, normally distributed variables were analyzed by Student's *t*-test. Analyses were performed with assistance of a computer program (JMP 5 Software). Differences were considered significant at $p \leq 0.05$.

RESULTS:

Expression of AT2R in normal and pancreatic lesions. Immunohistochemical analysis showed that in normal pancreas, AT2R protein was mainly detected in pancreatic islets. In intraductal papillary mucinous neoplasms (IPMN), AT2R staining could be detected in the mucin-filled ductal cells. In PDAC lesions, AT2R immunoreactivity was mainly localized to the ductal membrane and cytoplasm of the tumor epithelial cells and, to a lesser degree, in the stromal cells (Figure 1). These data show the active production and expression of AT2R in PDAC cells in vivo.

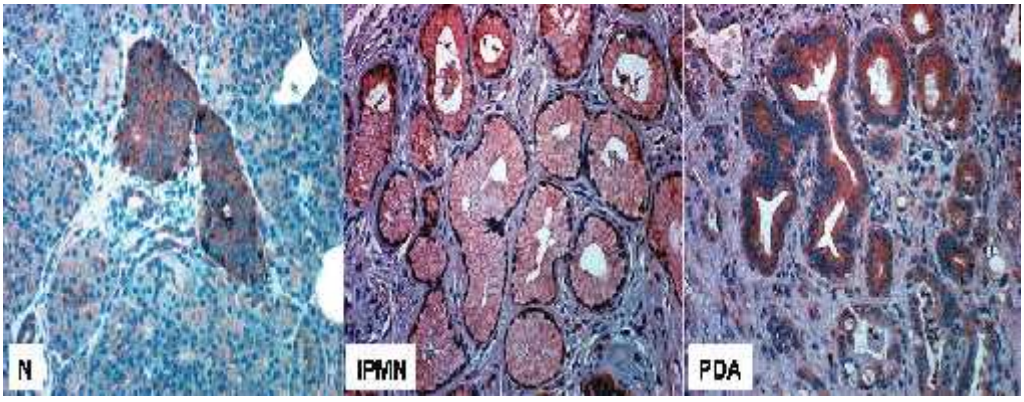

Figure 1: Representative immunohistochemical staining for AT2R in normal pancreatic islets (left panel), IPMN (middle panel), and PDAC (right panel). PDAC cells show more intense and darker staining compared to normal pancreatic islets and IPMN. (X200 original magnification).

AT2R blockade by AT2R antibody decreases cell proliferation. The addition of AT2R antibody significantly ($p<0.05$) decreased cell proliferation in MiaPaCa-2 cells after 48h (Figure 2A), and in AsPC-1 after 72h (Figure 2B).

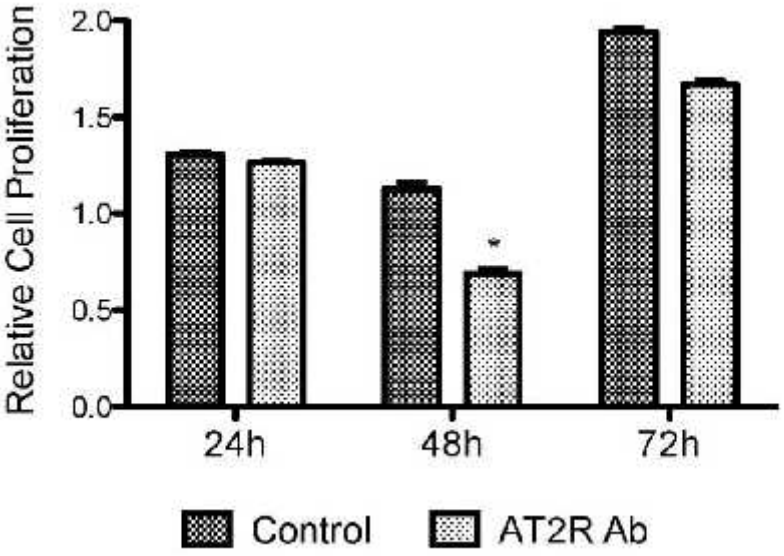

Figure 2A: AT2R blockade reduces cell proliferation in MiaPaca-2,*$p < 0.05$.

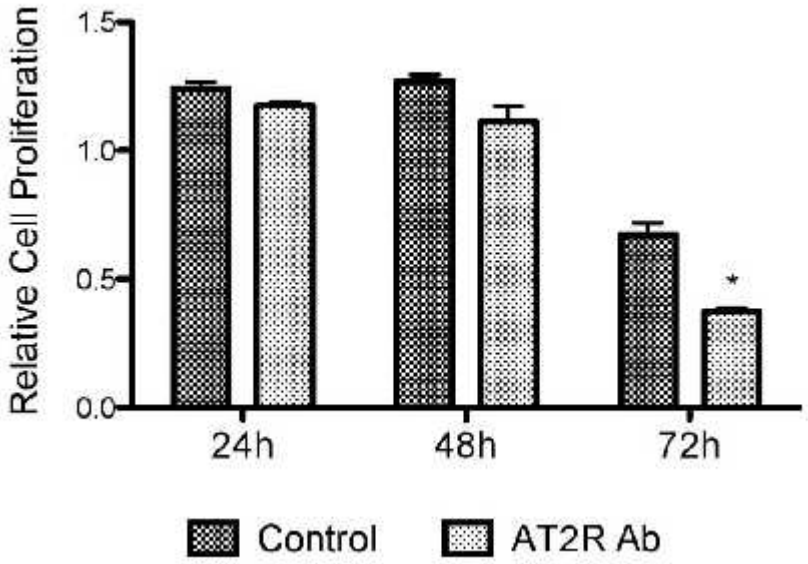

Figure 2B: AT2R blockade reduces cell proliferation in AsPC-1,*$p < 0.05$.

AT2R blockade reduces cell migration. AT2R blockade by AT2R antibody significantly ($p<0.05$) inhibited cell migration in both cell lines. It was evident in MiaPaCa-2 cells at all time points (Figure 3A), and in AsPC1 cells after 48h (Figure 3B).

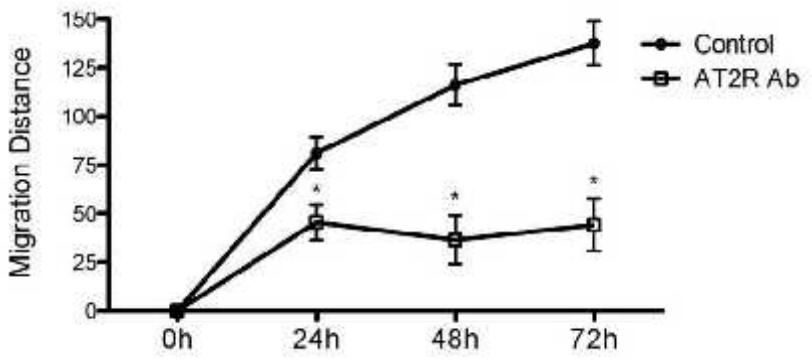

Figure 3A: AT2R blockade reduces cell migration in MiaPaca-2 cells,*p < 0.05.

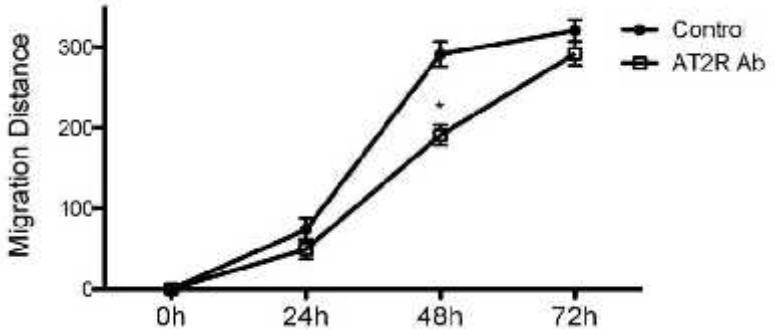

Figure 3B: AT2R blockade reduces cell migration in AsPC-1 cells,*p < 0.05.

AT2R blockade by AT2R antibody inhibits cell invasion. Adding AT2R antibody to the growth medium of MiaPaCa-2 cells significantly (p<0.05) reduced cell invasion (Figure 4).

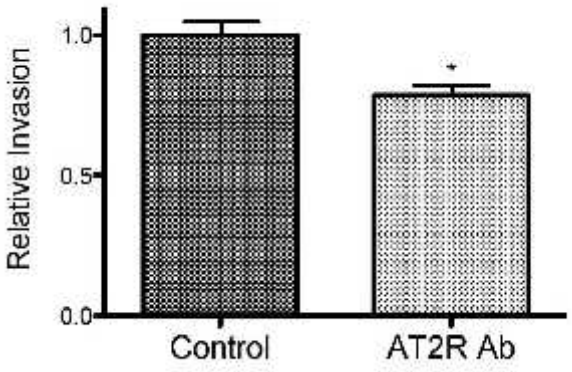

Figure 4: AT2R blockade causes significant decrease in MiaPaca-2 cells invasion at 48h,*p < 0.05.

Silencing AT2R reduces transcription of genes involved in oxidative stress, metastasis and inflammation.

MiaPaCa-2 cells were transfected with AT2R siRNA and tested for mRNA expression in panels of genes involved in oxidative stress, metastasis and inflammation. (Figure 5A, B, C). Silencing of AT2R significantly ($p<0.05$) reduced the expression of the ROS-inductive genes (Nox1, Nox3, Nox4, and Nox5) and pro-invasive genes (MMP-2, MMP-9, OPN, α5 integrin), whereas VEGF levels were not affected. In the panel of pro-inflammatory genes, silencing AT2R had an overall anti-inflammatory effect. AT2R siRNA significantly ($p<0.05$) reduced IL-1beta, IFN-γ, iNOS, IL-8 and ($p<0.01$) IL-6, IL-15 mRNA expression levels. TNF-α, MCP-1, IL-4 and IL-10 mRNA expressions were not significantly reduced. These data indicate that AT2R may play a role in PDAC inflammation, invasiveness and metastasis.

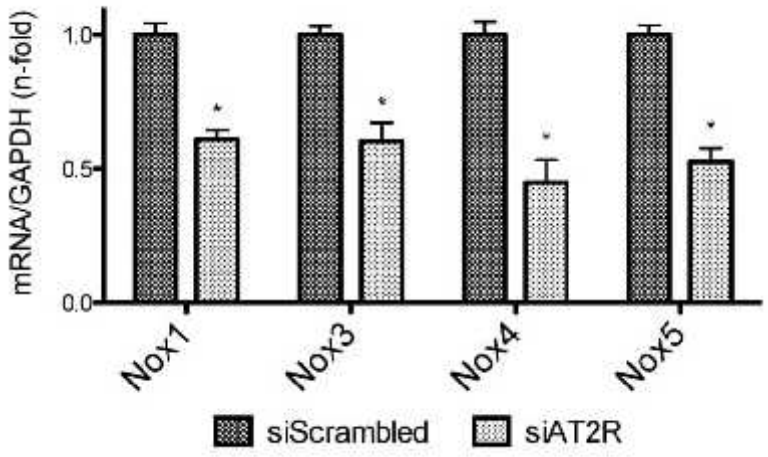

Figure 5A: AT2R silencing reduces the transcription of genes involved in oxidative stress, *p < 0.05

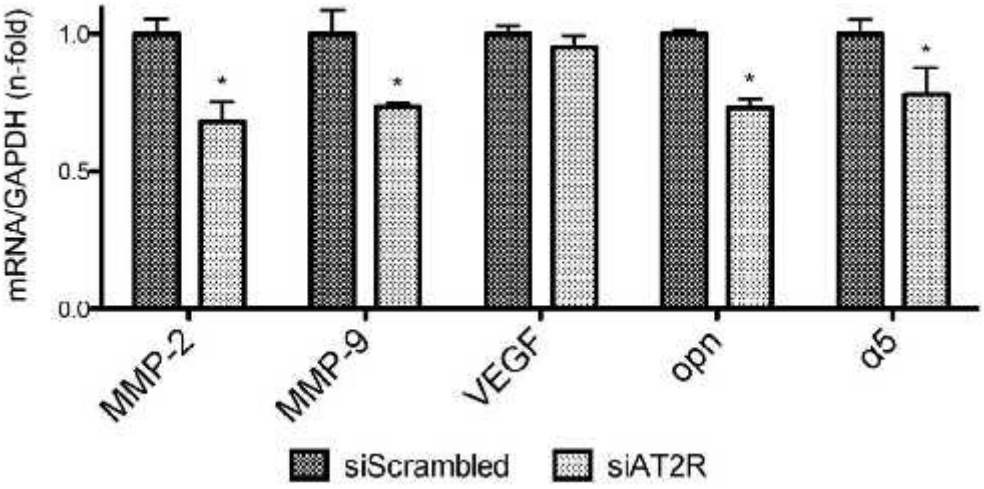

Figure 5B: AT2R silencing reduces the transcription of genes involved in oxidative metastasis, *p < 0.05

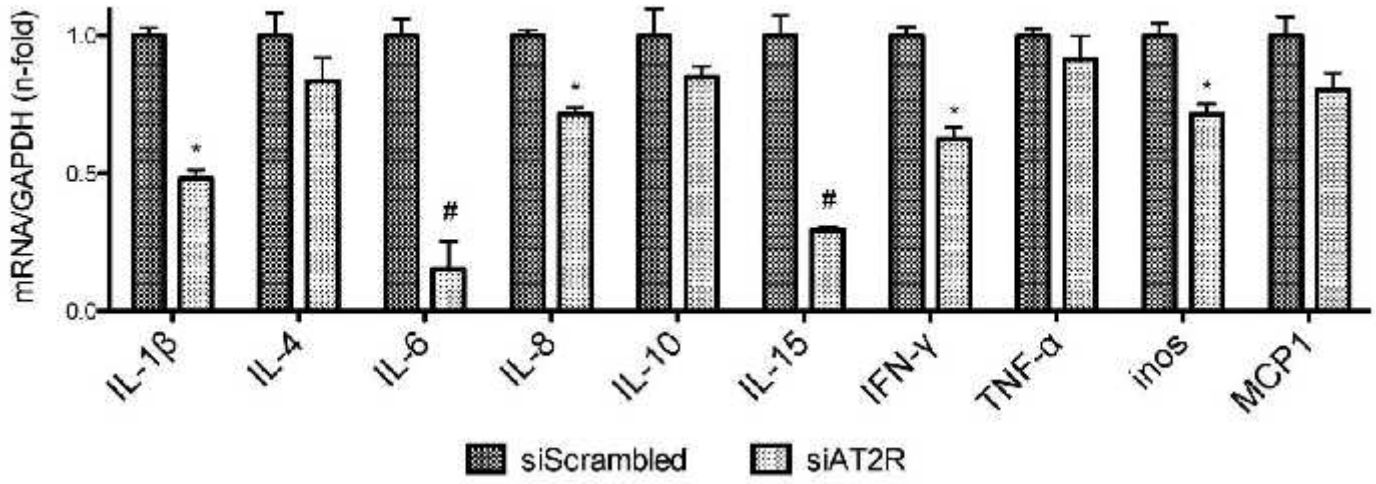

Figure 5C: AT2R silencing reduces the transcription of genes involved in inflammation,*p < 0.05 and #p < 0.01.

Silencing AT2R reduces constitutive and AngII-induced NF-κB activity. Since NF-kB is a major transcription factor activator of the panel of genes involved in inflammation that were affected by AT2R silencing, we examined whether AT2R stimulation or inhibition would directly affect NF-kB activity. Silencing of AT2R significantly ($p<0.01$) reduced the constitutive activity of NF-κB by 40% (Figure 6A) in MiaPaCa-2 cells. While AngII significantly ($p<0.05$) increased NF-κB activity, again this effect was reduced by more than 40% when AT2R was silenced In MiaPaCa-2 cells ($p<0.01$).

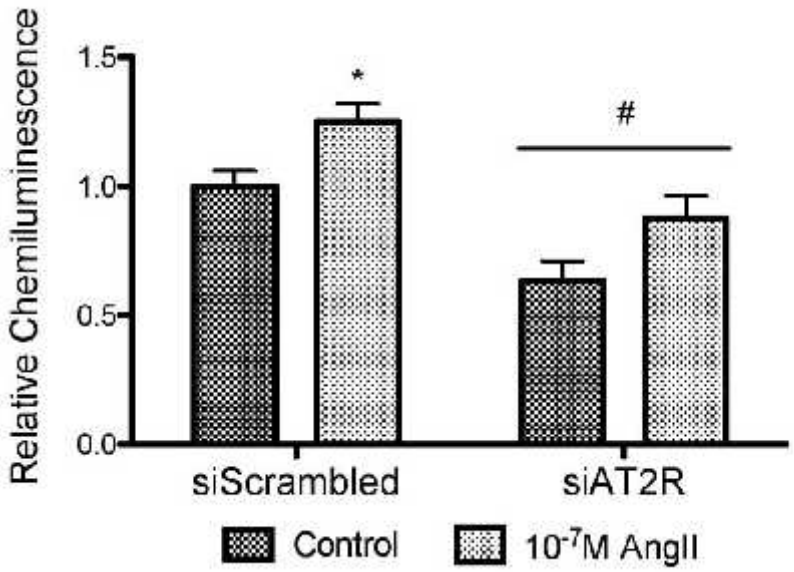

Figure 6A: AT2R silencing reduces constitutive, and AngII-induced NF-κB activity in MiaPaca-2 cells, *p < 0.05 and #p < 0.01.

AT2R agonist activates NF-κB. Activation ofAT2R with AT2R agonist resulted in significant ($p<0.05$) activation of NF-κB to levels similar to those obtained by AngII, an effect that was significantly reduced ($p<0.01$) when AT2R was

silenced. In MiaPaCa-2 cells (Fig 6B). These findings suggest that one mechanism by which AT2R is involved in promoting inflammation is through activation of constitutive and AngII-mediated NF-κB.

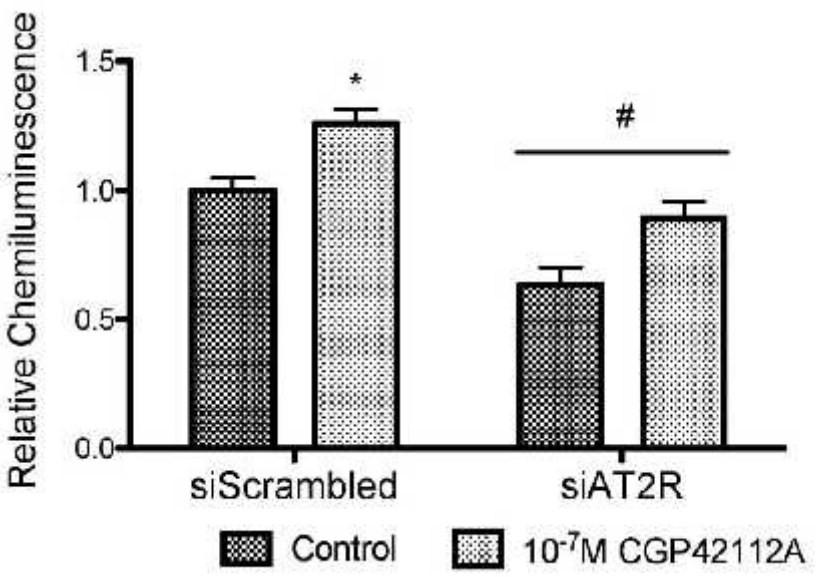

Figure 6B: AT2R silencing reduces CGP42112A-induced NF-κB activity in MiaPaca-2 cells, *p < 0.05 and #p < 0.01.

DISCUSSION:

Recent evidence indicates that angiotensin II is an important activator of cancer cell inflammation and angiogenesis via several signaling events mediated mainly through AT1R (11-19). AT2R's involvement has been controversial and not properly investigated (28). High levels of AT2R have been demonstrated in several types of cancer (32-36). Recently we showed that AT2R plays a critical role in PDAC lipogenesis through regulating AMPK activity (31).Proliferative effect of AT2R in PDAC cell lines seems to be consistent with other studies evaluating AT2R effect on various tumors (33-35). Similarly, the pro-migratory and invasive nature of AT2R appears to be validated by evidence demonstrating a dual (complimentary) role of AT1R and AT2R. In contrast, there have also been experiments detailing an opposite, anti-proliferative effects of AT2R in other tissue-specific tumors (28), a clear deviation from our findings in PDAC. Accordingly, AT2R might seem to generate differential proliferative effects depending on tissue origin. An explanation for such discrepancies has yet to be determined. Furthermore, more studies to discern the individual roles for AT1R and AT2R are necessary to determine whether their effects are complimentary.

Previous studies have implicated NAPDH oxidase in the development and progression of pancreatic cancer via the production of reactive oxygen species. ROS accumulation promotes pancreatic cancer survival by inhibiting apoptosis

(36) and enhances endothelial tumor growth in murine models (37).Furthermore, angiotensin II has been shown to induce ROS production via the type I receptor (38). Our findings indicate that blockade of AT2R reduces expression of NADPH isoforms Nox1, Nox3, Nox4, and Nox5, which ultimately decreases ROS levels. Hence, AT2R blockade may reduce cell survival through reduction of the Nox genes.

The lethality of pancreatic ductal adenocarcinoma is attributed to its metastasis through the extracellular matrix (ECM). Both tumor cell movement and growth are controlled by integrins that interact with ECM proteins, such as fibronectin. The major integrin responsible for fibronectin binding is α5β1, which is crucial for invasive and metastatic potential of cancer. Correlated with α5-subunit expression (39), matrix metalloproteinases help break down the extracellular matrix and further allow malignant tumors to invade surrounding tissue. Thus, the overexpression of MMPs has been intimately linked with highly aggressive cancers, such as ovarian, melanoma, breast, and colorectal. Another necessary component of metastasis and tumor progression is osteopontin (OPN), which has been shown to confer a migratory phenotype (40).Our study showed that silencing of AT2R significantly ($p<0.05$) reduced the expression of all those pro-invasive genes (MMP-2, MMP-9, OPN, α5 integrin) that resulted in reduction of invasive nature of MiaPaca-2 and AsPC-1 pancreatic cancer cell lines.

The main regulator of inflammation is Nuclear Factor kappa Beta (NF-κB), a transcription factor that regulates the expression of cytokines and ROS-inducing genes, among others. Dimerization and nuclear translocation of NF-κB lead to activation of such genes (41). Furthermore, NF-κB pathway is essential for many human cancers. Therapeutics that interfere with NF-κB signaling are of great clinical interest (42).We demonstrated that silencing AT2R blocked the expression of proinflammatory, prometastatic, and ROS-inducing genes – all genes that are transcriptionally activated by NF-κB. Our data also showed that AT2R regulated the activity of NF-κB. However, current evidence regarding AT2R's function in inflammation is conflicting. While it was originally thought that AT2R functioned to antagonize the effects of AT1R, the type 2 receptor activation has been linked to elevated oxidative stress and inflammation (4, 7, 25). Accordingly, further studies are required to elucidate the exact mechanism(s) through which AT2R mediates the regulation of NF-κB activity.Altogether, our results demonstrate a previously undescribed role of AT2R of promoting proliferative and invasive changes via the regulation of NF-κB activity, a central mediator of inflammation, in pancreatic ductal adenocarcinoma.

CONCLUSIONS:

In this research study, we demonstrated that blocking AT2R elicited anti-proliferative, anti-migratory, and anti-invasive properties of MiaPaca-2 and AsPC-1 pancreatic adenocarcinoma cell lines. Also, silencing AT2R correlated with lower mRNA expressions of key pro-inflammatory, pro-metastatic, and ROS-inductive genes. Lastly, AT2R silencing was

correlated with a significant reduction of NFκB activity while addition of AT2R agonist increased such activity. Based on these results, we introduce AT2R as a key player in PDAC progression that acts mainly by activating inflammation and invasion via NFκB regulation. Our results suggest that modulation of AT2R in RAS system could be a potential therapeutic target in PDAC, and AT2R antagonists might represent a novel and promising strategy for controlling and prevention of metastasis, and prolongation of survival in patients with primary or metastatic PDAC.

Acknowledgement: This study was supported by American Cancer Society grant RSG CSM-113191 and NIH grant 1R21 CA133753-02. Authors acknowledge research support and funding they have received from the Department of Surgery, Thomas Jefferson University Hospital, Philadelphia, PA, USA, and Department of Biomedical Sciences, University of New England, Biddeford, ME, USA, relevant to the work described.

Conflict of Interest: All named authors have no any financial interests in respect of this work and its publication as defined by **SDI**, or other interests that might be perceived to influence the results and/or discussion reported in this article.

Authors' contributions: Ankit V. Gandhi, Galina Chipitsyna and Hwyda A. Arafat design the study. Galina Chipitsyna designed and wrote protocols. Ankit V. Gandhi and Galina Chipitsyna performed the experiments. Ankit V. Gandhi wrote the first draft of the manuscript. Ankit V. Gandhi, Galina Chipitsyna, and Hwyda A. Arafat managed the literature searches. All authors participated in discussion and edited the manuscript. All authors read and approved the final manuscript.

Consent Disclaimer:

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

Disclaimer:

"Some part of this manuscript was previously presented and an abstract was published in the following conference. Conference name: 7thAnnual Academic Surgical Congress
Dates: February 14 – 16, 2012
Location: Thomas Jefferson University, Philadelphia, PA
Web Link of the proceeding: http://www.journalofsurgicalresearch.com/article/S0022-4804(11)01471-5/abstract

REFERENCES:

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics. CA Cancer J Clin. 2010;60:277-300.
2. Warshaw AL, Ferandez-Del Castillo C. Pancreatic carcinoma. N Engl J Med. 1992;326:455-65.

3. Dzau VJ, Mukoyama M, Pratt RE. Molecular biology of angiotensin receptors: target for drug research? J Hypertens Suppl. 1994;12:S1–S5.
4. Inagami T. Molecular biology and signaling of angiotensin receptors: an overview. J Am Soc Nephrol. 1999;10 (Suppl 11):S2–S7.
5. Kikkawa, F., Mizuno, M., Shibata, K. et al, Activation of invasiveness of cervical carcinoma cells by angiotensin II. Am J Obstet Gynecol. 2004;190:1258–63.
6. Escobar, E., Rodriguez-Reyna, T.S., Arrieta, O., Sotelo, J. Angiotensin II, cell proliferation and angiogenesis regulator: biologic and therapeutic implications in cancer. Curr Vasc Pharmacol. 2004;2:385–99.

7. Carl-McGrath S, Ebert MP, Lendeckel U, Röcken C. Expression of the local angiotensin II system in gastric cancer may facilitate lymphatic invasion and nodal spread. Cancer Biol Ther. 2007; 6(8):1218-26.
8. Tamarat R, Silvestre JS, Durie M, Levy BI. Angiotensin II angiogenic effect in vivo involves vascular endothelial growth factor- and inflammation-related pathways. Lab Invest. 2002;82:747–56.
9. Lever, A.F., Hole, D.J., Gillis, C.R. et al. Do inhibitors of angiotensin-I-converting enzyme protect against risk of cancer?. Lancet. 1998;352:179–84.
10. Yoshiji, H., Kuriyama, S., Noguchi, R., Fukui, H. Angiotensin-I-converting enzyme inhibitors may be an alternative anti-angiogenic strategy in the treatment of liver fibrosis and hepatocellular carcinoma (Possible role of VEGF). Tumour Biol. 2002;23:348–56.
11. Miyajima A, Kikuchi E, Kosaka T, Oya M. Angiotensin II Type 1 Receptor Antagonist as an Angiogenic Inhibitor in Urogenital Cancer.Rev Recent Clin Trials. 2009 May;4(2):75-8. Review.
12. Kosaka T, Miyajima A, Takayama E, Kikuchi E, Nakashima J, Ohigashi T, et al. Angiotensin II type 1 receptor antagonist as an angiogenic inhibitor in prostate cancer.Prostate. 2007;67(1):41-9.
13. Fujimoto, Y., Sasaki, T., Tsuchida, A., Chayama, K. Angiotensin II type 1 receptor expression in human pancreatic cancer and growth inhibition by angiotensin II type 1 receptor antagonist. FEBS Lett. 2001;495:197–200.
14. Fujita, M., Hayashi, I., Yamashina, S. et al. Blockade of angiotensin AT1a receptor signaling reduces tumor growth, angiogenesis, and metastasis. Biochem Biophys Res Commun. 2002;294:441–47.
15. Miyajima, A., Kosaka, T., Asano, T. et al. Angiotensin II type I antagonist prevents pulmonary metastasis of murine renal cancer by inhibiting tumor angiogenesis. Cancer Res. 2002;62:4176–79.
16. Rivera, E., Arrieta, O., Guevara, P. et al. AT1 receptor is present in glioma cells; its blockage reduces the growth of rat glioma. Br J Cancer. 2001;85:1396–99.
17. Arrieta, O., Guevara, P., Escobar, E. et al. Blockage of angiotensin II type I receptor decreases the synthesis of growth factors and induces apoptosis in C6 cultured cells and C6 rat glioma. Br J Cancer. 2005;92:1247–52.
18. Uemura, H., Ishiguro, H., Nakaigawa, N. et al. Angiotensin II receptor blocker shows antiproliferative activity in prostate cancer cells: a possibility of tyrosine kinase inhibitor of growth factor. Mol Cancer Ther. 2003;2:1139–47.
19. Suganuma T, Ino K, Shibata K, Kajiyama H, Nagasaka T, Mizutani S, Kikkawa F. Functional expression of

the angiotensin II type 1 receptor in human ovarian carcinoma cells and its blockade therapy resulting in suppression of tumor invasion, angiogenesis, and peritoneal dissemination. Clin Cancer Res 2005; 11:2686–94.

20. Lindholm,L et al. Relation between drug treatment and cancer in hypertensives in the Swedish Trial in Old Patients with Hypertension 2: a 5-year, prospective, randomised, controlled trial. Lancet. 2001:358:539–44

21. Friis,S et al. Angiotensin-converting enzyme inhibitors and the risk of cancer: a population-based cohort study in Denmark. Cancer. 2001:2462–70

22. Arafat HA, Gong Q, Chipitsyna G, Rizvi A, Saa CT, Yeo CJ. Antihypertensives as novel antineoplastics: angiotensin-I-converting enzyme inhibitors and angiotensin II type 1 receptor blockers in pancreatic ductal adenocarcinoma. J Am Coll Surg. 2007;20:996–1005.

23. Walther,T et al. Differential regulation of in vivo angiogenesis by angiotensin II receptors FASEB J. 2003:17:2061–67

24. Rizkalla,B et al. Increased renal vascular endothelial growth factor and angiopoietins by angiotensin II infusion is mediated by both AT1 and AT2 receptors. J. Am. Soc. Nephrol. 2003:14:3061–71

25. Zhang, X et al. Retinal expression of vascular endothelial growth factor is mediated by angiotensin type 1 and type 2 receptors. Hypertension. 2004:43:276–81

26. Tahmasebi M, Puddefoot JR, Inwang ER, Vinson GP. The tissue renin-angiotensin system in human pancreas. J Endocrinol. 1999;161:317–22.

27. Leung PS, Chappell MC. A local pancreatic renin–angiotensin system: endocrine and exocrine roles. Int J Biochem Cell Biol. 2003;35:838–846.

28. Deshayes F, Nahmias C. Angiotensin receptors: a new role in cancer? Trends Endocrinol Metab 2005; 16:293–9.

29. Anandanadesan R, Gong Q, Chipitsyna G, Witkiewicz A, Yeo CJ, Arafat HA. Angiotensin II induces vascular endothelial growth factor in pancreatic cancer cells through an angiotensin II type 1 receptor and ERK1/2 signaling. J Gastrointest Surg. 2008; 12:57-66.

30. Chehl N, Gong Q, Chipitsyna G, Aziz T, Yeo CJ, Arafat HA. Angiotensin II regulates the expression of monocyte chemoattractant protein-1 in pancreatic cancer cells. J Gastrointest Surg. 2009; 13:2189-200.

31. McGhee A, Sivarajah M, Gong Q, Lim S, Chipitsyna G, Yeo CJ, Arafat HA. Angiotensin II type 2 receptor blockade inhibits fatty acid synthase production through activation of AMP-activated protein kinase in pancreatic cancer cells. Surgery. 2011 Aug; 150(2):284-98.
32. Pawlikowski M. Immunohistochemical detection of angiotensin receptors AT1 and AT2 in normal rat pituitary gland, estrogen-induced rat pituitary tumor and human pituitary adenomas. Folia Histochem Cytobiol. 2006; 44:173–7.
33. Arrieta O, Pineda-Olvera B, Guevara- Salazar P, et al. Expression of AT1 and AT2 angiotensin receptors in astrocytomas is associated with poor prognosis. Br J Cancer 2008; 99:160–6.
34. De Paepe B, Verstraeten VM, De Potter CR, Bullock GR. Increased angiotensin II type-2 receptor density in hyperplasia, DCIS and invasive carcinoma of the breast is paralleled with increased iNOS expression. Histochem Cell Biol. 2002; 117: 13–19.
35. Fogarty DJ, Sanchez-Gomez MV, Matute C. Multiple angiotensin receptor subtypes in normal and tumor astrocytes in vitro. Glia. 2002; 39:304–13.
36. Vaquero EC, Edderkaoui M, Pandol SJ, et al. Reactive oxygen species produced by NADPH oxidase inhibit apoptosis in pancreatic cancer cells. J BiolChem. 2004;34643–54.
37. Bhandarkar SS, Jaconi M, Fried LE, et al. Fulvene-5 potently inhibits NADPH oxidase 4 and blocks the growth of endothelial tumors in mice. J Clin Invest. 2009;119, 2359–65
38. Sakurai T, Kudo M, Fukuta N, et al. Involvement of angiotensin II and reactive oxygen species in pancreatic fibrosis. *Pancreatology 2011;11:7-13*
39. Dozynkiewicz MA, Jamieson NB, Macpherson I, et al. Rab25 and CLIC3 Collaborate to Promote Integrin Recycling from Late Endosomes/Lysosomes and Drive Cancer Progression. Dev Cell. 2011;11:008
40. Lazar M, Sullivan J, Chipitsyna G, Gong Q, Ng CY, Salem AF, Aziz T, Witkiewicz A, Denhardt DT, Yeo CJ, Arafat HA. Involvement of osteopontin in the matrix-degrading and proangiogenic changes mediated by nicotine in pancreatic cancer cells. J Gastrointest Surg. 2010; 14(10):1566-77.
41. Stone KP, Kastin AJ, Pan W. NFκB is an Unexpected Major Mediator of Interleukin-15 Signaling in Cerebral Endothelia. Cell Physiol Biochem. 2011; 28(1):115-24.
42. Ramos JC, Sin SH, Staudt MR, et al. Nuclear factor kappa B (NFkB) pathway associated biomarkers in AIDS defining malignancies. Int J Cancer. 2011 Jul 25.