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ORIGINAL PAPER

IL-39 acts as a friend to pancreatic cancer

Alicia A. Manning[1] · Lei Zhao[2] · Ziwen Zhu[3] · Huaping Xiao[1] · Chase G. Redington[3] · Vivi A. Ding[1] · Theodore Stewart-Hester[1] · Qian Bai[3] · Jacob Dunlap[3] · Mark R. Wakefield[3] · Yujiang Fang[1,3,4]

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Abstract

Pancreatic cancer is the most lethal digestive cancer and the fourth leading cause of cancer death in the US. IL-39, a heterodimer of p19 and EBI3, is a newly found cytokine and its role in the pathogenesis of neoplasia has not been studied yet. This study was designed to investigate the direct role of IL-39 in the growth of pancreatic cancer. Clonogenic survival assay, cell proliferation, and caspase-3 activity kits were used to evaluate the direct effects of IL-39 on cell survival, proliferation and apoptosis of the widely studied pancreatic cancer cell line MiaPaCa-2. We further investigated the possible molecular mechanisms by using RT-PCR and IHC. The percentage of colonies of pancreatic cancer cells increased significantly in the presence of IL-39. This was paralleled with the increase in the OD value of cancer cells in the presence of IL-39. Interestingly, the relative caspase-3 activity in cancer cells decreased significantly in the presence of IL-39. Furthermore, the pro-tumor effect of IL-39 on pancreatic cancer cells correlated with decreased anti-proliferative molecule p21.The anti-apoptotic effect of IL-39 correlated with decreased pro-apoptotic molecule TRAILR1. These results suggest that IL-39 favors growth of pancreatic cancer by promoting growth and inhibiting apoptosis of cancer cells. This suggests that IL-39 acts as a friend to pancreatic cancer. Thus, inhibition of effect of IL-39 on cells might be a promising strategy to treat pancreatic cancer.

Keywords IL-39 · Apoptosis · Proliferation

Introduction

Pancreatic Cancer (PC) is the most lethal digestive cancer and one of the deadliest solid malignancies [1–3]. In United States, PC accounts for 7% of all cancer related death, correlating to the fourth leading cause of cancer related death [4, 5]. Devastatingly, PC is projected to increase dramatically and becoming the second most common cause of cancer related deaths by 2030, reaching 88,000 deaths per year [6–8]. The exceptionally high mortality rate and the 5-year relative survival rate not exceeding 8% is attributed to PC's aggressive nature of rapid propagation along with being asymptomatic until advance stages [1, 2, 6–8]. Currently, potential curative treatment is surgical resection with subsequent adjuvant therapy; however, only 20% of patient presentations are eligible for resection at diagnosis [2, 4, 8]. The present standard of care for patients with advanced stage PC is limited chemotherapies and immunotherapy leading to additional survival of approximately 5 weeks [5, 9]. Without effective universal primary screening or early clinical signs of PC, research efforts focused on identification of therapeutic targets for advanced disease are important [1, 2, 8]. Therefore, an urgency to discover effective therapies is essential to improve PC patient's quality of life and life expectancy.

Manipulation of the immune system with cytokines has been demonstrated as an effective therapy for cancer patients that warrants further investigation [10, 11]. Interleukin-39

Alicia A. Manning and Lei Zhao have contributed equally to this work.

✉ Yujiang Fang
yujiang.fang@dmu.edu

1 Department of Microbiology & Immunology, Des Moines University College of Osteopathic Medicine, Des Moines, IA 50312, USA

2 Department of Respiratory Medicine, The 2nd People's Hospital of Hefei and Hefei Hospital Affiliated to Anhui Medical University, Hefei, China

3 Department of Surgery, University of Missouri School of Medicine, Columbia, MO 65212, USA

4 Department of Microbiology, Immunology & Pathology, Des Moines University College of Osteopathic Medicine, Des Moines, IA 50312, USA

(IL-39) is the newest member in the interleukin-12 cytokine family. It is composed of the heterodimeric chain-pairing of alpha IL-23p19 and beta Ebi3; encoded on human chromosome 17q25.3 [12]. IL-39 has been studied in several body tissues under the names: Meteorin-like (Metrnl), Cometin, and Subfatin [13–16]. Under these unanimous names, the potential pathogenic role of IL-39 has been explored in a couple non-neoplastic diseases. In lupus-like mice, IL-39 is conferred to having an immunopathogenic effect in systemic lupus erythematosus (SLE) as well as other autoimmune diseases by contributing to the pro-inflammatory response [12]. Several inflammatory skin pathologies also have indications of IL-39 contributing to their inflammatory responses [16]. In addition, IL-39 has been studied for its function in adipose tissue as well as in barrier tissues including gut epithelium [13–16]. IL-39 serves as a functional cytokine correlated to influencing the immune system and having a role in various pathologies, however, has not been extensively studied in neoplasia. In the attempt to find more therapies for pancreatic cancer, due to the increasing incidence and dismal survival rates of pancreatic cancer, we investigated whether IL-39 has any direct effects on the growth and survival of MiaPaCa-2 human pancreatic cell line.

Materials and methods

Human pancreatic cell line

MiaPaCa-2 PC cell line, derived from human pancreatic carcinoma, was provided by Dr. Citrin (Radiation Oncology Branch, NIH). MiaPaCa-2 cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA) as described [17–20]. Cultures were incubated in 5% CO_2 humidified air at 37 °C.

Treatment of PC cell lines with IL-39

MiaPaCa-2 PC cells at 70–80% confluence were treated with IL-39 (50 ng/mL, MyBioSource Inc., San Diego, CA, USA) or DMEM medium alone for 3 days. IL-39 concentration and time of its incubation were based on our pilot experiments and our pervious cytokine studies [18].

Clonogenic survival assay

Following incubation with IL-39 for 3 days, MiaPaCa-2 PC cells were detached and counted with a hemocytometer. Clonogenic survival assay as performed previously described [17–20]. Colonies were counted and then expressed as a percentage of total colonies in the control.

Reverse transcription-polymerase chain reaction (RT-PCR)

MiaPaCa-2 PC cells with and without IL-39 treatment were washed with PBS and then homogenized in TRIzol (Invitrogen). RNA was extracted and the concentration determined via NanoDrop. Reverse transcription was performed utilizing 1 µg of RNA as described previously [17–20]. Primer sequences used in this study have been defined previously [17–20].

Immunohistochemistry (IHC)

IHC staining for proliferating cell nuclear antigen (PCNA) and TRAILR1 in MiaPaCa-2 PC cell line was previously described [18]. The PC cell slides were incubated for 30 min with 0.1% saponin in 1% BSA before incubation for 1 h at room temperature with the appropriate polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX). The slides were then incubated for 30 min with a secondary biotinylated antibody (Jackson, West Grove, PA). Immunoreactivity was confirmed with the avidin–biotin complex immunoperoxidase system (Vector, Burlingame, CA) and developed using NovaRED (Vector) as a chromogen. Counterstaining was demonstrated utilizing hematoxylin. Primary antibody was replaced with equal amounts of normal rabbit immunoglobulin G (IgG) as a negative control. As a negative control staining was all found to be negative at all times. All antibodies were obtained from Santa Cruz Biotechnology. The quantification of PCNA + cells was completed by manually counting all the cells in randomly selected high power fields utilizing the image analysis software MetaMorph (Molecular Devices Analytical Technologies). PCNA + cells were conveyed as a percentage of the total number of cells. MetaMorph image analysis software was also used for measuring a specific proteins' average immunostaining intensity within the area covered by all cells. Results are designated as the average integrated immunostaining intensity of 3 slides ± SEM relative to that in control cells.

Determination of proliferation with the Quick Cell Proliferation Assay Kit

Evaluation of MiaPaCa-2 PC cell proliferation was determined utilizing the Quick Cell Proliferation Assay Kit (BioVision), per the manufacturer's protocol [18].

Measurement of caspase-3 activity

Measurement of cellular caspase-3 activity was used to further evaluate apoptosis of PC cells by performing a caspase-3/CPP32 colorimetric assay kit (BioVision) as previously described [18].

Statistics

All experiments were repeated at least two to three times. Statistical analysis was performed utilizing an unpaired two-tailed Students's *t* test. A *p*-values < 0.05 was considered significant.

Results

IL-39 promotes growth and proliferation of MiaPaCa-2 cells

MiaPaCa-2 cell line was used to explore the direct effect of IL-39 on pancreatic cancer. Cells reaching 70–80% confluence were treated with 50 ng/mL of IL-39 or medium alone for 3 days, followed by the observation for cell survival by clonogenic survival assay. Comparison of cell colonies with and without treatment of IL-39 exhibited significant differences of growth rates. The percentage of MiaPaCa-2 colonies proliferation was significantly higher with IL-39 treatment compared to those treated with medium alone (Fig. 1a, $p < 0.05$). Stimulation of cell proliferation was further evaluated using a Quick Cell Proliferation Assay Kit (Fig. 1b). These results strongly indicate that IL-39 stimulates proliferation and survival of MiaPaCa-2 cells.

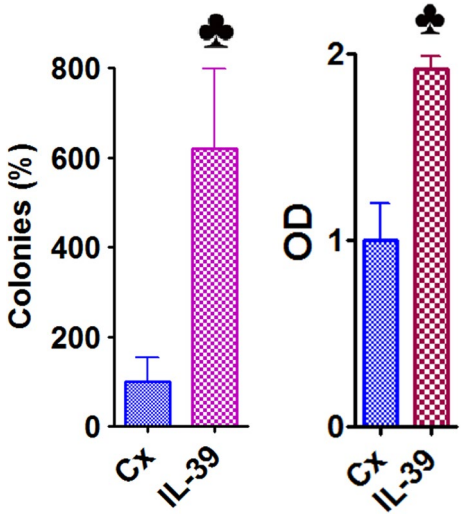

Fig. 1 IL-39 effects on growth and proliferation of MiaPaCa-2 cells. **a** Clonogenic survival assay of MiaPaCa-2 PC cells treated with IL-39 (50 ng/mL). The number of colonies were counted and expressed as a percentage of total colonies compared with Control (Cx, without IL-39). **b** Cell proliferation evaluated with a Cell Proliferation kit in MiaPaCa-2 cells treated with IL-39 (50 ng/mL) or medium alone. Results are expressed as the mean optical density (OD) + SEM of PC cells in each group. For all, error bars denote mean ± SD; Student's *t* test. Clover indicates significant difference ($p < 0.05$)

IL-39 alters expression of pro- and anti-proliferative molecules in MiaPaCa-2 cells

Cell proliferation and survival depends on balancing pro- and anti- proliferative molecules. Cyclin B, cyclin D, cyclin E, cdk2, and cdk4 are crucial pro-proliferative molecules, whereas p18, p21, p27, p53 are important anti-proliferative molecules. To investigate IL-39's potential molecular mechanisms for promoting MiaPaCa-2 PC cells survival, mRNA expression of stated pro- and anti-proliferative molecules in MiaPaCa-2 cells with the presence of IL-39 (50 ng/mL) or absence was determined by RT-PCR (Fig. 2). All but one mRNA molecule expression are comparable in both groups ($p < 0.05$), those treated with IL-39 and control group. P21 mRNA expression was significantly lower in cells treated with IL-39 than those treated with medium alone (Fig. 2, $p < 0.05$).

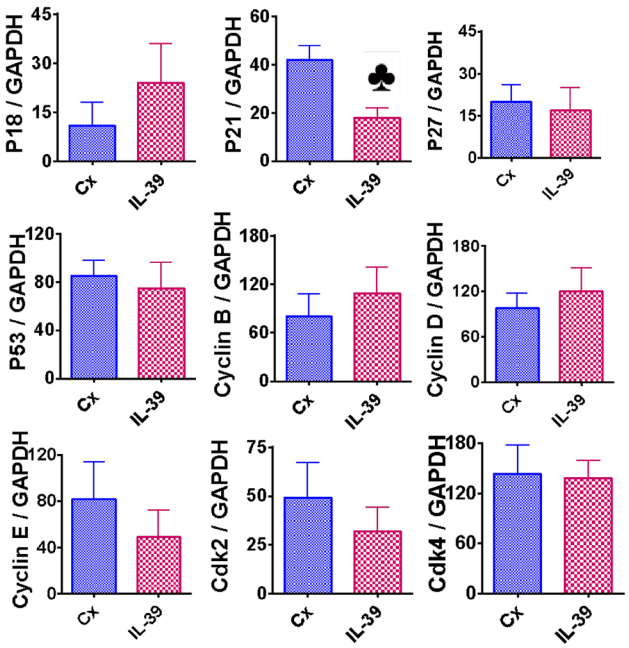

Fig. 2 Effects of IL-39 on pro- and anti-proliferative molecules evaluated by RT-PCR. mRNA was extracted as described in the Methods, and results were performed in triplicate and the results expressed as the mean ratio of proliferative molecule densitometric units/GAPDH + SEM. Significant difference in mRNA expression between cells treated with IL-39 and those in controls is indicated by the clover ($p < 0.05$)

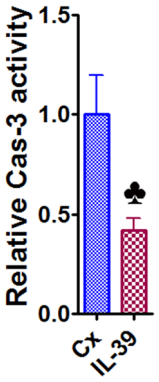

Fig. 3 IL-39 inhibits apoptosis of MiaPaCa-2 cells. MiaPaCa-2 cellular caspase-3 activity measured using caspase-3/CPP32 colorimetric assay kit. Results are expressed as a mean activity relative to controls + SEM. Assays were performed in triplicates. Error bars denote mean ± SD; Student's *t* test. Clover indicates significant difference ($p < 0.05$)

IL-39 inhibits apoptosis of MiaPaCa-2 cells

The stimulatory effect of IL-39 exposure to MiaPaCa-2 PC's proliferation and survival may be contributed to decreased apoptosis. To address the possibility, 70–80% confluent MiaPaCa-2 cells were treated with IL-39 or medium alone for 3 day. Apoptosis was evaluated by examining relative caspase-3 activity. The treated and untreated groups showed a difference in percentage being statistically significant, indicated by a $p > 0.05$ (Fig. 3); indicating IL-39's inhibition of MiaPaCa-2 cells apoptosis pathway supporting the stimulatory effect on survival of MiaPaCa-2 PC cells.

Effects of IL-39 on pro and anti-apoptotic molecules evaluated by RT-PCR and IHC

Apoptosis is controlled by the balance of pro- and anti-apoptotic cellular. The major pro-apoptotic molecules are Fas, FasL, TRAIL, TRAILR1, and Bax. Whereas the major anti-apoptotic molecules are FLIP, Bcl-2, and Survivin. To investigate the potential molecular mechanism by which IL-39 inhibits apoptosis of MiaPaCa-2 PC cells, the mRNA expression of major pro- and anti-apoptotic molecules exposed to IL-39 (50 ng/mL) were examined with RT-PCR (Fig. 4). A lower mRNA expression of the pro-apoptotic molecule TRAILR1 was observed in MiaPaCa-2 PC cells treated with IL-39 compared to cells treated with medium alone (Fig. 4, $p < 0.05$). Results from IHC staining further support decreased TRAILR1 protein expression in IL-39 treated cells, consistent with decreased mRNA expression of TRAILR1 (Fig. 5a, b, $p < 0.05$). These results indicate the downregulation of pro-apoptotic molecule TRAILR1 correlates with decreased apoptosis in MiaPaCa-2 PC cells induced by IL-39.

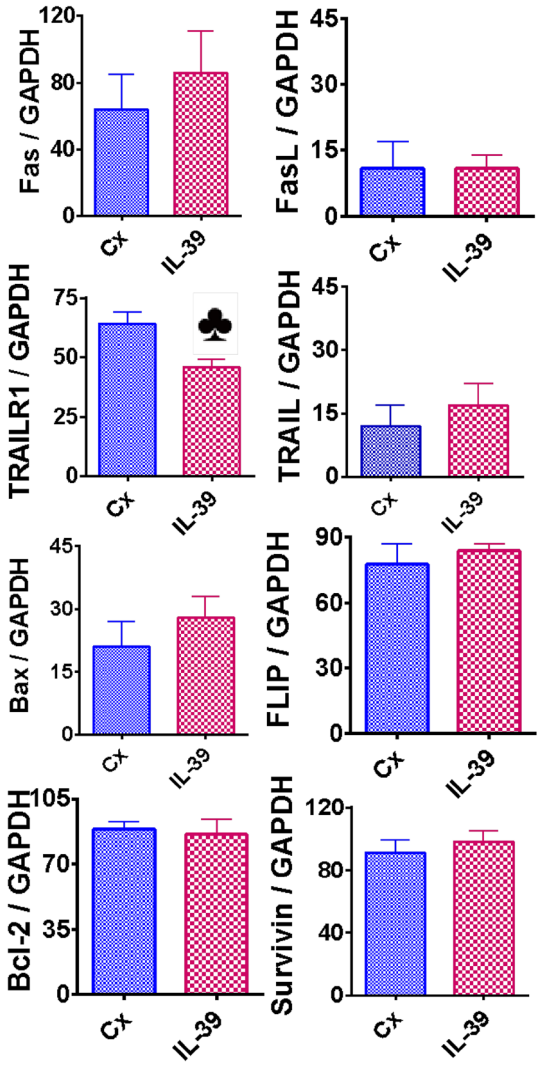

Fig. 4 Effect of IL-39 on expression of pro- and anti-apoptotic molecules evaluated by RT-PCR. Extraction of mRNA as described in Methods and experiments were performed in triplicate. Results are expressed as the mean ratio of pro- and anti-apoptotic molecule densitometric Units/GAPDH + SEM (×100). A significant difference in mRNA expression between cells treated with IL-39 and those in controls is indicated by the clover ($p < 0.05$)

Discussion

Pancreatic cancer incidence as well as mortality continues to rise with projections of becoming the second most common cancer related death by 2030 [1–3]. PC cancer's aggressive nature is partially due to its early and rapid propagation into the lymphatic system, liver, and peritoneal cavity [7]. Unfortunately, the majority of patients present at diagnosis with advanced, non-surgically treatable disease correlating to a poor prognosis because of undesirable response rates to both radiation and chemotherapy [21]. Thus, development of new therapy options is urgent in order to improve PC patient's outcomes. Recent progress with advances in immunotherapy and discovery of PC cancer specific biomarkers expands the opportunities for further research and primitive clinical use [21]. Therefore, it is reasonable to further investigate

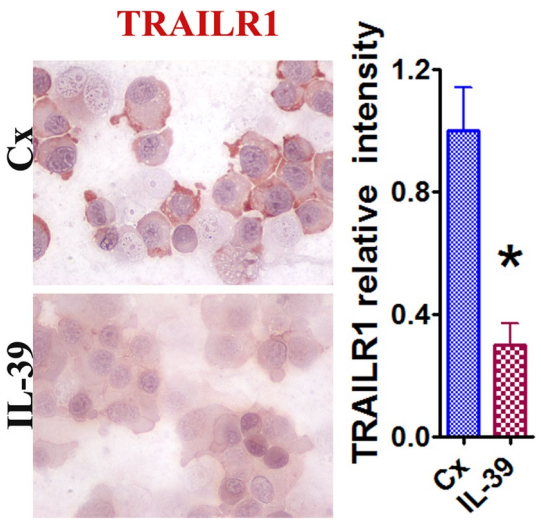

Fig. 5 Effect of IL-39 on expression of TRAILR1 evaluated by IHC. Shown are representative images of IHC. MetaMorph software was used to analyze the relative staining intensity in 3–5 randomly selected high power fields of three slides from each group. Results are expressed as the average integrated staining intensity of 3 slides + SEM relative to that in control cells. A significant difference in intensity between cells treated with IL-39 and those in controls is indicated by the clover ($p < 0.05$). Original magnification: ×400

cytokine immunotherapy as a potential efficacious treatment strategy.

IL-39, the newest of the interleukin-12 family, has been studied for its pathophysiological role in several diseases. Research of lupus-like mice found that activated B cells secret IL-39 causing an upregulation of neutrophils; which in turn increased the expression of IL-39 by activated B cell through positive feedback with B cell activation factor, BAFF [12, 22]. Several cytokines within the interkeukin-12 family, including IL-39, have already been pursued therapeutic targets in human autoimmune diseases [12, 22]. A recent study used anti-IL-39 polyclonal antibodies in lupus-like mice to ameliorate hallmark autoimmune symptoms by reducing inflammatory cell quantities and infiltrating inflammatory cells [23]. IL-39 has also been shown to be significantly over-expressed in inflammatory skin diseases such as psoriasis and atopic dermatitis as well as within synovial membranes of human rheumatoid arthritis [15]. IL-39 seems to play a pro-inflammatory role in autoimmune diseases and skin pathologies, however, its observed role in adipose tissue questions whether its function is tissue specific, due to influencing anti-inflammatory genes in adipose tissue [16]. In adipose tissue, IL-39 exhibited an insulin-sensitizing action as well as upregulation of genes within beige fat thermogenesis and anti-inflammatory cytokines [13, 14, 16]. Additionally, barrier tissues showed significant IL-39 levels especially in gut epithelium displaying its supporting role for secretion of antimicrobial peptides [13]. Observation of multiple functions throughout different tissues thus supports our suggestion to explore the direct effect of IL-39 on neoplasia, particularly pancreatic cancer.

While previous IL-39 research focused on non-neoplastic pathologies, the connection seen with the immune system supports our intent to explore its potential mechanism involving pancreatic neoplasia. Recent advances identifying PC's molecular biology and the correlation of cytokine prognostic biomarkers for gastrointestinal neoplasia support the interest to observe IL-39 interaction with PC [21]. In this study, we utilized PC cell line MiaPaCa-2 to investigate the direct effect of IL-39. Specifically, we found IL-39 causes stimulation of cell growth and inhibition of PC cell death. We observed significant increased percentage of colonies and OD value of PC cells treated with IL-39. IL-39 was also found to significantly decrease relative caspase-3 activity. To probe into the intracellular causes for PC cells uncontrolled growth, we explored the intracellular molecular expression controlling proliferation and apoptosis. Our data exhibited decreased expression of the anti-proliferative molecule p21 as well as decreased pro-apoptotic factor TRAILR1, both further support IL-39's observed stimulatory effect on PC cells. The pro-tumor effect IL-39 has is through downregulating apoptosis and upregulating proliferation by influencing intracellular molecular expression, thus overall supporting its role of acting as a friend to PC cells.

Uncontrolled cancer growth is well known to be attributed to unbalanced proliferative and apoptotic molecules; ordinarily tightly regulated within normal cells. The delicate balance between both pro- and anti-proliferative molecules as well as pro- and anti-apoptotic molecules are tightly regulated [24–26]. Intracellular molecules considered to promote cell proliferation include cyclin B, cyclin D, cyclin E, cdk2, and cdk4 as pro-proliferative molecules, whereas p18, p21, p27, p53 are considered anti-proliferative molecules due to their observed action blocking cell growth [27–30]. Intracellular molecules considered to promote cell death include Fas, FasL, TRAIL, TRAILR1, and Bax as the major pro-apoptotic molecules and FLIP, Bcl-2, and Survivin are considered anti-apoptotic molecules because their action of blocking cell death [31, 32]. A single balance disruption of either system's molecules influences whether cells undergo growth or death. Our results exhibited a decrease of the important anti-proliferative molecule p21 and a decrease of TRAILR1 whose role encourages apoptosis as a pro-apoptotic factor [31, 32]. The balance shift in both systems compounded together supports our observed uncontrolled cell proliferation in MiaPaCa-2 PC cells. In this study, the stimulatory effect of IL-39 on MiaPaCa-2 is supported by the decreased expression of both a molecule that inhibits cell proliferation and encourages apoptosis, p21 and TRAILR1, respectively.

In conclusion, IL-39 presents as a friend to MiaPaCa-2 pancreatic cancer for its pro-tumor effects through encouraging proliferation and reducing apoptosis. Decreased expression of anti-proliferative p21 correlates with increased proliferation and cell growth. The decreased expression of pro-apoptotic TRAILR1 correlates with decreased apoptosis. With current poor outcomes for PC patients, we believe our findings expanded our understanding of IL-39's role with neoplasia as well as highlight it as a potential therapeutic target that warrants further evaluation.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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