ORIGINAL PAPER

Inhibition of Notch3 enhances sensitivity to gemcitabine in pancreatic cancer through an inactivation of PI3K/Akt-dependent pathway

Jun Yao · Cuijuan Qian

Abstract Notch3 is one of the four Notch receptors identified in mammal, but its role in human pancreatic cancer remains poorly characterized. In this study, we sought to determine the effect of suppressing Notch3 expression on the chemosensitivity to gemcitabine in human pancreatic cancer cell lines BxPC-3 and PANC-1. RNA interference was used to suppress Notch3 expression. Gemcitabine-induced cytotoxicity was determined by MTT. Cell apoptosis was measured by flow cytometry. Caspase 3 activity was assayed using a Caspase Fluorescent Assay Kit. The effect of Notch3-specific siRNA on PI3K/Akt activity was also quantified. Notch3-specific siRNA suppressed Notch3 expression, and furthermore increased gemcitabine-induced, caspase-mediated apoptosis. The suppression of Notch3 expression decreased the average IC_{50} in BxPC-3 and PANC-1 cells treated with gemcitabine. PI3K/Akt activity was decreased by the suppression of Notch3 expression. Taken together, these data demonstrated that Notch3 is a potential therapeutic target for pancreatic cancer, and PI3K/Akt is a key signaling component by which activation of the Notch3 signal transduction pathway protects pancreatic cancer cells from chemotherapy-induced cell death.

Jun Yao and Cuijuan Qian contributed equally to this work.

J. Yao (✉)
School of Medicine, Taizhou University, Jiaojiang District,
318000 Taizhou, Zhejiang, China
e-mail: yaojuntzu@yeah.net

C. Qian (✉)
Department of Gastroenterology, Taizhou Municipal Hospital,
Jiaojiang District, 318000 Taizhou, Zhejiang, China
e-mail: cuijuanqian@yeah.net

Introduction

Pancreatic cancer is a leading cause of cancer-related death in the world and is characterized by rapid disease progression, highly invasive tumor phenotype, and resistance to chemotherapy [1, 2]. Despite significant advances in diagnosis, staging, and surgical management of the disease, overall 2 years survival in US, Europe, Australia is around 20%, and 50–70% of patients may survive 2 years if treated with chemotherapy and surgery [1, 2]. Chemotherapy is the primary treatment option for most patients with pancreatic cancer, but the standard agents, such as fluorouracils (5-FU), capecitabine, and gemcitabine have had limited impact on patient survival [1, 2]. Thus, the search for more effective chemotherapeutic agents is still ongoing, and new regimens are under active investigation.

Notch signaling is an evolutionarily conserved signaling pathway that plays an essential role in regulating cell fate determination, differentiation, proliferation, and apoptosis [3]. The Notch receptor is a transmembrane protein expressed on the cell surface as a heterodimer of an EGF-like repeat-rich extracellular domain and an intracellular domain with a single pass transmembrane domain [4]. There are four distinct isoforms of Notch (Notch1–4) in mammals, whereas five different ligands have been identified [5]. Notch signaling is initiated by ligand-receptor interaction, leading to two proteolytic cleavage events of the Notch receptor, by TACE and γ-secretase [6]. The binding of Notch receptor to its ligand results in proteolytic cleavage and the release of Notch intracellular domain (NICD), which translocates to the nucleus and

interacts with DNA-binding transcriptional repressor recombination signal-binding protein-Jκ(RBP-Jκ). The NICD/RBP-Jκ complex transactivates target genes including the hairy/enhancer-of-split (Hes-1) [7]. In humans, Notch receptor was first identified in T cell acute lymphoblastic leukemia (T-ALL), where chromosomal translocation resulted in the expression of truncated Notch under regulation of the TCR promoter in T lymphocytes [8]. Since then, aberrations in Notch signaling have been linked to various forms of neoplastic formation, such as T-cell leukemia, breast cancer, and lung cancer [9]. However, unlike other genes involved in tumorigenesis, Notch signaling functions in a context-dependent manner. It can function to promote tumor formation in one instance and limit tumor growth in another [10].

Akt is an important downstream target of PI3K and functions to regulate cell survival, proliferation, and protein synthesis. Experiments have shown a link between Notch and Akt pathways [11, 12]. In this study, we tested the hypothesis that Akt is a key downstream target by which Notch3 pathway attenuates the effects of chemotherapy in pancreatic cancer cells and that suppression of Notch3/PI3K/Akt pathway would enhance pancreatic cancer chemosensitivity to gemcitabine.

Materials and methods

Cell cultures and conditions

Human pancreatic cancer cells (BxPC-3 and PANC-1) were routinely cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco BRL, MD, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium) at 37°C in 95% air and 5% CO_2.

Apoptosis and cell survival analysis

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay (Trevigen Inc., Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. Logarithmically growing cells were plated at 5×10^3 cells per well in 96-well plates, and allowed to adhere overnight, then cultured in the presence or absence of gemcitabine (2′,2′-diflurodeoxycytidine; EliLilly & Co., Indianapolis, IN, USA). Gemcitabine-induced cytotoxicity was determined after 48 h of exposure. Plates were read using a Vmax microplate spectrophotometer (Molecular Devices) at a wavelength of 570 nm corrected to 650 nm and normalized to controls. Each independent experiment was performed three times, with ten determinations for each condition tested. The concentration of gemcitabine required to inhibit proliferation by 50% (IC_{50}) was calculated from these results. Alternatively, cells were harvested as above, and viable and dead cells were counted using trypan blue exclusion (Gibco BRL, MD, USA). After treatment, cells were washed, resuspended in 0.5 ml of PBS, and 1 μl/ml of the green fluorescent dye YO-PRO-1, and propidium iodide were added (Vybrant Apoptosis Assay Kit #4, Molecular Probes). Cells were incubated for 30 min on ice, then analyzed by flow cytometry (FACScan, Becton–Dickinson, San Diego, CA, USA), measuring fluorescence emission at 530 and 575 nm. Cells stained with YO-PRO-1 were counted as apoptotic; necrotic cells stained with propidium iodide. The number of apoptotic cells was divided by the total number of cells (minimum of 10^4 cells), giving the apoptotic fraction. Data were analyzed using CellQuest software (Becton–Dickinson, San Diego, CA, USA). The BD ApoAlert fluorometric Caspase Assay Plate (BD Biosciences Clontech, CA, USA) was then used for assessing caspase 3 activity. After harvesting the whole cell lysates, plates were read (excitation 360 nm; emission 480 nm) using a CytoFluor 4000 multiwell fluorescence plate reader (Applied Biosystems, Foster City, CA, USA). All measurements were performed in triplicate, each with four determinations for each condition.

Stable transfectants and small interfering RNAs

Human pancreatic cancer cells were transfected with DNA vector-based small interfering RNA (siRNA) using Genscript siRNA Expression Vector and Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA). The target insert is 5′-CACCUAUAACUGCCAGUGC-3′. Stable clones were selected using hygromycin.

Western blot analysis

Cells (2×10^6) were washed twice in ice-cold PBS and then incubated with 300 μl of lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM NaVO4, 0.1 mM benzamidine, 5 μg/ml leupeptin, and 5 μg/ml aprotinin] for 5 min on ice. Whole cell lysates were clarified by centrifugation at 15,000 rpm for 15 min at 4°C. The protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratory, Richmond, CA, USA) with bovine serum albumin as a standard protein. Thirty micrograms of protein was separated by electrophoresis on a sodium dodecyl sulfate–polyacrylamide gel (12% gel). After electrophoresis, proteins were transferred to nitrocellulose membrane. After 1 h incubation in a blocking solution (5% non-fat dry milk in PBS-0.5% Tween-20), the membrane was blotted with the anti-Notch3 polyclonal antibody (1:400; Santa Cruz, California,

CA, USA) and an anti-β-actin monoclonal antibody (1:1,000; Sigma, St. Louis, MO, USA). The blots were developed with peroxidase-labeled secondary antibodies. After extensive washing and incubating with the Enhanced Chemiluminescence Plus detection reagent for 5 min, protected from light, specific bands were detected using a developer, in dark by exposing it to autoradiography film: Hyperfilm Enhanced Chemiluminescence (Amersham Biosciences, NJ, USA) for suitable durations of time. Blots were performed in triplicate. Mean densitometric values ($\pm$SD) are shown.

Akt kinase assay

Akt activity was quantified using a commercially available non-radioactive in vitro kinase assay, in accordance with the manufacturer's instructions (Cell Signaling Technology, Beverly, MA, USA). Akt was immunoprecipitated from 200-μl cell lysates containing equal total protein. The resulting immunoprecipitates were incubated with glycogen synthase kinase-3 (GSK-3) fusion protein in the presence of ATP and kinase buffer. Phosphorylation of GSK-3, a physiologic target of Akt, was measured by Western blot using antiphospho-GSK-3α/β (Ser-21/9) antibody, and densitometric analysis of the stained bands was performed using ImageMaster software (Pharmacia Biotech, San Francisco, CA, USA). Mean values from three independent experiments with three samples per group are shown.

Statistical analysis

Statistical analyses were performed using SPSS-PC package (version 13.0; SPSS, Chicago, IL, USA). The data are expressed as means $\pm$ SD. Analysis was performed using ANOVA, unpaired t-test, and Mann–Whitney U test for non-parametric data. P-values of <0.05 were considered to be statistically significant.

Results

Notch3-specific siRNA suppresses Notch3 expression

RNA interference is emerging as a powerful technique for the specific inhibition of expression of individual genes at the post-transcriptional level [13]. Therefore, Notch3-specific siRNA was transfected with pancreatic cancer cell lines. We confirmed the suppression of Notch3 protein expression by Western blot analysis (Fig. 1). Notch3-specific siRNA robustly suppressed Notch3 expression after stable transfection. Control siRNA had no effect on Notch3 expression. Treatment with either siRNA did not affect expression of β-actin, indicating that non-specific suppression of protein

expression did not occur. These results demonstrated that Notch3-siRNA was effective in inhibiting the biological function of Notch3.

Inhibition of Notch3 expression enhances gemcitabine-induced cytotoxicity

After stable siRNA transfection, cells were exposed to 0–10 μM gemcitabine for further 48 h. The IC$_{50}$ was calculated from MTT cytotoxicity assay data. Notch3 siRNA suppressed the gemcitabine IC$_{50}$ in each of the two cell lines; control siRNA transfection had no effect on the gemcitabine IC$_{50}$ (Fig. 2). The increase in gemcitabine-induced cytotoxicity after transfection of Notch3 siRNA could be attributable to the induction of apoptosis. Therefore, we further analyzed the degree of apoptosis by flow cytometry. Gemcitabine significantly increased the induction of apoptosis in Notch3-siRNA transfected cells, and control siRNA had no significant effect on the gemcitabine-induced apoptotic fraction (Fig. 3). The feasibility of using siRNA as a mediator of chemo sensitization has been shown by previous studies [14], and the contemporary paradigm for anticancer drug development has moved toward target-directed therapies based on a more comprehensive understanding of cancer biology, rather than empirical discovery.

Gemcitabine induces apoptosis in tumor cells through caspase activation [15]. So we sought to determine the effect of Notch3-specific siRNA on activity of effector caspase-3 after exposure to gemcitabine for 48 h. Gemcitabine-induced caspase-3 activation was markedly increased after transfection of Notch3-specific siRNA, but was unaffected after transfection of control siRNA (data not shown). These results clearly demonstrated that inactivation of Notch3 sensitizes human pancreatic cancer cells to caspase-mediated apoptosis induced by gemcitabine. Akt is reported to inhibit activation of initiator caspase-9 and effector caspase-3, at a post-mitochondrial level, protecting cells from apoptosis [16]. The decrease in Akt activity that we observed after transfection of Notch3-specific siRNA may contribute to the increase in gemcitabine-induced, caspase-mediated activity and apoptosis as showed below.

Akt activity is suppressed by the inhibition of Notch3 expression

The potently antiapoptotic phosphatidyl inositol 3'-kinase (PI3K)-protein kinase B/AKT pathway is active in pancreas cancer [17]. Considering Akt is an important downstream target of PI3K and functions to regulate cell survival, proliferation, and protein synthesis, we examined
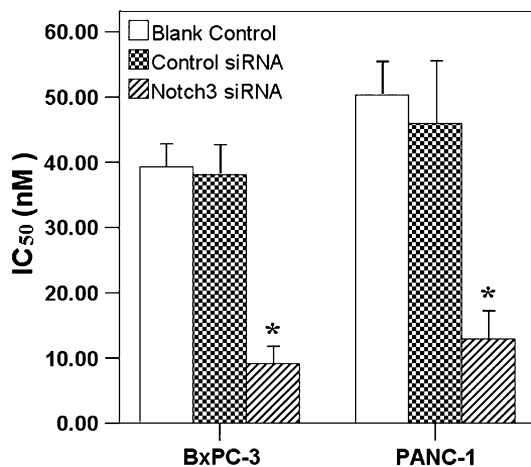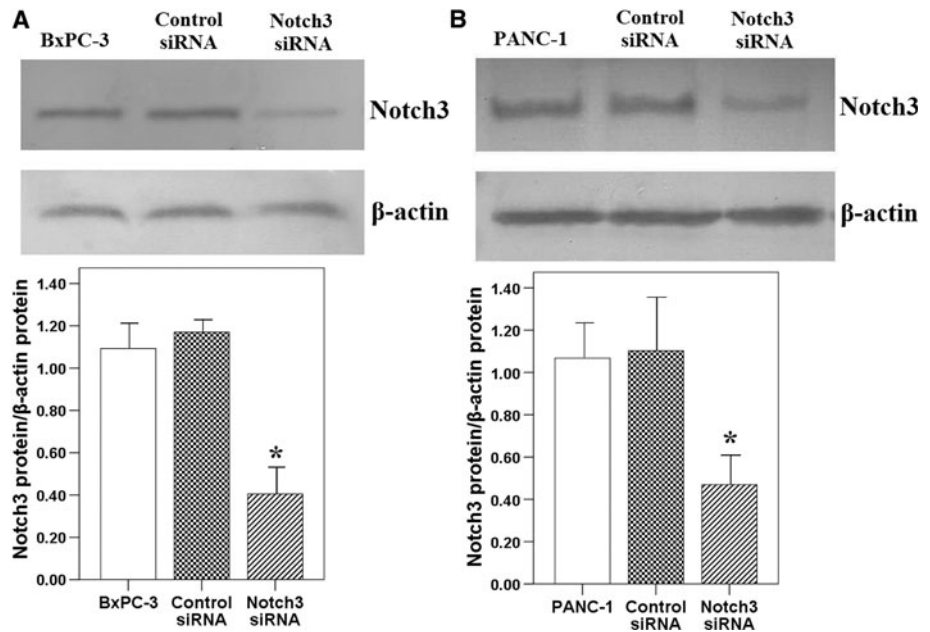
Fig. 2 Notch3-specific siRNA promotes gemcitabine-induced cyto-
toxicity. The gemcitabine IC_{50} was determined by MTT assay after
exposure to gemcitabine for 48 h. Treatment with Notch3-specific
siRNA decreased the gemcitabine IC_{50} of each cell line. Experiments
were performed in triplicate with 10 determinations per condition.
* $P < 0.05$ versus control siRNA. IC_{50}, concentration required to
inhibit proliferation by 50%; MTT, 3-(4,5-dimethylthiazol-2- yl)-2,5-
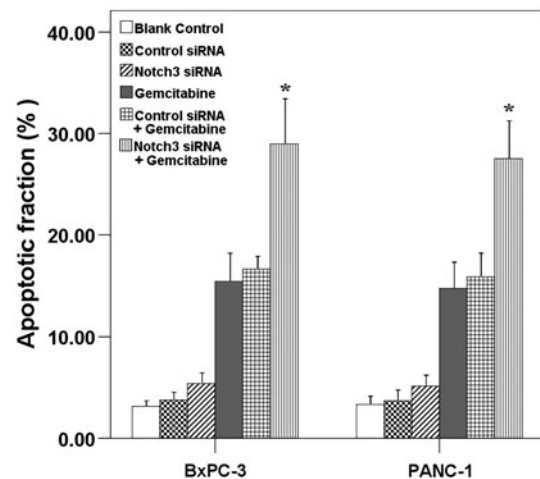diphenyltetrazolium bromide

Fig. 3 Notch3-specific siRNA increases gemcitabine-induced apop-
tosis. The apoptotic fraction of cells induced by exposure to 1 μM
gemcitabine for 48 h was quantified by flow cytometry after green
fluorescent dye YO-PRO-1 and propidium iodide staining. Control
siRNA did not affect gemcitabine-induced apoptosis. Four determi-
nations were used per condition, and experiments were performed in
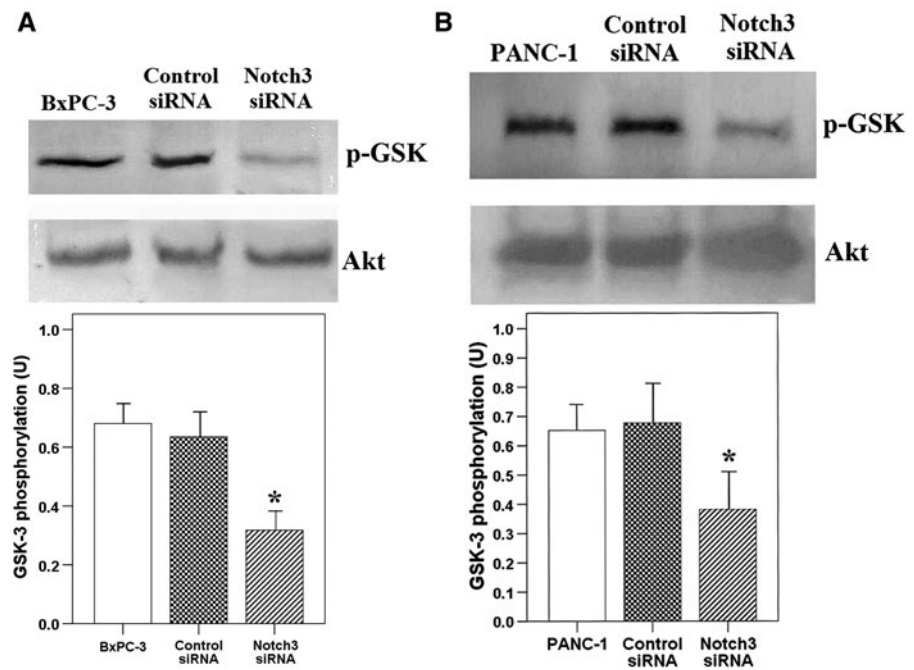triplicate. * $P < 0.05$ versus control siRNA

the effect of suppression of Notch3 expression on Akt
activity using a GSK-3 fusion protein phosphorylation
assay, after transfection of Notch3-specific and control
siRNA. Transfection of Notch3-specific siRNA resulted in
suppression of Akt activity in BxPC-3 and PANC-1 cells.
Control siRNA had no effect on Akt activity (Fig. 4).
Results indicated that inhibition of Notch3 expression
constitutively inactivated PI3K/Akt and significantly
enforced apoptosis induced by gemcitabine.

Discussion

In this study, we have demonstrated that suppression of
Notch3 expression by RNA interference enhances gem-
citabine-induced cytotoxicity in human pancreatic cancer
cells. Notch3 gene silencing increases gemcitabine-induced
caspase-mediated apoptosis in vitro and is associated with
decreased Akt activity.

Notch3 is overexpressed by a variety of human tumors
including hepatocellular carcinoma [18] and pancreatic

Fig. 4 Notch3 gene silencing suppresses Akt activity. Akt activity was assessed in BxPC-3 (**a**) and PANC-1 (**b**) cells after stable transfection with control and Notch3 specific siRNA. Suppression of Notch3 expression inhibited Akt activity, determined by in vitro kinase assay. Total Akt levels were unaffected. *Values* are means (±SD) of three independent experiments performed in triplicate. * $P < 0.05$ versus control siRNA

cancer [19], making Notch3 a rational target for therapeutic intervention. Notch3 has important roles in the control of cell-extracellular interactions such as spreading, migration, motility, and survival [20], but recently, attention has turned to Notch signaling as a determinant of chemoresistance. Akiyoshi et al. [21] demonstrated that γ-secretase inhibitors enhance taxane-induced mitotic arrest and apoptosis in colon cancer cells. Because γ-secretase is required for proteolytic cleavage of Notch receptors (including Notch3), Notch3 is overexpressed in pancreatic adenocarcinomas, and expression of nuclear Notch3 in pancreatic adenocarcinomas is associated with adverse clinical features, such as poorer overall survival [19], we hypothesized that RNA interference targeting Notch3 have been shown to induce apoptosis of pancreatic cancer cells and enhances sensitivity to chemotherapy. We chose siRNA to mediate Notch3 gene silencing as it is reported to have advantages over oligonucleotide-based techniques.

To investigate the role of Notch3 in the chemoresistance of human pancreatic cancer, we reduced the expression of Notch3 by RNAi and compared the changes in chemosensitivity among siRNA-Notch3 transfected pancreatic cancer cells and control group cells. Our results showed that siRNA against Notch3 combined with chemotherapy could significantly enhance cell apoptosis and the cytotoxicity of both BxPC-3 and PANC-1 pancreatic cancer cells, which means that targeting Notch3 by siRNA could significantly enhance the chemosensitivity to gemcitabine of pancreatic cancer cells in vitro. Gazzaniga et al. [22]

demonstrated gemcitabine-induced apoptosis in bladder cancer cells with the activation of caspase-3, caspase-8, and caspase-9. Notch signaling may resist apoptosis induced by chemotherapy agents in tumor cells [23]. We suggest that the inhibition of Notch3 expression could activate caspases and contribute to gemcitabine-inducing apoptosis in pancreatic cancer cells.

Although the mechanisms by which Notch3 overexpression serves such a protective role are not fully understood, our observations that Notch3 siRNA suppresses Akt activity may be important in this respect. Activation of Akt is common in pancreatic adenocarcinoma, and Akt inhibition has been shown to induce chemosensitization in pancreatic adenocarcinoma cells [17]. We chose BxPC-3 and PANC-1 to examine the effect of Notch3 siRNA on Akt activity as BxPC-3 and PANC-1 exhibit high basal Akt activity [24]. Akt activity is not completely inhibited by Notch3 siRNA treatment, which is consistent with the lack of effect on cell survival in the absence of gemcitabine. The degree of suppression of Akt activity following Notch3 siRNA treatment may however be sufficient to reduce the resistance of BxPC-3 and PANC-1 cells to gemcitabine.

To summarize, we showed that Notch3-specific siRNA enhances pancreatic cancer gemcitabine chemosensitivity by promoting caspase-mediated apoptosis through an inactivation of PI3K/Akt-dependent pathway. These findings raise the possibility that novel combined pharmacologic inhibitors of Notch3/PI3K/Akt may enhance the effectiveness of chemotherapeutic agents in the treatment of pancreatic cancer.

References

1. Fryer RA, Galustian C, Dalgelish AG. Recent advances and developments in treatment strategies against pancreatic cancer. Curr Clin Pharmacol. 2009;4(2):102–12.
2. Jonckheere N, et al. Tumour growth and resistance to gemcitabine of pancreatic cancer cells are decreased by AP-2alpha overexpression. Br J Cancer. 2009;101(4):637–44.
3. Nickoloff BJ, Osborne BA, Miele L. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. Oncogene. 2003;22(42):6598–608.
4. Miyamoto A, Lau R, Hein PW, Shipley JM, Weinmaster G. Microfibrillar proteins MAGP-1 and MAGP-2 induce Notch1 extracellular domain dissociation and receptor activation. J Biol Chem. 2006;281(15):10089–97.
5. Radtke F, Wilson A, Mancini SJ, MacDonald HR. Notch regulation of lymphocyte development and function. Nat Immunol. 2004;5(3):247–53.
6. Maillard I, Adler SH, Pear WS. Notch and the immune system. 1. Immunity. 2003;19(6):781–91.
7. Maillard I, Fang T, Pear WS. Regulation of lymphoid development, differentiation, and function by the Notch pathway. Annu Rev Immunol. 2005;23:945–74.
8. Real PJ, Ferrando AA. NOTCH inhibition and glucocorticoid therapy in T-cell acute lymphoblastic leukemia. Leukemia. 2009;23(8):1374–7.
9. Roy M, Pear WS, Aster JC. The multifaceted role of Notch in cancer. Curr Opin Genet Dev. 2007;17(1):52–9.
10. Dotto GP. Notch tumor suppressor function. Oncogene. 2008;27 (38):5115–23.
11. Baek SH, et al. Zinc-induced downregulation of Notch signaling is associated with cytoplasmic retention of Notch1-IC and RBP-Jk via PI3k-Akt signaling pathway. Cancer Lett. 2007;255(1): 117–26.
12. Meurette O, et al. Notch activation induces Akt signaling via an autocrine loop to prevent apoptosis in breast epithelial cells. Cancer Res. 2009;69(12):5015–22.
13. Kurreck J. RNA interference: from basic research to therapeutic applications. Angew Chem Int Ed Engl. 2009;48(8):1378–98.
14. Hu Y, et al. Inhibition of hypoxia-inducible factor-1 function enhances the sensitivity of multiple myeloma cells to melphalan. Mol Cancer Ther. 2009;8(8):2329–38.
15. Pauwels B, et al. The role of apoptotic cell death in the radio-sensitising effect of gemcitabine. Br J Cancer. 2009;101(4): 628–36.
16. Zhou H, Li XM, Meinkoth J, Pittman RN. Akt regulates cell survival and apoptosis at a postmitochondrial level. J Cell Biol. 2000;151(3):483–94.
17. Simon PO Jr, et al. Targeting AKT with the proapoptotic peptide, TAT-CTMP: a novel strategy for the treatment of human pancreatic adenocarcinoma. Int J Cancer. 2009;125(4):942–51.
18. Gramantieri L, et al. Aberrant Notch3 and Notch4 expression in human hepatocellular carcinoma. Liver Int. 2007;27(7): 997–1007.
19. Doucas H, et al. Expression of nuclear Notch3 in pancreatic adenocarcinomas is associated with adverse clinical features, and correlates with the expression of STAT3 and phosphorylated Akt. J Surg Oncol. 2008;97(1):63–8.
20. Dang L, et al. Notch3 signaling initiates choroid plexus tumor formation. Oncogene. 2006;25(3):487–91.
21. Akiyoshi T, et al. Gamma-secretase inhibitors enhance taxane-induced mitotic arrest and apoptosis in colon cancer cells. Gastroenterology. 2008;134(1):131–44.
22. Gazzaniga P, et al. Gemcitabine-induced apoptosis in 5637 cell line: an in vitro model for high-risk superficial bladder cancer. Anticancer Drugs. 2007;18(2):179–85.
23. Nefedova Y, Sullivan DM, Bolick SC, Dalton WS, Gabrilovich DI. Inhibition of Notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy. Blood. 2008; 111(4):2220–9.
24. Tassone P, et al. Zoledronic acid induces antiproliferative and apoptotic effects in human pancreatic cancer cells in vitro. Br J Cancer. 2003;88(12):1971–8.