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Delta like ligand 4 induces impaired chemo-drug delivery and enhanced chemoresistance in pancreatic cancer

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ABSTRACT

The stubborn chemoresistance of pancreatic ductal adenocarcinoma (PDA) is simultaneously influenced by tumor parenchymal and stromal factors, and the ctritical role of Notch ligand Delta-like 4 (DLL4) in the regulation of tumor malignancies has been observed. DLL4 positive expression ratio between duct cells from clinical tumor and adjacent tissues was statistically significant, and the overactivation of DLL4/Notch pathway enhanced the phenotype of EMT and cancer stem cell, even can induce multi-chemoresistance *in vitro*. Notably, the accompanied defective angiogenesis directly induced inefficient chemo-drug delivery *in vivo*. Collectively, overexpressed DLL4 on neoplastic cells can enhance chemoresistance through angiogenesis-dependent/independent mechanisms in PDA.

1. Introduction

Pancreatic ductal adenocarcinoma (PDA) is a hypovascular scirrhous carcinoma with highly chemoresistance, and carries a dismal 5-year survival rate of about 5% [1]. Since less than 20% of patients present with surgically resectable disease at initial diagnosis [2], chemotherapeutics remains the primary modality for advanced PDA [3]. Unfortunately, even with Gemcitabine, the current most effective chemo agent for advanced PDA, the tumor response rate remains poor (<10%) and the median survival is approximately only 6 months [3,4]. Thus, there is an urgent need for the design of new therapeutic strategies for conquering such refractory resistance.

Previous research has demonstrated that factors like genetic mutations, cancer stem cell (CSC) phenotype and drug metabolism are deeply involved in intrinsic and acquired chemoresistance in PDA [5,6]. In addition, a recently eminent argument indicated that inefficient drug delivery caused by poor vascularization is another important contributor to stubborn chemoresistance in PDA [7]. These research progressions point out the chemoresistance of PDA is simultaneously influenced by tumor parenchymal factors and tumor stromal factors, and further investigation in the mechanisms underlying the stubborn chemoresistance of PDA could lead to novel efficient treatment.

Cumulative research reveals that both aberrant expression and over activation of Notch pathway components are prerequisites for the angiogenesis, tumorigenesis, maintenance and progression of many solid tumors, including PDA [8,9]. The inactivation of Notch components could be a potential approach for overcoming the intractable chemoresistance of PDA [10,11]. In humans, the NOTCH signaling pathway is evolutionarily conserved, and consists of four receptors (Notch1–4) and five transmembrane ligands (Jagged1, 2, Delta like ligands 1, 3, 4). Previous studies have shown that among the five ligands of Notch, Delta like ligand 4 (DLL4), which mainly activates Notch1 and Notch4, is a key negative regulator of angiogenesis both in embryonic and postnatal development [12]. DLL4 was once found mainly expressed on endothelial cells lining the tumor vasculature [13,14], and significant progress has been made in understanding the key role of DLL4 in negative regulation of tumor vascularization [15–17]. But a recent study has reported the widespread DLL4 protein expression is observed in the cytoplasms of neoplastic cells from majority of tumors, including PDA, and stimulus like hypoxic condition can even promoted DLL4 translocating to the nuclei [18]. Meanwhile, it has been reported that DLL4 induced Notch activation is intimately related to tumor

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invasion [19] and tumor-initiating cell frequency [20,21]. Clinical study has also revealed that the high DLL4 expression is significantly associated with poor prognosis in PDA patients [13]. In combination with the commonly observed ligand-dependent Notch activation in PDA [22], these researches indicated the biological role of DLL4 is far beyond a regulator of angiogenesis.

In our present study, for the first time, we systematically evaluated the influence of DLL4/Notch pathway on drug delivery and chemosensitivity in PDA. We first investigated the expression level of DLL4 and its main receptors Notch1/4 in clinical PDA specimens by using IHC or qPCR, and then we assessed the pathological function of overexpressed DLL4 in the efficacy of chemotherapeutics in PDA. Collectively, our data showed the expressions of these Notch components are up-regulated within the tumor cells/tissues in different levels, and overactivation of DLL4/Notch pathway can simultaneously impair chemo drug delivery and enhance chemoresistance in PDA.

2. Materials and methods

2.1. Pancreatic carcinoma specimens preparation

The study was approved by the ethics committees of Zhejiang University. 42 tumor specimens (30 paraffin-embedded tissue blocks and 12 freshly-frozen tissues were collected from January 2007 to January 2010) and their corresponding normal tissues (pancreas tissue which was 2 cm apart from tumor tissue in each case) were randomly obtained from the tissue bank belonging to the Department of Surgery, Second Affiliated Hospital of Zhejiang University. All patients underwent resection for human sporadic pancreatic ductal adenocarcinoma, and the diagnosis was confirmed by at least two pathologists. All patients were classified according to the UICC 2002 edition.

2.2. Immunohistochemistry

The distribution and expression level of DLL4 protein in PDA samples was examined by immunohistochemical analysis of paraffin-embedded tissue sections as previously described [23], and the sections were incubated with primary antibody against DLL4 (Abcam) at a 1:100 dilution at RT. For scoring the protein expression ratio in neoplastic cells, tumor samples were considered to be positive for DLL4 when immunoreactivity was observed in >25% of the tumor parenchymal cells at any intensity. The staining results were evaluated by an experienced pathologist.

2.3. RNA extraction and quantitative PCR

Total RNA was extracted with Trizol (Invitrogen) reagent following the manufacturer's instructions as previously done with slight improvement [24]. All primer sequences were designed using Primer 5 software, and PCR amplification were done on the Light Cycle Real-time PCR thermocycler (Roche, USA) with SYBR Green PCR master mix kit (Takara). The relative gene mRNA levels of all target genes were calculated to the internal control gene GAPDH, by using ($=2^{-\triangle\triangle Ct}$) method [25], and all PCRs were done in triplicate.

2.4. Cells lines and culture conditions

Monolayer cultures of human pancreatic cancer cell lines (Bxpc-3 and Panc-1) were maintained in RPMI 1640 or DMEM medium (Gibco) with 10% fetal bovine serum (Thermo), and the suspension cultures of human burkitt's lymphoma cell line (Raji) were grown in DMEM with 10% fetal bovine serum. All cells were incubated at 37° in humidified air containing 5% CO_2, as described previously [24].

2.5. Plasmids and stable transfection

A full-length human DLL4 cDNA (GeneBank No. AF253468, 2.1 kb) was cloned from human placental cDNA using primers sense (5′–3′) CGGATCCACCATGGCGGCAGCGTCC, and antisense (5′–3′) GGAATTCTTATACCTCCGTGGCAATGACACA. This was followed by TA cloning into pGEM-T vector (Promega), then full length segments were cut by BAMH1 and ECOR1 (Takara) restriction enzymes and ligated into the plasmid pcDNA3.1 (+) vector (Invitrogen), cloning accuracy was verified by DNA sequencing.

One day before transfection, cancer cells were seeded at a density of 6×10^5/well (BxPC-3) or 4×10^5/well (Panc-1) in a six well plate. The cells were transfected with DLL4-pcDNA3.1 (+) or the native vector using Lipofectamine™2000 (Invitrogen). Stable expression clones were selected by using Geneticin (Gibco) for at least 3 weeks, then the single clone overexpressing human DLL4 was selected from survival cells by using limited dilution method.

Considering direct cell–cell interaction (receptor–ligand interactions) is required for the activation of notch signaling pathway, stable transduction cells were allowed to grow to confluence before the following *in vitro* experiments.

2.6. Flow cytometry

Cells were resuspended in 100 μl FACS buffer (1 × PBS, 0.2% FBS) and incubated with 5 μl anti-CD133–PE (Miltenyi Biotec) antibody for 15 min on ice. For analysis, cells were resuspendend in 500 μl FACS buffer and then sorted with a BD FACSCalibur (BD Biosciences), IgG1-PE (Miltenyi Biotec) was used as an isotype control.

2.7. VEGF-ELISA assay

The DLL4 stable transfected cells were plated in a six well plate (1×10^5) and incubated for 36 h in 2 ml of DMEM or 1640 medium containing 2% FBS. Then the supernatant was collected and the VEGF concentration was determined with the human VEGF ELISA kit (R&D) according to the instructions.

2.8. Cell proliferation and cytotoxicity assays

The cell proliferation was quantified every 24 h by using a cell counting kit-8 (Dojindo, Japan) according to the manufacturer's protocol. The viability of cell lines treated with varying chemo-drugs was determined by the MTT (Sigma) assay according to the instructions. All experiments were repeated three times to determine means and SDs.

2.9. Western blot analysis

Whole-cell lysates were prepared using RIPA lysis buffer (1%Triton X-100, 1 mmol/L EDTA, 100 mmol/L NaF, 1 mmol/L Na_3VO_4, and protease ingibitor cocktail), and Western-blot analyses were performed as described previously [23,24]. The following antibodies were used at the indicated dilutions: DLL4 (1:1000, Abcam), cleaved Caspase-3 (1:1000, CST), cleaved Caspase-9 (1:1000, CST), cleaved PARP (1:100, CST), ZEB (1:1000, CST), E-cadherin (1:1000, CST), Vimentin (1:1000, CST), Snail (1:1000, CST) and beta-Actin (1:5000, Sigma).

2.10. Administration of γ-secretase inhibitor DAPT

DAPT was dissolved in DMSO (Sigma–Aldrich) and stored at −80 °C until use. Cell lines were treated with 1 μM, 10 μM DAPT (Sigma–Aldrich) or the same volume of DMSO (0.1% v/v) to block the Notch activation.

2.11. Experimental animals and in vivo protocol

All animal experiments were approved by the ethics committee of ZheJiang University. SCID mice (BALB/c) were housed in a SPF environment. 32 four to six week old female mice received subcutaneous tumor implantations with stably expressing cell lines of mock-vector or DLL4-vector. Human pancreatic cancer cell lines (BxPC-3-EV and BxPC-3-DV) were harvested by trypsinization, washed twice in PBS and resuspended to a final concentration of 5×10^6 cells/200 μl. Cell suspensions were subcutaneously injected into the flank of SCID mice. A week later, tumor volume was measured twice weekly according to the formula: Tumor = $a \times b^2/2$, where a and b are the largest and smallest lengths of the palpable tumor, respectively. Four mice in each group were kept untreated until sacrificed, and when the tumor volume approximately reached 100 mm^3, another four mice from each group were randomly selected for MRI and perfusion assays (3rd week). Gemcitabine was administered intraperitoneally to other mice at a dose of 100 mg/kg every week. The mice were sacrificed when tumor size reached 15 mm, and if lost 15% of body weight.

2.12. Tumor perfusion studies with fluorescent lectin and doxorubicin

For the lectin and doxorubicin perfusion study, the experimental mice were anesthetized by intraperitoneal injection of Pentobarbital (60 mg/kg). 10 min prior to tumor tissue harvesting, doxorubicin was injected intravenously at a dose of 20 mg/kg for quantification of autofluorescence, and DyLight™-594 labeled lycopersicon esculenfum lectin (Vector Laboratories) was injected into the tail vein and allowed to circulate for 3 min before the mice were euthanized. Tumor specimens were embedded in O.C.T. matrix (Sakura finetek), snapped frozen in liquid nitrogen, and stored at −80 °C.

2.13. Immunofluorescence staining

Tumor sections (15 μm thickness) were fixed with cold acetone for 10 min in 4 °C, and blocked with goat serum, all subsequent steps were followed by a 3 × 5 min wash in PBS. Sections were incubated with rat anti mouse CD31 antibody

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