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The MUC1 oncomucin regulates pancreatic cancer cell biological properties and chemoresistance. Implication of p42–44 MAPK, Akt, Bcl-2 and MMP13 pathways

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ABSTRACT

MUC1 is an oncogenic mucin overexpressed in several epithelial cancers, including pancreatic ductal adenocarcinoma, and is considered as a potent target for cancer therapy. To this aim, we undertook to study MUC1 biological effects on pancreatic cancer cells and identify pathways mediating these effects. Our *in vitro* experiments indicate that inhibiting MUC1 expression decreases cell proliferation, cell migration and invasion, cell survival and increases cell apoptosis. Moreover, lack of MUC1 in these cells profoundly altered their sensitivity to gemcitabine and 5-Fluorouracil chemotherapeutic drugs. *In vivo* MUC1-KD cell xenografts in SCID mice grew slower. Altogether, we show that MUC1 oncogenic mucin alters proliferation, migration, and invasion properties of pancreatic cancer cells and that these effects are mediated by p42–44 MAPK, Akt, Bcl-2 and MMP13 pathways.

1. Introduction

The mucins are a family of large O-glycoproteins divided into two groups: secreted mucins and membrane-bounds mucins [1]. Secreted mucins are involved in the protection of epithelial cells to external influences such as inflammation, pathogens or pH variations by forming the mucus gel which is a physical protective barrier of epithelia. Membrane-bound mucins are involved in cell interactions and cell signaling by transmitting intracellular signals of cell survival and cell growth under stress condition. The mucin MUC1 is a membrane-bound mucin expressed at the apical pole of normal epithelial polarized cells [2,3]. In cancer cells, MUC1 is frequently overexpressed, internalized, circumferentially delocalized around the tumor cell surface when cells lose polarity. MUC1 becomes oncogenic as it is involved in several oncogenic pathways leading to altered cell properties such as cell growth, cell migration and invasion [4]. These profound modifications result from the interaction of MUC1 with tyrosine kinase receptors such as EGFR and the activation of oncogenic signaling pathways like MAPK, Akt or Wnt/β-catenin [5–7].

Pancreatic Ductal Adenocarcinoma (PDAC) is the fourth leading cause of death by cancer in Western countries and has a very poor prognosis due to a late diagnosis and a lack of efficient treatment. PDAC have a dramatic outcome with the 5 years survival rate lower than 5% [8]. Less than 20% of patients are entitled to surgical resection and the remaining 80% of patients present a locally advanced metastatic PDAC [9]. They may benefit from palliative chemotherapy based either on gemcitabine or FOLFIRINOX composed of 5-Fluorouracil (5-Fu), oxaliplatin and SN-38 [10]. In 90% of PDAC, the mucin MUC1 is overexpressed and MUC1 was proposed as one of the most robust predictive marker of PDAC survival [11,12]. It was shown that MUC1 induced EMT [13] and resistance to gemcitabine [14] and also increased cell invasion through Stat3 [15] and PDGR-β [16]. For these reasons MUC1 appears as an attractive target to moderate pancreatic cancer progression.

To better understand MUC1 biological effects on PDAC cells, we undertook to study its effects both *in vitro* and *in vivo* and the underlying cellular mechanisms mediating these effects. We generated stable cell lines knocked-down for MUC1 and showed *in vitro* that inhibiting MUC1 decreased cell proliferation ybb p42–44 MAPK, cell migration and invasion via MMP13, cell survival and apoptosis *via* Akt and Bcl2, and tumor growth *in vivo*. These results confirmed the potential of MUC1 as an attractive target to slow-down PDAC progression.

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2. Materials and methods

2.1. Cell culture and establishment of MUC1-KD stable cell lines

Capan-2 cell line was purchased from ATCC and was cultured as described previously [17]. Capan-2 stable cell lines knocked-down (KD) for MUC1 (Capan-2 MUC1-KD1/2) were generated by retroviral infection as previously described [18] using MUC1-Sh1 primers FP: GATCCCCACCTCCAGTTTAATTCCTCTTCAAGAGAGAGGAATTAAA CTGGAGGTTTTTTA and RP: GGGTGGAGGTCGAATTAAGGAGAAGTT CTCTCTCCTTAATTCGACCTCCAAAAAATTCGA. MUC1-Sh2 primers FP: GATCCCCATGTTTTTGCAGATTTATATTCAAGAGATATAAATCTGC AAAAACATTTTTTA and RP: GGGTACAAAAACGTCTAAATATAA GTT CTCTATATTTAGACGTTTTTGTAAAAAATTCGA.

2.2. Cell proliferation, migration and invasion assays

Stable cell lines were seeded at 10^5 cells per well in 6-well plates. Cells were counted daily using a Malassez counting chamber using Trypan Blue exclusion dye (Life Technologies) during 96 h. Invasion and migration assays were respectively performed using 24-well Boyden chambers (8 μm pores) coated or not with Matrigel™ (Pharmingen, BD Biosciences). Briefly, 5×10^4 cells were seeded on the top chamber and FBS 10% (v/v) was used as a chemoattractant in the bottom chamber for 24 h. Staining were performed using Vectashield hard set mounting medium with DAPI H-1500 (4′,6′-diamidino-2-phénylindole) (Vector labs). Three independent experiments were performed in triplicate.

2.3. Cytotoxicity assay

10^4 cells were seeded in 96-well plates during 24 h. Medium was refreshed with gemcitabine, 5-Fu, oxaliplatin or SN-38 for 72 h. The viability of cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay (MTT, Sigma–Aldrich, Saint Quentin Fallavier, France) as previously described [17]. Formazan crystals were solubilized in dimethylsulfoxide (Sigma–Aldrich) and analyzed at 570 nm with a microplate reader (Bio-Rad).

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using miRNeasy Mini Kit with Qiazol® (Qiagen) according to the manufacturer's instructions. cDNA was prepared as previously described. qPCR was performed using SsoFast Evagreen® Supermix (Bio-Rad) and the CFX96 real time PCR system (Bio-Rad). Expression levels of MUC1 (FP: TGCCGCCGA AAGAACTACG and RP: TGGGGTACTCGCTCATAGGAT) were normalized to GAPDH (FP: CCACATCGCTCAGACACCAT and RP: CCAGGCG CCCAATACG). Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Three independent experiments were performed.

2.5. Protein extraction and Western-blot analysis

Total proteins were extracted, electro-transferred, immunostained, and visualized as described before [19]. Antibodies used were MUC1 (M8, 1/250, from Pr. D. Swallow); β-actin (A5441 AC15, 1/5000) from Sigma–Aldrich; cyclin D1 (sc-718, 1/250), CDK6 (sc-177, 1/250) from Santa Cruz; Bcl-2 (#2872, 1/250), phospho p42–44 MAPK (#9101, 1/500), p42–44 MAPK (#9102, 1/500), β-catenin (#8480s, 1/1000), phospho AKT (#4060, 1/500), AKT (#4691s, 1/500) from Cell Signaling, Ozyme; MMP13 (Ab39012, 1/1000) from Abcam. Bands were quantified with image J analysis software three independent experiments were performed.

2.6. Subcutaneous xenografts

Subcutaneous (SC) xenografts (2×10^6 cells in 100 μl of RPMI 1640) of MUC1-Mock, MUC1-KD1 and MUC1-KD2 cell lines were carried out with 100 μl of Matrigel™ (BD Biosciences) into the flank of severe-combined immunodeficient (SCID) male mice (CB-17, Charles Rivers) that were bred and maintained under pathogen-free conditions (6 mice/group). Tumor development was followed weekly. The tumor volume (mm^3) was determined by calculating $V = W2 \times L$ in which W corresponds to the width (mm) and L to the tumor length (mm). Mice were sacrificed 28 days after inoculation. All procedures were performed in accordance with the guidelines and approved by the animal care ethical committee (Comité Ethique Expérimentation Animale Nord Pas-de-Calais, CEEA #122012).

2.7. Immunohistochemistry

Tumor xenografts were fixed in 10% (w/v) buffered formaldehyde, paraffin-embedded, cut at 4 μm thickness and applied on SuperFrost® slides (Menzel-Glaser, Thermo Scientific). Histology was assessed by staining tissues with Hematoxylin-Eosin. Automatic IHC was performed with an automated immunostainer (ES, Ventana Medical System, Strasbourg, France) as described [20]. Immunostainings in xenografts were carried out with anti-MUC1 M8 Mab (1/50) antibodies.

2.8. Gene Expression Omnibus microarray

Public PC microarrays were analyzed from the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nml.nih.gov/geo/). A set of gene-expression profiles from GEO containing both normal pancreas and PC tissue was used: 45 tumors and adjacent non-tumor tissues from PDAC cases (GSE28735). Data were analyzed using GEO2R software. The dataset GSE28735 used Affymetrix GeneChip Human Gene 1.0 ST array.

2.9. Statistical analyses

Statistical analyses were performed using Graphpad Prism 4.0 software (Graphpad softwares Inc.). Data are presented as mean ± SD or ±SEM. Differences in the mean of samples were analyzed by the student's t test or one way ANOVA test with selected comparison using Tukey's HSD post-hoc test. Differences less than 0.05 considered significant and were indicated with a *, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

3. Results

3.1. Silencing of MUC1 in Capan-2 PDAC cell line

In order to study the functional role of MUC1 in PDAC we generated two stable cell lines knocked-down for MUC1 by short-hairpin RNA (Sh-RNA) approach. The MUC1 decreased expression in MUC1-KD cells compared to control Mock cells was confirmed by qRT-PCR (Fig. 1A) ($p < 0.05$) and by Western-blot (Fig. 1B).

3.2. Inhibiting MUC1 decreased PDAC cell growth in vitro

Cells knocked-down for MUC1 showed a statistically significant decrease of cell proliferation (Fig. 1C), at 72 and 96 h ($p < 0.01$). To identify the mechanisms underlying the alterations of cell proliferation mediated by MUC1 knock-down, we measured the activation of the major signaling markers/pathways in the three cell lines. Accordingly to the decreased cell proliferation, we observed a