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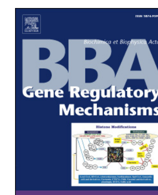

The mucin MUC4 is a transcriptional and post-transcriptional target of K-ras oncogene in pancreatic cancer. Implication of MAPK/AP-1, NF-κB and RalB signaling pathways

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ABSTRACT

The membrane-bound mucin MUC4 is a high molecular weight glycoprotein frequently deregulated in cancer. In pancreatic cancer, one of the most deadly cancers in occidental countries, MUC4 is neo-expressed in the preneoplastic stages and thereafter is involved in cancer cell properties leading to cancer progression and chemoresistance. K-ras oncogene is a small GTPase of the RAS superfamily, highly implicated in cancer. K-ras mutations are considered as an initiating event of pancreatic carcinogenesis and K-ras oncogenic activities are necessary components of cancer progression. However, K-ras remains clinically undruggable. Targeting early downstream K-ras signaling in cancer may thus appear as an interesting strategy and MUC4 regulation by K-ras in pancreatic carcinogenesis remains unknown. Using the Pdx1-Cre; LStopL-K-ras^{G12D} mouse model of pancreatic carcinogenesis, we show that the *in vivo* early neo-expression of the mucin Muc4 in pancreatic intraepithelial neoplastic lesions (PanINs) induced by mutated K-ras is correlated with the activation of ERK, JNK and NF-κB signaling pathways. *In vitro*, transfection of constitutively activated K-ras^{G12V} in pancreatic cancer cells led to the transcriptional upregulation of *MUC4*. This activation was found to be mediated at the transcriptional level by AP-1 and NF-κB transcription factors *via* MAPK, JNK and NF-κB pathways and at the post-transcriptional level by a mechanism involving the RalB GTPase. Altogether, these results identify MUC4 as a transcriptional and post-transcriptional target of K-ras in pancreatic cancer. This opens avenues in developing new approaches to target the early steps of this deadly cancer.

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1. Introduction

Pancreatic cancer is the 4th cause of death by cancer in the world [1] with a median survival lower than 6 months and an extremely low five-year survival rate (5%). This dramatic outcome can be explained by a late diagnosis and a lack of efficient therapies. At the time of diagnosis, only 10–15% of patients are eligible for surgical resection. For the other patients (85%), palliative gemcitabine-based chemotherapy remains the main option [2] but radio/chemo-resistance is frequent in pancreatic carcinoma (less than 30% of patients respond positively). Pancreatic carcinogenesis follows a hyperplasia/metaplasia/*in situ* carcinoma/invasive carcinoma development sequence. Pancreatic ductal adenocarcinoma (PDAC), the most frequent type of exocrine pancreatic cancer (85%), arises from ductal precursor lesions called Pancreatic Intraepithelial Neoplasia (PanIN), graded from hyperplasia (PanIN-1A/B) to *in situ* carcinoma (PanIN-3) [3]. Understanding mechanisms of initiating and progression of PanINs into adenocarcinoma may lead to identification of new therapeutic targets and diagnostic markers. Among histologic, cytologic and genetic alterations characterizing PanINs, K-ras mutations [4] and neoexpression of the mucin MUC4 have been described to occur as early as PanIN-1A stage in humans [5].

K-ras, a member of the Ras protein family is a small (21 kDa) guanosine tri-phosphate hydrolase protein (GTPase), ubiquitarily expressed [6]. Ras protein is a transducer of external stimuli from growth factors to signaling pathways through plasma membrane (notably tyrosine kinase receptors). Ras plays important roles in proliferation, apoptosis, senescence, migration, survival or angiogenesis. K-ras cycles between two states: an inactive state, bound to guanosine di-phosphate (GDP), and an active state, bound to GTP. Mutated K-ras is blocked into GTP-

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bound state and loses its ability to return to an inactivated state, inducing an upregulation of numerous downstream signaling pathways and cancer progression [7]. K-ras mutations are frequently observed in cancer (30% of cancer), especially in pancreatic cancer where mutations in K-ras (mainly at codons 12 and 13) are nearly systematic (>90%) [8]. Moreover, mutated K-ras is detected as early as PanIN-1A and participates in both initiation and progression of pancreatic carcinogenesis [9]. However, K-ras is still an undruggable clinical target [10].

Mucins are high molecular weight glycoproteins divided in two groups: (*i*) secreted mucins which participate in mucus architecture and epithelial protection and (*ii*) membrane-bound mucins that are implicated in cell–cell and cell–extra-cellular matrix interactions. Mucin aberrant overexpression is often associated with tumor progression [11]. MUC4 is a membrane-bound mucin expressed at the surface of epithelial cells in digestive-, respiratory- and reproductive tracts. Moreover, MUC4 is neo-expressed in pancreatic carcinogenesis as early as PanIN-1A stage with a gradual increase toward pancreatic adenocarcinoma, whereas it is not expressed in healthy pancreas [5].

In pancreatic cancer, MUC4 expression is associated with a poor prognosis [11]. It has been shown that MUC4 is involved in biological properties of cancer cells. Recent studies in our laboratory showed a direct interaction between MUC4 and its membrane partner, the oncogenic receptor ErbB2, also often overexpressed in pancreatic cancer [12]. Additionally, the involvement of MUC4 in gemcitabine resistance of pancreatic cancer cells [13] and its regulation by miRNAs [14] indicate that the oncogenic mucin MUC4 is a potential cancer biomarker and a promising therapeutic target. Understanding MUC4 regulation in the early steps of carcinogenesis will be useful for future therapies notably in pancreatic cancer.

MUC4 neo-expression and mutation in K-ras oncogene being early concomitant events and K-ras mutation being implicated in initiation of pancreatic carcinogenesis, we hypothesized that K-ras directly regulates MUC4. In this report, we show for the first time that indeed MUC4 is a target of K-ras signaling and that both transcriptional and post-transcriptional mechanisms are involved.

2. Material and methods

2.1. Mouse models

Pdx1-Cre (C57Bl/6 background) transgenic mice were obtained from the Mouse Models of Human Cancer Consortium (MMHCC, USA). LStopL-K-ras^{G12D} (C57Bl/6 background) transgenic mice were obtained from Dr. D. Tuveson (Cold Spring Harbor Laboratory, NY, USA). Mice were maintained as heterozygous lines and crossed to obtain Pdx1-Cre;LStopL-K-ras^{G12D} mice. Genomic DNA was extracted using the Nucleospin Tissue kit (Macherey Nagel, Hoerdt, Germany) and Cre and K-ras^{G12D} alleles were analyzed by PCR. [Cre: (forward) CCT GGA AAA TGC TTC TGT CCG, (reverse) CAG GGT GTT ATA AGC AAT CCC; K-ras: (forward K rasWT) GTC GAC AAG CTC ATG CGG GTG, (forward LStopL-K-ras^{G12D}) AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC A, (reverse) CCT TTA CAA GCG CAC GCA GAC TGT AGA]. Pdx1-Cre;LStopL-K-ras^{G12D} and Pdx1-Cre;K-ras^{WT} control mice were sacrificed by cervical dislocation at 3, 5, 9 and 12 months of age before dissection. All procedures were in accordance with the guideline of Animal Care Committee (Comité Ethique Expérimentation Animale Nord Pas-de-Calais, #AF042008 and #00422.01).

2.2. Immunohistochemistry

Sections of 5 μm of paraffin embedded pancreatic tissues from Pdx1-Cre;LstopL-K-ras^{G12D} mice and Pdx1-Cre;K-ras^{WT} were cut and deparaffinized using graded xylene–ethanol–water baths. Antigen sites were retrieved by heating in citrate buffer pH 6.0 (Dako Real™ Target Retrieval Solution) in a microwave for 20 min at 700 W or in a pressure cooker for 90 s at 100 °C followed by endogenous peroxidase inactivation in 3% (*v*/*v*) hydrogen peroxide for 30 min. A permeabilisation/saturation bath was performed by incubation in Teng-T 1X buffer (10 mM Tris–HCl pH = 8.0 containing 5 mM EDTA, 150 mM NaCl, 0.25% (*w*/*v*) gelatine, 0.01% (*v*/*v*) Tween® 20) (permeabilisation and saturation), or in D-PBS-BSA 3% (*w*/*v*) solution (saturation). Thereafter, sections were incubated overnight using anti-Muc4 1G8 (mouse, sc33654 Santa Cruz, 1/200), JNK and phospho JNK (rabbit, 9258 and 9251S, Cell Signaling, 1/100 and 1/50 respectively), p42/44 MAPK and phospho p42/44 MAPK (rabbit, 9102and 4377, Cell signaling, 1/500 and 1/100 respectively), p65 NF-κB and phospho p65 NF-κB (rabbit, sc7151X and sc33020, Santa Cruz, 1/500 and 1/100 respectively) antibodies at 4 °C. Then, the slides were incubated for 1 h with corresponding biotinylated secondary antibodies followed by incubation with Vectastain Elite solution (PK6100, Vector). Staining was done by incubation in 0.5 mg/ml of 3,3′diaminobenzidine (DAB), 0.02% (*v*/*v*) H_2O_2 in 30 mM imidazole, and 1 mM EDTA (pH 7.0). A counter-stain with hematoxylin (Sigma) and lithium carbonate was performed. Finally, after dehydration by graded H_2O–ethanol–xylene baths, sections were mounted using Pertex (Monting medium for light microscopy, Histolab). Controls were performed in the same conditions without primary antibodies. Each staining was performed on tissues from five different mice at every age. IHC stainings were scored by an expert gastrointestinal tract pathologist (FR). Results were evaluated as no/weak/strong staining and expressed as % of normal or PanINs stained tissue.

2.3. Cell culture

Human pancreatic cancer cell lines BxPC3 and CAPAN-2 were cultured as previously described [14,17]. The human immortalized pancreatic cell line HPNE was a gift from D. M. Ouellette (UNMC, Omaha, Nebraska) and was cultured in 1/3 of M-3 Base medium (INCELL) and 2/3 of Dulbecco's modified Eagle's medium (Gibco, Life Technologies) supplemented with 5% (*v*/*v*) of fetal bovine serum (Gibco, Life Technologies), 100 U/ml of penicillin, 100 μg/ml of streptomycin (Invitrogen, Life Technologies) and 1 ng/ml of recombinant EGF (Gibco, Life Technologies). All cell lines were maintained in an incubator at 37 °C with 5% CO_2.

2.4. Transfection and luciferase assays

Transient transfection experiments were performed 24 h after seeding using Effectene™ (Qiagen) as previously described [17] using 0.25 μg of expression vectors. PBABE-puro-K-ras^{G12V} was obtained from W. Hahn (Addgene plasmid # 9052). pBABE-puro was obtained from H. Land, J. Morgenstern and B. Weinberg (Addgene plasmid # 1764). PZIP and pZIP-ras15A plasmids were obtained from J. Merchant (University of Michigan, USA). NF-κB and AP-1 luciferase assays were performed as previously described using synthetic promoters containing respectively three κB and AP-1 binding sites [13]. In co-transfection studies, 1 μg of pGL3-*MUC4* promoter reporter vectors was co-transfected with 0.25 μg of either the expression vector pBABE-K-ras^{G12V} or corresponding empty vector. Results were expressed as fold induction of luciferase activity in cells transfected with K ras^{G12V} expression vector compared with that obtained with the empty vector. Transient inhibition of MUC4 (L-004577-00), K-ras (L005069-00), Erk1 (MAPK3, L-003514-00), Erk2 (MAPK1, L-003555-00), JNK1 (MAPK8, L-003592-00), JNK2 (MAPK9, L-003505-00) and NF-κB (p105 NF-κB, L-003520-00 and L-003918-00) and control non-targeting siRNA (NT, D-001,810-02) was carried out using siRNA from Dharmacon (Thermo Scientific) as previously described [13].

2.5. Western blotting

Total protein extracts were performed as previously described [13] and quantified using bicinchoninic acid method following the manufacturer's instruction (Pierce). Western blot experiments

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