Changes in myostatin signaling in non weight-losing cancer patients

CHANGES IN MYOSTATIN SIGNALING IN NON WEIGHT-LOSING CANCER PATIENTS

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Running title: Myostatin modulations in cancer patients

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Synopsis

The study shows that myostatin signaling is altered in non weight-losing cancer patients. Modulations at the molecular level in the skeletal muscle are different depending on tumor type, and occur even before cachexia becomes clinically apparent.

Abstract

Background Myostatin is a negative regulator of skeletal muscle mass. We recently demonstrated that myostatin expression is upregulated in an experimental model of cancer cachexia, suggesting that modulations of this pathway might play a pathogenic role in cancer-related muscle wasting. The present study was aimed at investigating whether myostatin signaling is modulated in the muscle of non weight-losing lung and gastric cancer patients.

Methods Myostatin signaling was studied in muscle biopsies obtained during surgical procedure from non weight-losing (nWL) patients affected by gastric (n=16) or lung (n=17) cancer. Western blotting was applied to test both the total expression of myostatin and the expression of phosphorylated form of GSK-3beta and Smad2/3.

Results In gastric cancer patients the expression of both myostatin and phosphorylated GSK-3beta (p-GSK3β) were significantly increased. By contrast, in lung cancer patients, myostatin levels were comparable to controls, while the expression of p-GSK3β significantly decreased in subjects with disease stage III/IV.

Conclusions Myostatin signaling is altered in nWL cancer patients. Different tumor types may give rise to different patterns of molecular changes within the muscle, that occur even before cachexia becomes clinically apparent.

Keywords: Cachexia, muscle wasting, myostatin, cancer, SMAD 2/3, GSK-3β

Introduction

Myostatin, also known as growth and differentiation factor-8 (GDF-8) is a negative regulator of muscle mass [1]. Myostatin is secreted as an inactive propeptide that is cleaved to generate a mature ligand, whose activity may be regulated *in vivo* by association and dissociation with binding proteins, including propeptide itself as well as follistatin or related molecules [2]. Active myostatin binds the Activin type II B receptor (ActRIIB) and, to a lesser extent, the related ActRIIA, resulting in the phosphorylation and consequent recruitment of the low-affinity type I receptor ALK (Activin Receptor Like-Kinase)-4 or ALK-5 [3,4]. This binding induces phoshorylation and activation of the transcription factors SMAD 2 and 3 [mammalian homolougue of *Drosophila* MAD (Mothers-Against-Decapentaplegic gene)], which translocate into the nucleus and together with SMAD 4 regulate the expression of target genes [5]. In addition myostatin has been suggested to exert its action through different pathway such as the Extracellular signal-Regulated Kinase (ERK)/Mitogen Activated Protein Kinase (MAPK) cascade [6].

Inactivating mutations of myostatin gene have been found in the “double-muscled cattle phenotype” [7] as well as in humans [8]. Myostatin null mice are characterized by marked muscle enlargement (~ 100%-200% more than controls), exhibiting both fiber hypertrophy and hyperplasia [1], while systemic administration of myostatin in adult mice induces profound muscle and fat loss [9]. Moreover, high myostatin protein levels have been reported in conditions associated with muscle depletion such as ageing, denervation atrophy or mechanical unloading [10,11]. Myostatin expression is upregulated in experimental models of cancer cachexia, suggesting that modulations of this pathway might have a pathogenic role in cancer-related muscle wasting [12, 13].

The present study was aimed at investigating whether myostatin signalling is modulated in the muscle of non-weight-losing gastric and lung cancer patients.

Materials and Methods

Patients. Thirty-three consecutive (16 stomach, 11 M, 5 F; 17 lung, 15 M, 2 F) cancer patients were studied, enrolled among those undergoing abdominal or thoracic surgery at the S.Andrea Hospital, II School of Medicine, Sapienza University of Rome and the Vito Fazzi Hospital, Lecce, Italy, respectively (see also Table 1). The study was approved by the local ethic committees and written informed consent was obtatined from all patients before enrollment in the study. Diagnosis of gastric cancer was performed by endoscopic biopsy, while diagnosis of lung cancer was performed by thoracoscopic or percutaneous biopsy. Age- and sex-matched control subjects were recruited among patients undergoing thoracic (3 males, 3 females, mean age 65±12 years) or abdominal (5 males, 5 females, mean age 63±10 years) surgery for non-neoplastic diseases. Reasons for thoracic surgery in controls were amartoma (n=4) and inflammatory non-tubercular granuloma (n=2). Reasons for abdominal surgery in controls were aneurisma of the abdominal aorta (n=3), inguinal hernia (n=2), laparocele (n=3), non-complicated cholelythiasis (n=2). Sex match was sub-optimal between lung cancer patients and thoracic controls due to the marked difference in prevalence of lung tumors in males and female subjects in Italy (14). Exclusion criteria for both cancer patients and controls were: acute and chronic renal failure, liver failure, diabetes, metabolic acidosis, sepsis, AIDS, inflammatory bowel disease, autoimmune disorders, acute and chronic hepatitis and chronic ostructive pulmonary disease.

Nutritional assessment. The nutritional assessment included anthropometric [height, actual body weight, % weight loss (WL) during the previous 6 months, body mass index (BMI), usual body weight], immunological (total lymphocyte count), and biochemical (serum albumin) indices.

Muscle biopsy. Biopsy specimens were obtained intraoperatively, during the initial phase of the operation, from the rectus abdominis muscle in patients undergoing abdominal surgery and from the dentatus anterior muscle in patients undergoing thoracic surgery. After skin incision and dissection through the subcutaneous fat, the anterior sheet of the rectus abdominis or dentatus anterior muscle was opened with scissors and a muscle biopsy specimen weighing about 0.5 g was obtained. Small bleeding vessels were carefully controlled with ligatures and cautery after the muscle biopsy had been obtained, whereafter the operation continued in a routine fashion. No complications occurred from the biopsy procedure. Biopsy specimens were immediately frozen in liquid nitrogen and stored at -70 °C until analysis; part of the specimens was used for the present study, part was kept stored for further, subsequent investigations.

Western blotting. About 100 mg of rectus abdominis or dentatus anterior were homogenized in 80 mm TRIS-HCl, pH 6·8 [containing 100 mm dithiothreitol (DTT), 70 mm Sodium Dodecyl Sulfate (SDS), and 1 mm glycerol], kept on ice for 30 min, centrifuged at 15 000 × g for 10 min at 4 °C, and the supernatant collected. Protein concentration was assayed according to Lowry [15], using Bovine Serum Albumine (BSA) as a working standard. Equal amounts of protein (30µg) were heat-denaturated in sample-loading buffer (50 mm TRIS-HCl,pH 6·8, 100 mM DTT, 2% SDS, 0·1% bromophenol blue, 10% glycerol), resolved on a SDS-Polyacrylamide Gel Electrophoresis (PAGE; 12% polyacrylamide, 0·1% SDS) and transferred to

nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Protein transfer was checked by Ponceau S staining. The filters were then blocked with Tris-Buffered-Saline (TBS) containing 0·05% Tween and 5% non-fat dry milk and incubated overnight with primary antibodies against myostatin (1:1000; Società Italiana Chimici, Rome, Italy), specific for the ~30 kDa processed myostatin, raised against a synthetic peptide (aa 133-148) representing a portion of human GDF-8 encoded within exon 3 (LocusLink ID 2660); a goat anti phospho-Smad2/3 polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against a short amino acid sequence containing phosphorylated Ser 423 and Ser 425 of Smad3 of human origin (~55 kDa); a rabbit polyclonal antibody against phospho-GSK-3β (Ser 9, 1:1000; Cell Signaling Technology, Danvers, MA, USA), binding the ~46 kDa phosphorylated form of the kinase.

Goat anti-rabbit, goat anti-mouse (Bio-Rad, Hercules, CA, USA) or rabbit-anti-goat (Millipore, Vimodrone, MI, Italy) peroxidase-conjugated IgG were used as secondary antibodies.

Filters were then stripped (30 min′, 50 °C, in 62·5 mm Tris-HCl, pH 6·7, containing 100 mm 2-mercaptoethanol and 2% SDS) and reprobed with a goat anti-GAPDH polyclonal antibody (~37 kDa; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to normalize sample loading. Membrane-bound immune complexes were detected by enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on a photon-sensitive film. Bands were quantified by densitometry scanning and elaborated as described below.

*Data analysis and presentation***.** Data are expressed as mean ± SD. Quantification of western blotting results was performed by densitometric analysis

(TotalLab, NonLinear Dynamics, Newcastle upon Tyne, UK). Significance of the differences was evaluated by the Student's 't' test. A p value < 0.05 was considered significantly different.

Results

Patients' characteristics are shown in Table 1. The mean age of gastric cancer patients was 66±3 years and the mean age of lung cancer patients was 65±10 years. According the to the UICC classification of gastric cancer [16], 13 patients were in stage I and II and 3 patients were in stage III/IV. According to the TNM staging for lung cancer, 10 patients were in stages I and II and 7 patients were in stage III and IV [17]. Percentage of body weight loss was 3.71±4.58 in lung cancer patients and 5.59±5.87 in gastric cancer patients. Stratifying lung cancer patients according to the disease stage, the percentage of body weight loss was 2.22±3.15 for early stages (I/II) and 6.49±5.74 for advanced stages (III/IV). Stratification has not been made for gastric cancer patients because of the small number of patients in advanced stage of the disease.

According to nutritional indices (Table 2) patients of both groups were not malnourished. Gastric cancer patients showed a mild anemia, with hemoglobin level significantly reduced with respect to lung cancer patients ($p= 0.008$).

Myostatin protein levels were significantly increased in the muscle of gastric cancer patients ($p<0.001$) (Fig. 1 A), while no differences were observed in the muscle of lung

cancer patients even when stratified for disease stage (Fig 1 B). The relative level of myostatin was higher in control muscles obtained by patients undergoing thoracic surgery in respect to patients undergoing abdominal surgery: although the reason of such difference is not known, it might possibly reflect individual differences among controls subjects recruited in the study or be due to the different anatomic districts used for skeletal muscle biopsies (dentatus anterior vs rectus abdominis). Phosphorylated-SMAD 2/3 (p-SMAD 2/3) did not change with respect to controls in gastric cancer patients (Fig. 2A). By contrast, in lung cancer patients p-SMAD 2/3 levels progressively increased, being about 150% and 400% of controls in stage I/II and III/IV, respectively (Fig. 2B). Phosphorylated-GSK-3β (p-GSK-3β) was significantly ($p<0.05$) increased in gastric cancer patients versus controls (Fig. 3A). In lung cancer patients only a trend towards increase could be observed in stage I/II patients. By contrast, a decrease was observed in stage III/IV subjects (Fig. 3B). The lack of significance is likely due to inter-group variability.

Discussion

Cachexia is a major issue in clinical oncology, negatively affecting morbidity, mortality and quality of life [18]. However, little attention is still paid to cancer-related weight loss and to its evolution into cachexia, its treatment being generally confined to terminal palliative cares. Conversely, cachexia should not be considered a late, pre-terminal event in the history of cancer patients [19]. The progressive loss of skeletal muscle mass is one of the most clinically relevant features of cancer cachexia. Enhanced protein breakdown is believed to be the main determinant of muscle depletion, with the ATP-ubiquitin-dependent system playing the major pathogenic role [19]. Indeed, a

considerable body of evidence indicates that this proteolytic system is upregulated in different experimental models of cancer cachexia, as well as in other conditions associated with muscle atrophy [20]. Conversely, human data are still scanty and inconsistent. In this respect, ubiquitin-dependent proteolysis was shown to be enhanced in the skeletal muscle of patients with upper g.i. tract cancer [21-23] but not in those with lung cancer [24]. The mechanisms ultimately leading to increased ubiquitin-dependent proteolysis in cancer-related muscle depletion, however, are not completely elucidated. Recently, it has been proposed that upregulation of myostatin-dependent signalling might result in increased expression of muscle-specific ubiquitin ligases [25], which in turn would be responsible for enhanced ubiquitin/proteasome-dependent proteolysis. This hypothesis is consistent with the observation of increased myostatin expression in several conditions associated with muscle wasting, such as AIDS, glucocorticoid treatment, unloading and disuse, aging [26]. Recently, upregulation of myostatin expression and bioactivity was reported by us and by others in different experimental models of cancer cachexia [12, 13]. To the best of our knowledge, no studies are available evaluating the state of activation of myostatin signalling in the skeletal muscle of cancer patients.

The present study is aimed at evaluating muscle myostatin signalling in two groups of patients, bearing gastric or lung cancer. The data obtained suggest that, similarly to what has been shown for ubiquitin-dependent proteolysis [21, 22, 24], myostatin signalling is differentially affected during gastric and lung cancer growth. In fact, in muscle biopsies from patients undergoing surgery for gastric cancer, a 6-fold increase in myostatin expression was observed with respect to controls. This increase was not accompanied by a consistent rise in p-SMAD 2/3 levels. Although the reason of

this result is not known at present, a possible explanation could be that myostatin increase is paralleled by a concomitant rise in the expression of follistatin, a physiological inhibitor of myostatin. This would result in a myostatin/follistatin ratio similar to controls, thereby maintaining the myostatin signalling in basal conditions. This reasoning is supported by the results obtained in experimental conditions [12]. In addition, unchanged levels of p-Smad 2/3, despite increased myostatin protein expression may also reflect a modulation of other molecules signaling through the activin receptor type IIB, such Activin A [27,28] and further investigations are needed to test also this hypothesis.

Recently, cross-talking between myostatin pathway and the IGF-1 axis, has been postulated [29]. According to this hypothesis, myostatin would target and inactivate Akt, one of the main kinases involved in the IGF-1-dependent signaling pathway. This would lead to increased atrogin-1 expression and hyperactivation of the ubiquitin proteasome pathway. Besides, myostatin could inhibit the IGF-1 signalling by activating GSK-3β [30]. Unlike these findings, however, in the present study, GSK-3β in gastric cancer patients was significantly hyperphosphorylated (i.e. inactivated) with respect to controls. These observations, which are similar to those obtained in the AH-130 model of cancer cachexia [Penna et al, submitted], suggest that in this clinical setting IGF-1 signalling is not down-modulated.

A different picture was observed in muscle biopsies from lung cancer patients. In fact, myostatin expression was not increased with respect to controls, irrespective of tumor stage, while SMAD 2/3 phosphorylation was > 4-fold increased in stage III/IV patients. Similarly to gastric cancer patients, also these findings may be explained by modulations of follistatin expression, that could be reduced with respect to controls, leading to an increased myostatin/follistatin ratio, in turn resulting in upregulation of

myostatin signalling. As for GSK-3β, no significant changes were observed with respect to controls.

Taken together, the results of the present investigation suggest that different tumor types may give rise to different patterns of molecular changes within the muscle, consistently with available data on the ubiquitin system modulation [21-24].

The modulations of follistatin postulated above might be at least in part responsible for these different molecular patterns. However, this hypothesis remains largely speculative, since, unfortunately, follistatin expression was not evaluated in this study. Besides the described discrepancies, the data obtained indicate that, independently of tumor type and stage, intramuscular IGF-1 pathway is not down-regulated, in agreement with data previously obtained in the cachectic rat. In summary, analogies and diversities exist in the pathogenesis of muscle depletion in cancer patients, strengthening the view that cachexia should be regarded to as a common, final phenotypic picture resulting from a combination of several mechanisms which may be different not only among diseases characterized by muscle atrophy, but also among different cancers.

Of particular interest is the observation that in the present study the reported changes in muscle molecular patterns were obtained in non-weight losing cancer patients. Indeed, mean weight loss was < 10% of usual body weight in both groups of patients examined. These findings are consistent with our previous data in gastric cancer patients showing that the upregulation of the ubiquitin-dependent proteolysis is an early phenomenon, occurring before the onset of significant weight loss, and further support the concept that early interventions, specifically targeting altered molecular pathways, should be entertained in order to prevent, rather than treat cancer cachexia [19, 31].

Molecules involved in the regulation of myostatin signaling pathway may be good candidates for the designing of targeted therapeutic strategies, which are currently under study. In particular, encouraging results were obtained in different models of muscle atrophy by myostatin gene disruption, treatment with anti-myostatin antibodies or myostatin antisense RNA, overexpression of a dominant negative form of the myostatin receptor, or administration of a soluble form of the same receptor [26]. It is likely that in the near future the myostatin pathway will become a suitable target for the prevention and treatment of disease-related muscle loss.

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Table 1. Characteristics of the subjects studied

	Lung cancer patients	**Thoracic Controls**	**Gastric cancer Patients**	**Abdominal Controls**
Subjects *(n)*	17 (15M, 2F)	6 (3M, 3F)	16 (11M, 5F)	10 (5M, 5F)
Age, years *(mean±SD)*	66±9	65±12	65±10	63±10
Type				
NSCLC	17	-	-	-
Adenocarcinoma *(n)*	-	-	15	
Lymphoma *(n)*	-	-	1	-
Stage				
I-II	10	-	13	-
III-IV	7	-	3	-
Percent Weight Loss *(mean±SD)*	3.7±4.6	No	5.6±5.9	No
Body Mass Index *(mean±SD)*	24.6±2.4	25.6±2.2	27.4±4.3	26.9±3.9

Table 2. Nutritional indices

	Lung cancer patients	Thoracic controls	Gastric cancer patients	Abdominal Controls
Serum total protein *(g/L)*	69.8±6.4	71±3.2	63.6±6.2	69.2±3.4
Serum albumin *(g/L)*	37.6±5.0	38.4±4.8	35.7±4.2	38.7±2.9
Serum creatinine *(mg/L)*	0.8±0.1	0.8±0.2	0.9±0.2	0.7±01
Haemoglobin *(g/dL)*	13.6±1.7	14.3±2.4	11.4±2.7 **	13.9±2.1
White cell count *(x10^3/cmm)*	8.9±2.4	6.9±1.8	6.5±1.1	6.6±1.3
Total lymphocyte count *(x10^3/cmm)*	2.1±0.9	2.2±0.8	1.9±0.7	2.0±0.9

Significance of the differences: **p<0.01 vs. lung cancer patients

Figure legends

Figure 1 Myostatin protein levels in *rectus abdominis* muscle of gastric cancer patients (A): representative western blots for myostatin and GAPDH (loading control) are shown on the right panel and densitometric quantifications of myostatin protein levels normalized to GAPDH are shown on the left panel.

Myostatin protein levels in *dentatus anterior* muscle of lung cancer patients (B): representative western blots for myostatin and GAPDH (loading control) are shown on the lower panel and densitometric quantifications of myostatin protein levels normalized to GAPDH are shown on the upper panel.Data (means ± SD) are expressed as percentage of controls (C = 100%). Significance of the differences: ***p<0.001

Figure 2 Phosphorylated-Smad 2/3 protein levels in *rectus abdominis* muscle of gastric cancer patients (A): representative western blots for p-Smad2/3 and GAPDH (loading control) are shown on the right panel and densitometric quantifications of p-Smad2/3 protein levels normalized to GAPDH are shown on the left panel.

Phosphorylated-Smad 2/3 protein levels in *dentatus anterior* muscle of lung cancer patients (B):: representative western blots for p-Smad2/3 and GAPDH (loading control) are shown on the lower

panel and densitometric quantifications of p-Smad2/3 protein levels normalized to GAPDH are shown on the upper panel.

Data (means ± SD) are expressed as percentage of controls (C = 100%). Significance of the differences: ***p<0.001

Figure 3 Phosphorylated GSK-3β protein levels in *rectus abdominis* muscle of gastric cancer patients (A): representative western blots for p- GSK-3β and GAPDH (loading control) are shown on the right panel and densitometric quantifications of p-GSK-3β protein levels normalized to GAPDH are shown on the left panel.

Phosphorylated GSK-3β protein levels in *dentatus anterior* muscle of lung cancer patients (B): representative western blots for p- GSK-3β and GAPDH (loading control) are shown on the lower panel and densitometric quantifications of p-GSK-3β protein levels normalized to GAPDH are shown on the upper panel. Data (means ± SD) are expressed as percentage of controls (C = 100%). Significance of the differences: *p<0.05

Fig.1

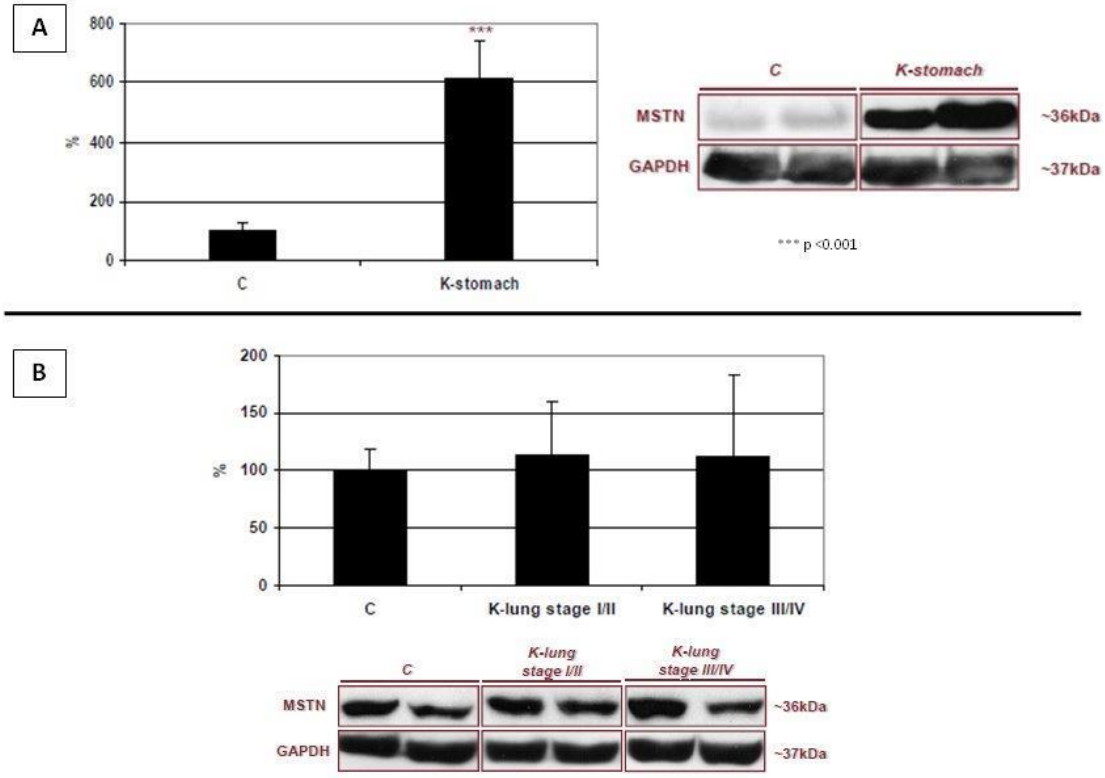
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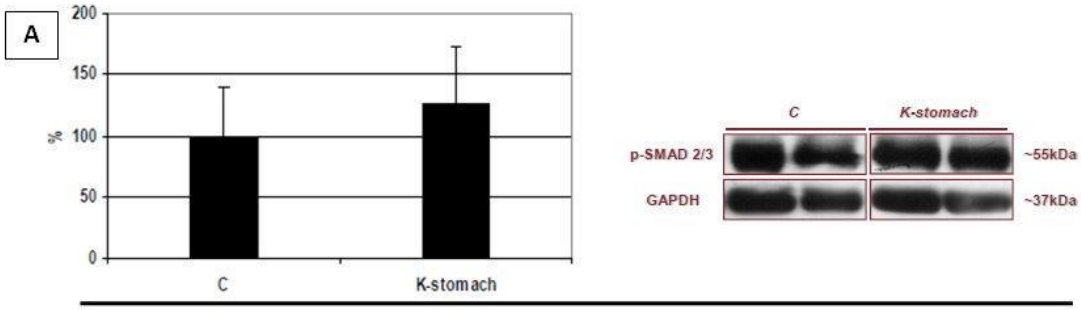
Fig.2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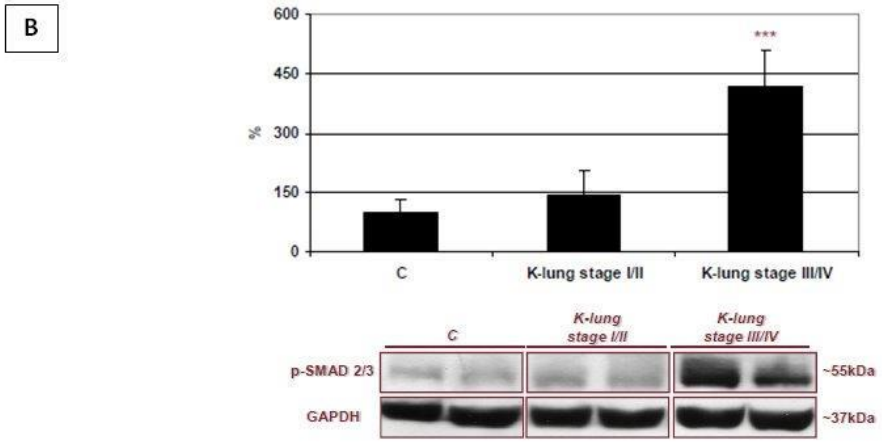
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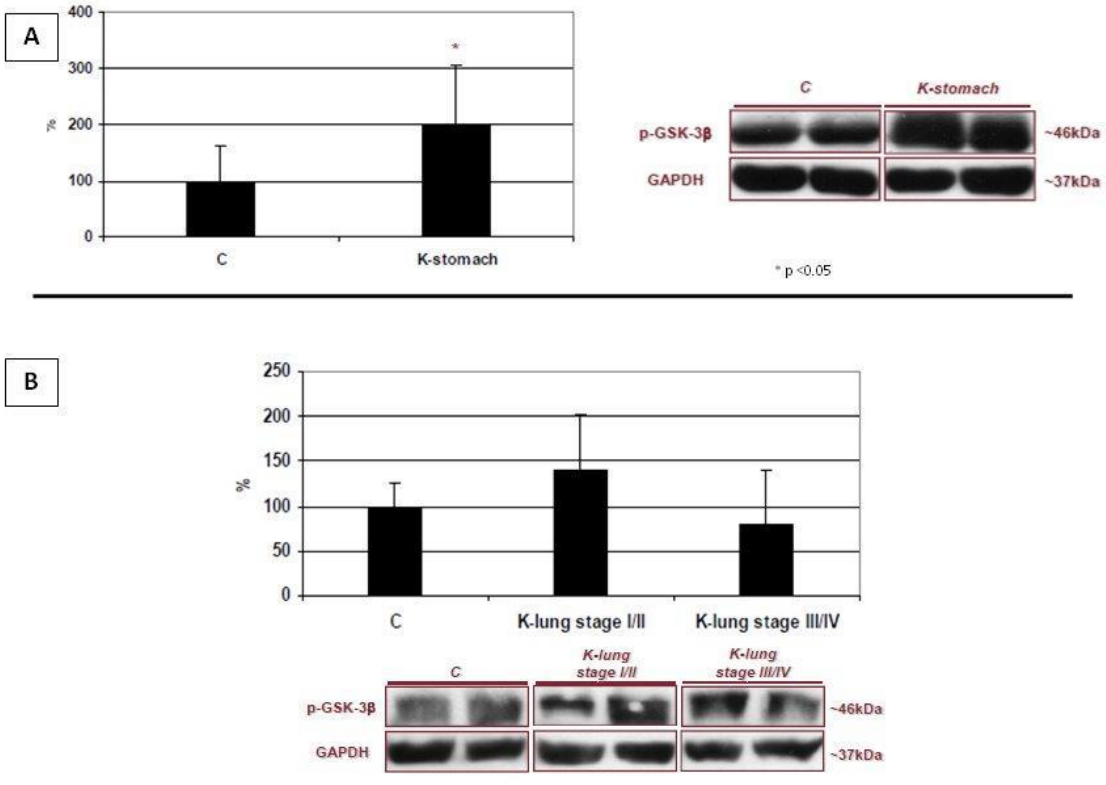
Fig.3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K-lung stage I/II
K-lung stage III/IV
C
K-lung stage I/II
K-lung stage III/IV
p-GSK-3β
GAPDH
~46kDa
~37kDa