Proteomic Differential Display Analysis Shows Up-regulation of 14-3-3 Sigma Protein in Human Scirrhous-type Gastric Carcinoma Cells

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Abstract. *This study performed proteomic differential display analysis of human scirrhous-type gastric carcinoma (SGC) cell lines and normal gastric mucosa (NGM) tissues by using two-dimensional gel electrophoresis (2-DE) and liquid chromatography–tandem mass spectrometry (LC-MS/MS). The human SGC cell lines were OCUM-1, OCUM-2M, OCUM-2MLN, OCUM-2D, OCUM-D3, OCUM-9 and OCUM-12. Among the SGC cell lines and the NGM tissues, 28 protein spots were found whose expression levels were different from the results of 2-DE: 19 protein spots appeared higher, and 9 other protein spots appeared lower in SGCs than in NGM tissues. These spots were analysed by LC-MS/MS analysis and identified by a peptide sequence tag. Identified increased spots included elongation factor 1-beta, 14-3-3 sigma, tropomyosin alpha-4 chain, protein DJ-1, nucleoside diphosphate kinase A, elongation factor Tu and peroxiredoxin-1. Western blot analysis showed increased protein level of 14-3-3 sigma in SGCs. Although OCUM-1 and AGS (gastric cancer) showed up-regulation of 14-3-3 sigma, MiaPaca-2 (pancreatic cancer), Huh-7 (HCC) and NCI-H2052 (malignant pleural mesothelioma) showed very weak expression of 14-3-3 sigma. The up-regulation of 14-3-3 sigma may play an important role in SGC carcinogenesis and progression and may be used as a diagnostic biomarker of SGC.*

Scirrhous-type gastric carcinoma (SGC) diffusely infiltrates into a broad region of the stomach accompanied by extensive stromal fibrosis and is frequently associated with metastasis to lymph nodes and peritoneal dissemination (1), showing a markedly poor prognosis with 5-year survival rates in the range of 10-15% (2). Although a novel oral fluorouracil derivative, S-1, has recently been proposed as first-line chemotherapy for SGC patients, still many patients with advanced SGC survive less than a year (3). Therefore, it is important to understand the biology of SGC and to find a molecular target for its therapy.

Proteome refers to the total protein complement of a genome (4). Anderson *et al*. defined proteomics as the use of quantitative protein-level measurements of gene expression to characterize biological processes (disease processes and drug effects) and decipher the mechanisms of gene expression control (5). The combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) is a sophisticated method of high-throughput proteomic analysis. Many proteomic studies using 2-DE and MS have identified various proteins that may be involved in the pathogenic mechanism of cancer in cancer cell lines, cancer tissues and sera from cancer patients (6, 7).

In the present study, proteomic differential display analysis using 2-DE and LC-MS/MS was performed to examine the difference in proteome between SGC cells and normal gastric mucosa (NEM) tissues, with the aim of identifying the protein(s) specific to SGC which may be useful for developing an appropriate therapy.

Materials and Methods

Scirrhous-type gastric carcinoma cell lines and culture conditions. The study used seven human SGC cell lines (OCUM-1, OCUM-2M, OCUM-2MLN, OCUM-2D, OCUM-D3, OCUM-9 and OCUM-12), AGS (a human gastric adenocarcinoma cell line), MiaPaca-2 (a human pancreatic adenocarcinoma cell line) and Huh-7 (a human hepatocellular carcinoma cell line). They were maintained in continuous *in vitro* culture in Dulbecco's modified Eagle's medium

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(DMEM) (high glucose; Wako, Osaka, Japan) with 2 mM L-glutamine and 10% FBS at 37˚C in a 5% CO_2 atmosphere. NCI-H2052 (a human malignant pleural mesothelioma cell line) was maintained in continuous *in vitro* culture in RPMI-1640 medium (Nissui, Tokyo, Japan) with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES and 90% 1.0 mM sodium pyruvate, and 10% FBS at 37˚C in a 5% CO_2 atmosphere.

Normal gastric mucosa tissues. Five non-cancerous gastric mucosa tissues were obtained from patients who were diagnosed with non-scirrhous-type gastric carcinoma and underwent surgical gastric resection at the Department of Surgery II, Yamaguchi University Hospital between January and December 2008. Written informed consent was obtained from all patients before surgery. None of the patients received any preoperative therapy such as chemotherapy or radiation. The study protocol was approved by the Institutional Review Board for Human Use of the Yamaguchi University School of Medicine.

Sample preparation. Cells and tissues were homogenised in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% NP-40) on ice. Suspensions were incubated for 1 h at 4˚C, centrifuged at 21,500 × *g* for 30 min at 4˚C and the supernatants were stored at –80˚C until use (8).

Two-dimensional gel electrophoresis (2-DE). The 2-DE was carried out according to the method described by Tanaka *et al*. (9). Briefly, for the first dimension, isoelectric focusing (IEF) was performed in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilised, pH 3-10 linear gradient strips (Bio-Rad, Hercules, CA, USA) at 50 μA/strip. In the second dimension, SDS-PAGE was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (Bio-Rad), run at 200 V.

Fluorescent gel staining. After 2-DE, the gels were subjected to fixing solution with 40% ethanol and 10% acetic acid for 2 h. The gels were stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (Bio-Rad) overnight. Stained gels were washed with Milli-Q (Millipore Bedford, MA, USA) water three times.

Image analysis and spot picking. The positions of the protein spots on the gels were recorded by the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA). Expression levels of the proteins were quantified by analysing the intensity of each spot with Progenesis PG240 software (PerkinElmer Inc.) (9). The differences in expression between SGC cell lines and NGM tissues were analysed statistically by the Student's *t*-test. The 2-DE analysis was performed five times. After statistical analysis, the gels were re-stained with See Pico™ (Benebiosis Co., Ltd, Seoul, Korea), and the selected spots, whose expression was significantly different between SGC cell lines and NGM tissues, were cut and removed for the MS analysis.

In-gel digestion. In-gel digestion was carried out according to the method described by Tanaka *et al*. (9). Briefly, the sample in the gel piece was reduced twice in 50% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel piece was dehydrated in 100% ACN twice for 30 min, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml sequencing-grade-modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT.

LC-MS/MS. An Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. Twenty-five μl of each sample was applied and separated on a column (Zorbax 300SB-C18, 75 μm, 150 mm, Agilent Technologies). Protein identification was performed in an Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine (http://kr.expasy.org/sprot/) and the MASCOT MS/MS ions search engine (http://www.matrixscience.com/search_form_select.html) (10, 11).

Western blot analysis. After electrophoresis, gels were transferred electrophoretically onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA, USA) and blocked for 1 h at room temperature with TBS containing 5% skimmed milk. Primary antibodies were an anti-14-3-3 sigma, which is an affinity-purified goat polyclonal antibody raised against a peptide mapping near the amino terminus of 14-3-3 sigma of human origin (dilution range 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an anti-actin goat polyclonal antibody as a loading control (dilution range 1:200) (Santa Cruz Biotechnology). Membranes were incubated with the primary antibodies overnight at 4˚C, washed three times with TBS containing 0.05% Tween-20 and once with TBS, and then incubated with a horseradish peroxidase-conjugated secondary antibody (dilution range 1:5,000; Jackson ImmunoResearch Lab., West Grove, PA, USA) for 1.5 h at room temperature, and developed with a chemifluorescence reagent (ECL Plus Western Blotting Detection Reagents; GE Healthcare). The immunoreactive protein bands were then obtained by using the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc.) (12).

Results

Detection and identification of the spots with altered expression between SGC cell lines and NGM tissues using 2-DE gels. Protein expressions in the human SGC cell lines (OCUM-1, OCUM-2M, OCUM-2MLN, OCUM-2D, OCUM-D3, OCUM-9 and OCUM-12) and five NGM tissues was assessed. Protein spots on the 2-DE gels were visualised and differences in spot intensities between SGC cell lines and NGM tissues were analysed with Progenesis PG240 software for each gel. The expression of 29 protein spots differed between SGC cell lines and NGM tissues. Of those, 19 protein spots appeared to have higher levels (Figure 1A) and 9 protein spots appeared to have lower levels (Figure 1B) in SGC cell lines than in NGM tissues. The spots whose expression levels were higher or lower by more than 1.5 fold ($p<0.05$) are indicated by circles. The expression levels of these spots are shown in Table I. The bar charts were developed by normalisation of the volume, which was calculated relative to total spot volume, and the error bars show standard deviations. The identification of these 28 protein spots with different expression levels was accomplished by measuring tryptic peptide masses using the Agilent 1100 LC-MS/MS Trap XCT system in the positive ion mode and

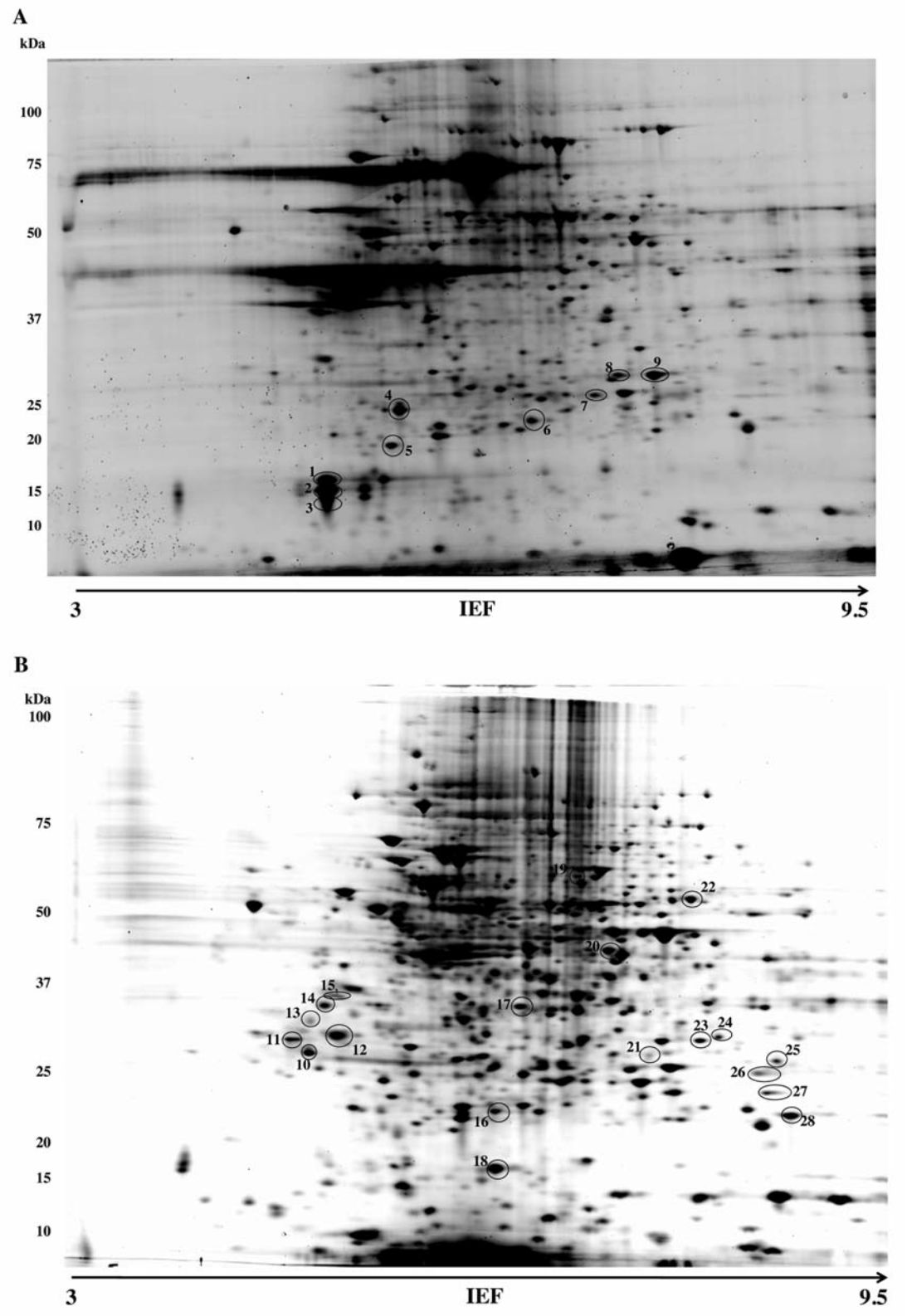

Figure 1. *2-DE patterns of NGM tissue and human SGC cell line stained with Flamingo Gel Stain. A: Protein spots whose expression levels were down-regulated in human SGC cell lines are shown (spots 1-9). B: Protein spots whose expression levels were up-regulated in human SGC cell lines are shown (spots 10-28).*

Table I. *Identification of proteins which are differentially expressed between scirrhous-type gastric cancer (SGC) cell lines and normal gastric mucosal tissues.*

Spot no.	Protein name	Accession no.	Theoretical		Distinct peptides	Sequence coverage (%)	MS/MS search score	Change in SGCs (fold)
			pI	Mr				
1	Gastrokine-1	Q9NS71	5.90	21,999	13	32	188.25	Decrease (0.13)
2	Gastrokine-1	Q9NS71	5.90	21,999	15	34	214.21	Decrease (0.03)
3	Gastrokine-1	Q9NS71	5.90	21,999	12	32	175.00	Decrease (0.00)
4	Apolipoprotein A-I	P02647	5.56	30,778	14	41	168.98	Decrease (0.13)
5	ATP synthase subunit d, mitochondrial	O75947	5.21	18,491	4	22	55.90	Decrease (0.08)
6	ATP synthase subunit alpha, mitochondrial	P25705	9.16	59,751	3	7	31.73	Decrease (0.70)
7	Ig lambda chain C regions	P01842	6.91	11,237	2	23	25.59	Decrease (0.18)
8	Carbonic anhydrase 1	P00915	6.59	28,870	4	18	58.24	Decrease (0.09)
9	Carbonic anhydrase 2	P00918	6.87	29,246	6	16	72.52	Decrease (0.23)
10	14-3-3 sigma protein	P31947	4.68	27,774	16	59	230.99	Increase (4.49)
11	Elongation factor 1-beta	P24534	4.50	24,764	7	25	95.06	Increase (26.84)
12	Tropomyosin alpha-4 chain	P67936	4.67	28,522	4	21	56.01	Increase (54.30)
13	Proliferating cell nuclear antigen	P12004	4.57	28,769	7	33	87.36	Increase (36.06)
14	Glucosidase 2 subunit beta	P14314	4.33	59,426	7	14	88.94	Increase (83.12)
15	Nucleophosmin	P06748	4.64	32,575	7	25	97.91	Increase (1.50)
16	Protein DJ-1	Q99497	6.33	19,891	6	34	78.77	Increase (5.65)
17	Carbonyl reductase 3	O75828	5.82	30,850	4	17	65.53	Increase (3.91)
18	Nucleoside diphosphate kinase A	P15531	5.83	17,149	5	41	72.89	Increase (3.65)
19	Bifunctional purine biosynthesis protein PURH	P31939	6.27	64,616	6	14	79.19	Increase (2.28)
20	Elongation factor Tu, mitochondrial	P49411	7.26	49,542	18	37	266.81	Increase (2.13)
21	*S*-Methyl-5'-thioadenosine phosphorylase	Q13126	6.75	31,236	6	19	84.66	Increase (3.32)
22	UDP-glucose 6-dehydrogenase	O60701	6.73	55,024	13	26	175.30	Increase (2.56)
23	Electron transfer flavoprotein subunit alpha, mitochondrial	P13804	8.62	35,080	11	45	165.67	Increase (5.89)
24	Pyrroline-5-carboxylate reductase 1, mitochondrial	P32322	7.18	33,361	5	16	65.06	Increase (3.86)
25	Proteasome subunit alpha type-4	P25789	7.58	29,484	1	4	13.83	Increase (2.42)
26	Eukaryotic translation initiation factor 4H	Q15056	6.67	27,385	2	12	31.33	Increase (2.61)
27	3-Hydroxyacyl-CoA dehydrogenase type-2	Q99714	7.65	26,923	4	20	62.91	Increase (2.39)
28	Peroxiredoxin-1	Q06830	8.27	22,111	5	27	71.87	Increase (1.71)

carrying out a database search in the Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine and the MASCOT MS/MS ions search engine, as summarised in Table I. Each sample provided good spectra of amino acid sequences. Figure 2 shows MS and MS/MS spectra of trypsin-digested spot 10 in Figure 1B. The MS/MS spectrum was identified as the partial tryptic peptide SAYQEAMDISK from 14-3-3 sigma processed with a spectrum MILL workbench. The spectrum of the precursor showed a double-charged peptide ion.

Up-regulation of proteins in SGC cell lines compared with NGM tissues. Western blot analysis of 14-3-3 sigma was performed in all SGC cell lines and NGM tissues. The expression tended to be higher (Figure 3A), although the expression of actin tended to be same or lower in SGC cell lines than in NGM tissues (Figure 3B). The up-regulation of 14-3-3 sigma was not detected in the human pancreatic cancer cell line MiaPaca-2, the human hepatocellular carcinoma cell line Huh-7 or the human malignant pleural mesothelioma cell line NCI-H2052 (Figure 4A), although the expression of actin tended to be same in all cell lines (Figure 4B). The non-scirrhous-type gastric carcinoma cell line AGS cells also showed up-regulation of 14-3-3 sigma, but this up-regulation was smaller than in SGC cells (Figure 4A).

Discussion

This study described a proteomic differential display analysis of seven human SGC cell lines compared to human NGM tissues. Nine protein spots were found to be down-regulated in SGC cell lines and these protein spots were identified as gastrokine-1, apolipoprotein A-I, mitochondrial ATP synthase subunit d, mitochondrial ATP synthase subunit alpha, Ig lambda chain C regions, carbonic anhydrase 1 and 2. Nineteen protein spots were found to be up-regulated in SGC cell lines (Table I). By Western blotting, up-regulation of 14-3-3 sigma was confirmed in the seven human SGC cell lines. Albeit OCUM-1 and AGS (gastric cancer) showed up-regulation of 14-3-3 sigma, MiaPaca-2 (pancreatic cancer), Huh-7 (HCC) and NCI-H2052 (malignant pleural mesothelioma) showed markedly weak expression of 14-3-3 sigma.

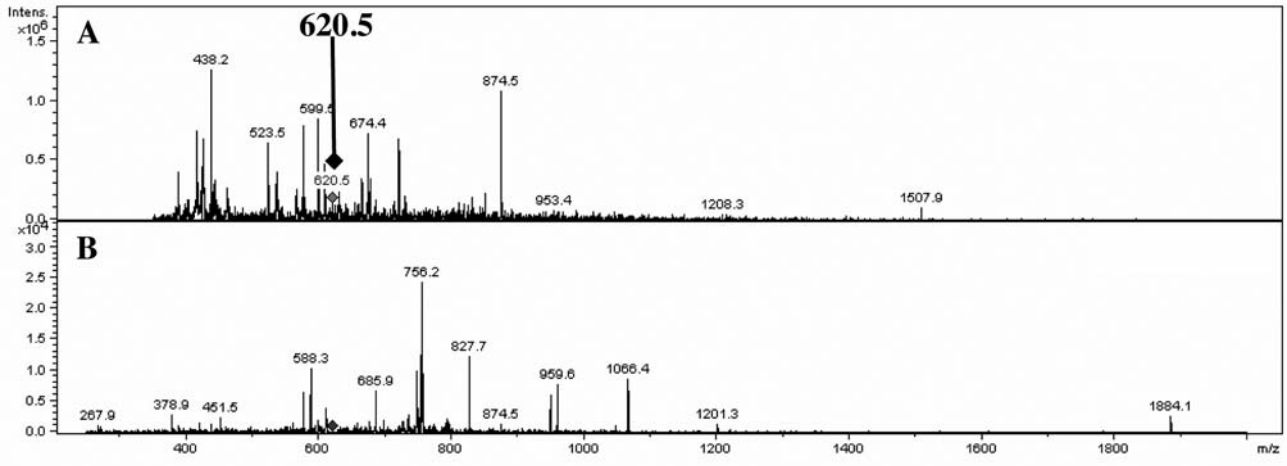

Figure 2. *MS and MS/MS spectra of trypsin-digested spot 10. A: LC-MS spectra of trypsin-digested spot 10, 14-3-3 sigma; precursor ion m/z is 620.5. B: LC-MS/MS spectrum of a precursor ion with m/z 620.5 marked by a diamond in (A). The MS/MS spectrum was identified as the partial tryptic peptide SAYQEAMDISK from 14-3-3 sigma processed with a spectrum MILL workbench.*

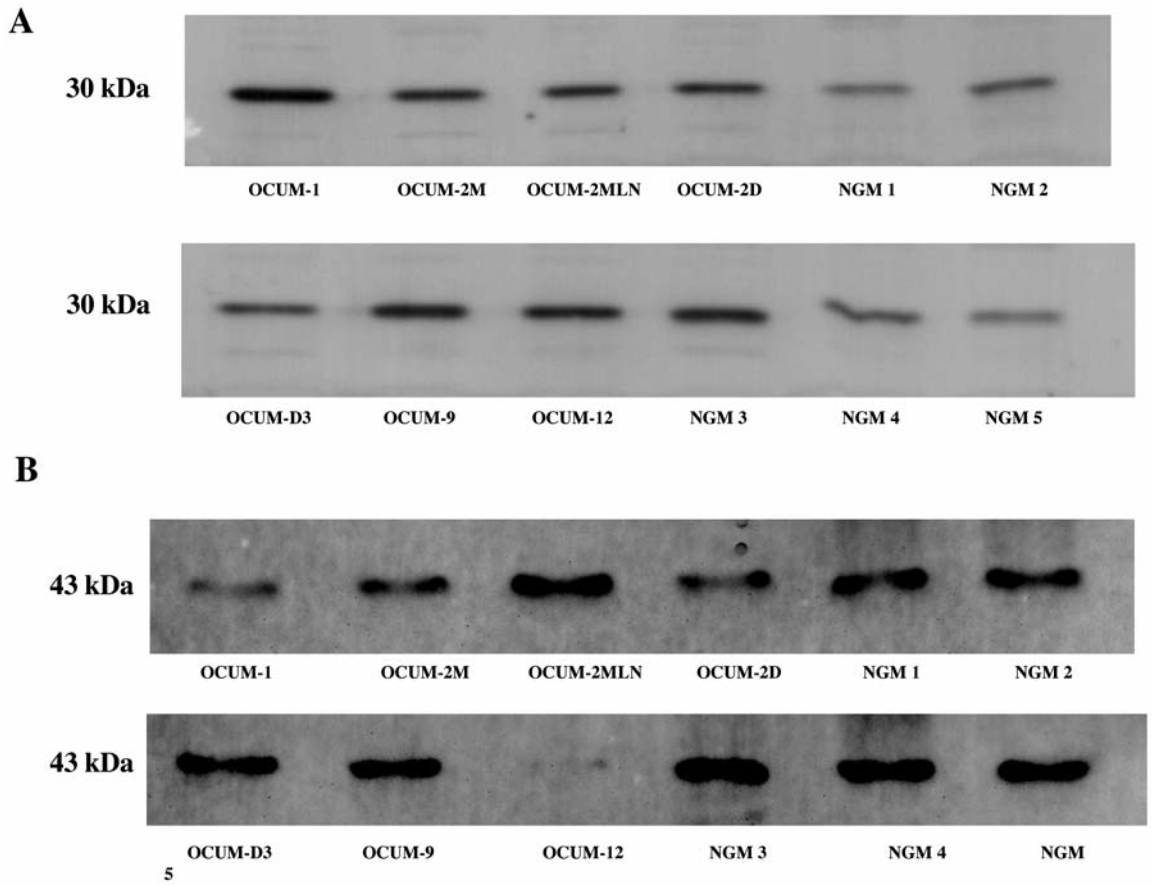

Figure 3. *Western blot analysis for 14-3-3 sigma protein in human SGC cell lines and NGM tissues. A: 14-3-3 sigma protein expression was significantly up-regulated in human SGC cell lines compared to NGM tissues. B: Actin expression levels were unchanged. Ten μg of protein were used.*

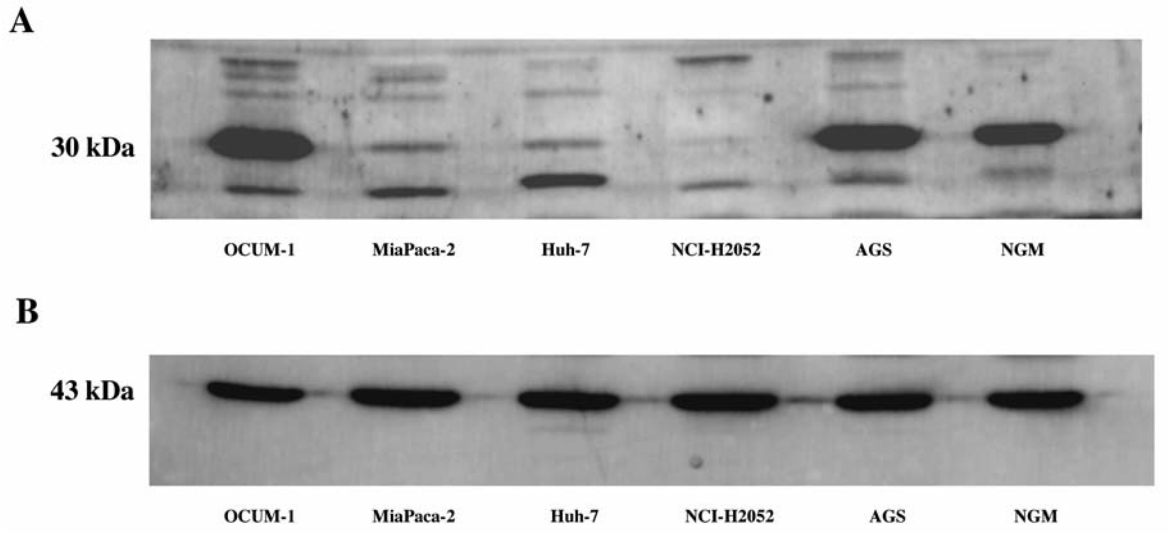

Figure 4. *Western blot analysis for 14-3-3 sigma protein in pancreatic cancer, hepatocellular carcinoma, malignant pleural mesothelioma and gastric cancer cell lines. A: 14-3-3 sigma protein expression level was not up-regulated in pancreatic cancer (MiaPaca-2), hepatocellular carcinoma (Huh-7) and malignant pleural mesothelioma (NCI-H2052) cell lines. B: Actin expression levels were unchanged. Ten μg of protein were used.*

14-3-3 Sigma protein that was only expressed in epithelial cells is a member of 14-3-3 family. It performs a checkpoint control by promoting G_2 arrest following DNA damage. Down-regulation of 14-3-3 sigma has been detected in human lung, prostate, ovary, urinary bladder, breast, liver, and skin cancer (13-19). In contrast, up-regulation of 14-3-3 sigma has also been detected in lung cancer, head and neck squamous cell carcinoma and oral squamous cell carcinoma (20-22). This controversy about the regulation of 14-3-3 sigma in cancer cells needs to be clarified in future studies.

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