Expression of Cystatin C in human stomach neoplasms

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Received March 15, 2010; Accepted May 7, 2010

DOI: 10.3892/mmr_00000304

Abstract. Cystatin C is a member of the cysteine protease inhibitor family and functions to decrease protease production. A recent study showed that it is aberrantly expressed in many malignant tumors in association with tumor invasion and metastasis. Our study aimed to detect Cystatin C expression in stomach neoplasm tissues and adjacent reparative normal tissues. Samples of cancerous and non-cancerous stomach tissue were obtained via surgery as matched pairs from 12 patients with stomach neoplasms and preserved in paraffin. The expression of Cystatin C in the samples was investigated by immunohistochemistry. Fisher's exact test was used to analyze the relationship between stomach neoplasms and adjacent normal tissues. Additionally, mRNA was extracted and analysed by reverse transcriptase-polymerase chain reaction. The intensity of Cystatin C immunostaining was increased in the tumor tissues compared to the adjacent normal tissues. Cystatin C mRNA expression was increased in stomach neoplasms compared to the normal tissues ($p<0.05$). The results indicate that the expression of Cystatin C may serve as a marker for stomach neoplasms.

Introduction

Stomach neoplasms are among the most common malignant tumors of the digestive system. In China, the mortality rate of this disease is 17.41/10,000, ranking them first in mortality among malignant tumors. Most stomach neoplasms show few symptoms in the early stages, but once tumors have formed, 80% of them metastasize. Despite the availability of treatment though surgery and combined therapy, the prognosis for stomach neoplasms remains poor.

The biological characteristics of tumor cells differ from those of normal cells. Tumor cells and their microenvironment have been found to contain numerous adhesion molecules, cell mobile molecules and various types of protease, which promote tumor growth. These enzymes include matrix metalloproteinases (MMPs), serine acid and cysteine proteinases (1-6). Cystatin C is the most important inhibitor of cysteine proteinase, and its major substrates, Cathepsin B, Cathepsin H and Cathepsin L (7), have also been reported to be related to the growth and invasion of various tumors (8-10). Therefore, the abnormal expression of Cystatin C is crucial for the investigation of invasion and metastasis in tumors. Several studies have found Cystatin C expression to be aberrant in numerous malignant tumors (11,12). To date, little is known about the expression of Cystatin C in stomach neoplasms. This study aimed to analyze the expression of Cystatin C in stomach neoplasms using clinical samples.

Materials and methods

Patients and samples. The stomach neoplasm samples were obtained from 5 female and 7 male patients, 38-73 years of age (mean 57.5). The samples were collected from a rural population at the First Affiliated Hospital of Xinxiang Medical College (Henan, China). None of the patients had received radiotherapy or chemotherapy. All the stomach neoplasms were gastric adenocarcinomas, including 6 cases of the diffuse infiltrative type and 6 cases of the ulcerative type. Samples were collected from tumor tissues and adjacent normal tissues at a distance of at least 6 cm. Small pieces were immediately snap frozen in the operating room and stored in liquid nitrogen until RNA isolation. The remaining tumor tissues were fixed in 4% paraformaldehyde.

Reverse transcriptase-polymerase chain reaction (RT-PCR). The frozen tumor tissues and adjacent normal tissues were removed from liquid nitrogen and immediately ground. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad,

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Key words: Cystatin C, immunohistochemistry, reverse transcriptase-polymerase chain reaction, stomach neoplasms

CA, USA). cDNA was obtained from 1 μg of the total RNA using the PrimeScript™ RT reagent kit (Perfect Real Time; Takara, Dalian, China) at 37˚C for 15 sec and 85˚C for 5 sec. Subsequently, the cDNA was diluted 5-fold with pure water, and 1 μl of this solution was used for each round of PCR amplification for the detection of mRNA. Amplification was performed with the following oligonucleotide primers: Cystatin C forward, 5'tccagtcccggcaagccgccgc3'; reverse, 5'ctaggcgtcctgacaggtggatttc3'. PCR was carried out with Takara Taq polymerase (Takara) in a thermal cycler (GeneAmp PCR System 9700; PE Applied Biosystems, Foster City, CA, USA). As an internal control, a fragment of human β-actin was amplified from parallel samples using the following primers: sense, 5'TGACGTGGACATCCGCAAAG'3; antisense, 5'CTGGAAGGTGGACAGCGAAGG3'. The thermocycling protocol was as follows: 94˚C for 4 min, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. Amplification products containing SYBR Green I Stain (Sigma, St. Louis, MO, USA) were resolved in 1.2% agarose gel. Gels were recorded with an Ultra-Lum system.

Immunohistochemistry. The samples were removed from paraformaldehyde and washed in flowing water overnight, then paraffin-embedded and routinely sectioned into 3 to 5-μm slices. The samples were dehydrated in ethanol (100, 90, 80 and 70%). The sections were washed in ddH_2O for 5 min, then in 1X PBS three times each for 5 min, 3% H_2O_2 for 5 min and 0.1% NaN_3 for 10 min. Antigen retrieval was conducted using a pressure cooker with 0.1 M citrate buffer (pH 6.0) for 10 min, then the samples were incubated in 10% BSA to block the non-specific reaction. Rabbit anti-human Cystatin C polyclonal antibody (Abcam, Cambridge, MA, USA; dilution 1:2,000) was added as the primary antibody, followed by incubation at 4˚C overnight. PBS was used to replace the primary antibody in the negative controls. The following day, the sections were washed three times in PBST (1% Tween-20), and goat-anti-rabbit IgG (Boster, Wuhan, China; dilution 1:400) as a second antibody was added for 40 min at room temperature, followed by washing again in PBST. The sections were stained with diaminobenzidine tetrahydrochloride (DAB) (Dako, Carpinteria, CA, USA) and the nuclei counterstained with hematoxylin, dehydrated and mounted with Permount. Positive Cystatin C staining was observed as brown in the cytoplasm and around the cell membrane (Fig. 2). The Cystatin C immunoreactivity score was calculated as the product of staining intensity and staining area. The signal intensity of Cystatin C was classified into four grades: 0, no immunostaining; 1, weak; 2, moderate; 3, strong. The percentage of staining area was also graded on a 4-point scale: 0, <5%; 1, 5-25%; 2, 25-50%; 3, >50%. The overall scores ranged from 0 to 9, thus immunoreactivity was scored as follows: -, 0; +, 1-2; ++, 3-5; +++, 6-9.

Statistical analysis. Statistical analysis was conducted using the Student's t-test for RT-PCR. The association between Cystatin C immunostaining and clinicopathological features was evaluated using Fisher's exact test when appropriate. $P<0.05$ was regarded as statistically significant. All statistical analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL, USA).

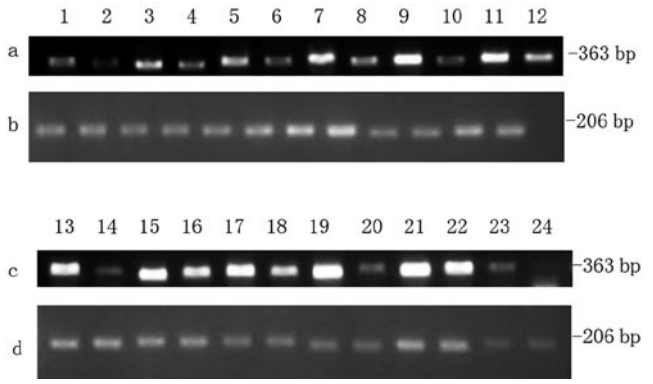

Figure 1. RT-PCR analysis was performed for Cystatin C and β-actin mRNA in 12 paired stomach neoplasm samples. Depressed expression of Cystatin C mRNA in adjacent reparative normal tissues and elevated expression of Cystatin C in the stomach neoplasms were observed. SN, stomach neoplasm tissues; NT, adjacent reparative normal tissues.

Results

RT-PCR analysis. To determine the relative amount of Cystatin C mRNA in the tumor and adjacent normal tissues, RT-PCR was performed with the housekeeping gene β-actin as an internal parameter. The results of RT-PCR are shown in Fig. 1. Expression of the Cystatin C gene was increased in the stomach neoplasm tissues compared to the adjacent normal tissues in 12 samples, with statistical significance ($P<0.01$).

Immunohistochemistry. Immunohistochemistry revealed that Cystatin C was expressed in the cytoplasm and on the cell membrane of the tumor cells (Fig. 2). Eight of the 12 stomach neoplasm samples showed strong staining, with up to 50% of the tumor cells expressing Cystatin C. Two samples showed moderate staining, but with a large number of tumor cells displaying positive expression of Cystatin C. Another 2 samples showed weak staining. In 1 of these 2, most of the tumor cells were stained, while in the other, only a small positively-stained area was observed. Although Cystatin C mRNA was expressed in all the nucleated cells, no expression was apparent in the adjacent reparative normal gastric gland tissues (Table I). No staining was observed in the control cells, validating our experiments. Six samples expressing Cystatin C were scored as ++ and another 6 as +++, while all adjacent normal tissues were scored as -, suggesting that the difference between the stomach neoplasm tissues and the adjacent reparative normal gastric tissues had statistical significance ($P<0.05$) (Table II).

Discussion

Cysteine protease is a type of proteolytic enzyme. Its active sites contain two cysteine residues, both found in lysosomes: exoproteinases, including cathepsin, and endopeptidases, of which the most studied are the cathepsins B, L and H, the major function of which is to hydrolyze proteins completely, so that parasites and viruses can use them to propagate and transmit. These are particularly found to destroy the proteins of the cellular matrix, such as collagen and lamina, and are therefore involved in the invasion and metastasis of some malignant tumors (13-16).

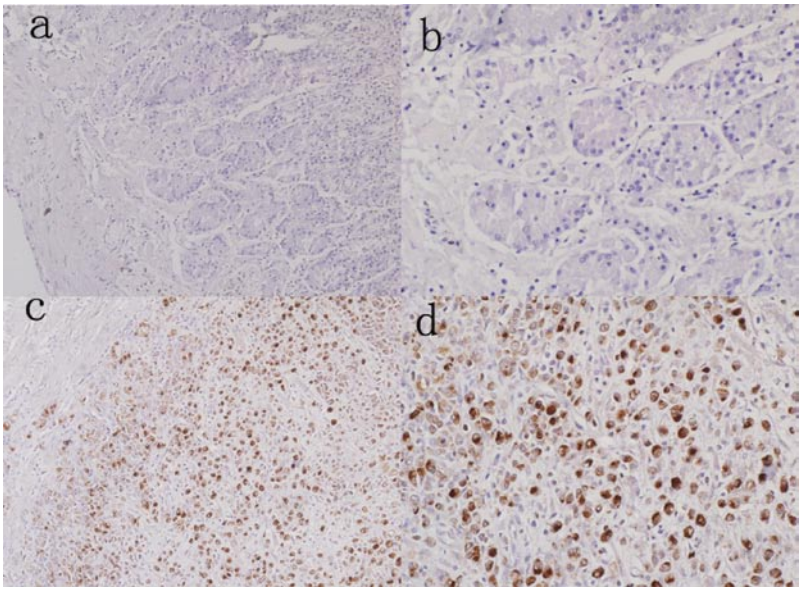

Figure 2. Immunohistochemistry to detect Cystatin C in paraffin-embedded sections of stomach neoplasm tissues incubated with polyclonal anti-Cystatin C antibody (Abcam-HRP staining). Cystatin C was expressed in the cytoplasm and cell membranes of tumor cells stained with DAB. Positive staining was observed in the stomach neoplasms, with little expression apparent in the adjacent reparative normal tissues, although Cystatin C mRNA is expressed in most human cells. (a and b) The adjacent reparative normal tissues (NT) showed no staining (blue). (c and d) Stomach neoplasm tissues (SN) showed strong staining (brown; Abcam-HRP staining). a and c magnification, x200; b and d, x400.

Table I. Cystatin C expression in stomach neoplasms (SN) and adjacent normal tissues (NT).

Patient no.	Gender	Age (years)	Cystatin C expression			
			Immunostaining intensity		Immunostaining area	
			SN	NT	SN	NT
1	Female	40	3	1	2	1
2	Male	47	3	0	3	0
3	Female	68	3	0	2	0
4	Female	58	3	0	3	0
5	Male	63	2	0	3	0
6	Male	68	2	1	3	1
7	Male	73	3	0	3	0
8	Male	61	3	0	3	0
9	Female	58	3	0	3	0
10	Female	71	2	1	2	2
11	Male	58	3	1	3	3
12	Female	38	1	0	3	0

Immunostaining intensity score: 0, no immunostaining; 1, weak; 2, moderate; 3, strong. Immunostaining area: the percentage area of stained cells in all tumor cells.

Numerous studies have reported that lysosomal cysteine protease cathepsin is highly expressed in human and murine tumor tissues (10,17,18) and sera (9). In certain studies, lysosomal cysteine protease cathepsin cDNA was transferred into tumor cells, increasing tumor cell metastasis, while in other studies, lysosomal cysteine protease cathepsin genes were knocked out or RNA interference technology was used for cysteine protease cathepsin genes (19-21), decreasing tumor cell invasion in matrigel *in vitro*, preventing solid tumor formation and inhibiting angiogenesis in a mouse model or in tumor cells. The expression of Cathepsin B can be detected in increasing levels in human glioblastomas (22), lung cancer (8,23), colon cancer (24), melanomas (9,17) and gastric carcinoma (10).

In 1960, Fossum isolated and purified the first cysteine protease inhibitor from egg whites, which at the time was named r-trace; cysteine protease inhibitors were named in the last decade. Based on molecular structure and biochemical

Table II. Cystatin C expression in stomach neoplasms and adjacent normal tissues.

Type of tissue	n	Cystatin C expression			
		-	+	++	+++
Ca	12	0	0	6	6
No	12	8	2	2	0

function, cysteine protease inhibitors are divided into three families: family one, the stefins, which comprises two members: Cystatin A and Cystatin B. Both are distributed in the cytoplasm. Cystatin A is expressed in epithelial cells, while Cystatin B is expressed ubiquitously. Family two, the cystatins, are composed of the salivary cystatins, Cystatin C and chicken egg white cystatin. These proteins have 120 amino acid residues and two intra-chain disulfide bonds. Family three consists of three members: low-molecular-weight (LMWK), high-molecular-weight (HMWK) and T-kininogens (25,26).

Cystatin C belongs to family two of the cysteine protease inhibitors, and its main function is to inhibit cysteine proteases, such as cathepsins B, L, H and K (7). In 1961, it was identified in the urine of patients with renal tubular dysfunction and named r-trace and then, in 1984, was formally named Cystatin C, due to its inhibition of cysteine protease. The protein has 120 amino acid residues and a signal peptide of 26 amino acids, forming an ~13 kDa protein without glycosylation; the human Cystatin C gene is located at chromosome 20 (24). The molecular weight is so small that it is easily filtered by the Glomerulus. The protein is expressed in all nucleated cells and is not affected by external factors. Moreover, the protein is degraded by renal tubular epithelial cells, so it is a better marker for the Glomerular filtration rate than the endogenous creatinine clearance rate (27,28).

As a member of cysteine protease inhibitors, Cystatin C decreases tumor invasion and metastasis (29-31). Previous studies have reported that the expression of Cystatin C was increased in some tumor tissues and cells, including prostate (11), ovarian (32) and non-small cell lung cancer (33).

In the sera of patients with malignant tumors, such as non-Hodgkin B-cell lymphoma (34), hepatocellular carcinoma (35), breast cancer (36), melanoma (9) and colon cancer (37), the expression level of Cystatin C was also up-regulated to a great extent compared to normal patients. By contrast, certain studies have shown that Cystatin C mRNA levels or immunostaining do not generally change significantly in human cancers, at least not in cancers of the brain, pituitary (38), pancreas and gut (39), kidney (40) and head and neck carcinoma (12,31). In malignant tumors, the balance between Cystatin C and its substrates cathepsin B, L, H and S is very important for tumor cell invasion and metastasis.

Wang *et al* (41) demonstrated that in *RIP1-Tag2/Cat S-/-* (deleted Cathepsin S gene) transgenic mice, tumor size was reduced, while in *RIP1-Tag2/Cyst C-/-* (absence of Cystatin C gene) mice, tumor size, the number of angiogenic islets and tumor microvessel density was increased. However, in *RIP1-Tag2/Cyst C+/+* mice, tumor growth was decreased. This study demonstrated that Cystatin C inhibits the growth, invasion and metastasis of malignant tumors depending on the Cystatin C inhibition of cathepsin stability.

In 2004, Skolt and Schiemann (42) transfected Cystatin C cDNA and mutant Cystatin C cDNA, which lacked the entire cysteine protease inhibitor signature located in the first hairpin loop, into human TH1080 fibrosarcoma cells. Both inhibited the invasion of HT1080 cells. The authors proved that Cystatin C inhibits the invasion of HT1080 cells depending on its inhibition of cysteine protease and TGF-β binding to TβR-II to impair the TGF-β signaling system. Cystatin C mRNA was down-regulated in cancers of the stomach (75%; 6/8 cases), uterus (71%; 5/7 cases), prostate (67%; 2/3 cases), colon (55%; 6/11 cases) and kidney (47%; 7/15 cases), although other authors found Cystatin C to be up-regulated in these tumors. In our study, we also found that Cystatin C was increased in the 12 paired samples of stomach cancer. Not only was Cystatin C protein up-regulated as detected by immunohistochemistry, but RT-PCR revealed Cystatin C mRNA to be up-regulated as well. Sokol *et al* (43) transferred Cystatin C cDNA into NmuMG (mouse breast cancer) cells, and found that Cystatin C was a new antagonist to TβR-II. That is to say, Cystatin C can prevent TGF-β binding to TβR-II, thus inhibiting TGF-β signaling, which is stimulated by TGF-β. TGF-β signaling decreases tumor cell proliferation, invasion and metastasis. This suggests that Cystatin C inhibits the progression of breast cancer and has potential therapeutic capacity in breast cancer.

Ogawa *et al* (44) induced the expression of recombinant Cystatin C in Pichia and transfected it into a cultured human colorectal adenocarcinoma cell line (Caco-2). The results showed that the growth of Caco-2 cells was significantly inhibited, and that cathepsin L activity was suppressed, which plays a direct role in promoting cell growth in these cells.

Cystatin C is expressed in the tumor tissues and inhibits the metastasis of some malignant tumors depending on its inhibition of cysteine protease, the TGF-β signaling system and tumor angiogenesis. In our study, we found that Cystatin C protein was intensively stained in stomach neoplasm cells, and not stained in their adjacent relative abnormal tissues. Although Cystatin C mRNA is expressed in all nucleated cells, its mRNA expression was up-regulated in the 12 paired stomach neoplasms and adjacent relative abnormal gland tissue samples.

Stomach neoplasms are among the most malignant tumors in China, with a very high associated mortality rate. To date, there is no specific marker for the diagnosis of stomach neoplasms, since the tumor cells easily metastasize though the blood or lymph nodes; furthermore, they directly implant into intraperitoneal cells, so the development of an earlier marker and treatment is crucial. Moreover, Cystatin C inhibits the growth of malignant tumors and their development, and thus may be a potential therapy for stomach neoplasms.

In conclusion, Cystatin C was strongly expressed in the stomach neoplasm tissues compared to adjacent normal tissues. Cystatin C mRNA expression was also higher in tumors than in normal tissues. This suggests that Cystatin C is a good marker for stomach neoplasms.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Nos. 30772498, 30772011 and 30972766), and the Guangdong Natural Science Foundation (Nos. 8151503102000022 and 9451503102003499). We thank Dr XinZhong Zhang from the first Affiliated Hospital of XinXiang Medical University for his generous help in designing the project.

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