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Expressions of chromogranin A and cathepsin D in human primary hepatocellular carcinoma

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Abstract

AIM To determine the expression and clinical significance of chromogranin A and cathepsin D in hepatocellular carcinoma (HCC).

METHODS Double immunofluorescence staining techniques combined with laser confocal scanning microscopy (LSCM) was used to investigate chromogr anin A and cathepsin D expressions in 85 HCC patients.

RESULTS Cathepsin D was expressed in 3 normal liver tissues, while in HCC the staining showed regional variation and the fraction of strongly stained cells increased as the tumors became less differentiated and usually clinically more malignant. Cells which showed strong positivity for cathepsin D were present in 71/85 (83.5%) cases. Strong expression of cathepsin D in cancer cells was related to histopathological features. They were more common in grade 3-4 (26/28, 92.9%) and grade 2 (46/53, 86.8%) tumors than in grade 1 tumors (1/4, 25.0%) ($P<0.01$). No significant correlation was found between age and cathepsin D expression. In patients with positive cathepsin D reaction, the mean age was 52.1± 2.8 years (range 32-68 years) and in the group with negative reaction, the mean age was 51.3± 4.5 years (range 28-71 years). No obvious relationship was observed between CgA expression in cancer cells and the histopathological features. The CgA positive rate was 75.0% (3/4) in grade 1, 71.7% (38/53) in grade 2, and 71.4% (20/28) in grade 3-4 ($P>0.05$) tumors. The coexpression of CgA and cathepsin D was found by double labeled immunofluorescence staining techniques. The processing of cathepsin D was disturbed in HCC cells and accumulated in the cells. Cathepsin D had proteolytic activity and autocrine mitogenic effect, suggesting their functions in invasion. These findings demonst rated that the expression of cathepsin D in HCC had prognostic value.

CONCLUSION Chromogranin A and cathepsin D are expressed in a high proportion of HCC and the existence of cathepsin D in HCC might be related to processing of CgA. This is clearly a subject for further studies because of its potential clinical applications.

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INTRODUCTION

The chromogranin A/secretogranin (CgA/Sg) acidic glycoprotein are widely distributed in vertebrate species. It has recently been proposed that CgA, a 50-kilodalton acidic glycoprotein, is costored and cosecreted with hormones and neurotransmitters in a variety of tissues. They thought to play a role in hormone packaging within secretory granules, in hormone secretion, and serve as prohormones for various proteolytic cleavage products[1]. CgA can be processed to several biologically active peptides such as pancreastatin. The single copy human CgA gene was isolated from a human fetal liver gene library[2]. The presence of CgA in hepatocellular carcinoma (HCC) was reported by Roskams *et al*[3]. They found that occasional positive cells or clusters of weakly CgA immunopositive cells were present in HCC. Cathepsin D is a lysosomal aspartyl proteinase[4], initially detected in breast cancer cell lines[5], which is widely distributed in normal tissues. The proteinase cathepsin D might be related to tumor invasion and metastasis through various mechanisms associated with its proteolytic activity. It was shown to degrade *in vitro* extracellular matrix and activate

latent precursor forms of other proteinase involved in such processes[6]. Experimental studies have already demonstrated that invasion of HCC cells can be abrogated by proteinase inhibitors. Growing evidence indicates that lysosomal cathepsin D may promote carcinogenesis and tumor progression. The metastatic activity of cathepsin D injected into athymic mice was significantly higher than that of control groups. These results show that overexpression of cathepsin D increased the transformed phenotype of malignant cells *in vitro* and their metastatic potency *in vivo*[7]. Higher cathepsin D serum mass concentrations were found in HCC group as compared to control patients[8]. Plasma and ascitic fluid of rats bearing the Yoshida ascites hepatoma AH-130 were shown to contain high levels of proteolytic enzymes belonging to different classes active at neutral and acidic pH. Compared with those measured in control rat plasma, and tumor-bearing animals, the activity levels of lysosomal cathepsin D activity was about 5-fold higher in both plasma and ascitic fluid[9]. The expression of cathepsin D was significant predictors of the prognosis[10]. The role of cathepsin D has been studied in human breast cancer progression[6-8], but the results were highly variable and no agreement has yet been reached on its effects on clinical behaviour and prognosis. There has been no report available on the role of cathepsin D expression in the progression of human HCC. To analyse this, the double immunofluorescence staining techniques combined with laser scanning confocal microscopy (LSCM) were used to investigate CgA and cathepsin D expression in HCC patients.

MATERIALS AND METHODS

Patients

The study group comprised 85 patients with HCC diagnosed in 117 Hospital and Xijing Hospital of the Fourth Military Medical University from 1984 to 1997. The mean age of the patients was 51.4±2.5 years (range 25-77 years). The female/male ratio was 28/57. The cohort was not entirely consecutive, since adequate tumor biopsy specimens for immunohistochemistry were not available in all cases.

Histological methods

The histological samples were surgically obtained biopsy specimens from the tumors which were fixed in buffered formalin (pH 7.0), embedded in paraffin, sectioned at 5 or 10 micrometer, and stained with haematoxylin and eosin. The samples were graded histologically into 1 (well differentiated, 4 cases), 2 (moderately differentiated, 53 cases), 3 (poorly differentiated, 26 cases), 4 (undifferentisted type, 2 cases) categories as described in detail before accor ding to the Edmondson-Steiner's criteria[11].

Immunohistochemistry

Five- or ten-micrometer sections from the primary HCC were employed in the fluorescent immunohistochemical analysis of cathepsin D protein, using rabbit anti-human cathepsin D antibody (Dako, Glostrup, Denmark) diluted 1:300 in 10mL/L bovine serum albumin (BSA)-phosphate-buffered saline (PBS). Several dilutions of the antibody were tested to find the optimal staining conce ntration, before the entire series was processed. The staining procedure was carried out as our previous reports[12], without protease treatment. Briefly, the steps included: ① the sections deparaffinized in xylene, hydrated in ethanol, and washed in 0.01mol/L PBS, then pretreated with 30m L/L normal goat serum for 40min and rinsed in 0.1mol/L PBS; ② incubation for 1h in a 1:1000 dilution of the primary monoclonal a ntibody of CgA and in a 1:300 dilution of rabbit anti-human cathepsin D antibody in 10mL/L BSA-PBS; ③ simultaneous incubation with 1:50 diluted secondary antibodies (biotin-conjugated goat anti-rabbit IgG and FITC - conjugated goat anti-mouse IgG) in 10mL/L BSA-PBS. All secondary ant ibodies were obtained from SABC (Luoyang, PRC); ④ incubation with 1:2000 diluted Texas -red-conjugated streptavidin (Sigma) for 30min. The sections were washed three times for 10min after incubation steps 2 to 4, respectively, and were finally mounted in 50g/L glycerin. The sections were examined with Bio-Rad 1024 LSCM. The specimens were excited with a laser beam at wavelengths of 568nm (Texas Red) and 488nm (FITC) and the emission light was focused through a pinhole aperture. The full field of view was scanned in square image formats of 512×512 pixels.

Scoring of CgA and cathepsin D protein expression

Firstly, the intensity of cytoplasmic fluorescence of the cancer cells in the entire section was scored into three categories. Cells showing no fluorescence for cathepsin D and CgA were considered as negative, cells with weak granular fluorescence (Figure 1) in the cytoplasm were scored as weak expressors, and cells with distinct cytoplasmic positivity (Figure 2) were scored as strong ones. Secondly, the fraction of cells in each of the staining categories in the entire section was also estimated in the areas with well-preserved tissue morphology. Areas with necrosis, or with distorted architecture, were excluded from the analysis. Thirdly, the presence in the invasion front of a distinct cathepsin D-positive cell zone composed of macrophage-like cells was scored positive or negative (determined in 65 cases). A breast cancer specimen showing intense uniform positivity for cathepsin D protein was used as a positive and negative control, with the expected results in all experiments. Since negative and weak

positive showed no association with other prognostic parameters, the cases were grouped into positive (strongly stained cells) or negative (negative or weakly positive cells only) for further analysis.

Controls

Primary antibodies were substituted by irrelevant antibodies and normal rabbit or goat serum as specific antibody control. PBS substituted for primary antibody as negative control. Primary antibody was omitted as blank control.

Statistical analysis

The Chi-square test was used in this study.

RESULTS

Relationship between expression of cathepsin D and histological features of HCC

Normal hepatocytes adjacent to carcinomas (n = 3) showed weak granular positivity for cathepsin D in the cytoplasm. Strong expression of cathepsin D in cancer cells was related to histopathological features (Table 1). Cells showing strong positivity for cathepsin D were present in 71/85 (83.5%) cases and were more common in grade 3-4 (26/28, 92.9%) and grade 2 (46/53, 86.8%) tumors than in grade 1 tumors (1/4, 25.0%, $P < 0.01$, Table 1). The positive reactivity was either granular or homogeneous in the cytoplasm (Figure 1). The positive cells distributed in disperse or patch pattern (Figures 1, 3,4).

Relationship between expression of cathepsin D and patients'age

We found no significant correlation between age and cathepsin D expression. The mean age of patients with positive cathepsin D reaction was 52.1 ±2.8 years (range 32-68 years) and 51.3±4.5 years (range 28-71 years, P>0.05) in the group with negative reaction.

Relationship between expression of CgA and histological grade of HCC

The CgA positive rate was 75.0% (3/4) in grade 1, 71.7% (38/53) in grade 2, and 71.4% (20/28) in grade 3-4 tumors (Table 2). No obvious relationship was observed between expression of CgA in cancer cells and the histopathological grades of HCC (P>0.05). The positive reactivity was homogeneous in the cytoplasm (Figures 2, 4, 5).

Correlation of expression of cathepsin D and CgA in HCC

Coexpression of cathepsin D and CgA was found in most of HCC (56/85, Table 3, Figures 3, 4, 6). It accounted for 91.8% of CgA-positive cases, and 78.9% (56/71) of cathepsin D-positive ones. Colocalization of cathepsin D and CgA was yellow observed by LSCM (Figures 3, 4, 6).

Table 1 Relationship between expression of cathepsin D and histologi cal features of HCC

Histologic grade	Expression of cathepsin D	
	Positive (%)	Negative (%)
1	1/4 (25.0)	3/4 (75.0)
2	46/53 (86.8)[a]	7/53 (13.2)
3+4	26/28 (92.9)[b]	2/28 (7.1)

[a]P<0.01 *vs* Grade 1; [b]P<0.01 *vs* Grade 2,1.

Table 2 Relationship between expression of CgA and histologic grade of HCC

Histologic grade	Expression of CgA	
	Positive (%)	Negative (%)
1	3/4 (75.0)	1/4 (25.0)
2	38/53 (71.7)[a]	17/53 (28.3)
3+4	20/28 (71.4)[b]	8/28 (28.6)

[a]P>0.05 *vs* Grade 1; P>0.05 *vs* Grade 2,1.

Table 3 Relationship between expression of cathepsin D and CgA in HCC

Expression of CgA	n	Expression of cathepsin D	
		Positive	Negative
Positive	61	56	5
Negative	24	15	9
Total	85	71	14

DISCUSSION

CgA is the major member of the granin family of acidic secretory glycoproteins that are expressed in all endocrine and neuroendocrine cells. Granins have been proposed to play multiple roles in the secretory process. Intracellularly, granins play a role in targeting peptide hormones and neurotransmitters to granules of the regulated pathway by virtue of their ability to aggregate in the low-pH, high-calcium environment of the trans-Golgi network. Extracellularly, peptides formed as a result of proteolytic processing of granins regulate hormone secretion. Some conserved features of the mature CgA protein are polyglutamic acids, calcium-binding sites, and several pairs of basic amino acids. The first two features are important for its intracellular functions, and the latter characteristic suggested that peptides could be released from the molecule by precursor processing enzymes. Several biologically active peptides encoded within the CgA molecule, such as vasostatin, beta-granin, chromostatin, pancreastatin, and parastatin act predominantly to inhibit hormone and neurotran smitter release in an autocrine or paracrine fashion. The biosynthesis of CgA is regulated by many different factors, including steroid hormones and agents that act through a variety of signalling pathways. CgA biosynthesis and that of the resident hormone or neurotransmitter can be regulated differentially. The widesp read distribution of CgA has made the measurement of circulating immunoreactive CgA a valuable tool in the diagnosis of neuroendocrine neoplasia, and CgA immuno histochemistry can help identify the neuroendocrine nature of tumors. Recent molecular biological studies are identifying those elements in the CgA gene promoter responsible for its specific neuroendocrine cell expression.

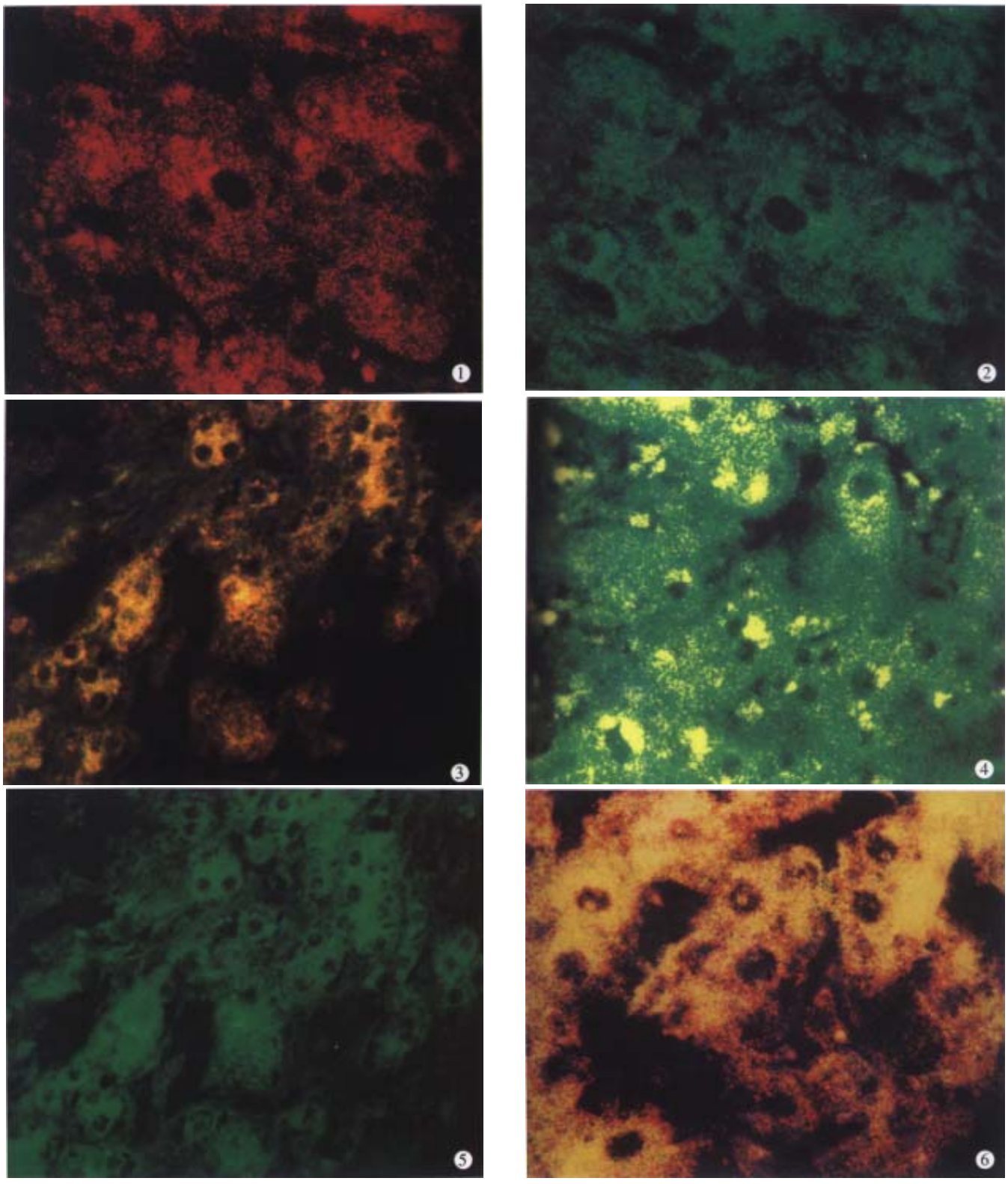

Figure 1 Distribution of cathepsin D in HCC (grade 3) The positive reactivity was granular in cytoplasm (red). TR-labelled ×400
Figure 2 Localization of CgA in HCC (grade 3) The positive reactivity was homogeneous in cytoplasm (green). The positive cells distributed in patch pattern. FITC-labelled×400
Figure 3 Coexpression of cathepsin D and CgA in HCC (grade 4). The positive cells distributed in disperse (yellow). TR-labelled cathepsin D, FITC-labelled CgA ×200
Figure 4 Coexpression of cathepsin D and CgA in HCC (grade 3). The positive cells distributed in disperse (yellow). TR-labelled cathepsin D, FITC-labelled CgA ×400
Figure 5 Expression of CgA in HCC (grade 3) The positive cells distributed in disperse or patch pattern (green). FITC-labelled ×200
Figure 6 Coexpression of cathepsin D and CgA in HCC (grade 2) The positive cells distributed in patch pattern (yellow). TR-labelled cathepsin D, FITC-labelled CgA ×400

Cathepsin D was purified to apparently homogeneous form from normal human liver and hepatoma. The puried enzyme could not be distinguished between normal liver and hepatoma in terms of specific activity, subunit composition, antigenicity, amino acid composition and tryptic peptides. However, the hepatom a enzyme was treated with endo-beta-N-acetylglucosaminidase H, the acidic variant forms disappeared and were converted into forms identical to those of normal liver. The content of mannose-6-phosphate in the hepatoma enzyme was twice as much as that in the normal liver enzyme. Thus, charge heterogeneity found in hepatoma cathepsin D is ascribed to increased phosphorylation on oligosaccharides bound to the enzyme, most probably due to cancer-associated, impaired processing in carbohydrate moiety. A significant elevation of cathepsin D activity per tissue proteins was observed in hepatoma as compared to normal liver. In contrast, true specific activity per cathepsin D protein in hepatoma was significantly lower than that of normal liver. The lower true specific activity in hepatoma tissue may be attributed to an increased content in an inactive, large-molecular precursor form of the enzyme[13].

Cathepsin D is encoded by a gene located on chromosome 11p15, but its increased expression is not usually due to gene rearrangement or amplification[14] . Instead, it can be induced by estrogens or several other factors in the cellular micro-environment[15,16]. Cathepsin D exists in the cell as a precursor for procathepsin D. In the normal hepatocytes, most of the procathepsin D is changed to an active form in the lysosomes, while in HCC cells the processing of procathepsin D is disturbed. Procathepsin D is accumulated in the cells, since smaller fraction is secreted than in normal liver tissues[17]. Procathepsin D has proteolytic activity and autocrine mitogenic effect[18], both of which suggest some functions in invasion and metastasis. Cathepsin D is a physiological regulator of other cathepsins (B and L) by activating their precusors[19], or possibly by inactivating endogenous inhibitors of cathepsins[20]. Cathepsin D was usually expressed in normal hepatocytes, while in malighant tumors the fluorescent staining showed regional variation and the fraction of strongly stained cells increased as the tumors became less differentiated and usually clinically more malignant. In HCC, increa sed expression was related to a high grade. The situation was also found in breast carcinomas[21]. However, the dissemination of the tumor to lymph nodes and to distant sites suggested a relationship between invasive potential and the expression of cathepsin D. Strong expression of cathepsin D was associated with cell proliferation and expression of growth factor receptors[22]. Our previous studies also proved that the hepatoma cells expressed epithelium growth factor and its receptors[22] and CgA[23]. Thus, it is likely that the strong expression of cathepsin D is due to induction of cathepsin D synthesis by epithelium growth factor. The higher proliferation rate of strongly cathepsin D-positive tumors may be partly related to a mitogenic effect of cathepsin D[18,24]. These findings, together with the clinical observations that a positive correlation between levels of expression of cathepsin D activity and malignant progression of some human neoplasms, seem to support this hypothesis[25].

The prognostic value of cathepsin D expression in neoplasms is a matter of controversy and the results of this study suggest that the expression of cathepsin D in HCC cells has prognostic value over already established prognostic factors.

In conclusion, cathepsin D is expressed in a high proportion of HCC. This is clearly a subject for further studies because of its potential clinical applications. Chromogenic peptides act as substrates for cathepsins[26], so the higher expression of cathepsin D, the lower expression of CgA in HCC and *vice versa*.

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