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Expression of programmed cell death 5 gene involves in regulation of apoptosis in gastric tumor cells

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The protein of programmed cell death 5 (PDCD5) is believed to participate in regulation of apoptosis. Although PDCD5 is reducibly expressed in various human tumors, it is not clear which expression level of PDCD5 is in gastric cancer (GC). In this study, we have systematically employed the approaches of RT-PCR, Real- time PCR, Immunohistochemistry (IHC), Immunofluorescence staining (IFS) and Western blot to determine the PDCD5 expression in GC cells and primary tumors, at mRNA and protein level, respectively. Our data revealed that the positive rate of PDCD5 expression in the gastric tumor tissues was significantly less than that of the normal tissues (14 out of 102 *vs* 36 out of 51), whereas, the decreased expression of PDCD5 protein was well correlated with the up-regulated expression of Bcl-2 in these tissues, and the up-regulated expression and nuclear translocation of PDCD5 protein were verified in the apoptotic GC cells induced by Diallyl trisulfide (DATS). Furthermore, the survival curve has suggested that the more PDCD5 expressions were found in the patients, the longer the survival periods were. Therefore, our observations lay down a reasonable postulation that PDCD5 may play a key role to regulate the apoptotic processes in the GC cells and gastric tumors.

Keywords: apoptosis; gastric cancer; nuclear translocation; programmed cell death 5.

Introduction

The balance between cellular proliferation and apoptosis is essential to maintain the physiological functions of organism. A number of genes are involved in proliferation and apoptosis, such as the TNF/Fas L family,[1,2] NF kappa B,[2] P53,[2,3] the ICE/CED-3 family,[2,4] ubiquitin/proteosome.[2,5] Recently, a novel apoptosis-related gene, PDCD5 (programmed cell death 5), was cloned from the apoptotic TF-1 cells.[6] Several investigations have shown that PDCD5 was able to enhance cell apoptosis,[6–9] and the anti-PDCD5 antibody could suppress the apoptotic effects of PDCD5 in HeLa cells.[7] Typically, PDCD5 translocated to cellular nucleus during apoptosis, and its accumulation of PDCD5 preceded the externalization of phosphatidylserine (PS) and the formation of DNA fragmentation.[8] PDCD5 has been found to be involved in facilitating the opening of mitochondrial membrane pores and the releasing of cytochrome C from the mitochondrion of mouse liver.[10] Recent study has also indicated that PDCD5 protein played an important enhancing role in TAJ/TROY triggered paraptosis-like cell death.[11]

The decreased expression of PDCD5 has been reported in various human tumors, such as breast cancer,[12,13] and hepatocellular carcinoma.[14] However, mechanisms through which PDCD5 exerts effects are currently not clear. In this study, we have determined the expression of PDCD5 in primary tumors and cell lines of stomach, and further characterized its roles in apoptosis.

Materials and methods

Cell culture and Diallyl trisulfide treatment

Gastric tumor cell lines of BGC823, MGC803, SGC7901 and PAMC82 were established in People's Hospital of Peking University, China. MKN45, AGS, RF-48, SNU-1, SNU-5 and SNU-16 were purchased from ATCC (American Type Culture Collection). The cells were cultured in complete DMEM (Hyclone) at 37°C in a humidified atmosphere containing 5% CO_2. When needed, the cells were treated with DMEM containing 25 μmol/L of DATS (commercial name as Allitridi, Shanghai Hefeng Pharmacy Company, China).

RT-PCR and real-time PCR

Total RNA from the tissues and cells were prepared by Trizol reagent (Invitrogen), and cDNA libraries were generated

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by reverse transcription using Moloney Murine Leukemia Virus reverse transcriptase (MMLV) and Oligo d (T) primers. The primers used for amplification of PDCD5 were 5′-CGGAATTCACCATGGCGGACGAGGAGC-3′ (forward) and 5′-CGGAATTCA ATAATCGTCATCTTCATC-3′ (reverse). The PCR reaction was initiated by 5 min incubation at 95°C, terminated after a 10 min extension at 72°C, 29–32 cycles for denaturation at 94°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min. The gene of β-actin was adopted as an internal control in these RT-PCR reactions.

Real-time PCR reactions were carried out using iCycler iQ™ Real-Time PCR Detection System (Bio-Rad). The conditions of gene amplification were similar to RT-PCR described above except GAPDH as an internal control. Data were analyzed according to the relative standard curve method with normalizing the values of GAPDH expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplified products.

Immunohistochemistry (IHC) and tissue microarray

Total of 153 human gastric specimens including 49 poor differentiated carcinomas, 53 moderate and well differentiated ones and 51 normal cases were collected by gastroscopy or surgery. The patients were fully informed and given the consents for collection of clinical samples. The sections were incubated with mouse anti-PDCD5 monoclonal antibody[7] and anti-bcl-2 monoclonal antibody followed by addition of anti-mouse Ig G as secondary antibody for color staining. The labeling index (LI) of PDCD5 gene was determined by percentage of positive stained cells under microscopy. Positive results were graded as LI > 5%.

Western blot analysis

Equal amounts of protein from different samples were electrophoresed on 15% SDS- PAGE and electro-transferred to PVDF membranes using Mini PROTEAN 3 system (Bio-Rad). PVDF membranes were blocked with phosphate-buffered saline (PBS) containing 5% milk powder for 2 h, and incubated with chick anti-PDCD5 polyclonal antibody at 4°C for overnight. The antibody against chick IgG conjugated with horseradish peroxidase (HRP) was adopted as a secondary antibody. Peroxidase activity was visualized with ECL kit (Amersham Pharmacia Biotech).

Immunofluorescence staining (IFS)

The treated cells were washed with cold PBS and fixed with 3% paraformaldehyde followed by addition of PBS containing 0.2% Tween 20 for 15 min at 37°C. The fixed cells were blocked with fetal calf serum (FCS), and then incubated with FITC-PDCD5 Ig G[8] for 30 min at 4°C. After thorough wash, the samples were subsequently analyzed by fluorescence microscope and FACScan flow cytometer.

Flow cytometric analysis of apoptosis

The cells (5×10^5) were harvested and washed with PBS, and fixed in cold 75% ethanol at 4°C overnight. After staining with propidium iodide (PI) solution for 30 min, cells were collected on a FACScan flow cytometer equipped with a 488 nm argon laser and analyzed using the CellQuest software (Becton-Dickinson).

Statistical analysis

Statistical analyses were conducted by SPSS software (version 11). Relationships between PDCD5 expression and GC were tested by χ^2 test. Follow-up studies were analyzed by Kaplan-Meier method. Survival difference between two groups of PDCD5 positive and negative expression was assessed by log-rank test. $P \leq 0.05$ was considered a threshold of significant difference.

Results

Comparison of PDCD5 protein expression in gastric primary tumors and normal tissues

To compare the expression levels of PDCD5 protein in primary tumors and normal gastric tissues, we stained tissue micro-array with 102 cases of GC and 51 normal using the antibodies of anti-PDCD5 and anti-bcl-2. On Figure 1, it is clear that positive staining in the normal tissues was obviously more intense than the stained tumor tissues. Furthermore, PDCD5 protein was expressed strongly in the neck and basal region of normal gastric glands (Figure 1). The rate of positive staining in the tumor tissues was 13.7% (14/102), as compared 70.6% (36/51) in the normal samples ($p = 0.001$) (Table 1). Kaplan-meier survival curve indicated that patients with positive PDCD5 expression had longer survival than the negative expression group ($p = 0.0287$) (Figure 2). In addition, to determine if PDCD5 was involved in apoptosis of GC, a typical apoptotic gene, bcl-2, was examined in these tissues. As listed in Table 2, the positive rate in tumor tissues was 45.2% (42/93), whereas 13.0% (6/46) positive in the normal group ($p = 0.001$). Thus, these data indicated that the decreased expression of PDCD5 protein coincided with the increased expression of Bcl-2 in these samples ($p = 0.018$) (Table 3).

Figure 1. Comparison of PDCD5 protein expression in primary gastric tumors by tissue array and IHC analysis (20×, 40× in the right down corner). (A) Positive staining of PDCD5 protein was strong in normal tissues; (B) (C) weak in well and poorly differentiated carcinoma; (D) PDCD5 protein was intensely expressed in the neck and basal region of normal glands; (E) PDCD5 protein was strongly expressed in normal glands, but negative in tumor cells (arrow); (F) Bcl-2 protein was strongly expressed in GC; (G) Bcl-2 protein was weaker in the normal.

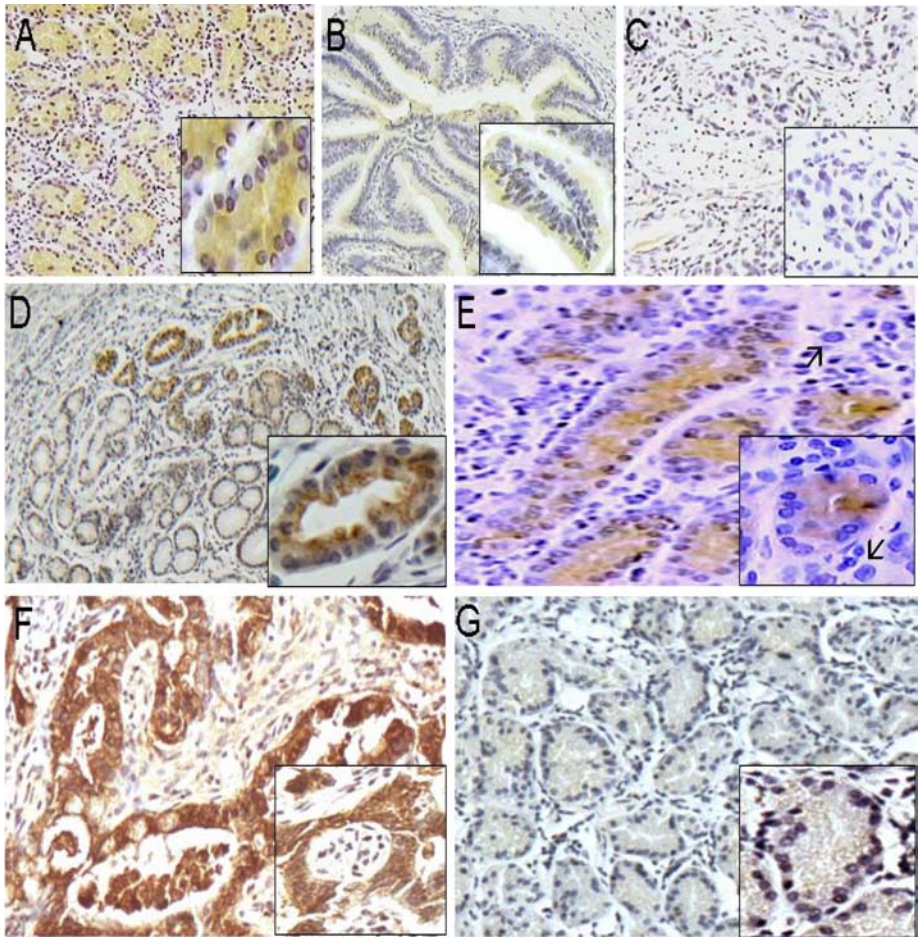


Table 1. Comparison of PDCD5 protein expression in gastric tumor and normal tissues

Histology	Total cases	Positive	Negative	*p* values
Normal	51	36 (70.6%)	15 (29.4%)	0.001
Carcinoma	102	14 (13.7%)	88 (86.3%)	
Poorly differentiated	49	4 (8.16%)	45 (91.84%)	0.116
Well differentiated	53	10 (18.9%)	43 (81.1%)	

Detection of differential expression of PDCD5 in gastric tumor cell lines

The approaches of RT-PCR and Real-time PCR were employed for detecting PDCD5 expression at mRNA level in the tumor cell lines. The results obtained from the amplification of PDCD5 gene demonstrated that the different GC cell lines contained various levels of PDCD5 mRNA. Of 10 GC lines, three cell lines, BGC823, SGC7901 and AGS, maintained higher expression of PDCD5, but the others with relatively low PDCD5 expression (Figure 3).

Table 2. Comparison of Bcl-2 protein expression in gastric tumor and normal tissues

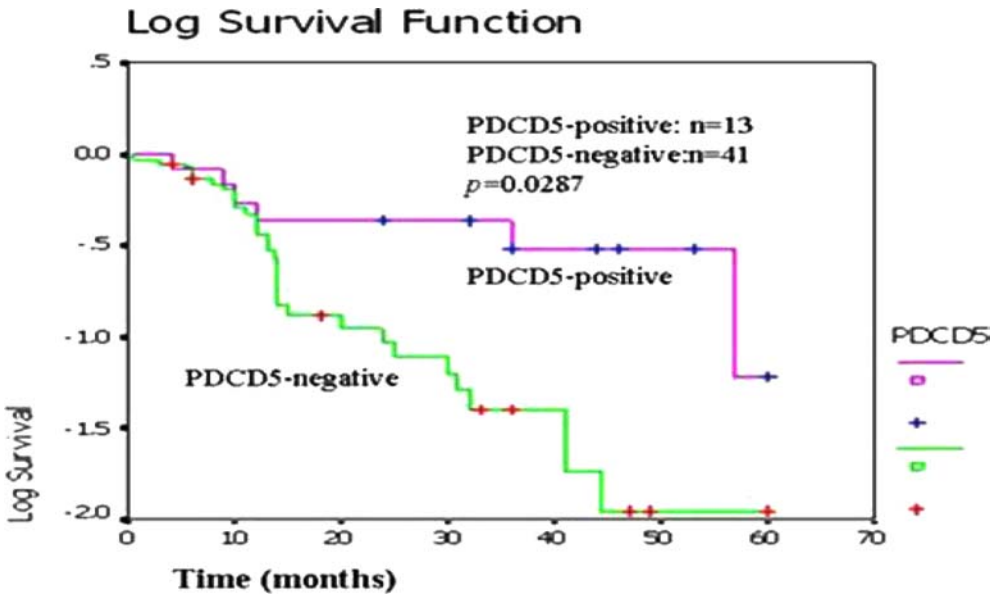
Histology	Total cases	Positive	Negative	*p* value
Normal	46	6 (13.0%)	40 (87.0%)	0.001
Carcinoma	93	42 (45.2%)	51 (54.8%)	

Table 3. Correlation of protein expression level between PDCD5 and Bcl-2

	PDCD5		
Bcl-2	Positive	Negative	*p* value
Positive	12	30	0.018
Negative	5	47	

Furthermore, IHC staining was used to determine PDCD5 protein expression level and localization in GC cell lines. The data revealed that PDCD5 protein mainly distributed in cytoplasm of these cells. As similar to mRNA

Figure 2. Correlation between PDCD5 protein and survival by Kaplan-meier analysis. The curve showed that patients with positive PDCD5 expression had longer survival than patients with negative expression ($P = 0.0287$).

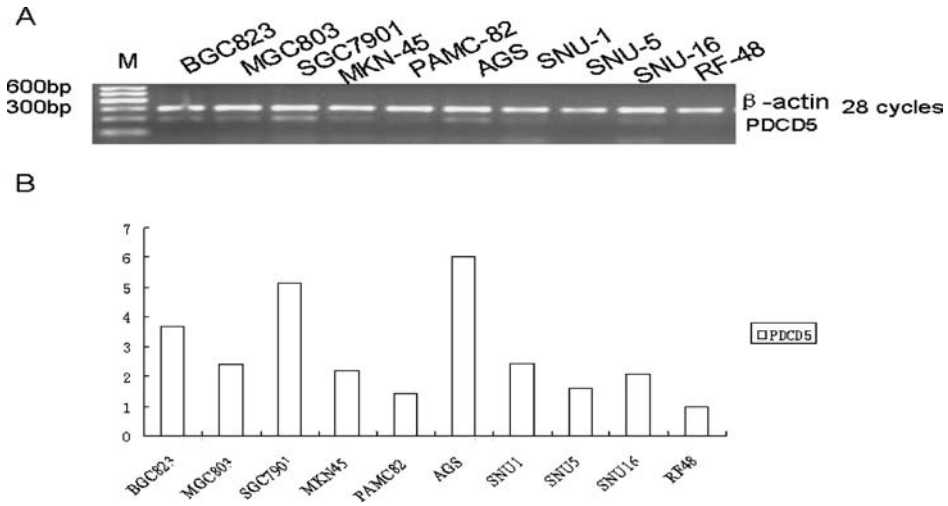


assay, the expression levels of PDCD5 protein in BGC823, SGC7901 and AGS cell lines were significantly higher than the other GC lines examined in this study (Figure 4).

Up-regulation and nuclear translocalization of PDCD5 protein in tumor cell lines treated with DATS

Previous study in our lab indicated that DATS, a main component of garlic, was able to induce G1/S arrest and to enhance apoptosis in tumor cell line BGC823.[15] In order to further investigate the role of PDCD5 in apoptosis in the tumor cells, we have treated BGC823 with DATS at 25 μmol/L, and used IFS to analyze its localization. Our data were shown that even within 0.5 h treatment with DATS, PDCD5 protein began to translocate from cytoplasm to nucleus of cells (Figure 5), and eventually it accumulated intensively around nucleus in most of the cells in 2 h and 6 h (Figure 5). The apoptotic bodies appeared after DATS-treatment for 48 h by staining of hochest33342 (Figure 5), and flow cytometric analysis demonstrated the apoptotic peak appeared after 72 h treatment (Figure 7). The nuclear translocation of PDCD5 preceded the formation of apoptotic bodies. On the basis of Western blot analysis, the expression of PDCD5 protein was elevated following DATS treatment

Figure 3. Detection of differential expression of PDCD5 mRNA in tumor cell lines. (A) PDCD5 mRNA was expressed in 10 tumor cell lines detected by RT-PCR analysis, and expression level of PDCD5 mRNA was higher in BGC823, SGC7901 and AGS than in other cell lines. (B) Data of Real-time PCR analysis was consistent with RT-PCR analysis.



at 2 h, 6 h and 12 h, but began to return to the base line after 24 h (Figure 6). We also treated cell line PAMC82, N87, MGC803 and SGC7901 with DATS. Endogenous PDCD5 level was measured with FITC-labeled anti-PDCD5 monoclonal antibody by flow cytometry at different time points. PDCD5 expression in these cell lines was up-regulated, and the apoptotic peak appeared after DATS treatment (Figures 6 and 7).

Figure 4. Distribution of PDCD5 protein in gastric cell lines detected by IHC analysis (20×, 40× in the right down corner). (A) Detection of PDCD5 protein in BGC823; (B) MGC803; (C) SGC7901; (D) PAMC82; (E) AGS; (F) N87. PDCD5 protein was mainly expressed in the cytoplasm of cells.

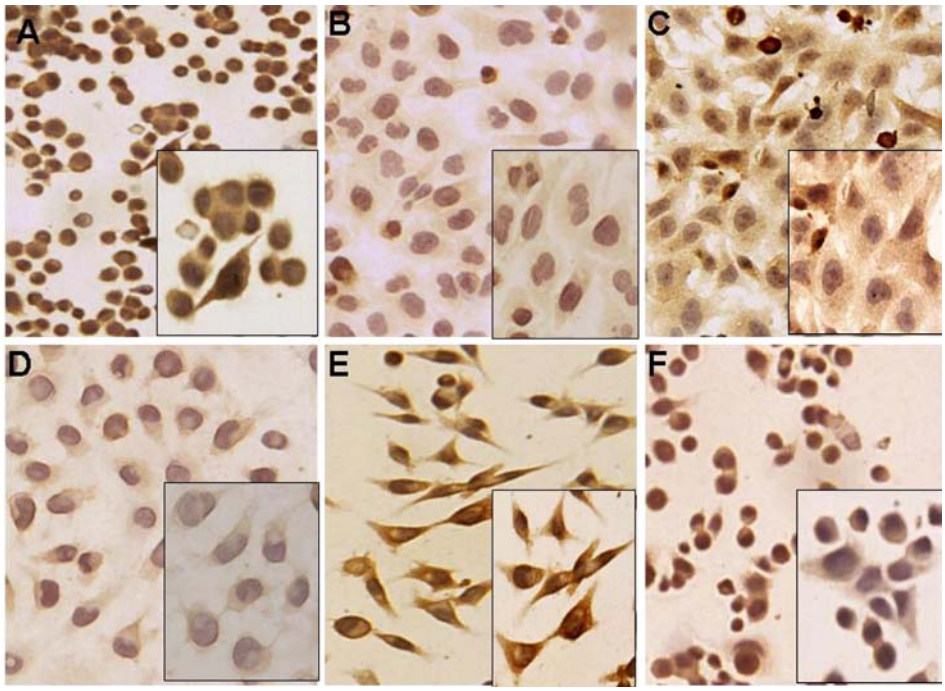


Figure 5. Nuclear translocalization of PDCD5 protein in tumor cell line BGC823 treated with DATS (20×). (A) PDCD5 protein was mainly expressed in the cytoplasm of BGC823 before DATS treat; (B) PDCD5 protein began to move to nucleus in BGC823 treated with DATS for 0.5 h (stained with FITC-labled anti-PDCD5); (C) (D) PDCD5 protein accumulated intensively around nucleus in most of the cells in 2 h and 6 h; (E) Typical apoptotic bodies were seen in BGC823 treated with DATS for 48 h by staining of hochest33342.

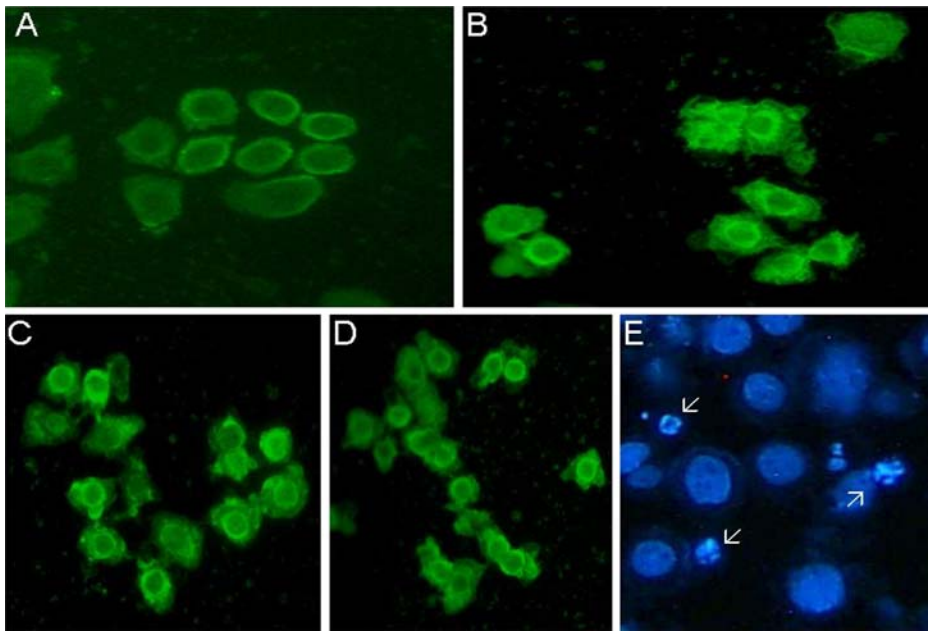
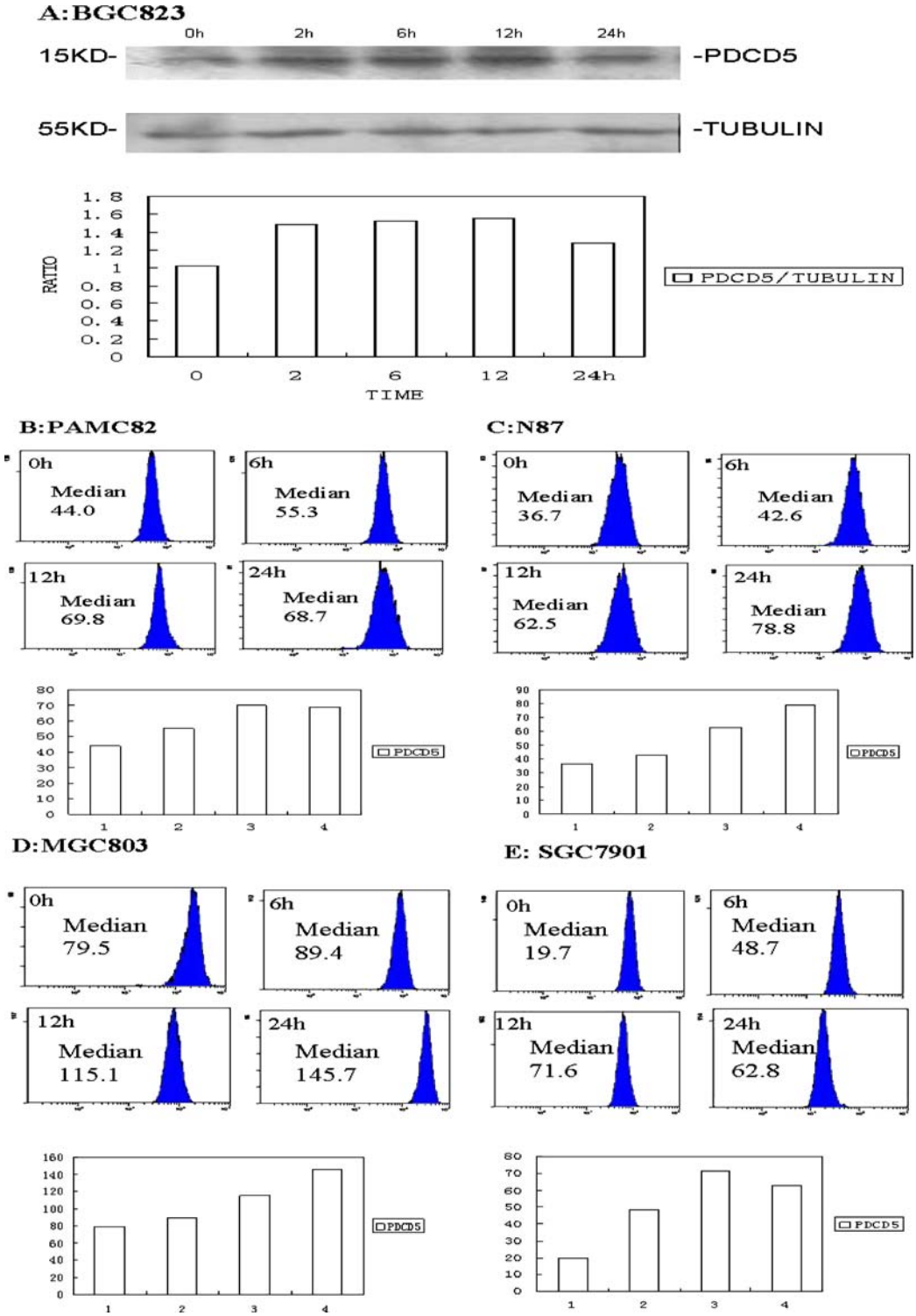


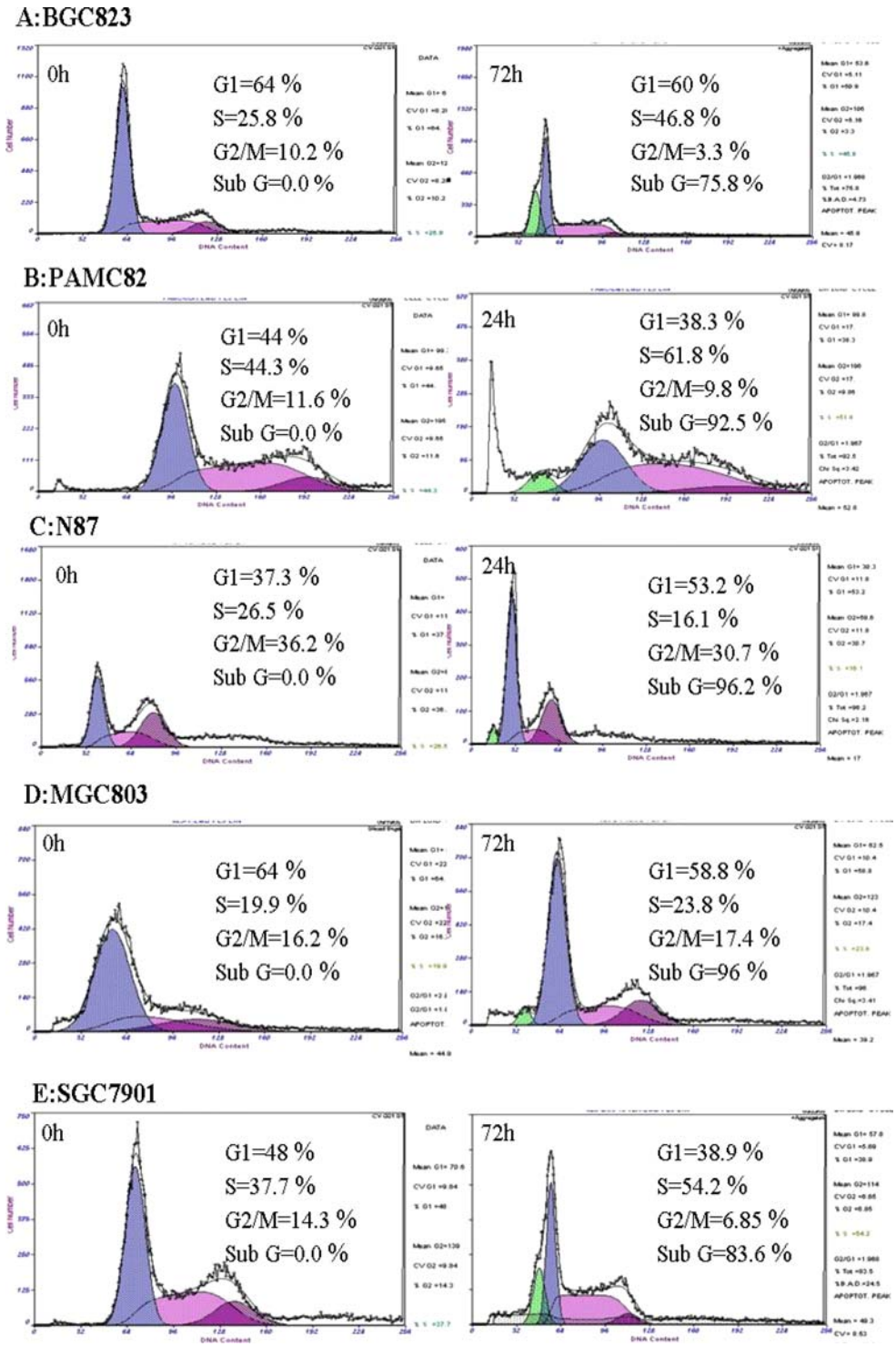
Figure 6. Upregulation of PDCD5 protein in cells treated by DATS. (A) PDCD5 protein expression level was increased in BGC823 treated with DATS for 2 h, 6 h and 12 h, and it began to be back to its basic level at 24 h by western blot analysis. (B) (C) (D) (E) At different time points after DATS treatment, endogenous PDCD5 levels were measured with a FITC-labeled anti-PDCD5 monoclonal antibody by flow cytometry. Median values of fluorescence intensity represent the expression of cellular PDCD5. Significant upregulation of endogenous PDCD5 was detected in PAMC82, N87, MGC803 and SGC7901 respectively.



Discussion

It is well known that apoptosis is a process of programmed cell death and dysfunction of this process leads to uncontrolled cell growth, such as cancer.[16,17] Our data displayed in this paper demonstrated that PDCD5, an apoptosis-associated protein, was significantly decreased in gastric tumors. Interestingly, the decreased PDCD5 expression was well correlated with the increased expression of Bcl-2 protein ($p = 0.018$). These results, therefore, have implied that PDCD5 could play an important role in the apoptotic regulations in GC. Some early reports have documented

Figure 7. Apoptosis peaks were observed in tumor cell lines treated with DATS by flow cytometric analysis. (A) BGC823; (B) PAMC82; (C) N87; (D) MGC803; (E) SGC7901.



that the PDCD5 expression either at mRNA or at protein level was abnormally changed in the specific human carcinomas. Hedenfalk *et al.* reported that PDCD5 was down-regulated in *BRCA2*-mutation cancer.[12] Xu *et al.* analyzed the gene expression profiles in human hepatocellular carcinoma/noncancerous samples and found that *PDCD5* was decreased in the patients with hepatocellular carcinoma.[13] Liu *et al.* also stated that PDCD5 protein was in low expression in the tissues of cervical cancer.[14] Taking together, these evidences strongly suggest that it is likely the involvement of PDCD5 in carcinoma formation.

Interestingly, we have also found that immunoactivity of PDCD5 protein appeared in the neck and basal region of normal gastric glands. Several investigators have found that a great number of genes related with proliferation and apoptosis were highly expressed in the neck region, such as p16[18] and TPR-MET.[19] We hypothesized that proliferation cells are susceptible to apoptosis. To prove the hypothesis, further experiments should be conducted to examine the functions of PDCD5 and whether this gene and its protein product could enhance the sensitivity of anticancer drugs or of radiation therapy adopted in the treatments of human cancers.

In this study, all of GC cell lines were found the expression of PDCD5 protein; however, only 14 of 102 tumor tissues were positive for PDCD5 immunostaining. It is not a surprising result. Since tumor cell lines are immortalized and activated for proliferation, but tumor tissues are consist of multiple cells in different phases of the cell cycle, usually tumor cell lines are different from tissues in many aspects.

The translocation of proteins is relevant to the functional performances. VP3/Apoptin protein was located in the cytoplasm of normal cells while it was relocalized to nucleus in tumor cells.[20] Caspase-3 was observed originally close to the inside surface of the cellular membrane, but transferred to cytoplasm, and finally translocated to the nuclear region in MOLT-4 cells due to exposure to X-rays.[21] In order to investigate the role and mechanism of PDCD5 in apoptosis, we treated tumor cell line BGC823 with DATS. Our data revealed that PDCD5 protein translocated from cytoplasm to nucleus within a short period, and this process preceded the formation of apoptotic bodies. This result was also consistent with previous observation.[8] So we concluded that the nuclear translocation of PDCD5 protein might result in the enhanced apoptosis. Based upon our observation that the expression of PDCD5 protein was increased in BGC823, PAMC82, N87, MGC803 and SGC7901 cells treated with DATS, and DATS was able to induce apoptosis in tumor cells, we postulated that the elevated expression of PDCD5 protein was in correspondence with apoptotic process activated in these cells. Moreover, short interfering RNA against PDCD5 could attenuate cell apoptosis and caspase-3 activity induced by Bax over-expression (personal communication, Li-na Chen *et al*.: Short interfering RNA against the PDCD5 attenuates cell apoptosis and caspase-3 activity induced by Bax overexpression. Apoptosis. 2005, in press). This finding partially confirmed our hypothesis that PDCD5 takes part in regulating apoptosis.

Conclusion

In this paper, we communicated that the gastric tumor tissues contained low expression of PDCD5 protein and the survival rate was dependent upon the expression level of PDCD5 in these tissues. Since the down-regulated expression of PDCD5 protein was well correlated with the up-regulated expression of Bcl-2 protein, and PDCD5 expression was enhanced in the GC cells treated with DATS, PDCD5 was reasoned to actively participate in apoptotic regulations. During this process PDCD5 was found to translocate from cytoplasm to the nucleus within a short time. Our findings suggest that PDCD5 really plays an important role in the carcinogenesis of stomach.

Acknowledgments

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References

1. Feng X. Regulatory roles and molecular signaling of TNF family members in osteoclasts. *Gene* 2005; **350**(1): 1–13.
2. Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. *CA Cancer J Clin* 2005; **55**(3): 178–194.
3. Gomez-Lazaro M, Fernandz-Gomez FJ, Jordan J. The role of protein p53 in neurodegenerative processes throughout the 25 years of its history. *Rev Neurol* 2004; **39**(3): 243–250.
4. Nicholson DW. ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. *Nat Biotechnol* 1996; **14**(3): 297–301.
5. Mani A, Gelmann EP. The ubiquitin-proteasome pathway and its role in cancer. *J Clin Oncol* 2005; **23**(21): 4776–4789.
6. Hongtao Liu, Yugang Wang, Yingmei Zhang, *et al*. *TFAR19,* a novel apoptosis-related gene cloned from human leukemia cell line TF-1, could enhance apoptosis of some tumor cells induced by growth factor withdrawal. *Biochem Biophys Res Comm* 1999; **254**(1): 203–210.
7. Min Rui, Yingyu Chen, Yingmei Zhang, Dalong Ma. Transfer of anti-TFAR19 monoclonal antibody into HeLa cells by in situ electroporation can inhibit the apoptosis. *Life Sciences* 2002; **71**: 1771–1778.
8. Yingyu Chen, Ronghua Sun. Wenling Han, *et al*. Nuclear translocation of PDCD5 (TFAR19): An early signal for apoptosis? *FEBS Letters* 2001; **509**: 191–196.
9. Dongsheng Liu, Yingang Feng, Yuan Cheng, Jinfeng Wang. Human programmed cell death 5 protein has a helical-core and two

dissociated structural regions. *Biochem Biophys Res Commun* 2004; **318**(2): 391–396.
10. Tian HK, Xia T, Jiang CS, Zhang HM, Wang K, Li XJ. TFAR19 enhances the opening of permeability transition pore in the mitochondrial membrane of mice liver. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* 2002; **34**(3): 279–284.
11. Ying Wang, Xianting Li, Lu Wang, *et al*. An alternative form of paraptosis-like cell death, triggered by TAJ/TROY and enhanced by PDCD5 overexpression. *J Cell Sci* 2004; **117**: 1525–1532.
12. Ingrid Hedenfalk, Dvid Duggan, Yidong Chen, *et al*. Gene-expression profiles hereditary breast cancer. *N Engl J Med* 2001; **344**(8): 539–548.
13. Xu XR, Huang J, Xu ZG, Qian BZ, Zhu ZD, Han ZG. Insight into hepatocellular carcinogenesis at transcriptome level by comparing gene expression profiles of hepatocellular carcinoma with those of corresponding noncancerous liver. *Proc Natl Acad Sci USA* 2001; **98**(26): 15089–15094.
14. Liu ZH, Zhang D, Li KM, Liao QP. Expression of PDCD5 in tissues of normal cervix, CIN I–III and cervical cancer. *J Pek Univer (Health sciences)* 2004; **36**(4): 407–410.
15. Lan H, Lu YY. Allitridi induces apoptosis by affecting Bcl-2 expression and caspase-3 activity in human gastric cancer cells. *Acta Pharmacol Sin* 2004; **25**(2): 219–225.
16. McCabe ML, Dlamini Z. The molecular mechanisms of oesophageal cancer. *Int Immunopharmacol* 2005; **5**(7–8): 1113–1130.
17. Kountouras J, Zavos C, Chatzopoulos D. Apoptotic and anti-angiogenic strategies in liver and gastrointestinal malignancies. *J Surg Oncol* 2005; **90**(4): 249–259.
18. Shirin H, Hibshoosh H, Kawabata Y, Weinstein IB, Moss SF. p16Ink4a is overexpressed in H. pylori-associated gastritis and is correlated with increased epithelial apoptosis. *Helicobacter* 2003; **8**(1): 66–71.
19. Soman NR, Correa P, Ruiz BA, Wogan GN. The TPR-MET oncogenic rearrangement is present and expressed in human gastric carcinoma and precursor lesions. *Proc Natl Acad Sci USA* 1991; **88**(11): 4892–4896.
20. Tavassoli M, Guelen L, Luxon BA, Gaken J. Apoptin: Specific killer of tumor cells? *Apoptosis* 2005; **10**(4): 717–724.
21. Feng Y, Hu J, Xie D, *et al*. Subcellular localization of caspase-3 activation correlates with changes in apoptotic morphology in MOLT-4 leukemia cells exposed to X-ray irradiation. *Int J Oncol* 2005; **27**(3): 699–704.