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Decreased Expression of Heat Shock Protein 20 in Colorectal Cancer and Its Implication in Tumorigenesis

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ABSTRACT

Heat shock protein 20 (HSP20), which is a member of the small heat shock protein family, is known to participate in many pathological processes, such as asthma, intimal hyperplasia, and insulin resistance. However, the function of HSP20 in cancer development is not yet fully understood. In this study, we identified HSP20 as a down-regulated protein in 20 resected colorectal cancer (CRC) specimens compared with their paired normal tissues. Because HSP20 proteins were barely detectable in HCT-116 cells (a human colorectal cancer cell line), recombinant adenovirus encoding HSP20 (Ad-HSP20) was used to induce HSP20 overexpression in HCT-116 cells. Infection of Ad-HSP20, but not control adenovirus (Ad-GFP), reduced viability, and induced massive apoptosis in a time-dependent manner. The forced expression of HSP20 enhanced caspase-3/7 activity and down-regulated the anti-apoptotic Bcl-xL and Bcl-2 mRNA and protein levels. In addition, immunohistochemical analysis of 94 CRC specimens for HSP20 protein showed that reduced HSP20 expression was related to advanced TNM stage, lymph node metastasis, and tumor recurrence. Our study shows, for the first time, that expression of the HSP20 protein has a pro-death role in colorectal cancer cells. Therefore, HSP20 may have value as a prognostic tumor marker and its overexpression might be a novel strategy for CRC therapy. J. Cell. Biochem. 116: 277–286, 2015.

KEY WORDS: APOPTOSIS; COLORECTAL CANCER; HEAT-SHOCK PROTEIN 20; TUMOR MARKER

Colorectal cancer (CRC) is one of the most common gastrointestinal tract cancers and the second-leading cause of cancer-related death in the world, with approximately 1.23 million new cases diagnosed annually [Ferlay et al., 2010]. In Korea, CRC is also the third-most common cancer in 2010, and the incidence has continued to increase in both sexes [Jung et al., 2013]. Although the use of colonoscopy for CRC screening has contributed to a reduction in mortality [Sonnenberg et al., 2000], 70% of newly discovered CRCs are detected at an advanced stage (stage III or IV according to the 7th International Union Against Cancer/American Joint Committee on Cancer (UICC/AJCC) TNM classification), which results in a poor prognosis, whereas that of stage I CRC is generally near 95% after radical surgery [O'Connell et al., 2004]. Therefore, research on the early diagnosis and prognosis of CRC has important clinical value.

It is well known that colorectal carcinogenesis is a multistep process involving genetic changes [Fearon and Vogelstein, 1990]. Nevertheless, at present, the mechanism of colorectal carcinogenesis remains unclear, and most recent studies have focused on the

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identification of different molecular genetic alterations during carcinogenesis [Toyota and Issa, 1999; Boland and Goel, 2010]. The increase of knowledge in the field of molecular genetics has led to an impressive increase in the number of putative biomarkers that are capable of predicting the response to specific adjuvant treatment, but it is not clear whether they have prognostic value or therapeutic implications.

Heat shock proteins (HSPs) are a diverse group of proteins that are ubiquitously expressed under normal physiological conditions to maintain a wide range of cellular processes. Under stress conditions, such as heat shock and some pathological states, the expression of these proteins is rapidly induced to protect the cell from various types of damage [Khalil et al., 2011]. Based on their molecular weight, HSPs are divided into six families, namely, small heat shock proteins (sHSPs), HSP40, HSP60, HSP70, HSP90, and HSP100. These proteins play a pivotal role in regulation of the cell cycle and apoptosis and are involved in many disease processes. Abnormal levels of these stress proteins have been found in a number of disorders [Whitley et al., 1999]. sHSPs comprise the most diverse family, with molecular masses ranging from 15 to 30 kDa, and share high sequence homology within their crystalline domain [Kappe et al., 2003]. These molecular chaperones have been implicated in cancer development. In particular, HSP27, whose expression is elevated in many types of human cancers, has been the most extensively studied. The tumorigenic potential of HSP27 has been observed in experimental models [Wang et al., 2009a], and its antiapoptotic effect has been well established [Garrido et al., 2006]. It has also been suggested that the enhanced expression of HSP27 is associated with poor prognosis in gastric cancer [Kapranos et al., 2002], hepatocellular carcinoma (HCC) [King et al., 2000] and prostate cancer [Cornford et al., 2000].

However, the precise role of HSP20, which is another member of the sHSPs, in tumor progression still remains unknown. HSP20 is an α-crystalline domain-containing protein that was originally described in the skeletal muscle [Kato et al., 1994], and it is constitutively expressed at high levels in cardiac [Fan and Kranias, 2011] and smooth muscle [Dreiza et al., 2010]. HSP20 shows versatile functions, such as suppression of platelet aggregation [Kozawa et al., 2002], association with insulin resistance [Wang et al., 2001], prevention of vasospasms [Flynn et al., 2005] and airway smooth muscle relaxation [Komalavilas et al., 2008]. In addition, a recent study reported that HSP20 has protective functions in the heart [Fan et al., 2006; Fan and Kranias, 2011]. These versatile functions of HSP20 are now being investigated in the field of cancer. Most studies to date have relied on proteomics, but evidence for HSP20 as an inhibitor of neoplastic growth is steadily accumulating. HSP20 expression in HCC was significantly reduced in comparison with surrounding non-cancerous tissue and inversely correlated with the tumor size and the TNM stage of HCC [Noda et al., 2007]. The most recent report indicated that HSP20 suppresses the growth of HCC cells via inhibition of the mitogen-activated protein kinase (MAPKs) and AKT signaling pathways [Matsushima-Nishiwaki et al., 2011].

In this study, we investigated the differential expression of proteins in CRC and paired normal tissues and identified significant down-regulation of HSP20 in CRC tissues. Given that HSP20 appears to impact multiple pathways related to tumor survival and progression, we hypothesized that HSP20 may function to suppress cancer cell growth or promote cancer cell death. To test this hypothesis, an adenoviral vector that produced HSP20 protein was constructed and used to induce HSP20 overexpression in HCT-116 cells (a human colorectal carcinoma cell line). The cells were then assessed for viability and apoptosis. In addition, this study aimed to investigate the clinical relationship between HSP20 and the prognosis of CRC.

MATERIALS AND METHODS

PATIENTS AND TISSUE SAMPLES

To determine tumor-specific protein expression, two-dimensional gel electrophoresis (2-DE) analysis was performed using colorectal cancer tissues and their corresponding normal colorectal tissues from 20 consecutive patients who underwent radical surgery for CRC from June to October 2007. Ninety-four patients (52 men and 42 women) who underwent radical surgery for CRC in Gyeongsang National University Hospital from January 2001 to December 2002 were randomly selected from the Institution's surgical database to investigate the relationship between HSP20 and the prognosis of CRC. Clinical data and the outcomes of the patients were retrospectively collected from the medical records. This study was approved by the committee for the conduct of clinical medical research at Gyeongsang National University Hospital (CRI5363). The pathologic stage of each patient was determined according to the 7th AJCC TNM classification, and informed consent was obtained from all of the patients. Cancerous lesions and the corresponding normal tissues were excised during surgery and stored at −70°C for proteomic analysis and further study.

TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE)

Tissue samples were homogenized in ice-cold 2-D sample buffer (8 M Urea, 4% CHAPS, 40 mM Tris, 100 mM DTT, 2% immobilized pH gradient buffer) containing protease inhibitors (Calbiochem/EMD Millipore, Billerica, MA). Total proteins (50 μg) were subjected to isoelectric focusing (pH 4–7) and sequentially to a gradient SDS–polyacrylamide gel (7.5–17.5%), as described previously [Jeong et al., 2012]. The gels were stained with silver nitrate followed by PDQuest analysis, and isolated protein spots were identified using mass spectrometry, as described in our previous work [Jeong et al., 2012].

CELL CULTURE

HCT-116 cells (human colorectal carcinoma cell line) were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antbiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate).

PREPARATION OF ADENOVIRUS

An adenovirus encoding HSP20 (Ad-HSP20) was created using the Virapower adenovirus expression system according to the

manufacturer's instruction (Invitrogen, Carlsbad, CA). Briefly, cDNA that encoded HSP20 was subcloned into the pENTR vector. After sequence verification, the HSP20 cDNA was transferred into the pAd/CMV/V5-DEST vector using the Gateway system with LR clonase (Invitrogen). The verified clone (Ad-HSP20) was linearized using PacI (New England Biolabs, Beverly, MA) and then transfected into 293 A cells using Lipofectamine 2000. The virus was prepared and amplified using the ViraPower adenoviral expression system (Invitrogen), and the viral titers were determined using a plaque-forming assay and serial dilution. An aliquot of the viral suspension was used to infect HCT-116 cells. A recombinant, replication-defective adenovirus containing green fluorescent protein (Ad-GFP) was used as a control.

CELL VIABILITY ANALYSIS

HCT-116 cells were seeded in 96-well plates at a concentration of 6×10^3 cells/well and incubated for 24 h. The cells were infected with Ad-GFP or Ad-HSP20 at a multiplicity of infection (MOI) of 10. At 24 h after viral infection, the growth media were replaced with normal culture media. After an additional 24- or 48-h incubation, the cell viability was assayed using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Briefly, 10 μl/well of the CCK-8 solution was added and incubated for 1 h at 37°C in a humidified, 5% CO_2 atmosphere. The amount of formazan dye that was generated by cellular dehydrogenase activity was measured as the absorbance at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). During the overexpression procedure, the cells were observed using an inverted microscope (Nikon, Tokyo, Japan) at different time intervals to check for phenotypic manifestations in the Ad-HSP20-infected HCT-116 cells compared to the control cells.

DETECTION OF APOPTOSIS

For Sub-G1 analysis, adenoviral-infected HCT-116 cells were washed twice with cold PBS, fixed with 70% ethanol for 1 h at 4 °C before treatment with 1 mg/ml RNase A (Sigma–Aldrich, St. Louis, MO) and then stained with 50 μg/ml PI (Sigma–Aldrich). The relative DNA content per cell was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed using the CellQuest Pro software (BD Biosciences). The cell cycle calculations were performed using the ModFit LT Software (Verity Software House, Topsham, ME). For Annexin V cell death assay, cells were stained with Annexin V and PI using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions. Apoptotic cells were analyzed under a FACSCalibur flow cytometer.

CASPASE ACTIVITY ASSAY

The cells were infected with Ad-HSP20 or Ad-GFP at an MOI of 10 and harvested 48 and 72 h after infection, and the cell lysates were subjected to caspase-3/7 and -9 activity measurement using the Caspase-Glo assay kit (Promega, Madison, WI). Briefly, the plates containing Ad-GFP- or Ad-HSP20-infected cells were removed from the incubator and allowed to equilibrate to room temperature for 30 min. Caspase-Glo reagent (100 μl) was added to each well, and the contents of the wells were incubated at room temperature with gentle agitation for 30 min. The luminescence of each sample was measured using a GloMax luminometer (Promega).

RNA EXTRACTION AND RT-PCR (REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION)

Total RNA was extracted from HCT-116 cells for RT-PCR using TRIzol reagent (Qiagen, Valencia, CA). For the reverse transcription reaction, a reaction mix (20 μl) containing 2 μg of total RNA, 5–pmoles of oligo(dT) 12–18, 1 mM of each dNTP and 200 U of Superscript III reverse transcriptase (Invitrogen) was incubated at 50° C for 60 min, followed by incubation at 70°C for 15 min and rapid cooling. The targeted genes were amplified using PCR with the following sets of primers (sense and antisense, respectively): 5′-CCCTTTTGCTTCAGGGTTTC-3′, 5′-GCCACTCGGAAAAAGACCTC-3′ for Bax; 5′-TGTGGCCTTCTTTG AGTTCG-3′, 5′-AGGTGCCGGTT-CAGGTACTC-3′ for Bcl-2; 5′-CCCAGAAAGGATACAGCTGG-3′, 5′-GC GATCCGACTCACCAATAC-3′ for Bcl-xL; and 5′-CGGAGT-CAACGGATTTGGTC-3′, 5′-AGCCTTCTCCAT GGTGGTGA-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR products (5 μl each) were separated by electrophoresis in a 1.5% agarose gel in Tris-acetic acid–EDTA (0.5× TAE) buffer and detected using ethidium bromide staining.

WESTERN BLOTTING

Cells were incubated in NP-40 lysis buffer (20 mM Tris (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 5 μM AEBSF, 1.5–nM Aprotinin, 10 nM E-64, and 10 nM Leupeptin) for 30 min. The cells were then sonicated and centrifuged at 12,000 *g* for 10 min at 4°C to remove insoluble debris. Total proteins (30 μg) were resolved in an SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane using the semi-dry technique. The membranes were blotted using specific antibodies against HSP20 (Novus Biologicals, Littleton, CO), Bax, Bcl-xL, and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and the proteins were identified using the ECL detection system (Pierce Biotechnology, Rockford, IL). β-actin was used as a loading control on the stripped blot.

IMMUNOHISTOCHEMISTRY

The formalin-fixed, paraffin-embedded tissue blocks from 94 patients with CRC were collected, sectioned at 4 μm and mounted on charged slides. The slides were deparaffinized, rehydrated in a graded alcohol series and pretreated with 10 mM sodium citrate. Immunostaining was performed using a rabbit polyclonal HSP20 antibody (1:5,000) and a LSAB kit (Dako, Cambridge, UK). Antigen retrieval was facilitated by microwaving the tissue for 15 min, and the staining procedure was carried out according to the standard avidin-biotinylated peroxidase complex system. Immunohistochemical staining was interpreted and categorized using an arbitrary semi-quantitative scale as 0 (negative staining), +1 (0–20% positive), +2 (20–40% positive) or +3 (more than 40% positive) by a pathologist. The staining intensity was simply categorized into "weak" (0 and +1) and "strong" (+2 and +3) groups.

STATISTICAL ANALYSIS

Statistical analysis was performed using the chi-squared test in SPSS version 18.0 (SPSS, Inc., Chicago, IL). All *P* values were based on a two-tailed statistical analysis, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

DOWN-REGULATION OF HSP20 IN CRC

We applied a typical comparative method (2-DE) to analyze the differential expression of the total proteins in CRC and the corresponding normal colorectal tissues from 20 consecutive patients. The clinical and pathological characteristics of the patients with CRC are shown in Supplementary Table SI. We found that many proteins in the CRC tissues were differentially expressed in comparison with normal colorectal tissues (listed in Supplementary Table SII). In particular, a protein spot, which was identified as HSP20 using mass spectrometry, was significantly down-regulated in CRC tissues (Figs. 1A and B). To verify the result obtained from 2-DE analysis, we performed Western blot analysis using lysates isolated from CRC and normal colorectal tissues. Consistent with the 2D-PAGE result, the HSP20 protein was down-regulated in CRC tissues (Fig. 1C).

HSP20 OVEREXPRESSION INDUCES APOPTOTIC CELL DEATH IN HCT-116 CELLS

Based on the finding that HSP20 expression was markedly decreased or absent in CRC tissue specimens, the effect of HSP20 on cell viability in human colon cancer HCT-116 cells was then investigated using recombinant adenovirus expressing HSP20 (Ad-HSP20) or GFP

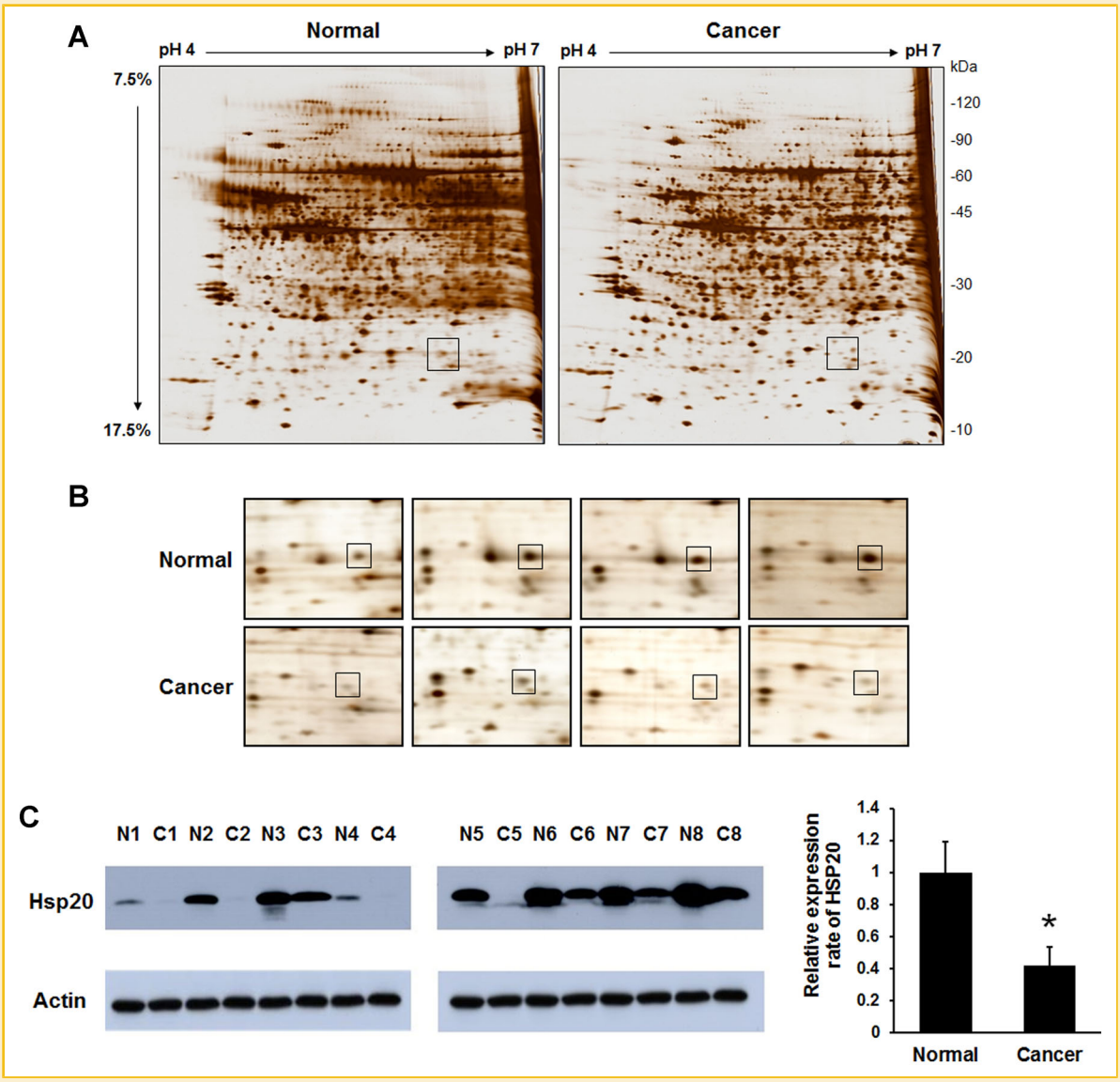

Fig. 1. Differential expression of the HSP20 protein in CRC. (A) Two-dimensional gel electrophoresis analysis. Total cellular proteins (50 μg) extracted from either CRCs or the surrounding noncancerous tissues were analyzed in a two-dimensional gel (focusing at pH 4.0–7.0, followed by 7.5–17.5% SDS-PAGE) and stained by silver nitrate as described previously [29]. A magnified image including the HSP20 protein is indicated by the box in (A) and was displaced (B). (C) The HSP20 protein level in CRC tissues (C) or adjacent normal tissues (N) was estimated by Western blotting using antibodies against HSP20. β-actin was used as a control for protein loading. Representative blots are shown (left panel). Values are plotted as mean ± SE (right panel). *P-< 0.05 versus normal tissues.

(Ad-GFP). Western blot analysis showed that HSP20 was over-expressed in Ad-HSP20-infected HCT-116 cells, but it was not detected in Ad-GFP-infected control cells (Fig. 2A). To clarify the relationship between the expression of the HSP20 protein and cell viability, a cell viability assay was performed using Ad-HSP20 or Ad-GFP-infected HCT-116 cells. The cell viability of the HSP20 over-expressing cells was remarkably decreased compared with that of the control, GFP-expressing cells in a time-dependent manner (Fig. 2B). Morphological changes were observed in HCT-116 cells infected with Ad-HSP20 using phase-contrast microscopy. Forty-eight hours post-infection, Ad-HSP20-infected cells underwent marked rounding, eventually detached from the culture dishes and displayed typical apoptotic morphology with cell retraction and plasma membrane blebbing (Fig. 2C). To investigate the involvement of HSP20 in apoptosis, we examined whether HSP20 expression induced spontaneous apoptotic cell death without challenge by apoptotic stimulators by measuring the cell number in the sub-G1 region. As shown in Figure 3A, the sub-G1 cell proportion of Ad-HSP20-infected cells was markedly higher than that of Ad-GFP-infected cells.

Furthermore, the type of cell death induced by HSP20 expression was mostly apoptotic as evaluated by Annexin V binding assay (Fig. 3B), demonstrating that the forced expression of HSP20 directly induced apoptosis in HCT-116 cells.

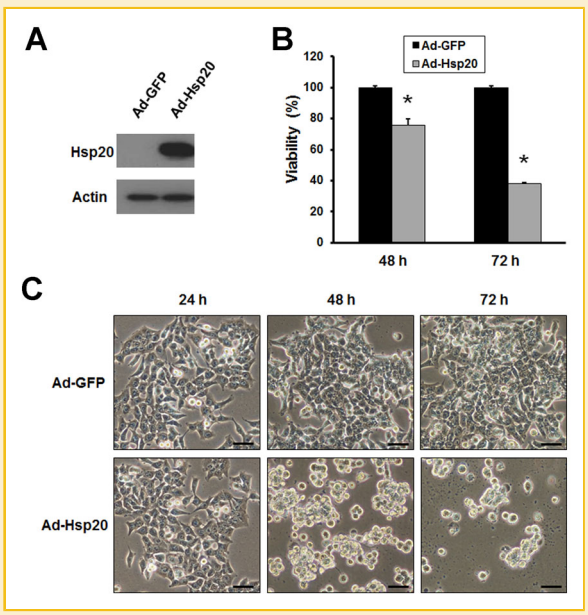

Fig. 2. HSP20 overexpression induces cell death in HCT-116 colon cancer cells. (A) HCT-116 cells were infected with Ad-HSP20 or control Ad-GFP adenovirus at an MOI of 10. Twenty-four hours after infection, the cells were harvested and subjected to Western blot analysis using the anti-HSP20 antibody. (B) HCT-116 cells were plated in 24-well dishes and infected (10 MOI) with Ad-GFP or Ad-HSP20 for the indicated time. Cell viability was measured using a Cell Counting Kit-8 (CCK-8). The viability of the control cells (Ad-GFP) was set at 100%, and viabilities relative to the control are presented. Data represent the means ± SD of three independent experiments. *Indicates $P < 0.05$. (C) HCT-116 cells were treated as in (B), and representative photographs of HCT-116 cells were taken using phase-contrast microscopy. Scale bar = 50 μm.

HSP20 OVEREXPRESSION ACTIVATES THE PRO-APOPTOTIC SIGNALING PATHWAYS

To explore the mechanism by which HSP20 induces apoptosis in HCT-116 cells, we examined the endogenous expression of Bcl-2 family members, including Bax, Bcl-2, and Bcl-xL. RT-PCR and Western blotting data showed that the levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL were down-regulated in Ad-HSP20-infected HCT-116 cells (Figs. 4A and B). Control adenovirus did not affect the levels of the Bax, Bcl-2, or Bcl-xL proteins. The best recognized hallmark of the early and late stages of apoptosis is the activation of cysteine proteases (caspases). Caspase-3/7 activity in cells is an important feature for detecting apoptosis induced by a wide variety of apoptotic signals. Relative caspase-3/7 activity was significantly increased by 2.5-fold at 72 h post-infection by Ad-HSP20 (Fig. 4C), whereas control adenovirus Ad-GFP resulted in >90% infectivity at an MOI of 10 (data not shown) but did not induce significant caspase-3/7 activation. It has been recognized that caspase-3/7 is activated by caspase-9 activation, which is a part of the intrinsic apoptosis pathway. Therefore, caspase-9 activity was also measured under the same conditions. Caspase-9 activity assay showed similar results to those obtained by caspase-3/7 assay (Fig. 4C). Collectively, these data suggested that HSP20 expression increases HCT-116 cell caspase-3/7 and -9 activity.

HSP20 EXPRESSION IN CRC TISSUES IS INVERSELY ASSOCIATED WITH TNM STAGE, LYMPH NODE METASTASIS AND RECURRENCE

To investigate the clinical significance of HSP20 expression, immunohistochemical analysis was performed. A total of 94 patients who underwent radical surgery for colorectal cancer were enrolled. HSP20 was mainly found in the cytoplasm of the epithelium of cancerous and noncancerous tissues (Fig. 5). Examination of CRC sections for the immunoreactivity of HSP20 showed weak HSP20 staining (0 or +1) in 74 of the 94 cases, and these were classified as the weak-positive group. The remaining 20 cases exhibited strong HSP20 staining (+2 or +3) and were classified as the strong-positive group. The immunoreactivity of HSP20 in CRC specimens was markedly lower than that in noncancerous tissues. The expression levels of HSP20 were compared with the clinical and pathologic characteristics of 94 patients with CRC (Table I). The levels of HSP20 expression were negatively correlated with TNM stages ($P = 0.002$) and were significantly lower in patients with lymph node metastasis ($P = 0.008$) and with tumor recurrence ($P = 0.03$).

DISCUSSION

Colorectal carcinogenesis requires the accumulation of multiple genetic alterations during which a normal cell is transformed into a malignant cell and successive clones are established that are each characterized by a relative growth advantage. This growth advantage may be conferred through an increased rate of proliferation, evasion of cell death or both. Hence, reduced apoptosis or apoptosis resistance plays a vital role in carcinogenesis [Hanahan and Weinberg, 2000]. Recently, many studies have focused on the

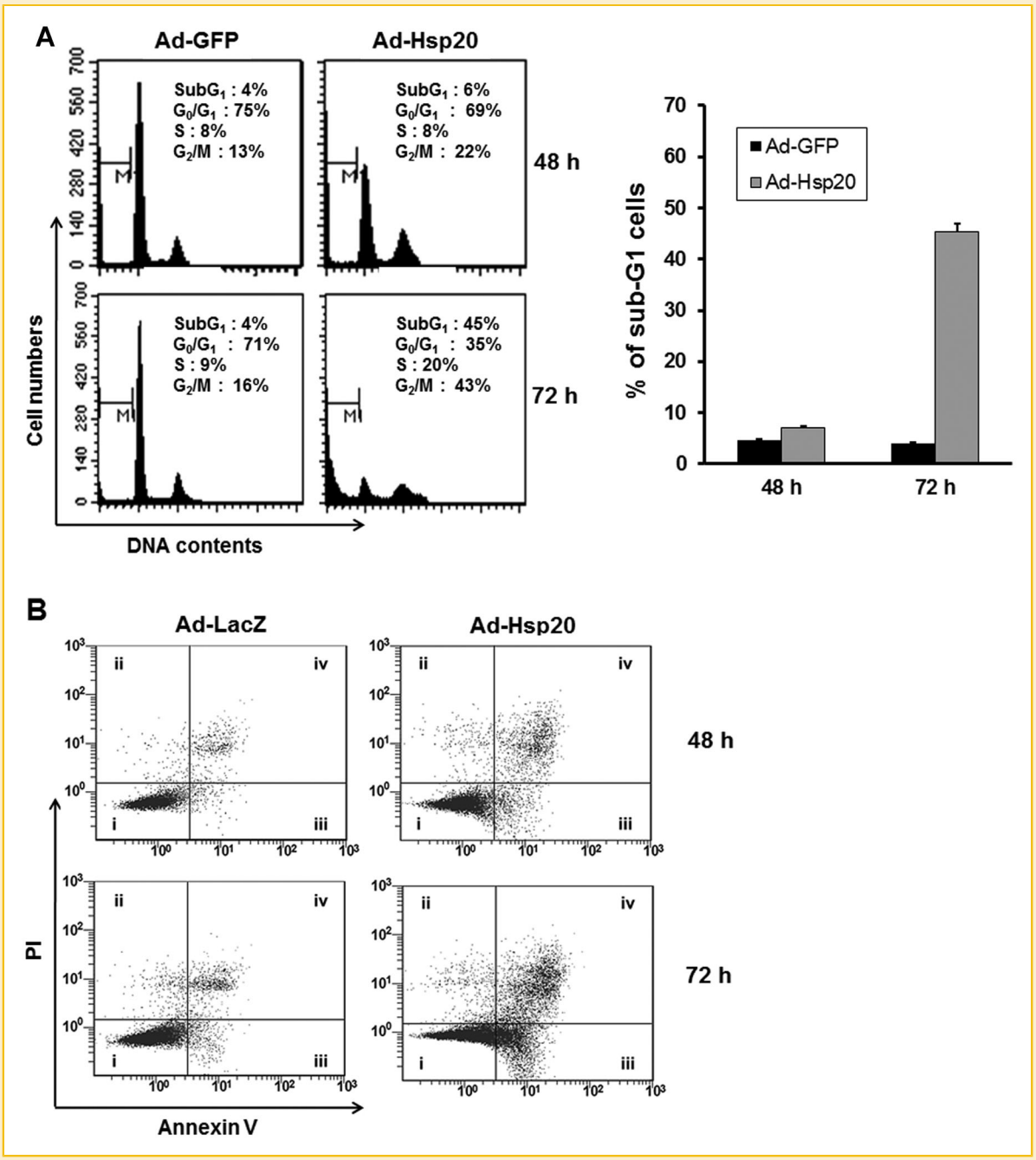

Fig. 3. HSP20 overexpression induces apoptotic cell death in HCT-116 cells. (A) Plated HCT-116 cells were exposed to Ad-HSP20 or Ad-GFP at an MOI 10. Apoptotic cells (Sub-G1) were determined 48 h or 72 h post-infection by propidium iodide (PI) staining using FACS. Original FACS plots are represented in the left panel, and the mean values of three independent experiments ± SD are shown in the graph on the right. * indicates $P < 0.05$ compared with Ad-GFP-infected cells. (B) HCT-116 cells were infected with Ad-HSP20 or Ad-LacZ at an MOI of 10 for 48 h or 72 h. Apoptosis was measured by Annexin V and PI double staining. i, ii, iii, and iv denote viable (live), necrotic, early apoptotic, and late apoptotic regions, respectively.

identification of different molecular genetic alterations in carcinogenesis [Boland and Goel, 2010]. Several studies have identified heat shock proteins that were involved with tumorigenesis in CRC. Kanazawa et al. [2003] demonstrated that Hsp70 and Hsp40 are overexpressed in cancer tissue samples compared with normal tissues. However, no significant correlation between their expression and various clinicopathological parameters of colorectal cancer were found. Hwang et al. [2003] demonstrated that the levels of Hsp70 and Hsp110 were highly correlated with advanced clinical stages and positive lymph node involvement. Cappello et al. [2005] found that the expression of Hsp60 in primary CRC and lymph node metastasis was correlated with the tumor grade, while Hsp10 expression was

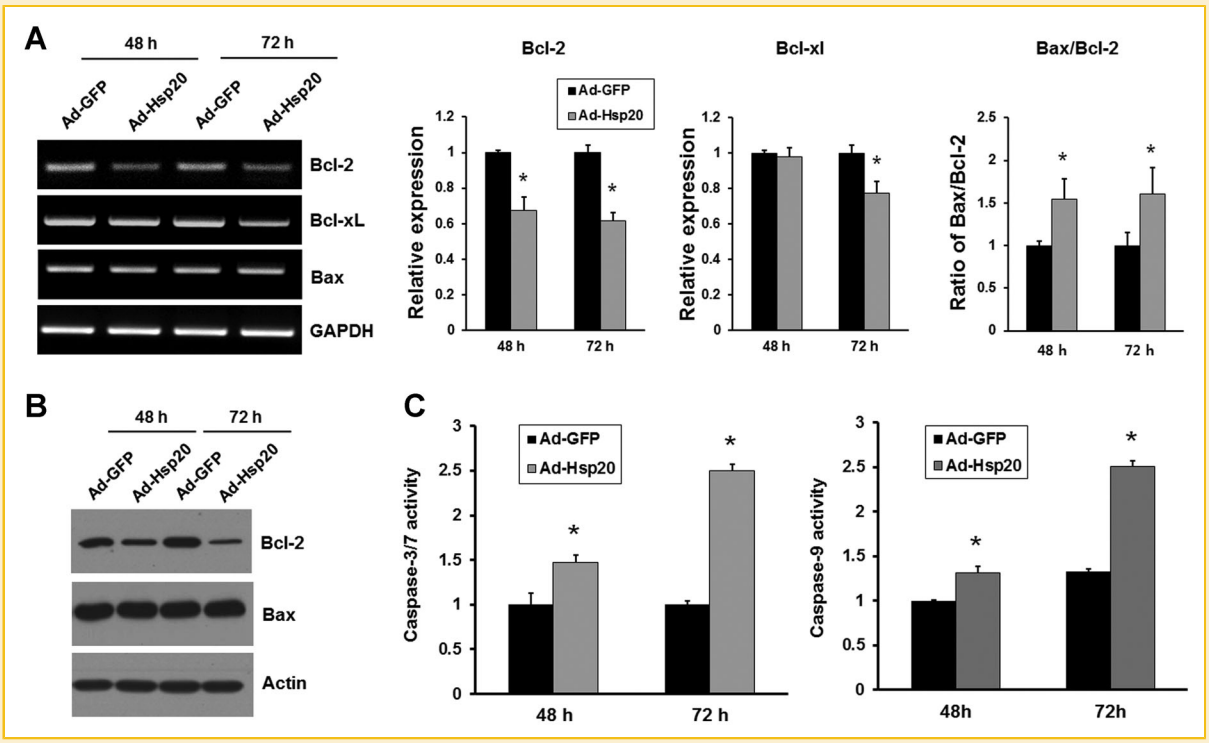

Fig. 4. HSP20 overexpression alters Bcl-2 and Bcl-xL expression and promotes caspase-3/7 activation. (A) Semi-quantitative PCR was used to detect Bcl-2, Bcl-xL, and Bax mRNA expression. Total RNA was extracted from Ad-HSP20- or Ad-GFP-infected HCT-116 cells, and RT-PCR analyses were performed using Bcl-2, Bcl-xL, Bax, and GAPDH-specific primers (left panel). Bcl-2, Bcl-xL, and Bax transcript levels were normalized relative to the housekeeping gene GAPDH. The resulting Bcl-2/GAPDH, Bcl-xL/GAPDH, or Bax/GAPDH mRNA ratios are represented as a percentage of the Ad-GFP ratio (right panel). The Bax/Bcl-2 protein ratio, which reflects the apoptotic cell status, was determined using densitometric analysis. (B) Total cellular proteins were extracted from HCT-116 cells infected with GFP- or HSP20-expressing adenovirus, and protein levels were detected using Western blot analysis with the indicated antibodies. β-actin was used as a control for protein loading. (C) HCT-116 cells were infected with Ad-HSP20 or Ad-GFP at an MOI of 10, and, at the indicated time, the caspase-3/7 and -9 activity in the Ad-HSP20 and Ad-GFP cell lysates was measured as described in Section 2. The data are presented as the mean ± SD from three independent experiments for each group. * indicates a significant difference from Ad-GFP-infected cells ($P < 0.05$).

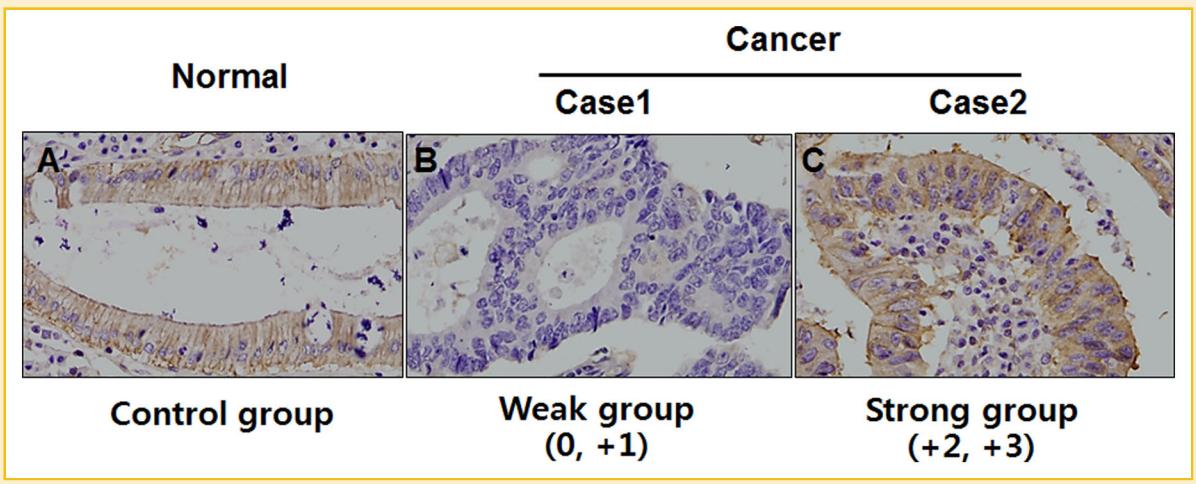

Fig. 5. Immunohistochemical analysis of HSP20 expression in normal and CRC tissues. Representative examples of HSP20 expression in normal colon tissue, with positive HSP20 staining scored as +3 (A), colon cancer with negative HSP20 staining scored as 0 (B), and colon cancer with positive HSP20 staining scored as +2 (C). Original magnification, ×200.

TABLE I. Relationship Between Hsp20 Expression and Clinicopathological Characteristics of Colorectal Cancer

Clinicopathological characteristics	Level of expression (n = 94)		
	Weak[a] (0, +1)	Strong[b] (+2, +3)	*P*-value
Differentiation			
Mucinous, poor	11 (14.9%)	1 (5.0%)	0.126
Moderate	51 (68.9%)	12 (60.0%)	
Well	12 (16.2%)	7 (35.0%)	
T stage			
T1, 2	16 (21.6%)	7 (35.0%)	0.217
T3, 4	58 (78.4%)	13 (65.0%)	
N stage			
L/N (−)	43 (58.1%)	18 (90.0%)	0.008
L/N (+)	31 (41.9%)	2 (10.0%)	
M stage			
Metastasis (−)	65 (87.8%)	20 (100.0%)	0.101
Metastasis (+)	9 (12.2%)	0 (0%)	
TNM stages			
Stage I, II	39 (52.7%)	18 (90.2%)	0.002
Stage III, IV	35 (47.3%)	2 (10.0%)	
Recurrence[c]			
Recur (−)	52 (80.0%)	20 (100.0%)	0.030
Recur (+)	13 (20.0%)	0 (0%)	

Immunointensity was scored on an arbitrary scale of 0, negative; +1, 0–20% positive; +2, 20–40% positive; and +3, more than 40% intense positive ([a]Weak, 0 and +1; [b]Strong, +2 and +3). [c] Recurrence during the follow-up period after the operation. Stage IV was excluded.

not. In this study, to the best of our knowledge, we identified, for the first time, the down regulation of HSP20 in HCT-116 cells. In addition, this study demonstrated that the HSP20 levels correlated inversely with lymph node metastasis, advanced stage, and recurrence of CRC. A recent study reported that the expression levels of HSP20 in human HCC are inversely correlated with the tumor stage based on the TNM classification, the presence of microvascular invasion, and tumor size [Noda et al., 2007]. Although the multifunctional protective abilities of HSP20 have been reported in various research fields, it is still unknown whether HSP20 exerts its biological functions in human cancers such as CRC. However, decreased HSP20 expression has been recently reported in HCC, melanoma, cervical carcinoma, lung adenocarcinoma, and glioma [Edwards et al., 2011], indicating that HSP20 can serve as an inhibitor of neoplastic growth. This study has provided the first evidence that HSP20 overexpression is toxic to HCT-116 cells and that this toxicity is a consequence of HSP20-driven apoptotic cell death in HCT-116 cells.

Fan et al. [2005] suggested that HSP20 overexpression may stabilize the apoptosis-related proteins Bcl-2 and Bax in cardiomyocytes. Bcl-2 overexpression or Bax ablation correlated with a reduction of cardiomyocyte apoptosis [Chen et al., 2001; Hochhauser et al., 2003]. In this study, HSP20 overexpression decreased the expression of the anti-apoptotic protein Bcl-2 and Bcl-xL and activated caspase 3/7, which, finally, led to the activation of the pro-apoptotic signaling pathway. A recent study reported that HSP20 suppresses the growth of HCC cells via the MAPK and AKT signaling pathways [Matsushima-Nishiwaki et al., 2011]. In those series, the activities of extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and AKT were negatively correlated with the HSP20 protein levels in human HCC tissues. Furthermore, it has been reported that HSP20 directly associates with phosphatidylinositol 3-kinase (PI3K) and suppresses its activity in HCC, resulting in the inhibition of the AKT pathway [Matsushima-Nishiwaki et al., 2013]. PI3K signaling pathway is activated by several different mechanisms in cancers, and it is considered to be an effective target for therapeutic intervention in cancer [Courtney et al., 2010]. HSP20 has also been reported to protect mesenchymal stem cells against oxidative stress-induced cell death, and this effect is associated with enhanced AKT activation and increased secretion of growth factors [Wang et al., 2009b].

HSP20 may interact with various proteins in multiple pathophysiological processes in CRC development. Phosphorylated HSP20 has been reported to interact with AKT, actin, apoptosis signal-regulating kinase 1(ASK1), Bax, Bag3, Beclin-1, HSP22, and HSP27 [Fan and Kranias, 2011]. HSP20 was also recently reported to form a complex with the 14–3-3 protein, and this association affects the activities of other 14–3-3 binding proteins [Chernik et al., 2007; Dreiza et al., 2010]. Binding of phosphorylated HSPB6 to 14–3-3 can induce the displacement of certain binding partners of 14–3-3, such as pro- or antiapoptotic factors, protein kinases, protein phosphatases, and proteins involved in regulation of the actin cytoskeleton [Seit-Nebi and Gusev, 2010]. Matsushima-Nishiwaki et al. [2008] reported that phosphorylated HSP27 represses the growth of HCC cells via inhibition of the ERK signaling pathway, and they proposed that the interaction between HSP20 and HSP27 might affect ERK signaling in HCC. In addition, wild-type HSP27 has been reported to associate with and inhibit the rate of phosphorylation of HSP20 [Bukach et al., 2009]. In the field of CRC, the role of HSP27 was reported in several studies. HSP27 was found to be up-regulated in colorectal cancers with aggressive behavior and metastasis [Morino et al., 1997], and its expression was correlated with TNM stage [Yu et al., 2010]. Tweedle et al. [2010] found a highly significant association between high HSP27 expression and incomplete resection margins in rectal cancer. Additionally, elevated HSP27 was associated with poor survival [Tweedle et al., 2010; Wang et al., 2012]. Further studies are necessary to identify the HSP20 interactome to

investigate the precise role of HSP20 in CRC. These efforts will help us to use HSP20 as a target for cancer therapy.

Our study demonstrated, for the first time, that HSP20 overexpression markedly inhibited cell progression by inducing apoptosis in HCT-116 cells. This event is likely to be associated with multiple HSP20-regulated targets in HCT-116 cell proliferation. HSP20 decreased the expression of Bcl-2 and Bcl-xL and subsequently mediated an increase in the ratio of Bax over Bcl-2, leading to activation of caspase-3/7. HSP20 expression in CRC tissues was inversely associated with the TNM stage, lymph node metastasis, and recurrence. Our findings suggest that HSP20 has value as a prognostic tumor marker and that its overexpression might be a potential approach for treatment of CRC.

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