Expression of Type 2 Hexokinase and Mitochondria-related Genes in Gastric Carcinoma Tissues and Cell Lines

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Abstract. *Background: The constant overexpression of glycolysis and active mitochondrial function are critical for productive energy required for the immortal proliferation of cancer cells. The genes related to glycolysis and mitochondrial respiration might have some function during stomach carcinogenesis. Materials and Methods: The expression of hexokinase 2 (HK2), Bcl-2 and several mitochondria-related gene products were investigated by immunohistochemistry in 257 consecutive human gastric carcinomas, and the results were compared with the clinicopathological characteristics. In addition, transcriptional change of HK2 and Bcl-2 was investigated in the hypoxic state or with mitochondrial inhibitors. Results: In immunohistochemical analysis, HK2 was overexpressed in 43 out of 257 stomach cancer patients. Bcl-2 was not expressed in cases with HK2 positive cancer tissues except for one case, while the voltage-dependent anion channel, complex II and pyruvate dehydrogenase, located in mitochondria, were expressed in all cases. The patients with HK2 expression showed a worse prognosis compared to the HK2 negative cases, and patients who were negative in Bcl-2 and positive in HK2 represented the lowest survival rate. HK2 and Bcl-2 responded to hypoxia, but not to mitochondrial dysfunction while the cellular growth was severely repressed by mitochondrial inhibitors, indicating that transcriptional regulation of HK2 and Bcl-2 proceeds upstream of dysfunctional mitochondria. Conclusion: HK2 was overexpressed in 16.7% of gastric carcinomas, and reciprocal expression pattern with Bcl-2. The HK2 positive cases showed a worse prognosis and aggressive character.*

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For rapidly growing tumors, a high rate of glycolysis is as important as a major carbon catabolic pathway for energy production in hypoxic or even in normoxic conditions (1, 2). Thus, the activation of glucose transporter 1 (Glut1) and hexokinase 2 (HK2) could be the first choice for keeping the intracellular glucose level and ensuring the continuous generation of ATP energy (3). HK2 (ATP: D-hexose-6-phosphotransferase, EC2.7.1.1) catalyzes the first step in the glycolytic pathway which phosphorylates glucose into glucose-6-phosphate in the glycolytic pathway (3). The translocation of HK2 from the cytoplasm to the mitochondrial outer membrane is required for cellular proliferation (4, 5). Thus, mitochondria-bound HK2 could acquire the required ATP for glucose phosphorylation, which would accelerate the glycolytic rate and stimulate the consecutive TCA cycle in the mitochondria. Glutamine can also be an important energy source in cancer (6). Moreover, the majority of the HK2 in cancer cells is the mitochondria-bound type (7), and presents a target for cancer therapy (3). Mitochondria play a crucial role in the regulation of cell survival and cell death. Interactions between the voltage-dependent anion channel (VDAC) and the HK2 on the mitochondrial outer membrane not only increase mitochondrial energy metabolism, but also down-regulate the apoptotic pathway by suppressing cytochrome c release (8). Moreover, interactions between HK2, Bax and Bcl-2 anchored on the mitochondrial membrane also regulate apoptosis (9, 10).

The cellular oxygen tension regulates glycolysis and mitochondrial respiration, because hypoxia-induced factor 1 (HIF-1) increases the intracellular glucose concentration by increasing Glut1 activity (11), and the HK2 gene responds to hypoxia *via* hypoxia responsive element (2). In growing tumor cells oncogenic products stimulate HIF-1 and c-Myc, which in turn activate hypoxia/glucose responsive elements of the genes and increase glycolytic metabolism (12).

In terms of the genes involved in mitochondrial function, tumor suppressor p53 has a direct apoptogenic role in mitochondria. Translocation of p53 from the

Table I. *Expression frequencies of mitochondria-related gene products in gastric carcinoma by immunohistochemistry (n=257).*

Antibody	Retrieval methods	Dilution	Source	Positive expression (%)
HK2	None	1:100	Santa Cruz (Santa Cruz, CA)	43 (16.7)
p53	Microwave	1:100	DAKO (Carpinteria, CA)	91 (35.4)
Akt	Pressure cooker	1:50	Cell Signaling (Beverly, MA)	190 (73.9)
Bcl-2	Microwave	1:100	DAKO (Glostrup, Denmark)	31 (12.1)
PDH	Microwave	1:200	Molecular Probes (Leiden, Netherlands)	257 (100)
VDAC	None	1:2000	Molecular Probes (Leiden, Netherlands)	257 (100)
Complex II	Microwave	1:800	Molecular Probes (Leiden, Netherlands)	257 (100)

cytoplasm into mitochondria can launch apoptosis in cancer cells, by inducing the permeabilization of the mitochondrial outer membrane by forming a complex with Bcl-2 (13). It is also known that the serine/threonine kinase Akt/PKB increases the intracellular ATP level by relocating HK2 activity to the mitochondria to activate glycolysis and oxidative phosphorylation (OXPHOS) (9). It was hypothesized that HK2, pyruvate dehydrogenase (PDH), Akt, VDAC, complex II, p53 and Bcl-2, which directly or indirectly participated in the glycolytic pathway and in the mitochondrial respiration pathway, might be expressionally related, and that they could compose an expressional network in cancer cells. To characterize gastric cancer at the molecular level, the expressions of these mitochondria-related genes in human stomach cancer were evaluated.

Materials and Methods

Primary gastric cancer samples. A total of 257 consecutive cases of gastric carcinomas, surgically resected over a period of six months (January 1995 - June 1995), were collected from the files of the Department of Pathology, Seoul National University College of Medicine, Korea (14). The age, gender, tumor location, gross type according to Borrmann's classification, tumor size, lymphatic invasion and pTNM class were evaluated by reviewing medical charts and pathological records, and glass slides were reviewed to determine histological types according to the WHO (World Health Organization) and Lauren classifications. The average age of patients was 54.6 years. No patient had received pre-operative chemo- or radiotherapy, and 92.6% (238/257) of the patients had undergone curative resection (R0 according to the UICC guideline). Clinical outcome of the patients was followed from the date of surgery until either the date of death or December 1, 2000, which resulted in a follow-up period ranging from 1–72 months (mean: 50 months). Cases lost to follow-up and those ending in death from any cause other than gastric cancer were regarded as censored data during the survival analysis.

Six array blocks containing a total of 257 cases were prepared, as described previously (14). An adequate case was defined as one with a tumor occupying more than 10% of the core area. Each slide contains non-neoplastic gastric mucosa from body and antrum with/without intestinal metaplasia.

Immunohistochemistry and analysis. Immunohistochemical staining was performed using the streptavidin peroxidase procedure, after an antigen retrieval process. For statistical analysis the immunostaining result was considered to be positive if 10% or more of the neoplastic cells were stained by visual analysis of the immunohistochemical staining of paraffin sections from the tumor specimens using each commercial antibody (Table I).

The Chi-square test or Fisher's exact test (2-sided) was used to determine the correlation between immunohistochemical staining result and clinicopathological parameters. Survival curves were estimated using the Kaplan-Meier product-limit method, and the significance of differences between the survival curves was determined using the log-rank test. Results were considered to be statistically significant at *p*-values of less than 0.05. All statistical analyses were conducted using the SPSS 11.0 statistical software program (SPSS, Chicago, IL, USA).

Stomach cancer cell lines. The stomach cancer cell lines used for this study, SNU668 and SNU216, were obtained from the Korean Cell Line Bank, Seoul, Korea. Both cell lines were maintained and cultured in RPMI1640 medium (Life Technologies/GIBCO-BRL, Paisley, Scotland) supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin/penicillin (Gibco, Rockville, MD) in 5% CO_2, 95% air at 37˚C for normoxia, or in 0.2% O_2 and 5% CO_2 at 37˚C for hypoxia.

Growth inhibition of stomach cancer cells by mitochondrial respiratory inhibitors was determined using a WST-1™ assay kit (Roche, Mannheim, Germany). The assay was repeated at least in triplicate.

For immunocytochemistry, cancer cells were collected and cultured on an 8-well HTC glass slide™ (Cell-Line/Erie, Portsmouth, NH, USA) overnight. After each chemical treatment at the indicated concentration, they were cultured for 1 day. The cells were then washed with sterilized PBS, fixed in 0.5% paraformaldehyde for 30 min, washed with PBS several times, and the proteins were detected using specific primary antibodies.

RNA extraction and RT-PCR. Total RNA was extracted from cultured cells ($5x10^6$) using RNA isolation kits (RNeasy Mini Kit, Qiagen, Valencia, CA, USA). cDNA synthesized from 1 μg of total RNA with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) was subject to amplification according to a standard program for 30 cycles (30 sec at 94˚C for denaturation, 30 sec at 57˚C for annealing, 1 min at 72˚C for elongation) using PCR SuperMIX™ (Invitrogen, Carlsbad, CA, USA). The designed primers were as follows: HK2, forward, 5'-

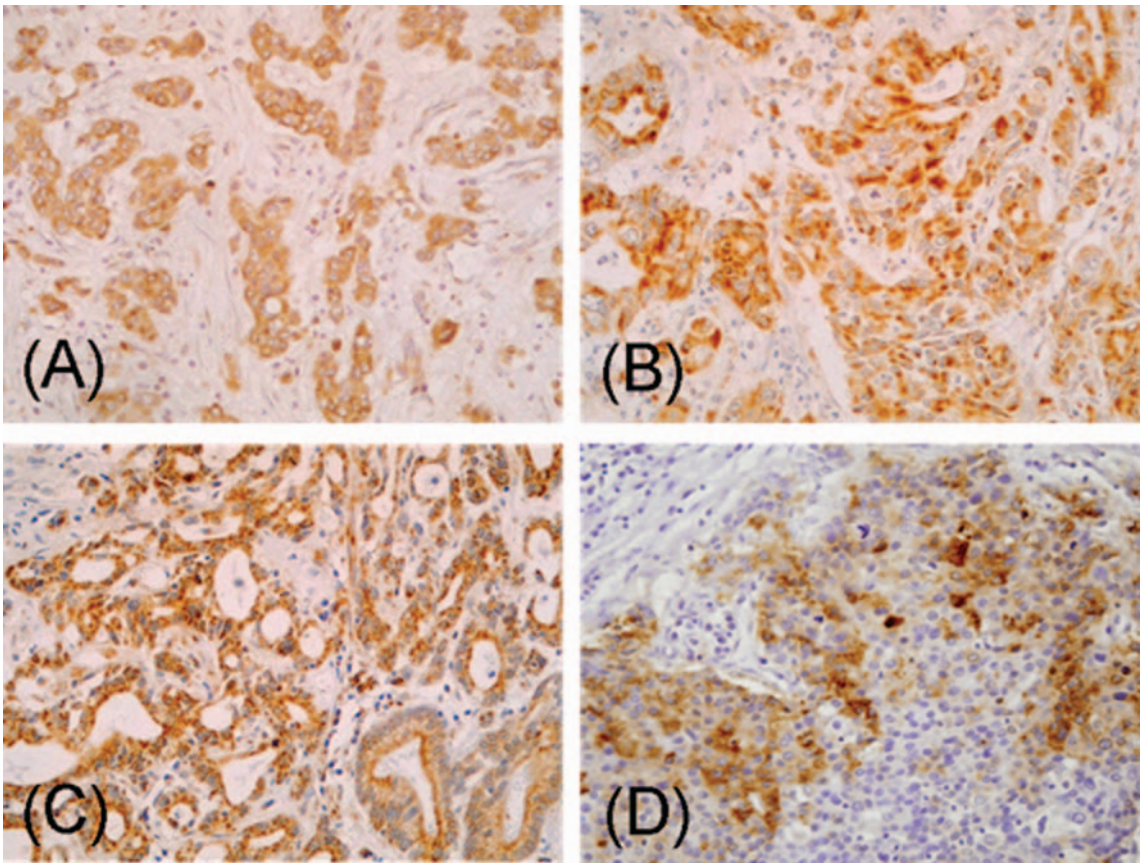

Figure 1. *Immunohistochemical staining for stomach cancer tissues. Stomach cancer tissue sections were stained with an indicated primary antibody. (A) VDAC, (B) complex II, and (C) PDH was uniformly expressed in all cases of stomach cancer tissue. (D) HK2 expression in stomach cancer cells in their cytoplasm (x200).*

ggtggaaggcgatgaggggcggatgtgtat-3', reverse, 5'-tatgtagacgcttggcaa aatgggggtgtt-3'; Bcl-2, forward, 5'-gaggagctcttcagggacgg-3', reverse, 5'-ccaggtgtgcaggtgccggt-3'; beta-actin, forward, 5'-gcccgggagctatgcat gggagtgatttcac-3', reverse, 5'-gcccgggtcagctcattctcaccttaatggc-3'.

Results

Gene expression in stomach cancer tissue. In order to investigate the expressions of HK2, PDH, complex II, Bcl-2, p53 and Akt, which are associated with mitochondrial function, 257 samples of the primary human stomach cancer tissues arrayed on glass slides were evaluated by immunohistochemistry.

On immunohistochemistry with HK2 antibody, normal gastric mucosa showed negative or weakly positive staining. Strong cytoplasmic staining of HK2 in cancer cells were noted in 16.7% (43/257) of the gastric carcinomas (Figure 1 and Table I). In our previous study, the positive rate of Bcl-2 was 12% (15). Most of the carcinomas with positive Bcl-2 expression were negative in HK2 expression except for one case ($p=0.032$), indicating a reciprocal expression pattern (Table II). The tumor suppressor gene product p53 was positive in 35.4% (91/257) of the stomach carcinoma. It was positive in 46.5% (20/43) of the HK2 overexpression cases, and 33.2% (71/214) of the HK2 negative cases, and no statistical correlation between HK2 and p53 was found.

Table II. *The relationship between hexokinase 2 positive gastric carcinoma and Bcl-2, p53 and Akt expression.*

	Number of Bcl-2 expression (%)	Number of p53 expression (%)	Number of Akt expression (%)
HK2 positive (n=43)	1 (2.3)	20 (46.5)	23 (53.5)
HK2 negative (n=214)	30 (14.0)	71 (33.2)	167 (78.0)
p-value	0.032	N.S	0.001

N.S., not significant.

Table III. *Clinicopathological characteristics of hexokinase 2 expression in stomach cancer.*

Characteristics	Total	HK2 expression		*p*-value
		Positive	Negative	
Age	257	52.7±14.7	55.0±12.8	N.S.
Gender				N.S.
Male	176	28	148	
Female	81	15	66	
WHO classification				0.037
Well-differentiated	22	5	17	
Moderately-differentiated	70	12	58	
Poorly-differentiated	108	24	84	
Mucinous	14	0	14	
Signet ring cell	43	2	41	
Depth of invasion				0.047
Early	67	6	61	
Advanced	190	37	153	
pT class (invasion depth)				N.S.
T1	67	6	61	
T2	132	22	110	
T3	55	14	41	
T4	3	1	2	
Lymph node metastasis				N.S.
Absent	85	9	76	
Present	172	34	138	
Distant metastasis				N.S.
Absent	244	40	204	
Present	13	3	10	
Lymphatic invasion				0.017
Absent	177	23	154	
Present	80	20	60	

N.S., not significant.

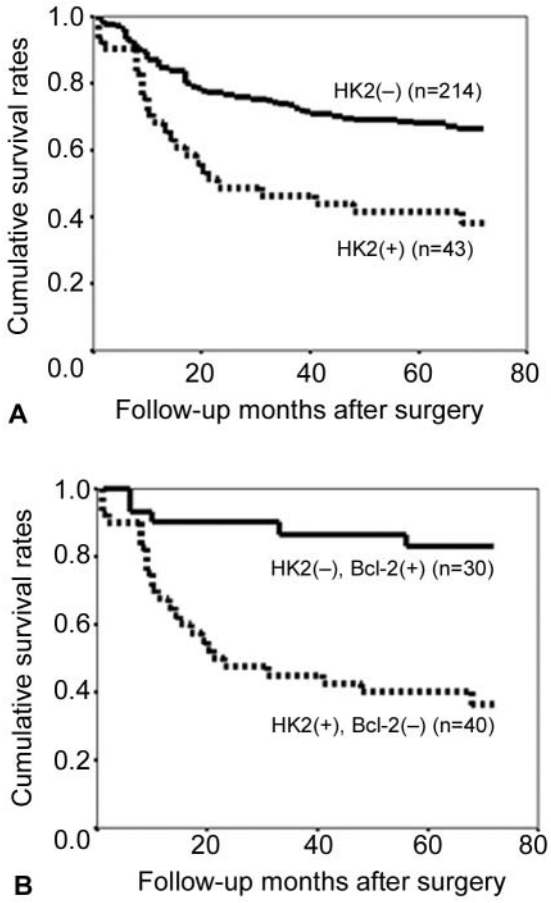

Figure 2. *Kaplan-Meier survival curves of stomach cancer patients. (A) Patients with HK2 overexpression showed significantly worse prognosis than patients without expression of HK2 (p=0.0002). (B) HK2(+)/Bcl-2(–) expression status was associated with the worst prognosis among four groups (p=0.0002).*

Expression of the Akt protein was found to be less frequent in the HK2 overexpression cases than in the HK2 negative cases with statistical significance ($p=0.001$). Mitochondria-located proteins including complex II, PDH and VDAC, were uniformly expressed in all 257 cases of stomach cancer, suggesting the requirement of intact mitochondrial respiratory function in cancer cells (Table I).

The advanced carcinomas more frequently showed positive HK2 expression ($p=0.047$) compared to early gastric cancer, as shown in Table III. The expression status of HK2 was associated with lymphatic invasion ($p=0.017$), but not with lymph node or distant metastasis.

Kaplan-Meier survival curves showed that the patients with HK2 overexpression had a worse prognosis than those with negative HK2 ($p=0.0002$) (Figure 2A). The worst prognosis was seen in patients with HK2-positive/Bcl-2-negative status compared to other groups ($p=0.0002$) (Figure 2B). By multivariate analysis, the HK2 expression showed some association with survival independently of pTNM (tumor-lymph node-metastasis) class in gastric carcinomas, but did not quite reach the statistical significance ($p=0.090$) (Table IV).

Table IV. *Multivariate logistic regression analysis for tumor stage and hexokinase 2 expression in stomach cancer.*

Parameter	Hazard ratio (95% CI)	*p*-value
HK2 expression		0.090
Positive *vs.* negative	1.468 (0.942-2.288)	
Pathological stage		<0.001
II *vs.* I	4.575 (2.139-9.788)	
III *vs.* I	10.865 (5.411-21.813)	
IV *vs.* I	25.270 (12.459-51.255)	

HK2 and Bcl-2 expression in stomach cancer cells. Semi-quantitative RT-PCR was performed to investigate the

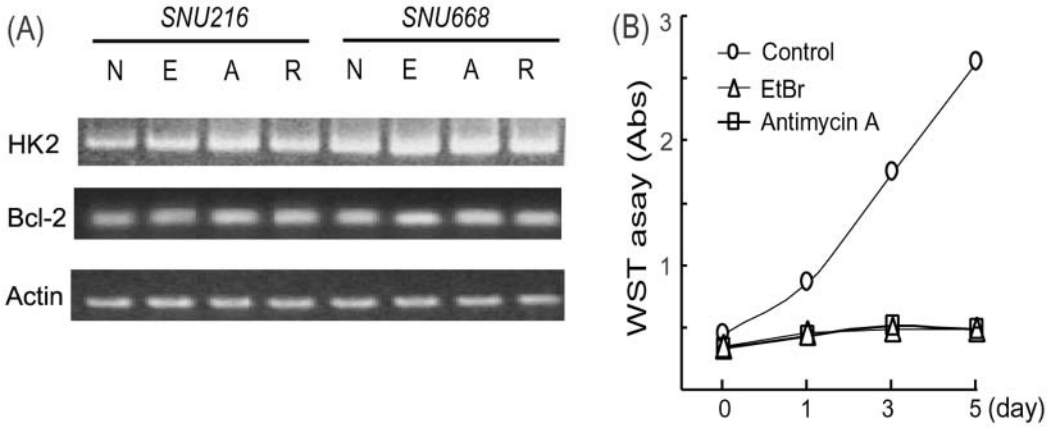

Figure 3. *Mitochondrial respiratory inhibitor does not control HK2 or Bcl-2 transcriptions. A) HK2 and Bcl-2 transcripts were amplified by RT-PCR. Each mitochondrial inhibitor was added to the growing cells for 24 h, and the cells were harvested for total RNA extraction. N, no addition; E, ethidium bromide (1 μg/ml); A, antimycin A (1 μg/ml); R, rotenone (500 nM). B) Growth suppression of the SNU668 stomach cancer cell line in mitochondrial respiratory deficient conditions by the addition of ethidium bromide (1 μg/ml), or Antimycin A (1 μg/ml) was determined by WST assay as indicated.*

effect of the mitochondrial respiratory state on *HK2* and *Bcl-2* transcriptional expression. The addition of mitochondrial respiration inhibitors, such as ethidium bromide (1 μg/ml), antimycin A (1 μg/ml), or rotenone (500 nM) did not alter the level of *HK2* transcription (Figure 3A), even though the growth of the cells was severely repressed by these inhibitors (Figure 3B). Addition of the above mitochondrial inhibitors did not significantly alter the *Bcl-2* expression level .

The hypoxic state up-regulates the *HK2* transcription (538 bp) in both SNU216 and SNU668 stomach cancer cells as well as in non-cancerous 293 cells (Figure 4). On the other hand, the level of *Bcl-2* mRNA was significantly down-regulated by hypoxia.

Immunocytochemistry of stomach cancer cells (Figure 5) showed that HK2 products were highly expressed under normal condition, visualized as dark cytoplasmic staining. However, the addition of mitochondrial inhibitors including antimycin A, ethidium bromide, or rotenone severely obstructed cell growth and produced stained protein clumps in the cytoplasm. Bcl-2 products were localized throughout the cytoplasm under normal condition, but with mitochondrial inhibitors, Bcl-2 products were also improperly localized in the shrunken cellular morphology.

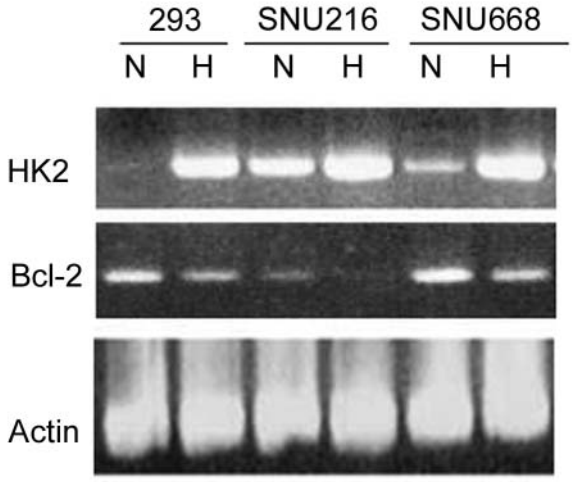

Figure 4. *Hypoxia regulates HK2 and Bcl-2 transcriptions in stomach cancer cell lines. RT-PCR was carried out for HK2 and Bcl-2 transcripts. Hypoxic condition up-regulated the HK2 transcription in both SNU216 and SNU668 stomach cancer cells, as well as in non-cancerous 293 cells. On the other hand, Bcl-2 was significantly down-regulated by hypoxia. Actin was used for loading control. N, normoxic condition; H, hypoxic condition.*

Discussion

The continuous consumption of energy for immortal cell proliferation requires the activation of glycolysis and mitochondrial function to generate ATP by the use of any available carbon sources (16). Growing cancer cells in the tumor mass are under relatively hypoxic condition. Therefore, they induce more fermentative energy production by glycolysis (17). It was found that HK2, which functions within the metabolic pathway, and Bcl-2 which participates in apoptosis were inversely expressed in stomach cancer. Under hypoxic conditions *HK2* levels were increased whereas *Bcl-2* was decreased, although their expressional levels were not found to be dependent on mitochondrial dysfunction. Moreover, damage to the mitochondrial

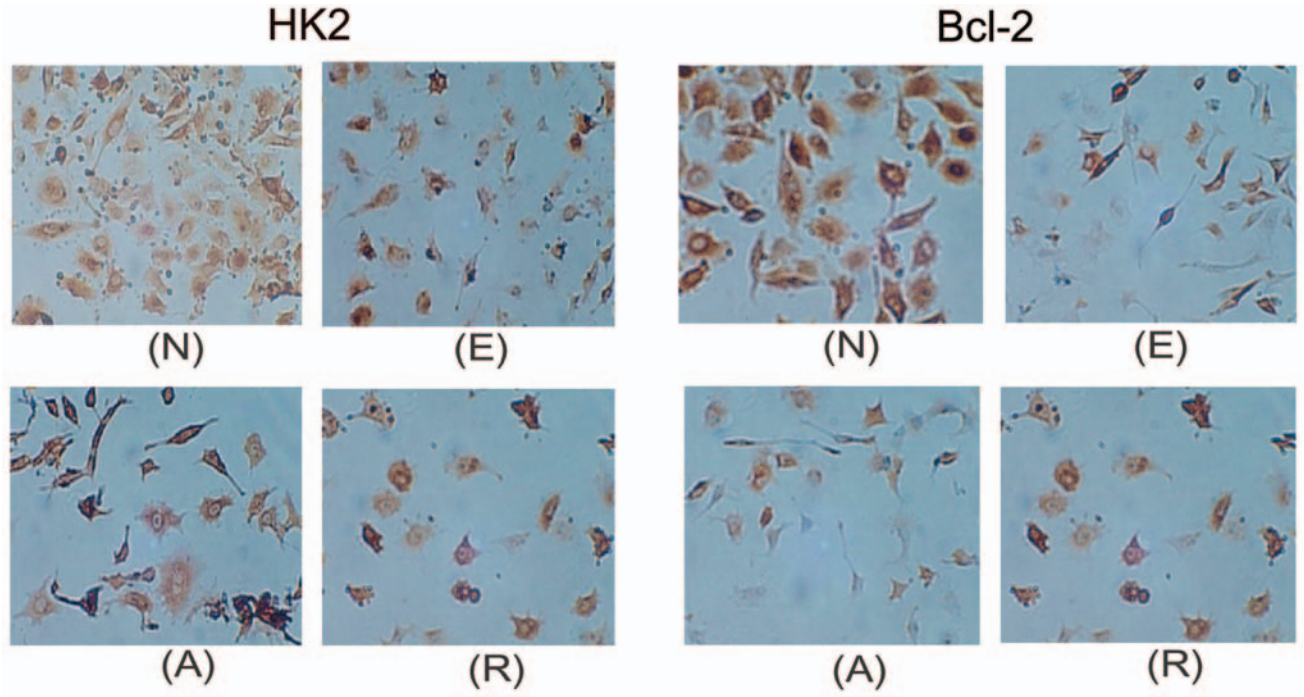

Figure 5. *Immunocytochemistry of a stomach cancer cell line in mitochondrial respiratory deficient condition. The SNU668 stomach cancer cell line was treated with (N) no treatment, (E) ethidium bromide (1 μg/ml), (A) antimycin A (1 μg/ml), or (R) rotenone (500 mM), for 1 day, and the cells were subjected to immunocytochemistry with HK2 or Bcl-2 antibody (x400). The cells treated with mitochondrial respiratory inhibitors were reduced in number but the staining intensity was not changed considering their shrunken cytoplasm.*

respiratory pathway by ethidium bromide, antimycin A, or rotenone did not inhibit *HK2* or *Bcl-2* transcription. The growth of stomach cancer cell lines was severely suppressed by mitochondrial respiration inhibitors, probably due to the disruption of cellular energy homeostasis caused by lost ATP production (5). Thus, mitochondrial function and nucleo-mitochondrial interactions might not be related to HK2 or Bcl-2 expression in stomach cancer cell lines. Under hypoxic conditions, transcription factor HIF-1 binds to the upstream region of HK2 to activate gene expression, but HIF-1 activation does not require functional mitochondria (18).

Mitochondria are critical energy producing units and functionally comprised of complex I (NADH, ubiquinone oxidoreductase), complex III (cytochrome c oxidoreductase, cytochrome Bcl-complex), complex IV (cytochrome c oxidase), complex V (ATP synthase), and complex II (succinate oxidoreductase). Mitochondrial damage by ethidium bromide (a mitochondrial DNA mutagen), antimycin A (a complex III inhibitor), or rotenone (a complex I inhibitor) severely inhibits ATP production (19). Moreover, glucose limitation induces the loss of mitochondrial membrane potential, and subsequently induces the release of cytochrome c into the cytosol to activate apoptotic caspase. However, an active glycolytic metabolism may down-regulate cellular apoptosis, because exogenously expressed HK2 exerts an anti-apoptotic effect as growth factors do (20), and because the overexpression of Glut1 also markedly delays apoptosis (21). Mitochondrial homeostasis is critical for the maintenance of the anabolic pathway required for normal cellular growth (22). Moreover, cancer cell growth depends on mitochondria, which regulate the apoptotic pathway and electron transit chain (19).

The expression of PDH in the mitochondria supports the higher energy state in stomach cancer. In addition, VDAC is a critical component of the mitochondria-dependent apoptotic pathway, because its interaction with the Bcl-2 family controls the rate of cytochrome c release for further progress of apoptosis (23). The ubiquitous expressions of PDH, complex II and VDAC in stomach cancer tissue emphasize the importance of their role in the conservation of mitochondrial integrity. Akt also maintains mitochondrial integrity by promoting physical and functional interactions between mitochondria and HK2, to inhibit cytochrome c release and apoptosis (9). However, the expressional frequencies of HK2 (16.7%), Bcl-2 (12.1%) and Akt (73.9%) in stomach cancer illustrate reduced association with gene expression regulation.

Tumor suppressor p53 inhibits Bcl-2, and their functional antagonism occurs at both the transcriptional and post-

translational levels. Following cytochrome c release, p53 which is located in the mitochondria induces permeabilization of the mitochondrial outer membrane by forming complexes with Bcl-2. p53 function on the mitochondria is sufficient to launch apoptosis in cancer cells as a primary anti-neoplastic activity (24). However, in the present study, no correlation between Bcl-2 and p53 expression was found.

HK2 is often highly-expressed in poorly-differentiated and rapidly growing tumors that exhibit a high rate of aerobic glycolysis. In addition, HK2 inhibits apoptosis by binding to VDAC and suppressing the release of intermembrane space proteins of the mitochondria (25). In this study, VDAC was constantly expressed in gastric cancers and HK2 was overexpressed in 16.7% of them. HK2 overexpression was associated with poorly-differentiated adenocarcinoma ($p=0.037$) and advanced stage ($p=0.047$). The patients with gastric cancers showing HK2 overexpression showed significantly worse prognosis than the patients with negative HK2 ($p=0.0002$). Furthermore, a trend was shown for HK2 expression as an independent prognostic marker ($p=0.090$) by multivariate analysis.

The overexpression of Bcl-2 restores cellular growth at very low levels of cellular ATP, and also protects cells from the hypoxic injury associated with a decrease in mitochondrial membrane potential reduction (26). The introduction of Bcl-2 into most eukaryotic cells protects them from a wide variety of stresses that causes apoptosis (27), and Bcl-2 expression might affect survival in cancer patients (28). However, Bcl-2 expression in tumors is not consistently correlated with a poorer outcome, or resistance to anticancer therapies (29). Although Bcl-2 controls apoptosis, Bcl-2 modulation does not affect aggressive cancers, which do not require Bcl-2-mediated antiapoptotic machinery. As with stomach cancer, Bcl-2 expression is low in highly advanced metastatic prostate and breast cancers (30).

Though HK2, p53, Akt, HK2, Bcl-2, PDH, VDAC and complex II seem to be independently expressed in stomach cancer, a reciprocal relationship between HK2 and Bcl-2 was found, and the Bcl-2/HK2 ratio could affect the prognosis of stomach cancer patients. Moreover, enhanced energy production by HK2 overexpression could promote tumor growth in the presence of intact mitochondria especially in those tumors lacking Bcl-2 expression.

In this study, it was found that the HK2 was expressed in a small fraction of gastric carcinomas, and those carcinomas showed worse prognosis. Furthermore, the Bcl-2/HK2 expression status could predict the patients' outcome. Further study is needed to determine whether HK2 can be useful as a predictive marker for therapy or as a direct therapeutic target.

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