Network analysis of genes involved in the enhancement of hyperthermia sensitivity by the knockdown of BAG3 in human oral squamous cell carcinoma cells

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Abstract. BCL2-associated athanogene 3 (BAG3), a co-chaperone of the heat shock 70 kDa protein (HSPA) family of proteins, is a cytoprotective protein that acts against various stresses, including heat stress. The aim of the present study was to identify gene networks involved in the enhancement of hyperthermia (HT) sensitivity by the knockdown (KD) of BAG3 in human oral squamous cell carcinoma (OSCC) cells. Although a marked elevation in the protein expression of BAG3 was detected in human the OSCC HSC-3 cells exposed to HT at 44˚C for 90 min, its expression was almost completely suppressed in the cells transfected with small interfering RNA against BAG3 (siBAG) under normal and HT conditions. The silencing of BAG3 also enhanced the cell death that was increased in the HSC-3 cells by exposure to HT. Global gene expression analysis revealed many genes that were differentially expressed by >2-fold in the cells exposed to HT and transfected with siBAG. Moreover, Ingenuity® pathways analysis demonstrated two unique gene networks, designated as Pro-cell death and Anti-cell death, which were obtained from upregulated genes and were mainly associated with the biological functions of induction and the prevention of cell death, respectively. Of note, the expression levels of genes in the Pro-cell death and Anti-cell death gene networks were significantly elevated and reduced in the HT + BAG3-KD group compared to those in the HT control group, respectively. These results provide further insight into the molecular mechanisms involved in the enhancement of HT sensitivity by the silencing of BAG3 in human OSCC cells.

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Abbreviations: AIM2, absent in melanoma 2; ATF2, activating transcription factor 2; BAG, BCL2-associated athanogene; BNIP3, BCL2/adenovirus E1B 19 kDa interacting protein 3; bZIP, basic-region leucine zipper; CEBPB, CCAAT/enhancer binding protein β; CLU, clusterin; DDIT3, DNA damage inducible transcript 3; DDX58, DEAD box polypeptide 58; DNAJ, DnaJ (Hsp40) homolog; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCH1, GTP cyclohydrolase 1; GSK3B, glycogen synthase kinase 3β; HSPA, heat shock 70 kDa protein; HSPB, heat shock 27 kDa protein; HSPD, heat shock 60 kDa protein; HSPH, heat shock 105 kDa/110 kDa protein; HSPs, heat shock proteins; HT, hyperthermia; ISG15, ISG15 ubiquitin-like modifier; KD, knockdown; MAP1LC3B, microtubule-associated protein 1 light chain 3 beta; MAPK12, mitogen-activated protein kinase 12; OSCC, oral squamous cell carcinoma; qPCR, quantitative polymerase chain reaction; RPS6KA2, ribosomal protein S6 kinase, 90 kDa, polypeptide 2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; SMAD2, SMAD family member 2; SQSTM1, sequestosome 1

Key words: hyperthermia, BCL2-associated athanogene 3 knockdown, microarray, gene network, human oral squamous cell carcinoma cell

Introduction

Hyperthermia (HT) therapy in combination with either chemotherapy, radiotherapy or both are used for patients with cancer in various organs. The anticancer effects of these combination therapies have been verified in many clinical trials (1-4). However, the acquisition of thermotolerance in cancer cells, which is at least partly due to an increase in the levels of heat shock proteins (HSPs), attenuates the therapeutic effects of HT (5,6). HSPs function as molecular chaperones, and their epxression is induced by various stresses, particularly heat. Moreover, it has been recognized that these proteins exert potent cytoprotective effects, which prevent cell death (7,8). HSPs consist of several family members, including DnaJ (Hsp40 homolog (DNAJ), heat shock 70 kDa protein (HSPA), heat shock 27 kDa protein (HSPB), heat shock 60 kDa protein (HSPD) and heat shock 105 kDa/110 kDa protein (HSPH), and among these, HSPA1A plays a major role as a molecular chaperone (9,10).

BCL2-associated athanogene (BAG) family proteins, an ubiquitous family of chaperone regulators, have been found to be associated with the anti-apoptotic protein, BCL2, and also to interact with HSPA proteins, such as HSPA1A and

HSPA8 (11,12). Among the BAG proteins, the expression of BAG3 has been reported to be regulated, at least in part, by the activation of heat shock transcription factor 1 as in the cases of HSPs (13,14). Under normal conditions, the expression level of BAG3 is relatively low, whereas a significant elevation in its protein level is observed in cells exposed to stressors, such as heavy metals (15), heat (16-18), oxidative stress (19) and ultrasound (20). It has also been indicated that BAG3 is abundantly expressed in a variety of cancers, and is involved in cellular processes such as cell growth and cell death (11,12,16,21-24). Liu *et al* (25) previously reported that silencing the BAG3 gene sensitizes leukemic cells to compound-induced cell injury. Recently, we clearly demonstrated that the inhibition of BAG3 improves cell death sensitivity to HT in cancer cells (17,18). However, the detailed molecular mechanisms underling the enhancement of HT sensitivity by BAG3 knockdown (KD) in cancer cells have not yet been elucidated.

In the present study, we examined gene expression patterns in human oral squamous cell carcinoma (OSCC) HSC-3 cells exposed to HT and transfected with small interfering RNA (siRNA) against BAG3 using a global-scale microarray system. In addition, gene network analysis of differentially expressed genes was performed using computational gene expression analysis tools.

Materials and methods

Cell culture and exposure to HT. Human OSCC HSC-3 cells were obtained from the Human Science Research Resources Bank, Japan Health Sciences Foundation (Tokyo, Japan). The HSC-3 cells were cultured in E-MEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) at 37˚C in humidified air with 5% CO_2 and 95% air. Exposure to HT was were performed by immersing plastic culture vessels containing the attached cells in a water bath at 44˚C for 90 min. Following exposure to HT, the cells were incubated for 6-24 h at 37˚C, as previously described (26).

siRNA transfection. A siRNA (siBAG; GGUGGAUUCUAAA CCUGUU) targeting BAG3 for BAG3-KD was designed by Nippon EGT Co., Ltd. (Toyama, Japan). Luciferase siRNA (siLuc; CGUACGCGGAAUACUUCGA) was used as a negative control siRNA. The cells were incubated in Opti-MEM® I Reduced Serum Medium containing 20 nM siRNA and Lipofectamine™ RNAiMAX (both from Life Technologies Japan, Ltd., Tokyo, Japan) at 37˚C. Six hours following transfection, the medium was exchanged for E-MEM supplemented with 10% FBS, and the cells were then maintained at 37˚C for 42 h, as previously described (18).

Measurements of cell growth and cell death. The number of cells was counted using a hematocytometer. When the cell death was evaluated, the cells were treated with 0.2% trypan blue solution (NanoEnTek Inc., Seoul, Korea) at room temperature for 5 min. The number of dead cells (stained) and viable cells (unstained) was counted using an EVE™ automatic cell counter (NanoEnTek Inc.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. The cells were dissolved in lysis buffer (150 mM NaCl, 1% Nonidet P-40 and 50 mM Tris-HCl, pH 8.0) containing a protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan). SDS-PAGE and western blot analysis were carried out as previously described (27,28). The primary antibodies used were as follows: a rabbit monoclonal anti-BAG3 antibody (GTX62327; GeneTex Inc., Irvine, CA, USA) and a mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (MAB374; Millipore Co., Temecula, CA, USA). Immunoreactive proteins were visualized using a luminescent image analyzer (LAS-4000 mini; GE Healthcare, Tokyo, Japan) using an enhanced chemiluminescence detection system. GAPDH served as a loading control.

RNA isolation. Total RNA was extracted from cells using a NucleoSpin® RNA isolation kit (Macherey-Nagel GmbH & Co., Düren, Germany) along with on-column DNase I treatment. The RNA quality was analyzed using a Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA). RNA samples with RNA integrity number (RIN) values >9.5 were considered acceptable.

Quantitative (real-time) polymerase chain reaction (qPCR). qPCR was performed on a Real-Time PCR system Mx3005P (Agilent Technologies, Inc.) using SYBR® Premix Ex Taq™ II (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. Reverse transcriptase reaction was carried out with total RNA using a random 6 mers and an oligo dT primer (PrimeScript RT reagent kit; Takara Bio, Inc.). The reaction was carried out using the specific primers: human BAG3 forward and reverse, CGACCAGGCTACATTCCCAT and TCTGGCT GAGTGGTTTCTGG, respectively; human GAPDH forward and reverse, AAGGCTGGGGCTCATTTGCA and ATGACC TTGCCCACAGCCTT, respectively. The temperature cycling conditions for each primer consisted of 10 min at 95˚C followed by 40 cycles for 10 sec at 95˚C and 40 sec at 60˚C. The mRNA expression level of BAG3 was normalized with respect to the mRNA expression level of GAPDH, as described in a previous study of ours (18).

Microarray gene expression analysis. Microarray gene expression analysis was performed using a GeneChip® system with a Human Genome U133-plus 2.0 array, which was spotted with 54,675 probe sets (Affymetrix, Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. In brief, 500 ng of total RNA was used to synthesize cRNA with a GeneChip® 3' IVT Express kit (Affymetrix, Inc.). Fragmentated biotin-labeled cRNA was hybridized to the array at 45˚C for 16 h. After the staining with streptavidin-phycoerythrin, the array was scanned using a probe array scanner. The obtained hybridization intensity data were analyzed using GeneSpring® GX software (Agilent Technologies, Inc.) to extract the significant genes. To examine gene ontology, including biological processes, cellular components, molecular functions and gene networks, the obtained data were analyzed using Ingenuity® pathway analysis tools (Ingenuity Systems, Inc., Mountain View, CA, USA), as previously described (29,30).

Statistical analysis. Data are shown as the means ± SD. The Student's t-test was used for statistical analysis and

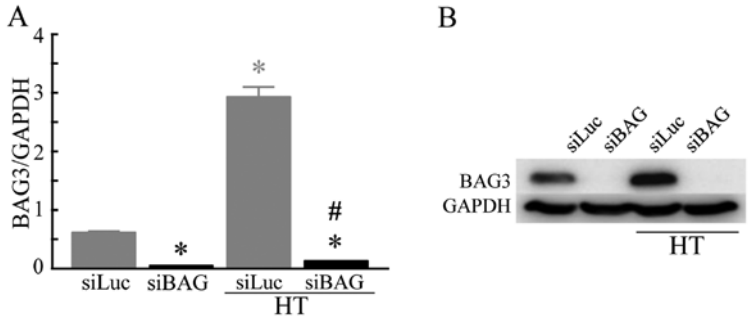

Figure 1. Effects of BCL2-associated athanogene 3 (BAG3)-knockdown (KD) on the mRNA and protein expression levels of BAG3 in HSC-3 cells exposed to hyperthermia (HT). HSC-3 cells transfected with luciferase siRNA (siLuc) or BAG3 siRNA (siBAG) were incubated at 37˚C or 44˚C for 90 min (HT exposure). Six hours later, the cells were harvested. (A) qPCR was performed with specific primers for BAG3 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The mRNA level of BAG3 was normalized to the expression level of GAPDH. Data are presented as the means ± SD (n=4); *P<0.05 vs. the control (siLuc transfection only); #P<0.05 vs. the HT-exposed group. (B) Western blot analysis was performed using specific primary antibodies for BAG3 and GAPDH. GAPDH served as a loading control.

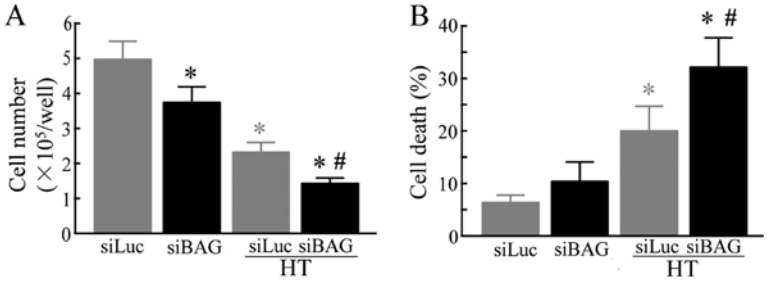

Figure 2. Effects of BCL2-associated athanogene 3 (BAG3)-knockdown (KD) on the growth and cell of HSC-3 cells exposed to hyperthermia (HT). HSC-3 cells transfected with luciferase siRNA (siLuc) or BAG3 siRNA (siBAG) were incubated at 37˚C or 44˚C for 90 min (HT exposure). (A) The cell number and (B) cell death were evaluated 24 h after HT exposure. Data are presented as the means ± SD (n=4); *P<0.05 vs. the control (siLuc transfection only); #P<0.05 vs. the HT-exposed group.

P-values <0.05 were considered to indicate statistically significant differences.

Results

Effects of BAG3-KD on the growth and death of HSC-3 cells exposed to HT. Although the mRNA expression level of BAG3 was relatively low in the HSC-3 cells transfected with siLuc (control), in the cells subjected to both siLuc transfection and HT exposure at 44˚C (HT control), a significantly increased expression level of BAG3 was observed. A significant decrease in the mRNA expression level of BAG3 was detected in the cells transfected with siBAG under both the control (siLuc) and HT conditions (Fig. 1A). The results of western blot analysis clearly demonstrated that the protein expression level of BAG3 was significantly increased in the cells exposed to HT. Transfection of the cells with siBAG almost completely inhibited the protein expression level of BAG3 under either condition (Fig. 1B). We then evaluated whether BAG3-KD affected the growth and death of HSC-3 cells exposed to HT. At the normal temperature, transfection of the cells with siBAG significantly suppressed the cell number compared to the control group. HT markedly decreased cell growth, and a further decrease in the number of cells was observed in the cells subjected to both siBAG transfection and exposure to HT to those exposed to HT alone (Fig. 2A). HT significantly enhanced cell death. Moreover, a significant increase in cell death was observed in the cells subjected to both siBAG transfection and exposure to HT compared to those exposed to HT alone. These results indicate that the silencing of BAG3 enhances the sensitivity of human OSCC HSC-3 cells to HT (Fig. 2B).

Global gene expression analysis. To identify genes involved in the enhancement of HT sensitivity by BAG3-KD, global-scale gene expression analysis was carried out using a GeneChip® system with a Human Genome U133-plus 2.0 array, which was spotted with 54,675 probe sets. Complete lists of probe sets from all samples are available on the Gene Expression Omnibus, a public database (accession number, GSE75127). GeneSpring software was used to analyze gene expression in the HSC-3 cells subjected to both HT exposure and siLuc (HT control) or siBAG transfection (HT + BAG3-KD), and revealed that many genes were differentially regulated by a factor of ≥2.0. The Venn diagram in Fig. 3 summarizes the numbers of specifically and commonly expressed genes in each group. The total numbers of genes that were found to be differentially expressed were 913 (331 up- and 582 downregulated genes) and 1,892 (679 up- and 1,213 downregulated genes) in the HT control and HT + BAG3-KD groups, respectively. The numbers of commonly up- and downregulated genes were 204 and 303, respectively (Fig. 3A and B).

Identification of biological functions and gene networks. In order to identify the biological functions and gene networks in differentially expressed genes involved in the enhancement of HT sensitivity by BAG3-KD, functional category and gene

Table I. Top three biological functions in differentially expressed genes.

Name	P-value	Number of molecules
Upregulated		
HT control only (75)[a]		
Cell growth and proliferation	5.02E-05-4.19E-02	36
Post-translational modification	1.20E-04-3.51E-02	6
Protein folding	1.20E-04–2.12E-02	4
HT + BAG3-KD only (263)[a]		
Cell growth and proliferation	1.47E-05-2.73E-02	123
Cell death and survival	3.35E-05-2.73E-02	121
Cellular development	1.93E-04-2.73E-02	92
Commonly regulated (133)[a]		
Cell death and survival	2.03E-17-2.70E-03	82
Cell growth and proliferation	4.39E-14-2.70E-03	84
Cell cycle	1.26E-12-2.70E-03	39
Downregulated		
HT control only (62)[a]		
Cell cycle	7.27E-04-4.99E-02	17
Gene expression	3.52E-03-4.63E-02	6
Protein synthesis	4.88E-04-1.57E-02	3
HT + BAG3-KD only (432)[a]		
Cellular development	2.20E-06-2.64E-02	123
Cell growth and proliferation	2.20E-06-2.70E-02	121
Cell death and survival	1.28E-05-2.70E-02	92
Commonly regulated (108)[a]		
RNA post-transcriptional modification	4.19E-05-3.58E-02	9
Cell cycle	2.90E-04-4.74E-02	32
Cell morphology	2.90E-04-3.58E-02	26

[a]Numbers of functionally annotated genes. HT, hyperthermia; BAG3, BCL2-associated athanogene 3; KD, knockdown.

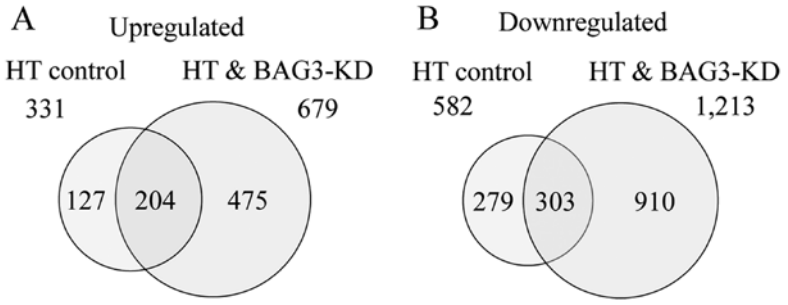

Figure 3. Venn diagram of genes that were differentially expressed in BCL2-associated athanogene 3 (BAG3)-knockdown (KD) HSC-3 cells under hyperthermia (HT) exposure conditions. HSC-3 cells transfected with luciferase siRNA (siLuc) or BAG3 siRNA (siBAG) were incubated at 37˚C or 44˚C for 90 min (HT exposure). Six hours following exposure to HT, total RNA was extracted. Gene expression analysis was carried out using a GeneChip microarray system and GeneSpring software. (A) The numbers of upregulated and (B) downregulated genes are shown. HT control indicates cells subjected to both HT exposure and transfection with siLuc; HT + BAG3-KD indicates both exposure to HT and transfection with siBAG.

network analyses were conducted by use of the Ingenuity Pathways Knowledge Base. We identified many functionally annotated genes, and the top 3 biological functions in each group are summarized in Table I. In the upregulated genes, biological functions including cell death and survival, and/or cell growth and proliferation were observed in all 3 groups: i) the HT control only; ii) the HT + BAG3-KD only; and iii) the commonly regulated groups. On the other hand, these 2 biological functions were observed only in the downregulated genes of the HT + BAG3-KD only group. In addition, we identified 2 unique gene networks, and these are designated as Pro-cell death and Anti-cell death, that were obtained from the upregulated genes (Fig. 4). The Pro-cell death gene network included several transcription factors, such as activating transcription factor 2 (ATF2), CCAAT/enhancer binding protein β (CEBPB), DNA damage inducible tran-

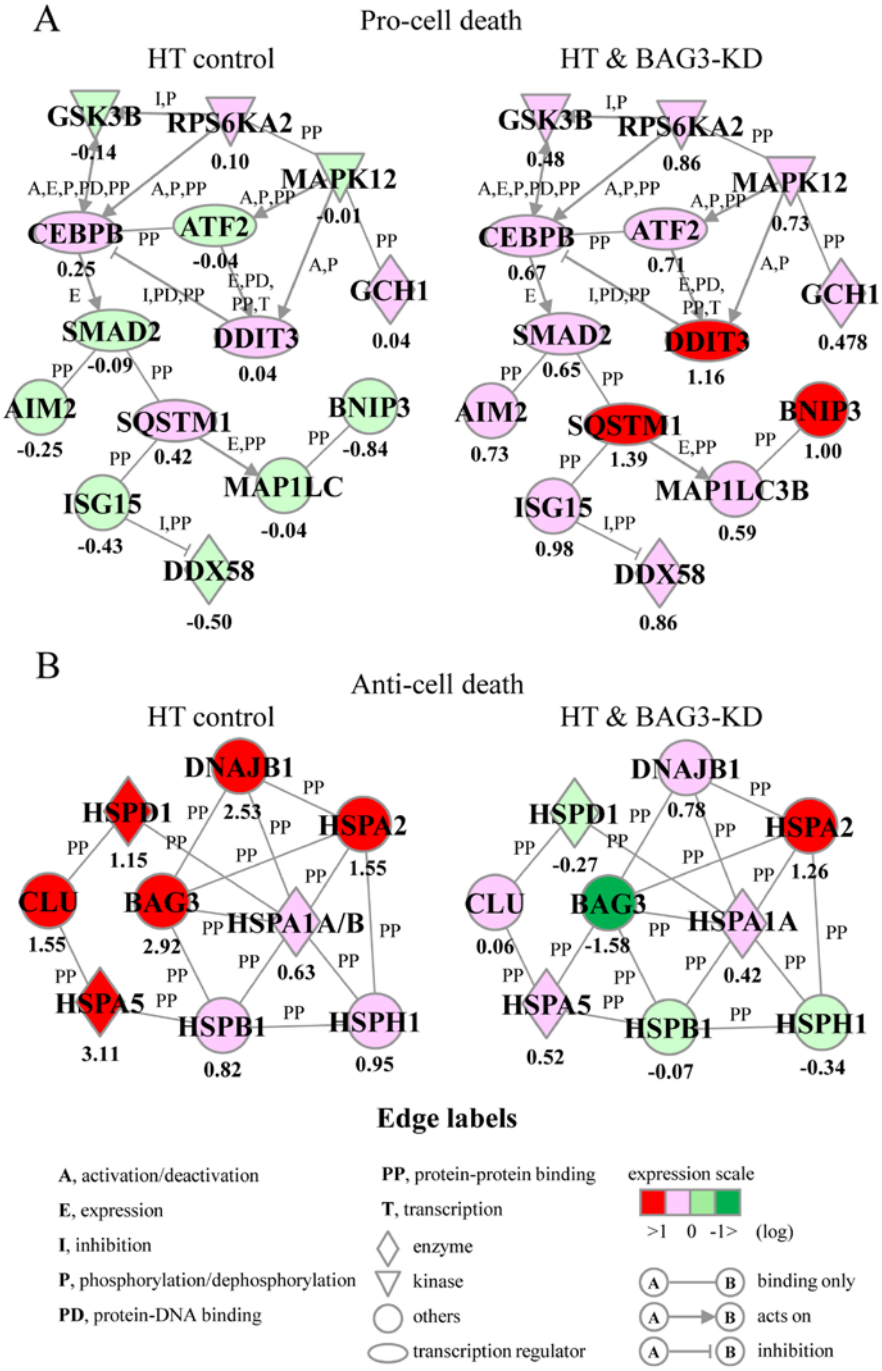

Figure 4. Gene networks, (A) Pro-cell death and (B) Anti-cell death. Upregulated genes in HSC-3 cells subjected to both hyperthermia (HT) and transfection with small interfering RNA against BAG3 (siBAG) were analyzed using the Ingenuity pathway analysis tools. Logarithmic scales of the expression levels of genes are shown. The network is represented graphically with nodes (genes) and edges (the biological associations between the nodes). HT control indicates exposure to HT and transfection with luciferase siRNA; HT + BAG3-KD indicates exposure to HT and transfection with siBAG.

script 3 (DDIT3), SMAD family member 2 (SMAD2) and sequestosome 1 (SQSTM1), as well as BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), and was associated with the biological function of the induction of cell death (Fig. 4A). The Anti-cell death gene network contained several HSPs, such as DNAJB1, HSPA1A, HSPA5, HSPB1, HSPD1, and HSPH1, as well as BAG3 and clusterin (CLU), and was associated with the biological function of the prevention of cell death (Fig. 4B). The expression levels of genes in the Pro-cell death and Anti-cell death gene networks were significantly elevated and reduced in the HT + BAG3-KD group compared to those in the HT control group, respectively (Fig. 4A and B). As expected, the mRNA expression level of BAG3 was markedly decreased in the HT + BAG3-KD group as detected by the microarray system (Fig. 4B).

Discussion

BAG3, a co-chaperone of the HSPA family of proteins, is well known as a cytoprotective protein that acts against various stresses, including heat stress (11,12,16,21-25). In the present study, the almost complete silencing of BAG3 significantly

enhanced sensitivity of human OSCC HSC-3 cells to HT. This finding is compatible with those of our previous studies (17,18). In addition, using global-scale microarray and bioinformatics analyses, we herein identified genes and gene networks involved in the enhancement of HT sensitivity in BAG3-KD OSCC cells.

Our functional category analysis demonstrated that biological functions including cell death and survival, and cell growth and proliferation were observed in the upregulated genes in the cells from the HT + BAG3-KD group (Table I). Of note, we also successfully identified 2 unique gene networks, designated as Pro-cell death and Anti-cell death (Fig. 4). The Pro-cell death gene network consisted of 14 genes and was principally associated with the biological function of the induction of cell death. A marked induction of genes in this network was observed in the HT + BAG3-KD group compared to the HT control group (Fig. 4A). This network included 3 basic-region leucine zipper (bZIP) transcription factors, ATF2 (31), CEBPB (32) and DDIT3 (33), which have been reported to induce cell death. Homo- or hetero-dimeric protein complexes of the bZIP protein function as repressors and activators of transcription (34); associations have been identified between DDIT3 and both ATF2 and CEBPB (34-36). The activation of these bZIP transcription factors has also been reported to be regulated by kinases, such as glycogen synthase kinase 3β (GSK3β) (37), ribosomal protein S6 kinase, 90 kDa, polypeptide 2 (RPS6KA2) (38) and mitogen-activated protein kinase 12 (MAPK12) (39). Moreover, absent in melanoma 2 (AIM2) (40), BNIP3 (41), DEAD box polypeptide 58 (DDX58) (42), GTP cyclohydrolase 1 (GCH1) (43), ISG15 ubiquitin-like modifier (ISG15) (44), microtubule-associated protein 1 light chain 3 beta (MAP1LC3B) (45), SMAD2 (46), and SQSTM1 (47) have been reported to exert cell-damaging effects.

On the other hand, the expression levels of genes in the Anti-cell death gene network were significantly decreased in the HT + BAG3-KD group compared to those in the HT control group (Fig. 4B). This gene network consisted of 9 chaperone genes, 7 HSPs, CLU and BAG3. It is well known that HSPs protect cells both by protein chaperoning and refolding and by directly interfering with the cell death pathway (7,8). HSPs such as DNAJB1 (48), HSPA1A (48,49), HSPA2 (50), HSPA5 (51), HSPB1 (52), HSPD1 (49) and HSPH1 (53) were found to be associated with the prevention of cell death. Of note, BAG3 silencing markedly decreased the expression levels of CLU, DNAJB1, HSPA5, HSPB1, HSPD1 and HSPH1 in HSC-3 cells induced by HT exposure (Fig. 4B). CLU is a secreted or cytosolic chaperone that is expressed under certain stress conditions such as heat shock (54), and secretory human CLU has been reported to decrease the rate of cell death of human breast cancer cells (55). In addition, protein-protein interactions have been reported between BAG3 and DNAJB1 (12), HSPA5 (56) and HSPB1 (12) under *in vitro* experimental conditions.

Taken together, our results suggest that an increase in gene expression in the Pro-cell death gene network, and the decrease in gene expression in the Anti-cell death gene network may be closely associated with the enhancement of HT-induced cell death by BAG3-KD in OSCC cells. However, the interaction between gene expression and the enhancement of the HT effects remains a subject for further study. In clinical fields, HT combined with radiotherapy and/or chemotherapy has been used as a possible treatment modality for various types of cancer (1-4). However, the thermotolerance resulting from the elevation of HSP expression and other cytoprotective proteins in some cancer cells remains a disadvantage, diminishing the effects of HT (5,6). The functional silencing of BAG3, a co-chaperone of the HSPA family of proteins, may effectively enhance the sensitivity of cancer cells to HT. Therefore, the targeting of BAG3 in combination with HT may become a promising therapeutic approach for the treatment of cancer (17,18).

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