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Gene networks related to the cell death elicited by hyperthermia in human oral squamous cell carcinoma HSC-3 cells

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Abstract. Local hyperthermia (HT) for various types of malignant tumors has shown promising antitumor effects. To confirm the detailed molecular mechanism underlying cell death induced by HT, gene expression patterns and gene networks in human oral squamous cell carcinoma (OSCC) cells were examined using a combination of DNA microarray and bioinformatics tools. OSCC HSC-3 cells were treated with HT at 44˚C for 90 min or mild hyperthermia (MHT) at 42˚C for 90 min, followed by culturing at 37˚C for 0-24 h. Treatment of cells with HT prevented cell proliferation (62%) and induced cell death (17%), whereas these alterations were not observed in cells treated with MHT. Microarray analysis revealed substantial differences with respect to gene expression patterns and biological function for the two different hyperthermic treatments. Moreover, we identified the temperature-specific gene networks D and H that were obtained from significantly up-regulated genes in the HT and MHT conditions, respectively, using Ingenuity pathway analysis tools. Gene network D, which contains 14 genes such as ATF3, DUSP1 and JUN, was associated with relevant biological functions including cell death and cellular movement. Gene network H, which contains 13 genes such as BAG3, DNAJB1 and HSPA1B, was associated with cellular function and maintenance and cellular assembly and organization. These findings provide a basis for understanding the detailed molecular mechanisms of cell death elicited by HT in human OSCC cells.

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Abbreviations: ATF3, activating transcription factor 3; BAG3, BCL2-associated athanogene 3; CLU, clusterin; DNAJA1, DnaJ (Hsp40) homolog, subfamily A, member 1; DNAJB1, DnaJ (Hsp40) homolog, subfamily B, member 1; DUSP1, dual specificity phosphatase 1; GADD45B, growth arrest and DNA-damage-inducible, β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HES1, hairy and enhancer of split 1, (Drosophila); HSPA1B, heat shock 70 kDa protein 1B; HSPH1, heat shock 105/110 kDa protein 1; HSPs, heat shock proteins; HT, hyperthermia; JUN, jun proto-oncogene; MHT, mildhyperthermia; OSCC, oral squamous cell carcinoma; PGF, placental growth factor; PRKCD, protein kinase C, δ; qPCR, quantitative polymerase chain reaction

Key words: hyperthermia, microarray, gene network, cell death, human oral squamous cell carcinoma HSC-3

Introduction

In the treatment of oral squamous cell carcinoma (OSCC), treatment outcomes have improved in the past two decades due to progress in reconstructive techniques, stereotactic radiotherapy, new anticancer drugs such as taxanes, and combinations of these therapeutic modalities (1). Nonetheless, it is well known that patients with advanced tumors or tumor recurrence have a poor prognosis with a median survival period less than one year (2). Local hyperthermia (HT) for various malignant tumors including OSCC has been recognized as an effective and attractive tool with the advantages of relatively few side effects and slight damage to normal tissue. Combinations of HT with chemotherapy, radiotherapy or both have been clinically used for patients with cancer in various organs, and their antitumor effects have been verified by many clinical trials (3-7).

In general, HT elicits a wide spectrum of stress responses such as induction of heat shock proteins (HSPs), protein aggregation, an imbalance of protein homeostasis, DNA and RNA damage, reactive oxygen species production, cell growth arrest and cell death in mammalian cells (8,9). In particular, HSPs, which are induced by heat, behave as molecular chaperones and exert strong cytoprotective effects that prevent cell death. The treatment of cells with HT induces numerous signal transduction pathways which contribute variously to cell death and survival (10-12). Although many biological processes are affected by HT, the overall responses to HT in mammalian cells remain unknown.

Recent DNA microarray and computational microarray analyzing technologies have provided a view of the expression

profiles of many genes, and their relevant biological functions and gene networks based on the gene-expression profiles (13). To date, several investigators have demonstrated that gene-expression profiles, biological functions and gene networks in a wide variety of cell types are affected by mild hyperthermia (MHT) or HT (9,14-25).

It is well known that cancer cells exposed to HT of more than 42.5˚C will undergo cell death, whereas exposure to MHT conditions, i.e., temperatures up to 42.5˚C, produces only slight or no cytotoxicity (26). The aim of this study was to better understand the detailed molecular mechanism by which HT induces cell death; thus, we compared the differences in the gene expression patterns of cells under HT conditions at 44˚C (with cell death) and MHT conditions at 42˚C (without cell death) using a combination of DNA microarray and bioinformatics tools.

Materials and methods

Cell culture. Human OSCC HSC-3 cells were obtained from the Human Science Research Resources Bank, Japan Health Sciences Foundation (Tokyo, Japan). The cells were cultured in E-MEM medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum at 37˚C in humidified air with 5% CO_2 and 95% air.

MHT and HT treatments. MHT and HT were performed by immersing plastic vessels containing the attached cells in a water bath at 42˚C (±0.05˚C) or 44˚C (±0.05˚C) for 90 min, respectively. The temperature was monitored with a digital thermometer (no. 7563, Yokogawa, Tokyo, Japan) during heating. After heat treatment, the cells were incubated for 0-24 h at 37˚C.

Analyses of cell growth and cell death. The number of cells was counted using a hemocytometer. For the detection of cell death, flow cytometry was used with propidium iodide and fluorescein isothiocyanate (FITC)-labeled Annexin V (Annexin V-FITC kit, Immunotech, Marseille, France) (27).

RNA isolation and gene expression analysis. Total-RNA was isolated from cells using an RNeasy total-RNA extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA samples were treated with RNase-free DNase I for 15 min at room temperature. Gene expression analysis was performed by a GeneChip® system with a Human Genome U133-plus 2.0 array, which was spotted with 54,675 probe sets (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, 500 ng of total-RNA was used to synthesize cRNA with a GeneChip® 3' IVT Express kit (Affymetrix). After fragmentation, biotin-labeled cRNA was hybridized to the array at 45˚C for 16 h. The arrays were washed, stained with streptavidin-phycoerythrin, and scanned using a probe array scanner. The scanned arrays were analyzed using the GeneChip® Analysis Suite software (Affymetrix). The obtained hybridization intensity data were analyzed using the GeneSpring software (Silicon Genetics, Redwood City, CA, USA) 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 the Ingenuity Pathway Analysis tools (Ingenuity Systems, Mountain View, CA, USA), a web-delivered application that enables the identification, visualization, and exploration of molecular interaction networks in gene expression data (28).

Real-time quantitative polymerase chain reaction (qPCR) assay. Real-time qPCR assay was performed on a real-time PCR system (Mx3000P, Stratagene Japan, Tokyo, Japan) using SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan) or Premix Ex Taq (for the use of TaqMan probes; Takara Bio) according to the manufacturer's protocols. Reverse transcriptase reaction was carried out with total-RNA using an oligo(dt) primer and random hexamers. Real-time qPCR assay was performed using the specific primers listed in Table I. The transcript levels of these genes were estimated from the respective standard curves. Each expression level was normalized to the transcript level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (21).

Statistical analysis. Data are shown as means ± SD. The Student's t-test was used for statistical analysis and significance was assumed for P-values <0.05.

Results

Effects of MHT and HT on cell growth and cell death in OSCC HSC-3 cells. The cell number of HSC-3 cells after MHT or HT for 90 min followed by culture at 37˚C for 24 h is shown in Fig. 1A. MHT at 42˚C did not inhibit the growth of cells. On the other hand, significant growth inhibition was observed in cells treated with HT at 44˚C. The number of cells at 37˚C (control), 42˚C and 44˚C were 20.8±2.0x10^4, 19.6±2.6x10^4 and 7.9±1.7x10^4 cells (mean ± SD), respectively. Moreover, effects on cell death of MHT or HT for 90 min followed by culture at 37˚C for different periods were examined. The control percentage of cell death was 3.1±1.0 (mean ± SD). Treatment by MHT at 42˚C for 90 min did not change the percentage of cell death, the levels being 3.1±2.2, 3.1±1.2 and 5.0±1.5, (mean ± SD) for 0, 6 and 12 h, respectively. On the other hand, when HSC-3 cells were treated with HT at 44˚C for 90 min, cell death was significantly and time-dependently elevated, the levels being 4.4±1.0, 9.5±0.9 and 17.4±0.6, (mean ± SD) for 0, 6 and 12 h, respectively (Fig. 1B). The data indicate that either cell growth inhibition or cell death was observed only in cells treated with HT at 44˚C.

Global gene-expression analysis and identification of gene networks. In order to analyze gene-expression patterns of HSC-3 cells treated with HT, we compared the differences in the gene expression patterns of cells under HT conditions at 44˚C (with cell death) and MHT conditions at 42˚C (without cell death) using a GeneChip® system. Of the 54,675 probe sets analyzed, we identified the many probe sets that were differentially expressed by 2.0-fold or more. We identified 443, 225 and 353 up-regulated and 784, 963 and 1,129 down-regulated probe sets at 0, 6 and 12 h after MHT, respectively, and 344, 266 and 107 up-regulated and 580, 403 and 323 down-regulated probe sets at 0, 6 and 12 h after HT, respectively (Table II). The complete list of probe sets from all samples is available on the Gene Expression Omnibus, a public database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24783).

Table I. Nucleotide sequences of primers and a TaqMan probe for target genes.

Genes	Orientation	Nucleotide sequence (5' to 3')	GenBank accession no.
ATF3	Sense	ACCTGACGCCCTTTGTCAAG	AB066566
	Antisense	GGCACTTTGCAGCTGCAATC	
BAG3	Sense	CGACCAGGCTACATTCCCAT	NM_004281
	Antisense	TCTGGCTGAGTGGTTTCTGG	
DNAJB1	Sense	ACCCGGACAAGAACAAGGAG	NM_006145
	Antisense	GCCACCGAAGAACTCAGCAA	
DUSP1	Sense	GAGCTGTGCAGCAAACAGTC	NM_004417
	Antisense	CAGGTACAGAAAGGGCAGGA	
GADD45B	Sense	CAGAAGATGCAGACGGTGAC	NM_015675
	Antisense	ACCCGCACGATGTTGATGTC	
GAPDH	Sense	AAGGCTGGGGCTCATTTGCA	NM_002046
	Antisense	ATGACCTTGCCCACAGCCTT	
HSPA1B	Sense	AGGTGCAGGTGAGCTACAAG	NM_005346
	Antisense	ATGATCCGCAGCACGTTGAG	
HSPA1H	Sense	ACCATGCTGCTCCTTTCTCC	NM_006644
	Antisense	CTGGGTTTTCTGGTGGTCTC	
JUN	Sense	CTGCAAAGATGGAAACGACCTT	J04111
	Antisense	TCAGGGTCATGCTCTGTTTCAG	
	Probe	FAM-TATGACGATGCCCTCAACGCCTCGT-TAMRA	

ATF3, activating transcription factor 3; BAG3, BCL2-associated athanogene 3; DNAJB1, DnaJ (Hsp40) homolog, subfamily B, member 1; DUSP1, dual specificity phosphatase 1; GADD45B, growth arrest and DNA-damage-inducible, β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSPA1B, heat shock 70 kDa protein 1B; HSPA1H, heat shock 105/110 kDa protein; JUN, jun proto-oncogene.

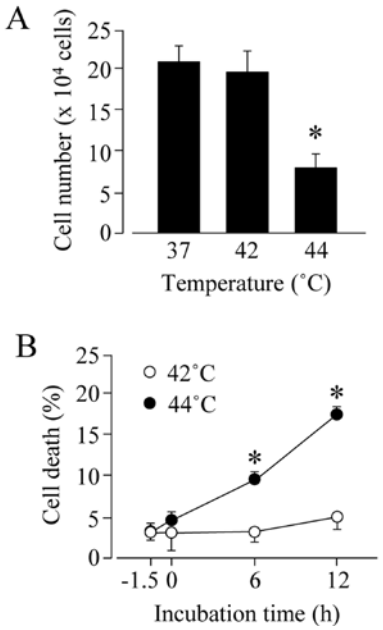

Figure 1. Effects of mild hyperthermia (MHT) and hyperthermia (HT) on cell growth and cell death in human OSCC HSC-3 cells. The cells were incubated at 37˚C (control), 42˚C (MTH) or 44˚C (HT) for 90 min and then cultured at 37˚C for 24 h. (A) Cell numbers were counted. The cells were incubated at 42 or 44˚C for 90 min and then cultured at 37˚C for 12 h. (B) Cell death was evaluated by flow cytometry using an Annexin V-FITC kit. Data are presented as means ± SDs (n=4). *P<0.05 vs. control (cells treated at 37˚C). *P<0.05 vs. control (non-treated cells).

We next carried out a functional category analysis of the probe sets at each time point using the Ingenuity Pathways Knowledge Base. The top biological functions at each time point and each condition are shown in Table II. In MHT-treated cells, the biological functions including cellular function and maintenance, and cellular growth and proliferation, or cell cycle and cellular development were observed in up- or down-regulated probe sets, respectively. In HT-treated cells, the biological functions including gene expression and cell death, or post-transcriptional modification and gene expression were observed in up- or down-regulated probe sets, respectively. Moreover, two gene networks D and H were identified in the up-regulated probe sets affected by HT and MHT, respectively. Gene network D contained 14 up-regulated genes, such as activating transcription factor 3 (ATF3), dual specificity phosphatase 1 (DUSP1), growth arrest and DNA-damage-inducible, β (GADD45B), hairy and enhancer of split 1, (Drosophila) (HES1), and jun proto-oncogene (JUN), and was associated with relevant biological functions including cell death (significance, 2.49E-10 to 1.57E-1) and cellular movement (2.3E-11 to 9.04E-4) (Fig. 2). Gene network H contained 13 up-regulated genes, such as BCL2-associated athanogene 3 (BAG3), DnaJ (Hsp40) homolog, subfamily B, member 1 (DNAJB1), heat shock 70 kDa protein 1B (HSPA1B), and heat shock 105/110 kDa protein 1 (HSPH1), and was associated with relevant biological functions including cellular function and

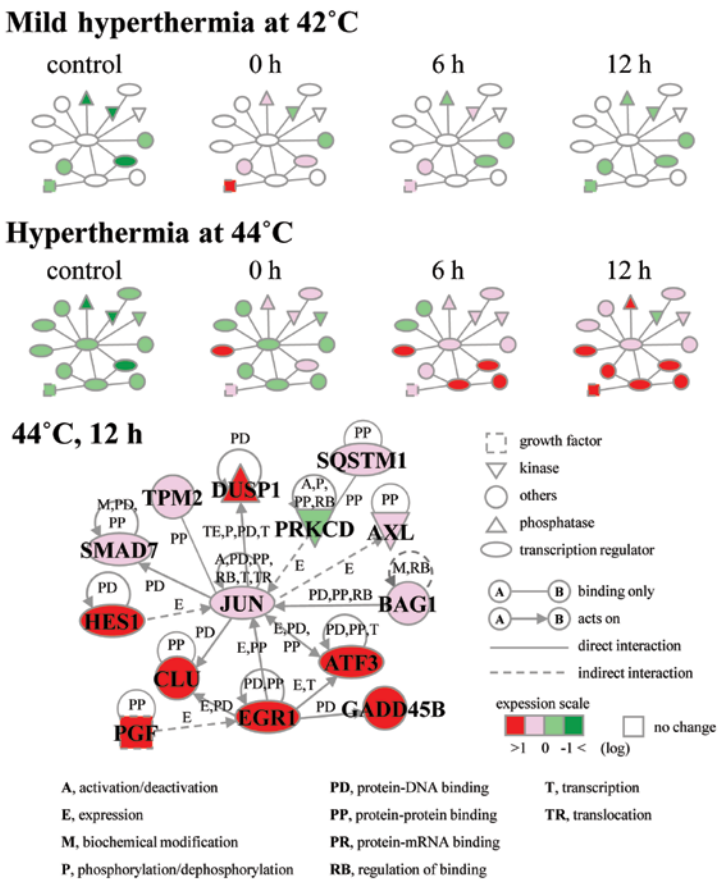

Figure 2. Gene network D. Up-regulated probe sets for the HT condition at 44˚C (peak expression, 6 and/or 12 h) were analyzed by the Ingenuity Pathways Analysis software. The network is shown graphically as nodes (genes) and edges (the biological relationships between the nodes).

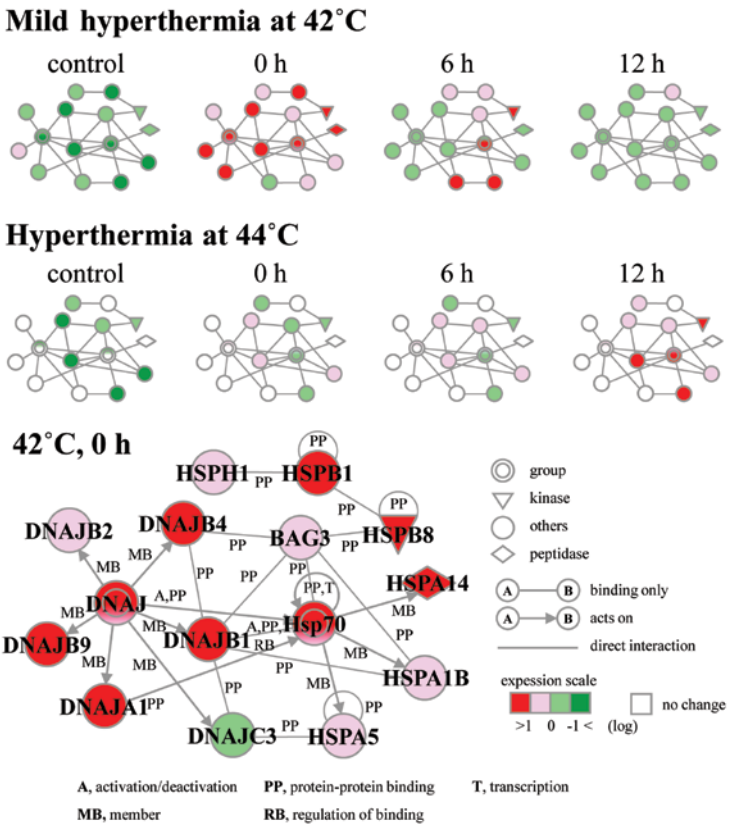

Figure 3. Gene network H. Up-regulated probe sets for the MHT condition (peak expression, 0 and/or 6 h) were analyzed by the Ingenuity Pathways Analysis software. The network is shown graphically as nodes (genes) and edges (the biological relationships between the nodes).

Table II. Top biological functions.

	Gene expression	Altered probe sets, n	Functions probe sets
Heat stress at 42˚C			
0 h	Up-regulation	443	Cellular functions and maintenance
	Down-regulation	784	Cell cycle
6 h	Up-regulation	225	Cellular growth and proliferation
	Down-regulation	963	Cellular development
12 h	Up-regulation	353	Cellular growth and proliferation
	Down-regulation	1,129	Cell cycle
Heat stress at 44˚C			
0 h	Up-regulation	344	Gene expression
	Down-regulation	580	Post-transcriptional modification
6 h	Up-regulation	266	Cell death
	Down-regulation	403	Gene expression
12 h	Up-regulation	107	Cell death
	Down-regulation	323	Gene expression

HSC-3 cells were treated with heat stress at 42 or 44˚C for 90 min and then cultured at 37˚C for 0, 6 or 12 h. Gene expression was monitored using the GeneChip microarray system. Many probe sets were up- and down-regulated by more than 2-fold were identified. Functional category analysis was performed using the Ingenuity Pathways Knowledge Base. The top biological functions are shown.

maintenance (significance, 8.43E-14 to 3.74E-2) and cellular assembly and organization (9.31E-8 to 3.74E-2) (Fig. 3).

Verification of differentially expressed genes. To verify the microarray results, real-time qPCR was performed. The expression levels of eight selected genes were comparable to those determined by GeneChip® gene expression analysis. Among the genes in gene network D, the expression levels of ATF3, DUSP1, GADD45B and JUN were markedly and time-dependently increased after HT-treatment. On the other hand, in MHT-treated cells, the expression levels of ATF3 and DUSP1 were transiently increased at time point 0 h, and the levels of GADD45B and JUN did not increase over the culture period. Among the genes in gene network H, the expression levels of BAG3, DNAJB1, HSPA1B and HSPH1 were transiently and significantly elevated at time point 0 and/or 3 h in MHT-treated cells. On the other hand, the expression levels of these 4 genes were gradually and significantly elevated when peaks were observed at the 12-h time point (Fig. 4).

Discussion

DNA microarray and computational data analysis technologies are powerful technologies for elucidating genome-wide gene expression signatures in various life science research areas (13). In the present study, a unique gene network for cell death elicited by HT treatment at 44˚C in human OSCC cells was identified using these technologies. To our knowledge, this is the first report regarding the identification of gene networks influenced by HT in OSCC cells.

The temperature of 42.5˚C for cancer cells is well known to be the inflection point of HT, that is, cancer cells exposed to temperatures >42.5˚C undergo cell death, but exposure of up to 42.5˚C induces slight or no cytotoxicity (26). In the present study, the treatment of human OSCC HSC-3 cells with HT at 44˚C, higher than the inflection point, significantly induced cell death while treatment with MHT at 42˚C, lower than the inflection point, did not. These results were comparable to our previous studies using human leukemia U937 cells (25,27). As expected, substantial differences with respect to gene expression patterns and biological functions between HT and MHT treatments were observed in HSC-3 cells. Furthermore, Ingenuity pathway analysis identified the significantly-altered and temperature-specific gene networks D and H that were obtained from up-regulated genes in HT- and MHT-treated cells, respectively. In addition, real-time qPCR assay demonstrated that the expression levels of eight selected genes were comparable to those detected by the microarray experiment. In this study, gene network H contained many HSPs such as DNAJA1, DNAJB1 and HSPA1B, and was mainly associated with the relevant biological function of cellular function and maintenance (Fig. 3). Microarray and real-time qPCR experiments clearly indicated that the up-regulation of HSPs in the MHT condition at 42˚C occurred at earlier time points than those in the HT condition at 44˚C. Under MHT without cell death, early induction of HSPs may exert central cytoprotective effects that prevent cell death (10-12). HSPs including DNAJA1 (29), DNAJB1 (30) and HSPA1B (31), and BAG3 (32) have been previously described as anti-cell-death molecules. A previous report indicated that the specific protein interaction between BAG3 and Hsp70 occurred and that silencing of BAG3 attenuated apoptosis in the colonic cancer cell line RKO (32). It is also possible that HSPs may play a role in cytoprotection from HT-induced cell death at 44˚C. Induction of HSPs and/or BAG3 is a common response to heat with or without cell death, as described in several microarray studies (14-19,21-25).

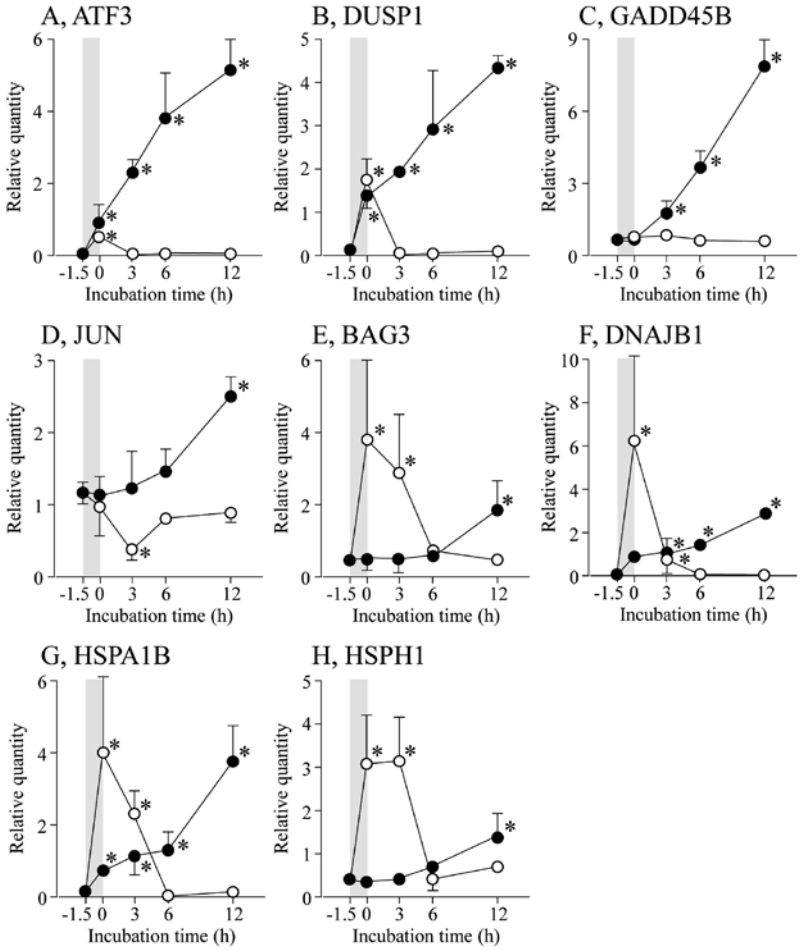

Figure 4. Verification of microarray results by real-time qPCR. HSC-3 cells were incubated at 42˚C (mild hyperthermia, open circles) or 44˚C (hyperthermia, closed circles) for 90 min and then cultured at 37˚C for 0-12 h. Real-time qPCR was performed. (A) ATF3; (B) DUSP1; (C) GADD45B; (D) JUN; (E) BAG3; (F) DNAJB1; (G) HSPA1B; (H) HSPH1. Each expression level was normalized to the GAPDH expression level. The shadows indicate heat treatment. Data are presented as means ± SDs (n=4). *P<0.05 vs. control (non-treated cells).

We successfully identified gene network D that was associated with relevant biological functions including cell death from up-regulated genes in the HT condition at 44˚C (Fig. 2). The gene network D had JUN at the center and contained ATF3, DUSP1, HES1 and GADD45B. The up-regulation of almost all genes in network D was remarkable and was relatively sustained at the HT condition, whereas a transient and slight increase in the expression of several genes including ATF3, CLU (clusterin), DUSP1, PGF (placental growth factor), and PRKCD (protein kinase C, δ) was detected only at the 0- or 6-h time point at the MHT condition. It has been indicated that DUSP1 protein increases the death of Jurkat T cells (33). Kannan *et al* (34) recently demonstrated that Notch/HES1-mediated poly (ADP-ribose) polymerase 1 activation leads to apoptosis in human precursor B-leukemia cell lines. It has also been reported that ectopic overexpression of GADD45B protein is apoptogenic in HeLa cells (35). JUN and ATF3, the basic-region leucine zipper transcription factors, have been reported to participate in cell death in human cell lines (36,37). These cell death-associated genes in gene network D may be correlated with the cell death induced by HT in HSC-3 cells. In several microarray experiments, heat stress induced elevation of the expression of ATF3, DUSP1, GADD45B and JUN (18,19,21,23-25).

In conclusion, the present results indicate that the differentially expressed genes and gene networks identified here are likely to be related to the cell death induced by HT in OSCC cells. HT in combination with other treatments has been considered a promising approach in cancer therapy (3-7). Thus, the current data will help enable the rational design of more effective strategies for future HT therapy in OSCC cells.

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