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Gene expression in enhanced apoptosis of human lymphoma U937 cells treated with the combination of different free radical generators and hyperthermia

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Abstract

The effects of various free radicals derived from 6-formylpterin (6-FP), α-phenyl-*tert*-butyl nitrone (PBN) and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) combined with hyperthermia, on gene expression in similarly enhanced apoptosis of human lymphoma U937 cells were investigated using cDNA microarrays containing approximately 16,600 genes and computational gene expression analysis tools. When the cells were treated for 10 min at 44°C (15% apoptosis level), 39 up-regulated and 3 down-regulated genes were identified. In the up-regulated genes, apoptosis- and unfolded protein response-associated genes were contained. The combined treatment with heat and either chemical enhanced apoptosis level (approximately 30%) and showed a chemical-specific gene expression pattern. Furthermore, the expression levels of selected genes were confirmed by a real-time quantitative PCR. The present results will provide a basis for further understanding the molecular mechanisms in enhancement of heat-induced apoptosis by different intracellular oxidative stress.

Keywords: *Oxidative stress, hyperthermia, heat stress, DNA microarray, gene expression*

Introduction

Local hyperthermia for various malignant tumors has been recognized as a useful therapy with the advantages of relatively low side effects and slight damages to normal tissue. Combined treatments with hyperthermia and either chemotherapy, radiotherapy or both have been clinically used for patients with cancer in various organs, and their anti-tumor effects have been verified by many clinical trials [1,2]. However, the problems of uniformity of temperature distribution and difficulty of heating deep areas still remain as disadvantages reducing the effect of cancer cell killing. To overcome these problems, epoch-making chemicals enhancing heat-induced apoptosis, so called "apoptosis thermosensitizers", should be urgently developed.

Oxidative stresses such as reactive oxygen species (ROS) and free radicals are involved in many signal

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transductions of apoptosis [3,4]. In addition, it has also been reported that oxidative stresses including ROS play an important role as intracellular mediators of heat-induced apoptosis [5]. Recently, we have reported that heat-induced apoptosis of human lymphoma U937 cells is due to the intracellular generation of superoxide, rapid formation of lipid peroxidation, and an increase in the intracellular Ca^{2+} concentration [6–9]. Moreover, the heat-induced apoptosis was also enhanced by load of intracellular oxidative stress due to a hydrogen peroxide generator, 6-formylpterin (6-FP) [10], an oxidative stress specific nitric oxide donor, α-phenyl-*tert*-butyl nitrone (PBN) [11], and a temperature-dependent alkyl and alkoxyl free radical generator, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) [6,9]. Thus, these three chemicals are suggested to be effective sensitizers for heat-induced apoptosis because they can display their potency for cell killing only when they are combined with hyperthermia at nontoxic concentrations. However, the molecular mechanisms by which these chemicals act to produce different intracellular oxidative stress enhance heat-induced apoptosis are largely unknown. Here, to elucidate the mechanism of signal transduction related to the enhancement, we tried direct comparison of gene expression patterns in human lymphoma U937 cells treated with hyperthermia combined with 6-FP, PBN or AAPH using cDNA microarrays containing approximately 16,600 genes at similar level of apoptosis. Moreover, we explored the functional relationships of the candidate genes using computational gene expression analysis tools.

Materials and methods

Cell culture

A human myelomonocytic lymphoma cell line U937 was obtained from Human Sciences Research Resources Bank (Japan Human Sciences Foundation, Tokyo, Japan). The cells were grown in RPMI1640 medium containing 10% fetal bovine serum, and maintained in a humidified incubator maintained at 37.0°C with 5% CO_2 and 95% air.

Chemical and heat treatments

6-FP was synthesized by our research group and its purity was determined as previously mentioned [12]. PBN and AAPH were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA) and Wako Pure Chemical Industries Ltd (Tokyo, Japan), respectively. For heat treatment, the cells were seeded at 3×10^6/sample in plastic tubes and exposed to 44.0 ± 0.05°C by immersing tubes containing 3 ml of cell suspension into a precision controlled water bath for 10 min. The temperature of the culture medium was monitored with a digital thermometer with a 0.8-mm thermocouple. For chemical treatment, the culture media containing various concentrations of the chemical were added to the cells in plastic tubes just before heating. After these treatments, the cells were incubated at 37°C for 3 h.

Determination of DNA fragmentation

The cellular DNA fragmentation was analyzed using the method of Sellins and Cohen [13] with minor modifications. Briefly, the cells were lysed with a lysis buffer (1 mM EDTA, 0.2% Triton X-100 and 10 mM Tris–HCl, pH 7.5) and centrifuged at 13,000 *g* for 10 min. Subsequently, each DNA in the supernatant and the pellet were precipitated in 12.5% trichloroacetic acid at 4°C and quantified using a diphenylamine reagent after hydrolysis in 5% trichloroacetic acid at 90°C for 20 min. The absorbance at 600 nm in each sample was determined after overnight color development with diphenylamine reagent. The percentage of fragmented DNA in each sample was calculated as the amount of DNA in the supernatant divided by total DNA for that sample (supernatant plus pellet) [7].

Separation of total RNA

Total RNA was extracted from the cells using an RNeasy Total RNA Extraction Kit (Qiagen K.K., Tokyo, Japan). RNA samples were treated with RNase-free DNase (Qiagen K.K.).

cDNA microarray analysis

cDNA microarray analysis was performed by IntelliGene HS Human Expression glass microarrays (Takara Bio Inc., Shiga, Japan) which were spotted with approximately 16,600 cDNA fragments of human genes. A list of these genes is available at Takara's website (http://www.takara-bio.co.jp/). Antisense RNA was labeled with Cy3 (control group) or Cy5 (treated group) from mRNAs from cells at 3 h after treatment by using an RNA Transcript SureLABEL Core Kit (Takara Bio). In some experiments, control sample was labeled with Cy5; and in others, it was labeled with Cy3, with essentially identical results. Hybridization and washing of the microarray were carried out according to the manufacturer's instructions. The microarrays were scanned in both Cy3 and Cy5 channels with a ScanArray Lite (Packard BioChip Technologies, Billerica, MA, USA). QuantArray software (Packard BioChip Technologies) was used for image analysis. Genes were considered to be positive-expressed if the signal/background ratio was >2.0. The average of GAPDH Cy3 and Cy5 signal (12 spots each) gives a ratio that was used to balance or normalize the signals [14].

Computational gene expression analysis

To examine the gene ontology, including biological processes, cellular components, molecular functions and genetic networks, the data were analyzed using Ingenuity Pathways Analysis tools (Ingenuity Systems, Mountain View, CA, USA), a web-delivered application that enables the discovery, visualization and exploration of molecular interaction networks in gene expression data. The gene lists identified by microarray analysis containing gene identifier were uploaded into the Ingenuity Pathways Analysis [15].

Real-time quantitative PCR

Real-time quantitative PCR (RT qPCR) was performed on a real-time PCR system (Mx3000P, Stratagene Japan K.K., Tokyo, Japan) using Brilliant SYBR Green QPCR Master Mix (Stratagene Japan K.K.) or qPCR Master Mix (for the use of TaqMan probes; Eurogentec, Seraing, Belgium) according to the manufacturer's protocols. Reverse transcriptase reaction was carried out with total RNA by using an oligo $d(T)_6$ primer. RT qPCR was performed by using the specific primers listed in Supplemental Material Table SI online. Each mRNA expression level was normalized with respect to the mRNA expression of *GAPDH* [14,16].

Statistical analysis

Data are shown as means ± SD. Statistical analysis was carried out using Student's *t*-test and *p* values less than 0.05 were regarded as significant.

Results

Apoptosis induced by heat treatment alone and the combination with heat treatment and chemical

When human lymphoma U937 cells were treated for 10 min at 44°C (heat treatment alone), the index of apoptosis was approximately 15% (concentration: 0 mM). For the combination with heat treatment and 6-FP, the DNA fragmentation, that was the index of apoptosis, was 22.3 ± 2.9, 31.4 ± 10.5 and 35.0 ± 14.7% at the concentrations of 0.1, 0.3 and 0.4 mM, respectively. For the combination with heat treatment and PBN, the index of apoptosis was 25.7 ± 4.1, 36.5 ± 10.2, 45.6 ± 5.3 and 62.8 ± 15.2% at the concentrations of 1, 3, 5 and 10 mM, respectively. For the combination with heat treatment and AAPH, the index of apoptosis was 18.0 ± 11.1, 24.3 ± 9.8, 29.2 ± 4.7 and 46.3 ± 11.7% at the concentrations of 2, 10, 20 and 50 mM, respectively (Figure 1). Based on these results, the optimum concentrations of each chemical required to enhance the DNA fragmentation up to 30% (C30% DF) was used for the gene expression analyses. The C30% DF of 6-FP, PBN and AAPH was 0.3, 2 and 20 mM, respectively.

Changes of gene expression by heat treatment alone and the combination with heat treatment and chemical

To identify specific genes related to three chemicals that can enhance apoptosis induced by the heat treatment, cDNA microarray analysis was performed on the cells treated with these combinations. Genes were considered up- or down-regulated, if each value and the average fold change in two different experiments were 1.5 or greater. Of approximately 16,600 genes analyzed, 42 genes (39 up- + 3 down-regulated genes) that differentially expressed were identified in heat-treated cells (heat treatment alone) in comparison with control cells. Up-regulated genes included BCL2-associated athanogene 3 (*BAG3*), DnaJ (Hsp40) homolog, subfamily A, member 1 (*DNAJA1*), DnaJ (Hsp40) homolog, subfamily B, member 1 (*DNAJB1*), heat shock 70 kDa protein 1B (*HSPA1B*), heat shock 70 kDa protein 6 (*HSPA6*), heat shock 105/110 kDa protein 1 (*HSPH1*), selenoprotein W1 (*SEPW1*) and heme oxygenase (decycling) 1 (*HMOX1*) while down-regulated genes included chemokine (C—C motif) ligand 2 (*CCL2*) (Supplemental Material Table SII online).

When the cells were treated with heat shock and 0.3 mM 6-FP, two up-regulated genes were detected in comparison with gene expression after heat treatment. On the other hand, there was no down-regulated gene observed (Supplemental Material Table SIII). When the cells were treated with heat shock and 2 mM PBN, 19 down-regulated genes including *DNAJA1*, *SEPW1* and *HMOX1* were identified in comparison with gene expression after heat treatment, but up-regulated gene was not detected. Moreover, when the cells were treated with heat shock and 20 mM AAPH, two up-regulated genes including *GADD45B* (growth arrest and DNA-damage-inducible beta) and 17 down-regulated genes including *DNAJA1* and *SEPW1* were identified in comparison with gene expression after heat treatment (Supplemental Material Table SIII online).

Quantitative analysis of the candidate genes by RT qPCR

To verify the results of the microarray experiments, RT qPCR was performed. Among the genes identified by the microarray analysis, heat shock-, apoptosis- or oxidative stress-related genes were chosen in this experiment, adding analysis for *HMOX1* [17] and *JUN* (v-jun sarcoma virus 17 oncogene homolog) [18] which were speculated to have a close relation to heat-induced apoptosis (Supplemental Material Table SI). As in the case of the heat treatment alone, the expression levels of *BAG3*, *DNAJA1*, *DNAJB1*, *HSPA1B*, *HSPA6*, *HSPH1*, *SEPW1*, *HMOX1* and

Table SI. Nucleotide sequences of primers and TaqMan probes for target genes.

Genes	Orientation	Nucleotide sequence (position)	GenBank accession no	Function
BAG3	Sense	5′-CGACCAGGCTACATTCCCAT-3′ (574–593)	NM_004281	A
	Antisense	5′-TCTGGCTGAGTGGTTTCTGG-3′ (749–730)		
CCL2	Sense	5′-TGAAAGTCTCTGCCGCCCTT-3′ (75–94)	NM_002982	A
	Antisense	5′-CTTTGGGACACTTGCTGCTG-3′ (257–238)		
DNAJA1	Sense	5′-GCCGAGGTACTGGAATGCAA-3′ (641–660)	NM_001539	H
	Antisense	5′-CCTGGTTCTTGGTCTCCTTC-3′ (881–862)		
DNAJB1	Sense	5′-ACCCGGACAAGAACAAGGAG-3′ (135–154)	NM_006145	H
	Antisense	5′-GCCACCGAAGAACTCAGCAA-3′ (364–345)		
GADD45B	Sense	5′-CAGAAGATGCAGACGGTGAC-3′ (140–159)	NM_015675	A
	Antisense	5′-ACCCGCACGATGTTGATGTC-3′ (375–356)		
GAPDH	Sense	5′-AAGGCTGGGGCTCATTTGCA-3′ (394–413)	NM_002046	C
	Antisense	5′-ATGACCTTGCCCACAGCCTT-3′ (737–718)		
HSPA1B	Sense	5′-AGGTGCAGGTGAGCTACAAG-3′ (509–528)	NM_005346	A, H
	Antisense	5′-ATGATCCGCAGCACGTTGAG-3′ (722–703)		
HSPA6	Sense	5′-GGCCATGACCAAGGACAACA-3′ (1760–1779)	NM_002155	H
	Antisense	5′-AACCATCCTCTCCACCTCCT-3′ (1976–1957)		
HSPH1	Sense	5′-ACCATGCTGCTCCTTTCTCC-3′ (1666–1685)	NM_006644	H
	Antisense	5′-CTGGGTTTTCTGGTGGTCTC -3′ (1974–1955)		
SEPW1	Sense	5′-ATTGTGGCGCTTGAGGCTAC-3′ (227–246)	NM_003009	O
	Antisense	5′-TCCACGTAGCCATCGCCTTT-3′ (404–385)		
JUN	Sense	5′-CTGCAAAGATGGAAACGACCTT-3′ (1265–1286)	J04111	A, O
	Antisense	5′-TCAGGGTCATGCTCTGTTTCAG-3′ (1381–1360)		
	Probe	5′-FAM-TATGACGATGCCCTCAACGCCTCGT-TAMRA-3′ (1288–1312)		
HMOX1	Sense	5′-GAGGGAAGCCCCCACTCA-3′ (841–858)	NM_002133	A, O
	Antisense	5′-AACTGTCGCCACCAGAAAGCT-3′ (923–903)		
	Probe	5′-FAM-ACCCGCTCCCAGGCTCCGCTTC -TAMRA-3′ (861–882)		

A, apoptosis-related gene; C, internal control gene; H, heat shock-related gene; O, oxidative stress-related gene.

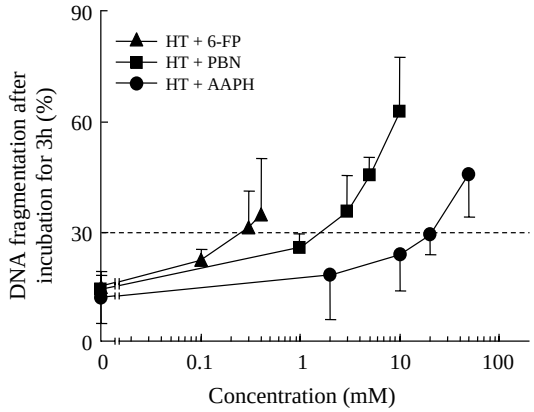

Figure 1. Enhancement of heat shock-induced apoptosis in U937 cells by AAPH, 6-FP and PBN at various nontoxic concentrations. The cells were treated with the combination of heat shock (44.0°C, for 10 min) and each chemical, and then harvested after incubation for 3 h. DNA fragmentation rate was analyzed. Bars in figure indicate standard deviation ($n = 3$).

JUN were significantly increased, with the expression levels being 16.0-, 5.9-, 10.9-, 23.8-, 62.3-, 15.4-, 2.7- and 9.2-fold, respectively. In contrast, the expression level of *CCL2* was significantly decreased, with the expression level being 0.15-fold (Figure 2A). As in the case of the combination with heat treatment and 6-FP, the expression level of *HMOX1* was significantly decreased, with the expression level being 0.54-fold. In contrast, the expression level of *JUN* was significantly increased, with the expression level being 5.4-fold (Figure 2B). As in the case of the combination with heat treatment and PBN, the expression levels of *DNAJA1*, *SEPW1* and *HMOX1* were significantly decreased, with the expression levels being 0.73-, 0.61- and 0.49-fold, respectively. In contrast, the expression level of *JUN* was significantly increased, with the expression level being 3.3-fold (Figure 2C). As in the case of the combination with heat treatment and AAPH, the expression levels of *DNAJA1*, *SEPW1* and *HMOX1* were significantly decreased, with the expression levels being 0.58-, 0.36- and 0.56-fold, respectively. In contrast, the expression levels of *GADD45B* and *JUN* were significantly increased, with the expression levels being 2.3- and 3.9-fold, respectively (Figure 2D).

Computational gene expression analysis

To examine the gene ontology, including biological processes, cellular components, molecular functions and genetic networks, the data identified here were analyzed using the Ingenuity Pathways Analysis tools. For the genes identified by the heat treatment alone, a genetic network including *DNAJA1*, *DNAJB1*, heat shock protein (HSP) 27 kDa protein 1 (*HSPB1*), prion protein p27–30 (*PRNP*), suppression of tumorigenicity 13 (Hsp70 interacting protein) (*ST13*) and stress-induced phosphoprotein 1 (Hsp70/Hsp90 organizing protein) (*STIP1*) was identified. The gene interaction between *CCL2* and *JUN* was also observed. Moreover, apoptosis- or unfolded protein response-related genes whose names or edges were highlighted red or blue color were identified, respectively (Figure 3). For the genes identified by the combinations of heat and chemicals, the gene interaction between DEAD box polypeptide 5 (*DDX5*) and v-myc myelocytomatosis viral oncogene homolog (*MYC*) was identified in the heat plus PBN group. The gene interaction between *IL16* and *JUN* was identified in the heat plus AAPH group. However, no reliable gene interaction was observed in the heat plus 6-FP group. In the heat plus chemical groups, apoptosis-related genes whose names were highlighted red color were observed (Figure 4).

Discussion

Here, we utilized heating at 44°C for 10 min as a hyperthermia treatment and found the enhancement of hyperthermia-induced apoptosis by various oxidative stresses. In order to elucidate the mechanism of signal transduction related to the enhancement and to explore specific genes responding to different oxidative stresses, we tried direct comparison of gene expression patterns in human lymphoma U937 cells treated with hyperthermia combined with 6-FP, PBN, or AAPH using cDNA microarrays at similar level of enhancement on apoptosis. The present results indicate that extra oxidative stress can enhance the hyperthermia-induced apoptosis with accompanying specific change of gene expression.

In the present study, direct comparison of the gene expression pattern in U937 cells treated with hyperthermia using cDNA microarrays containing approximately 16,600 genes was made. We identified 39 up-regulated and 3 down-regulated genes. In addition, the change of expression levels of selected genes was confirmed by the RT qPCR. There are several reports that utilize DNA microarray system for analyzing change of gene expression by hyperthermia. First, 23 of 1046 genes were found as heat responding genes in Jurkat cells after hyperthermia at 43°C for 4 h [19]. Second, 28 of 1176 cancer related genes were observed in squamous cell carcinoma IMC-3 cells after hyperthermia at 43°C for 30 min [20]. In addition, 664 of 12,814 genes were identified in HeLa cells after hyperthermia 44°C for 1 h [21]. These results commonly show up-regulation of HSP-related genes as early response to heat treatment. In the present study, 44°C was selected as an ideal temperature for clinical local heating in hyperthermic cancer therapy, and 10 min as minimal period and mild condition for apoptosis induction in U937 cells [22]. Most of the 39 up-regulated genes induced by hyperthermia alone are HSP-related genes and the evidence was accorded with previous findings [19–21]. Furthermore, the

Table SII. Regulated genes by heat treatment alone.

Genes	Fold change			Genbank accession no.	RT qPCR
	Exp.1	Exp.2	Average		
Cont. vs. HT					
Up-regulated					
PSAT1	1.7	1.9	1.8	NM_058179	
HMOX1	1.8	1.7	1.8	NM_002133	⋆
HSPC138	1.6	2.0	1.8	NM_016401	
GPR89	1.8	1.9	1.9	NM_016334	
LENG4	1.7	2.0	1.9	NM_024298	
CDC6	1.6	2.1	1.9	NM_001254	
MRPS6	1.7	2.1	1.9	NM_032476	
EIF1	1.9	2.0	1.9	NM_005801	
NARF	1.9	2.0	1.9	NM_031968	
ST13	1.9	2.0	2.0	NM_003932	
MAST3	1.9	2.1	2.0	XM_038150	
YT521	1.6	2.5	2.0	NM_133370	
C20ORF111	2.2	1.9	2.1	NM_016470	
RBM23	1.6	2.6	2.1	NM_018107	
ADFP	1.8	2.5	2.1	NM_001122	
ATP6V1B2	2.2	2.1	2.1	NM_001693	
ALAS1	1.9	2.5	2.2	NM_000688	
MOBKL2A	1.7	2.7	2.2	NM_130807	
STIP1	1.9	2.5	2.2	NM_006819	
LOC220763	2.0	2.6	2.3	XM_055551	
HSPB1	3.0	2.2	2.6	NM_001540	
MRPL18	2.7	2.5	2.6	NM_014161	
FKBP4	1.5	3.9	2.7	NM_002014	
SEPW1	1.9	3.8	2.9	NM_003009	⋆
AHSA1	2.0	3.9	2.9	NM_012111	
LOC347400	2.5	3.5	3.0	XM_293315	
PRNP	2.7	3.3	3.0	NM_000311	
LOC341473	2.0	4.1	3.1	XM_292092	
HSPD1	3.1	3.2	3.2	NM_002156	
LOC338801	2.6	4.0	3.3	XM_290583	
DNAJA1	3.5	4.6	4.0	NM_001539	⋆
HSPA1B	4.0	4.5	4.2	NM_005346	⋆
HSPA1A	4.3	4.7	4.5	NM_005345	
DNAJA4	4.6	4.7	4.6	NM_018602	
BAG3	6.5	3.2	4.9	NM_004281	⋆
HSPA6	7.4	2.8	5.1	NM_002155	⋆
IER5	7.1	5.1	6.1	NM_016545	
HSPH1	6.1	8.2	7.2	NM_006644	⋆
DNAJB1	13.7	9.2	11.5	NM_006145	⋆
Down-regulated					
CCL2	0.32	0.25	0.29	NM_002982	⋆
C1QR1	0.49	0.59	0.54	NM_012072	
DKFZP566N034	0.54	0.62	0.58	NM_030923	

Microarray analysis was performed according to the experimental condition described in Materials and methods; ⋆ heat shock-, apoptosis- or oxidative stress-related genes chosen in RT qPCR.

functional relationships of the candidate genes were examined using the computational gene expression analysis tools. Interestingly, we were able to identify a genetic network that centered the *STIP1* gene interacting with the other heat shock-related genes (Figure 3). It has been reported that *STIP1* is regulated by cell cycle kinases and mediates the assembly of the Hsp70/Hsp90 chaperone heterocomplex [23]. A relationship between *STIP1* and *DNAJA1* [24], *DNAJB1* [24], *ST13* [25] or *PRNP* [26] has been demonstrated. Furthermore, the computational gene expression analysis has indicated that apoptosis- and unfolded protein response-related genes were differentially expressed in the hyperthermia condition (Figure 3). It has been well known that HSPs are induced by various stresses and function as molecular chaperones [27]. Most of HSPs also have been known to possess anti-apoptotic action [28,29]. Of the identified genes, up-regulation of *BAG3* appears to be due to cellular stress response against hyperthermia to induce apoptosis, since this gene has been known to act as anti-apoptotic molecules by interacting with *HSP70* and/or by binding with *BCL-2* [30,31]. Up-regulation of either *SEPW1* or *HMOX1* is presumably

Table SIII. Regulated genes by the combinations of heat treatment and chemicals.

Genes	Fold change			Genbank accession no.	RT qPCR
	Exp.1	Exp.2	Average		
HT vs. HT + 6-FP					
Up-regulated					
LOC219962	2.0	1.6	1.8	XM_166920	
NEUROD4	2.4	2.6	2.5	NM_021191	
Down-regulated	Not detected				
HT vs. HT + PBN					
Up-regulated	Not detected				
Down-regulated					
LOC151103	0.24	0.60	0.42	XM_098004	
C15ORF12	0.39	0.53	0.46	NM_018285	
ITGA5	0.50	0.48	0.49	NM_002205	
SEPW1	0.32	0.67	0.50	NM_003009	*
NARF	0.45	0.56	0.50	NM_031968	
CHERP	0.41	0.63	0.52	NM_006387	
SEPHS2	0.41	0.63	0.52	NM_012248	
GPR89	0.48	0.59	0.53	NM_016334	
SPAG5	0.44	0.64	0.54	NM_006461	
NULP2	0.44	0.64	0.54	NM_007342	
MYC	0.56	0.53	0.55	NM_002467	
NP	0.56	0.54	0.55	NM_000270	
DDX5	0.45	0.66	0.55	NM_004396	
SLC3A2	0.46	0.64	0.55	NM_002394	
HMOX1	0.46	0.65	0.55	NM_002133	*
LENG	0.48	0.63	0.55	NM_024298	
DNAJA1	0.52	0.63	0.58	NM_001539	*
RAC1	0.59	0.57	0.58	NM_018890	
MRPL14	0.51	0.65	0.58	NM_032111	
HT vs. HT + AAPH					
Up-regulated					
GADD45B	2.1	1.9	2.0	NM_015675	*
IER3	2.6	2.0	2.3	NM_003897	
Down-regulated					
IL16	0.36	0.47	0.42	NM_004513	
CKS2	0.27	0.62	0.44	NM_001827	
LOC338801	0.33	0.64	0.48	XM_290583	
C15ORF12	0.30	0.67	0.49	NM_018285	
AP4B1	0.47	0.51	0.49	NM_006594	
IFNGR2	0.32	0.67	0.49	NM_005534	
LOC90670	0.42	0.58	0.50	XM_033352	
EIF5	0.48	0.54	0.51	NM_001969	
SLBP	0.49	0.54	0.52	NM_006527	
HSPD1	0.51	0.55	0.53	NM_002156	
NOTCH1	0.39	0.67	0.53	NM_017617	
GPR89	0.53	0.62	0.57	NM_016334	
KIAA0174	0.50	0.65	0.58	NM_014761	
TCTEL1	0.51	0.66	0.58	NM_006519	*
SEPW1	0.53	0.65	0.59	NM_003009	
SPAG5	0.60	0.58	0.59	NM_006461	*
DNAJA1	0.56	0.65	0.60	NM_001539	

Microarray analysis was performed according to the experimental condition described in Materials and methods; * heat shock-, apoptosis- or oxidative stress-related genes chosen in RT qPCR.

due to cellular response against oxidative stress, since *SEPW1* or *HMOX1* has been known as a molecule of free radical scavenging [32,33] or anti-oxidizing and anti-apoptotic one [34,35], respectively. Recently, transient increase of intracellular superoxide formation induced by hyperthermia at 44°C for 10 min and its maintaining for 1 h in U937 cells has been already shown [11]. As *JUN* has been reported as an early and sensitive gene to hyperthermia treatment in U937 cells [18], RT qPCR revealed up-regulation of this gene induced by 44°C hyperthermia for 10 min. In this study, *CCL2* was identified as a down-regulated

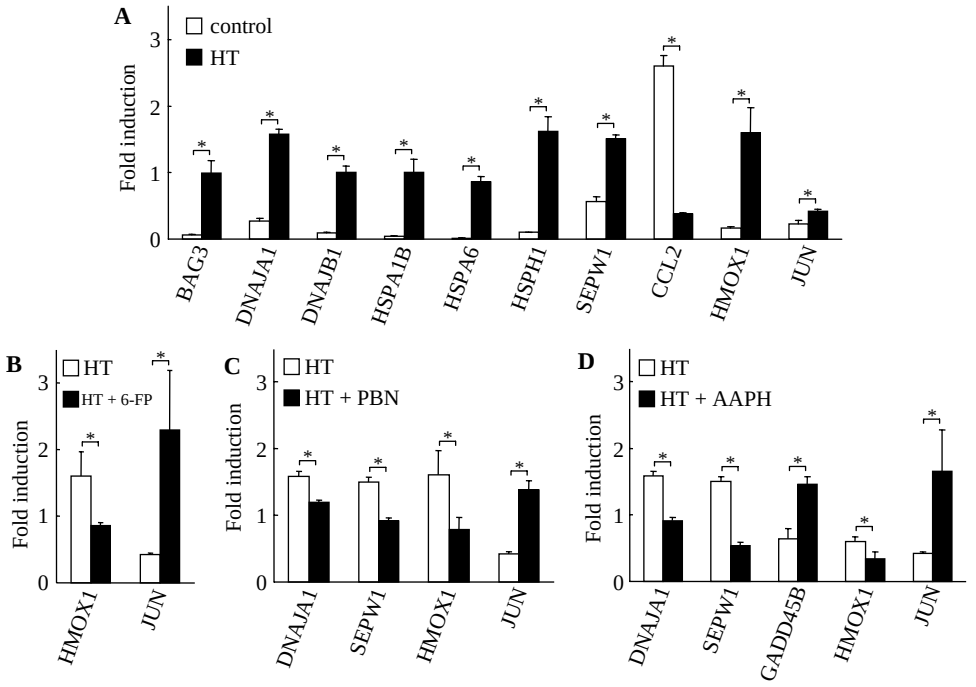

Figure 2. Verification of DNA microarray results with RT qPCR. U937 cells were treated with the combination of heat shock (44.0°C, 10 min) and 6-FP (0.3 mM), PBN (2 mM), or AAPH (20 mM) and then harvested after incubation for 3 h. RT qPCR was performed according to the manufacture's instructions. Each mRNA expression level was normalized by GAPDH. (A) heat treatment vs. control; (B) 6-FP vs. heat treatment; (C) PBN vs. heat treatment; (D) AAPH vs. heat treatment. Data indicate mean ± SD for four different experiments. $^{*}p < 0.05$ (Student's *t*-test).

gene by hyperthermia. It has been reported that *CCL2* suppresses apoptosis induced by hypoxia in rat cardiac myocytes [36]. Taken together, the genetic network and differentially expressed genes identified here may be closely associated with the hyperthermia-induced apoptosis in U937 cells.

Our previous findings have shown that alkyl radicals from AAPH and hydrogen peroxide due to 6-FP significantly enhance heat-induced apoptosis due to mitochondria dysfunction, intracellular superoxide formation, lipid peroxidation, and activation of caspases [6,9,10]. Recently, we also found enhancement of apoptosis by nitric oxide released from PBN under hyperthermic condition where intracellular oxidative stress was generated [11]. Although PBN has been widely used as a spin trap for electron paramagnetic resonance-spin trapping studies and antioxidant *in vitro* and *in vivo* because PBN react with oxygen radicals to produce less reactive species [37–41], it has been shown that PBN can act as a nitric oxide donor under oxidative condition [42,43]. In the present study, very interestingly, the combined treatment with heat and either chemical enhanced apoptosis level and showed a chemical-specific gene expression pattern. However, unexpectedly, only modification of change of gene expression was observed. The number of changes of gene expression in the cells treated with hyperthermia and chemicals were lower than those in the cells with hyperthermia alone. The evidence could be explained as follows. Roles of different oxidative stresses are considered to be only modification of change of gene expression due to hyperthermia, because most of genes observed changes in heat-treated cells are heat-responding genes and none of new genes was found in the cells treated with heat and chemicals. The chemical concentrations used in this study were lower than those that induce apoptosis, and these conditions infer not to influence gene expression directly. Particularly, the number of change of gene expression in the cells treated with hyperthermia and 6-FP was much lower than those in the heat-treated cells with AAPH and PBN. It has been demonstrated that the toxicity of 6-FP, a metabolite of folic acid, is extremely low [44,45]. Microarray and RT qPCR analyses revealed the down-regulation of *MYC* gene in the heat-treated cells combined with PBN. MYC, a transcription factor, is reported to be a multifunctional protein concerning stress response, apoptosis and cell cycle. Mu et al. [46] previously indicated that MYC protein decreases the hydrogen peroxide-enhanced oxidative stress of cells. Previous report also demonstrated that down-regulation of MYC mRNA increases apoptosis of leukemic cell lines [47]. Decrease in expression of *MYC* may

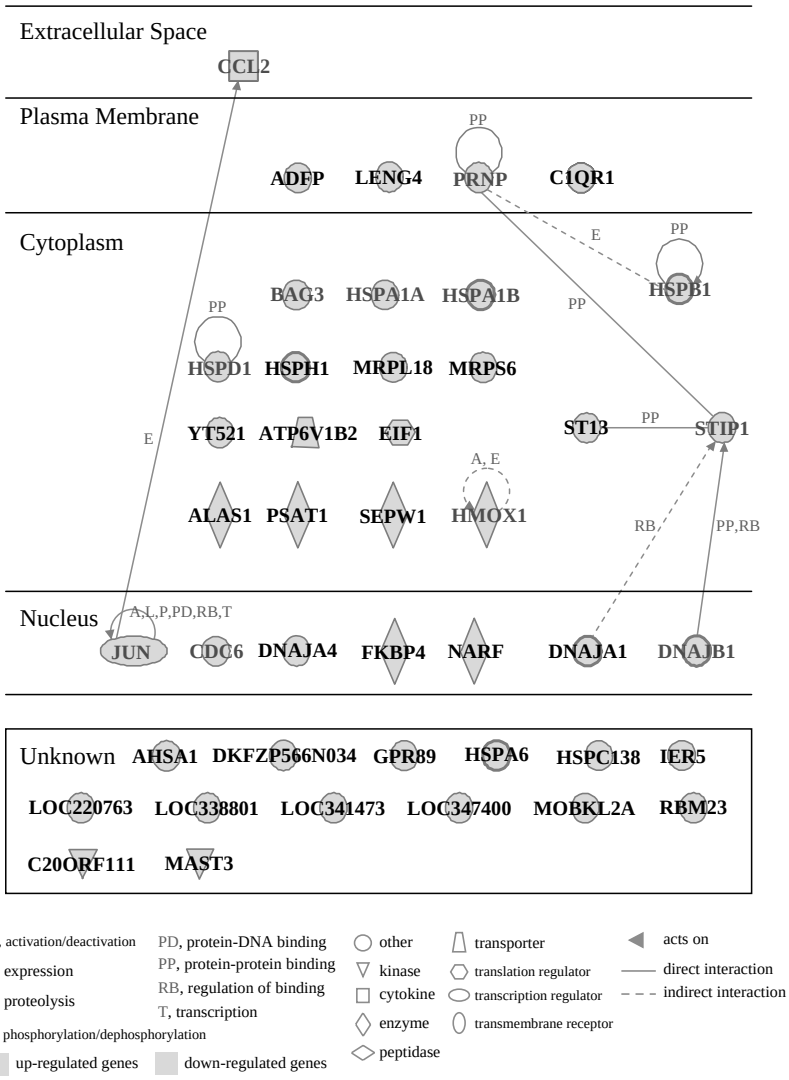

Figure 3. Relationship of genes associated with heat shock alone. The genes associated with heat shock (44.0°C, 10 min) alone were analyzed by the Ingenuity Pathways Analysis tool. The network is displayed graphically as nodes (genes) and edges (the biological relationships between the nodes). Location of the gene is also presented. Nodes and edges are displayed in various shapes and labels that present the functional class of genes and the nature of the relationship between the nodes, respectively. The names or edges of genes associated with apoptosis or unfolded protein response are highlighted.

participate in enhancement of apoptosis in heat-treated cells with PBN. In the heat-treated cells with AAPH, the up-regulation of gene expression for *GADD45B* was detected. *GDD45B* is known to protect cells by regulation of cell cycle and suppression of apoptosis in damaged cells under various stresses [48]. Up-regulation of *GDD45* may be specific heat stress response combined with AAPH to generate alkyl radicals and alkyl peroxyl radicals. Interestingly, decrease of *HMOX1* expression and increase of *JUN* were commonly observed in heat-treated cells under different oxidative stresses. *HMOX1* (inducible Hsp32), one of HSP families, is known to function against oxidative stress [34,35]. A major component of AP1 transcription factor, JUN, has been reported to promote apoptosis induced by ultraviolet and heat [49,50]. Since it has been reported that MAP kinase which is important for activation of *JUN* in JNK pathway, plays a role for the regulation of transcription of *HMOX1* expression [51], the detailed interaction between *HMOX1* and *JUN* remains a crucial subject in future study for better understanding the mechanism of enhancement of heat-induced apoptosis by free radical generators.

The data presented here indicate that under hyperthermic condition intracellular excess oxidative stresses from three chemicals modify the signal transduction system of apoptosis induced by hyperthermia and contribute to enhancement. These evidences will provide significant information on molecular pharmacological actions of hyperthermic sensitizers on apoptosis to the development of

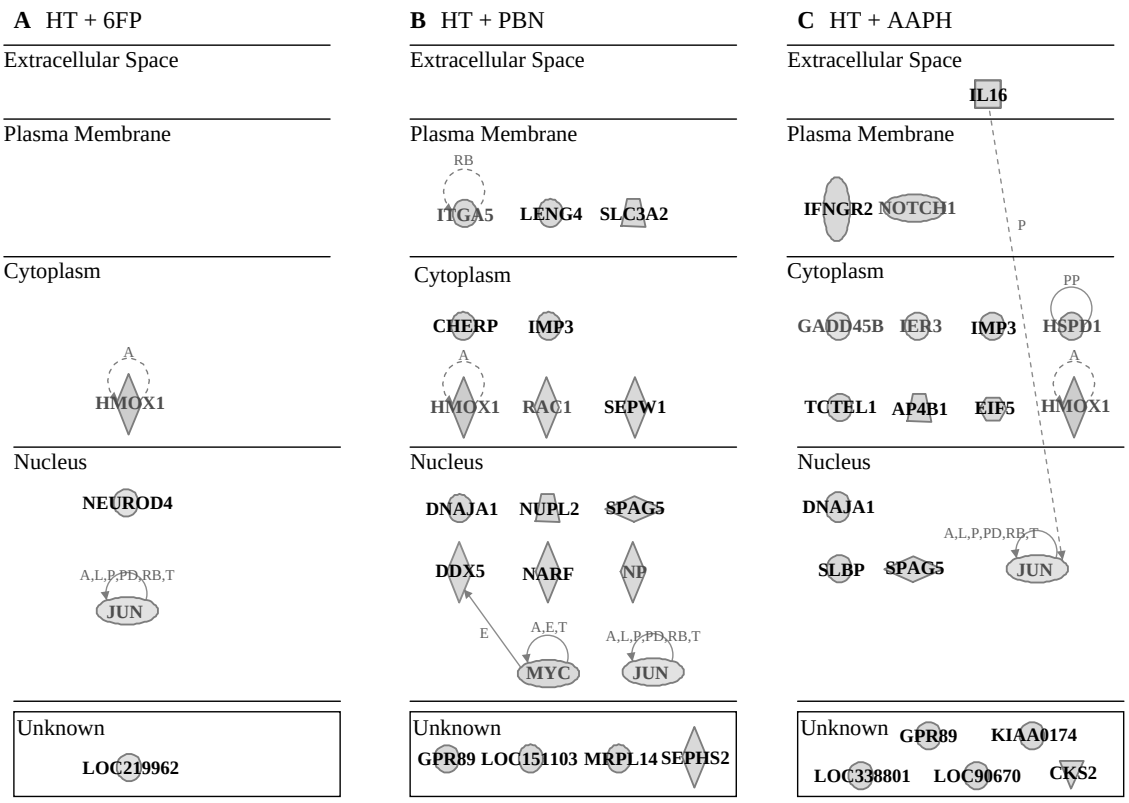

Figure 4. Relationship of genes associated with the combinations of heat shock and chemicals. The genes associated with each condition were analyzed by the Ingenuity Pathways Analysis tool. For the explanation of the symbols and letters, see Figure 3. The relationship is displayed graphically as nodes (genes) and edges (the biological relationships between the nodes). Location of the gene is also presented. Nodes and edges are displayed in various shapes and labels that present the functional class of genes and the nature of the relationship between the nodes, respectively. The names of genes associated with apoptosis or cell growth are highlighted.

therapeutic agents for future hyperthermic cancer therapy. This study is the first to report, using cDNA microarray system the different and common genes related to the iso-enhancement of apoptosis induced by hyperthermia. Since these results have been derived from a human lymphoma cell line, U937, which is relatively sensitive to hyperthermia-induced apoptosis, the evidence does not explain the comprehensive mechanism and gene network of apoptotic enhancement by hyperthermia. However, the current data appear to contribute to the better understanding of the sensitization mechanism on hyperthermia-induced apoptosis due to intracellular oxidative stress.

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