

Genomic Discovery Reveals a Molecular System for Resistance to Oxidative and Endoplasmic Reticulum Stress in Cultured Glioma

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Oxygen is required for respiration and the energetic processes that enable aerobic life. Reactive oxygen species and free radicals, by-products of oxygen use, cause DNA damage and induce endoplasmic reticulum (ER) stress and apoptosis. However, rapidly multiplying cancer cells are resistant to ER and oxidative stress-induced apoptosis. The present article reports the results of highly specific genome-scale expression discovery used to identify genes differentially expressed in cultured glioma cells vs normal brain tissue. The discovered states of expression reveal a cohesive molecular system that protects rapidly growing glioma cells from ER and oxidative stress-induced apoptosis. *Arch Neurol.* 2005;62:233-236

Oxidative and endoplasmic reticulum (ER) stress are physiologic mechanisms that prevent aberrant growth by the activation of programmed cell death. A cost associated with oxygen use is the formation of reactive oxygen species (ROS) that activate oxidative and ER stress responses, which lead to apoptosis. However, cancer cells can survive both high oxygen demand and misfolded proteins and still maintain rapid growth.

The ER is one of the largest cell organelles: its membrane constitutes more than half of the total membrane present in the cell, and its lumen makes up more than 10% of the cell volume. The ER has 2 essential functions: (1) folding, glycosylating, and sorting proteins to their proper destination and (2) synthesizing of lipids and cholesterol of the cell membranes. A quality-control mechanism ensures that only correctly folded proteins exit the ER. Incorrectly folded proteins are retained and ultimately degraded. Disruption of ER homeostasis interferes with protein folding and leads to the accumulation of unfolded and misfolded proteins in the ER lumen. This condition has been designated *ER stress*. Endoplasmic reticulum stress may arise from any of the follow-

ing: (1) accumulation of unfolded or misfolded proteins, (2) starvation of glucose (important for glycosylation), (3) oxidative stress, and (4) starvation of cholesterol. Activation of the ER stress response leads to protein synthesis inhibition and apoptosis.¹

Recent reports have described a mathematical algorithm for highly specific discovery (MASH) from the genome-scale expression profiling of 2 samples.^{2,3} In the present report, MASH is applied to analyze the expression data sets of 19200 complementary DNAs in cultured glioma cells as compared with normal brain tissue, which appears to best represent genetic expression in normal adult glial cells. Embryonal human glial cultures are not readily available, and genetic expression differs between embryonal and mature cells. The discovered states of genetic expression reveal a cohesive system of molecular interactions that protect glioma cells from ROS and ER-induced apoptosis.

METHODS

GLIOMA CELL LINES

The present experiments profile RNA isolated from 6 glioma cell lines and from normal brain tissue. Two glioma cell lines were

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Table. Genes Relevant to the Phenotype of Resistance to Oxidative and Endoplasmic Reticulum Stresses Discovered to Be Up-regulated in Cultured Glioma Cells Compared With Normal Brain Tissue

IMAGE Identification	Symbol	Expanded Name
501617	<i>AKR1A1</i>	Aldo-keto reductase family 1, member A1
209473	<i>AKR1C2</i>	Aldo-keto reductase family 1, member C2
268145	<i>NQO1</i>	NAD(P)H dehydrogenase, quinone 1
236069	<i>GSTP1</i>	Glutathione S-transferase pi
304962	<i>GCLM</i>	Glutamate-cysteine ligase, modifier subunit
356962	<i>PRDX1</i>	Peroxiredoxin 1
120292	<i>TXNRD1</i>	Thioredoxin reductase 1
234385	<i>PGD</i>	Phosphogluconate dehydrogenase
261610	<i>TALDO1</i>	Transaldolase 1
116454	<i>AFG3L1</i>	AFG3 ATPase family gene 3-like 1 (yeast)
135961	<i>ANT2</i>	Solute carrier family 25
130269	<i>PAI1</i>	Plasminogen activator inhibitor type 1
380727	<i>GAPD</i>	Glyceraldehyde-3-phosphate dehydrogenase
135782	<i>CYPB</i>	Peptidylprolyl isomerase B (cyclophilin B)
146910	<i>KDELRL1</i>	KDEL endoplasmic reticulum protein retention receptor 1
322626	<i>SEC61A1</i>	Protein transport protein Sec61 α subunit isoform 1
298590	<i>S1P</i>	Membrane-bound transcription factor protease, site 1
364351	<i>PP5</i>	Protein phosphatase 5, catalytic subunit
364936	<i>MCP1</i>	Chemokine (C-C motif) ligand 2
341045	<i>GRP94</i>	Tumor rejection antigen (gp96) 1
147814	<i>ATF4</i>	Activating transcription factor 4

Abbreviations: IMAGE, integrated molecular analysis of genomes and their expression; KDEL, receptor of endoplasmic reticulum proteins that share the carboxy-terminal sequence Lys-Asp-Glu-Leu; NAD(P)H, nicotinamide adenine dinucleotide phosphate.

purchased from American Type Culture Collection, Manassas, Va (T98G and U373MG). The others were cultured from a glioblastoma, an oligodendrogloma, and 2 astrocytoma tumor samples (provided by Herbert Engelhard, MD, PhD, University of Illinois at Chicago).

MICROARRAYS

Normal brain RNA was obtained by pooling RNA from human occipital lobes harvested from 4 individuals with no known neurologic disease whose brains were frozen less than 3 hours after death. Tumor RNA samples were extracted from 6 cultured glioma cell lines. The quality of RNA was assayed by gel electrophoresis; only high-quality RNA was processed. Tumor RNA was profiled in comparison with aliquots from the same normal brain RNA. Microarray experiments used 19K microarrays (Ontario Cancer Institute, Ontario, Canada); the design included probe switching (dye swapping), as previously described.^{3,4} Each 19K microarray contained 19200 complementary DNAs spotted in duplicates. The experiments generated 4 replicate measurements per gene and tumor.

DATA ANALYSIS

We applied MASH to analyze the data sets² and discovered the states of genetic expression, up- or down-regulation. The false discovery rate for MASH in 19K microarrays is 1 in 192000 measurements. We followed the following steps in the listed order: (1) applied MASH to find the genes differentially expressed in each cell line compared with the normal brain sample;

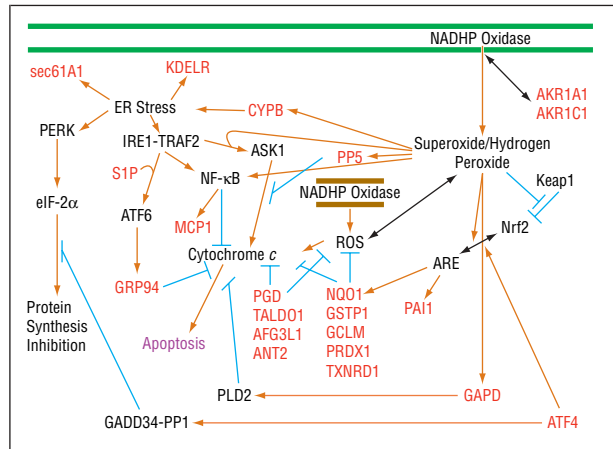


Figure. The genes listed in the Table predict a molecular system that enables glioma cells to resist oxidative and endoplasmic reticulum (ER) stress. Genes that are up-regulated in glioma cells compared with normal brain tissue are shown in red. Inhibitory and stimulatory (facilitating) “interactions” are depicted as cyan and orange, respectively. Double arrows indicate translocation or binding. Double green lines depict the plasma membrane. Double brown lines depict the mitochondrial membrane. PERK indicates double-stranded RNA-dependent protein kinase-like ER kinase or pancreatic ER kinase.

(2) found the set of genes, S, that were extracted by MASH in at least 1 of the 6 cell lines; and (3) identified the 4 “raw” replicate ratios of each of the genes of S in each cell line. We then applied a filter consisting of the following “fuzzy logic” rules in sequence: (1) all 4 replicate log₂ (ratios) of a gene in any cell line to be of the same sign and different than 0 (all 4 show either up- or down-regulation); (2) the mean of the 4 replicate ratios to be either greater than 1.5 or less than 0.67; (3) if rules 1 and 2 both apply, compute the mean of the replicate log₂ expression values; otherwise, exclude the genes by transforming the log₂ expression to 0; (4) exclude genes that are not resistant to both rules 1 and 2 in at least 5 of 6 cell lines; and (5) exclude genes that are simultaneously up-regulated in one cell line and down-regulated in another.³

RESULTS

The data revealed that 268 genes were consistently up- or down-regulated in at least 5 of 6 cultured glioma cell lines compared with normal brain tissue. The **Table** shows 21 up-regulated genes related to ER and oxidative stress.

OXIDATIVE STRESS

High ROS activity initiates a cascade of events that culminate in commitment to apoptosis through activation of the mitochondrial pathway and release of cytochrome c.^{5,6} The expression data (Table and **Figure**) reveal that gliomas adapted to high ROS activity by activating several pathways that protect them against apoptosis.

AKR1A1 and *AKR1C1* are up-regulated in glioma cells; they belong to the aldo-keto reductase superfamily of enzymes, which bind to the nicotinamide adenine dinucleotide (NAD⁺) as cofactors.⁷ Oxidative stress is associated with degradation of lipid peroxides, which generates toxic lipid aldehydes. *AKR1A1* and *AKR1C1* protect cells by efficiently detoxifying and reducing aldehydes and ketones.⁸

Several antioxidant genes are up-regulated in glioma cells. These include *PDG*, *TALDO1*, *AFG3L1*, and *ANT2* and the phase 2 genes *NQO1*, *GSTP1*, *GCLM*, *PRDX1*, and *TXNRD1*.⁹⁻¹¹ The antioxidant proteins protect against ROS-induced apoptosis by preventing the release of cytochrome c.^{12,13} Reactive oxygen species induce the expression of phase 2 genes by disrupting the cytoplasmic complex between the actin-binding protein Keap-1 (Kelchlike ECH-associated protein 1) and the transcription factor Nrf2 (NF erythroid 2-related factor 2), thereby releasing Nrf2 to migrate to the nucleus where it activates the antioxidant response element.¹⁴⁻¹⁸

MCPI and *GAPD* are up-regulated in gliomas. Nuclear factor κ B (NF- κ B) is directly activated by application of oxidizing agents, particularly hydrogen peroxide.¹⁹ Several laboratories have reported constitutive activation of NF- κ B in cultured glioma cells and glioblastoma surgical samples.^{20,21} Activated NF- κ B induces *MCPI* and protects against apoptosis by regulating several antiapoptotic proteins including *BCL2* (B-cell lymphoma/leukemia 2).²² *GAPD* is transcriptionally up-regulated by hypoxia²³; it mediates hydrogen peroxide-dependent activation of phospholipase D2, which protects against apoptosis.²⁴

ER STRESS RESPONSE

Glioma cells appear to acquire pathways to (1) recover from ER stress-induced protein inhibition and (2) prevent apoptosis. Chaperones within the ER lumen are responsible for folding newly synthesized proteins into their tertiary structures prior to their export to the Golgi. *KDEL1*, *CYPB*, and *Sec61A1* are up-regulated in gliomas. The receptor of ER proteins that share the carboxy-terminal sequence Lys-Asp-Glu-Leu (KDEL) contributes to a quality control system where newly synthesized misfolded or partially assembled proteins are retrieved to the ER.²⁵ The ER molecular chaperone *CYPB* is susceptible to oxidation by ROS.^{26,27} *Sec61A1* is a subunit of the *Sec61p* channel that mediates the retrograde export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation.²⁸

The ER stress response initiates several signaling pathways, including the phosphorylation of PERK (double-stranded RNA-dependent protein kinase-like ER kinase or pancreatic ER kinase) and the oligomerization and autophosphorylation of IRE1 (inositol requiring kinase 1) on the ER membrane leading to the formation of the IRE1-TRAF2 (tumor necrosis factor receptor-associated factor 2) complex (Figure). *ATF4* is up-regulated in glioma cells. The oligomerization and autophosphorylation of PERK sets off a phosphorylation cascade leading to inactivation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2 α) resulting in switching off protein synthesis. *ATF4* opposes ER stress-induced protein inhibition by inducing *GADD34* (growth arrest and DNA damage-inducible protein), which dephosphorylates eIF-2 α causing protein synthesis recovery.²⁹ *GADD34* recruits type 1 protein serine/threonine α to the ER where it dephosphorylates eIF-2 α .²⁹⁻³² *ATF4* also interacts with Nrf2 to regulate the expression of the genes induced by the antioxidant response element.^{33,34}

PP5 is up-regulated in glioma cells. The up-regulation of *PP5C* appears to grant glioma cells a survival advantage by neutralizing the proapoptotic effects of apoptosis signal-regulating kinase 1 (ASK1). Ubiquitously expressed, ASK1 is a MAPKKK (mitogen-activated protein kinase kinase kinase) that binds to IRE1 and TRAF2 to generate the IRE1-TRAF2-ASK1 complex, which activates the JNK (c-Jun N-terminal protein kinase) and p38 pathways and induces apoptosis through mitochondria-dependent caspase activation.³⁵⁻³⁹ *PP5* is a binding partner of ASK1; it directly dephosphorylates ASK1 and thereby inactivates its activity both in vitro and in vivo.

Paradoxically, the IRE1-TRAF2 complex may also protect against apoptosis by activating NF- κ B.^{1,40} Both *S1P* and *GRP94* are up-regulated in glioma cells. IRE1 activation appears to be upstream of ATF6 in the ER stress-signaling pathway.²⁵ ATF6 contains a single transmembrane domain with 272 amino acids oriented in the lumen of the ER, which senses ER stress and causes translocation to the Golgi, where it is cut in its luminal domain by S1P.^{41,42} S1P-mediated cleavage releases ATF6 from cell membranes for translocation to the nucleus, where it binds to DNA and induces the expression of several glucose-regulated proteins including tumor rejection antigen (gp96) 1. The latter stabilizes calcium homeostasis in the ER and protects against oxidative stress-mediated death.^{43,44}

COMMENT

These results reveal a molecular system in cultured glioma cells that protects against oxidative and ER stress-induced apoptosis. The rapid multiplication rate of cancer cells generates high levels of ROS, but a cohesive system of several molecular pathways protects rapidly growing glioma cells from ROS and ER stress-mediated apoptosis (Figure). Lincoln et al⁴⁵ and Perquin et al⁴⁶ find that antioxidant genes are up-regulated in aggressive thyroid, prostate, colorectal, and breast carcinomas. Furthermore, patients with cancer show changes in their plasma and urine consistent with excessive oxidative stress.⁴⁷ For example, patients with ovarian cancer have elevated plasma levels of thiobarbituric acid-reactive substances and conjugated dienes and low levels of antioxidants such as superoxide dismutase, catalase, vitamin C, and vitamin E.⁴⁸

The survival of patients with malignant astrocytomas is now about the same as it was 30 years ago.⁴⁹ The resistance of gliomas may stem from the redundancy and multiplicity of their molecular systems (Figure). The data present additional support to the idea that biological phenotypes are created by complex systems of gene-to-gene and gene-to-protein molecular interactions.³ Perturbation experiments and mathematical modeling of the dynamic behavior of this system may identify therapeutic targets that are best suited to reverse the resistant phenotype and kill glioma cells.

Accepted for Publication: July 20, 2004.

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REFERENCES

- Pahl HL. Signal transduction from the endoplasmic reticulum to the cell nucleus. *Physiol Rev.* 1999;79:683-701.
- Fathallah-Shaykh H, He B, Zhao L-J, Badruddin A. A mathematical algorithm for discovering states of expression from direct genetic comparison by microarrays. *Nucleic Acids Res.* 2004;32:3807-3814.
- Fathallah-Shaykh HM, He B, Zhao L-J, et al. Genomic expression discovery predicts pathways and opposing functions behind phenotypes. *J Biol Chem.* 2003;278:23830-23833.
- Fathallah-Shaykh H, Rigen M, Zhao L-J, et al. Mathematical modeling of noise and discovery of genetic expression classes in gliomas. *Oncogene.* 2002;21:7164-7174.
- Filomeni G, Aquilano K, Rotilio G, Ciriolo MR. Reactive oxygen species-dependent c-Jun NH2-terminal kinase/c-Jun signaling cascade mediates neuroblastoma cell death induced by diallyl disulfide. *Cancer Res.* 2003;63:5940-5949.
- Petrosillo G, Ruggiero FM, Paradisi G. Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. *FASEB J.* 2003;17:2202-2208.
- Sanli G, Blaber M. Structural assembly of the active site in an aldo-keto reductase by NADPH cofactor. *J Mol Biol.* 2001;309:1209-1218.
- Srivastava S, Chandra A, Bhatnagar A, Srivastava SK, Ansari NH. Lipid peroxidation product, 4-hydroxynonenal and its conjugate with GSH are excellent substrates of bovine lens aldose reductase. *Biochem Biophys Res Commun.* 1995;217:741-746.
- Shih AY, Johnson DA, Wong G, et al. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potentially protects neurons from oxidative stress. *J Neurosci.* 2003;23:3394-3406.
- Sekhar KR, Crooks PA, Sonar VN, et al. NADPH oxidase activity is essential for Keap1/Nrf2-mediated induction of GCLC in response to 2-indol-3-yl-methylenequinolindin-3-ols. *Cancer Res.* 2003;63:5636-5645.
- Lee JM, Calkins MJ, Chan K, Kan YW, Johnson JA. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. *J Biol Chem.* 2003;278:12029-12038.
- Nonn L, Berggren M, Powis G. Increased expression of mitochondrial peroxiredoxin-3 (thioredoxin peroxidase-2) protects cancer cells against hypoxia and drug-induced hydrogen peroxide-dependent apoptosis. *Mol Cancer Res.* 2003;1:682-689.
- Berggren MI, Husbeck B, Samulitis B, Baker AF, Gallegos A, Powis G. Thioredoxin peroxidase-1 (peroxiredoxin-1) is increased in thioredoxin-1 transfected cells and results in enhanced protection against apoptosis caused by hydrogen peroxide but not by other agents including dexamethasone, etoposide, and doxorubicin. *Arch Biochem Biophys.* 2001;392:103-109.
- Dinkova-Kostova AT, Holtzclaw WD, Cole RN, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A.* 2002;99:11908-11913.
- McMahon M, Itoh K, Yamamoto M, Hayes JD. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem.* 2003;278:21592-21600.
- Zipper LM, Mulcahy RT. The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J Biol Chem.* 2002;277:36544-36552.
- Wakabayashi N, Itoh K, Wakabayashi J, et al. Keap1-null mutation leads to post-natal lethality due to constitutive Nrf2 activation. *Nat Genet.* 2003;35:238-245.
- Velichkova M, Hasson T. Keap1 in adhesion complexes. *Cell Motil Cytoskeleton.* 2003;56:109-119.
- Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.* 1991;10:2247-2258.
- Wang H, Wang H, Zhang W, Huang HJ, Liao WS, Fuller GN. Analysis of activation status of Akt, NFkappaB, and Stat3 in human diffuse gliomas. *Lab Invest.* 2004;84:941-951.
- Robe PA, Bentires-Alj M, Bonif M, et al. In vitro and in vivo activity of the nuclear factor-kappaB inhibitor sulfasalazine in human glioblastomas. *Clin Cancer Res.* 2004;10:5595-5603.
- Bharti AC, Aggarwal BB. Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochem Pharmacol.* 2002;64:883-888.
- Yamaji R, Fujita K, Takahashi S, et al. Hypoxia up-regulates glyceraldehyde-3-phosphate dehydrogenase in mouse brain capillary endothelial cells: involvement of Na+/Ca2+ exchanger. *Biochim Biophys Acta.* 2003;1593:269-276.
- Kim JH, Lee S, Park JB, et al. Hydrogen peroxide induces association between glyceraldehyde 3-phosphate dehydrogenase and phospholipase D2 to facilitate phospholipase D2 activation in PC12 cells. *J Neurochem.* 2003;85:1228-1236.
- Zuzak TJ, Steinhoff DF, Sutton LN, Phillips PC, Eggert A, Grotzer MA. Loss of caspase-8 mRNA expression is common in childhood primitive neuroectodermal brain tumour/medulloblastoma. *Eur J Cancer.* 2002;38:83-91.
- van der Vlies D, Pap EH, Post JA, Celis JE, Wirtz KW. Endoplasmic reticulum resident proteins of normal human dermal fibroblasts are the major targets for oxidative stress induced by hydrogen peroxide. *Biochem J.* 2002;366:825-830.
- Derckx PM, Madrid SM. The foldase CYPB is a component of the secretory pathway of *Aspergillus niger* and contains the endoplasmic reticulum retention signal HEEL. *Mol Genet Genomics.* 2001;266:537-545.
- Pilon M, Schekman R, Romisch K. Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *EMBO J.* 1997;16:4540-4548.
- Ma Y, Hendershot LM. Delineation of the negative feedback regulatory loop that controls protein translation during ER stress. *J Biol Chem.* 2003;278:34864-34873.
- Brush MH, Weiser DC, Shenolikar S. Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Mol Cell Biol.* 2003;23:1292-1303.
- Connor JH, Weiser DC, Li S, Hallenbeck JM, Shenolikar S. Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor. *Mol Cell Biol.* 2001;21:6841-6850.
- Novoa I, Zeng H, Harding HP, Ron D. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J Cell Biol.* 2001;153:1011-1022.
- He CH, Gong P, Hu B, et al. Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein: implication for heme oxygenase-1 gene regulation. *J Biol Chem.* 2001;276:20858-20865.
- Rutkowski DT, Kaufman RJ. All roads lead to ATF4. *Dev Cell.* 2003;4:442-444.
- Ichijo H, Nishida E, Irie K, et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science.* 1997;275:90-94.
- Tobiume K, Matsuzawa A, Takahashi T, et al. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep.* 2001;2:222-228.
- Hatai T, Matsuzawa A, Inoshita S, et al. Execution of apoptosis signal-regulating kinase 1 (ASK1)-induced apoptosis by the mitochondria-dependent caspase activation. *J Biol Chem.* 2000;275:26576-26581.
- Takeda K, Matsuzawa A, Nishitoh H, Ichijo H. Roles of MAPKKK ASK1 in stress-induced cell death. *Cell Struct Funct.* 2003;28:23-29.
- Urano F, Wang X, Bertolotti A, et al. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science.* 2000;287:664-666.
- Kaneko M, Niinuma Y, Nomura Y. Activation signal of nuclear factor-kappaB in response to endoplasmic reticulum stress is transduced via IRE1 and tumor necrosis factor receptor-associated factor 2. *Biol Pharm Bull.* 2003;26:931-935.
- Chen X, Shen J, Prywes R. The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. *J Biol Chem.* 2002;277:13045-13052.
- Ye J, Rawson RB, Komuro R, et al. ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol Cell.* 2000;6:1355-1364.
- Wang Y, Shen J, Arenzana N, Tirasophon W, Kaufman RJ, Prywes R. Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. *J Biol Chem.* 2000;275:27013-27020.
- Berridge MJ. The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium.* 2002;32:235-249.
- Lincoln DT, Ali Emadi EM, Tonissen KF, Clarke FM. The thioredoxin-thioredoxin reductase system: over-expression in human cancer. *Anticancer Res.* 2003;23:2425-2433.
- Perquin M, Oster T, Maul A, Froment N, Untereiner M, Bagrel D. The glutathione-related detoxification system is increased in human breast cancer in correlation with clinical and histopathological features. *J Cancer Res Clin Oncol.* 2001;127:368-374.
- Banerjee D, Madhusoodanan UK, Nayak S, Jacob J. Urinary hydrogen peroxide: a probable marker of oxidative stress in malignancy. *Clin Chim Acta.* 2003;334:205-209.
- Senthil K, Aranganathan S, Nalini N. Evidence of oxidative stress in the circulation of ovarian cancer patients. *Clin Chim Acta.* 2004;339:27-32.
- Fathallah-Shaykh H. Darts in the dark cure animal but not human brain tumors. *Arch Neurol.* 2002;59:721-724.