J Huazhong Univ Sci Technol［Med Sci］
DOI 10.1007/s11596-009-0606-2 29(6):705-709,2009

Suppressive Effects of Genomic Imprinted Gene PEG10 on Hydrogen Peroxide-induced Apoptosis in $L0_2$ Cells*

Yao LIU (刘 瑶)[1], Huanjun HUANG (黄焕军)[2#], Jusheng LIN (林菊生)[1], Qiang ZHANG (张 强)[1], Jinquan TAN (谭锦泉)[3], Jinghua REN (任精华)[4]
[1]*Department of Liver Diseases, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*
[2]*Department of Gastroenterology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*
[3]*Department of Immunology, School of Medicine, Wuhan University, Wuhan 430071, China*
[4]*Cancer Center, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430023, China*

Summary: The effects of PEG10 on hydrogen peroxide (H_2O_2)-induced apoptosis in human normal liver cell line $L0_2$ were investigated. The PEG10 gene was transfected into $L0_2$ cells by lipofectamine, the positive clone was screened by G418 and defined as $L0_2$/PEG10, while the cell transfected with empty expression vector (pEGFP-N1) was defined as $L0_2$/vector. $L0_2$/vector and parental $L0_2$ cells served as control. RT-PCR and Western blotting were employed to detect the expression of target genes. H_2O_2 (50–400 mmol/L) was administered to induce the apoptosis of $L0_2$ cells. Cells viability was measured by MTT and the morphological changes of apoptotic cells were determined by fluorescence microscopy using hoechst33342 nuclei staining. DNA fragmentation was observed by agarose gel electrophoresis. PEG10 mRNA and protein levels in $L0_2$/PEG10 cells were significantly increased as compared with those in the control cells. After treatment with 400 mmol/L H_2O_2 for 24 h, the cellular growth inhibition rate of $L0_2$/PEG10 cells was significantly lower (58.2%) than that of $L0_2$ (92.5%) and $L0_2$/vector (88%). Distinct morphological changes characteristic of cell apoptosis such as karyopyknosis and conglomeration were not observed in $L0_2$/PEG10. Ladder-like DNA fragmentation in a dose-dependent manner was observed in both $L0_2$ and $L0_2$/vector cell lines, but not in $L0_2$/PEG10. PEG10 over-expression significantly inhibited cytotoxicity induced by H_2O_2 on human normal liver cell line $L0_2$ by antagonizing H_2O_2-induced apoptosis.
Key words: genetic imprinting; gene PEG10; $L0_2$ hepatocytes; hydrogen peroxide; apoptosis

Paternally expressed gene 10 (PEG10) was identified on the basis of its location in an imprinted domain on human chromosome 7q21 and characterized as paternally expressed/maternally silenced[1–3]. Data from manipulating the expression of this gene in cancer cell lines indicated that PEG10 was over-expressed in hepatocellular carcinoma (HCC), including those with normal serum AFP and of small size[4–10]. These results suggested that PEG10 has growth-promoting activity and may play an important role in the carcinogenesis of human hepatocytes. Understanding the molecular basis of the abnormal imprinting of PEG10 will shed new light on the processes leading to liver disease.

The objective of this study was to investigate whether PEG10 is involved in H_2O_2-mediated cellular apoptosis. The $L0_2$ cells stably expressing PEG10 was established and designated as the $L0_2$/PEG10 cell strain, while the cells transfected with the empty expression vector was dubbed the $L0_2$/vector cell strain. We demonstrated that PEG10 promotes the proliferation rate of $L0_2$/PEG10 and inhibits H_2O_2-induced cytotoxicity and DNA fragmentation.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Genes, Cells and Main Reagents Eukaryotic vector pPEG10/neo containing a full-length human PEG10 cDNA (1027 bp) and human normal liver cell line $L0_2$ were stocked in our laboratory. The plasmid pEGFP-N1 was kindly provided by Dr. Shen Wenzhuang from the Department of General Surgery of Tongji hospital, Wuhan, China. The solution of H_2O_2 was diluted immediately prior to the start of the experiments and stored at 4°C during the experiments. RPMI 1640 and fetal bovine serum (FBS) were procured from Gibco, USA. Lipofectamine™ was the product of Invitrogen Co. Ltd., USA. Hoechst33342 was bought from Beyotime Biotechnology, China. MTT and dimethylsulfoxide (DMSO) were purchased from Sigma, USA. PCR primer was synthesized by Shanghai Shengong Co. Ltd., China.

1.1.2 Cell Culture Cells were cultured in RPMI 1640 medium supplemented with penicillin/streptomycin (100

Yao LIU, E-mail: hxb3114@yahoo.com.cn
#Corresponding author
*This project was supported by grants from National Natural Sciences Foundation of China (No. 30471983, 30872237) and Major State Basic Research Development Program of China (973 Program) (No. 2007CB512900).

U/mL and 100 μg/mL respectively) and 10% FBS at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 and passaged every three days.

1.2 Methods

1.2.1 Gene Transfection and Establishment of Stable Cell Lines $L0_2$ cells were seeded into a 24-well culture plate at a cell density of about 1×10^4 cells. At the same time, non-transfected (normal $L0_2$), pEGFP-N1 and pPEG10/neo transfected groups were designated. Gene transfection was conducted in accordance with the protocol of lipofectamine2000™ recommended by the manufacturer. After incubation at 37°C for 24 h, the cells were digested by 0.25% trypsinase and seeded into a 6-well plate at 1×10^8 cell density, and then selected with 400 mg/L G418 for 3 weeks. When most of the non-transfected cells were dead, the concentration of G418 was decreased to 200 mg/L and maintained for another 3 weeks. After formation of cellular clones, subclones were chosen at random and amplified. The subclone cells were named $L0_2$/vector and $L0_2$/PEG10, respectively.

1.2.2 Detection of Cellular PEG10 mRNA Expression The fragment of PEG10 was amplified from RNA of $L0_2$/PEG10 by RT-PCR, and the primers were as follows: 5'-GTC GAC ACC ATG ACC GAA CGA AGA AG-3' (sense), 5'- GGA TCC TTA TAT AGG GCC GGG GAG TTT-3' (antisense). The PCR conditions were as follows: 95°C for 5 min, 95°C for 45 s, 58.5°C for 30 s, 72°C for 1 min, 35 cycles and 72°C for 10 min. β-actin served as an internal standard and the primers were as follows: 5'-GGC ATC GTG ATG GAC TCC G -3' (sense), 5'-GCT GGA AGG TGG ACA GCG A -3' (antisense). The PCR reactions were performed for 30 cycles at 94°C for 1 min, 58.5°C for 30 s, 72°C for 50 s. PCR fragments were separated electrophoretically on 1.5 % agarose gel.

1.2.3 Detection of Cellular PEG10 Protein Expression Protein (50 μg) from each sample was eletrophoresed on 10% SDS-polyacrylamide gel and transferred to PVDF membranes. Membranes were blocked with 5% (w/v) non-fat milk in Tween-TBS (TBST) overnight at 4°C and incubated with the anti-PEG10 antibody diluted at 1:1000 in TBST for 1 h at room temperature. Membranes were washed three times with TBST and incubated with a secondary antibody at a dilution of 1:5000 at room temperature for 1 h. Immunodection was carried out by using an ECL kit.

1.2.4 Cellular Proliferation Assay $L0_2$, $L0_2$/vector and $L0_2$/PEG10 cells were seeded onto 96-well plates at $3–5\times10^5$ cells/mL. For each cell line, 50 mmol/L H_2O_2 group, 100 mmol/L H_2O_2 group, 200 mmol/L and 400 mmol/L H_2O_2 group were designed, with each group having 5 wells. After treatment with H_2O_2 for 24 h, 10-μL MTT solution (5 mg/mL) was added to each well and the samples were cultured for another 4 h. The formazan crystals were dissolved in 100-μL DMSO and the absorbance was measured at 570 nm using an ELISA plate reader. The growth inhibitory rate was calculated by the following formula: Growth inhibition rate (%)=(1–Average A values of the experimental group/ Average A values of the control group)×100%. All the experiments were repeated at least three times separately to confirm their reproducibility, and data from one representative experiment were used.

1.2.5 Hoechst33342 Staining $L0_2$, $L0_2$/vector and $L0_2$/PEG10 cells (1×10^5/mL) were plated onto 25 mL culture flasks. The next day the cells were treated with 50, 100, 200 and 400 mmol/L H_2O_2. At 24 h following initiation of the culture, apoptosis was assessed by Hoechst33342 staining. The changes in nuclei were observed with a fluorescent microscope (Olympus, USA) through a UV-filter.

1.2.6 Agarose Gel Electrophoresis for Analysis of DNA Fragmentation Cells (1×10^6/ mL) after treatment were washed and suspended in 400 μL cell lysis buffer (5 mmol/L Tris–HCl, 10 mmol/L NaCl, 100 mmol/L EDTA, 1 % (w/v) SDS, 50 mg/mL proteinase K, pH 7.4). After incubation overnight at 50°C for complete digestion, DNA was extracted twice with equal volume of phenol, which was followed by two extractions with chloroform. The DNA was then precipitated by adding two volumes of absolute ethanol, which was followed by centrifugation at 14 000 r/min for 20 min. Extracted DNA was dissolved in 20 μL TE buffer. Samples were then loaded onto a 1.5 % agarose gel and electrophoresis was carried out at 60 V in TBE buffer.

1.3 Statistical Analysis

Data were expressed as $\bar{x}\pm s$ and analyzed by using SPSS12.0 software package.

2 RESULTS

2.1 RT-PCR Analysis

Fig. 1 shows that, after electrophoresis of RT-PCR products, a PEG10 (996 bp) amplification band was observed in $L0_2$/PEG10. $L0_2$ and $L0_2$/vector cells had no bands. β-actin (613 bp) served as internal controls.

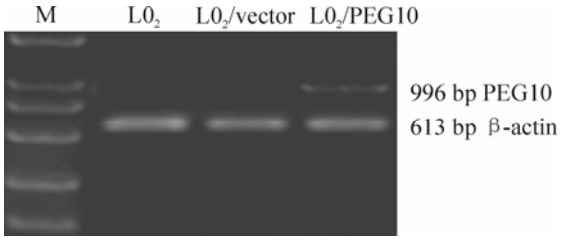

Fig. 1 Cellular PEG10 expression detected by RT-PCR
M: DNA marker DL2000

2.2 PEG10 Protein Detection by Western Blotting

The expression of PEG10 in cells was confirmed by Western blotting. The PEG10 protein was detected in $L0_2$/PEG10 cells, suggesting a stable transfection. In contrast, the wild $L0_2$ cells and the $L0_2$/vector cells did not express PEG10 (fig. 2). GAPDH served as internal controls.

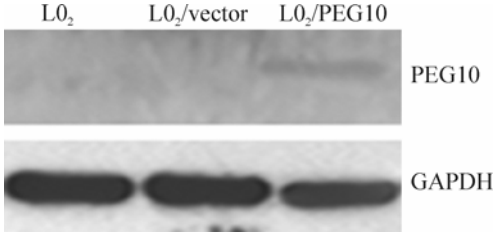

Fig. 2 Cellular PEG10 expression detected by Western blotting

2.3 Assessment of Cell Viability by MTT

MTT assay showed that, after exposure of exponentially growing cells to 50, 100, 200, 400 mmol/L H_2O_2 for 24 h, the growth of $L0_2$, $L0_2$/vector and $L0_2$/PEG10 cells was reduced in a dose-dependent manner. After treatment with 400 mmol/L H_2O_2 for 24 h, the inhibitionn rate of $L0_2$/PEG10 cells was 58.2%, while that of $L0_2$ cells reached 92.5%, and the difference between these two groups was significant (t=6.42, P<0.01). The difference in growth inhibitory rate between $L0_2$ and $L0_2$/vector cells (88 %) was not significant (P>0.05) (fig. 3).

2.4 Detection of Apoptosis by Fluorescence Microscopy

Apoptotic cell death in $L0_2$, $L0_2$/vector and $L0_2$/PEG10 cells were examined by fluorescent microscopic examination of hoechst33342-stained cells. After $L0_2$/PEG10 cells were treated with H_2O_2 (400 mmol/L) for 24 h, a significant decrease in the percentage of apoptotic cells was observed (fig. 4).

2.5 Formation of 'DNA Ladder' during Apoptosis in $L0_2$/Vector Cells

To test whether H_2O_2 induced cytotoxic effects in $L0_2$, $L0_2$/vector and $L0_2$/PEG10 cells via apoptosis, we detected intranucleosomal DNA fragmentation, a hallmark of apoptosis, by agarose gel electrophoresis. As shown in fig. 5, ladder-like DNA fragmentation was noted in a dose-dependent manner in $L0_2$, $L0_2$/vector cell lines 24 h after H_2O_2 treatment. In the $L0_2$/PEG10 cells, DNA fragmentation was significantly reduced.

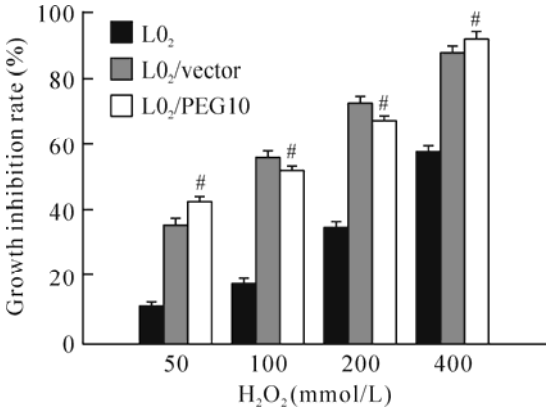

Fig. 3 Cell growth inhibition rate of stably transfected $L0_2$ ($L0_2$/PEG10) cells and control cells ($L0_2$, $L0_2$/vector) after the treatment of H_2O_2

Data are expressed as $\bar{x}\pm s$ of 3 independent experiments. $^{\#}P$<0.01 *vs* $L0_2$ and $L0_2$/vector groups

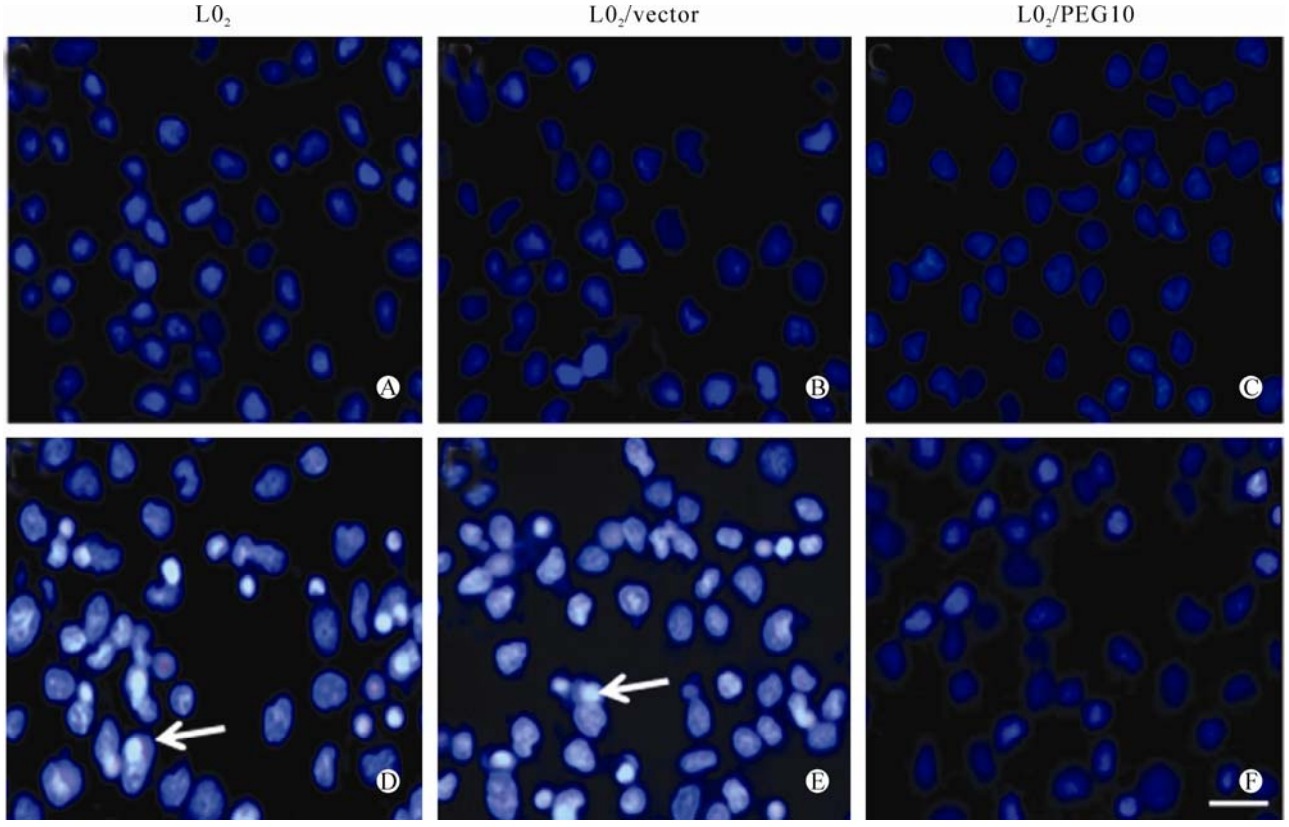

Fig. 4 Morphological changes of cell apoptosis detected by Hoechst33342 staining before (A, B, C) and after (D, E, F) treatment of H_2O_2 (400 mmol/L)

Arrows denote condensed, fragmented nuclei. Magnification in (A) to (F) was 200 times. Scale bar in (F) equals 40 μm and applies to all panels.

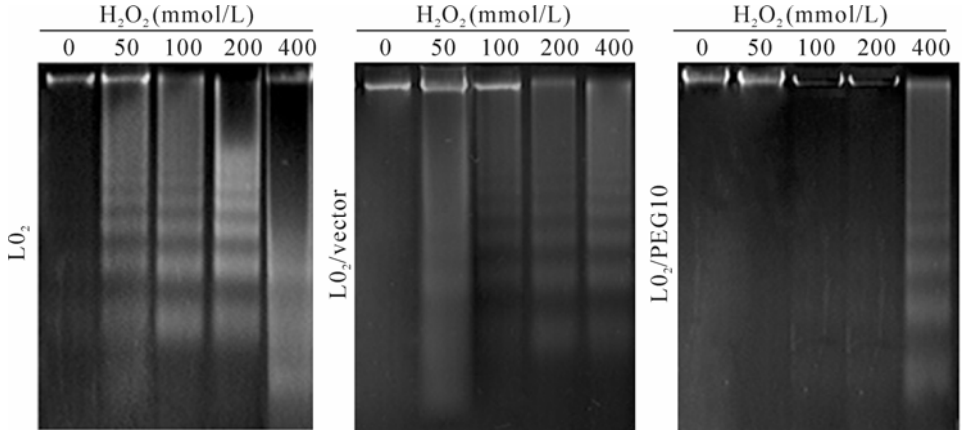

Fig. 5 Formation of DNA ladder during apoptosis

Cells were treated with H_2O_2 at the concentration as indicated at 37°C, 5% CO_2 for 24 h. After treatment, DNA was extracted and analyzed by agarose gel electrophoresis.

3 DISCUSSION

HCC is a leading cause of death worldwide and is especially prevalent among Asian populations. Similar to other human cancers, HCC is caused by multiple mechanisms among which chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), intake of food contaminated with chemical carcinogens and excessive consumption of alcoholic beverages are considered to be the major risk factors for HCC development[1, 5, 8, 9].

Compelling evidence suggests that most known environmental risk factors for HCC development lead to generation of reactive oxygen species (ROS) and are associated with oxidative stress[11–13]. Moreover, ROS production is part of the inflammatory processes. As we all known it, inflammation is a common response in the human liver and is involved in chronic hepatitis, cirrhosis, steatosis, ischemia-reperfusion damage, hepatocarcinomas and metastasis[12]. Liver infiltration by activated phagocytic cells provides an additional source of ROS production that promotes oxidative stress via interleukin or NO production that can damage proteins, lipids, DNA and mitochondrial transmembrane potential ($\Delta\Psi m$)[14]. Increasing evidence indicates that mitochondrial dysfunction often participates in the induction of apoptosis and in many system, decreased $\Delta\Psi m$ is an early requirement for apoptosis[15–18].

Expression of PEG10 was found in the brain, kidney, lung, testis and placenta, but not in the liver and a number of other tissues[1–3]. Transfer of PEG10 into hepatoma cells that expressed non-detectable endogenous PEG10 protein elicited significant growth promotion activity [6]. Okabe H *et al* found the imbalance between the expression of PEG10 and SIAH Ⅰ, a mediator of apoptosis, may be involved in hepatocarcinogenesis through inhibition of apoptosis[19]. Huang *et al* demonstrated that SiRNA eukaryotic expression vectors targeting PEG10 gene can induce HepG2 cell growth inhibition and apoptosis through down-regulating PEG10 and Cyclin D1[20–22].

Our study demonstrated that PEG10, a cell apoptosis inhibitor, could inhibit the apoptosis-inducing effects of H_2O_2. The MTT assay showed that cell survival was markedly decreased in $L0_2$ and $L0_2$/vector, while in $L0_2$/PEG10 cells, no such remarkable changes took place after treatment with H_2O_2. When $L0_2$ and $L0_2$/vector cells were exposed to 400 mmol/L H_2O_2 for 24 h, typical apoptotic morphological changes of the $L0_2$ nuclei was observed by Hoechst33342 staining. Agarose gel electrophoresis of DNA extracted from H_2O_2-treated $L0_2$ and $L0_2$/vector cells showed ladder-like DNA fragmentation in a dose-dependent manner in $L0_2$, $L0_2$/vector cell lines and PEG10 reduced H_2O_2-induced DNA fragmentation.

It seems that an increase in the cellular H_2O_2 may be an important event of cell apoptosis. On the other hand, evidence suggests that the prolonged production of cellular oxidants has been linked to modification of gene expression[13]. In particular, signal transduction pathways are known to be activated by ROS, and they lead to the transcription of genes involved in cell growth regulating pathways[23, 24]. A recent study suggested that common genomic gains of PEG10 in HCC were derived from HBV carriers[25]. It is speculated that PEG10 gene may be directly activated by ROS or HBx which could inhibits cell apoptosis.

Although a substantial body of data from clinical and laboratory studies indicates that over-expression of PEG10 in cancer is associated with inhibited apoptosis, the roles of PEG10 are complicated and not fully understood. Our findings may help to gain insight into the hepatocarcinogenesis.

REFERENCES

1 Yuen MF, Cheng CC, Lauder IJ, *et al*. Early detection of hepatocellular carcinoma increases the chance of treatment: Hong Kong experience. Hepatology, 2000, 31(2):330-335

2 Ono R, Kobayashi S, Wagatsuma H, *et al*. A retrotransposon-derived gene, PEG10, is a novel imprinted gene located on human chromosome 7q21. Genomics, 2001,73(2):232-237

3 Ono R, Shiura H, Aburatani H, *et al*. Identification of a large novel imprinted gene cluster on mouse proximal chromosome 6. Genome Res, 2003,13(7):1696-1705

4 Ono R, Nakamura K, Inoue K, *et al*. Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. Nat Genet, 2006,38(1):101-106

5 Ip WK, Lai PB, Wong NL, *et al*. Identification of PEG10

as a progression related biomarker for hepatocellular carcinoma. Cancer Letters, 2007,250(2):284-291
6 Tsou AP, Chuang YC, Su JY, *et al*. Overexpression of a novel imprinted gene, PEG10, in human hepatocellular carcinoma and in regenerating mouse livers. J Biomed Sci, 2003,10(6 Pt 1):625-635
7 Li CM, Margolin AA, Salas M, *et al*. PEG10 is a c-MYC target gene in cancer cells. Cancer Res, 2006,66(2): 665-672
8 Jia HL, Ye QH, Qin L X, *et al*. Gene expression profiling reveals potential biomarkers of human hepatocellular carcinoma. Clin Cancer Res, 2007,13(4):1133-1139
9 Wang XW, Hussain SP, Huo TI, *et al*. Molecular pathogenesis of human hepatocellular carcinoma. Toxicology, 2002,43(7):181-182
10 Chang Y, Tao LW, Chen XP, *et al*. Specificity and significance of expression of imprinted gene PEG10 in hepatocellular carcinoma. World Chin J Digestol, 2005, 13(12):1408-1411
11 Toyokuni S. Novel Aspects of Oxidative Stress-Associated Carcinogenesis. Antioxid Redox Signal, 2006,8(7-8): 1373-1377
12 Olaya N. Oxidative stress and inflammation in liver carcinogenesis. Iatreia, 2007,20(1):S-20
13 López-Lázaro M. Dual role of hydrogen peroxide in cancer: Possible relevance to cancer chemoprevention and therapy. Cancer Letters, 2007,252(1):1-8
14 Ly JD, Grubb DR, Lawen A. The mitochondrial membrane potential (deltapsi(m)) in apoptosis; an update. Apoptosis, 2003,8(2):115-128
15 Hale AJ, Smith CA, Sutheland LC, *et al*. Apoptosis: molecular regulation of cell death. Eur J Biochem, 1996, 236(1):1-26
16 Jiang B, Liu JH, Bao YM, *et al*. Catalpol inhibits apoptosis in hydrogen peroxide-induced PC12 cells by preventing cytochrome C release and inactivating of caspase cascade, Toxicon, 2004,43(1):53-59
17 Clement MV, Hirpara JL, Pervaiz S. Decrease in intracellular superoxide sensitizes Bcl-2-overexpressing tumor cells to receptor and drug-induced apoptosis independent of the mitochondria. Cell Death Differ, 2003, 10(11):1273-85
18 Wei YH, Lee HC. Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. Exp Biol Med, 2002,227(9):671-682.
19 Okabe H, Satoh S, Furukawa Y, *et al*. Involvement of PEG10 in human hepatocellular carcinogenesis through interaction with SIAH1. Cancer Res, 2003,63(12): 3043-3048
20 Huang J, Lin JS, Dong XY, *et al*. Effects of siRNA enkaryotic expression vectors targeting PEG10 gene on cell cycle in HepG2 cells. Chin J Gastroenterol Hepatol, 2007,16(1): 33-35
21 Huang J, Lin JS, Chang Y, *et al*. The construction and identification of siRNA eukaryotic expression vector targeting genetic imprinted gene PEG10. Cancer Res Prev Treat (Chinese), 2007,34(2):86-88
22 Jie X, Lang C, Jian Q, *et al*. Androgen activates PEG10 to promote carcinogenesis in hepatic cancer cells. Oncogene, 2007,26(39):5741-5751
23 Baud O, Greene AE, Li J, *et al*. Glutathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. J Neurosci, 2004,24(7):1531-1540
24 Shinkai M, Shinomiya N, Kanoh S, *et al*. Oxygen stress effects on proliferation rates and heat shock proteins in lymphocytes. Aviat Space Environ Med, 2004,75(2): 109-113
25 Huang J, Sheng HH, Shen T, *et al*. Correlation between genomic DNA copy number alterations and transcriptional expression in hepatitis B virus-associated hepatocellular carcinoma. FEBS Lett, 2006,580(15):3571-3581

(Received Dec. 3, 2008)