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산화성 손상에 대한 MTH1 역할에 관한 연구

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지도교수 유 호 진

이 논문을 이학석사 학위신청논문으로 제출함.

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위 원 조선 대학교 교수 인

2005 년 11 월 일

조선대학교 대학원

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산화적 손상에 대한 MTH1 역할에 관한 연구

송 승 희

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정상 세포의 호흡과정중에 발생되는 Oxygen radicals 은 genomic 과 mitochondrial DNA 에서의 mutations 을 일으킬수 있다. Human MTH1 은 8-oxo-dGTP 나 2-hydroxy-dATP 와 같은 산화된 purine nucleoside triphosphates를 가수분해하여, 복제 동안 이들 산화 nucleotide의 삽입 착오를 방지한다. MTH1에 대해서 선택적인 small interfering RNAs (siRNAs) 가 설계, 합성, 사용되었고, DNA 손상에 대한 세포의 생존능력 전환 및 H_2O_2에 대한 세포의 저항성에 있어서의 효율성이 연구되었다. 인간 섬유아세포에서 MTH1 발현 억제는 H_2O_2에 대항하는 세포 생존능력을 감소시킨다. 게다가 MTH1 발현 억제는 H_2O_2 농도에 따라서 세포 죽음을 증가시킨다. 또한 MTH1 결핍세포에서 H_2O_2 투여 후 caspase 와 PARP 의 절단이 증가함을 관찰하였다. 이상의 연구결과 산화성 유전자 손상 복구 효소인 MTH1 은 산화성 스트레스에 의한 세포 사멸사 억제에도 관여할 것으로 사료된다.

ABSTRACT

Oxygen radicals generated through normal cellular respiration process can cause mutations in genomic and mitochondrial DNA. Human MTH1 hydrolyzes oxidized purine nucleoside triphosphates, such as 8-oxo-dGTP and 2-hydroxy-dATP, to monophosphates, thereby preventing the misincorporation of these oxidized nucleotides during replication. We designed, synthesized, and utilized small interfering RNAs (siRNAs) that were selective for MTH1 and investigated their effectiveness in altering the viability of the cells against DNA damage as well as the resistance of the cells to H_2O_2. The suppression of MTH1 expression in the human fibroblast cells lead to a decrease in the cell viability against H_2O_2. In addition, the cell death was increased concentration-dependent in the suppression of MTH1 expression. Moreover, MTH1 knockout cells have shown that caspase and PARP are fragmented after exposure of $H_2O_2$2 but, not control cells. These results suggest that MTH1, which is well known to repair the oxidative bases, also has an antiapoptotic function against oxidative stress.

I. INTRODUCTION

It has been estimated that around 2×10^4 DNA damaging events occur in every cell of the human body every day [1]. A significant portion of the damage is caused by reactive oxygen species (ROS). The effect of excessive production of ROS and/or the inadequacy of the anti-oxidant cellular defense systems to neutralize them commonly referred to as oxidative stress. ROS are constantly produced in the living cell [2-4]. They are generated as by-products of respiration, ROS constitute the major class of endogenous toxic agents in aerobic organisms. 8-Hydroxydeoxyguanosine (8-OHdG), an oxidized form of guanine, is the major oxidative DNA-damage product that can produce mutations—A:T to C:C or G:C to T:A transversion mutations—because of its base pairing with adenine as well as cytosine (reviewed in [5]). The oxidized bases and AP sites, as well as DNA single-strand breaks induced by ROS with 3′ phosphoglycolate blocked ends, are repaired predominantly by the DNA base excision repair (BER) pathway. The human homologes mutM (OGG1), mutY (MYH) and mutT (MTH) have been identified (reviewed in [6]). MTH1, a counterpart of *E. coli* MutT, possesses 8-oxo-dGTPase activity, which degrades oxidized nucleotide 8-oxo-dGTP to sanitize the nucleotide pool [7]. The *MTH1* gene consists of five major exons and is mapped on 7p22 [8]. An alteration of MTH1-expression with the accumulation of 8-OH-G in DNA is observed in neurodegenerative diseases [9].

Recently, several groups have reported that RNA interference (RNAi) is a form of post-transcriptional control in which the introduction of a double-stranded RNA (dsRNA) into a cell leads to the homology-dependent degradation of its cognate mRNA. Biochemical studies have revealed that cells contain evolutionary conserved RNAi machinery that triggers dsRNA cleavage into the 21-or 22-nucleotide small interfering RNAs (siRNAs). The siRNAs then hybridize into their cognate mRNA, which induces the specific degradation of the target mRNA [10-13]. Although the physiological significance of post-transcriptional gene silencing and RNA interference is currently under investigation, a powerful new technology for the selective inhibition of the specific gene expression employing siRNAs is rapidly evolving [14-17].

This study presents the data demonstrationg that MTH1 gene expression in human fibroblast cells is specifically suppressed by MTH1 siRNA, resulting in a significant decrease in the DNA viability capacity as well as an increase in the cell death to H_2O_2. These results show the feasibility of using siRNAs to specifically reduce the MTH1 expression level in human fibroblast cells and highlight the potential use of MTH1 siRNA as a new and highly effective strategy to H_2O_2.

II. MATERIALS AND METHODS

Cell culture

The human fibroblast cells were cultured in EMEM medium containing 10%FBS, 100U/ml penicillin, and 100 μg/ml streptomycin at 37℃ in a humidified atmosphere of 5% CO_2 .

Preparation of the constructs and clones

Human MTH1 cDNA was amplified by RT–PCR using the MTH1 oligo primer (5'-GGA TCC ATG AGT GGA ATT AGC CCT CAG C-3', 5'-GCG GCC GCC TAG ACC GTG TCC ACC TCG CG-3') from human fibroblast GM00637 cells. After confirming the DNA sequences, the MTH1 cDNA was cloned into a pcDNA3.1(+) mammalian expression vector(Promega). The transfections were performed using the LipofectAMINE method (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were incubated in complete medium containing 400 μg/ml G418 for 4–5 weeks. The cell clone that was resistant to G418 (for pc DNA3.1/MTH1) was isolated and analyzed.

siRNA design, synthesis, and expression vector transfected cell clones

Sequence information regarding the human MTH1 mRNA was extracted from the NCBI Entrez nucleotide data base. Three target sites within the human MTH1 genes were chosen from the human MTH1 mRNA sequences (GenBank™ accession no. AB025240). Following selection, each target site was searched with NCBI BLAST to confirm specificity only to the human MTH1. The sequences of the 21-nucleotide sense and antisense RNA are as follows: hMTH1-siRNA, 5'- GAAGAAAUUCCACGGGUACUU-3'(sense) and 5'-GUACCCGUG- GAAUUUCUUCUU-3' (antisense) for the hMTH1 gene (nt 540-560, Target sequence AAGAAGAAATTCCACGGGTAC); LacZ siRNA, 5'-CGUACGCGGAAUACU- UCGAUU-3'(sense), 5'-AAUCGAAGUAUUCCGCGUACG- UU-3' (antisense) for the LacZ gene. These siRNAs were prepared by a transcription-based method using a Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. LacZ siRNA was used as a negative control. siRNA expression vectors (pSilencer hygro) for MTh1 and a control vector were employed. The construction of siRNA-expression plasmids was base on the pSilencer hygro vector (Ambion). The vector included a human U6 promoter, a hygromycin resistance gene. We purchased synthetic oligonucleotides (XENOTECH, Korea). After annealing, DNA fragments were ligated into the pSilencer hygro. Cells were transfected with the siRNA duplexes by using Oligofectamine (Invitrogen).

Western blotting

The cells were washed with PBS and lysed at 0°C for 30 min in a lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4 and 5 mM NaF). The protein content was determined using the Bio-Rad dye-binding microassay (Bio-Rad, Hercules, CA), and 20 μg protein per lane was electrophoresed on 10% SDS polyacrylamide gels. The proteins were blotted onto Hybon ECL membranes (Amersham-Phamacia, Biotech) and immunoblotting was carried out with anti-一 MTH1, anti-cleaved caspase3, anti-cleaved caspase9, anti-cleaved PARP (Cell Signaling Technology), and anti--tubulin antibodies (Pharmingen). The blotted proteins were then detected using an enhanced chemiluminescence detect system (iNtRON, Biotech, Seoul, Korea).

Semiquantitative Reverse Transcriptase Polymerase Chain Reaction

RNA extraction from the GM00637 cell line was conducted using the RNA-STAT-60 according to the manufacturer's instructions (TEL-TEST, Inc., Friendswood, TX). Briefly, cells in the medium were exposed to 80uM H_2O_2 for 12h. After homogenizing cells in the RNA STAT-60, the homogenate was mixed with chloroform (5:1; v/v), shaken vigorously for 20 s, and then centrifuged at

13,000 rpm for 15 min at 4 °C. The RNA presented in the upper colorless aqueous phase was precipitated by adding isopropyl alchohol, washed twice with 70% ethanol, and air-dried for 5 min. The RNA was then resuspended in DEPC. 10-μl RNA aliquots were prepared and stored at –70 °C until needed. 2 μg of the total RNA were reverse-transcribed using a M-MLV cDNA synthesis system (Invitrogen), and the reverse-transcribed DNA was subjected to PCR. The profile of the replication cycles was denaturation at 94 °C for 2 min, annealing at 55 °C for 30 s, and polymerization at 72 °C for 2 min. In each reaction, the same amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. All of the primers were commercially synthesized by ZENOTECH ,Korea. (Table 1). The PCR products were resolved on 1.5% agarose gels, stained with ethidium bromide, and then photographed.

Flow cytometry

Cells (2×10^6) were centrifuged at 1000rpm for 5min, decanted and washed in PBS, centrifiged, decanted and resuspended in cold 80% ethanol with vigorous mixing to a final density of 1×10^6cells/ml. The cells were incubated at 4℃ for a minimum of 30min. The ethanol-fixed cells were then centrifuged, decanted, blotted to remove remaining ethanol. cells were washed in ice-cold PBS and resuspended in 25 μg of propidium iodide/ml and 50 μg of RNasa A/ml in PBS.

Sample were incubated at 37℃ for 2h and DNA profiles were analyzed by FACS using a FACScan flow cytometer(BD Biosciences).

Microarray analysis

The GM00637-sihygro, GM00637-siMTH1 cells were placed in 100mm plate at 1×10^6 cells. Sixteen hours after plating, the cells were treated with 80uM of H_2O_2. After 12h, the total RNA was isolated using a TriReagent (Sigma-Aldrich, St. Louis, MO) and further purified with RNeasy (Qiagen, Valencia, CA) according to the manufacturer's instructions. Hybridization was done with the cDNA from the H_2O_2 treated cells (GM00637-sihygro, GM00637-siMTH1) labeled with Cy5 and those from the control samples labeled with Cy3. Scanning was carried out using a GenePix 4000A scanner (Axon Instruments, Inc., Foster City, CA), and image acquisition was done using Axon GenePix image software. Analysis of the gene expression data was done using the GeneSpring software (Silicon Genetics, Inc., Redwood CITY, CA) and PathwayAssist (Ariadne Genomics, Rockvile, MD). Intensity-dependent normalization was also applied, where the ratio was reduced to the residual of the Lowess fit of the intensity versus the ratio curve. Statistical analysis was done using Student's *t* test with a *P* of 0.05 with the additional criteria of the Ras-expressing cells being either 1.5-fold higher or lower than the control transfected cells. The genes that met these variables were classified by a molecular function using the annotations from Silicon Genetics or Ariadne

Genomics.

Cytotoxicity assays

cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertraxoli- um bromide (MTT) assay. Cells were seeded at a concentration of 6000cells/well on a 96-well plate. After 24 h, cells were treated with H_2O_2 at concentrations of 40, 60, 80, 100, 120, 160, and 200uM for 24 h. After treatment, MTT(10mg/ml) was incubated with cells in a 96-well plate for 4 h at 37℃. The medium containing MTT was removed, and 100 $\mu\ell$ of dimethyl sulfoxide (DMSO) was added. cells were incubated for 15min at 37℃ with gentle shaking. The absorbance was read on a scanning elisa reader (BIO-TEK INSTRUMENTS,INC.) using a 540nm filter.

Table 1 Sequences of the primers used in RT-PCR analysis

Gene	Primer(20Mer)	GenBank database No.	PCR product size (bp)/Optimal annealing temperature)(℃)
GAPDH	5'-tgaccacagtccatgccatc-3'(sense) 5'-ttactccttggaggccatgt-3'(antisense)	NM_002046	492/58
BAD	5'-tgagccgagtgagcaggaag-3'(sense) 5'-aagttccgatcccaccagga-3'(antisense)	NM_004322	456/53
SULF1	5'-cagacacgttccttgtccgt-3'(sense) 5'-tcatgcgtgaagcaagtgag-3'(antisense)	NM_015170	626/54
PRDX2	5'-aagccagcccctgacttc-3'(sense) 5'-attccttgctgtcatccacg-3'(antisense)	DQ231563	550/55
HSPA1L	5'-cagcggctgcttcaggacta-3'(sense) 5'-ccagaaccatgcgctcaatc-3'(antisense)	NM_005527	523/53
MGMT	5'-gtgagcagggtctgcacgaa-3'(sense) 5'-ttcatgggccagaagccatt-3'(antisense)	NM_002412	446/53
SEPP1	5'-catcagcaccttggcagcagt-3'(sense) 5'-caactggcactggcttctgtg-3'(antisense)	NM_005410	457/55

III. RESULTS

siRNA-mediated down-regulation of MTH1

To investigate the effects of MTH1 siRNA on the MTH1 expression levels, the cell viability, four different 21-basepair siRNA constructs, MTH1-siRNA1, MTH1-siRNA2, MTH1-siRNA3, and control siRNA, were transfected into the GM00637 cells to test their ability to suppress MTH1 expression. The MTH1 mRNA level in the GM00637 cells transfected with 200nM each for the three different MTH1 siRNAs and the control siRNA. In order to obtain the quantitative MTH1 mRNA expression values, quantitative RT-PCR experiments were performed 24h, 48h after treating the cells with either the MTH1 siRNAs or control siRNA. This result indicated that the MTH1 siRNA3 inhibited MTH1 mRNA expression 24h, 48h after transfection (Fig.1). This result demonstrates that the MTH1 siRNA3 was specific to MTH1. This result suggests that MTH1 silencing in the GM00637 cells results from a reduction in the amount of MTH1 mRNA available for translation. This suggests that the MTH1-siRNA was highly specific and efficient in MTH1 gene silencing in the GM00637 cells. To better understand the mechanisms involved in this process, we have focused on siMTH1 gene. To that end, we first established a GM00637 fibroblast cell line that stably expresses the MTH1 and siRNA silencing of the MTH1. After transfection and

selection for neomycin and hygromycin resistance, respectively, cells were characterized by RT-PCR by using an MTH1 specific primer (Fig. 2).

Silencing of MTH1 expression via siRNA leads to a decrease in the cell viability capacity in response to H_2O_2

The cells were treated with H_2O_2 for 24h, after which time the cell number/viability was determined by the colorimetric MTT assay. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystal which is largely impermeable to cell membranes, thus resulting in its accumulating within healthy cells. The addition of a tensioactive agent(detergent) resulted in the liberation of the crystals which got solubilized. The number of surviving cells is directly proportional to the level of the formazan formed. The colour was then measured by using a simple colorimetric assay.

Plots of Fig. 3 show cell viability versus compound dose(uM). As show in plots, overexpression of MTH1 resulted in elevated and down-regulation of MTH1 resulted in reduced cell viability after exposure to H_2O_2, compared to the control vector-transfected cells, respectively. These results suggested that the transfection and subsequent expression of the MTH1-targeted siRNAs resulted in the

suppression of the cell viability capacity in response to H_2O_2.

Silencing of MTH1 expression via siRNA leads to a increase in the cell death in response to H_2O_2

GM00637-siMTH1 induced a decrease in cell viability of approximately 35%, we next investigated whether this effect could be due to apoptosis. Thus, we analyzed cell death by flow cytometry using propidium iodide in GM00637-MTH1 and GM00637-siMTH1 cell lines exposed to doses of H_2O_2 ranging from 40 to 120uM, from 20 to 80uM for 24hr, respectively.

Fig. 4 shows a concentration-dependent cell death. After treatment of H_2O_2, cell death was decreased in GM00637-MTH1 cells, compared with the control. And cell death was increased in GM00637-siMTH1 cells, compared with the control. These results indicated that the suppression of MTH1 gene expression leads to a increase in the cell death in response to H_2O_2.

Cell death by the suppression of MTH1 gene expression related the apoptosis

GM00637-siMTH1 cell line was analyzed caspase 3, 9 and PARP cleavage by western analysis with GM00637-sihygro control. Caspases, members of aspartate-specific cysteine proteases, play a pivotal role in the execution of programmed cell death[18,19]. It is believed that caspase 9 is initiator caspases activated in apoptosis induced by mitochondrial stress. Caspase 3 is one of the

effector caspases, which are activated by initiator caspases[20]. Caspase 3 has been reported to be the most grequentiy activated caspase protease in apoptotic cells, indicating its crucial role in the cell death process. PARP is a 116-kDa constitutive nuclear protein involved in DNA repair and a wellcharacterized substrate for caspase 3. Activated caspase3 cleaves PARP, generating 89-and 24-kDa inactive fragments. Fig. 5 is show the cleavage of caspase 3,9 and PARP. After treatment of H_2O_2 , the cleaved fragments of caspase3 and 9 were increased concentration-dependent in GM00637-siMTH1 cell line. In addition, the activation of cleaved PARP demonstrated that the caspase were functionally activated. These results showed that the cell death related the apoptosis in GM00637-siMTH1 cell line.

Analysis of gene expression by biological function using microarray and RT-PCR

In order to asses the expression profiles in GM00637-siMTH1 cell line treated with H_2O_2 (80uM, 12h), cDNA microarray that contained duplicate cDNA probes from 8K human clones was performed to be screened at a time.

To normalize intensity ratin of each gene expression pattern, global M method was used in this study. First, the primary data were normalized by the total spots of intensity between two groups, and then normalized by the intensity ratio of reference genes, such as housekeeping genes in both groups. Finally, the

expression ratio of control and H_2O_2 group was converted to $\log_2$ ratio of each gene.

After normalizing the data, a difference in the normalized intensity ratio was selected. The genes of expression ratio were observed to be lower than -1 for which the observed expression was downregulated and raise than 1 for which the observed expression was upregulated by H_2O_2 (Table 2). The genes regulated by H_2O_2 were divided into three categories by biological function ; apoptosis – related gene (Table 2-1), cell death – related gene (Table 2-2) and response to stress – related gene (Table 2-3).

We selected BAD, SULF1, PRDX2, HSPA1L, SEPP1 and MGMT among the downregulated genes by H_2O_2 treatment, and observed mRNA expression of BAD, SULF1, PRDX2, HSPA1L, SEPP1 and MGMT (Table 1) using RT-PCR that reproduced the results of cDNA microarray. The efficiency of the reaction was adjusted by GAPDH amplification. As shown in Fig.6, the expressions of BAD, SULF1, PRDX2, HSPA1L, SEPP1 and MGMT were decreased by H_2O_2 treatment (80uM, 12h).

Figure Legends

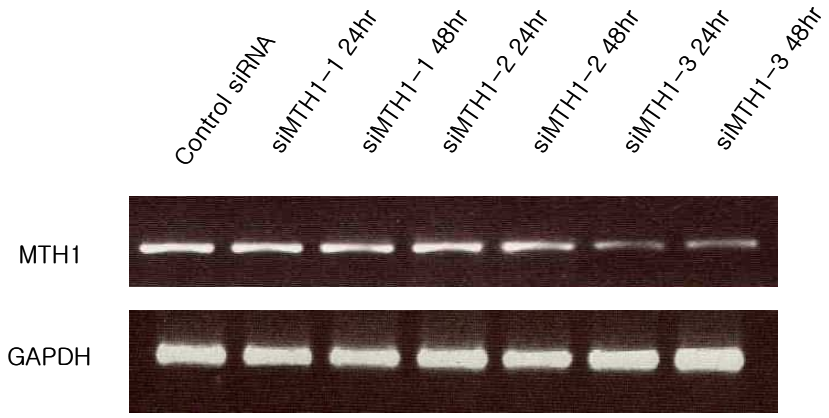


Fig.1

Fig. 1. Down-regulation of the MTH1 expression

The MTH1 mRNA level in the GM00637 cells transfected with 200nM each for the three different MTH1 siRNAs and the control siRNA. In order to obtain the quantitative MTH1 mRNA expression values, quantitative RT-PCR experiments were performed 24h, 48h after treating the cells with either the MTH1 siRNAs or control siRNA.

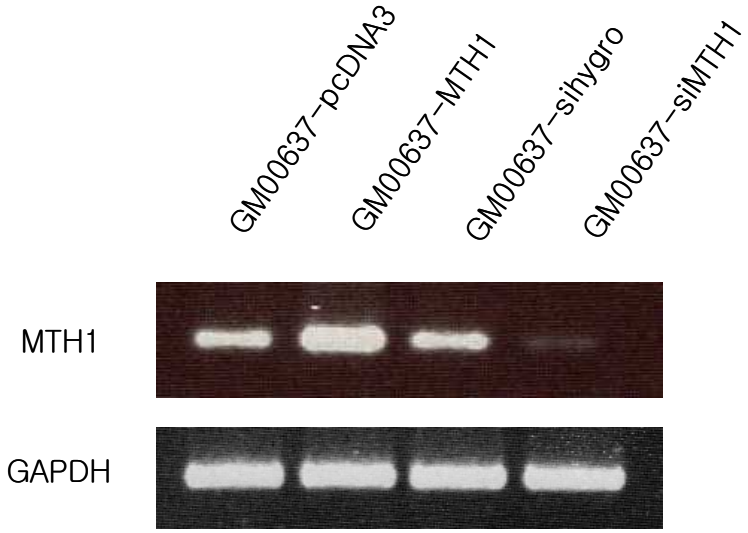


Fig.2

Fig. 2. Establishment of stable cell lines

We first established a GM00637 fibroblast cell linr that stably expresses the MTH1 and siRNA silencing of the MTH1. After transfection and selection for neomycin and hygromycin resistance, respectively, cells were characterized by RT-PCR by using an MTH1 specific primer.

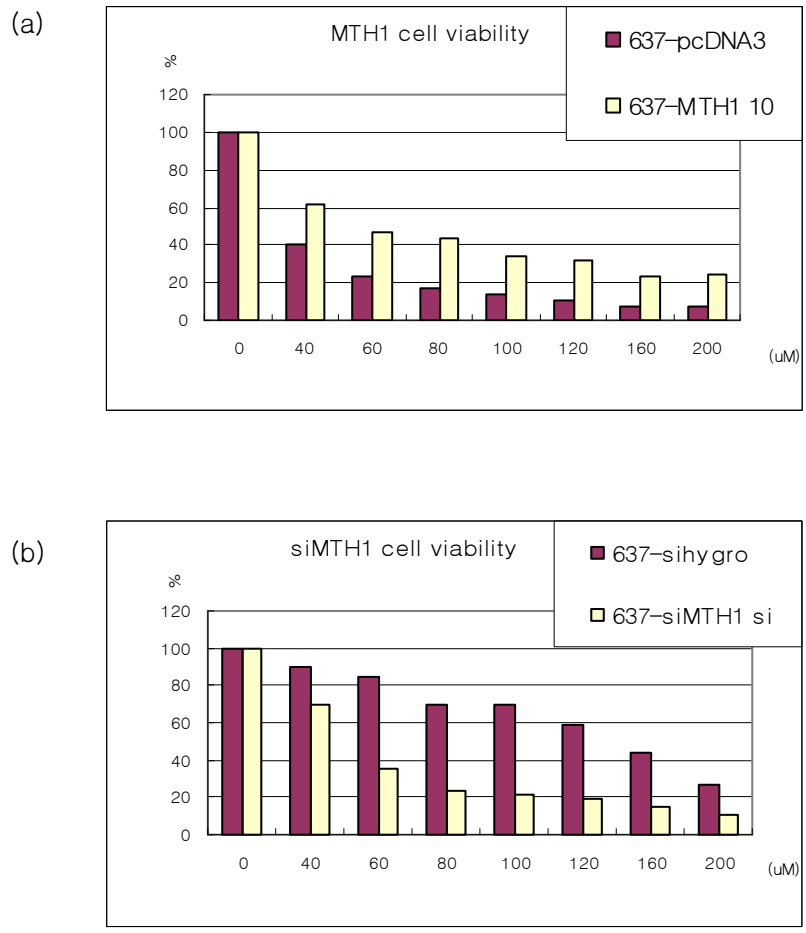
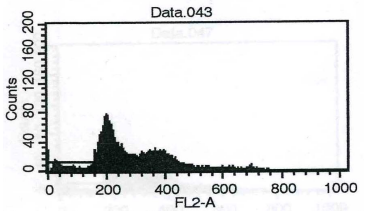


Fig.3.

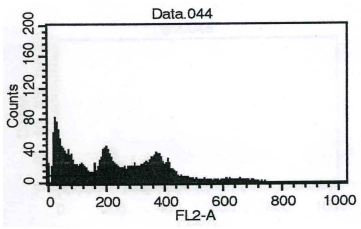
Fig. 3. Cytotoxicity of H_2O_2.

(a) GM00637-pcDNA3, GM00637-MTH1, (b) GM00637-sihygro, GM00637-siMTH1 cells were treated with various concentration (40, 60, 80, 100, 120, 160, and 200uM) of H_2O_2 for 24h prior to the determination of cellular viability through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Independent experiment was repeated thrice. Results are presented as mean ±SE (P<0.01 *vs* control group).

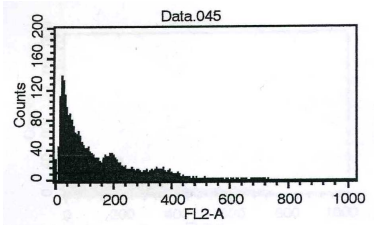
(a)



H2O2 0㎛ 24h



H2O2 40㎛ 24h



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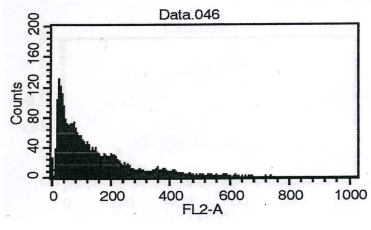
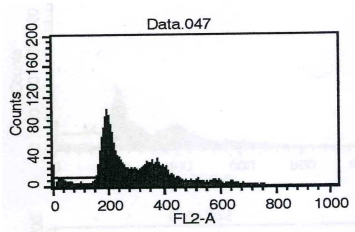
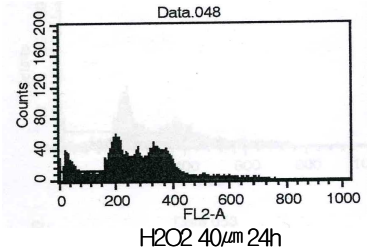


H2O2 120㎛ 24h

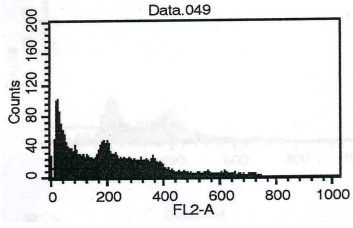
(b)



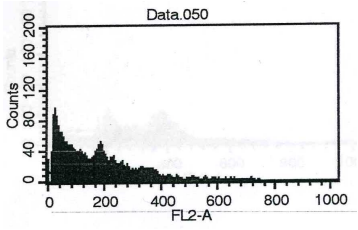
H2O2 0㎛ 24h



H2O2 40㎛ 24h



H2O2 80㎛ 24h



H2O2 120㎛ 24h

Fig. 4

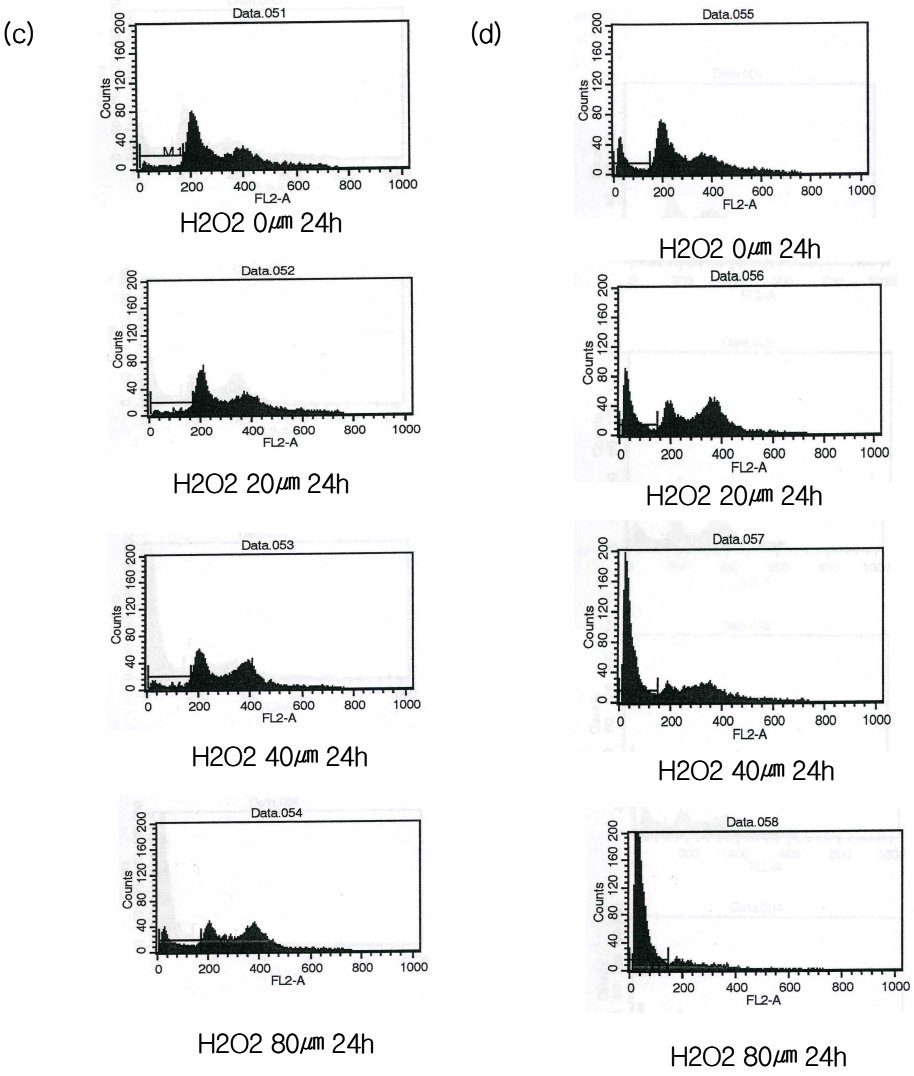


Fig. 4. The effect of H_2O_2-induced cell cycle arrest and apoptosis.

Cell cycle distribution of (a) GM00637-pcDNA3, (b) GM00637-MTH1, (C) GM00637-sihygro, and (d) GM00637-siMTH1 cells assessed by Flow Cytometry after staining with propidium iodide. Cell were pre-incubated with H_2O_2 (a,b) 40,

80, 120uM, (c,d) 20, 40, 80uM for 1hours and incubated for additional 24hours in the absence of H_2O_2. prevented cell cycle progression and, consequently, dose-dependent H_2O_2-induced G2/M-arrest. Additionally, H_2O_2 treatment decreased the number of cells in late S-phase.

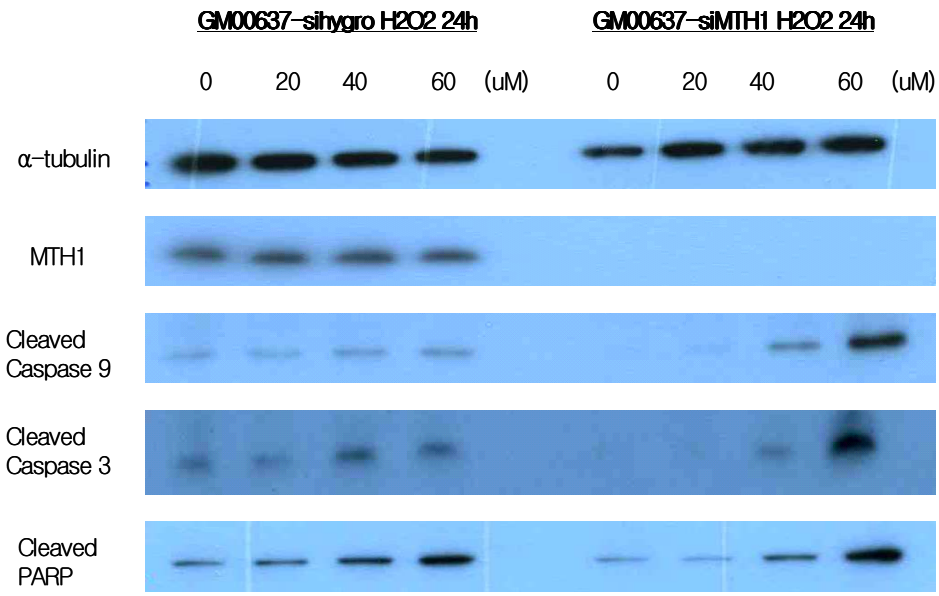


Fig. 5

Fig. 5. Analysis of caspase 3, 9 and PARP cleavage by western blot.
GM00637-siMTH1 cell line was analyzed caspase 3, 9 and PARP cleavage by western analysis with GM00637-sihygro control. After treatment of H_2O_2, 30ug of the total protein were loaded on SDS-polyacrylamide gels for immunoblot analysis. Antibodies against MTH1, Cleaved Caspase 3, Cleaved Caspase 9, and Cleaved PARP were used. The detection of α-tubulin was used as the loading control.

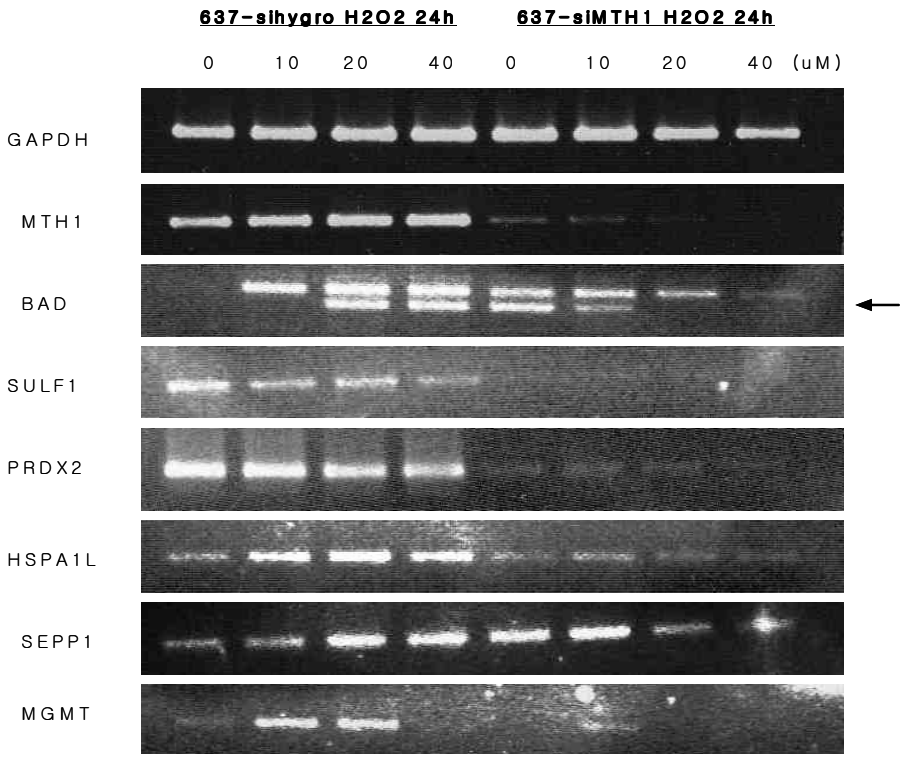


Fig.6

Fig. 6. Confirmation of cDNA microarray results of downregulated genes by RT-PCR.

Six genes, BAD, SULF1, PRDX2, HSPA1L, SEPP1, and MGMT, were analyzed by RT-PCR with total RNA from control and H_2O_2 (80uM, 12h)-treated human fibroblast cells. As an internal control, GAPDH was amplified.

Table 2-1 List of genes apoptosis by H2O2 treatment

Gene	Title	GenBank	sihygro global.M	siMTH1 global.M
BAD	BCL2-antagonist of cell death	AW007022	-1.00	0.16
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	AA481712	2.56	4.29
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	AA285065	-0.79	-2.76
ERN1	Endoplasmic reticulum to nucleus signalling 1	AI950065	-1.15	-2.52
INHA	Inhibin, alpha	AI220927	-1.03	-2.07
LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	AI193092	0.11	1.28
NOTCH2NL	Notch homolog 2 (Drosophila) N-terminal like	AI382438	-0.82	-2.02
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	U43195	-1.30	-2.71
S100B	S100 calcium binding protein, beta (neural)	AI459281	-1.06	-2.92
SON	SON DNA binding protein	X63071	-0.11	-1.10
SULF1	Sulfatase 1	AI961332	-1.00	-2.03
UNC13B	Unc-13 homolog B (C. elegans)	AF020202	-0.36	-1.98
UNC5C	Unc-5 homolog C (C. elegans)	AF055634	-0.17	1.19
ZNF443	Zinc finger protein 443	AB011414	-0.35	-1.52

Table 2-2 List of genes cell death by H2O2 treatment

Gene	Title	GenBank	sihygro global.M	siMTH1 global.M
BAD	BCL2-antagonist of cell death	AW007022	-1.00	0.16
C8G	Complement component 8, gamma polypeptide	NM_000606	-0.99	-2.19
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	AA481712	2.56	4.29
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain	AA285065	-0.79	-2.76
ERN1	Endoplasmic reticulum to nucleus signalling 1	AI950065	-1.15	-2.52
INHA	Inhibin, alpha	AI220927	-1.03	-2.07
LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	AI193092	0.11	1.28
NOTCH2NL	Notch homolog 2 (Drosophila) N-terminal like	AI382438	-0.82	-2.02
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	U43195	-1.30	-2.71
S100B	S100 calcium binding protein, beta (neural)	AI459281	-1.06	-2.92
SON	SON DNA binding protein	X63071	-0.11	-1.10
SULF1	Sulfatase 1	AI961332	-1.00	-2.03
UNC13B	Unc-13 homolog B (C. elegans)	AF020202	-0.36	-1.98
UNC5C	Unc-5 homolog C (C. elegans)	AF055634	-0.17	1.19
ZNF443	Zinc finger protein 443	AB011414	-0.35	-1.52

Table 2-3 List of genes response to stress by H2O2 treament

Gene	Title	GenBank	sihygro global.M	siMTH1 global.M
ABCF2	ATP-binding cassette, sub-family F (GCN20), member 2	AL050291	0.97	2.01
BLM	Bloom syndrome	U39817	-0.48	-1.92
C8G	Complement component 8, gamma polypeptide	NM_000606	-0.99	-2.19
CCL17	Chemokine (C-C motif) ligand 17	D43767	-1.02	-2.32
CD4	CD4 antigen (p55)	NM_000616	-0.93	-2.60
CD59	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344)	AI127949	-1.83	-4.26
CTGF	Connective tissue growth factor	X78947	2.37	1.04
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	AI207061	0.70	1.98
EPHX2	Epoxide hydrolase 2, cytoplasmic	AI301066	-0.10	-1.17
ERCC5	Excision repair cross-complementing rodent repair deficiency,complementation group 5 (xeroderma pigmentosum, complementation group G (Cockayne syndrome))	X69978	0.14	-1.21
ERN1	Endoplasmic reticulum to nucleus signalling 1	AI950065	-1.15	-2.52
FPR1	Formyl peptide receptor 1	NM_002029	-0.62	-1.62
HLA-DPB1	Major histocompatibility complex, class II, DP beta 1	AA731863	-0.99	-2.20
HLXB9	Homeo box HB9	AF107457	-0.83	-2.12
HOXB13	Homeo box B13	AI884491	2.15	0.98
HSPA1L	Heat shock 70kDa protein 1-like	AA622726	-0.83	-1.84
IL10RB	Interleukin 10 receptor, beta	Z17227	-0.09	1.01
INHA	Inhibin, alpha	AI220927	-1.03	-2.07
KIAA1018	KIAA1018 protein	AI383494	-0.63	-3.34
KLRC3	Killer cell lectin-like receptor subfamily C, member 2	X54869	-0.17	-1.51
KLRC4	Killer cell lectin-like receptor subfamily C, member 4	NM_013431	0.07	-2.55
LOC493869	Similar to RIKEN cDNA 2310016C16	AI351012	-0.61	-2.13
LY64	Lymphocyte antigen 64 homolog, radioprotective 105kDa (mouse)	D83597	-0.38	-1.59
MAPK8	Mitogen-activated protein kinase 8	L26318	0.62	-1.09
MGMT	O-6-methylguanine-DNA methyltransferase	M29971	4.78	-0.33
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	NM_000249	0.46	-1.03
NEIL1	Nei endonuclease VIII-like 1 (E. coli)	AI925612	-0.60	-2.73
PFC	Properdin P factor, complement	M83652	-1.00	-2.14
PRDX2	Peroxiredoxin 2	AI298463	1.01	0.01
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1	AF038603	-1.40	-3.46
PTE1	Peroxisomal acyl-CoA thioesterase	X86032	-0.20	-1.30
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	NM_000963	-0.73	-2.63
RAD1	RAD1 homolog (S. pombe)	AF011905	-0.57	-1.99
S100B	S100 calcium binding protein, beta (neural)	AI459281	-1.06	-2.92
SCAP	SREBP cleavage-activating protein	AL040564	0.64	1.65
SEPP1	Selenoprotein P, plasma, 1	AA845625	-0.92	-2.82
SERPINF2	Serine (or cysteine) proteinase inhibitor, clade F(alpha-2 antiplasmin, pigment epithelium derived factor), member 2	D00174	-1.50	-3.85
SLAMF1	Signaling lymphocytic activation molecule family member 1	U33017	-1.61	-0.41
TFPI2	Tissue factor pathway inhibitor 2	C17799	0.26	-1.29
TLR3	Toll-like receptor 3	U88879	-0.43	-1.85
TRIF	TIR domain containing adaptor inducing interferon-beta	AI560708	0.11	-1.00
UBE2A	Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	AI609766	0.33	-1.09
WDR33	WD repeat domain 33	AA827926	0.14	-1.10
ZNF443	Zinc finger protein 443	AB011414	-0.35	-1.52

IV. DISCUSSION

Mammalian MTH1 protein, a homolog of bacterial MutT protein, plays an important antimutagenic role in cells by preventing incorporation of oxidatively modified purine nucleotides into DNA during replication [review in 21]. To investigate the effects of down-regulation on the MTH1 expression levels, the cell viability, we designed and synthesized siRNA silencing of the MTH1. In addition, to better understand the mechanisms involved the function of MTH1, we have focused on the siMTH1 (siRNA silencing of the MTH1).

The cells were treated with H_2O_2 for 24h, after which time the cell viability was determined by the colorimetric MTT assay. Overexpression of MTH1 resulted in elevated and down-regulation of MTH1 resulted in reduced cell viability after exposure to H_2O_2, compared to the control vector-transfected cells, respectively. These results suggested that the tranfection and subsequent expression of the MTH1 – targeted siRNAs resulted in the suppression of the cell viability capacity in response to H_2O_2. We next investigated whether this effect could be due to apoptosis. Thus, we analyzed cell death by flow cytometry using propidium iodide in GM00637-MTH1 and GM00637-siMTH1 cell lines exposed to dose of H_2O_2. The cell death of GM00637-MTH1 cells were decreased and GM00637-siMTH1 cells were increased, compared with the control, respectively. These results indicated that the suppression of MTH1 gene expression leads to a increase in the

cell death in response to H_2O_2. We next analyzed caspase 3, 9 and PARP cleavage by western analysis. After treatment of H_2O_2, the cleaved fragments of caspase 3 and 9 were increased concentration-dependent in GM00637-siMTH1 cell line. In addition, the activation of cleaved PARP demonstrated that the caspase were functionally activated. These results showed that the cell death related the apoptosis in GM00637-siMTH1 cell line. In order to asses the expression profiles in GM00637-siMTH1 cell line treated with H_2O_2 (80uM, 12h), cDNA microarray was performed. We selected the downregulated genes by H_2O_2 treatment, and observed mRNA expression of the selected genes using RT-PCR that reproduced the results of cDNA microarray. However, the correlation of between the obove mentioned genes and MTH1 was not identified.

It has been estimated that around 2×10^4 DNA damaging events occur in every cell of the human body every day [1]. A significant portion of the damage is caused by reactive oxygen species (ROS). As early as 1952, Conger and Fairchild [22] demonstrated that increased oxygen pressure could lead to the accumulation of chromosomal aberrations. The effect of excessive production of ROS and/or the inadequacy of the anti-oxidant cellular defense systems to neutralize them commonly referred to as oxidative stress. ROS are constantly produced in the living cell [2-4]. Major endogenous sources are metabolic processes, primarily oxidative metabolism in the mitochondria, and pathological processes such as inflammation. Exogenous sources of ROS include exposure to ionizing radiation

(IR) or radiomimetic chemicals, and ROS produced by neighboring cells. Because they are generated as by-products of respiration, ROS constitute the major class of endogenous toxic agents in aerobic organisms. These include all partially reduced oxygen species, namely, the superoxide anion radical ($.O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (.OH).

8-Hydroxydeoxyguanosine (8-OHdG), an oxidized form of guanine, is the major oxidative DNA-damage product that can produce mutations—A:T to C:C or G:C to T:A transversion mutations—because of its base pairing with adenine as well as cytosine (reviewed in [5]). It has been proposed that, unlike bulky DNA lesions, oxidized base lesions such as 8-OHdG and 5-hydroxyuracil are incorporated by DNA polymerases in the nascent DNA strand from the deoxynucleotide pool. Thus, 8-OHdG can be incorporated opposite A in the template strand [23]. The oxidized bases and AP sites, as well as DNA single-strand breaks induced by ROS with 3′ phosphoglycolate blocked ends, are repaired predominantly by the DNA base excision repair (BER) pathway. In *Escherichia coli*, the mutM, mutY and MutT DNA-repair specific enzymes protect the highly mutagenic effect of the oxidated guanine. MutM removes the oxidized base from 8-oxoG:C base pairs in duplex DNA. The DNA glycolase mutY excises adenine misincorporated opposite 8-oxoG during replication. The 8-oxodGMPase mutT prevents the incorporation of 8-oxo-dGMP into nascent DNA. The human homologes mutM (OGG1), mutY (MYH) and mutT (MTH) have been identified (reviewed in [6]).

MTH1, a counterpart of *E. coli* MutT, possesses 8-oxo-dGTPase activity, which degrades oxidized nucleotide 8-oxo-dGTP to sanitize the nucleotide pool [7]. When the cDNA for MTH1 is expressed in *E. coli mutT* cells, the elevated level of spontaneous A:T to C:G mutation frequency reverts to normal [8]. Although bacterial MutT does not hydrolyze oxidized dATP, MTH1 degrades 2-OH-dATP and 2-OH-ATP more efficiently than 8-oxodGTP [24]. The pool size of ATP in human cells is 100 times larger than dGTP pool, what supports the idea that the physiological substrates for MTH1 may be oxidized ATP or dATP [25]. The *MTH1* gene consists of five major exons and is mapped on 7p22 [8]. The mRNA is abundant in thymus, testis and the embryonic tissues. The *MTH1* gene produces four different polypeptides (p18, p21, p22, p26) through alternative splicing, alternative initiation of translation or SNP of splice site [26]. mRNA with a polymorphic alteration (GU to GC) at the beginning of exon 2C produces p26. Among them, p18 is the major form of the products and is identical to the purified 8-oxo-dGTPase in Jurkat cell [27] and [26]. The p18 protein is mainly localized in cytoplasm, and about 5% is in mitochondrial matrix [28]. An alteration of MTH1-expression with the accumulation of 8-OH-G in DNA is observed in neurodegenerative diseases [9].

A G to A change at codon 83 of exon 4 of *MTH1* has been identified as an SNP, and this change results in the substitution of Val to Met [29]. Interestingly, this polymorphism is strongly linked with the polymorphism (GU to GC) at exon 2C

[26]. Met83-MTH1 has GC at exon 2C while Val83-MTH1 has GT at the site. 8-OH-dGTPase activity of MTH1-Met83 is more thermolabile than that of MTH1-Val83 [30]. The allele frequency of Val83 and Met83 in Japanese healthy volunteers is 0.91 and 0.09, respectively [26]. Met83 was detected in nine hereditary non-polyposis colorectal cancer (HNPCC) patients [29]. However, the same change was detected in 5 of 30 unrelated healthy individuals and it was concluded that the polymorphism is not associated with a marked HNPCC predisposition. MTH1is expressed aberrantly in the brains of patients with Parkinson's disease (PD) [31]. Val83Met polymorphism was studied in 73 patients with sporadic PD and 151 age-matched non-PD controls. The frequency of either Val83 or Met83 allele was not statistically different between PD patients (92.5 or 7.5%) and the controls (88.7 or 11.3%). MTH1 is not directly associated with ovarian cancer, either [32]. However, the polymorphism is suggested to be involved in the development of type 1 diabetes mellitus in the Japanese female population [33].

This study presents the data demonstrationg that MTH1 gene expression in human fibroblast cells is specifically suppressed by MTH1 siRNA, resulting in a significant decrease in the DNA viability capacity as well as an increase in the cell death to H_2O_2. The suppression of MTH1 expression in the human fibroblast cells lead to a decrease in the cell viability against H_2O_2. In addition, the cell death was increased concentration-dependent in the suppression of MTH1 expression.

Moreover, MTH1 knockout cells have shown that caspase and PARP are fragmented after exposure of H_2O_2, but not control vector transfected cells. These results suggest that the MTH1 has a protective role against oxidative stress and may be an important defense protein after exposure oxygen free radicals.

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