

Matrix metalloproteinase gene expressions might be oxidative stress targets in gastric cancer cell lines

Salih Gencer^{1,2}, Anil Cebeci³, Meliha Burcu Irmak-Yazicioglu³

¹Department of Genetics and Bioengineering, Faculty of Engineering, Fatih University, Istanbul 34500, Turkey; ²Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Uskudar University, Istanbul 34662, Turkey; ³Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Halic University, Istanbul 34381, Turkey

Corresponding to: Meliha Burcu Irmak-Yazicioglu, Assoc. Prof. Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Halic University, Siracevizler Str., No. 29 Bomonti, Istanbul 34381, Turkey. Email: burcuyazicioglu@halic.edu.tr.

Objective: Oxidative stress is linked to increased risk of gastric cancer and matrix metalloproteinases (MMPs) are important in the invasion and metastasis of gastric cancer. We aimed to analyze the effect of the accumulation of oxidative stress in the gastric cancer MKN-45 and 23132/87 cells following hydrogen peroxide (H₂O₂) exposure on the expression patterns of *MMP-1*, *MMP-3*, *MMP-7*, *MMP-9*, *MMP-10*, *MMP-11*, *MMP-12*, *MMP-14*, *MMP-15*, *MMP-17*, *MMP-23*, *MMP-28*, and β -catenin genes.

Methods: The mRNA transcripts in the cells were determined by RT-PCR. Following H₂O₂ exposure, oxidative stress in the viable cells was analyzed by 2',7'-dichlorofluorescein diacetate (DCFH-DA). Caffeic acid phenethyl ester (CAPE) was used to eliminate oxidative stress and the consequence of H₂O₂ exposure and its removal on the expressions of the genes were evaluated by quantitative real-time PCR.

Results: The expressions of *MMP-1*, *MMP-7*, *MMP-14*, *MMP-15*, *MMP-17* and β -catenin in MKN-45 cells and only the expression of *MMP-15* in 23132/87 cells were increased. Removal of the oxidative stress resulted in decrease in the expressions of *MMP* genes of which the expressions were increased after H₂O₂ exposure. β -catenin, a transcription factor for many genes including *MMPs*, also displayed decreased levels of expression in both of the cell lines following CAPE treatment.

Conclusions: Our data suggest that there is a remarkable link between the accumulation of oxidative stress and the increased expressions of *MMP* genes in the gastric cancer cells and *MMPs* should be considered as potential targets of therapy in gastric cancers due to its continuous exposure to oxidative stress.

Key Words: Oxidative stress; hydrogen peroxide (H₂O₂); matrix metalloproteinases (MMPs); expression; caffeic acid phenethyl ester (CAPE)



Submitted Mar 19, 2013. Accepted for publication May 24, 2013.

doi: 10.3978/j.issn.1000-9604.2013.06.05

Scan to your mobile device or view this article at: <http://www.thejcjr.org/article/view/2202/3062>

Introduction

To live in homeostasis, every single organism must control the regulatory mechanism of itself against the changing conditions of the environment. From bacteria to mammals, oxidative stress has devastating effects which are the protein dysfunction, the lipid peroxidation, the breakdown of DNA, the change of the genetic code via mutations, and finally apoptosis (1,2).

Oxidative stress can trigger many cell signaling pathways to protect the normal cell life. Low levels of oxidative stress

activate the transcription of genes encoding proteins that participate in the defense against oxidative injuries, oxidative damage repair mechanisms, and apoptosis (3). Nuclear accumulation of Nrf-2 and NF- κ B causes transcriptional activation of antioxidant enzymes including superoxide dismutases, glutathione peroxidases and thioredoxin, respectively (3-6). If these first defense attempts are not sufficient to prevent oxidative stress-induced DNA damage, several other signaling processes such as base excision repair (7), cell cycle arrest at G1-S transition,

S phase, and G2-M transitions (8) serve as protective mechanisms. In MCF-7 breast cancer cells, oxidative stress causes the expressions of *CYGB*, *FOXMI*, *NOX5*, *NUDT1* and *SEPP1* genes which can be affected differentially. Especially, the expression of *FOXMI* may suppress cancer development and increases the effects of anticarcinogenic agents (9). In MDA-MB-231 breast cancer cells, oxidative stress responses of the cells are increased by NRP/B, a nuclear matrix protein, via the Nrf2 pathway. Nrf2 pathway associates with antioxidant response element (ARE) sourced detoxifying and antioxidant genes (10). Oxidative stress also regulates the role of β -catenin which is a key element of canonical Wnt pathway through binding and activating T cell factor (TCF) transcription factor. Under oxidative stress, the transcriptional activity of β -catenin/TCF complex is inhibited (11,12). FOXO, another transcription factor that is modulated by β -catenin, interacts with β -catenin in increased oxidative stress resulting with increased expression of anti-oxidant genes. FOXO can also inhibit the cell cycle progress under this condition because of its regulator role in G1 transition of the cell cycle (12). Additionally, HIF-1 and HIF-2, transcription factors that are responsible for the response of cells to hypoxia, directly bind to β -catenin and regulate the adaptation of cells to hypoxia (13).

The balance between pro-oxidants and antioxidants determines the extent of oxidative damage induced by reactive oxygen species (ROS). The shift from antioxidants to pro-oxidants in the tissue has been linked to increased risk of cancer (14,15). Chronic inflammation as a consequence of persistent oxidative stress has been linked to various steps involved in carcinogenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (16,17). Our insight in the molecular pathways involved in these biological responses to different oxygen radicals is still incomplete.

Matrix metalloproteinases (MMPs) are zinc-dependent proteins that are responsible for degrading extracellular matrix. Due to their capabilities, they can play important roles in growth, development and pathological processes. In carcinogenesis, they may provide suitable growth, invasion and finally metastasis conditions for tumor cells. Different MMP types can take action in different stages of carcinogenesis. These all depend on the cell environment and the needs of tumor progression (18).

Gastric cancer is the seventh most common cancer and the second most common cause of cancer-related death worldwide (19). The gastric epithelium is continuously exposed to toxic ROS within the gastric lumen due to

ingested food and cigarette smoke and inflammation due to *Helicobacter pylori* infection. The dynamic balance between cell proliferation and apoptosis is essential for maintaining mucosal homeostasis. Decreased apoptosis as well as increased proliferation may favor the carcinogenic process. Prolonged survival of abnormal cells can support the accumulation of sequential genetic mutations, changes in gene expression profiles and protein structure and function which can result in tumor promotion (20-24). Consequently, the link between oxidative stress and gastric cancer cannot be ignored. However, the molecular mechanisms behind it are still not clear.

In this study, the changes in the expression levels of *MMP* genes were analyzed following H_2O_2 exposure of gastric carcinoma MKN-45 and 23132/87 cell lines to understand whether a relation exists between oxidative stress formation in gastric cancer cells and the expressions of *MMP* genes.

Materials and methods

Cell culture

Human gastric adenocarcinoma cell lines, MKN-45 (DSMZ ACC409) and 23132/87 (DSMZ ACC201), were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). The cells were cultured in a standard RPMI-1640 (Biochrom, Berlin, Germany) medium supplemented with 10% (V/V) fetal bovine serum (FBS) and 1% (V/V) penicillin and streptomycin (10,000 μ g/mL, Biochrom) in a humidified chamber at 37 °C in the presence of 5% CO_2 .

H_2O_2 treatment of cell lines

The cells were plated at a density of 3×10^3 - 5×10^3 cells per well and cultured in the standard medium overnight. The medium was aspirated and washed with phosphate buffered saline (PBS); the cells were starved in the culture medium supplemented with 0.01% FBS overnight and they were treated with indicated concentrations of H_2O_2 ranging from 50-200 μ mol/L for indicated time points. The control cells were only starved and were not exposed to H_2O_2 . The morphological changes were analyzed under microscope (Axiovert 40 CFL, Zeiss, Jena, Germany).

Determination of 8-hydroxydeoxyguanosine (8-OHdG) in cell lines

MKN-45 and 23132/87 cell lines that were exposed to H_2O_2

ranging from 50 to 200 $\mu\text{mol/L}$ for 24 h were fixed with 4% paraformaldehyde for 15 min at room temperature. After the fixation, the presence of 8-OHdG in the cells was determined using DakoCytomation LSAB2 System-HRP kit (Dako, Hamburg, Germany). Mouse monoclonal 8-OHdG antibody (Cosmobio, Tokyo, Japan) was used as primary antibody. The sections were treated for 30 min at room temperature first with biotinylated goat anti-rabbit and goat anti-mouse IgG (Cosmobio) and then with streptavidin-horseradish peroxidase (HRP) solution. Between incubations, the sections were washed with $1\times$ PBS (pH 7.4). 3-3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, Mo., USA) was used as HRP substrate. The sections were incubated with substrate-chromogen solution for 5 min and counterstaining was performed with Mayer's hematoxylin to visualize the nucleus (Zeiss, Axiovert 40 CFL).

Reverse transcription-polymerase chain reaction (RT-PCR)

mRNA transcripts encoding *MMP-1*, *MMP-3*, *MMP-7*, *MMP-9*, *MMP-10*, *MMP-11*, *MMP-12*, *MMP-14*, *MMP-15*, *MMP-17*, *MMP-23*, *MMP-28*, β -catenin, and glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) genes were determined by RT-PCR. Samples were homogenized to break them up. RNA was extracted from the cell lines using NucleoSpin RNA II kit (Macherey-Nagel, Oensingen, Switzerland). RNA concentration and purity of each extract were determined by A_{260}/A_{280} absorptions using Shimadzu UV-1202 spectrophotometer. RNA extracts were stored at -80°C for long-term use. cDNA was synthesized using oligo(dT)₁₈ primer according to the manual of RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Primers for amplification of selected *MMPs*, β -catenin, and *GAPDH* and the PCR conditions are shown in *Table 1*. To determine the optimum number of cycles required for the amplification of these genes, an aliquot of first strand cDNA was amplified with the respective primers using an increasing number of PCR cycles (20-35). Initial denaturation was performed at 94°C for 4 min. The subsequent cycling programs consisted of denaturation at 95°C for 30 s, annealing for 30 s (annealing temperatures, see *Table 1*), and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The amplified products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed using Gel Doc 2000 imaging system (Bio-Rad, Hercules, CA). PCR reactions in which the first strand cDNA was omitted served as negative

controls. To avoid technical error, each PCR experiment was repeated three times.

Determination of oxidative stress in cell lines

Oxidative stress in the cell lines was assessed using an oxidative stress marker, 2',7'-dichlorofluorescein diacetate (DCFH-DA) (25). The non-fluorescent DCFH-DA (Sigma) is a cell-permeable compound that can enter into the cells, where it is deacetylated and entrapped as 2',7'-dichlorofluorescein (DCFH) of which by ROS produces a highly fluorescent product, DCF, which can be visualized under a fluorescent microscope. DCFH-DA is freshly prepared in 10 mmol/L HEPES (pH 7.5), 10 mmol/L glucose and 1 $\mu\text{mol/L}$ DCFDA (dissolved in methanol) in PBS. MKN-45 and 23132/87 cells were treated with 200 $\mu\text{mol/L}$ H_2O_2 for 24 h and after this time point, the medium was aspirated, and the cells were washed two times with PBS, and incubated with DCFH-DA solution at 37°C for 15 min, and then were washed two times with PBS and observed under fluorescent microscope (Axio-skop, Zeiss, Jena, Germany). The live cells that were under oxidative stress were counted in five $20\times$ fields per culture (typically 70-100 cells/ $20\times$ field), and the percentage of the cells under oxidative stress in each culture was calculated.

Removal of stress with caffeic acid phenethyl ester (CAPE) in cell lines

The generated oxidative stress with 200 $\mu\text{mol/L}$ H_2O_2 was eliminated with CAPE. Both of the cell lines were grown to 70% confluency in RPMI medium supplemented with 10% FBS. Three hours after the addition of 3 $\mu\text{g/mL}$ CAPE (Sigma) to the growth medium, 200 $\mu\text{mol/L}$ H_2O_2 was applied to the cells. After 24 h of incubation of the cells in CO_2 incubator, the presence of oxidative stress in the live cells was demonstrated using DCFH as mentioned above. CAPE-untreated controls with only 200 $\mu\text{mol/L}$ H_2O_2 exposure were also used in the experiment.

Quantitative real-time PCR

Total RNA was isolated and cDNA was synthesized from the cell lines using RNA extraction kit (NucleoSpin RNA II, Macherey-Nagel) and cDNA synthesis kit (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas), respectively, according to the manufacturer's instructions. Associated genes, their primers and annealing temperatures are listed

Table 1 Specific primers for *MMP*, β -catenin and *GAPDH* genes

Primer	Gene accession number	Sequence	Amplicon size (bp)	Annealing temperature (°C)
<i>MMP-1</i>	AY769434	Forward: 5-ATGCTGAAACCCTGAAGGTG-3 Reverse: 5-GAGCATCCCCTCCAATACCT-3	348	55
<i>MMP-3</i>	AF405705	Forward: 5-GTCTCTTTCACTCAGCCAAC-3 Reverse: 5-ATCAGGATTTCTCCCCTCAG-3	250	55
<i>MMP-7</i>	AY795972	Forward: 5-TCCCGCGTCATAGAAATAATG-3 Reverse: 5-AGGAATGTCCCATACCCAAAG-3	451	57
<i>MMP-9</i>	AF538844	Forward: 5-CCTTCCTTATCGCCGACAAG-3 Reverse: 5-TGAACAGCAGCATCTTCCCC-3	225	60
<i>MMP-10</i>	AY744675	Forward: 5-CATTCCTTGTGCTGTTGTGTC-3 Reverse: 5-TGTCTAGCTTCCCTGTCACC-3	225	63
<i>MMP-11</i>	AY899208	Forward: 5-AGACACCAATGAGATTGCAC-3 Reverse: 5-GCACCTTGAAGAACCAAATG-3	249	57
<i>MMP-12</i>	AY856072	Forward: 5-ACTACACATTCAGGAGGCAC-3 Reverse: 5-CAAATTGGGGTCACAGAGAG-3	270	56
<i>MMP-14</i>	AY795074	Forward: 5-ATAAACCCAAAACCCACC-3 Reverse: 5-AAACACCCAATGCTTGTCTC-3	251	61
<i>MMP-15</i>	EF032329	Forward: 5-ACAACATCCCATGCCCATC-3 Reverse: 5-ACCTGTCCTTGAAGAAG-3	242	57
<i>MMP-17</i>	AB021225	Forward: 5-TCCAGATCGACTTCTCCAAG-3 Reverse: 5-CCACATGGCTTAACCCAATG-3	225	62
<i>MMP-23</i>	AB031068	Forward: 5-CGACTTCTGCTACGAATCCC-3 Reverse: 5-TTGGCGATGATGCTCAGGTG-3	321	57
<i>MMP-28</i>	AF219624	Forward: 5-AGGCATTCTAGAGAAGTACG-3 Reverse: 5-CTTACGCCTCATTTTGGTCC-3	338	58
β -catenin	AY463360	Forward: 5-CAGAAGCTATTGAAGCTGAGG-3 Reverse: 5-TTCCATCATGGGGTCCATAC-3	326	63
<i>GAPDH</i>	M33197	Forward: 5-GACCTGCCGTCTAGAAAAAC-3 Reverse: 5-TTGAAGTCAGAGGAGACCAC-3	126	60

in *Table 1*. As an internal control, the *GAPDH* mRNA was also amplified in PCR reactions. The reactions were carried out with 2 μ L cDNA template in a total volume of 25 μ L, containing 1 \times iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) with primers for whole genes in a Rotor-Gene real-time PCR instrument (Corbett Research, Sydney, Australia). After initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 10 s, annealing at different temperatures from 55 to 63 °C (see *Table 1*) for 25 s and extension at 72 °C for 25 s were carried out. Finally, melting analysis was performed in the temperature range of 55 to 95 °C to verify product homogeneity. Real-time PCR reactions were carried out in triplicate for each sample as a technical replicate. Each cDNA sample was tested in three different reactions

with three technical replicates and negative controls. Three biological replications have been performed for each transcript in order to determine whether there are significant differences in the expressions at the different time points.

Statistical analysis

Each of the data is the mean of three independent values and all data are expressed as $\bar{x} \pm s$. Student's *t*-test is used in order to assess whether the means of mRNA expression rates of untreated control cells and treated cells are statistically different from each other by using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

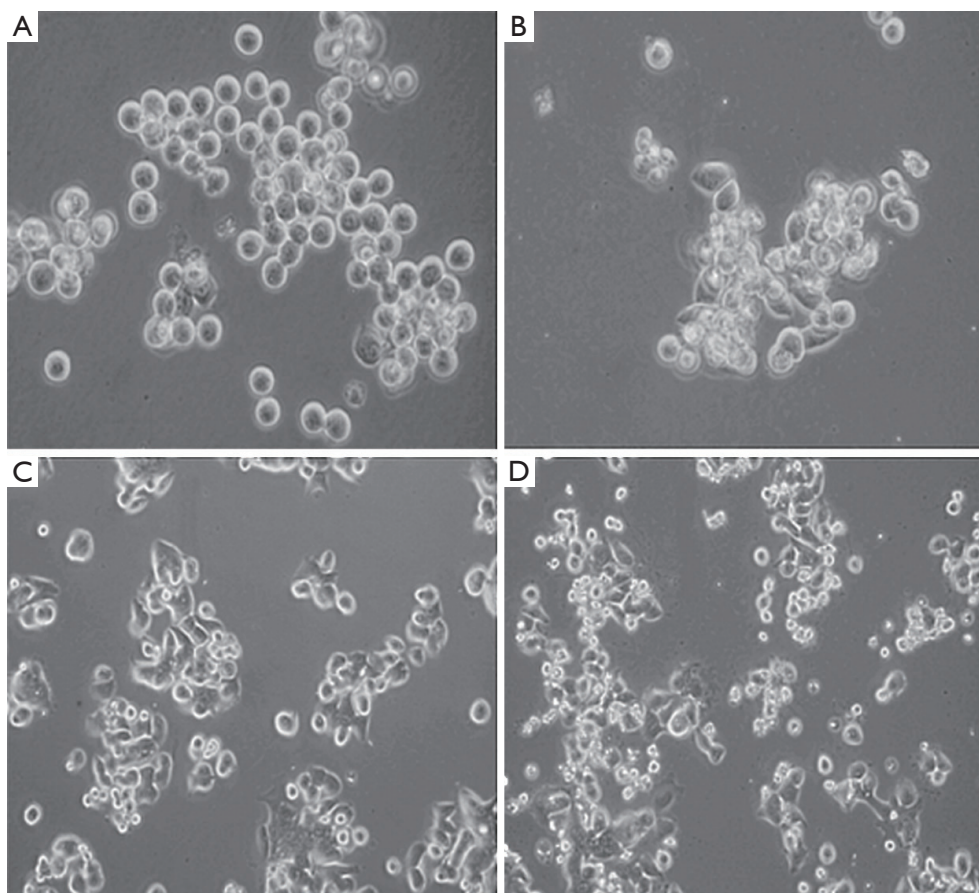


Figure 1 The inverted light microscope views of morphological changes in MKN-45 and 23132/87 cell lines treated with 200 $\mu\text{mol/L}$ H_2O_2 after 24 h (200 \times). A. control MKN-45; B. MKN-45 with 200 $\mu\text{mol/L}$ H_2O_2 treatment; C. control 23132/87; D. 23132/87 with 200 $\mu\text{mol/L}$ H_2O_2 treatment

Results

Morphological changes of cell lines

Gastric cancer cell lines, MKN-45 and 23132/87, exhibited a remarkable change in their morphologies and loss of surface attachment following H_2O_2 exposure when they were compared with their H_2O_2 -untreated controls as shown by inverted light microscopic views with the presence of 8-OHdG (data not shown). The unexposed control cells exhibited distinctive cell shape having regular plasma membrane boundaries with apparent cytoplasm and nucleus whereas H_2O_2 exposure resulted in irregular cell membrane with nuclear and cytoplasmic shrinking, and chromatin condensation in the cell lines (Figure 1).

Determination of MMP expressions

MKN-45 and 23132/87 cell lines showed different expression

patterns of MMP genes (Table 2). MMP-1 (26), MMP-7 (26), MMP-10, MMP-14 and MMP-17 genes were only expressed in MKN-45 cells, while MMP-11, MMP-15 (26), MMP-23 and MMP-28 genes were expressed in both of the cell lines. On the other hand, MMP-3 (26), MMP-9 and MMP-12 gene expressions were determined in neither of them. Moreover, β -catenin expression was positive in both of the cell lines (Figure 2).

Effects of H_2O_2 exposure on expressions of MMP genes

Among the four MMP genes that were expressed in 23132/87 cells, only the expression of MMP-15 gene was determined to be increased following H_2O_2 exposure. However, in MKN-45 cells, there was an increase in the expressions of MMP-1, MMP-7, MMP-14, MMP-15, MMP-17, and β -catenin (Table 3). As seen in Figure 3, 12 h exposure to H_2O_2 resulted in a more increase in the

Table 2 Expression profiles of *MMP* and β -*catenin* genes at the transcription level in MKN-45 and 23132/87 cell lines

Gene	MKN-45	23132/87
<i>MMP-1</i>	+	-
<i>MMP-3</i>	-	-
<i>MMP-7</i>	+	-
<i>MMP-9</i>	-	-
<i>MMP-10</i>	+	-
<i>MMP-11</i>	+	+
<i>MMP-12</i>	-	-
<i>MMP-14</i>	+	-
<i>MMP-15</i>	+	+
<i>MMP-17</i>	+	-
<i>MMP-23</i>	+	+
<i>MMP-28</i>	+	+
β -catenin	+	+
<i>GAPDH</i>	+	+

+, expression; -, no expression

expression of *MMP-14* gene compared to 24 h exposure to H_2O_2 . The same pattern was observed for *MMP-7* gene as we have previously reported (27). Conversely, *MMP-1*, *MMP-15* and *MMP-17* exhibited more expression when MKN-45 cells were exposed to H_2O_2 for 24 h. β -catenin expression was also affected positively by the application of H_2O_2 to MKN-45 cells for 12 and 24 h.

β -catenin expression was significantly 0.75 (80%), 1.60 and 1.45 fold increased ($P < 0.05$) after 12 h of H_2O_2 exposure while it was 1.80, 1.45 and 0.90 (90%) fold increased significantly ($P < 0.05$) after 24 h of oxidative stress treatment at 50, 100 and 200 $\mu\text{mol/L}$ H_2O_2 concentrations, respectively, compared with the untreated control cells. Following 12 h of H_2O_2 application, *MMP-14* displayed a significant up-regulation of 1.40, 4.40 and 2.03 fold ($P < 0.05$) and 24 h of H_2O_2 application 2.45, 1.70 and 0.80 (80%) fold significant increase ($P < 0.05$) at 50, 100 and 200 $\mu\text{mol/L}$ H_2O_2 concentrations, respectively, compared with the untreated control cells. The analysis of the *MMP-14* gene

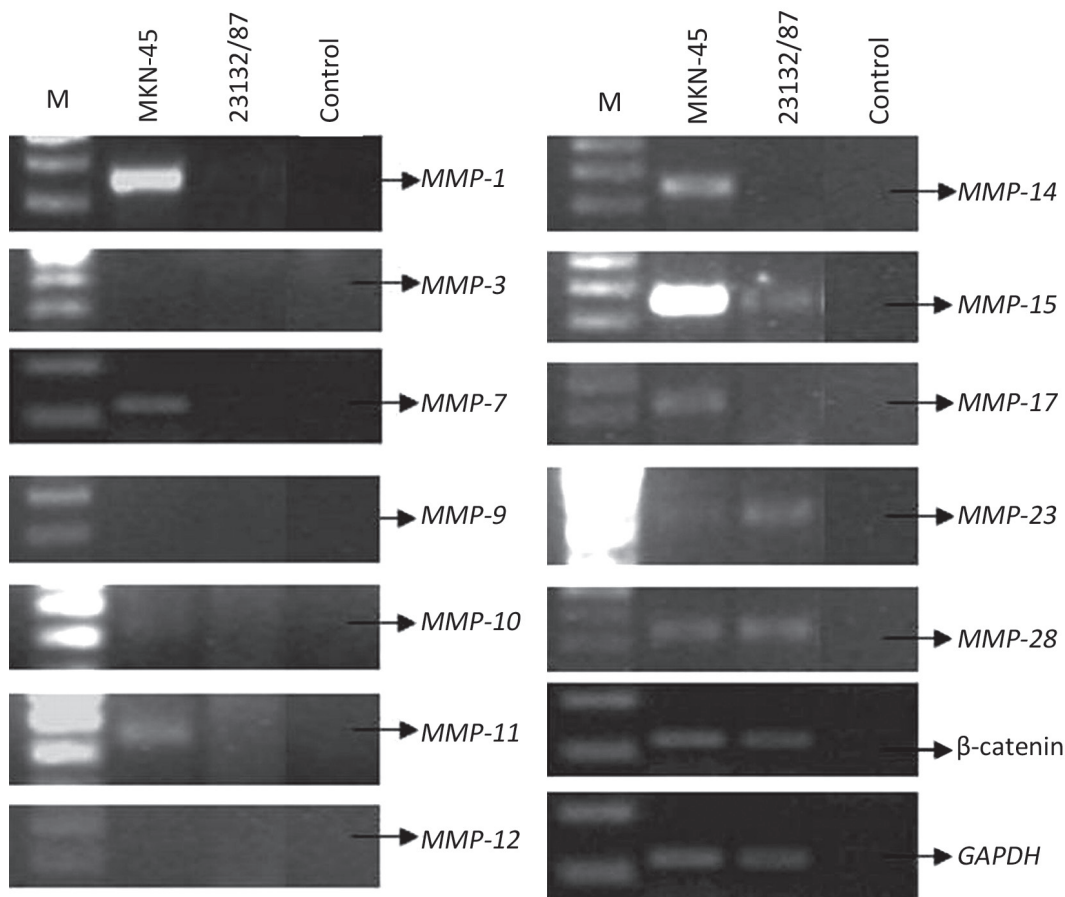
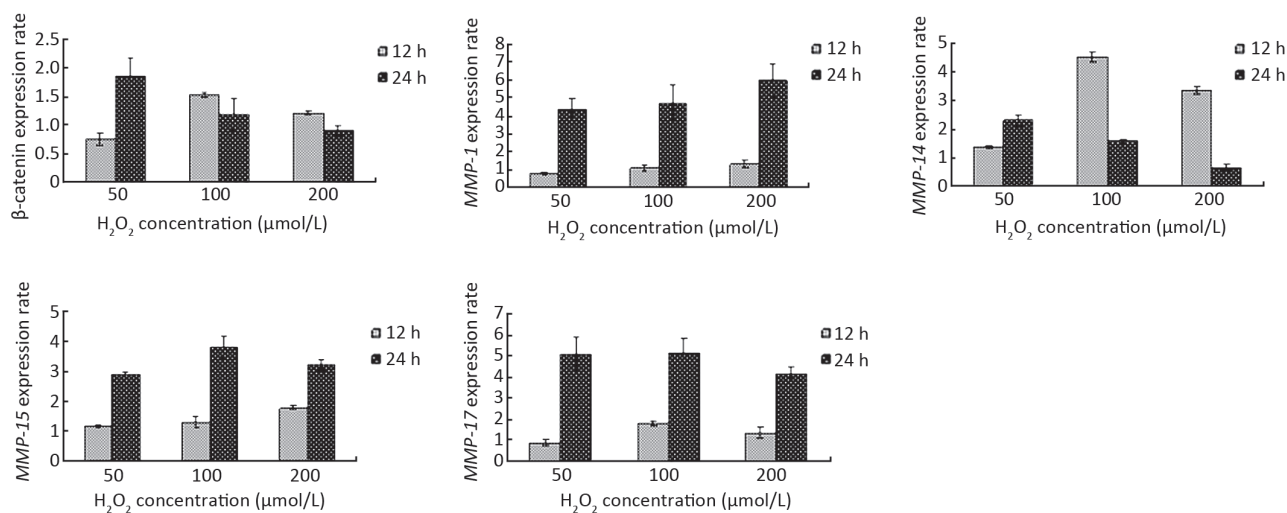


Figure 2 RT-PCR analysis of several *MMP* genes, β -catenin and *GAPDH* in MKN-45 and 23132/87 cell lines. M, 100 bp molecular weight marker

Table 3 The change of gene expression which intracellular oxidative stress is removed with CAPE in cell lines

Gene	MKN-45 (H ₂ O ₂)	MKN-45 (CAPE)	23132/87 (H ₂ O ₂)	23132/87 (CAPE)
<i>MMP-1</i>	↑	↓	–	–
<i>MMP-7</i>	↑	↓	–	–
<i>MMP-10</i>	–	–	–	–
<i>MMP-11</i>	–	–	–	–
<i>MMP-14</i>	↑	↓	–	–
<i>MMP-15</i>	↑	↓	↑	↓
<i>MMP-17</i>	↑	↓	–	–
<i>MMP-23</i>	–	–	–	–
<i>MMP-28</i>	–	–	–	↓
β-catenin	↑	↓	–	↓

↑, increase; ↓, decrease

**Figure 3** The expression rates of β-catenin, *MMP-1*, *MMP-14*, *MMP-15* and *MMP-17* genes in MKN-45 cells treated with H₂O₂ compared to the untreated control cells at 12 and 24 h under indicated H₂O₂ concentrations. Each of data is the average of three independent values and standard deviations are indicated above (σ_{n-1}) data values. All the up-regulations observed in the expressions were taken as statistically significant ($P < 0.05$) as determined by student *t*-test

expression gave parallel patterns of expression profiles with β-catenin. There was a 0.76 (76%), 1.69 and 1.35 fold significant increase ($P < 0.05$) after 12 h H₂O₂ exposure and 1.82, 1.33 and 0.92 (92%) fold significant increase ($P < 0.05$) after 24 h H₂O₂ treatment at 50, 100 and 200 μmol/L H₂O₂ concentrations, respectively, compared with the control cells in *MMP-14* gene (Figure 3). Likewise, *MMP-7* gene showed a similar pattern of increase with different values at the same order of the concentrations (27).

The expression of *MMP-1* gene in MKN-45 cells

after 12 h of H₂O₂ exposure was nearly similar at all the three concentrations, but 24 h exposure to H₂O₂ caused a significant up-regulation of 4.6, 4.9 and 6.1 fold ($P < 0.05$) at 50, 100 and 200 μmol/L concentrations, respectively. The same profile was observed in the *MMP-15* and *MMP-17* genes. Subsequent to 24 h H₂O₂ exposure, *MMP-15* expression was significantly increased by 3.0, 3.9 and 3.4 fold ($P < 0.05$) and *MMP-17* expression was significantly up-regulated by 5.05, 5.10 and 4.30 fold ($P < 0.05$) at 50, 100, and 200 μmol/L concentrations of H₂O₂, respectively (Figure 3).

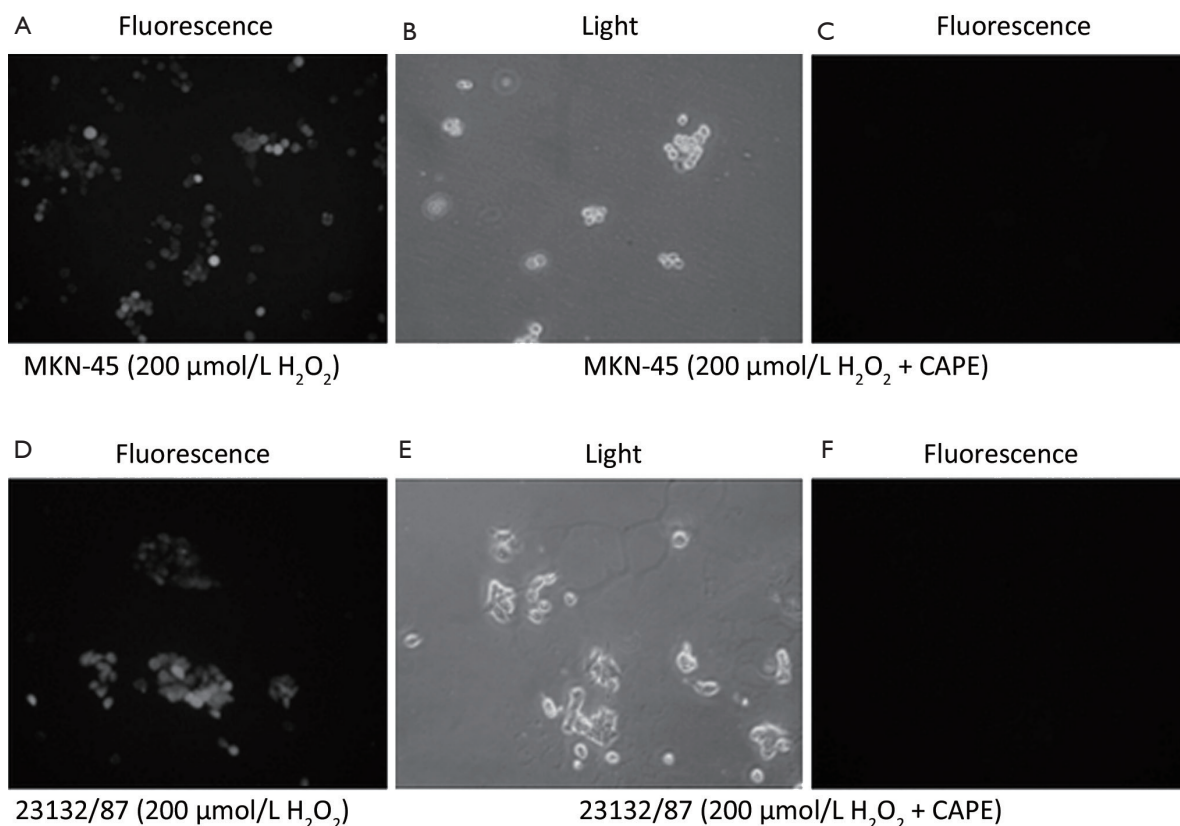


Figure 4 Analysis of internal oxidative stress in only 200 μmol/L H₂O₂ treated MKN-45 and 23132/87 (A, D) and CAPE treated cells which were exposed to 200 μmol/L H₂O₂ initially (B, C, E, F). B and E show the light microscopic views while A, C, D and F show the fluorescent microscopic views of MKN-45 and 23132/87 cells lines under indicated conditions (200×)

Removal of oxidative stress with CAPE exposure

In both MKN-45 and 23132/87 cells, 24 h exposure to CAPE resulted in the removal of oxidative stress as shown by DCFH-DA method (Figure 4). And 200 μmol/L H₂O₂ exposure revealed an intracellular accumulation of oxidative stress in both of the cell lines (Figure 4A,D). However, the fluorescent microscopic view of the cells (Figure 4C,F) of which light microscopic views were demonstrated (Figure 4B,E) clearly reveals that CAPE treatment removed the accumulated oxidative stress in the viable MKN-45 and 23132/87 cells.

Effect of CAPE treatment on expressions of MMP genes compared to oxidative stress conditions

In MKN-45 cells, the expressions of *MMP-1*, *MMP-7*, *MMP-14*, *MMP-15*, *MMP-17* and β -catenin genes that were up-regulated consequent to H₂O₂ exposure were down-regulated following inclusion of CAPE compared to

the expression levels of the H₂O₂ exposed cells. Likewise, in 23132/87 cells, a decrease only in the expression of *MMP-15* gene after CAPE application was observed since it was the only *MMP* in this cell line that was affected by H₂O₂ exposure (Table 3). β -catenin expression was 1.0 fold down-regulated significantly ($P < 0.05$) following CAPE treatment compared to H₂O₂ treated MKN-45 cells, while it was 2.4 fold decreased significantly ($P < 0.05$) in 23132/87 cells. Moreover, the *MMP-1* and *MMP-7* genes that were expressed in MKN-45 but not in 23132/87 cells showed a significant decrease 4.7 and 8.7 ($P < 0.05$), respectively, in MKN-45 cells following CAPE treatment compared to the H₂O₂ exposed cells (Figure 5). Also, 1.3 and 10.8 fold significant decrease ($P < 0.05$) in the expression of *MMP-15* gene in MKN-45 and 23132/87 cells, respectively, was determined (Figure 5). The expressions of *MMP-14* and *MMP-17* genes that were only expressed in MKN-45 cells were decreased significantly by 11.20 and 0.38 (38%)

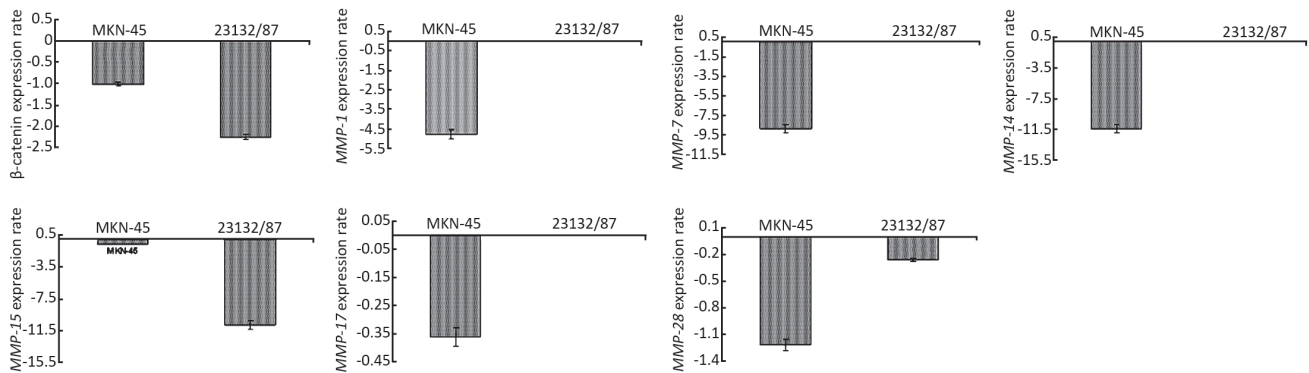


Figure 5 The expression rates of β -catenin, *MMP-1*, *MMP-7*, *MMP-14*, *MMP-15*, *MMP-17* and *MMP-28* genes in the cell lines treated with CAPE after 24 h of 200 $\mu\text{mol/L}$ H_2O_2 exposure compared with 200 $\mu\text{mol/L}$ H_2O_2 exposed control cells. Each of data is the average of three independent values and standard deviations are indicated above (σ_{n-1}) data values. All the down-regulations observed in the expressions were taken as statistically significant ($P < 0.05$) as determined by student *t*-test

fold ($P < 0.05$), respectively, after the removal of oxidative stress. On the other hand, *MMP-28* gene of which the expressions was demonstrated in both of the cell lines but not affected by H_2O_2 exposure, showed 1.20 and 0.27 (27%) fold significant decrease ($P < 0.05$) in expression after CAPE treatment (Figure 5).

Discussion

Extensive research to date suggests that continued oxidative stress can lead to chronic inflammation, which in turn could mediate most chronic diseases such as diabetes, cardiovascular, neurological, pulmonary diseases and cancers. Oxidative stress is linked to various steps involved in carcinogenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis and metastasis (16,17).

As a tumor promoter, it leads to increased levels of oxidatively modified bases such as 8-OHdG, in the tissues which are exposed to oxidative stress (28-32). Gastric tissue is the primary site where continuous oxidative stress and chronic inflammation appear. In this study, likewise, we demonstrated the presence of 8-OHdG, an oxidative stress induced DNA base modification, in MKN-45 and 23132/87 cells lines suggesting the involvement of oxidative stress in gastric tumor promotion with abnormality in their morphologies and detachment from the surface. We also showed that H_2O_2 exposure causes an accumulation of oxidative stress in the viable MKN-45 and 23132/87 cells and the removal of the oxidative stress with CAPE resulted in removal of the accumulated oxidative stress in these cells.

NF- κ B, AP-1, P53, HIF-1 α , PPAR- γ , β -catenin/Wnt, and Nrf2 are a variety of transcription factors which are activated by oxidative stress. The studies revealed that activation of these transcription factors can lead to the expression of over 500 different genes, including those for growth factors, cell cycle regulatory molecules, anti-inflammatory molecules, inflammatory cytokines, and chemokines (1,2,10,11). Here, we analyzed the change in the expression pattern of β -catenin, an adhesion molecule and a transcription factor, after H_2O_2 exposure of the gastric cancer cell lines, and we report that β -catenin expression increases in gastric cancer MKN-45 cells following H_2O_2 exposure but not in 23132/87 cells.

Among the studied MMPs, *MMP-1*, *MMP-7*, *MMP-10*, *MMP-14* and *MMP-17* genes were specifically expressed in MKN-45 cells. In these cells, parallel to β -catenin up-regulation following H_2O_2 treatment, a remarkable increase in the expressions of these MMP genes were also illustrated. Moreover, the removal of oxidative stress with CAPE caused a decrease in the expressions of the same genes. All these information suggests *MMP-1*, *MMP-7*, *MMP-14* and *MMP-17* up-regulation is directly related with the exposure to oxidative stress. In addition to this, increase and decrease in β -catenin expression under oxidative stress and non-oxidative stress conditions, respectively, might be related to the increase in the expressions of these MMP genes. On the other hand, *MMP-11*, *MMP-15*, *MMP-23* and *MMP-28* were expressed in both of the cell lines. Among these, the expressions of *MMP-15* was increased after exposure of MKN-45 and 23132/87 cells to H_2O_2 while CAPE treatment reversed this effect and caused a

decrease in *MMP-15* gene expression in both of the cell lines studied, which also reveals the link between oxidative stress and *MMP-15* gene expression. Interestingly, the down-regulation of *MMP-28* expression in 23132/87 cells after CAPE treatment even though there was not an up-regulation following H₂O₂ exposure suggested an internal oxidative stress in this cell line prior to external H₂O₂ exposure took role in *MMP-28* expression and removal of internal oxidative stress consequent to CAPE treatment resulted in the down-regulation of *MMP-28* gene compared to the untreated control cells.

β-catenin-independent up-regulation of Wnt5A induced *MMP-1* expression was observed in temporomandibular joint condylar chondrocytes (33). Here, we suggest that β-catenin might augment tumor invasion by increasing the rates of cell migration under oxidative stress conditions by promoting up-regulation of *MMP-1* in gastric cancer MKN-45 cells. It is well established that *MMP-7* is a Wnt-β-catenin target (34,35) and our results about the parallel up-regulation of β-catenin and *MMP-7* following H₂O₂ exposure of MKN-45 cells is expected. Up-regulation of *MMP-14* can also be related to the up-regulation of β-catenin since there exist data that demonstrated the detection of Fhit and β-catenin at the *MMP-14* promoter in MCF-7 cells to regulate growth (36). On the other hand, there is no information about the regulation of *MMP-17* by β-catenin, thus our results suggest a possible relation between the regulation of *MMP-17* and β-catenin since under oxidative stress conditions, the expression of the genes increased, while the removal of oxidative stress caused the opposite.

In support of our data with change in the expressions of *MMP* genes, *MMP-13*, *MMP-3* and *MMP-10* were remarkably up-regulated by oxidant directly, and their activities were implicated in the invasive potential induced in NMuMG cells (37). Likewise, *MMP-2* and *MMP-9* were activated post-transcriptionally by prolonged oxidative treatment (38). The activation of *MMPs*, such as *MMP-2*, probably occurs by the reaction of ROS with thiol groups in the protease catalytic domain (39). In addition to their role as key regulators of *MMP* activation, ROS have been implicated in *MMP* gene expression (40). Both H₂O₂ and nitric oxide donors, as well as the increased expression of iNOS, stimulate the expression of several *MMPs* (*MMP-1*, *MMP-3*, *MMP-9*, *MMP-10* and *MMP-13*) (40). In fibroblastic cells, the sustained production of H₂O₂ recently was shown to activate *MMP-2* and to increase cell invasion (41). Oxidative stress may also modulate *MMP* expression by activation

of Ras, or direct activation of the MAPK family members ERK1/2, P38, and JNK, or inactivation of phosphatases that regulate these proteins (42).

In conclusion, our data related to the change in the expressions of β-catenin and several *MMP* genes following oxidative stress exposure to gastric cancer cell lines give an idea about the effect of oxidative stress on the expressions of these genes which will be a hot spot research in gastric carcinogenesis at the molecular level in near future and take the attention of the researchers in this area since gastric mucosa is continuously exposed to oxidative stress and these genes are the main modulators of invasion and metastasis.

Acknowledgements

This project was granted by the Scientific and Technical Research Council of Turkey (TUBITAK) under the project number 105S352 (SBAG-K-110) and by the Scientific Research Fund of Fatih University under the project number P50030703.

Disclosure: The authors declare no conflict of interest.

References

1. Reuter S, Gupta SC, Chaturvedi MM, et al. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* 2010;49:1603-16.
2. Cortes DF, Sha W, Hower V, et al. Differential gene expression in normal and transformed human mammary epithelial cells in response to oxidative stress. *Free Radic Biol Med* 2011;50:1565-74.
3. D'Autréaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 2007;8:813-24.
4. Morel Y, Barouki R. Repression of gene expression by oxidative stress. *Biochem J* 1999;342:481-96.
5. Dinkova-Kostova AT, Holtzclaw WD, Kensler TW. The role of Keap1 in cellular protective responses. *Chem Res Toxicol* 2005;18:1779-91.
6. Kobayashi M, Yamamoto M. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid Redox Signal* 2005;7:385-94.
7. Slupphaug G, Kavli B, Krokan HE. The interacting pathways for prevention and repair of oxidative DNA damage. *Mutat Res* 2003;531:231-51.
8. Clopton DA, Saltman P. Low-level oxidative stress causes cell-cycle specific arrest in cultured cells. *Biochem Biophys Res Commun* 1995;210:189-96.

9. Chua PJ, Yip GW, Bay BH. Cell cycle arrest induced by hydrogen peroxide is associated with modulation of oxidative stress related genes in breast cancer cells. *Exp Biol Med (Maywood)* 2009;234:1086-94.
10. Seng S, Avraham HK, Jiang S, et al. The nuclear matrix protein, NRP/B, enhances Nrf2-mediated oxidative stress responses in breast cancer cells. *Cancer Res* 2007;67:8596-604.
11. Almeida M, Han L, Martin-Millan M, et al. Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. *J Biol Chem* 2007;282:27298-305.
12. Hoogeboom D, Essers MA, Polderman PE, et al. Interaction of FOXO with beta-catenin inhibits beta-catenin/T cell factor activity. *J Biol Chem* 2008;283:9224-30.
13. Kaidi A, Williams AC, Paraskeva C. Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nat Cell Biol* 2007;9:210-7.
14. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer* 2003;3:276-85.
15. Sanders LM, Henderson CE, Hong MY, et al. Pro-oxidant environment of the colon compared to the small intestine may contribute to greater cancer susceptibility. *Cancer Lett* 2004;208:155-61.
16. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860-7.
17. Mantovani A. Cancer: Inflammation by remote control. *Nature* 2005;435:752-3.
18. Gialeli C, Theocharis AD, Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. *FEBS J* 2011;278:16-27.
19. Farinati F, Cardin R, Cassaro M, et al. Helicobacter pylori, inflammation, oxidative damage and gastric cancer: a morphological, biological and molecular pathway. *Eur J Cancer Prev* 2008;17:195-200.
20. Sternlicht MD, Bissell MJ, Werb Z. The matrix metalloproteinase stromelysin-1 acts as a natural mammary tumor promoter. *Oncogene* 2000;19:1102-13.
21. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet* 2002;3:737-47.
22. Ito E, Yana I, Fujita C, et al. The role of MT2-MMP in cancer progression. *Biochem Biophys Res Commun* 2010;393:222-7.
23. Lowy AM, Clements WM, Bishop J, et al. beta-Catenin/Wnt signaling regulates expression of the membrane type 3 matrix metalloproteinase in gastric cancer. *Cancer Res* 2006;66:4734-41.
24. Tang CH, Yamamoto A, Lin YT, et al. Involvement of matrix metalloproteinase-3 in CCL5/CCR5 pathway of chondrosarcomas metastasis. *Biochem Pharmacol* 2010;79:209-17.
25. Bass DA, Parce JW, Dechatelet LR, et al. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* 1983;130:1910-7.
26. Gencer S, Cebeci A, Irmak-Yazicioglu MB. Silencing of the MMP-3 gene by siRNA transfection in gastric cancer AGS cells. *J Gastrointest Liver Dis* 2011;20:19-26.
27. Gencer S, Irmak Yazicioglu MB. Differential response of gastric carcinoma MKN-45 and 23132/87 cells to H₂O₂ exposure. *Turk J Gastroenterol* 2011;22:145-51.
28. Wei H, Frenkel K. Suppression of tumor promoter-induced oxidative events and DNA damage in vivo by sarcophytol A: A possible mechanism of anti-promotion. *Cancer Res* 1992;52:2298-303.
29. Hattori-Nakakuki Y, Nishigori C, Okamoto K, et al. Formation of 8-hydroxy-2'-deoxyguanosine in epidermis of hairless mice exposed to near-UV. *Biochem Biophys Res Commun* 1994;201:1132-9.
30. Ohshima H, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res* 1994;305:253-64.
31. Rosin MP, Saad el Din Zaki S, Ward AJ, et al. Involvement of inflammatory reactions and elevated cell proliferation in the development of bladder cancer in schistosomiasis patients. *Mutat Res* 1994;305:283-92.
32. Weitzman SA, Gordon LI. Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis. *Blood* 1990;76:655-63.
33. Ge X, Ma X, Meng J, et al. Role of Wnt-5A in interleukin-1 beta-induced matrix metalloproteinase expression in rabbit temporomandibular joint condylar chondrocytes. *Arthritis Rheum* 2009;60:2714-22.
34. Crawford HC, Fingleton BM, Rudolph-Owen LA, et al. The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. *Oncogene* 1999;18:2883-91.
35. Brabletz T, Jung A, Dag S, et al. beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am J Pathol* 1999;155:1033-8.
36. Weiske J, Albring KF, Huber O. The tumor suppressor Fhit acts as a repressor of beta-catenin transcriptional activity. *Proc Natl Acad Sci USA* 2007;104:20344-9.

37. Mori K, Shibamura M, Nose K. Invasive potential induced under long-term oxidative stress in mammary epithelial cells. *Cancer Res* 2004;64:7464-72.
38. Westermarck J, Kahari V. Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J* 1999;13:781-92.
39. Rajagopalan S, Meng XP, Ramasamy S, et al. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* 1996;98:2572-9.
40. Nelson KK, Melendez JA. Mitochondrial redox control of matrix metalloproteinases. *Free Radic Biol Med* 2004;37:768-84.
41. Yoon SO, Park SJ, Yoon SY, et al. Sustained production of H₂O₂ activates pro-matrix metalloproteinase-2 through receptor tyrosine kinases/phosphatidylinositol 3-kinase/NF-kappa B pathway. *J Biol Chem* 2002;277:30271-82.
42. Storz P. Reactive oxygen species in tumor progression. *Front Biosci* 2005;10:1881-96.

Cite this article as: Gencer S, Cebeci A, Irmak-Yazicioglu MB. Matrix metalloproteinase gene expressions might be oxidative stress targets in gastric cancer cell lines. *Chin J Cancer Res* 2013;25(3):322-333. doi: 10.3978/j.issn.1000-9604.2013.06.05