Expression of the DNA repair enzyme, N-Methylpurine-DNA glycosylase (MPG) in astrocytic tumors

Jung Yong Ahn

Brain Korea 21 Project for Medical Science
The Graduate School, Yonsei University

Expression of the DNA repair enzyme, N-Methylpurine-DNA glycosylase (MPG) in astrocytic tumors

Directed by Professor Joong Uhn Choi

The Doctoral Dissertation
submitted to the Brain Korea 21
Project for Medical Science
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

Jung Yong Ahn

June, 2002

This certifies that the Doctoral Dissertation
of Jung Yong Ahn is approved.

This Supervisor: Joong Uhn Choi

Tai Seung Kim: Thesis Committee Member

Jin Woo Chang: Thesis Committee Member

Jeon-Soo Shin: Thesis Committee Member

Won-Jae Lee: Thesis Committee Member

The Graduate School
Yonsei University

June, 2002

ACKNOWLEDGEMENTS

I am grateful to Professor Joong Uhn Choi for meticulous guidance and warm encouragement. Special thanks go to Professor Tai Seung Kim, Jin Woo Chang, Jun-Soo Shin, and Won-Jae Lee for generous advice and critical reading the manuscript.

I am greatly indebted to Dr. Kyu Sung Lee and Dr. Jin Yang Joo at Yongdong Severance Hospital for providing surgical specimens, to Professor Nam Keun Kim, Doyeun Oh, Jeong Yun Shim, and Hee Jung An at Pundang CHA Hospital for sustained and significant contributions to research, and to the staff of the institute for clinical research for extensive collaboration and technical guidance. I thank Jin Hyun Kwon for his help in the preparation of surgical specimens and the operating room staff for their continued support in the acquisition of surgical specimens.

I dedicate the dissertation to my wife; Eun Wan Choi, my daughter; Soo Bin Ahn, my father; Tai Sik Ahn, and my mother; Jung Rye Uh.

Contents

List of Figures

List of abbreviations

3MeA	3-methyladenine
7MeG	7-methylguanine
AP	apurinic-apyrimidinic
BER	base excision repair
cDNA	complementary DNA
CHO	Chinese hamster ovary
FEN	5'-flap endonuclease
HPRT	hypoxanthine-guanine phosphoribosyltransferase
MGMT	O^6-methylguanine-DNA methyltransferase
MMS	methyl methanesulfonate
MPG	N-methylpurine DNA glycosylase
mRNA	messenger RNA
NTH	thymine glycol DNA glycosylase
OGG	8-oxo-guanine DNA glycosylase
PCNA	proliferating cell nuclear antigen
RT-PCR	reverse transcriptase-polymerase chain reaction
SSC	standard saline citrate

Abstract

Expression of the DNA repair enzyme, N-Methylpurine-DNA glycosylase (MPG) in astrocytic tumors

Jung Yong Ahn

Brain Korea 21 Project for Medical Science
The Graduate School, Yonsei University

(Directed by Professor Joong Uhn Choi)

Background: DNA is continuously damaged due to exposure to alkylating compounds or oxygen free radicals generated during normal cellular metabolism and as environmental mutagens. Several studies found that MPG mRNA levels were lower in adult brain than in other tissues. Terminally differentiated and nonproliferating cells have a lower DNA repair capacity than proliferating cells from organs, embryo, ovary and testis. The accumulation of damage in nondividing tissues would be more acute and more serious. If the DNA repair enzyme does not work properly, the damaged DNA lead to tumorigenesis or cell death. This study was designed to investigate the association of tumorigenesis with DNA repair enzyme, N-methylpurine-DNA-glycosylase (MPG) in astrocytic tumors.

Materials and Methods: MPG mRNA expression and localization in the 30 astrocytic tumors and 7 tumor-adjacent brain tissues was examined by reverse transcriptase-polymerase chain reaction and RNA *in situ* hybridization. Expression and intracellular localization of MPG protein was determined by immunohistochemistry and western blot analysis.

Results: MPG mRNA expression in reverse transcriptase-polymerase chain reaction was significantly higher in grade IV tumor tissues than in brain tissues adjacent to tumor or in grade II-III tumor tissues ($p < 0.05$). MPG mRNA in *in situ* hybridization was detected both in brain tissues adjacent to tumor and in astrocytic

tumor tissues, regardless of the tumor grades. MPG mRNA expression in RT-PCR was higher in glioblastomas (grade IV) than in diffuse astrocytomas (grade II) and anaplastic astrocytomas (grade III). However, MPG protein localization in immunohistochemical study was detected only in the nucleus of all tumor tissues. In brain tissues adjacent to tumor, immunohistochemical staining for MPG was not stained both in the nucleus and in cytoplasm. MPG protein expression in western blot analysis was higher in grade IV tumor tissues than in grade II-III astrocytomas. Increased MPG expression was found in malignant astrocytic tumors. And the intracellular localizations of MPG mRNA in tumors or adjacent brain tissues were different from one of MPG protein in tissues. However, the intracellular localization of MPG in the tissues was not heterogeneous among the tumor grades.

Conclusions: These results suggest MPG's role in human astrocytic tumors and raise the possibility that the increased mRNA level and intracellular localization could be associated with astrocytic tumorigenesis. Further studies about control of MPG gene expression in astrocytic tumors are warranted.

Key Words: astrocytic tumors, repair gene, N-methylpurine-DNA glycosylase (MPG), expression, tumorigenesis

Expression of the DNA repair enzyme, N-Methylpurine-DNA glycosylase (MPG) in astrocytic tumors

Jung Yong Ahn

Brain Korea 21 Project for Medical Science
The Graduate School, Yonsei University

＜Directed by Professor Joong Uhn Choi＞

I. INTRODUCTION

DNA in all somatic and germ cells in the body are continuously damaged causing mutation, chromosome aberration, aging, and carcinogenesis. DNA repair that is a universal and ubiquitous process is essential for survival of life.[1-3] Mice lacking a precise DNA repair activity have been generated, and these mutants show various combinations of defective embryogenesis, tissue-specific dysfunction, hypersensitivity to DNA-damaging agents, premature senescence, genetic instability, and elevated cancer rates.[4] DNA repair enzymes have been reported to play an important role in the carcinognesis of several cancers.[5-9] N-Methylpurine-DNA glycosylase is one of the key enzyme in DNA repair and it removes N-alkylpurine and other related adducts including 8-hydroxyguanine, 1,N^6-ethanoadenine and hypoxanthine.[10-12] Expression of MPG is known to be induced by viral damage and a variety of DNA-damaging agents such as physical agents like UV light and γ-rays, and chemical compounds including alkylating, intercalating drugs and oxygen free radicals.[7,13] This enzyme repairs N-alkyladducts via a base excision repair pathway in which the first step is the removal of the alkyladduct by MPG and AP-endonuclease.[7,14-17] Mitchell and Hartman[18] reported that proliferating cells from organs and embryos have a

greater DNA repair capacity than terminally differentiated and nonproliferating cells. Kim et al.[19] also reported that transcription of MPG is maximum in rapidly dividing and growing tissues. Increased expression of MPG gene was studied in breast cancer, cervical neoplasia and thymic carcinoma in SV 40 T-antigen expressing transgenic mice.[5,6,9] In breast cancer, MPG was overexpressed up to 24-fold as compared to normal primary breast epithelium, suggesting the role of MPG in breast carcinogenesis.[5]

In brain cells that are not undergoing DNA replication, DNA repair is maintaining nucleotide sequences of genomic DNA over time. Brain tissues may be protected from exogenous damage by blood/brain barrier. However, cellular metabolism, mitochondrial respiration and ionizing radiation generate reactive oxygen species which are extremely reactive and interact with DNA.[10,20] Nitrosamines are also produced in mammals from ingested nitrites and secondary or tertiary amines. Of particular significance are the ubiquitous N-nitroso compounds that include dietary N-nitrosodialkylamines, volatile and nonvolatile nitrosamines found in tabocco smoke, and chemotherapeutic alkylnitrosoureas. Endogenous processes such as nonenzymatic methylation by *S*- adenosylmethionine or reaction with products of lipid peroxidation may also contribute to alkylation-induced DNA damage.[21,22] This type of damage would be particularly deleterious for terminally differentiated cells. Therefore, DNA damage may be a more serious problem for brain cells than other somatic cells despite available mechanisms for avoiding and repairing such damage.[8,20,23,24]

In this study, we tested whether the expression and intracellular localization of MPG could be altered in astrocytic tumors. This is the first report on MPG expression in astrocytic tumors.

II. MATERIALS AND METHODS

1. Tissues

The Institutional Review Board of Pundang CHA Hospital approved this study in May

2001. Through June 2001 to May 2002, astrocytic tumor patients consented to use of their tissues. Tumor and tumor-adjacent brain tissue (tumor-adjacent brain tissues; N=7; tumor tissues; N=30) were collected from Department of Neurosurgery, Pundang CHA General Hospital at the Pochon CHA University and Department of Neurosurgery, Yongdong Severance Hospital at the Yonsei University College of Medicine. The patient material consisted altogether of 30 patients - 13 females and 17 males, aged 41 to 72 years. Mean age at diagnosis was 54.6 years±7.5 (SD). All patients underwent craniotomy and the study was done on diagnostic material received for frozen section and histology. The histological diagnosis was made by two neuropathologist. The tissues were classified as brain tissues adjacent to tumor, diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (grade IV) according to World Health Organization (WHO) classification of the astrocytic tumors. Of the 30-biopsy samples, 11 were diagnosed as grade II, 9 as grade III, and 12 as grade IV.

2. DNA probe and primer preparation

Semi-quantitative RT-PCR was used to assess mRNA amounts of MPG in tissues, based on the relative expression of 2 mRNAs: MPG and HPRT. To normalize mRNA amounts of MPG between tissue samples, HPRT, a moderately expressed housekeeping gene, was used as the reference gene.[25] Primers of HPRT were designed as previously described.[26] Primers used were MPG sense, 5'-GTCCTAGTCCGGCGACTTCC-3', and anti-sense, 5'-CTTGTCTGGGCAGGCCCTTTGC-3', leading to a 603-bp PCR product, and HPRT sense,5'-GCCGGCTCCGTTATGGCG-3',andanti-sense,5'-AGCCCCCCTTGAGCACACA-3', leading to a 225-bp PCR product. RT-PCR was carried out using at least triplicates for each condition.

3. Reverse transcriptase-polymerase chain reaction

To compare the level of MPG mRNA between adjacent brain tissues and astrocytic

tumors, the total RNA was transcribed into cDNA, and polymerase chain reaction was carried out using RT-PCR one batch system (Bioneer Co., Korea). The reaction mixture contained 1μg total RNA, 20 U M-MLV RTase, 10 U RNasin, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dNTP, 1 pmole each of 5' and 3' primers and 1.0 U Taq polymerase. The reverse transcription and PCR amplification was simultaneously performed using Thermal Cycler (Perkin Elmer 2400) in one batch tube. The reverse transcription was carried out for 60 minutes at 42°C and was heated to 95°C for 5 minutes to terminate the reverse transcription. The amplification was performed for 30 cycles of denaturation at 95°C for 30 seconds, annealing at 64°C for 30 seconds and extension at 72°C for 30 seconds. A final extension step for 5 minutes at 72°C followed. Aliquots of each product were electrophoresed in 1.2% agarose gel containing 0.5μg/ml ethidium bromide. Relative levels of target gene (MPG/HPRT) were quantitated by a computerized imaging analysis system (Viiber Lourmat, France).

4. RNA *in situ* hybridization

The non-radioactive *in situ* hybridization was performed as described by Roche protocol with slight modification.[27] Tissues were formalin fixed and paraffin embedded. Cryostat sections of 5μm were collected on ProbeOn Plus slides (Fisher Biotech, USA) and proteinase K (1μg/ml) treatment in TE buffer (pH 7.4) for 10 min and fixed in 4% paraformaldehyde in PBS for 5 minutes. Prehybridization was performed in a solution of 50% formamide, 5× SSC, 5× Denhardt's solution (1 mg/ml Ficoll, 1 mg polyvinylpyrrolidone and 1 mg/ml BSA), 250μg/ml tRNA and 500μg/ml salmon sperm DNA at 62°C for 2 hours. Hybridization was performed in the same solution containing 1μg/ml digoxigenin-labeled riboprobe at 62°C overnight. After hybridization, the slides were washed in 2× SSC at 37°C for 30 minutes and in 50% formamide/ 2× SSC at 60°C for 30 minutes, and then incubated with NTE solution (500 mM NaCl, 10 mM Tris, 1 mM EDTA) containing RNase A (20μg/ml). And then the slides incubated with blocking buffer (10% heat-inactivated normal goat serum, 0.1 M Tris, pH 7.5 and 0.15 M NaCl) for 2 hours at room temperature, before the addition of alkaline phosphate-conjugated

antidigoxigenin antibody (1：1,000 dilution in blocking buffer 1：10 diluted with maleic acid). After 2 h incubation at room temperature, the slides were washed three times in maleic acid buffer. NBT/BCIP were added as a substrate for alkaline phosphatase, after overnight incubation the color reaction was stopped with 10 mM Tris/1 mM EDTA. To monitor background level and specificity of hybridization, the sense strand of MPG probe was used.

5. Immunohistochemistry

Surgical specimens were fixed in formalin, dehydrated in ethanol, embedded in paraffin, and sectioned by 5μm. Sections were deparaffinized with xylene for 3 minutes, quickly rehydrated, and microwaved in citrate buffer for 20 minutes to enhance antigen retrieval. Endogenous peroxidases were blocked by 0.5% peroxide in ethanol. Sections were preincubated with nonimmune goat serum for 10 minutes at the room temperature. The primary antibody, monoclonal human anti-MPG, kindly provided from R. Roy (University of Texas Medical Branch, TX, USA), was diluted to 1：100 before use. The sections were incubated with the primary antibody for 2 hours at room temperature. The secondary antibody (rabbit anti-mouse IgG antibody, 1：250) was then applied for 10 min at the room temperature, and binding of the primary antibody was detected and visualized by streptavidine-biotin immunoperoxidase with the use of a polyvalent immuno-peroxidase kit (DAKO, CA, USA) with diaminobenzidine, following the manufacturer's procedure. Sections were counterstained with Mayer's hematoxylin.

6. Western blot analysis

To analyze tumor cells overexpressing nuclear MPG cells were collected from a confluent plate, pelleted, washed with Dulbecco's phosphate-buffered saline (Gibco BRL Life Technologies) and lysed with protein loading dye (0.0313 M Tris, 0.1 M SDS, 3.6 mM bromophenol blue, 2.75 mM β-mercaptoethanol, 50% glycerol, final concentration 1×). The samples were boiled to denature the proteins and quantitated using the Bradford Protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (20μg) were separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The Roche Chemiluminescence kit (Indianapolis, IN) was used for the blocking and detection reagents. Antihuman MPG antibody was used at a dilution of

1 : 300 for western blots and rotated overnight at 4°C. Following primary antibody incubation the filter was washed and the rabbit secondary antibody was added for 45 60 minutes at room temperature. Again the filter was washed and the detection solutions were added. The Roche kit contained a horseradish peroxidase-labeled secondary antibody and a luminal substrate solution for the detection of bands. After these solutions were added to the filter it was exposed to high performance autoradiography film (Amersham Pharmacia Biotech, Little Chalfont, UK).

7. Statistical analysis

Statistical analysis was performed by Student's *t* test and ANOVA test using the Statistical Package for Social Science Statview Package (SPSS Inc., Chicago, IL) with a p value <0.05 considered statistically significant. RT-PCR was carried out using at least triplicates for each condition.

III. RESULTS

1. Expression of MPG mRNA by RT-PCR

To investigate the role of MPG, we examined its mRNA expression between tumor tissues and adjacent brain tissues (Figure 1). The level of MPG mRNA expression in grade IV increased about 2.3-fold above brain tissues adjacent to tumor (Figure 2), which was significant statistically

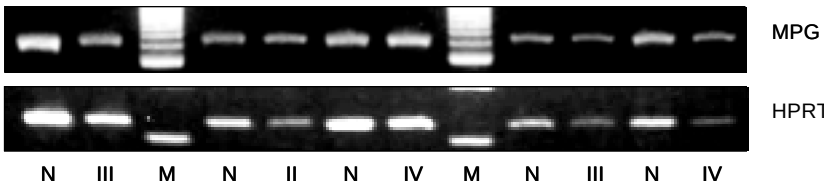

Figure 1. RT-PCR products of MPG and HPRT mRNA from cDNA. The same amount of cDNA was amplified at different cycles. The bands corresponding to MPG is 603 bp and HPRT is 225 bp. Lane "N" represents PCR products of brain tissues adjacent to tumor at cycle numbers 30. Lane "II, III, and IV" represents PCR products of tumor tissues according to grade. "M" represents DNA size marker.

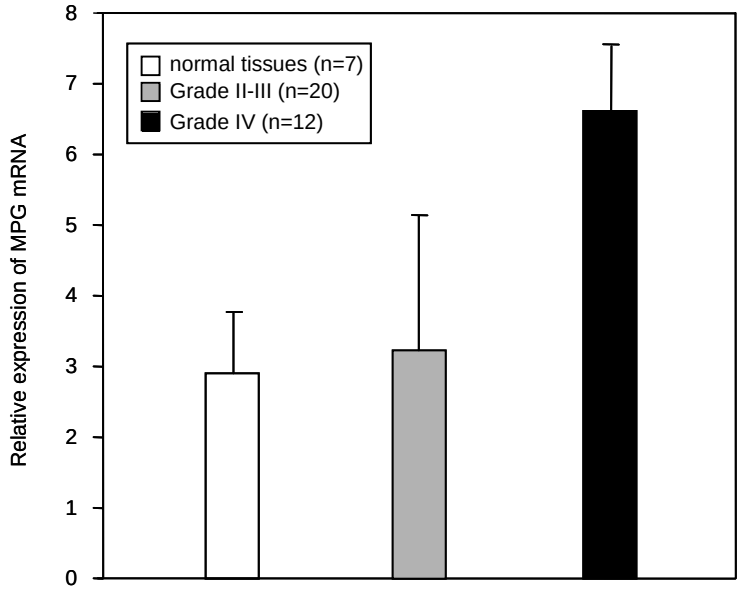

Figure 2. mRNA ratios of MPG over HPRT in astrocytic tumor tissues according to grade and adjacent brain tissues. The relative mRNA level was calculated by laser densitometer. The level of MPG transcripts in grade IV increased about 2.3-fold above adjacent brain tissues, which was significant statistically ($p<0.05$). As compared with grade II and III astrocytic tumors, grade IV tumors showed a significantly higher level of MPG expression ($p<0.05$).

($p<0.05$).

As compared with grade II and III astrocytic tumors, grade IV tumors showed a significantly higher level of MPG expression ($p<0.05$) (Figure 2).

2. Cellular localization of MPG mRNA by *in situ* hybridization technique

Although RT-PCR assay showed the average mRNA levels of MPG in the biopsy tissues, it has limitation in determining the distribution of MPG mRNA in individual cells. To examine the cellular distribution of MPG, RNA *in situ* hybridization technique was used. Surprisingly, MPG mRNA was constantly expressed in cytoplasms of tumor tissues and adjacent brain tissues (Figure 3A, B). The expression of MPG mRNA in normal glial

tissues was confirmed by *in situ* hybridization techniques.

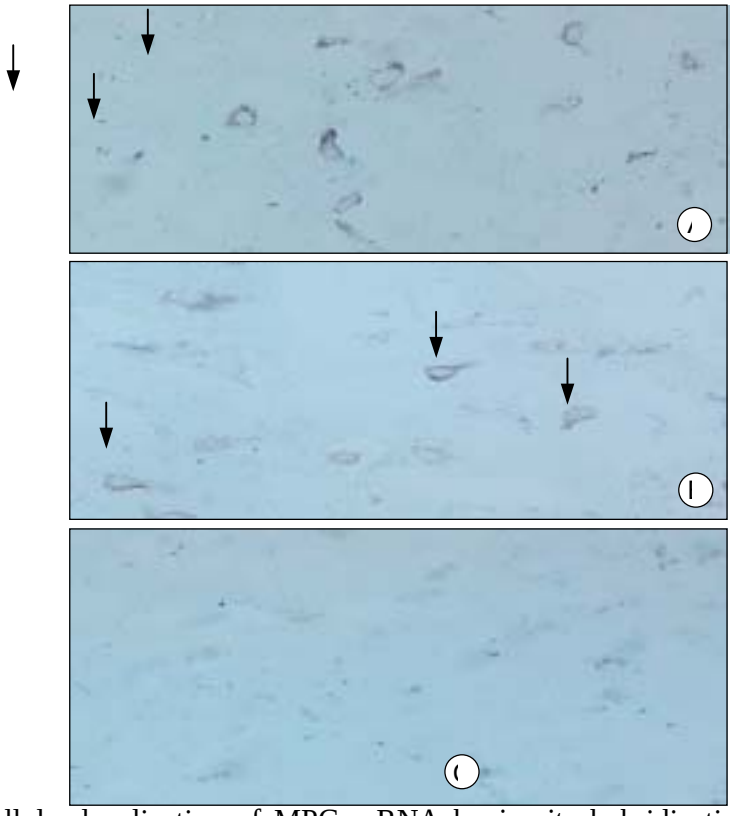

Figure 3. Cellular localization of MPG mRNA by *in situ* hybridization technique. MPG mRNA is constantly expressed in adjacent brain tissue (A) and astrocytic tumor tissues (B) (Original magnification ×400). However, it is not detected in the adjacent brain tissues using sense strand probe (C). Dark arrows indicate expression of MPG mRNA in the cytoplasm.

3. Intracellular localization & expression of MPG protein by immunohistochemistry

Immunostaining of MPG protein was not observed both in the nucleus and cytoplasm of brain tissues adjacent to tumor (Figure 4A, C, and E). However, MPG was strongly stained in the nucleus of astrocytic tumor tissues regardless of tumor grade, not in the cytoplasm (Figure 4B, D, and F). Therefore, we could not detect the significant difference of MPG localization and the expression level between different grades of astrocytic tumors. MPG protein localization was strikingly altered in brain tumor tissues. The translocation of

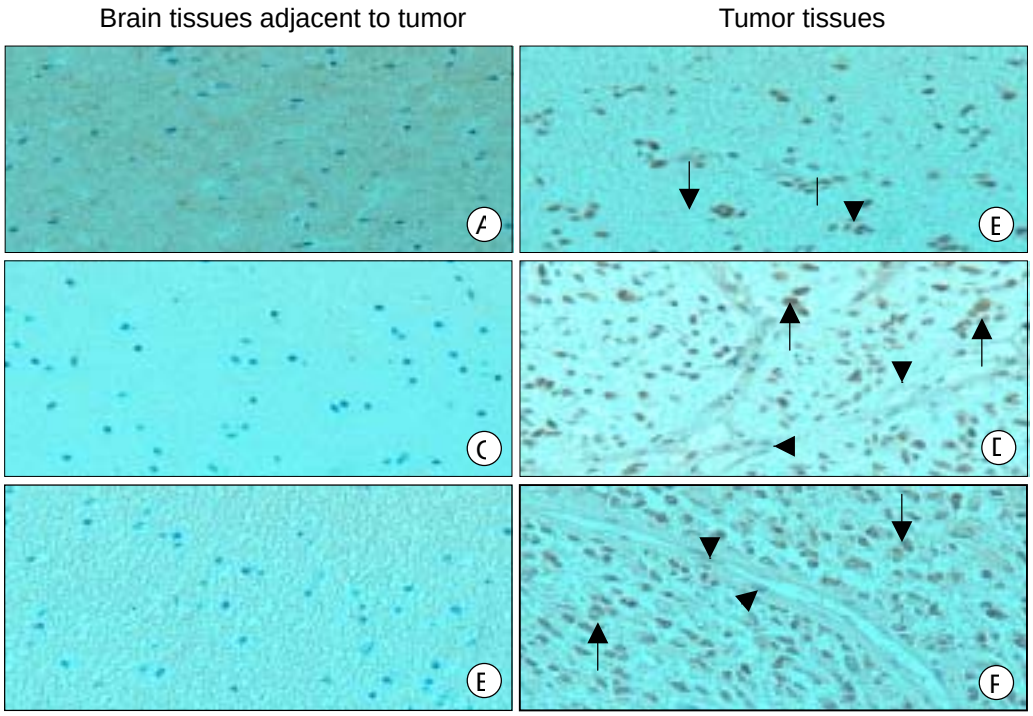

Figure 4. Immunohistochemical stains for MPG in tumors and corresponding brain tissues adjacent to tumor of the same patients. A, C, and E are the adjacent brain tissues of tumor case B, D, and F. Brain tissues adjacent to tumor are negative for MPG protein (Original magnification ×200). B. Grade II astrocytoma. Tumor cells show nuclear positivity for MPG protein (Original magnification ×200). D. Grade III astrocytoma. The nuclei of tumor cells demonstrate strong positivity for MPG protein, contrasted to negative staining of endothelial cells (arrowhead) in intervening blood vessels (Original magnification ×200) F. Atypical tumor cells of grade IV astrocytoma show diffuse, strong nuclear positivity (Original magnification ×200). Dark arrows indicate expression of MPG protein in the nucleus.

MPGfrom the cytoplasm to the nucleus in astrocytic tumor tissues raises the possibility that MPG participates in DNA repair during tumorigenesis.

4. Western blot analysis of MPG protein

The size of MPG protein was about 35 kDa. MPG expression was present in grade II, III, and IV astrocytic tumors. Grade IV tumor cells expressed higher levels of MPG protein than grade II-III tumor cells and brain tissues adjacent to tumor (Figure 5).

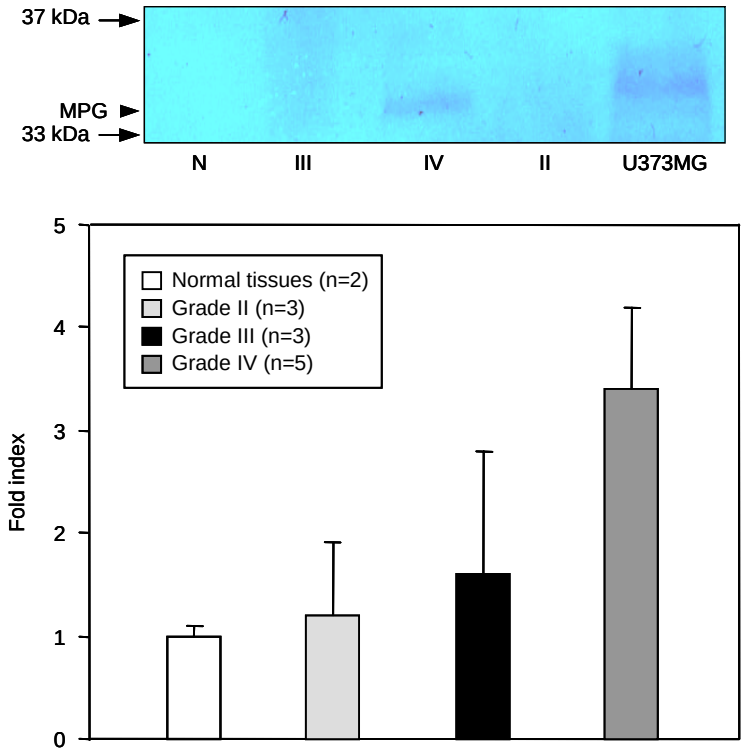

Figure 5. Western blot analysis of MPG protein expression. Arrowhead indicates the MPG protein, 35 kDa in size. MPG expression is determined in grade II, III, and IV astrocytic tumors. Grade IV tumor cells expressed higher levels of MPG protein than grade II-III tumor cells and adjacent brain tissues. U373MG indicates tumor saline of grade III.

IV. DISCUSSION

MPG is one of a growing list of enzymes responsible for the recognition and excision of altered bases in the first step of the BER pathway.[15,28] In the simplest form of BER, the resulting abasic site is then repaired by the sequential action of an AP en donuclease that generates a single-strand break, the removal of the 5'-terminal deoxyribose phosphate residue, insertion of a single nucleotide by DNA polymerase β, and finally ligation of the repaired patch by DNA ligase I or XRCC1-DNA ligase III. Mammalian MPG has been

shown to be active against a wide range of modified bases in vitro, many structurally unrelated to 3MeA, the substrate after which the enzyme was first named.[14] Engelward et al.[29] have genetically engineered animals deficient in MPG, a DNA glycosylase that removes a broad spectrum of base damages, including, but likely not limited to, 3MeA, 7MeG, 1-N^6-ethenoadenine, hypoxanthine, and 8-oxo-7,8-dihydroguanine.

Cells are under constant threat from a great variety of induced structural alterations to their DNA, which could lead to high and deleterious mutation rates as well as to cytotoxic effects. Endogenous damage to DNA occurs in consequence to hydrolysis, lipid peroxidation events and formation of other reactive small molecules intracellularly. Since endogenous DNA damage occurs at a frequency of about 20,000 events per human cell per day,[30] a high spontaneous mutation frequency might be expected to result from such error-prone repair, in addition to the mutagenic threat resulting from tardy DNA glycosylase excision of miscoding lesions, such as deaminated adenines. The significant turnover of DNA due to the excision of endogenous lesions might to expect to contribute to the carcinogenic process in mammalian cells, unless special mechanisms exist to increase the accuracy of BER.[31]

Mammalian cell actively regulate DNA repair enzymes and genes during cell proliferation. DNA repair pathways are expressed in the cell cycle in a defined temporal pattern relative to the induction of DNA replication.[32] The MPG expression was initially identified by virtue of temporal and spatial regulation in protein level[33,34] and spatial regulation in mRNA level.[19,35] The MPG expression is known tissue-specific and developmental stage-specific variation.[19] The MPG mRNA level in whole embryo is the highest in day 14.5 post coitum and apparently declined until the end of gestation.[19] These results suggest that the levels of MPG mRNA in embryonic tissues during embryogenesis may reflect the levels of cell proliferation in such tissues. Engelward et al.[35] reported the MPG mRNA levels in various tissues of adult mice with the testis and heart showing the highest and lowest level among tested organs in adults. XPBC/ERCC-3, another DNA repair gene, has shown relatively

high levels of mRNA in the testis, suggesting that there may be a general increase in the level of DNA repair in germline cells.[36] Mitchell and Hartman[18] reported that proliferating cells from organs and embryos have a greater DNA repair capacity than terminally differentiated and nonproliferating cells. In brain cells that are not undergoing DNA replication, DNA repair is maintaining nucleotide sequences of genomic DNA over time. Kim et al.[19] reported MPG expression in the brain was relative high in 1-week after birth, and the level remained low in day 400 mature adults, suggesting that brain tissue is terminally differentiated and nonproliferating tissues. However, DNA damage from exogenous or endogenous mutagens, above remarked, may be a more serious problem for brain cells than other somatic cells despite available mechanisms for avoiding and repairing such damage.[8,20,23,24] DNA repair enzymes, including AP endonuclease, DNA polymerase β, XRCC1/DNA ligase III, PCNA, FEN1, MGMT, NTH, and OGG, may be associated with brain tumorigenesis. Herein, we first report that MPG mRNA level in astrocytoma was slightly higher than that in brain tissues, but not significant statistically. However, MPG mRNA level in grade IV astrocytoma was definitely higher than that in grade II-III or tumor-adjacent brain tissues. These facts mean that the increased MPG expression of malignant astrocytic tumors is closely associated with brain tumorigenesis. Even if tumor-adjacent brain tissues were nearly normal brain tissue in gross examination, histological examination invariably revealed areas of local invasion. Infiltrative extension of the malignant gliomas is characteristic and accounts for the frequent appearance of multicentricity.[37] We think our result is significant and meaning because brain tissues adjacent to tumor are not purely normal. Direct estimation of MPG enzyme activity in tumor tissues will be helpful for association with tumorigenesis.

Surprisingly, we found that the MPG mRNA localization and the level of brain tissues adjacent to tumor were similar to that of tumor tissues by RNA *in situ* hybridization techniques. Moreover, the difference of MPG mRNA level between adjacent brain tissues and grade II-III astrocytomas by RT/PCR was not statistically significant ($p > 0.05$). MPG

mRNA might be ready for defense to DNA damage in normal astrocytic cells as well as astrocytic tumor cells. MPG protein was expressed only in the nucleus of the astrocytic tumor tissues. However, MPG expression in adjacent brain tissues was not found both in the nucleus and cytoplasm. These same results were obtained from Western blot anlaysis. These results suggest that MPG gene expression might be controlled by posttranscriptional or translational level at least in the astrocytic tissues of brain. However, our knowledge of the level and sites of MPG expression in patients are limited currently.

Recently, Sohn et al.[9] reported that the intracellular expression and localization of MPG altered in cervical neoplsia. Granular positivity of MPG was notable in the perinuclear regions of the cytoplasm in human papilloma virus-infected invasive cervical carcinoma. However, the expression of MPG has not been studied yet in the brain tumor patients. Therefore, in this study, it was examined whether MPG protein was expressed in the cytoplasm or the nucleus of brain tumor tissues. We provide evidence that MPG protein is not found in tumor-adjacent brain tissues, but rather is found in nuclear localization of the astrocytic tumor tissues. The nuclear localization means that MPG maybe transported from cytoplasm to nucleus for DNA repair. Nuclear localization of MPG protein in astrocytic tumors is different from cytoplasmic localization of MPG protein in cervical carcinoma. How can it explain the difference? There is no definite clue for proper explanation of the nuclear MPG protein localization. Cool and Sirover[38] reported that the immunocytochemical localization of BER enzyme uracil DNA glycosylase was examined as a function of cell proliferation. During the proliferation, there was an increase in glycosylase activity in each of the subcellular fractions. These results suggest a correlation between the proliferative state of normal human cells and the preferential nuclear or perinuclear localization of an immunocytochemically reactive glycosylase protein. Further, immunofluorescence of the nuclear enzyme may be dependent on defined conformational states of that nuclear glycosylase in the cell cycle. In MGMT, Lim and Li[39] reported a study showing a possible two step-model for the nuclear localization of the 21 kDa human protein. The first step is the translocation of the protein from the cytosol to the nucleus.

This appears to require the nuclear targeting property associated with the holoprotein in combination with a cellular factor to effect the nuclear translocation of MGMT. The second step involves the nuclear retention of MGMT to prevent its export from the nucleus. This requires a basic region (PKAAR, codons 124-128) that can bind to the non-diffusible DNA elements in the nucleus. However, there has not been known holoprotein or specific region of codons in MPG. Further studies will be needed.

In many commonly used chemotherapy protocols, the dose-limiting factor is the bone marrow. By transferring genes that confer resistence to chemotherapeutic agents into the dose-limiting organs, the patient will be able to better tolerate harmful drugs. By providing protection to the bone marrow through the expression of DNA repair genes, higher doses of drugs could be used, which would lead to increased patient response in brain tumors.[40] There are a lot of studies about MGMT to chemotherapeutic effect. MGMT inhibits the killing of tumor cells by alkylating agents. MGMT activity is controlled by a promoter. Methylation of MGMT promoter is associated with regression of tumor and prolonged overall and disease-free survival and acts as independent prognostic factor.[41] DNA repair MGMT knockout mice are sensitive to the lethal effects of chemotherapeutic alkylating agents.[42] Kaina and associates[43] demonstrated that the overexpression of MPG in CHO cells renders them more sensitive to alkylating agents, such as MMS and *N*-methyl-*N*'-nitro-*N*-nitrosoguanindine, and gave a higher incidence of sister chromatid exchange. Van Zeeland and coinvestigators[44] overexpressed the rat MPG gene in CHO cells and demonstrated that the overexpression of MPG results in more sensitivity to MMS and mutation induction. Because of the increased number of AP sites that result from overexpression of MPG, it is believed that the rest of the BER pathway is unable to keep up and repair the damage.[43] As a result, the cells cannot correct the damage and die. These findings indicate that MPG may not be beneficial for gene therapy usage alone, but possibly may be effective when coupled with other BER pathway members. MPG may also be beneficial in gene therapy experiments in which the tumor cell is targeted for killing. Given that the current data indicate that MPG causes a sensitization to alkylating agents, this sensitization could be exploited to kill tumor cells if methods of precise targeting could be achieved.

V. CONCLUSION

DNA is continuously damaged due to exposure to alkylating compounds or oxygen free radicals generated during normal cellular metabolism and as environmental mutagens. Several studies found that MPG mRNA levels were lower in adult brain than in other tissues. Terminally differentiated and nonproliferating cells have a lower DNA repair capacity than proliferating cells from organs, embryo, ovary and testis. The accumulation of damage in nondividing tissues would be more acute and more serious. This study was designed to investigate the association of tumorigenesis with DNA repair enzyme, N-methylpurine-DNA-glycosylase (MPG). Expression and localization of mRNA/protein of MPG in the 30 astrocytic tumors and 7 brain tissues adjacent to tumor was examined by RT-PCR, RNA *in situ* hybridization, immunohistochemistry, and western blot analysis. We obtained several conclusions as follow.

1. The level of MPG mRNA expression in grade IV increased about 2.3-fold above brain tissues adjacent to tumor, which was significant statistically ($p < 0.05$).

2. As compared with grade II and III astrocytic tumors, grade IV tumors showed a significantly higher level of MPG expression ($p < 0.05$).

3. The MPG mRNA expression was found in the both tissues of tumor tissue and adjacent brain tissues with similar level by RNA *in situ* hybridization.

4. The MPG protein expression showed only in the nuclear region of tumor tissues by immunohistochemistry. The MPG protein expression was not found in adjacent brain tissues.

5. The MPG protein expression in grade IV tumors was higher than that in grade II-III tumors by western blot analysis.

In conclusion, the level of MPG mRNA and intracellular localization in astrocytic tumor tissues was changed. The increased expression and subcellular localization was shown to

be associated with tumorigenesis as well as increased susceptibility to DNA damage. Direct estimation of MPG enzyme activity in tumor tissues will be helpful for association with tumorigenesis. The MPG expression in brain may be controlled by posttranscriptional or translational regulation. However, our knowledge of the level and sites of MPG expression in patients are limited currently. Further studies in vitro are warranted for documentation of control of MPG gene in astrocytic tumors.

REFERENCES

1. Diamond JM. Causes of death before birth. Nature 1987;329:487-8.
2. Lindahl T, Sedgwick B, Sekiguchi M, Nakabeppu Y. Regulation and expression of the adaptive response to alkylating agents. Annu Rev Biochem 1988;57:133-57.
3. Walter CA, Grabowski DT, Street KA, Conrad CC, Richardson A. Analysis and modulation of DNA repair in aging. Mech Ageing Dev 1997;98:203-22.
4. Friedberg EC, Meira LB, Cheo DL. Database of mouse strains carrying targeted mutations in genes affecting cellular responses to DNA damage. Mutat Res 1997;383:183-8.
5. Cerda SR, Turk PW, Thor AD, Weitzman SA. Altered expression of the DNA repair protein, N-methylpurine-DNA glycosylase (MPG) in breast cancer. FEBS Lett 1998;431:12-8.
6. Kim NK, Lee SH, Seo JS, Cha KY. Tissue-specific expression and activation of N-methylpurine-DNA glycosylase (MPG) in SV40 T-antigen gene expressing transgenic mice. Mol Cells 1998;8:383-7.
7. Mitra S, Kaina B. Regulation of repair of alkylating damage in mammalian genomes. Progr Nucleic Acids Res Mol Biol 1993;44:109-142.
8. Silber JR, Blank A, Bobola MS, Mueller BA, Kolstoe DD, Ojemann GA, et al. Lack of the DNA repair protein O^6-methylguanine-DNA methyltransferase in histologically normal brain adjacent to primary human brain tumors. Proc Natl Acad Sci USA 1996;93:6941-6.
9. Sohn TJ, Kim NK, An HJ, Ko JJ, Hahn TR, Oh D, et al. Gene amplification and expression of the DNA repair enzyme, N-methylpurine-DNA glycosylase (MPG) in HPV-infected cervical neoplasias. Anticancer Res 2001;21:2405-11.
10. Bessho T, Roy R, Kasai YKH, Nishimura S, Tano K, Mitra S. Repair of 8-hydroxyguanine in DNA by mammalian N-methylpurine-DNA glycosylase. Proc Natl Acad Sci USA 1993; 90:8901-4.

11. Dosanjh MK, Roy R, Mitra S, Singer B. 1,N-6-Ethanoadenine is preferred over 3-methyladenine as substrate by a cloned human N-methylpurine-DNA glycosylase (3-methyladenine-DNA glycosylase). Biochemistry 1994;33:1624-8.
12. Saparbaev M, Kleibl K, Laval J. Escherichia coli, Saccharomyces cerevisiae, rat and human 3-methyladenine DNA glycosylases repair 1,N-6-ethanoadenine when present in DNA. Nucleic Acids Res 1995;23:3750-5.
13. Hatahet Z, Kow YW, Purmal AA, Cunningham RP, Wallace SS. New substrates for old enzymes. 5-Hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine are substrates for Escherichia coli endonuclease III and formamidopyrimidine DNA N-glycosylase, while 5-hydroxy-2'- deoxyuridine is a substrate for uracil DNA N-glycosylase. J Biol Chem 1994;269:18814-20.
14. Lindahl T. DNA repair enzymes. Annu Rev Biochem 1982;51:61-87.
15. Mitra S, Hazra TK, Roy R, Ikeda S, Biswas T, Lock J, et al. Complexities of DNA base excision repair in mammalian cells. Mol Cells 1997;7:305-12.
16. Samson L, Derfler B, Boosalis M, Call K. Cloning and characterization of a 3-methyladenine DNA glycosylase cDNA from human cells whose gene maps to chromosome 16. Proc Natl Acad Sci USA 1991;88:9127-31.
17. Tatsuka M, Ibeanu GC, Izumi T, Narayan S, Ramana CV, Kim NK, et al. Structural organization of the mouse DNA repair gene, N-methylpurine-DNA glycosylase. DNA Cell Biol 1995; 14:37-45.
18. Mitchell DL, Hartman PS. The regulation of DNA repair during development. Bioessay 1990; 12:74-9.
19. Kim NK, Lee SH, Sohn TJ, Roy R, Mitra S, Chung HM, et al. Spatial expression of a DNA repair gene, N-methypurine-DNA glycosylase (MPG) during development in mice. Anticancer Res 2000;20:3037-44.
20. Cardozo-Pelaez F, Brooks PJ, Stedeford T, Song S, Sanchez-Ramos J. DNA damage, repair, and antioxidant systems in brain regions: a correlative study. Free Radic Biol Med 2000; 28:779-85.
21. Barrows LR, Magee PN. Nonenzymatic methylation of DNA by S-adenosylmethionine in vitro. Carcinogenesis 1982;3:349-51.
22. Bartsch H, Montesano R. Relevance of nitrosamines to human cancer. Carcinogenesis 1984;5: 1381-93.
23. Mineura K, Yanagisawa T, Watanabe K, Kowada M, Yasui N. Human brain tumor O(6)-methylguanine-DNA methyltransferase mRNA and its significance as an indicator of selective chloroethylnitrosourea chemotherapy. Int J Cancer 1996;69:420-5.
24. Srivenugopal KS, Mullapudi SR, Shou J, Hazra TK, Ali-Osman F. Protein phosphorylation is a regulatory mechanism for O^6-alkylguanine-DNA alkyltransferase in human brain tumor cells. Cancer Res 2000;60:282-7.

25. Rey JM, Pujol P Callier P, Cavailles V, Freiss G, Maudelonde T, et al. Semiquantitative reverse transcription-polymerase chain reaction to evaluate the expression patterns of genes involved in the oestrogen pathway. J Mol Endocrinol 2000;24:433-40.
26. Jolly DJ, Okayama H, Berg P, Esty AC, Filpula D, Bohlen P, et al. Isolation and characterization of a full-length expressible cDNA for human hypoxanthine phosphoribosyl transferase. Proc Natl Acad Sci USA 1983;80:477-81.
27. Schummer B, Hauptfeisch S, Siegsmund M, Schummer M, Lemmer B. High accurate quantification of mRNA expression by means of Titan one tube RT-RCR and capillary electrophoresis. In: Zeibolz B, editor. Biochemica. 2nd ed. Mannheim, Germany: Roche Molecular Biochemicals; 1987. p.31-3.
28. Hansen WK, Kelley MR. Review of mammalian DNA repair and translational implications. J Pharmacol Exp Ther 2000;295:1-9.
29. Engelward BP, Weeda G, Wyatt MD, Broekhof JL, de Wit J, Donker I, et al. Base excision repair deficient mice lacking the Aag alkyladenine DNA glycosylase. Proc Natl Acad Sci USA 1997;94:13087-92.
30. Lindahl T. Instability and decay of the primary structure of DNA. Nature 1993;362:709-15.
31. Lindahl T. Suppression of spontaneous mutagenesis in human cells by DNA base excision repair. Mutat Res 2000;462:129-35.
32. Weng Y, Sirover MA. Developmental regulation of the base excision repair enzyme uracil DNA glycosylase in the rat. Mutat Res 1993;293:133-41.
33. Washington WJ, Foote RS, Dunn WC, Generoso WM, Mitra S. Age-dependent modulation of tissue-specific repair activity for 3-methyladenine and O^6-methylguanine in DNA in inbred mice. Mech Ageing Dev 1989;48:43-52.
34. Washington WJ, Dunn WC, Generoso WM, Mitra S. Tissue-specific variation in repair activity for 3-methyladenine in DNA in two stocks of mice. Mutat Res 1988;207:165-9.
35. Engelward BP, Boosalis MS, Chen BJ, Deng Z, Siciliano MJ, Samson LD. Cloning and characterization of a 3-methyladenine/7-methylguanine/3-methylguanine DNA glycosylase cDNA whose gene maps to chromosome 11. Carcinogenesis 1993;14:175-81.
36. Weeda G, Ma L, van Ham RC, Bootsma D, van der Eb AJ, Hoeijmakers JH. Characterization of the mouse homolog of the XPBC/ERCC-3 gene implicated in xeroderma pigmentosum and Cockayne's syndrome. Carcinogenesis 1991;12:2361-8.
37. Burger PC. Classification, grading and patterns of spread of malignant gliomas. In: Appuzzo MLJ, ed. Malignant cerebral glioma. Park Ridge, IL: American Association of Neurosurgeons; 1990. p.3-17.
38. Cool BL, Sirover MA. Immunocytochemical localization of the base excision repair enzyme uracil DNA glycosylase in quiescent and proliferating normal human cells.

Cancer Res 1989;49:8693-7.
39. Lim A, Li BF. The nuclear targeting and nuclear retention properties of a human DNA repair protein O6-methylguanine-DNA methyltrasferase are both required for its nuclear localization: the possible implications. EMBO J 1996;15:4050-60.
40. Limp-Foster M, Kelley MR. DNA repair and gene therapy: implications for translational uses. Environ Mol Mutagen 2000;35:71-81.
41. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 2000;343:1350-4.
42. Glassner BJ, Weeda G, Allan JM, Broekhof JL, Carls NH, Donker I, et al. DNA repair emthyltransferase (Mgmt) knockout mice are sensitive to the lethal effects of chemotherapeutic alkylating agents. Mutagenesis 1999;14:339-47.
43. Coquerelle T, Dosch J, Kaina B. Overexpression of N-methylpurine DNA glycosylase in Chinese hamster ovary cells renders them more sensitive to the production of chromosomal aberrations by methylating agents-a case of imbalanced DNA repair. Mutat Res 1995;336:9-17.
44. Calleja F, Jansen JG, Vrieling H, Laval F, van Zeeland AA. Modulation of the toxic and mutagenic effects induced by methyl methanesulfonate in Chinese hamster ovary cells by overexpression of rate N-alkylpurine-DNA glycosylase. Mutat Res 1999;425:185-94.

국문요약

성상세포종에서의 **DNA** 복구 유전자인 **N-methylpurine-DNA glycosylase (MPG)**의 발현

연구 배경: DNA는 환경적인 돌연변이 요소들과 정상적인 세포 대사과정에서 생성되는 알킬 화합물이나 산소 유리기 등에 계속적으로 노출되어 손상이 일어난다. 몇몇 연구에서 MPG의 mRNA발현 정도는 타 장기보다 성인 뇌조직에서 낮다고 알려지고 있다. 즉 분화가 끝나고 더 이상 증식하는 않는 세포들은 배아 조직, 난소, 정소와 같은 증식 세포들 보다 DNA 복구 능력이 낮다. 이러한 비분열 조직에서의 DNA 손상의 축적은 더 심각할 것이다. 이러한 손상은 DNA 복구과정이나 세포 자연사 과정을 거치게 되고, 그렇지 않을 경우 뇌종양으로 발전할 지도 모른다. 본 연구는 DNA 복구 효소의 일종인 MPG와 종양형성과의 관련성을 알고자 하였다.

재료 및 방법: 30례의 신경 교종들과 7례의 종양 주위의 정상 뇌조직에서의 역전사-중합효소 연쇄 반응(reverse transcriptase-polymerase chain reaction)과 RNA *in situ* hybridization을 이용하여 MPG의 mRNA 발현을 보았다. 또한 면역조직화학염색 (immunohistochemistry)과 western blot 분석 방법을 이용하여 MPG의 단백질의 발현과 세포내 국재화 (localization)를 알아보았다.

결과: 역전사-중합효소 연쇄 반응에서의 IV 등급 종양 조직의 MPG mRNA의 발현은 종양 주위의 뇌조직 또는 II-III 등급 종양 조직의 발현보다 통계학적으로 의미 있게 높았다 ($p<0.05$). RNA *in situ* hybridization에서의 MPG mRNA는 종양의 등급과 관계없이 종양 조직과 종양 주위의 뇌조직 모두에서 발현되었다. 역전사-중합효소 연쇄 반응에서의 IV 등급 종양 조직의 MPG mRNA의 발현은 II-III등급의 종양들의 발현보다는 높았다. 그러나, 면

역조직화학 염색에서의 MPG 단백질 발현은 모든 종양 조직에서만 핵에서 발현이 되었다. 종양 주위의 뇌조직에서는 핵과 세포질 모두에서 발현이 되지 않았다. Western blot 분석에서의 MPG단백질 발현은 II-III등급의 종양보다 IV등급 종양 조직에서 높았다. 악성 성상세포종에서 증가된 MPG 발현 양상을 관찰하였고, 종양과 종양 주위의 뇌조직에서의 MPG mRNA의 세포내 발현 양상은 MPG 단백질의 발현 양상과 달랐다. 그러나 종양 조직 내에서의 MPG의 세포내 국재화는 이질적이지는 않았다.

결론: 본 연구의 결과들로 성상세포종에서의 MPG와의 관련성을 제시하고, 변화된 mRNA의 발현 및 세포내 국재화는 종양 형성과정과 관련이 있음을 암시한다. 향후 성상세포종에서 MPG 유전자의 조절에 대한 더 많은 연구가 필요하다.

핵심되는 말: 성상세포종, 복구 유전자, N-methylpurine-DNA glycosylase (MPG), 발현, 종양 형성화