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Expression of the DNA Repair Gene, N-Methylpurine-DNA Glycosylase in Astrocytic Tumors

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Objective : This study is designed to investigate the association of tumorigenesis with DNA repair gene, N-methylpurine-DNA-glycosylase (MPG) in astrocytic tumors.

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 (RT-PCR) and RNA *in situ* hybridization. Expression and intracellular localization of MPG protein was determined by immunohistochemistry. Statistical analysis was performed by ANOVA with a p value <0.05 considered statistically significant.

Results : MPG mRNA expression in RT-PCR was significantly higher in grade IV tumor tissues than in brain tissues adjacent to tumor or in grade II-III astrocytic 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. However, MPG protein localization in immunohistochemical study was detected only in the nucleus of all tumor tissues. In brain tissues adjacent to tumor, immunohistochemical study for MPG was not stained both in the nucleus and in cytoplasm.

Conclusion : 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 · Expression · Tumorigenesis.

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[6,17,32]. 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[10]. DNA repair enzymes have been reported to play an important role in the carcinogenesis of several cancers[4,13,23,28,29]. N-Methylpurine-DNA glycosylase (MPG) is one of the key enzyme in DNA repair and it removes N-alkylpurine and other related adducts including 8-hydroxyguanine, 1,N^6-ethenoadenine and hypoxanthine[1,7,26]. 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[12,23]. 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[16,22,25,26,31]. Mitchell and Hartman[21] reported that proliferating cells from organs and embryos have a greater DNA repair capacity than terminally differentiated and nonproliferating cells. Kim *et al.*[14] 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[4,13,29]. 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[4].

In this study, we tested whether the expression and intracellular localization of MPG could be altered in different grade of astrocytic tumors.

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Methods

Tissues

Through June 2001 to May 2002, patients with newly diagnosed astrocytic tumor consented to use of their tissues. All surgical specimens were primary resections and no patients had received prior radiation or chemotherapy. Tumor and tumor-adjacent brain tissue(tumor-adjacent brain tissues ; N=7 ; tumor tissues ; N=30) were collected. The patient materials 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 neuropathologists. 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.

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(hypoxanthine-guanine phosphoribosyltransferase). To normalize mRNA amounts of MPG between tissue samples, HPRT, a moderately expressed housekeeping gene, was used as the reference gene[24]. The sequence of human MPG was obtained from R. Roy(University of Texas Medical Branch, TX, USA). 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', and anti-sense, 5'-AGCCCCCCTTGAGCACACA-3', leading to a 225-bp PCR product. RT-PCR was carried out using at least triplicates for each condition.

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., Taejon, South Korea). The reaction mixture contained 1 μg total RNA, 20U M-MLV RTase, 10U RNasin, 10mM Tris-Cl(pH 8.3), 50mM KCl, 10mM $MgCl_2$, 0.5mM dNTP, 10 pmole each of 5' and 3' primers and 1U Taq polymerase. The reverse transcription and PCR amplification was simultaneously performed using Thermal Cycler(PerkinElmer 2400, PerkinElmer Life Sciences Inc., Boston, USA) in one batch tube. The reverse transcription was carried out for 60 minutes at 42℃ and was heated to 95℃ for 5 minutes to terminate the reverse transcription. The amplification was performed for 30 cycles of denaturation at 95℃ for 30 seconds, annealing at 64℃ for 30 seconds and extension at 72℃ for 30 seconds. A final extension step for 5 minutes at 72℃ followed. Aliquots of each product were electrophoresed in 1.2% agarose gel containing 0.5 μg/ml ethidium bromide. Relative levels of target gene(MPG/HPRT) were quantitated by a computerized imaging analysis system(Vilber Lourmat, Marne-la-Vallee Cedex 1, France).

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 5 μm were collected on ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA) and proteinase K(1 μg/ml) treatment in TE buffer(pH 7.4) for 10 minutes 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(1mg/ml Ficoll, 1mg polyvinylpyrrolidone and 1mg/ml BSA), 250 μg/ml tRNA and 500 μg/ml salmon sperm DNA at 62℃ for 2 hours. Hybridization was performed in the same solution containing 1 μg/ml digoxigenin-labeled riboprobe at 62℃ overnight. After hybridization, the slides were washed in 2× SSC at 37℃ for 30 minutes and in 50% formamide/2× SSC at 60℃ for 30 minutes, and then incubated with NTE solution(500mM NaCl, 10mM Tris, 1mM EDTA) containing RNase A(20 μg/ml). And then the slides incubated with blocking buffer (10% heat-inactivated normal goat serum, 0.1M Tris, pH 7.5 and 0.15M 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 hours incubation at room temperature, the slides were washed three times in maleic acid buffer. NBT/BCIP was added as a substrate for alkaline phosphatase, after overnight incubation the color reaction was stopped with 10mM Tris/1mM EDTA. To monitor background level and specificity of hybridization, the sense strand of MPG probe was used.

Immunohistochemistry

Surgical specimens were fixed in formalin, dehydrated in ethanol, embedded in paraffin, and sectioned by 5 μm. Se-

ctions 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 mouse 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 minutes 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 Co., Carpinteria, CA, USA) with diaminobenzidine, following the manufacturer's procedure. Sections were counterstained with Mayer's hematoxylin.

Statistical analysis

Statistical analysis was performed by ANOVA using the Statistical Package for Social Science Statview Package(SPSS Inc., Chicago, IL, USA) with a p value <0.05 considered statistically significant.

Results

Expression of MPG mRNA by RT-PCR

The level of MPG mRNA expression in grade IV tumors increased about 2.3-fold above brain tissues adjacent to tumor(Fig. 1, 2), 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 mRNA expression($p<0.05$)(Fig. 2). However, there was no significant difference of MPG mRNA expression between grade II and III astrocytic tumors and brain tissues adjacent to tumor ($p>0.05$).

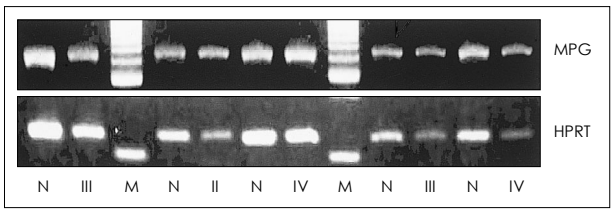

Fig. 1. RT-PCR products of N-methylpurine DNA glycosylase (MPG) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA from cDNA. The same amount of cDNA was amplified at different cycles. The bands corresponding to MPG is 603bp and HPRT is 225bp. Lane "N" represents PCR products of brain tissues 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.

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

MPG mRNA was constantly expressed in cytoplasms of tumor tissues and adjacent brain tissues (Fig. 3A, B). The expression of MPG mRNA in normal glial tissues was confirmed by *in situ* hybridization techniques.

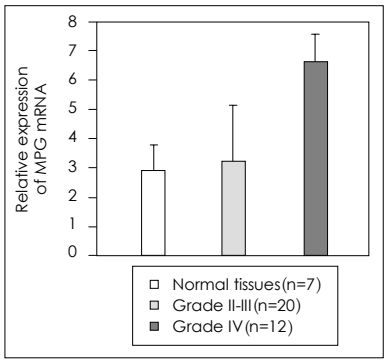

Fig. 2. mRNA ratios of N-methylpurine DNA glycosylase(MPG) over hypoxanthine-guanine phosphoribosyltransferase (HPRT) in astrocytic tumor tissues according to grade and adjacent brain tissues. The relative mRNA level was calculated by laser densitometer. The level of MPG transcripts in grade IV increased 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($p<0.05$).

Intracellular localization and 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(Fig. 4A, C, E). However, MPG was strongly stained in the nucleus of astrocytic tumor tissues regardless of tumor grade, not in the cytoplasm(Fig. 4B, D, F). We could not detect the significant difference of MPG localization and the expression level between different grades of astrocytic tumors.

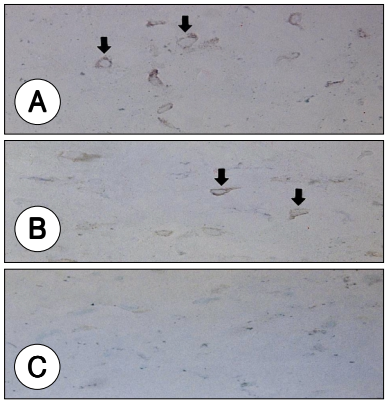

Fig. 3. Cellular localization of N-methylpurine DNA glycosylase(MPG) mRNA by *in situ* hybridization technique. MPG mRNA is constantly expressed in adjacent brain tissue(A) and astrocytic tumor tissues(B)(Original magnification ×400). However, it is not detected in the adjacent brain tissues using sense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

Discussion

DNA repair enzyme, N-methylpurine-DNA glycosylase (MPG) and carcinogenesis

MPG is one of a growing list of enzymes responsible for

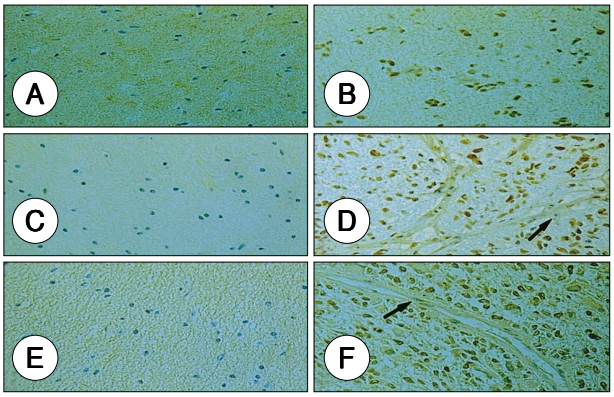

Fig. 4. Immunohistochemical stains for N-methylpurine DNA glycosylase(MPG) in tumors and corresponding brain tissues adjacent to tumor of the same patients. A, C and E are the adjacent brain tissues of tumor case B, D and F respectively. Brain tissues adjacent to tumor are negative for MPG protein (Original 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 in intervening blood vessels(Original magnification×200). F : Atypical tumor cells of grade IV astrocytoma show diffuse, strong nuclear positivity (Original magnification×200). (arrow) : endothelial cell.

the recognition and excision of altered bases in the first step of the base excision repair(BER) pathway[11,22]. In the simplest form of BER, the resulting abasic site is then repaired by the sequential action of an AP endonuclease 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[16]. Engelward et al.[9] have genetically engineered animals deficient in MPG, a DNA glycosylase that removes a broad spectrum of base damages, including, but likely not limited to, 3-methyladenine, 7-methylguanine, 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[18], 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[19].

Spatial expression of MPG in brain and its tumorigenesis

Mammalian cells 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[36]. The MPG expression was initially identified by virtue of temporal and spatial regulation in protein level[33,34] and spatial regulation in mRNA level[8,14]. The MPG expression is known tissue-specific and developmental stage-specific variation[14]. The MPG mRNA level in whole embryo is the highest in day 14.5 post coitum and apparently declined until the end of gestation[14]. 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.[8] 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[35]. Mitchell and Hartman[21] 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.[16] 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[3,20,28,30]. DNA repair enzymes, including AP endonuclease, DNA polymerase β, XRCC1/DNA ligase III, PCNA(proliferating cell nuclear antigen), FEN1(5'-flap endonuclease), MGMT(O^6-methylguanine-DNA methyltransferase), NTH(thymine glycol DNA glycosylase), and OGG(8-oxo-guanine DNA glycosylase), may be associated with brain tumorigenesis. Herein, we first report that MPG mRNA level in grade IV astrocytoma was significantly higher than that in tumor-adjacent brain tissues. Also, MPG mRNA level in grade IV astrocytomas was definitely higher than that in grade II-III astrocytomas.

These facts means that altered MPG mRNA expression is closely associated with brain tumorigenesis, perhaps advanced stage. Direct estimation of MPG enzyme activity in tumor tissues will be helpful for association with tumorigenesis.

Intracellular localization and regulation of MPG in brain

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, we found that the MPG mRNA localization and the level of tumor adjacent brain tissues were similar to that of tumor tissues by RNA *in situ* hybridization techniques. Moreover, the difference of MPG mRNA level between tumor-adjacent brain tissues and grade II-III tumor tissues by RT/PCR was not statistically significant. 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 protein expression in tumor-adjacent brain tissues was not found both in the nucleus and cytoplasm. 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. It is useful to know the control level of MPG gene expression by investigation of the changes in DNA repair activities both mitochondrial and nuclear extracts.

Recently, Sohn et al.[29] reported that the intracellular expression and localization of MPG altered in cervical neoplasia. 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 evidences that MPG protein is not found in brain tissues adjacent to tumor, 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[5] 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[15] reported a study showing a possible two step-model for the nuclear localization of the 21kDa 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 about nuclear localization of MPG protein are warranted.

Conclusion

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. Further studies *in vitro* are warranted for documentation of control of MPG gene in astrocytic tumors.

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