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Methylation-specific PCR method for MGMT coding gene silencing evaluation and its prognostic significance in alkylating antitumor treatment

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ABSTRACT

MGMT (O6-methyl-guanine-DNA methyltransferase) is a protein with a specific enzyme activity that is involved in repair of DNA alkylation alterations introduced by classical chemotherapy, such as involving temozolomide (TMZ) use for glioma and Ewing sarcoma tumors. The *MGMT* methylation biomarker is frequently used for the evaluation of the treatment prognosis in such type of classical anticancer approach. In this article, optimized analytical conditions of a nested MS-PCR method for the methylation status of the MGMT coding gene promoter are described and the clinical significance of the reaction results are discussed in relation with the prognostic value for the tumor tissues treatment with an alkylating drug.

Key words: *epigenetic, methylation-specific PCR, MGMT, prognostic, treatment.*

1. INTRODUCTION

MGMT (O6-methyl-guanine-DNA methyltransferase) is a protein with a specific enzyme activity that is involved in DNA repair. It is also named O6-alkylguanine DNA alkyltransferase (AGT or AGAT). The gene encoding this protein is usually named after the protein name, *MGMT*. Since the first report of this enzyme of the TANO [1] this protein and its encoding gene was implemented as a prognostic biomarker for cancer treatment (NATARAJAN [2], SHIRAISHIN[3], KAINA [4]).

Alkylation reactions are commonly occurring during normal cell division, when molecular processes of DNA replication and transcription take place. However, the effects of such alterations in DNA processing should be normally buffered by specific repair enzymes. O^6-methylguanine DNA methyltransferase is crucial for genome stability. It depletes the methyl groups from modified guanine, hence repairing the naturally occurring mutagenic DNA lesion O^6-methylguanine back to guanine. This process therefore prevents mismatch and errors during DNA replication and transcription. Literature has initially reported studies that demonstrated that loss of *MGMT* activity increases the carcinogenic risk in mice after exposure to alkylating agents. Presently it is well established that this enzyme is involved in human genomic stability maintenance and its activity should be maintained in normal range. Any disturbance in its activity, such as its repression through methylation processes, may alterate the genome stability and introduce carcinogenic risk. However there is actually more information that allows specific interpretation of MGMT involvement in specific pharmaco-genomic and pharmaco-epigenomic approaches in oncology. This new interpretation suggests that methylation of MGMT gene may be indicative of a better reactivity towards specific treatments.

MGMT methylation test is an epigenetic test. As a common gene feature, the gene expression in its encoded protein, depends on its regulatory regions status. One of such regulatory regions of a gene is its promoter: commonly, when it is methylated, it acquires a silenced state, when the protein is no more expressed and hence cannot contribute to the phenotype. This type of regulation does not depend on the DNA sequence and therefore is named "epigenetic regulation".

Epigenetic inactivation by promoter methylation of the *MGMT* gene is very well established. This gene is epigenetically silenced in a variety of cancers (ESTELLER [5]). Specifically, *MGMT* methylation is found in glioblastomas (ESTELLER [6], MELLAI [7], SHAMSARA [8]), colon cancer (HERFATHN[9], and OGINO [10]), non-small cell lung cancer (NSCLC) (WOLF [11] and WU [12]), gastric carcinoma (OUE [13]), head and neck squamous cell carcinoma (HNSCC) (GOLDENBERG [14], MARUYA [15] and STEINMANN [16]), and many other cancer types. Epigenetic inactivation of DNA repair genes in cancer has been reported for several DNA repair pathways and it has been assumed that these epigenetic inactivation processes can result in an increase in genetic instability during tumorigenesis that can be directly attributed to the deficiencies in DNA repair. Therefore,

inactivation of DNA repair genes can be seen as an important event in cancer initiation and/or progression by reducing genomic stability thus leading to genetic aberrations at other important gene loci in the tumor genome. However, reduced repair capacity for alkylated guanines by promoter methylation of the *MGMT* gene has provided a therapeutic benefit in patients with certain tumor tissues, like those of glioma (ESTELLER [6]). Methods commonly used for the epigenetic MGMT test were frequently reported as Methylation specific (MS) PCR, RealTime (RT) PCR for qualitatively estimation of the methylation state in the *MGMT* gene promoter; pyrosequencing for the qualitatively and quantitatively estimation of the number and type of the CpG sites that are methylated in the *MGMT* gene promoter.

2. EXPERIMENTAL SECTION

Ewing sarcoma tumors (2 specimens) and glioblastoma tumors (2 specimens) were collected from diagnosed patients. Extracted DNA from the FFPE tissues (Qiagen QiAmp) was analyzed for the methylation pattern of the MGMT coding gene promoter after the bisulphite conversion step (EpiTect Qiagen) by the methylation specific (MS) PCR method. Nested MS-PCR was used in comparison with classical MS-PCR in order to increase the sensibility and specificity of the method. The results are linked with the MGMT coding gene transcription state and their value for the prognostics of the alkylating antitumor treatment with temozolomide (TMZ) are discussed. **Patients:** Two types of tumor tissues were obtained from Timisoara Pediatric Clinical Hospital Louis Turcanu (two glioma tissues and one Ewing osteosarcoma tissues) and from Colentina Hospital Bucuresti (one Ewing osteosarcoma tissue). The archived tissues were obtained according to the bioethic rules that comprised the consent of the patient and patient's family and the physicians). The tissues were archived since the period of 2010-2014. The formalin fixed paraffined embedded (FFPE) tissues were cut in strips of 10 µm mesh (8 strips for each sample for DNA extraction). **DNA extraction** from the FFPE archived tissues was performed with QiAmp-tissue (Qiagen) according to the kit-indicated protocol and the optimizations according to Int (SENGUVEN [17]. The final concentration of DNA of more than 150ng/µL was achieved in order that the nested multistep MS-PCR to be approached. The DNA concentration and the sample quality was checked on the Nanodrp (LaRoche). **Methylation Specific PCR (MS-PCR).** The methylation status of the MGMT coding gene was determined by MS-PCR method (HERMAN [18]), modified by using **nested primers** together with the usual pairs of primers targeting the methylated and unmethylated forms of the 289-bp DNA relevant fragment (containing the richest CpG density of the promoter). The two stages MS-PCR approach was tested in order to optimize the detection of methylated regions in the context of prevalent unmethylated gene alleles (one methylated allele in over 50,000 unmethylated alleles (PALMISANO [19]). The *MGMT* gene promoter fragments vary from the methylation pattern and not by the nucleotide sequence, hence any classical MS-PCR method comprises first the bisulfite conversion step (Epitect, Qiagen), in order to selectively mutate the unmethylated cytidine (C) residues, while preserving unchanged the methylated cystidine (mC) residues. Also, the choice of the primers has been done based on the MethPrime program selection of the CpG-rich promoter regions and verified by the PerlPrimer actual program (LONG-CHENG LI [20]; Li LC [21]; MARSHALL [22]). Thus the primers recognize and initiate the polymerisation and amplification only the bisulfite-modified template. Moreover, any DNA polymerase cannot discriminate between methylated and unmethylated alleles, which do not differ from the nucleotide sequence. For the second step, the step one PCR products were diluted (50 fold) and a new substrate volume was subjected to a new, second step MS-PCR. The reaction mixture for both steps contained the following components: 20 µL Master Mix with GoldStar DNA Polymerase (Qiagen), 0,1µL forward primer (0,25 µM), 0,1µL backward primer (0,25 µM), 17,8 µL H20 and 2µL DNA (more than 50ng/µL), The conditions for the first step were as follows: initial denaturation (95°C / 30 sec), 40 cycles comprising: aligning (52°C / 30 sec), elongation (72°C / 30 sec) and a final elongation (72°C / 10 min). The conditions for the second step were as follows: initial denaturation (95°C / 15 min), 35 cycles comprising: aligning (59°C / 50 sec), elongation (72°C / 50 sec) and a final elongation (72°C / 10 min). The primers for the nested MS-PCR reactions were as follows: the first round: 5'-GGA TAT GTT GGG ATA GTT-3' (MGMT-F) and 5'-CCA AAA ACC CCA AAC CC-3' (MGMT-R); the second round: 5'-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3' (MGMT-UF) and 5'-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3' (MGMT-UB); 5'-TTT CGA CGT TCG TAG GTT TTC GC-3' (MGMT-MF) and 5'- GCA CTC TTC CGA AAA CGA AAC G-3' (MGMT-MB), where "M" mark indicates the methylated form, "U" mark indicates the unmethylated form and "B", "F" marks indicate the backward and forward primers. For the control MS-PCR reaction for the PWS patient the following usual primers were used for the SNRPN encoding gene: Maternal: 5′-TAT TGC GGT AAA TAA GTA CGT TTG CGC GGT C-3′; Paternal: 5′-GTG AGT TTG GTG TAG AGT GGA GTG GTT GTT G-3′ and Common : 5′-CTC CAA AAC AAA AAA CTT TAA AAC CCA AAT TCC-3′ primers. The amplicons were analyzed on 3% agarose and visualized through the UV/VIS N760.

3. RESULTS SECTION

The MS-PCR method proved its efficiency in characterizing the methylation pattern of *MGMT* gene. The nested MSPCR gave better and clear results as compared with classical MSPCR method. The optimized conditions are referred to as the

use of a GoldStar TAQ Polymerase, able to activate the polymerisation at high temperature, where the specific annealing takes place; also the preliminary step of the first round of MSPCR with only methylated form amplification (using only the primer pair designed for the methylated form) could enrich the methylated form as compared with the unmethylated one- thus, the next MSPCR step, with both primer pairs, for unmethylated and methylated forms, resulted in clear amplicon bands on electrophoregrams as compared with the electrophoregram obtained by a classical MSPCR described elswhere (UDRISTE in press). The modifications were according to the indications from literature in order to raise the sensitivity of the MS-PCR method (TOMASZ [23]).

The methylation test results. From the Fig. 1 the two types of tumors behaved differently in terms of the methylation state of the *MGMT* gene promoter methylation: both glioma tumor tissues presented both methylated and unmethylated, heterogenous, states of the *MGMT* gene, while the EWS tissues presented only unmethylated *MGMT* gene state. These results are in accordance with the literature data pointing to the rare methylated MGMT gene detected so far, in EWS tumor tissues (JENS [24]).

The clinical significance of this estimation is linked with the repair capacity of the MGMT enzyme of the tumour damage introduced by classical (alkylating) chemotherapy and radiotherapy. The gene encoding the MGMT enzyme is epigenetically silenced in a variety of cancers by the epigenetic process of DNA promoter methylation. The interpretation of the biomarker depends on the type of tumor: the lack of the DNA repair activity may be dramatic for the initiation of the neoplasic transformation, but is beneficial for the late tumors during classical alkylating based treatment. The silenced/active transcriptional state of the MGMT coding gene is epigenetically correlated with the methylated/unmethylated state of its promoter (ESTELLER [5]). Further, the repair activity of the gene is correlated with the resistance to the alkylating affect of the antitumor drug TMZ, and by contrast to the frequent efficiency in the treatment of the glioma with the same drug.

This study aims at the analytical validation of the optimized nested MSPCR method for the estimation of the methylation state in the promoter of the MGMT encoding gene. Also, the clinical validation was also performed by linking the tumor diagnosis with the presence of a specific methylation pattern in the studied glioma and EWS tissues. The variation of the tumor type resulted in the variation of the methylation state in the MGMT encoding gene promoter. The significance of the biomarker is linked with the ability of the MGMT enzyme to repair the DNA damages introduced by the chemo- and radiotherapies in tumor cells. In the case MGMT is active, hence unmethylated, it may negatively interfere with the anticancer action of the alkylating drugs such as TMZ or of the radiation inducing double strand DNA breaks. Therefore, while inactivation of DNA repair genes can be seen as an important event in cancer initiation, the reduced repair capacity for alkylated guanines by promoter methylation of the *MGMT* gene may provide a therapeutic benefit in patients with tumor tissues prepared for classical alkylating drugs combined with radiotherapy, like those of glioma (ESTELLER [6]). Similar correlations with an epigenetic biomarker was reported in literature, where inactivation of the mismatch repair (MMR) system comprising also MGMT enzyme, has been associated with resistance of cells to cisplatin treatment , also an alkylating drug for colon cancer (FINK [25]).

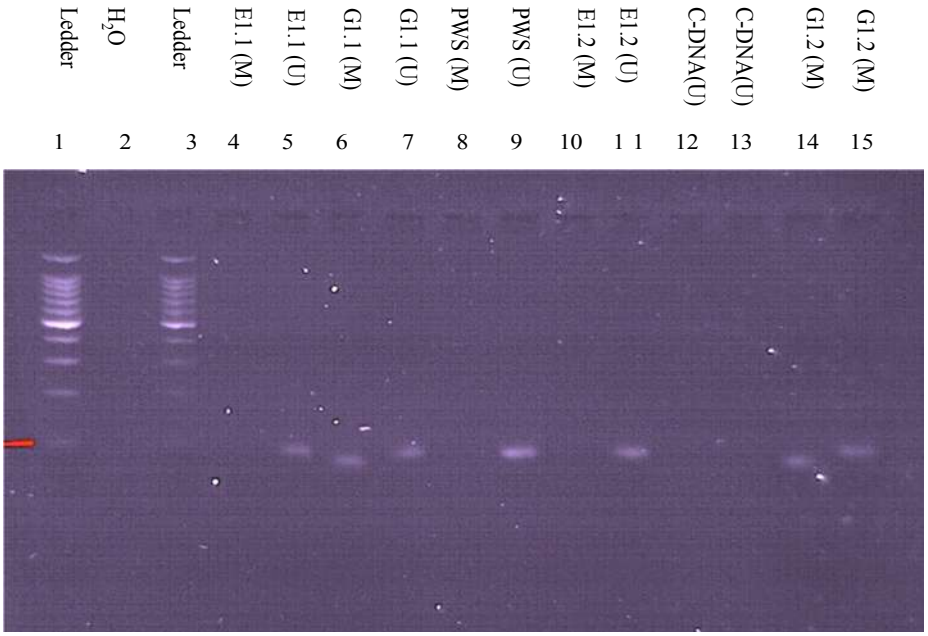

Figure 1. Electrophoregram of the amplicons obtained in the second step of the nested MS-PCR protocol for analyzis of the methylation status of the MGMT encoding gene promoter.

The presence of a visible PCR product in the "U"marked lanes is indicating an amplicon of the initial unmethylated allele of the MGMT gene promoter, while, the presence of those "M" marked DNA fragments are indicating amplicons of the initial methylated alleles. The H_2O control was included in the MS-PCR protocol as seen in the flanking electrophoregrams (lane 2). Also, the type of the analyzed tumor tissues are assigned as follows: G.1/lanes 15, 14 (G 1.2) and laines 7,6 (G 1.1)- glioma tissue in

different reactions conditions; E 1/ lanes 11,10 (E 1.2) and laines 5,4 (E 1.1): EWS tissue in different normal conditions. A normal unmethylated MGMT encoding gene from a Prader-Willi Syndrome patient was included in the lane nr. 9,8 PWS. The molecular weight marker on the ladder, the red line, correspond to the 100bp.

4. CONCLUSIONS

The MGMT methylation test described here was optimized by the nested primers approach added to the classical, two primers pairs, MS-PCR method. The optimized amplification reaction of the different DNA forms, the unmethylated versus methylated form, could result in clear bands for the two methylation patterns. These ones should be very clearly linked with the active/inactive form of the MGMT encoding gene. Our results could differentiate therefore the two types of tumors: one that is frequently detected as inactive for MGMT, thus presenting the methylated form of its promoter, and the other type of tumors, the EWS, that was found active for MGMT and thus explained its frequently observed resistance to the classical chemotherapy and radiotherapy. As a prognostic biomarker, the methylation of the MGMT encoding gene promoter indicated that glioma patients with a methylated and inactivated *MGMT* gene who were treated by chemotherapy with alkylating agents, such as temozolomide, would have a better survival relative to patients with an unmethylated and active *MGMT* gene (ESTELLER [6], HEGI [26], KAINA [4]). In our cases, the glioma tissues had a good prognostic regarding their progression through a TMZ treatment, however, was not indicated for the treatment of the two EWS tumors, based on the methylation biomarker.

This study will be continued for the identification of other genes that may play an important role in gliomagenesis and, through their methylator phenotype may be involved in a more complex and precise diagnosis and treatment prognosis algorithm that correlates gene mutations (such as 1p19q locus and *IDH1* gene) with epigenetic modifications (LAFFAIRE [27]). The correct interpretation of the information provided by particular genetic and epigenetic tests should be carefully examined and used. With ever-increasing knowledge of the genome and epigenome of specific cancer types, there is now the opportunity to develop chemotherapy regimens tailored to a patient's DNA repair gene status by incorporating information on epigenetic silencing of the relevant genes in the tumor.

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