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MS-MLPA method for the analysis of the glioma tumor MGMT encoding gene promoter methylation: treatment predictive considerations

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ABSTRACT

The epigenotype of a tumor tissue containing methylated promoter of the MGMT encoding gene is considered presently as a good prognostic marker signifying an increase in the sensitivity to its treatment with alkylating agents such as temozolomide (TMZ). The principle of this biomarker application in pharmaco-epigenetics as well as its correct clinical significance is based on the correlation between the silencing methylation process in gene promoters and the DNA damaging activity of the classical alkylating drugs. Two methods that are currently used in genetic laboratories for methylation pattern based biomarkers: Methylation Sensible (MS) – Multiplex ligation-dependent probe amplification (MLPA) and Methylation Specific (MS) -PCR are described comparatively on two tumor types (glioma tumors and Ewing sarcoma-EWS) whose treatment with the alkylating drug TMZ requires an evaluation of its efficiency. The first method was chosen for its semiquantitative capacity as compared with the classical MS-PCR method, whose poor sensibility requires an optimization in order that its only qualitative result to be sensible. The two types of tumors were estimated to behave differently due to their MGMT enzyme activity: for methylated glioma tissues a positive prognostic was established, while for the unmethylated EWS the future evolution of the tumor progression was negative.

Key words: *epigenetic, MLPA, MGMT, treatment prognostic, glioma*.

1. INTRODUCTION

Assessment of the methylation status of the *O*^{6}-*methylguanine-DNA methyltransferase* (*MGMT*) gene promoter in malignant tumors, such as glioma (G) and Ewing sarcoma (EWS) has been recognized as an important molecular assay in clinical oncology. The landmark study by HEGI [1], followed by numerous clinical trials in glioblastoma of the same research group (HAU [2] and BADY [3]), have confirmed that hypermethylation of the *MGMT* promoter serves as a strong prognostic factor for progression-free survival and overall survival specifically in glioma tissues. For Ewing's sarcoma this type of methylation pattern in MGMT encoding gene was detected only very rare (12%) (JAHROMI [4]). Also, using this methylation biomarker for the management of EWS patients during their treatment is still a matter of debate since the first studies using the classical toxic drugs, such as temozolomide TMZ.(MISER [5], VIETTI [6], CANGIR [7]).

The *MGMT* gene encodes a native DNA repair enzyme that has a counteracting activity towards the lethal effects of alkylating agents on tumor cells. It acts by removing alkyl adducts from the O^6-position of guanine (PEGG [8]). The alkylating effect of the classical drugs results in O^6-alkylated guanine, that causes base mispairing and double-strand breaks, which induce apoptosis and cell death (KARRAN [9]). Based on this DNA repair activity, the MGMT protein is believed to provide resistance against cytotoxic effects of alkylating agents, such as TMZ (KONDO [10]). By contrast, MGMT repair activity should be constantly efficient for the normal cells, however, when its activity is silenced through the epigenetic process of methylation, the neoplasic transformation may be initiated by the increased genomic instability and genetic reprogramming of certain housekeeping or tumor suppressor genes, Therefore, even that MGMT methylation may be dramatic for the primary tumorigenesis through continuously increase in genomic instability it may turn in a benefic state for the late progressed tumor during its treatment with specific, alkylating drugs, which interfere with the repair process catalyzed by the MGMT enzyme. The alkylating drug such az TMZ induces tumor DNA damages which are rapidly repaired by MGMT enzyme. This therapeutically disadvantageous protective effect of the active, unmethylated MGMT gene is thought not to be present in tumors where MGMT is silenced through methylation. Such epigenotype was frequently observed in many human cancers including glioblastoma, head and neck cancers and colon cancers,

thus rendering cells more sensitive to alkylating drugs. Promoter methylation of MGMT encoding gene became therefore a good prognostic and predictive marker for patients suffering from malignant glioma, and it is commonly assessed both in clinical trials and routine diagnostics (PREUSS [11]). EWS tissues however were resistant to numerous alkylating drugs and radiotherapy, both inducing DNA double strands breaks, hence introducing MGMT methylation biomarker may explain this behaviour by the above described principle.

As for the analytical approach of the MGMT methylation biomarker, there are several diagnostic methods described and established in genetic testing laboratories. The first commonly used one is MS-PCR, methylation-specific polymerase chain reaction (HERMANN [12]), described elsewhere (UDRISTE in press). It is a qualitative method described for *MGMT* methylation in glioma by Esteller*et al.* (ESTELLER [13,14]). An alternative, methylation specific but quantitative technique, included later real-time MSPCR (RT-MSP) (VLASSENBROEK [15]) bisulfite sequencing (FROMMER [16]), pyrosequencing (PSQ) (RONAGHI [17]), combined bisulfite restriction analysis (COBRA) (XIONG [18]), SNuPE ion pair-reverse phase high-performance liquid chromatography (SIRPH) (ELMAARI [19]). Certain current alternative methods of determining the *MGMT* status of a tumor are not approaching the promoter methylation analysis, but rather are referring to the function of the MGMT coding gene and its protein phenotype: quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the estimation of mRNA expression by (TANAKA [20]), immunohistochemistry (IHC) for protein detection (CAPPER [21]), and direct assessment of MGMT activity (PREUSSER [22]). One of the most approached method in study is methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) (NYGREN [23]), however there is still a lack in a generally accepted consensus as to most suitable method to be applied for routine activity (WELLER [24]) One of the reason there is still a matter of choice the routine approach is linked with the analytical aspects of the DNA extraction and processing from the archived tissues (FFPE, formalin-fixed, paraffin-embedded), such that the DNA concentration and purity to be proper for the multistep and error prone above methods and also with the variable robustness of the listed techniques.

In the present study, we analyzed 4 FFPE tumour specimens derived from two types of patients. One group of 2 patients which were diagnosed with glioblastoma and who were treated according to an alkylating approach, a TMZ-based chemoradiotherapy protocol; the second group comprised two patients, who were diagnosed with EWS and their treatment was approached by a combined inefficient chemo- and radio-therapy.

We compared two usual assays for *MGMT* promoter methylation analysis: one based on MS-PCR as a qualitative method and MS-MLPA as a semi-quantitative method, in order to determine which of the methods is able to predict clinical outcome most reliably.

Also, the importance of our study regarding the choice of the diagnostic method is linked with the actual disease *management during the anticancer-alkylating* (TMZ) treatment of cancers. Current treatment protocols involving concurrent chemoradiotherapy for the specific tumors, such as malignant gliomas demonstrated an increased incidence of *pseudoprogression* or radiation necrosis (ROLDAN [25])| Inaccurate diagnosis of pseudoprogression in the early period of chemoradiation may lead to an unwanted discontinuation of effective treatment. Several reports have demonstrated that some clinical, radiological, and biological indicators can be helpful in predicting pseudoprogression (YANG [26]). One reason for the lack of definite diagnostic criteria for pseudoprogression is that variables associated with pseudoprogression are difficult to quantify. Therefore, the prognostic value of O^6-methylguanine DNA methyltransferase (MGMT) promoter methylation in glioblastoma patients treated with concurrent radiotherapy and temozolomide followed by adjuvant temozolomide is critical and its association with an incidence of pseudoprogression has also been demonstrated (BRANDES [27], PARK [28,29]).

Therefore, the need for the quantitative results together with the qualitative ones regarding the MGMT methylation biomarker in such approaches is critical for the choice of the method: MSPCR method is easy and cost efficient, but it is only qualitative as compared with the most complex MS-MLPA method. In the case an optimized and more sensible nested MS-PCR is performed, a combination of both methods may avoid certain inaccuracy in determining the prognosis, including the occurrence of pseudoprogression.

2. EXPERIMENTAL SECTION

2.1.Multiplex ligation-dependent probe amplification (MLPA) reaction. MS-MLPA analysis for MGMT methylation status determination was carried out using *the SALSA MLPA ME011 Mismatch Repair genes probemix* (MRC-Holland) according to manufacturer protocol. This kit contains 6 probes specific for the MGMT promoter region (10q26) developed to detect aberrant CpG island methylation. These probes contain HhaI recognition site.To evaluate the methylation of MGMT promoter region, the sample was analyzed twice: undigested and digested. For this purpose, the sample DNA was denaturated and the probes were allowed to hybridize. The reaction products were splitted in two: one part was ligated, while for the other part the ligation was combined with *Hha*I digestion. *Hha*I enzyme is methylation sensitive, digesting unmethylated probes that will not be exponentially amplified by PCR and hence will not generate a signal when analyzed by capillary electrophoresis. In contrast, if the sample DNA is methylated, the hemi-methylated probes are prevented from being digested by *Hha*I and the ligated probes will generate a signal. The methylation pattern is determined by comparing undigested sample with its digested counterpart. After MS-MLPA reaction, the samples were loaded onto an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems).

2.2.Methylation-Specific Polymerase Chain Reaction. The methylation status of the MGMT promoter was also analyzed by MSPCR, and the results were compared with those of MS-MLPA. Prepared DNA was modified by sodium bisulfite treatment using an EZ DNA Methylation-Gold Kit (Catalog No. D5005, Zymo Research). The primer sequences for the MGMT were as follows: methylated forward: 5′-TTT CGA CGT TCG TAG GTT TTC GC-3′, methylated reverse: 5′-GCA CTC TTC CGA AAA CGA AAC G-3′, unmethylated forward: 5′-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3′, unmethylated reverse: 5′-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3′. The annealing temperature was 64°C. The obtained PCR products were electrophoresed in 2% agarose gels and visualized under ultraviolet illumination after staining with ethidium bromide.

The results were interpreted as positive if the MGMT gene promoter methylation was detected as a fragment of 100 bp observed in the gel, and they were negative if MGMT gene promoter methylation was not detected with the methylated primers.

3. RESULTS SECTION

MSMLPA Results. The methylation status of the probe has been determined by comparing the peak pattern of digested DNA sample to the undigested one (figure 1). The percentage of the methylation has been done with Coffalyser.Net software by dividing the normalization constant of each probe obtained on the digested test sample by the normalization constant of each MS-MLPA probe obtained on the corresponding undigested sample and by multiplying this value by 100 (see Fig. 1). In the analyzed sample, the MS-MLPA analysis for MGMT methylation status evidenced the methylation of MGMT promoter region from 14% to 70%, depending on specific position of probes (table 1). According with recommendations of manufacturer, the results obtained for two probes, the 346 nt probe (located between the methylation hot spots and showing methylation in only rare cases), which showed no methylation in our case, and the 409 nt probe (which shows frequent methylation, but at times in cases where the other five probes do not show any methylation), which was 80% methylated, were disregarded.The MSMLPA results showed semiquantitatively that the MGMT gene promoter was methylated in glioma tissues and unmethylated in EWS tissues. These results were in accordance with the literature, that is indicating that EWS are rarely methylated in the MGMT gene promoter and thus suggesting that the prognostic for the tumor progression under the treatment with alkylating agesnt such as TMZ would be negative (JEUKEN [30], [31]).

MSPCR Results. The classical MSPCR method proved poor sensitivity and was later optimized by a nested MSPCR approach after addition of a preliminary step of MSPCR with a pair of nested primers in order to enrich the DNA fragments initially methylated for the MGMT promoter (UDRISTE in press). In the electrophoregram of the classical MSPCR (seeFig.2) there may be seen as sharp bands the amplicons for only the glioma tissue (lanes 1,2), as compared with the poor bands corresponding for the EWS tissue (lanes 7,8). One explanation may reside from the fact that the concentration and the quantity of the processed DNA extracted from EWS tissue was insufficient (less then 50ng) in order to be correctly processed through the numerous steps of MSPCR, hence the quantity of the resulted amplified unmethylated form was insufficient to yield a sharp band. However the quality was poor, the method proved that, also according to the literature, the EWS tissue was unmethylated for the MGMT encoding gene promoter.

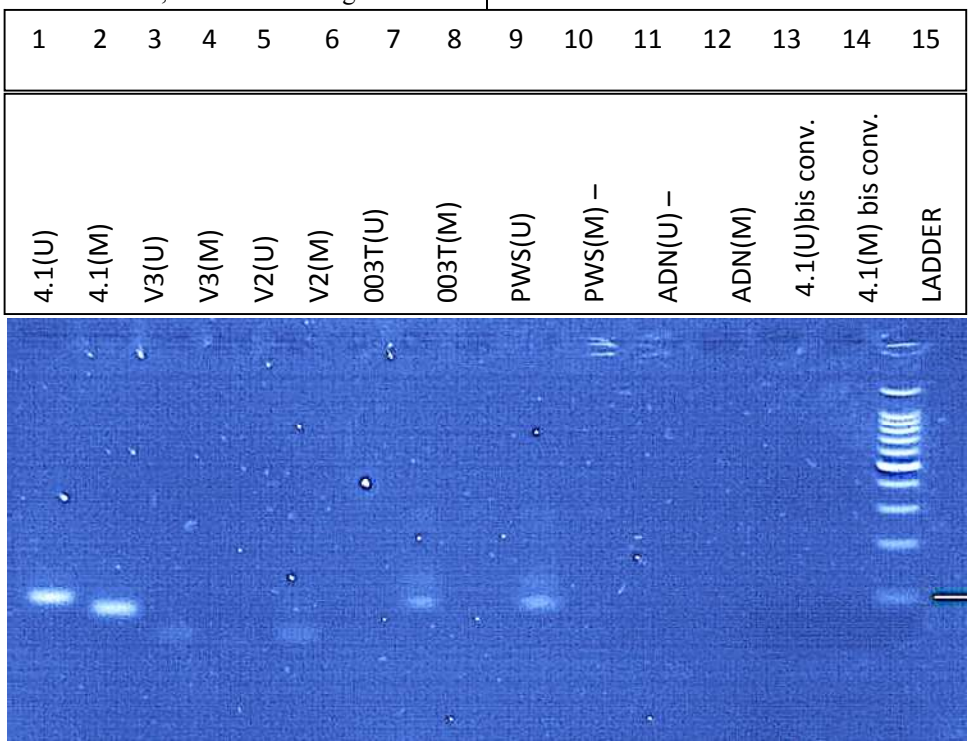

Figure 1. Processed electropherograms of analyzed sample: (A) undigested sample and (B) digested sample.

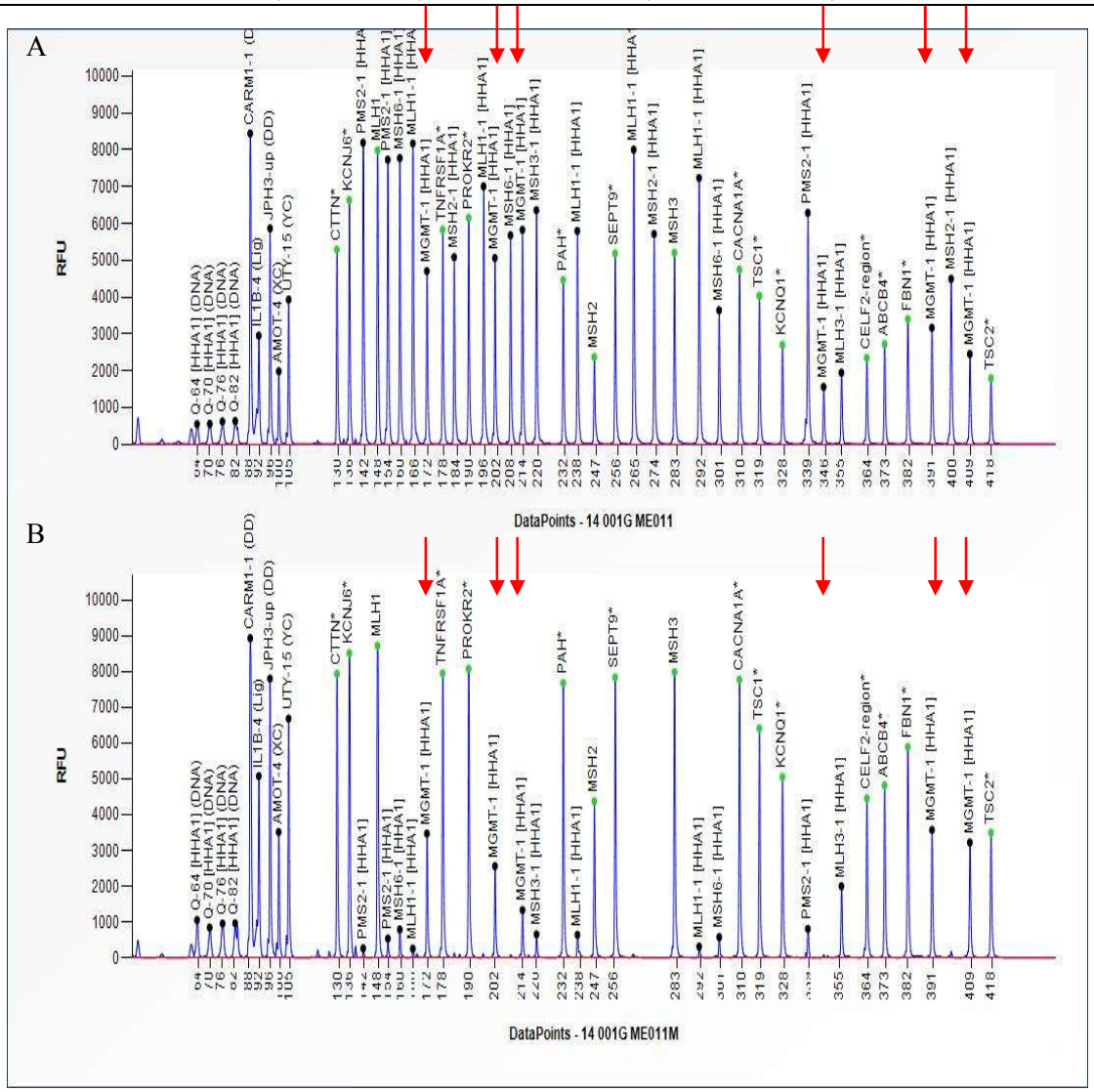

Figure 2. Electrophoregram of the classical MS-PCR amplicons: Lanes 1,2- Glioma tissue: the methylated (M) and unmethylated (U) MGMT promoter fragments in one glioma tissue; lanes 7,8: the unmethylated (U) forms only in one EWS tissue; lane 15-the ladder control, lanes 11,12 controls without DNA, lanes 9,10: controls with amplification of MGMT promoter state in PWS patient DNA. The lanes 3,4,5,6 (V3) represents the amplicons with false methylated and unmethylated results for EWS tissues DNA proving the lack of the robustness of the classical method in our approach. The 100bp is marked with a line.

Extensive studies on the role of MGMT repair enzyme activity in genome stability over the past decade have led to the accumulation of proofs suggesting that it could serve as a biomarker for tumor prognosis and treatment, especially for glioma tissues. *MGMT* gene methylation is a biomarker that has recently been shown to be able to stratify or even select glioblastoma patients for clinical trials (WICK [32], MOLENAAR [33]). In general, MSPCR has been first accepted as the available clinical method for the analysis of the *MGMT* promoter methylation status in cancers. However, its prognostic value of the classical MSPCR is still not sufficient to provide alone a solid background for clinical decision making due to the lack of sensibility and inaccuracy as well as the obtaining of the nonquantitative data from the classical approach of the technique. The results of this study have shown that MS-MLPA data are concordant with those obtained from MSPCR.This method can detect aberrant methylation at specific CpG sites based on digestion with a methylation-sensitive restriction enzyme and yields semiquantitative results.

The advantages of MS-MLPA include the following important analytical aspects: (1) it can bypass the bisulfite conversion reaction, the most time consuming and error prone step of most assays for the methylation status, (2) different CpGdinucleotides can be analyzed simultaneously, (3) multiple genomic DNA analyses can be performed during a single session within a day, (4) copy number analysis is possible by comparison with control DNA, and (5) only a small volume of DNA (20 ng) is required, as compared with MS-PCR method, which required a much greater volume (over 50ng).

Also, this study demonstrates the capacity of the MS-MLPA technique to provide semiquantitative results. This is important in the correct diagnostic especially when pseudoprogression such as that observed in in glioblastoma patients, is suspected (BRANDES 27). It is generally recognized therefore, that the specificity of the chosen test remains a problem in the prediction of pseudoprogression, Therefore, the *MGMT* promoter methylation test by MS-MLPA can provide important information for clinical decision making on early-response to

classical treatment evaluation. However, in the case an optimized MS-PCR is developed, the combination of MS-MLPA and MS-PCR can further improve the qualitative and quantitative diagnostic accuracy of pseudoprogression for some patients, for an accuracy rate that can clearly help to make clinical decisions.

Table 1. The estimation of the percentage of methylation of MGMT promoter region with MS-MLPA.

Gene	Probe position	Methylation %
MGMT	Upstream – 405 nt before exon 1; 432 nt before ATG (= 406 nt before transcription start)	70
	Upstream – 319 nt before exon 1; 346 nt before ATG	31
	Upstream – 66 nt before exon 1; 93 nt before ATG reverse	0
	Intron 1 – 72 nt after exon 1; 151 nt after ATG	14
	Intron 1 – 154 nt after exon 1; 233 nt after ATG reverse	46
	Intron 1 – 385 nt after exon 1; 464 nt after ATG reverse	81

4. CONCLUSIONS

Hypermethylation in the promoter region of the *MGMT* gene encoding the DNA repair protein O^6-methylguanine-DNA methyltransferase for DNA double- stranded breaks is among the most important prognostic factors for patients with glioblastoma and predicts response to treatment with alkylating agents like temozolomide and radiotherapy; both interventions efficiency is based on the inducing DNA double-strand breaks.

Hence, the *MGMT* status is widely determined in most clinical trials and frequently requested in routine diagnostics of tumor capacity for repair the DNA damages introduced by chemo- and radiotherapies during anticancer treatemnt. MS-MLPA and MSPCR were two methods that gave results regarding the state of the methylation in the MGMT gene promoter.

The two types of tumors were found heterogenously methylated (in glioma tissues) and nonmethylated in the EWS tissues. However, the classical method MSPCR was not accurately able to distinguish the methylated and unmethylated forms especially in the case of the EWS DNA, requiring its optimization further. MSMLPA was a useful method that can simultaneously produce semiquantitative and qualitative results on the methylation status of MGMT gene promoters. This method has a great predictive value in glioblastoma management outcomes, including the cases of pseudoprogression.

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