Author's response to reviews

Title: O6-Methylguanine-DNA methyltransferase protein expression by immunohistochemistry in brain and non-brain systemic tumours: systematic review and meta-analysis of correlation with methylation-specific polymerase chain reaction

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Author's response to reviews: see over
IN REPLY TO REFEREE 1

REFEREE’S REPORT:
Loss of MGMT activity is likely to underlie much of the sensitivity to temozolomide and other alkylating agents in brain and other cancers. In this communication, the authors have performed a review of the literature and a meta-analysis of the correlation between two methods determining absent MGMT function; promoter methylation as measured by methylation specific PCR (MSP) and immunohistochemistry.

Important methodological problems seem to exist with both assays. MHMT IHC does not have well defined cut off values between positive and negative. The non-quantitative nature of MSP is a major problem. However, as the authors mention, more quantitative methylation assessment methods will need validation.

* Minor Essential Revisions

- The authors do not mention another important confounding factor. Low levels of methylation are present in the normal tissue of certain individuals in particular those with the T allele of the rs16906252 polymorphism. This may lead to false positive calls for methylation of the tumour.

RESPONSE:
We completely agree with the reviewer and appreciate his interesting comments. Although some authors (ref. 8, 10, 92) state that the presence of a methylated MGMT allele can be attributed solely to the neoplastic cells, it has been demonstrated by Candiloro et al. (ref. 94) that low levels of methylation may be present in peripheral blood of certain individuals, in particular those with the T allele of the rs16906252 polymorphism. Likewise, Shen et al. (ref. 93) reported MGMT promoter methylation in normal-appearing colonic mucosa several centimetres away from colorectal tumours, and Zhang et al. (ref. 56) describe similar findings in normal epithelium obtained from oesophageal squamous cell carcinoma patients. Similarly, an absence of detectable MGMT activity in
histologically normal brain adjacent to primary brain tumours has been demonstrated (ref. 3).

Accordingly new information has been added in the new version of the manuscript, page 15, 2nd paragraph (Discussion):

"... there are other confounding factors that may lead to false positive methylation results. Although it has been stated that the presence of a methylated MGMT allele can only be attributed to neoplastic cells [8,10,92], some authors have demonstrated that MGMT promoter methylation may occur in non-neoplastic central nervous system tissue [3] or in normal-appearing mucosa several centimetres away from digestive tumours [56,93]. Moreover, Candiloro et al. [94] have shown low levels of methylation in peripheral blood of healthy individuals with the T allele of the rs16906252 polymorphism”.

- In addition, further false positives may arise because of poor primer design, insufficiently stringent reaction conditions and incomplete conversion by bisulfite.

RESPONSE:

MSP is indeed technically complex with a high percentage of non-conclusive results even in experienced hands. Due to the fact that it relies on the differential susceptibility of methylated versus unmethylated cytosines to sodium bisulfite modification, which leads to selective amplification with specific primers (ref. 19), it is highly dependent on tissue quality and quantity. In addition, the specificity of the selected primers, and the adequacy of bisulfite treatment and PCR conditions may lead to further false results.

This information has been included in the new version of the manuscript, page 15, 1st paragraph (Discussion):

"Third, due to the fact that MSP relies on the different susceptibility of methylated versus unmethylated cytosines to sodium bisulfite modification and subsequent selective primers amplification, it is highly
dependent on tissue quality and quantity, primer design, bisulfite treatment adequacy and PCR conditions [19]. “

The reference by Candiloro et al. (ref. 111) concerning methylation-sensitive high resolution melting (MS-HRM) methodology has been included in the Discussion, on page 17, 2nd paragraph.

• The second sentence of the abstract urgently needs revision.

RESPONSE:
The sentence has been corrected, page 2, 1st paragraph (Abstract), and the full text has been edited by a native English speaker:

“Several methods have been applied to its analysis, with methylation-specific polymerase chain reaction (MSP) the most commonly used for promoter methylation study, while immunohistochemistry (IHC) has become the most frequently used for the detection of MGMT protein expression.”

• Typos that need to be corrected:

  “Cutt-off” (table 1)
  polyclonal (table 2)
  The I of monoclonal is often on a new line in the tables

RESPONSE:
All typos have been corrected and the full text has been edited by a native English speaker:

Additional file 2: “Cutt-off has been replaced by Cut-off”
Additional file 3: “Polyclonal has been replaced by polyclonal”
The I of monoclonal has been checked in the tables

IN REPLY TO REFEREE 2
REFEREE’S REPORT:

Brell et al.: This is a careful review on MGMT immunohistochemistry (IHC) and methylation specific PCR (MSP), notably for gliomas but also for other tumour groups. Lots of data on MGMT expression have been published in the last years, and data are quite conflicting especially as to the predictive and progressive value of the different methods applied. This study arrives at the conclusion that MGMT IHC does not provide the same information as MSP regarding MGMT expression. Therefore, both methods are not interchangeable. The same conclusion was previously drawn by some other authors based on their glioma studies, such as Maxwell et al (ref. 68). This study goes somewhat further since the Meta-analysis shows lack of concordance between IHC and MSP if relevant published studies were compared.

- Unfortunately, the authors do not draw a conclusion as to the usability of IHC as a predictive/prognostic marker. They should refer at least to the recent study of Preusser et al. (ref. 20) in which important limiting factors were identified such as inter-observer differences in specimen evaluation and the quality of the antibody.

- I also recommend to add some data comparing the clinical response with the IHC. Are Kaplan-Meier curves for gliomas available on the basis of IHC and are they as significant as those published for MSP?

RESPONSE:

Following reviewer’s suggestions, comments on the prognostic and predictive value of MGMT protein expression assessed by immunohistochemistry and whether it may be useful as a clinical biomarker have been added to the revised manuscript.

The prognostic and predictive value of MGMT protein expression determined by immunohistochemistry or quantitative immunofluorescence in patients with glioma has been evaluated in several studies, with contradictory results (ref. 3, 21, 113). Although differences in the design of the studies could explain at least in part these contradictory results, other possibilities should be
considered. While those neoplastic cells that do not express MGMT may not be able to correct DNA damage induced by chemotherapy, loss of MGMT expression can also be a negative prognostic factor because of increased susceptibility to acquiring other mutations (ref. 120, 121, 122). Furthermore, due to variable interobserver agreement, insufficient correlation with MGMT promoter methylation status and the lack of a firm association with patient outcome (ref. 18, 20, 29, 103), MGMT IHC has not proved to be a clinically usable biomarker for routine diagnostic purposes and clinical decision-making (ref. 20). This information has been added to the revised manuscript, page 17, 3rd paragraph:

“Both MGMT status at protein level and promoter methylation have been correlated with prognosis and chemosensitivity in glioma patients. As is shown in Additional file 2, the prognostic and predictive value of protein expression has been evaluated in some studies with contradictory results. Several authors have reported a significant association of MGMT expression assessed by immunohistochemistry with patients’ overall or progression-free survival [22,23,31,88,113-117]. Some of them have even shown MGMT protein expression to be an independent predictor in the multivariate analysis [31,84,85,115,116,118,119], whilst others have demonstrated a lack of correlation [29,46,58,74]. However, most published data were obtained from heterogeneous groups of patients with different grades and histologies, as well as distinct treatment protocols [31]. Although differences in study design could explain, at least in part, these contradictory results, other possibilities should be considered. In this sense, while those neoplastic cells that do not express MGMT may not be able to correct DNA damage induced by chemotherapy, loss of MGMT expression can also be a negative prognostic factor because of an increased susceptibility to acquiring other mutations [120-122]. Furthermore, due to variable interobserver agreement, insufficient correlation with MGMT promoter methylation status and the lack of a firm association with patient outcome [20,29,103], MGMT IHC has not proved to be a clinically usable biomarker for routine diagnostic purposes and clinical decision-making.”
• Finally, it would be interesting to compile those papers in which pre-treatment (primary) tumors was compared with recurrences. This has been recently done for gliomas (Christmann et al., Int.J.Cancer, 127, 2106-2118). Are similar data available for MGMT IHC for gliomas and other tumor groups and is there any correlation between pre-treatment tumors and recurrences in IHC and MSP?

RESPONSE:
Following the reviewer’s comment, papers in which MGMT status in pre-treatment tumours has been compared with the status in recurrences have been included in the new version, (page 16, 1st paragraph)

“Interestingly, some factors, such as glucocorticoids, ionizing radiation and chemotherapy, can induce MGMT expression [26,102]. Thus, a further question to be addressed is whether tumour recurrences exhibit the same MGMT status as the pre-treatment tumour or a different one. Unfortunately, data on this are limited and contradictory [103]. While some studies have demonstrated an increase in MGMT immunostaining [84] or a lower frequency of MGMT promoter methylation [87,104,105] in recurrent gliomas after chemotherapy, other series have failed to demonstrate any change [84,103,106]. Finally, both an increase and a decrease in MGMT expression have also been described for recurrent tumours [22,76,87,107-109]. A higher protein expression might indicate that the MGMT gene has been up-regulated by the treatment, although other possible explanations, such as selection of chemoresistant cells with high MGMT protein levels or intratumoral regional variations, can not be excluded [26,84,109].”

• The last paragraph (Conclusions p.17) should end up with a recommendation regarding the use of both methods. If they are not interchangeable, should both methods be applied or is one of them better suited for the purpose of prediction of the patient’s response (see comment above)?
RESPONSE:
Following the reviewer’s comment, a new paragraph recommending the use of MSP as the reference test has been included. We therefore agree with Karayan-Tapon (ref. 46) that MSP is the best approach for MGMT status assessment in glioma patients.

This information has been added in the revised version page 19, 2nd paragraph, Conclusions:

“Despite all the above mentioned aspects, MSP currently remains the most established method and the best approach to assessing MGMT status. It is also the technique for which the most convincing clinical correlations have been reported, and thus it should be considered the reference test. Unfortunately, it is a relatively complex and time-consuming method not apt for routine clinical implementation in many centres [19].

However, the analytical and clinical performance of MGMT immunoassaying seems to be inappropriate for routine diagnostic purposes. This fact, along with the lack of a robust association with MGMT promoter methylation as demonstrated in the present meta-analysis, precludes its use as a valuable biomarker for clinical decision making. However, it remains to be determined whether novel anti-MGMT antibodies directed against other epitopes would improve its performance [20].

Accordingly, some authors have suggested the feasibility of using MSP combined with IHC for prognostic and predictive purposes [104,116]. Immunohistochemistry may represent a useful preliminary test to identify methylated cases while MSP should be performed in non-immunoreactive cases to identify truly methylated tumours [70]. Again, this issue deserves further investigation.