MGMT promoter methylation: assays, implications

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October 2020—With *MGMT* gene promoter methylation observed in about 50 percent of glioblastomas, it remains a biomarker of strong clinical interest in routine practice, even though it's not the sole determinant in decisions related to therapy.

PCR and pyrosequencing are the most commonly used assays, and there's a technique that is not yet mainstream but gaining interest, said Tejus A. Bale, MD, PhD, assistant attending pathologist in the Department of Neuropathology and Diagnostic Molecular Pathology, Memorial Sloan Kettering Cancer Center.

Dr. Bale spoke June 30 in the first of a series of Association for Molecular Pathology webinars on emerging and evolving biomarkers.

"Clinical trials have started to use *MGMT* status as a patient selection criterion as well as for stratification of various treatment arms," she said. "So in planning the care for the patient overall, it's definitely still important, and it's a component of the overall clinical decision-making."

O^6-methylguanine-DNA-methyl-transferase, or MGMT, is encoded by the *MGMT* gene, located on chromosome 10q26.3. It's a highly evolutionarily conserved and widely expressed enzyme and involved in DNA repair. When *MGMT* is deficient, "and not able to do its job," Dr. Bale said, "there is persistence of the O^6-methylguanine DNA lesion."

Unrepaired O^6MeG frequently mispairs with thymine during DNA replication, resulting in G:C to A:T transition mutations. "In a low-*MGMT* state, you have this mutation phenotype, so the DNA is taking hits, and on the one hand, that's a setup for cancer," Dr. Bale said.

On the other hand, "the cell does not like all the hits that it's taking to its DNA." There are pathways in place, including mismatch repair, that attempt to repair the ongoing cell damage and excise the base mispairing. During DNA replication, these attempts lead to sister chromatid exchange and single- and double-strand breaks. Eventually, "the cell will say enough is enough" and activate the cell death cycle, she said. "This is the so-called Achilles' heel that we're trying to capitalize on" with the use of alkylating chemotherapy.

To date, there is no consensus on the optimal testing method for *MGMT* gene promoter methylation. There are a couple of options, Dr. Bale said, and they follow the same general outline: genomic DNA extraction followed by qualitative and quantitative checks, a bisulfite conversion check followed by additional quality checks, and because "the bisulfite conversion doesn't give you any information on its own, you need to pair it with a downstream assay of your choice"—most commonly PCR or pyrosequencing.

A pathologic examination is critical to ensuring maximal tumor purity, she said. *MGMT* testing is frequently reliant on formalin-fixed, paraffin-embedded samples, so sample DNA will already have some degree of damage and fragmentation. In addition, *MGMT* expression and promoter methylation status can be affected by tumor heterogeneity. “You want to avoid regions of necrosis, and to avoid contaminating non-tumor, normal cells.” For that, laboratories can consider using laser capture microdissection or H&E-guided macrodissection. And obtaining a tumor purity estimate can aid in downstream test interpretation. “Rely on histopathology to set yourself up for success,” she said.

[dropcap]T[/dropcap]he bisulfite conversion deaminates cytosines in the DNA transcript into uracil, while methylated cytosines are protected from the reaction. With PCR amplification, the deaminated uracils are converted to thymine, “and now you have a bisulfite-induced mutation,” she said, which can be detected using a downstream assay. “That’s the way you can separate your methylated transcripts from your unmethylated transcripts.”

But bisulfite conversions are harsh and thus come with DNA degradation and fragmentation and a high degree of sample loss. There’s also the problem of partial conversion. Cytosines that remain in the transcript post conversion are presumed to be methylated, but if the reaction isn’t complete, “it’s impossible to know whether those cytosines are actually there because they weren’t converted appropriately.”

An optimized bisulfite protocol is therefore key, “and that involves being rigorous about quantifying and assessing the quality of your input DNA.” And to avoid false-positives resulting from partial conversion, the bisulfite conversion efficiency should be checked routinely. The best way to do this, she said, is to run methylated and unmethylated controls in parallel with patient samples.

A review published in *The Journal of Molecular Diagnostics* details the laboratory methods for detecting *MGMT* gene promoter methylation (Cankovic M, et al. *J Mol Diagn.* 2013;15[5]:539–555). In addition to PCR and pyrosequencing, several other assays can be used. Dr. Bale summarized these testing approaches:

- In methylation-specific PCR (“garden variety” PCR, as she put it), methylated and unmethylated *MGMT* gene promoter components from the same DNA sample are selectively amplified to separate methylated from unmethylated transcripts. “It’s mainly qualitative,” she said, “but it’s highly standardized, reliable, and quick.” The downsides: “You’re going to have issues resolving positive cases at the low percentage and purity threshold,” or cases that have heterogeneous levels of methylation. “And there is capacity to look at the band intensities and translate this into semiquantitative use, but that is imprecise.”
- A step up from MS-PCR, she said, is quantitative real-time PCR analysis, in which amplification of methylated and unmethylated *MGMT* gene promoter is done by PCR but its detection is obtained using fluorophores. Methylation-sensitive quantitative Locked Nucleic Acid PCR, MethyLight, and many other assays can be used. While this method has increased sensitivity over standard PCR, it requires greater technical skill and specific instruments and reagents, so costs are higher.
- In pyrosequencing, after the DNA is isolated, it undergoes bisulfite treatment and PCR amplification, and then a sequencing-by-synthesis system is used to query methylation at each individual CpG site. “Essentially, you’re detecting a signal of light every time the nucleotide is incorporated,” she said. A defined cutoff value, commonly 10 percent, is used to classify cases as methylated or unmethylated. Pyrosequencing has greater sensitivity than PCR, particularly in low methylated cases and heterogeneously methylated patterns. And while costs are higher than for

PCR, it “may be an excellent setup for laboratories that have a higher volume.”
- Multiplex ligation-dependent probe amplification and immunohistochemistry, unlike the other methods, do not have a bisulfite conversion component. MLPA involves endonuclease activity, “so again you have to worry about the completion of your digestion in order to validate how accurate your result will be.” IHC queries expression of MGMT protein rather than test for *MGMT* promoter methylation, and “the clinical implications of IHC-related MGMT expression are not as clear as *MGMT* promoter methylation.”

MS-PCR and pyrosequencing would be best considered gold standard, and several studies compare single methods with each other. “What’s reassuring is that for the most part, in most cases, you’re able to resolve methylated or unmethylated results from the *MGMT* promoter, but what’s disheartening is that there is a significant amount of discordant results between patients and between sites,” Dr. Bale said, adding that this is likely the result of interlaboratory differences and technical issues surrounding DNA bisulfite treatment and tissue processing. Additionally, some of the discordance is related to the techniques themselves, because different methods can query different CpG sites.

[dropcap]H[/dropcap]igh-throughput profiling of cancer DNA methylome using DNA methylation array is a technique of increasing interest and clinical relevance, Dr. Bale said. In methylome profiling, tumor DNA from FFPE tissue is hybridized to specially designed bead chips. “You’re able to obtain a methylation profile that encompasses anywhere from 450,000 to 850,000 CpG sites across the genome,” depending on the bead chip used. This technique, particularly the Illumina Infinium MethylationEPIC BeadChip kit, is gaining popularity because it is overall high throughput with good accuracy, has a small sample requirement, and is relatively low cost, she said, although that’s in comparison with other whole genome methylation analyses.

Dr. Bale

Bady, et al., reported in 2012 on use of the Infinium methylation BeadChip to identify two distinct CpG regions with high importance for gene silencing and survival outcome (Bady P, et al. *Acta Neuropathol*. 2012;124[4]:547–560). From this discovery, they developed the STP-27 algorithm, which uses a stepwise logistic regression model incorporating the M-values (log ratio of the beta value) of the two CpG sites to output a probability of *MGMT* status. “By using your validated probability cutoff against a gold standard method,” she said, “you can then cut off and determine a methylated, unmethylated, and an unsure probability for the *MGMT* promoter methylation.”

A study published this year compared methylation-specific PCR with high-density DNA methylation array using the STP-27 algorithm to assess *MGMT* gene promoter methylation status in 39 glioblastoma cases. Contradictory results were validated by pyrosequencing. When cases with an inconclusive result in one or the other method were taken into account, the inter-method reliability reached 77 percent. When only cases with conclusive results in both methods were considered, inter-method reliability was 91 percent (Braczynski AK, et al. *Pathol Res Pract*. 2020; 216[1]:152728).

Dr. Bale and colleagues are using the array-based method at MSK to determine *MGMT* promoter

methylation, and, she predicts, “it will catch on in more ways than one.”

Why choose this method if it requires an iScan, reagents, dedicated bead chip arrays, and time (four to five days)? “It’s a robust assay with highly reproducible results, and since you’re looking at 450,000 to 850,000 CpG sites, there is a lot of additional data that’s coming along for the ride,” Dr. Bale said. And with patient tissue itself the most expensive or nonrenewable aspect of testing, leaving additional data on the table “may be a bargain in time we can’t afford to make, particularly if it has other useful applications.”

One such application is DNA methylation-based classification of central nervous system tumors (Capper D, et al. *Nature*. 2018;555[7697]:469–474). To develop the classifier (https://tinyurl.com/y2z44xyj), researchers at the German Cancer Research Center performed unsupervised clustering on 2,801 CNS tumor methylation profiles across 76 WHO-defined histopathologic CNS tumors and normal/reactive tissue. These were incorporated into a Random Forest type of algorithm to assign a diagnostic class to a CNS tumor based on its methylation profile.

“This is becoming important as an objective diagnostic method,” Dr. Bale said. “There are levels of granularity in terms of making a diagnosis that aren’t available any other way.” A similar classifier has been created for sarcoma, “and there’s a lot of attention around doing this for other tumor types”—and that’s one way to add value despite the higher costs.

[dropcap]C[/dropcap]urrently, standard-of-care treatment for GBM consists of maximum safe resection, followed by radiotherapy and concomitant and adjuvant chemotherapy with the alkylating agent temozolomide (TMZ).

MGMT was originally investigated as a biomarker of sensitivity to alkylating chemotherapy, including TMZ. The first evidence associating *MGMT*-methylation status with response to TMZ emerged in 2005, and from that a new standard of care was established (Hegi ME, et al. *N Engl J Med*. 2005;352[10]:997–1003; Stupp R, et al. *N Engl J Med*. 2005;352[10]:987–996). “One of the important things that came out of the ‘Stupp trial’ data is that *MGMT* appears to be a very strong prognostic marker,” Dr. Bale said. Regardless of whether patients were treated with TMZ plus radiotherapy or radiotherapy alone, *MGMT* gene promoter methylation was found to be the strongest predictor of survival.

In the 2013 RTOG0525 trial, newly diagnosed GBM patients were randomized to two different sequential treatments of TMZ after completion of standard TMZ with concurrent radiotherapy. The *MGMT*-unmethylated patients received a more intensive maintenance schedule of TMZ, while the *MGMT*-methylated patients received standard maintenance therapy as in the Stupp trial. In both arms, *MGMT* status was found to be a strong prognostic factor, with the median survival rate in the *MGMT*-methylated cases 21.2 months, versus 14 months in the unmethylated cases.

MGMT may be a predictive biomarker as well, Dr. Bale said. The survival benefit observed in the Stupp trial with the addition of TMZ to radiotherapy was much larger in the *MGMT*-methylated patients than in the unmethylated patients (https://bit.ly/3bnwqyA), “and that has been borne out in the long-term data of the Stupp trial as well as in other trials,” she said.

MGMT status has been found to have a predictive role in elderly GBM patients. In the NOA-08 and Nordic trials, which compared TMZ monotherapy with radiotherapy alone in patients 65 and older and 60 and older, respectively, longer survival was seen in *MGMT*-methylated patients treated with TMZ

monotherapy compared with *MGMT*-unmethylated patients. No survival difference was seen according to *MGMT* status among patients in the radiotherapy arm (Perry JR, et al. *N Engl J Med*. 2017;376[11]:1027–1037).

But, in general, almost all GBM patients are treated with TMZ. The Stupp trial found a slight benefit in favor of TMZ plus radiotherapy versus radiotherapy alone even in *MGMT*-unmethylated patients. An overall survival advantage was also observed in *MGMT*-unmethylated elderly patients treated with TMZ plus short-course radiation compared with short-course radiation alone. "We're looking at 10 months versus 7.9 months, but that's still clinically meaningful when you're out in the weeds like we are with glioblastoma," she said, adding that new treatment options are urgently needed.

"So we're interested," she said of *MGMT* gene promoter methylation status, "but it's not *the* jumping off point that determines one treatment versus another."

[dropcap]I[/dropcap]n 2016, the WHO incorporated molecular data into neuropathology diagnosis, moving away from diagnosis made on histomorphology alone. This shift has profoundly affected the way gliomas are diagnosed and graded, Dr. Bale said, with the new way "shaped by our understanding that the lower-grade gliomas—astrocytomas and oligodendrogliomas—are mediated by the first hit mutations in *IDH1* and *IDH2*."

A study published in 2015 found that patients who had lower-grade gliomas with *IDH*-wildtype had substantially shorter overall survival than did those with lower-grade gliomas with mutated *IDH* (Brat DJ, et al. *N Engl J Med*. 2015;372[26]:2481–2498). The study also found that patients who had lower-grade gliomas with an IDH mutation and no 1p/19q codeletion had shorter overall survival than did patients who had lower-grade gliomas with an *IDH* mutation with codeletion. But both groups had substantially longer overall survival than did those who had lower-grade gliomas with *IDH*-wildtype.

IDH-mutant GBMs demonstrate a hypermethylator phenotype, and that has a significant association with methylated *MGMT*. "So both of these are going to confer a relatively favorable clinical course," according to the data, she said. As a result, the impact of *MGMT* methylation on survival may have been overestimated in earlier studies. About 10 percent of GBMs are estimated to harbor an *IDH* mutation, "so without significantly accounting for that," Dr. Bale said, "it's impossible to know how much of that has been overestimated. There have been many attempts to resolve this and to look at pure *IDH*-wildtype cohorts, and for the most part *MGMT* status does remain supported as a predictive and prognostic indicator, particularly in *IDH*-wildtype glioblastoma tumors."

In low-grade gliomas, on the other hand, the true prognostic or predictive value of *MGMT* status is unclear. "Moving forward, the most effective strategy is to use *MGMT* in conjunction with histology and molecular findings," she said, citing a study that found the superior predictions came from combining *IDH* status and *MGMT* methylation status (Molenaar RJ, et al. *Neuro Oncol*. 2014;16[9]:1263–1273).

The clinical consensus, then, is that *MGMT* promoter methylation status is accepted as a prognostic and promising predictive biomarker and should be assessed in all patients with a histologic diagnosis of GBM according to the 2016 WHO classification.

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