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CORRESPONDENCE

Biallelic *MGMT* loss in a case of IDH-wild-type adult glioblastoma: a case for concurrent epigenomic and molecular karyotype testing

To the Editor,

Molecular genetic investigation has become established practice for many solid tumours, including low and high grade gliomas.[1] Both acquired somatic DNA sequence and copy number variants are gaining increasing importance to further refine the histological diagnosis by identifying the underlying genetic features that have important diagnostic, prognostic and therapeutic implications. This includes identifying epigenetic changes such as altered methylation of the gene *MGMT* [O(6)-methylguanine-DNA methyltransferase] in glioblastoma, with its status well documented in the literature as an independent favourable prognostic marker and indicator of increased responsiveness to alkylating chemotherapy agents (e.g. temozolomide).[2,3] This has led to *MGMT* promoter methylation status driving treatment decision making in cases of newly diagnosed glioblastoma and those aged 60 years and over.[5] Treatment standard for glioblastoma is currently surgical excision, followed by radiotherapy and alkylating chemotherapy. In older patients, the presence of *MGMT* promoter methylation has resulted in monotherapy with alkylating chemotherapy (temozolomide), rather than dual chemotherapy and radiotherapy, or radiotherapy alone in unmethylated tumours.

MGMT encodes a DNA damage repair enzyme which reverses mutagenic alkylation of the O^6-guanine in DNA.[4] Temozolomide, an alkylator, induces DNA crosslinking and causes single- and double-strand DNA breaks, resulting in cell cycle arrest, apoptosis and tumour cell death. Hypermethylation of its 5′ CpG island promoter region results in *MGMT* gene silencing. Paradoxically, a *MGMT* hypermethylated tumour can accumulate cancer promoting mutations over time but also has enhanced sensitivity to alkylating agents as *MGMT* based DNA damage repair is inhibited. Therefore, epigenetic silencing of *MGMT* increases the effectiveness of temozolomide.[5]

MGMT promoter methylation is the main pathway by which *MGMT* gene expression is reduced in glioblastoma. The mainstay of determining *MGMT* methylation status is by standard bisulfite pyrosequencing, which has been described as the gold standard for correlating methylation with overall patient survival and progression free survival in published major clinical trials.[6] However, there are alternate pathways through which reduction of *MGMT* gene expression can occur. Herein, we describe a rare case of glioblastoma with biallelic loss of *MGMT*, and describe the divergent results obtained by traditional pyrosequencing and cytogenomic methods.

Single nucleotide polymorphism (SNP) chromosome microarray (CMA) is an important tool to assess chromosomal copy number abnormality in both low grade and high grade gliomas. In glioblastoma, hallmark cytogenetic features involve co-occurring chromosome 7 gain/chromosome 10/10q loss (including *PTEN)*, *CDKN2A/2B* biallelic loss, and *EGFR* amplification (without chromosome 7 gain). Loss or copy neutral loss of heterozygosity (cnLOH) of chromosome 17p (with *TP53* loss-of-function somatic variants) is also important.[6] Of interest, *MGMT,* located within chromosome 10q, can also be lost but single copy loss is not usually considered prognostic.[7]

The recent World Health Organization (WHO) Classification of Tumours of the Central Nervous System[1] now integrates the recommendations of the previously published cIMPACT-NOW guidelines, whereby molecular events must be integrated with demographic factors (patient age, tumour location) and histopathological findings to determine tumour diagnosis and grade.[1,8] Therefore, understanding the molecular information gained by utilising molecular testing is imperative to arriving at an 'integrated diagnosis', as encouraged by the WHO.

We present a case of a 70-year-old male with new irregularly enhancing lesions of the high left paramedian and parietal lobe with distant non-contiguous lesions in the left parietal occipital junction, left corpus callosum splenium and the left major forceps. Histology and immunohistochemical findings showed an IDH-wild-type glioblastoma. Fresh tissue was sent for chromosome microarray testing at time of specimen receipt, as per protocol in our laboratory for all gliomas (a diagnosis of glioblastoma usually results in cancellation of array testing, but this was not cancelled in time). Subsequent *MGMT* promoter methylation testing was performed on formalin fixed, paraffin embedded tissue (FFPE) after histological diagnosis.

Molecular karyotyping by SNP-CMA: DNA was obtained using the Wizard Genomic DNA purification kit (Promega, USA) with modification involving overnight 56°C digestion with protease (Sigma, USA), followed by 37°C RNAse treatment. The Infinium 850K CytoSNP Beadchip v1.2 microarray (Illumina, USA) was analysed using NxClinical v6.2 (BioDiscovery, USA) and genome reference GRCh37, according to the manufacturer's instructions. Copy number abnormality (CNA) loss or gain was called when >50 kb and probe Log R values were below −0.2 and above 0.1, respectively. cnLOH was called when >5 Mb. Tumour load (%) was estimated from the B-allele frequency (BAF).[9] Chromothripsis was defined as alternating CNAs in a chromosome arising from ≥10 breakpoints.

Pyrosequencing of MGMT promoter region: DNA was extracted from FFPE tissue using the Qiagen QIAmp DNA FFPE Tissue Kit as per manufacturer's protocol (Qiagen, Germany), after adequacy of tumour cellularity was assessed by a pathologist. Sodium bisulfite modification of the tumour DNA was then performed. Quantitative methylation measurement was undertaken with the Qiagen Therascreen MGMT Pyro Kit, on the Qiagen PyroMark Q24 system. Data from four CpG island sites in exon 1 of the *MGMT* promoter region were analysed using PyroMark Q24 Software. Both external methylated and unmethylated controls were run simultaneously with expected results.

Results of cytogenetics and pyrosequencing: SNP-CMA showed a near-tetraploid (4n) tumour and complex molecular karyotype at an estimated 70−80% tumour load (Fig. 1A). The more significant abnormalities included tetrasomy 7

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(including *EGFR)*, *CDKN2A/CDKN2B* biallelic loss due to overlapping deletions within chromosome 9p, 17p cnLOH (including *TP53)* as part of chromosome 17 complex structural changes, and chromosome 10 chromothripsis. The latter resulted in *MGMT* biallelic loss due to complete loss of bands 10q26.2 to 10q26.3 (Fig.1B,C). *PTEN* was retained.

MGMT promoter methylation status by pyrosequencing reported the tumour to be unmethylated (Fig. 1D). As per the SNP array result, the tumour cells no longer harboured the *MGMT* gene or its promoter region due to biallelic loss. Therefore, the *MGMT* promoter region assessed by pyrosequencing reflected the background proportion of germline (normal) cells with an intact *MGMT* promoter region, within the sample submitted for pyrosequencing.

Interrogation of publicly available cancer genomic databases: Genomic datasets pertaining to glioblastoma (Columbia[10] and TCGA[11]) were identified via cBioPortal for Cancer Genomics (https://www.cbioportal.org/).[12] Six hundred IDH-wild-type glioblastoma samples were identified. Data including single nucleotide variants (*IDH1/2* status), copy number variants and methylation data were collected and analysed.[10–12] Three of 600 cases (0.5%) demonstrated biallelic (homozygous) deletion of *MGMT*. *MGMT* promoter status for all three cases was unmethylated, much like our case.

In neuropathology, as with other areas which require molecular features for histological diagnosis, triage of tissue, molecular testing techniques and integration of molecular findings are important in providing standard of care for patients. Clinicians and scientists within the neuropathology sphere are now required to be versed in a range of techniques available in order to elicit clinically relevant and personalised pathological information from tumours.

SNP-CMA is an important diagnostic tool in many cancers, including glioma. It has the advantage that it can detect CNA (loss, gain, amplification) and cnLOH; the latter frequently harbour activating/inactivating gene mutations. It also allows assessment of tumour ploidy, tumour load (purity) and genomic content containing cancer-related genes within identified chromosomal aberrations relevant for the tumour type under investigation. However, it cannot detect balanced structural rearrangements or DNA sequence variants and is limited in detection of CNAs when <20 kb in size or present in <20% subclonal fraction/tumour load. SNP-CMA performed in most clinical diagnostic laboratories cannot detect DNA methylation, however methylation microarrays are available in the research setting.

In our case, taken together with the pyrosequencing results, we theorise that this tumour would behave like a methylated tumour due to biallelic loss of *MGMT* repair genes. Examination of publicly available cancer genomic databases revealed three cases with the same histological and molecular phenomena; however, a review of the literature failed to find other reports describing biallelic loss of chromosome bands containing *MGMT*, or even 10q in glioblastoma. Therefore, to our knowledge, this is the first describing such a case.

This case illustrates the advantages and limitations of both techniques, the outcome of which have implications for clinical management and prognosis. This tumour could be expected to respond as a methylated tumour with a better response to temozolomide. Unfortunately, the patient died prior to initiation of therapy; correlation with clinical management and response would have been informative in this case.

This case also raises issues with specific genetic testing: genome wide molecular cytogenetic karyotyping (e.g., for detection of chromosome 7 gain/10 loss), versus sequencing technologies (e.g., for *MGMT* promoter methylation, *IDH1/2* status). There are practical deliberations to consider. SNP-CMA works best on fresh tissue, so there are limitations for specimen handling and transportation. There is also a higher cost associated with this test in Australia, in terms of specialised processing, laboratory expertise and monetary cost. Pyrosequencing is performed on FFPE tissue meaning this technique can be embedded into current pathology workflow, similar to next generation sequencing. There is also an associated lower cost compared to cytogenetics. Our case demonstrates that any molecular test has both advantages and disadvantages, and anatomical pathologists are best placed to balance the tissue requirements necessary for diagnosis.

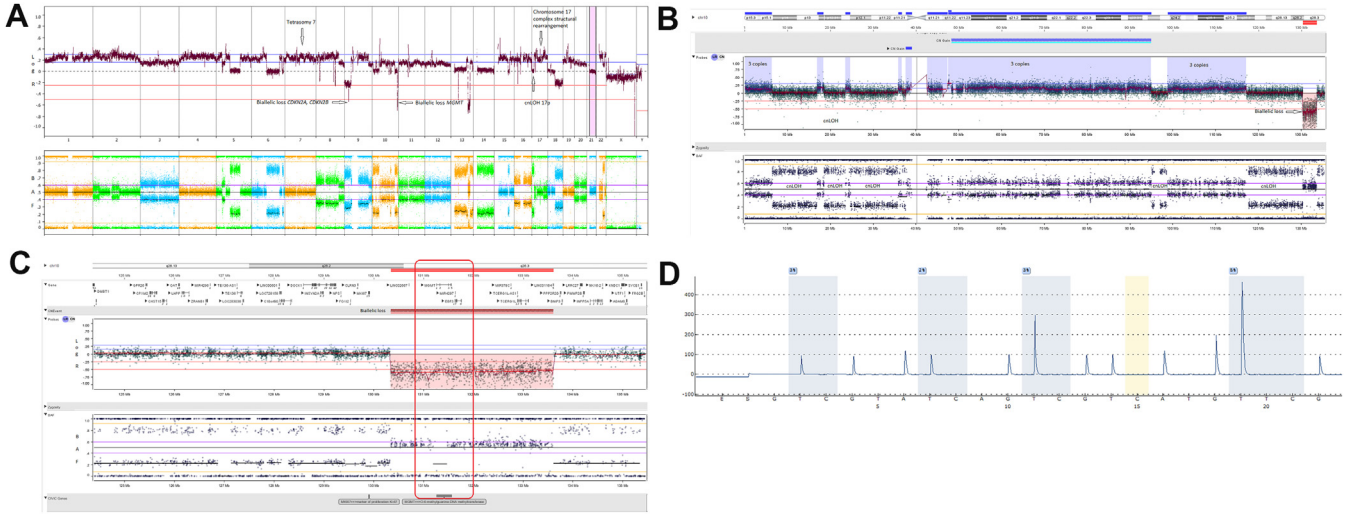

Fig. 1 (A) Genome-wide 850K CytoSNP Beadchip microarray data showing a near-tetraploid tumour with a likely count of 101−102 chromosomes. A complex molecular karyotype is present with the more significant abnormalities indicated: tetrasomy 7, complex loss within 9p, chromothripsis of chromosome 10, and cnLOH 17p. The top panel shows the Log R plot (copy number) and the bottom panel the B-Allele Frequency (BAF) plot; (B) chromosome 10 chromothripsis involving alternating copy number change of three copies and two copies (cnLOH), and biallelic loss of bands q26.2 to q26.3; (C) chromosome 10q indicating biallelic loss that includes *MGMT*; (D) pyrosequencing trace of *MGMT* promoter region, demonstrating unmethylated status (CpG sites highlighted in blue, with % methylation given).

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There is a growing recognition of a need to test for epigenetic changes as well as genetic changes in gliomas, and technologies such as methylation SNP microarrays, may find a more routine place in tumour molecular pathology. The case illustrates biallelic loss of *MGMT* in glioblastoma, a novel finding in literature with potential management ramifications, and has demonstrated the need for complementary molecular testing in glioma with regards to tumour prognostication and management determination.

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