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SHORT REPORTS

Unrepresentative astrocytoma biopsy sampling is partly overcome by assessment of the MIB-1-labelled growth fraction

Representative sampling of gliomas at biopsy is essential for correct assignment of histological grade and subsequent patient management. If sampling is unrepresentative, tissue devoid of mitoses or necrosis may be obtained and result in a falsely low glioma grade. The objective of the present study was to assess whether the MIB-1 labelling index of glioma tissue cores specifically constructed so as to replicate ‘unrepresentative’ astrocytoma biopsies would predict the real or actual glioma grade. Tissue microarrays were prepared from 134 samples of low-grade astrocytoma (LGA; WHO grade II), anaplastic astrocytoma (AA) and glioblastoma multiforme (GBM). Donor blocks were sampled in such a way as to avoid mitoses, necrosis and endothelial hyperplasia. Immunohistochemistry was performed using the Ki-67 (MIB-1) proliferation marker, and the percentage of MIB-1-positive cells per core was calculated. Mean MIB-1% values for LGA (n = 47), AA (n = 38) and GBM (n = 46) were mean (SD) 0.54 (0.82), 5.68 (6.69) and 7.21 (7.98); ranges 0–3.07, 0–30.08 and 0–29.08, respectively. An MIB-1% count of >3.07 excludes LGA, but an MIB-1% count of <3.07

does not exclude GBM or AA. It is concluded that in unrepresentative glioma biopsy material, MIB-1 labelling may be used to exclude LGA, but cannot be used to distinguish between GBM and AA.

Astrocytomas in adults demonstrate considerable cellular heterogeneity, and pathological grading is the most significant predictor of clinical outcome.¹ While grading may be suggested radiologically, it is recommended that all astrocytomas be biopsied to establish a correct diagnosis and grade. The presence of mitoses and necrosis is a key adjunct in the grading of astrocytomas. Mitoses are a feature of high-grade gliomas, including anaplastic astrocytomas (AAs), and would not generally be seen on a biopsy of a low-grade astrocytoma (LGA). Moreover, the presence of palisaded necrosis in an astrocytoma distinguishes GBM from AA.

Inadequate or unrepresentative tissue sampling at biopsy may lead to erroneous diagnosis of an astrocytoma having a lower grade than is actually the case, with adverse consequences in terms of failure to administer supplementary treatments including radiotherapy. If sampling is inaccurate, glioblastoma multiforme (GBM) may be devoid of mitoses and necrosis, and AAs may be devoid of mitoses.

Evaluation of proliferative activity using the Ki-67 (MIB-1) antibody has been used as an adjunct in the assessment of tumour grade in human astrocytomas,^{2,3} and its prognostic value in distinguishing subtypes is recognised.^{4,5} We sought to evaluate the ability of this marker to predict the underlying histological grade of gliomas using tissue microarray (TMA) technology to simulate inadequately sampled biopsy specimens. TMA methodology is now widely used in cancer research⁶ and is a validated method for tumour immunoprofiling.⁷ More recently, it has been used in the study of non-neoplastic disease of the central nervous system.⁸ In this study, we hypothesised that the MIB-1 labelling index in tissue cores designed to replicate unrepresentative astrocytoma biopsies would predict the histological grade in three astrocytoma subtypes.

Patients and methods

Subject material

Adult astrocytomas were selected from the neuropathology tissue archives in Beaumont Hospital, Dublin, Ireland. The material that was chosen consisted of surgically resected formalin-fixed paraffin-embedded tumour tissue. Astrocytomas were graded according to the WHO International Classification of brain tumours.¹ Forty-eight were classified as LGA (WHO grade II); 39 as AA (WHO grade III); and 47 as GBM (WHO grade IV), yielding 134 cores. WHO grade I or pilocytic astrocytomas, glioneuronal tumours and mixed oligoastrocytomas were specifically excluded.

Tissue microarray construction

TMAs were constructed by extracting ‘core biopsies’ from a precise morphologically

representative area of the original paraffin ‘donor’ block, with subsequent re-embedding of carefully selected cores into a ‘recipient’ microarray paraffin block. This was done using a precision instrument (Beecher Instruments, Silver Spring, MD, USA), employing separate core needles for punching donor and recipient blocks. Glioma TMAs were prepared using cores designed to replicate intraoperative inadequate astrocytoma sampling. Cores (0.6 mm diameter) were prepared from resected LGA, AA and GBM. During core sampling, tissue areas were targeted to avoid mitoses, necrosis and endothelial hyperplasia. Specifically, two experienced neuropathologists examined haematoxylin and eosin-stained sections of the original tumour resection, and astrocytoma grade was agreed. Core biopsies were not used as they did not provide sufficient tissue for sampling nor could we have been certain that such true in vivo biopsies accurately reflected correct tumour grade. Areas of highest cell density but without mitoses were marked with a Nikon optical circular marker having a diameter of 1 mm. With grade II astrocytomas, which by definition are devoid of mitoses, we sought to sample the most cellular areas. The donor block was overlaid with the marked slide; cores were obtained from within the marked circle and subsequently arranged in the recipient tissue block together with location and immunocytochemical controls. Later, all cores in the recipient block were again examined to ensure that mitoses were not inadvertently included.

Immunohistochemistry

TMAs were immunostained with the Ki-67 (MIB-1) antibody (DakoCytomation, Glostrup, Denmark) using a standard avidin–biotin peroxidase technique with the Vectastain® Elite ABC kit (Vector Labs, Burlingame, CA, USA) with diaminobenzidine as the chromogenic substrate. The sections were counterstained with haematoxylin.

Scoring of MIB-1 immunostaining

The tissue arrays were labelled so that the investigator (K.M.S.) was blinded as to the origin of the individual cores. Using image analysis (Olympus DP-soft version 3) with a grid overlay, the individual cores were evaluated for total cell count and total number of MIB-1-positive cells. The results were then expressed as the percentage of MIB-1-positive tumour cells per core. MIB-1 positivity was defined as definitive brown nuclear staining. The mean number of cells per core analysed was 516, 504 and 942 for LGA, AA and GBM, respectively. Statistical analysis was performed using a t test. A p value of <0.05 was considered statistically significant.

Results

LGA tumours had significantly lower MIB-1% positivity in comparison with GBM (T = –5.7; p<0.0001) and AA (T = –4.6; p<0.0001)

Table 1 Mean MIB-1% labelling indices in each of the glioma subtypes

	GBM (n = 46)	AA (n = 38)	LGA (n = 47)
Mean	7.21	5.68	0.54
SD	7.9	6.69	0.82
Range	0–29.08	0–27.16	0–3.07

GBM, glioblastoma multiforme; AA, anaplastic astrocytoma; LGA, low-grade astrocytoma

Figure 1 Representative cores from each of the three glioma subtypes. (A) Low-grade astrocytoma: total cell count 728; total MIB-1-positive cells 2; MIB-1% index 0.27%. (B) Anaplastic astrocytoma: total cell count 985; total MIB-1-positive cells 67; MIB-1% index 6.8%. (C) Glioblastoma multiforme: total cell count 1350; total MIB-1-positive cells 256; MIB-1% index 18.9%.

(table 1, fig 1). AA was marginally lower than GBM, but this did not reach statistical significance ($T = 0.6$; $p = 0.54$).

Mean (SD) MIB-1% values for LGA ($n = 48$), AA ($n = 39$) and GBM ($n = 47$) were 0.54 (0.82), 5.67 (6.69) and 7.21 (7.98); ranges 0–3.07, 0–30.08 and 0–29.08, respectively.

An MIB-1 count $>3.07\%$ excludes an LGA but, conversely, an MIB-1 count of $<3.07\%$ does not exclude either AA or GBM (fig 2). Thus, GBM and AA have similar proliferation rates in unrepresentative biopsies and are not distinguishable by this method.

A summary of the MIB-1 labelling indices obtained in each of the tumour types is given in table 1.

Discussion

In this study, which was designed to simulate unrepresentative astrocytoma sampling, we demonstrated that an MIB-1 index $>3.07\%$ excludes LGA. An MIB-1 index of $<3.07\%$ does

Figure 2 Distribution of MIB-1% positivity in each of the three glioma types. GBM, glioblastoma multiforme; AA, anaplastic astrocytoma; LGA, low-grade astrocytoma.

not exclude a higher grade astrocytoma. Furthermore, the significant overlap in MIB indices between AA and GBM renders distinction between these two grades of astrocytoma unreliable in unrepresentative biopsies.

In the post-surgery management of astrocytomas in adults, it is vital to distinguish between LGA and AA, whereas distinction between AA and GBM is less critical in that there is no debate about the need for maximum tumour reduction followed by whole brain irradiation (RT). The benefits of RT for LGA are less evident, as is the benefit/risk ratio of surgery in LGA. Many centres adopt an expectant approach to LGA—that is, biopsy with interval imaging, reserving RT for tumours which show clinical or radiological evidence of progression. A false diagnosis of LGA could result in a patient being denied the possible benefits of early RT. In practice, the presence of enhancement with contrast in an astrocytoma over-rides the predictive value of a diagnosis of LGA on biopsy. In cases of adult supratentorial astrocytoma without contrast enhancement, an MIB-1 index of $>3.07\%$ is, in our practice, used to sway a treatment decision in favour of early RT. We did not

attempt to relate the MIB-1 index to outcome as we would have required a larger patient sample to control for other variables such as patient age, residual post-surgical tumour volume and degree of neurological disability, factors which of themselves are independent determinants of outcome in astrocytoma.

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Take-home messages

- Biopsy sampling of gliomas is essential for correct classification of histological grade.
- Unrepresentative sampling that lacks mitoses or necrosis may result in a falsely low grade.
- Ki-67 (MIB-1) labelling is a useful adjunct in the assessment of glioma grade and is of prognostic value.
- In this study, we assessed whether the MIB-1 labelling index in tissue cores designed to replicate unrepresentative biopsies would predict the histological grade in three astrocytoma subtypes.
- Our findings indicate that in unrepresentative glioma biopsy material, MIB-1 labelling may be used to exclude low-grade astrocytoma, but cannot be used to distinguish between glioblastoma multiforme and anaplastic astrocytomas.

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Homozygous frameshift mutation in the *SLC22A12* gene in a patient with primary gout and high levels of serum uric acid

Idiopathic renal hypouricaemia (IRH), an autosomal recessive disorder, is an uncommon disease that presents with an increase in uric acid excretion. Most patients with IRH have homozygous deleterious mutations in the *SLC22A12* gene that encodes for URAT1 protein. Gout is a multifactorial metabolic disease caused by the deposition of urate crystals. This study describes a patient with high levels of uric acid, primary gout and a novel *SLC22A12* gene mutation, which is associated to IRH. The patient showed a 1 bp homozygous insertion (680insG) that resulted in substitution of threonine instead of alanine and in a premature stop codon. This finding provides information about the influence of environmental and/or epigenetic factors in Mendelian inheritance.

Idiopathic renal hypouricaemia (IRH) is an autosomal recessive disorder characterised by increased uric acid excretion.¹ The main biochemical defect is abnormal uric acid transport at the proximal tubule. URAT1 protein seems to be the major mechanism regulating blood urate levels.^{2,3} Most patients with IRH (Online Mendelian Inheritance in Man 607 096) harbour homozygous deleterious mutations in the *SLC22A12* gene, which encodes for URAT1 protein.² The major clinical manifestation of IRH is the presence of uric acid stones in the urinary tract.⁴ Around 50% of patients with IRH are Japanese.

Gout is a multifactorial metabolic disease caused by the deposition of urate crystals that produce characteristic joint inflammation. Hyperuricaemia is the most important risk factor for gout and, in 90% of patients, is secondary to renal uric acid under-excretion. *SLC22A12* has been studied in patients with gout, the mutation W258X is observed in a healthy population but in none of the patients with gout; the mutation W258X seems to be a suppressing factor for the development of gout.⁵ *SLC22A12* polymorphisms have been associated with reduced uric acid excretion and hyperuricaemia in a German population.⁶ In a previous study,⁷ authorised by the patients and the ethics committee, we did molecular analysis of the *SLC22A12* gene in patients with primary gout, in first-grade relatives and in healthy controls. We found mutations in 16 patients with gout, 3 in first-grade relatives and none in healthy controls. Most of the mutations were heterozygous with one exception, the subject of this report. This finding provides information about the influence of environmental and/or epigenetic factors in the Mendelian inheritance.

Case report and molecular analysis

A 39-year-old man was referred to our rheumatology department with a history of arthritis in the lower limbs. His parents were healthy and non-consanguineous. The other members of his family (wife and two daughters 8 and 10 years old) were healthy. He had no familial history of gout or hyperuricaemia.

Table 1 Primers used in the PCR analysis of exons 3–6 of the *SLC22A12* gene

Region	Primers	length (bp)
Ex3 F Ex3 R	5'-TGG CAA GCC ACA GAC CCT GC-3' 5'-AGC CCT GGA GCC TGC ATG GA-3'	250
EX4 F EX4 R	5'-TCC ATG CAG GCT CCA GGG CT-3' 5'-GGC AGG ATC TCC TCT GAG GA-3'	254
EX5 F EX5 R	5'-GCC ACA GGC AAT GAC CCC TC-3' 5'-ACC TTC TTC CCA GGG AGC TG-3'	163
EX6 F EX6 R	5'-TCA GAG AGG AGG AGG TGC CT-3' 5'-CCA GGT TCC CCT GTG GAG GT-3'	220

The patient had a 6 year history of podagra, acute attacks affecting the knees and ankles, and tophi. At physical examination his height was 1.70 m, weight 73 kg and waist circumference 83 cm; he had no hypertension, history of lithiasis, or chronic renal failure nor previous treatment with diuretics or salicylates.

Laboratory evaluation reported serum uric acid 11.8 mg/dl (702 µmol/l), urinary uric acid 403 mg/24 h (2.39 mmol/d), and uric acid clearance 3.7 ml/min/1.73 m², serum creatinine 0.89 mg/dl (78.8 µmol/l), serum urea 36.4 mg/dl (6.07 mmol/l), glucose 102 mg/dl (5.67 mmol/l), triglycerides 413 mg/dl (4.66 mmol/l), cholesterol 203 mg/dl (5.25 mmol/l) and creatinine clearance 82.8 ml/min/1.73 m². The patient had hyperuricaemia and hypertriglyceridemia, but no other criteria for metabolic syndrome according to ATP III criteria.

Gout diagnosis was made according to the American College of Rheumatology criteria. The patient received treatment with allopurinol 300 mg/day, colchicine 1 mg/day and fenofibrate 200 mg/day. Laboratory test of his daughters were within normal limits, but unfortunately it was not possible to determine blood and urinary laboratory values in his siblings because they lived in another city.

As part of our previously mentioned study,⁷ *SLC22A12* gene was analysed in this patient. DNA extraction from peripheral blood was

performed as described elsewhere.⁸ All exons of the *SLC22A12* gene were initially analysed through PCR. Conditions and primers to amplify exons 1–2 and 7–10 are described elsewhere,⁹ and conditions to amplify exons 3–6 are shown in table 1. In general, PCR consisted of 30 cycles of denaturation at 94°C for 60 s, annealing at 62°C and extension at 72°C for 60 s. PCR products were purified with a PCR purification kit (Qiagen, Valencia, California, USA). DNA sequence analysis was performed using ABI PRISM 310 genetic analyser (Applied Biosystems, Foster City, California, USA) according to manufacturer's conditions. All procedures were performed twice in patients and healthy controls.

Results and discussion

Hyperuricaemia is defined as serum urate concentration >7 mg/dl in men. Genetic and/or environmental factors such as diuretics, obesity and high alcohol intake are necessary for gout expression.¹⁰ In this study, we describe a patient with primary gout, hyperuricaemia and a novel *SLC22A12* gene mutation. *SLC22A12* mutations were first associated with hypouricaemia and IRH.

Our patient showed a 1 bp homozygous insertion (680insG) within exon 4 (fig 1) that resulted in substitution of threonine instead of alanine and in frameshift of the open reading frame; this 1 bp insertion generated a TGA

Figure 1 Forward and reverse primers of the partial DNA sequence of exon 4 of the *SLC22A12* gene of the patient with primary gout and the normal control, only a small fragment is shown. Codon 227 with the 1 bp insertion 680Gins is shown underlined.