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ORIGINAL PAPER

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Low-level microsatellite instability phenotype in sporadic glioblastoma multiforme

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Abstract *Purpose*: Genetic instability is a hallmark of glioblastoma multiforme (GBM). Microsatellite instability (MSI) is a significant event in the tumorigenesis of many sporadic malignancies. The aim of our investigation was to study microsatellite instability in newly diagnosed glioblastomas. *Methods*: MSI was investigated in 109 GBMs with 15 microsatellite markers. Immunohistochemistry was performed for the mismatch repair (MMR) proteins hMLH1, hMSH2, hPMS2, and hMSH6 in cases showing MSI. Sequence and promoter methylation status of *hMLH1* were analyzed in the case of a decreased hMLH1 protein expression as well. To further investigate MSI(+) GBMs we carried out studies of LOH at selected chromosome regions, *EGFR* amplification, and sequence of *p53* and *PTEN*. *Results*: MSI was observed in six GBMs (5.5%) and it was more frequent in GBMs with a previous lower grade astrocytoma (18.8% vs. 3.2%). MMR protein staining was positive in all MSI(+) GBMs except in one case, which showed an aberrant expression of hMLH1 and hPMS2 without *hMLH1* inactivation. Among MSI(+) GBMs, one tumor corresponded to the GBM molecular type 1 (*p53* mutation, no *EGFR* amplification), another tumor to type 2 (wild-type *p53*, *EGFR* amplification), and four tumors to neither type (wild-type *p53*, no *EGFR* amplification). None of the six tumors carried a *PTEN* mutation. *Conclusions*: MSI in GBM might be caused by inactivation of minor MMR genes rather than by a deficiency of *hMLH1* or *hMSH2* and it appears not to play a decisive role in the pathogenesis of these tumors. MSI(+) GBMs predominantly showed a profile which included wild-type of *p53* and *PTEN* and absence of *EGFR* amplification but MSI occurred in all GBM molecular subtypes.

Keywords Glioblastoma multiforme · Microsatellite instability · Mismatch repair · hMLH1

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Introduction

Glioblastoma multiforme (GBM) is the most frequent intracranial tumor in adults and accounts for approximately 15% of all brain neoplasms (Kleihues et al. 2002). The prognosis is dismal with a median survival time of 12 months after diagnosis despite aggressive therapy (Lacroix et al. 2001). In the pathogenesis of GBM two molecular pathways are characterized, type 1 and type 2 glioblastomas (von Deimling et al. 1993). Type 1 GBMs typically harbor *p53* inactivation and most of type 2 GBMs carry *EGFR* amplification without *p53* mutations (von Deimling et al. 1995; Reifenberger et al. 1993). However, in about one-third of all GBMs neither of these molecular alterations can be found (von Deimling et al. 1995; Louis 1997).

Genetic instability in GBMs is characterized by chromosomal instability with multiple imbalances (Hui et al. 2001), an event observed in the tumorigenesis of numerous solid tumors (Kinzler and Vogelstein 1997). A second form of instability, denominated microsatellite instability (MSI), was first described in patients with hereditary non-polyposis colorectal cancer (HNPCC) syndrome, which is caused by a deficiency of the

mismatch repair (MMR) system and shows length mutations of microsatellite sequences (Thibodeau et al. 1993; Ionov et al. 1993). MSI was observed in HNPCC-associated malignancies in 80–90% of the cases (Ionov et al. 1993) as well as in many sporadic neoplasms such as colorectal, gastric, pancreatic, endometrial, and breast carcinomas (Ionov et al. 1993; Peltomäki et al. 1997; Wooster et al. 1997) in up to 40% of these tumors. HNPCC-related colorectal cancers show distinct pathological features as compared to their MSS homologues (Dietmaier et al. 1997; Jass et al. 1999), and MSI in those malignancies was observed to be predominantly associated with an inactivation of *hMLH1* or *hMSH2* (Boland et al. 1998). To our knowledge, an investigation of the MMR system in a large series of more than 100 glioblastomas has not been systematically performed. Furthermore, since 30% of GBMs show molecular alterations which do not conform to one of the known pathways, the analysis of the genetic events associated with MSI is of high interest. Therefore, we studied MSI in 109 sporadic newly diagnosed GBMs, evaluated the expression of the MMR proteins in MSI(+) cases, and performed an analysis of *hMLH1* including sequencing and promoter methylation status in the case of hMLH1 reduced expression. Additionally, we characterized the molecular features of MSI(+) GBMs according to type 1 and 2 pathways.

Material and methods

GBM samples

One hundred and nine paired samples of blood and tumor tissue were obtained from GBM patients treated at the Department of Neurosurgery of the University of Dresden. Portions of the fresh tumor samples were frozen in liquid nitrogen and stored at −80 °C. Tumor tissue was evaluated at the Institute of Pathology, University of Dresden, Germany, according to the World Health Organization (WHO) criteria (Kleihues et al. 2002). Formalin-fixed, paraffin-embedded specimens were used for immunohistochemistry. This study was conducted with the consent of the institutional ethics committee and informed consent from each patient or patient caretaker was obtained. Sixteen patients had a previous astrocytoma, 11 patients an astrocytoma grade II, and further five patients had an astrocytoma grade III. Seven of the 109 GBM had a survival time of more than 36 months after surgery (so-called long-term survivors). Four patients had a personal history of cancer (three colorectal carcinoma and one breast cancer). There was no evidence for an association between MSI and family history of cancer (Table 1).

MSI analysis

Matched genomic tumor and blood DNA samples ($n = 109$) were amplified by PCR using 15 primer pairs (Dietmaier et al. 1997): mononucleotides (BAT25, BAT26, BAT40, TGFß-RIIpA, GTBPIn5, GTBPIn9), dinucleotides (D5S346, D17S250, D10S197, D2S123, D18S58, D13S153, D3S1300, D3S1619), and tetranucleotide (MYCL1). PCR was performed containing 50–100 ng DNA. Amplified PCR products were electrophoresed on a 6.5% Long Ranger polyacrylamide gel on an Automated Laser Fluorescence (A.L.F. express) sequencing device (Amersham Pharmacia Biotech, Freiburg, Germany) and analyzed using the ALLELELINKS 1.00 software (Amersham Pharmacia Biotech). MSI was defined by the presence of novel alleles in tumor DNA, which were absent in corresponding leukocyte DNA and confirmed in two independent analyses. Informative dinucleotide repeats without MSI were assessed for LOH as described (Cawkwell et al. 1994). Three categories were defined according to the MSI findings: high-level MSI (MSI-H, ≥40% of studied loci altered), low-level MSI (MSI-L, <40%), and MSS (no analyzed marker unstable) (Dietmeier et al. 1997; Boland et al. 1998).

Immunohistochemistry of MMR proteins

Immunostaining for hMLH1 (clone G168–15, PharMingen Int., San Diego, Calif., USA), hMSH2 (clone FE11, Oncogene Res. Products, Cambridge Mass., USA), hMSH6 (clone 44, Transduction Lab., Lexington, UK), and hPMS2 (A16–4, PharMingen Int.) was performed in all six MSI(+) tumors as described (Plaschke et al. 2002). The relative densitometry of

Table 1 MSI status of the analyzed GBMs and clinical data of the patients. (*A-II* astrocytoma WHO grade II, *A-III* astrocytoma WHO grade III, *CRC* colorectal cancer, *NF-2* neurofibromatosis type 2)

Age range	Male/female	Previous astrocytoma	Family or patient tumor history	Long-term survival patients(months)	MSI-L
<18 years($n = 1$)	1/0	0	None	0	0
18–55 years($n = 45$)	29/16	8 A-II3 A-III	1 pinealoma	5/45 (11.1%) (mean: 55range: 37–70)	2/45 (4.4%)
>55 years($n = 63$)	40/23	3 A-II2 A-III	3 CRC1 breast cancer1 NF-2	2/63 (3.2%) (mean: 59range: 48–70)	4/63 (6.3%)

MMR protein expression (compared to respective negative controls) was performed using the Meta-Vue software (Universal Imaging Systems, Penn., USA).

Mutational and epigenetic analyses of hMLH1

We sequenced all coding exons and exon-intron boundaries of hMLH1 from leukocyte and tumor DNA in the case of a decreased hMLH1 protein expression. After PCR amplification, the products were electrophoresed in 0.8% agarose gel and subjected to cycle sequencing reactions using the Thermo Sequenase Fluorescent Cycle Sequencing kit (Amersham Pharmacia Biotech). After denaturation, the cycle sequencing products were resolved using a denaturing 6.5% Long Ranger polyacrylamide gel on a sequencing device. Primer sequences and amplification conditions were as described elsewhere (Kolodner et al. 1995). To evaluate the *hMLH1* promoter methylation in the case mentioned above, DNA were digested with methylation-sensitive or insensitive restriction endonucleases, *HpaII* (Life Technologies, Karlsruhe, Germany) or *MspI* (New England Biolabs, Frankfurt am Main, Germany), respectively. After digestion, 25 ng from each digest was used as the template for PCR amplification of an 829-bp fragment which contains the promoter region, the exon 1 and a part of the intron 1 of the *hMLH1*, and harbors five *HpaII* sites. The amplification products were visualized using a 1% agarose gel. Primer sequences and PCR conditions were as described previously (Kane et al. 1997). The breast cancer cell line CAL51 was used as positive control.

Characterization of type 1 and type 2 pathways in MSI(+) GBMs

LOH at 10q23–24 was studied with the markers D10S215 and D10S541 (flanking *PTEN*), PTENCA (intragenic marker), D10S583 and D10S579 (telomeric and centromeric to *PTEN*, respectively). Analysis of LOH at 17p13 (*p53*) was done with the TP53 and a *p53* intragenic marker. Allelic losses at 9p21 (*p16/CDKN2A*, $p14^{ARF}$ and *p15/CDKN2B*) and 13q14 (*RB1*) were studied with the primers D9S1748, D9S171, D9S1749, D13S153, and D13S267, respectively. Loss of heterozygosity at 1p35–36 and 19q13 were assessed with the markers D1S468, D1S482, D19S112, and D19S412 (Smith et al. 2001). Primer sequences and PCR amplification conditions are based on Genome Database entries (www.gdb.org). To detect *EGFR* amplification a differential PCR with the CFTR- (cystic fibrosis transmembrane regulator) gene was carried out, as previously described (Hunter et al. 1995). Sequencing of all coding exons and intron-exon boundaries of *p53* and *PTEN* was performed using primers and conditions previously described, which are available from the authors upon request (Liaw et al. 1997; Steck et al. 1997).

Statistical analysis

Contingency analyses between variables were performed using a two-tailed Fisher's exact test and *t*-test. The relationship between MSI and other variables was evaluated by multiple regression analysis. Odds ratios (OR) and 95% confidence intervals (CI) were obtained through logistic regression. A value of $P < 0.05$ was considered to be statistically significant.

Results

MSI analysis

MSI-L was observed in six glioblastomas (5.5%) (Fig. 1). Nine of the 872 evaluable dinucleotides (1.03%) and one of 109 tetranucleotides (0.92%) were unstable. Instability of mononucleotide repeats was not evidenced. The most sensitive markers were D3S1619, D13S153 and D17S250, comprising 60% of the observed length mutations.

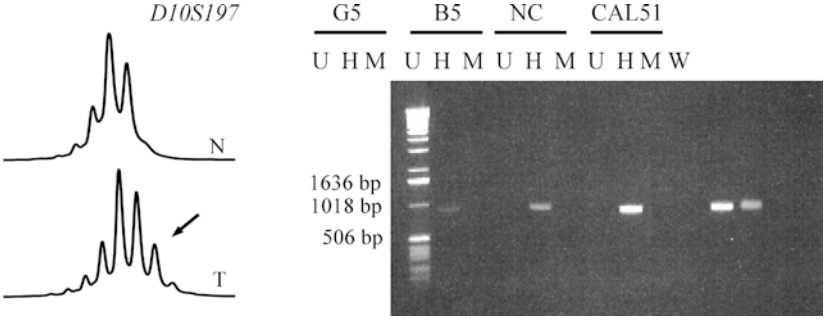

Fig. 1 *Left*: microsatellite instability at marker D10S197 (case G1). N and T mean constitutional and tumor DNA, respectively. The *arrow* indicates a novel peak observed in tumor DNA. *Right*: analysis of *hMLH1* promoter methylation of the giant-cell GBM (case G5). The *HpaII*- and *MspI* digested DNA (H and M, respectively) showed no amplification of the promoter fragment (829 bp) of *hMLH1*, indicating no methylation of the five cytosine residues within the CpG islands of the five restriction sites within the fragment. Leukocyte DNA (B5) from the patient was also analyzed. As negative control (*NC*) we used leukocyte DNA from a healthy Caucasian adult. The breast cancer cell line CAL51 was used as positive control. *U* and *W* stand for undigested DNA and water, respectively

We plotted the relative frequency of microsatellite mutation in respect to patient age at tumor resection. The MSI-L rate in patients older than 55 years was 9.5% (this cut-off level showed the lowest *P*-value), whereas in patients younger than 55 years 4.4% ($P = 0.26$). No difference was observed between MSI-L and MSS groups in regards to age ($P = 0.32$) or gender ($P = 0.23$). The incidence of MSI-L in newly diagnosed GBMs was 3.2% but 18.8% in GBM patients who previously showed an astrocytoma grade II or III ($P = 0.15$).

The relationship between MSI status and LOH pattern was evaluated as well. Overall, 108 instances of LOH were observed among 63 tumors. No significant differences between the MSI-L and MSS GBMs could be detected regarding allelic losses at chromosome regions mapping *hMLH1* (3p21, $P = 0.51$), *hPMS2* (7p22, $P = 0.54$), *hMSH6* (2p16, $P = 0.42$), and *hMSH2* (2p22, $P = 0.43$).

Immunohistochemical evaluation of MMR proteins

Five MSI-L GBMs had a positive nuclear staining for all MMR proteins. A loss of hPMS2 and a reduced staining of hMLH1 was found in a giant cell GBM (Fig. 2). Interestingly, only the giant cell phenotype showed this altered staining pattern, whereas the non-giant tumor cells had a positive pattern. There was a significant difference of the relative densitometry of hMLH1 expression (compared to respective negative controls) between giant ($n = 50$) and non-giant ($n = 50$) cells ($P = 0.0005$, *t*-test). No significant differences in the relative densitometry of MMR proteins was found in twenty MSI(-) GBMs as compared to MSI(+) GBMs ($P > 0.05$).

Sequence and promoter methylation analysis of hMLH1

Neither germline nor somatic genetic alterations were detected by sequencing *hMLH1* in the giant cell GBM with reduced hMLH1- and loss of hPMS2 expression. By the methylation analysis of the promoter of *hMLH1*, no fragment were obtained from tumor DNA after digestion with *HpaII*. These findings indicated that the 5-tested cytosine residues in the promoter region of *hMLH1* were not hypermethylated (Fig. 1).

Characterization of the molecular profile of MSI(+) GBMs

Mutations of *p53* were found only in one giant cell GBM consisting in two transitions, on exon 5 (codon 175,

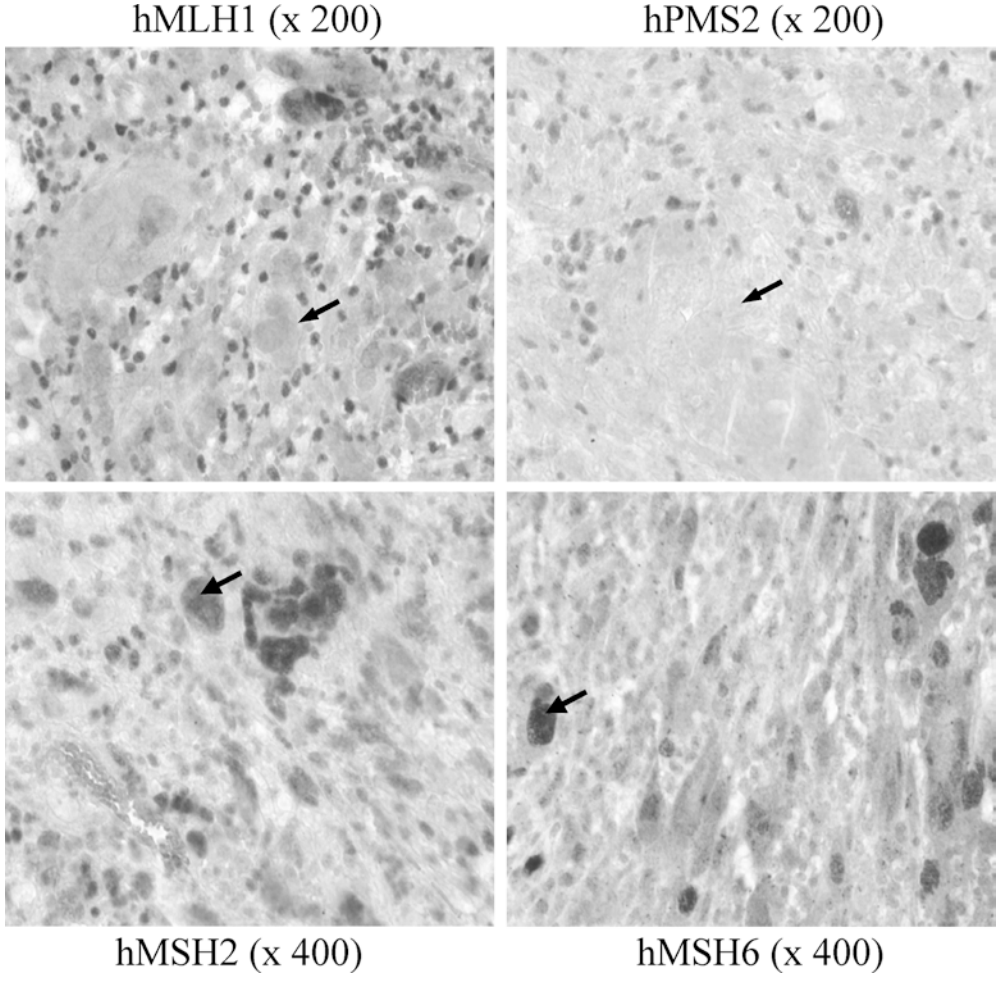

Fig. 2 Immunohistochemical studies of hMLH1 (×200), hPMS2 (×200), hMSH2 (×400), and hMSH6 (×400) of the giant-cell GBM (case G5). They show a reduced expression of hMLH1 and a loss of expression of hPMS2 in the giant tumor cells. The non-giant tumor cells show a positive expression of both gene products. Positive nuclear patterns of hMSH2 and hMSH6 are observed in both giant and non-giant glioblastoma cells. *Arrows* indicate giant cells

Table 2 Molecular findings in MSI(+) glioblastomas. EGFR/ CFTR ratios >3.91 were indicative of *EGFR* amplification. (*MSI* microsatellite instability, *LOH* loss of heterozygosity, *m* male, *f* female, *Het* allelic balance, *n.i.* not informative, *Mut.* mutation)

Case	Gender/age[a]	MSI	EGFR/CFTR	p53	PTEN	p15/p16	RB1
G1[b]	m/63	D13S153 D10S197	1.5	HetNo mutation	HetNo mutation	LOH	LOH
G2	f/73	MYCL1	1.2	HetNo mutation	HetNo mutation	Het	n.i.
G3[2]	f/56	D17S250	18.4	HetNo mutation	LOH 10q23No mutation	LOH	LOH
G4[2]	m/27	D5S346	1.9	n.i.	LOH 10q23No mutation	Het	Het
G5	f/55	D3S1300 D3S1619 D13S153 D17S250	1.3	LOH 17p13Mut. codons 175, 199	LOH 10q23No mutation	Het	Het
G6	m/78	D3S1619	1.6	LOH 17p13No mutation	HetNo mutation	Het	LOH

[a]Age (years) at surgical procedure
[b]Patients with previous lower grade astrocytoma

CGC → CAC) and exon 6 (codon 199, GGA → AGA) predicting the amino acid substitution of arginine and glycine by histidine and arginine, respectively. In this specimen, an LOH at 17p13 was observed as well. An *EGFR* amplification was detected in one GBM. *PTEN* mutations were not found in any case. However, LOH at 10q23 was evidenced in three cases. According to the results of *p53* and *EGFR* analyses, four MSI(+) GBM had a profile which did not correspond either to type 1 or to type 2 pathways, another GBM was classified as type 1, and a further tumor as type 2 GBM. Additional molecular features of the MSI(+) GBMs are shown in Table 2.

Discussion

In the pathogenesis of neoplasms, mechanisms responsible for the maintenance of the fidelity of DNA replication play a crucial role (Kinzler and Vogelstein 1997). A deficient MMR was observed to be the underlying mechanism in HNPCC-associated colon cancers and up to 40% of sporadic colon cancers. Such a deficiency leads to MSI and accumulation of somatic and/or germline alterations in target genes (Thibodeau et al. 1998; Boland et al. 1998). However, in most non-colonic sporadic cancers MSI is not caused by inactivation of MMR genes (Boland et al. 1998; Parc et al. 2000). Some reports described MSI in GBMs by either investigating small series (Dams et al. 1995; Zhu et al. 1996; Sobrido et al. 2000) or using few microsatellite markers (Alonso et al. 2001). Furthermore, different instability criteria and methods were used. We investigated MSI with 15 repeats as recommended by the National Cancer Institute (Boland et al. 1998) and observed low-level MSI in six of 109 (5.5%) GBMs. However, out of the six MSI(+) tumors, only one GBM showed a deficient MMR protein expression. This is in concordance with the observations in other MSI(+) sporadic non-colonic cancers such as gastric or endometrial neoplasms (Wu et al. 1997; Katabuchi et al. 1995). Thus, our findings suggest that MSI in the analyzed GBMs might rather be explained by inactivation of minor MMR genes (such as hMSH3 or hPMS1). This was supported by the results of our LOH studies in MSI and MSS GBMs at chromosome regions mapping the MMR genes. Alternatively, MSI might be caused by MMR genes mutations leading to a dominant negative phenotype as reported for *hPMS2* (Nicolaides et al. 1998) or an imbalance in the relative amount of MMR proteins as shown for hMSH3 and hMSH6 (Marra et al. 1998).

The correlation between the clinicopathological data and MSI status revealed that the incidence of MSI was higher in patients who previously had an astrocytoma WHO grade II or III than in those clinically de novo glioblastomas (18.8% vs 3.2%, $P=0.15$). This might indicate a trend between MSI and progression of lower grade astrocytoma to glioblastoma multiforme, although this finding should be confirmed by investigating a larger number of GBMs. A similar observation was reported in gastric cancers since MSI was observed to be more frequent in advanced than in early cancers (Strickler et al. 1994).

According to our results of *p53*, *EGFR*, and *PTEN*, MSI(+) GBMs showed wild-type *p53* and *PTEN* more frequently as well as an absence of *EGFR* amplification. Mutations of *p53* were detected only in one MSI(+) giant cell GBM, a subgroup which typically shows a higher rate of *p53* mutations than common GBMs (Meyer-Puttlitz et al. 1997). Additionally, only missense *p53* mutations were detected but not insertions/deletions at iterated bases, thus making unlikely the possibility that MSI caused *p53* alterations. A similar observation was found in MSI(+) gastric carcinomas (Renault et al. 1996). In the studied GBMs, *PTEN* mutations and MSI were mutually exclusive events.

We have shown a reduced hMLH1 expression and a loss of hPMS2 expression in a giant cell GBM. This GBM subtype is characterized by specific clinicopathological and molecular features (Kleihues et al. 2002). Typically, they show numerous multinucleated giant cells. They usually arise de novo (like type 2 GBMs) but carry genetic alterations which are different from those corresponding to type 1 and type 2 GBMs (Meyer-Puttlitz et al. 1997). In the studied specimen, the reduced hMLH1 expression was not associated with mutational

or epigenetic inactivation of *hMLH1*. Furthermore, LOH at 3p21 (hMLH1) and 7p22 (hPMS2) was not evidenced. Taking into account the histological characteristics of giant cell GBMs, the possibility that *hMLH1* mutations and/or hypermethylation of the *hMLH1* promoter only affected the giant cell phenotype cannot be excluded. Alternatively, a transcriptional silencing affecting only the giant cells might lead to this condition as well.

In summary, our data show that MSI-L is infrequent in sporadic newly diagnosed GBMs. This instability phenotype was not associated with a deficient expression of MMR proteins in most of the cases. Although MSI(+) GBM predominantly showed a molecular profile with wild-type *p53* and *PTEN* and absence of *EGFR* amplification, MSI was present in all different glioblastoma molecular subtypes.

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