

MGMT promoter methylation status in clival chordoma

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Abstract Chordomas are rare, slow-growing neoplasms, characterized by locally aggressive growth patterns and high local recurrence rates. To the best of our knowledge, the *MGMT* promoter methylation status has not been studied in a population of patients with chordomas to determine if a biologic rationale exists to support the use of temozolomide. We here show for the first time that methylation of *MGMT* promoter is present in a significant portion or recurring clival chordomas; on the contrary in clival chordomas without recurrence *MGMT* promoter was always unmethylated ($p = 0.0317$). Although these observations need to be confirmed in a larger study population, our results (1) indicate that methylation of *MGMT* promoter is present in a significant portion of recurring chordomas, and (2) prompt further investigation into the potential role of temozolomide as an adjuvant treatment of these tumors.

Keywords Chordoma · Clivus · *MGMT* · Methylation · Temozolomide

Introduction

Chordomas are rare, slow-growing neoplasms, characterized by locally aggressive growth patterns and high local recurrence rates. Chordomas are believed to arise from vestigial or ectopic notochordal remnant [1–3] and involving the axial skeleton from clivus to sacrum. In the clivus these tumors destroy bone, incorporating vital neurovascular structures, therefore a wide curative surgical excision is rarely allowed. Nevertheless surgery remains the best standard treatment [3, 4], because chordomas are typically largely resistant to conventional chemo- and radiotherapy. The endoscopic endonasal approach is a competitive alternative to transcranial approaches with minimal morbidity and high success rates of gross total resection when performed by experienced cranial base surgeons [5].

Outcome of chordomas is often poor and recurrent disease is a common event [1].

The molecular and genetic events involved in the development and progression of chordomas are not well understood and some common cancer associated genes, like *KRAS* and *BRAF*, failed to show a consistent genetic profile [6].

IDH1/2 mutations have not been observed in intracranial chordomas; on the contrary they are frequent in chondrosarcomas and may constitute a diagnostic clue for differential diagnosis [7].

DNA methylation is a tightly regulated process during normal development and it becomes deregulated during neoplastic transformation and disease development [8]. DNA methylation of cytosine at CpG islands can function as transcription repressor, which subsequently leads to the silencing of the associated genes. It is well known that promoter methylation of the *MGMT* gene represents a well

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established molecular feature in gliomas, with prognostic and predictive significance: in fact the efficaciousness of temozolomide (TMZ) is linked to the presence of methylated *MGMT*, whereas the absence of such feature would predict a poor response [9]. The assessment of *MGMT* promoter methylation status is usually achieved using methylation-specific PCR [10].

To the best of our knowledge, the *MGMT* promoter methylation status has not been studied in a population of patients with chordomas. In this paper we assessed the *MGMT* promoter methylation status in a group of chordomas patients, to determine if it may play a role and if a biologic rationale could suggest the use of TMZ for treatment of chordomas.

Materials and methods

Patients

Cases of patients operated for clival chordoma between 1997 and 2011 were retrieved from the files of the Section of Pathology of the Department of Biomedical and Neuro-motor Sciences of the University of Bologna at Bellaria Hospital. The same neurosurgeons (GF, DM) operated on all the patients using the endoscopic extended trans-sphenoidal approach.

The cases meeting the following criteria were selected: (1) the availability of enough material to allow molecular characterization, (2) total/subtotal surgical excision, (3) cases of chondroid subtype were excluded, (4) no radiation therapy before and after surgery, (5) no treatment with imatinib after surgery, and (6) the availability of follow-up information. A comprehensive written informed consent was signed for the surgical treatment that produced the tissue samples and the related diagnostic procedures. All information regarding the human material used in this study was managed using anonymous numerical codes and samples were handled in compliance with the Helsinki declaration. Data about age, sex and recurrence were collected.

Histology and immunohistochemistry

Tissue was fixed in 10 % buffered formalin and embedded in paraffin. Blocks were serially cut and underwent haematoxylin and eosin (H&E). For immunohistochemical analysis the avidin–biotin complex immunohistochemical method was followed. An epitope retrieval procedure was used. Before immunostaining, sections were steamed in citrate buffer for 5 min and cooled for 5 min. All specimens were immunostained with anti-cytokeratin (Cell Marque, clone AE1+AE3, prediluted), anti-S100 (Ventana, clone 4C4.9, prediluted) and anti-EMA (Cell

Marque, clone E29, prediluted) antisera. The Ki 67 (Ventana, clone 30-9, prediluted) labelling index was calculated as the percentage of positive cells per 10 HPF (400×; Zeiss NeoFluar 0.25 mm²). The number of mitoses was measured in each case per 10 HPF.

MGMT status assessment

Tissue blocks were selected for DNA extraction after careful examination on H&E staining of corresponding sections to rule out the presence of contaminating necrotic debris. Molecular genetic analyses were performed on samples showing an estimated tumor cell content of at least 90 % from five sections of 10 μm from paraffin embedded tissue blocks. Two incubations with xylene at 60 °C and two incubations with absolute ethanol at room temperature for 10 min each were used to eliminate paraffin. DNA was purified by High Pure PCR Template Preparation kit (Roche Applied Science, Mannheim) according to the manufacturer's instructions and quantified by Quant-iTTM dsDNA BR kit (Invitrogen, Carlsbad, California). At least 200 ng of DNA was then treated with bisulfite using the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *MGMT* methylation status was evaluated by means of the methylation sensitive—quantitative locked nucleic acid PCR (MS-qLNAPCR) protocol described by Morandi et al. [10].

In brief, Real Time PCR analysis was performed using an SDS-ABI Prism 7000 (Applied Biosystems, Foster City, CA) with 3'-locked nucleic acid (LNA) modified primers recognizing the methylated and unmethylated alleles of *MGMT* (Fig. 2b) and *SNURF* promoter as a reference for normalization. Relative quantification of the methylated and unmethylated allele ratio (M/U ratio) was calculated according to $\Delta\Delta C_t$ method [11] using an equal amount of *SssI*-treated wild type DNA (fully methylated) mixed with the same amount of untreated DNA as a calibrator [10].

Amplicons were detected by SeaKem LE agarose gel (3 %, Lonza, Basel, Switzerland) by the use of GelStar (Lonza, Basel, Switzerland) as intercalator.

IDH1 mutation analysis

Isocitrate dehydrogenase 1 (*IDH1*) analysis for the p.R132H mutation was performed on paraffin sections, using allele specific locked nucleic acid quantitative PCR (ASLNAqPCR), according to previously described protocol [12] from all cases. The forward primers used for the analysis were designed for the wild type allele (5'-TTGATCCCCATAAGCATGA[+C]-3') and for the p.R132H allele (5'-TTGATCCCCATAAGCATGA[+T]-3') (LNA bases are preceded by +); the reverse primer (5'-GTGGCACGGTCTTCAGAGA-3') allowed to amplify a

Table 1 Clinical-pathological and bio-molecular features of chordomas without recurrence

Case	Age	Sex	Mitoses	Ki67 (%)	Necrosis	IDH1	MGMT
1	47	F	<1	1	No	wt	U
2	57	M	<1	2	No	wt	U
3	30	M	<1	2	No	wt	U
4	68	M	<1	1	No	wt	U
5	73	F	<1	3	No	wt	U
6	33	F	<1	2	No	wt	U
7	73	F	<1	4	No	wt	U
8	46	F	<1	3	No	wt	U
9	41	F	1	8	No	wt	U
10	64	F	<1	4	No	wt	U
11	47	M	<1	1	No	wt	U
12	40	M	<1	2	No	wt	U
13	61	M	1	5	No	wt	U
14	42	F	<1	1	No	wt	U
15	43	M	<1	4	No	wt	U

F female, M male, IDH1 Isocitrate dehydrogenase 1, wt wild type, MGMT: O⁶-methylguanine DNA-methyltransferase, U unmethylated

segment of 104 bp long. The ASLNAqPCR IDH1 results were confirmed by sequencing using the 454 GS-Junior next generation sequencer (Roche, Branford, CT, USA).

Statistical analysis

For statistical evaluation features of the 2 groups were compared using Fisher exact test (sex), Mann–Whitney test (Ki67) and Student’s t test (age, recurrence time).

Correlation of recurrence with MGMT promoter methylation status was evaluated using χ^2 -test.

A p value less than .05 was considered statistically significant.

Results

Forty-nine patients with diagnosis of clival chordomas were operated during the 15-year considered period. Thirty cases met the inclusion criteria and were subdivided in two groups, those without recurrences (Group 1) and those with recurrences (Group 2). Clinico-pathological data are summarized in Table 1 (Group 1) and in Table 2 (Group 2). Follow-up ranged from 24 to 198 months.

Group 1

The study population contained 7 male and 8 female patients. Mean age at surgery was 51 years (range 30–73 years). None of these patients developed recurrence after a mean follow-up of 8.5 years (range 2–15 years).

On histology, the lesions were composed of mucin-rich lobules, consisting of ribbons and cords of epithelioid cells, associated with a fibrous stroma. Tumor samples were positive for cytokeratin, EMA and S100 protein. All cases were diagnosed as conventional chordoma. None case presented dedifferentiated or sarcomatous features. In all cases necrosis was absent. Mitotic figures were rare, in 13 cases less than 1 mitosis per 10 HPF was observed, in 2 cases 1 per 10 HPF. Mean Ki67 label index (LI) was 2.8 %.

Table 2 Clinical-pathological and bio-molecular features of chordomas with recurrence

No.	Age	Sex	Rec ^a	Mitoses		Ki67		Necrosis		IDH1		MGMT	
				FT	Rec	FT (%)	Rec (%)	FT	Rec	FT	Rec	FT	Rec
16	51	F	25	<1	<1	2	3	No	No	wt	wt	Met	Met
17	67	M	8	<1	<1	1	2	No	No	wt	wt	U	U
18	65	F	56	<1	<1	2	3	No	No	wt	wt	U	U
19	49	F	43	<1	<1	2	2	No	No	wt	wt	Met	Met
20	31	M	13	<1	<1	3	4	No	No	wt	wt	U	U
21	48	M	21	<1	<1	2	2	No	No	wt	wt	U	U
22	68	M	11	<1	<1	3	3	No	No	wt	wt	U	U
23	50	M	5	<1	<1	2	2	No	No	wt	wt	Met	Met
24	32	M	28	1	1	4	7	No	No	wt	wt	U	U
25	40	F	25	<1	1	3	4	No	No	wt	wt	U	U
26	42	M	19	<1	1	3	5	No	No	wt	wt	U	U
27	58	F	39	1	1	5	7	Yes	Yes	wt	wt	U	U
28	64	M	12	1	1	5	5	No	No	wt	wt	U	U
29	79	M	18	<1	<1	2	2	No	No	wt	wt	Met	Met
30	38	F	17	1	3	7	20	No	Yes	wt	wt	U	U

F female, M male, Rec recurrence, IDH1 Isocitrate dehydrogenase 1, FT first tumor, wt wild type, MGMT O⁶-methylguanine DNA-methyltransferase, Met methylated, U unmethylated
^a Months

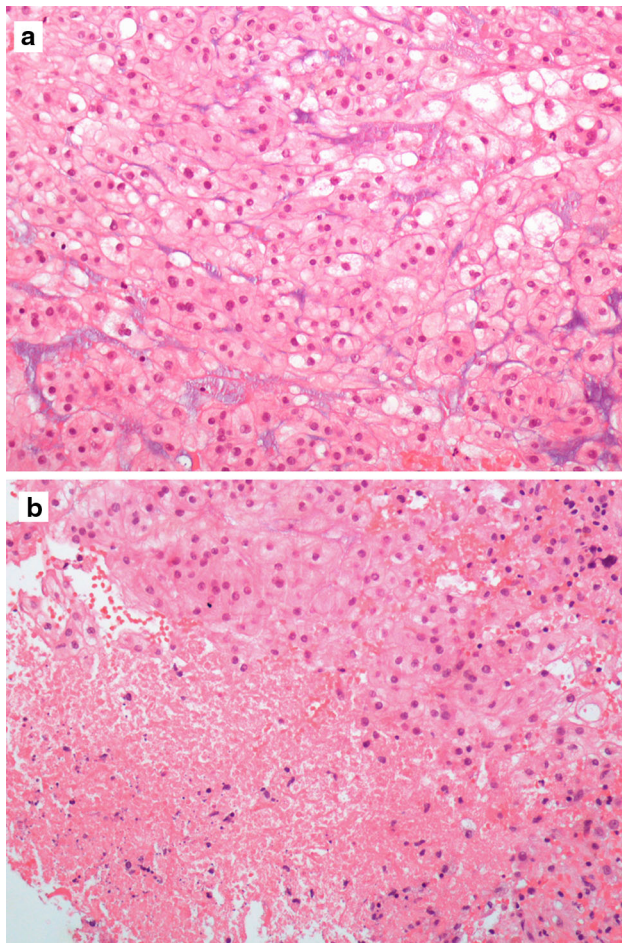


Fig. 1 **a** Case no. 16 (methylated recurring chordoma). The tumor is composed of mucin-rich lobules, consisting of ribbons and cords of epithelioid cells (H&E, $\times 200$ magnification). **b** Case no. 27 (unmethylated recurring chordoma). Neoplastic cells are similar to those of the case no. 16, but an area of necrosis is evident on the left side (H&E, $\times 200$ magnification)

In no case *MGMT* promoter was found to be methylated. All cases did not harbor *IDH1* mutations.

Group 2

The study population contained 9 male and 6 female patients. Mean age at surgery was 52.1 years (range 31–79 years). Mean follow-up was 9.1 years (range 2–16.5 years). All these patients presented tumor recurrence and thus were submitted to a second surgery. Mean recurrence time was 22.6 months (range 5–56 months).

On histology, the lesions showed the same histological and immunohistochemical findings observed in Group 1, and such features were similar in first tumors and recurrences, except for case no. 30.

All first tumors were diagnosed as conventional chordoma (Fig. 1a). In the first lesion 11 cases showed less than

1 mitosis per 10 HPF, while in 4 cases 1 mitosis per 10 HPF was present. In the recurrences 9 cases presented less than 1 mitosis per 10 HPF, 5 cases 1 mitosis per 10 HPF, while case no. 30 showed 3 mitoses per 10 HPF. Mean Ki67 LI was 3 % in first tumors, and 4.73 % in recurrences.

Case no. 27 showed foci of necrosis in both first tumor (Fig. 1b) and recurrence, while in case no. 30 necrosis appeared in recurrent specimen. In all the remaining cases necrosis was absent. Only case no. 30 showed dedifferentiated features in recurrent lesion. All cases did not harbor *IDH1* mutations.

Analysing the first tumors *MGMT* promoter was found to be methylated in 4 samples (26.7 %) (Fig. 2) and unmethylated in 11 samples (73.3 %). Also the tumor recurrences in those 4 cases were found to be methylated.

Correlations

Comparison of age, sex and Ki67 LI between Groups 1 and 2 did not evidence any statistically significant difference.

On the contrary, the association between promoter methylation of *MGMT* gene and recurring chordomas (Group 2) resulted statistically significant ($p = 0.0317$).

Inside the Group 2, methylated cases and unmethylated cases did not show significant differences in age, sex, recurrence time and Ki67 LIs of first tumor.

Comparing Ki67 LIs observed in recurrence specimens, the mean value in was 2.25 in methylated cases and 5.63 in unmethylated cases ($p = 0.0386$).

Discussion

We here show for the first time that methylation of *MGMT* promoter is present in a significant portion of recurring clival chordomas; on the contrary in clival chordomas without recurrence *MGMT* promoter was always unmethylated ($p = 0.0317$).

Malignancy of chordomas is due to local invasiveness, tendency of recurrence and potential to metastasize. They have two distinct biological behaviors: the first group consists of slow growing lesions which, in rare cases, may not even grow at all. These tumors remain locally confined rather than metastasize to other areas; the second group is composed by tumors with a more aggressive behavior, rapid local recurrence, spread to other areas of the neuro-axis or metastasis to the lung, liver or bone [13].

Clival chordomas destroy bone, incorporating vital neurovascular structures, therefore a wide curative surgical excision is rarely allowed and recurrent disease is a common event [1]. Nevertheless surgery remains the best standard treatment [3, 4], because chordomas are typically

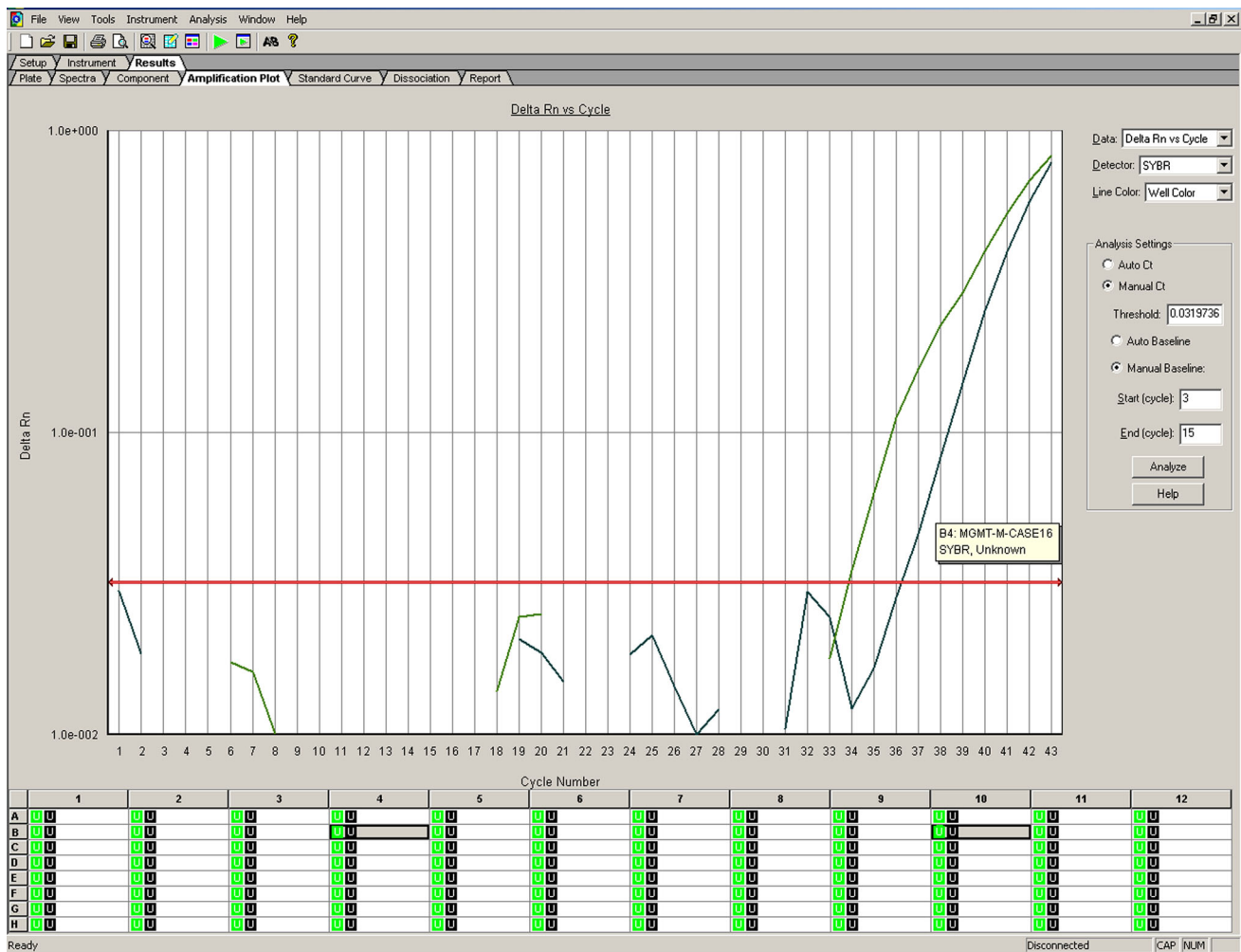


Fig. 2 Amplification plot of case 16. *MGMT* methylation status was evaluated by methylation sensitive—quantitative locked nucleic acid PCR (MS-qLNAPCR) protocol. The unmethylated allele (green curve) precedes the methylated allele (black curve) of about two *Ct*

largely resistant to conventional chemo- and radiotherapy. Therapeutic advances are therefore urgently required for improving the prognosis.

Molecular and genetic events involved in pathogenesis of chordomas have been explored in the last years. Recent studies evidenced loss of *CDKN2A* and *PTEN* expression [14] and significant changes of the DNA methylation pattern [6]. Furthermore patients harboring skull base chordomas were found to demonstrate positive expression of *PDGFRB* [15]. Inhibition of *PDGFRB* is likely to mediate antitumour activity of imatinib in chordoma [16, 17].

In the present study, as expected [7], we did not find any case with *IDH1* mutations, confirming that none case of chondrosarcoma was present in our series.

Assessment of *MGMT* promoter methylation status evidenced promoter methylation of the *MGMT* gene in 26.6 % of recurring chordomas; all observed methylated cases recurred.

The results of this study suggest a prognostic role of promoter methylation of the *MGMT* gene, as this molecular feature seems to label cases prone to show a more aggressive behaviour and could help clinicians in predicting the postoperative outcome and planning a better follow-up schedule.

Furthermore it is well known that promoter methylation of the *MGMT* gene may predict the efficaciousness of TMZ (9). Chordomas with an aggressive behaviour, which are a higher priority for chemotherapy, could benefit from TMZ, as a biologic rationale exists to support the use of such treatment.

In conclusion, although these observations need to be confirmed in a larger study population, our results (1) indicate that methylation of *MGMT* promoter is present in a significant portion of recurring chordomas, and (2) findings prompt further investigation into the potential role of TMZ as an adjuvant treatment of these tumors.

Conflict of interest The authors declare that they have no conflict of interest.

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