The O⁶-methylguanine-DNA methyltransferase (MGMT) promoter methylation status and clinical outcomes of Ewing sarcoma patients treated with irinotecan and temozolomide

Samer Salah¹, Walid Naser², Omar Jaber³, Yacob Saleh¹, Rawan Mustafa¹, Ramiz Abuhijlih⁴, Fawzi Abuhijla⁵, Taleb Ismaeel³, Sameer Yaser¹, Yiad Sultan³, Nour Mustafa⁶, Abdelghani Tbakhi²

¹Department of Medical Oncology, King Hussein Cancer Center, Amman, Jordan
²Department of Cell Therapy and Applied Genomics, King Hussein Cancer Center, Amman, Jordan
³Department of Pathology, King Hussein Cancer Center, Amman, Jordan
⁴Department of Radiation Oncology, King Hussein Cancer Center, Amman, Jordan
⁵Department of Pediatrics, King Hussein Cancer Center, Amman, Jordan
⁶Department of Pharmacy, King Hussein Cancer Center, Amman, Jordan

ABSTRACT

Background: There remains an unmet need to identify molecular biomarkers in Ewing sarcoma (ES). We sought to assess the influence of the O⁶-methylguanine-DNA methyltransferase (MGMT) promoter methylation on response and progression-free survival (PFS) following initiation of irinotecan and temozolomide (IT), PFS following initiation of vincristine, doxorubicin, and cyclophosphamide alternating with ifosfamide and etoposide (VDC-Ie), and overall survival (OS).

Materials and methods: Data of advanced ES patients, treated with IT were retrospectively collected. Patients were required to have progression after prior VDC-Ie. MGMT promoter methylation was assessed on non-decalcified Formalin-fixed paraffin embedded (FFPE) tissue using methylation sensitive restriction enzyme-quantitative PCR (MSRE-qPCR). Survival was estimated by the Kaplan-Meier method.

Results: A total of 20 ES patients underwent MGMT promoter methylation testing, and were eligible for analysis. Five patients (25%) had methylated MGMT, whereas the remaining (75%) had unmethylated promoter. Five (25%) had objective response to IT, with no observed difference by promoter methylation (p = 0.76). Median PFS from initiation of IT for methylated vs. unmethylated MGMT patients was 4.9 and 1.2 months, respectively, p = 0.69. Median PFS from date of initiation of VDC-Ie was significantly superior in the methylated group; 27.8 vs. 8.6 months, p = 0.034. Median OS was superior but not statistically significant in the methylated group.

Conclusion: MGMT promoter methylation did not correlate with clinical activity or outcomes following the IT regimen for advanced ES. However, methylated MGMT predicted significantly superior PFS following initiation of the standard VDC-Ie protocol.

Key words: Ewing sarcoma; biomarkers; MGMT methylation; prognosis; survival

Rep Pract Oncol Radiother 2022;27(5):759–767
Introduction

Ewing sarcoma (ES) is a rare malignant bone neoplasm, and is the second most common bone malignancy in children and adolescents [1]. This neoplasm is characterized by a balanced translocation involving the \( EWSR1 \) gene on chromosome 22 with one of the members of the ETS family of transcription factors [2]. Localized ES is treated with a multimodal approach that integrates chemotherapy and local control with surgery or radiotherapy [1, 3, 4]. Recent data has shown superiority of VDC-IE over VIDE as the primary chemotherapy regimen and is likely to become the most practiced regimen worldwide [5].

Optimal regimen at relapse remains undefined. Final analysis of the rEECur randomized trial that compares 4 regimens in relapsed ES is awaited [6]. In the first interim analysis of rEECur, gemcitabine and docetaxel were dropped off the 4 arms due to inferior outcomes [7]. Following the second interim analysis, the irinotecan and temozolomide (IT) regimen was dropped off randomization due to inferior overall survival (OS); randomization remains ongoing between cyclophosphamide plus topotecan and high-dose ifosfamide [6]. Nevertheless, the IT remains a well-tolerated regimen that is associated with clinical activity for many patients. Overall response rate (ORR) of 25–63% has been reported [8–11]. There remains an unmet need to identify subgroups that could benefit most from the IT or other regimens. Molecular biomarkers might be the key to guide future therapy selection.

The \( O^\prime \)-methylguanine-DNA methyltransferase (\( MGMT \)) gene codes for a repair enzyme that combats the genetic damage induced by alkylating agents including temozolomide [12–14]. Methylation of \( MGMT \) promoter causes gene silencing, making tumor cells more susceptible to the effect of alkylating agents [12–14]. Methylation status correlates with clinical outcomes of temozolomide-treated glioblastoma multiforme (GBM) patients [12, 14]. Other data show that the \( MGMT \) status correlates with response to alkylating agents in some other neoplasms [15]. Nevertheless, it is not clear if \( MGMT \) methylation status is predictive of outcomes following temozolomide-based regimens in relapsed ES. Recent data did not show association between \( MGMT \) expression and clinical outcomes of patients treated with IT [16]. Noteworthy, \( MGMT \) methylation in that study was assessed by immunohistochemistry expression and did not involve molecular testing. In the current project we sought to assess the \( MGMT \) promoter methylation status utilizing Methylation Sensitive Restriction Enzyme-Quantitative PCR (MSRE-qPCR). In addition, we sought to assess if the methylation status is predictive of response and progression free survival (PFS) of patients with relapsed ES following the salvage IT regimen, PFS from time of initiation of the primary VDC-IE regimen, and OS from time of diagnosis.

Materials and methods

Patients

Included patients were required to have a pathologically confirmed diagnosis of ES. Patients should have received the IT regimen (second-line or beyond) after progression following prior VDC-IE chemotherapy. To be eligible, patients were required to have FFPE non-decalcified tissue blocks that are sufficient (≥ 40% tumor abundance) for \( MGMT \) promoter methylation testing. IT chemotherapy was given in one of two protocols:

- Protocol #1: irinotecan 40 mg/m\(^2\) D1–D5 and temozolomide 100 mg/m\(^2\) D1–D5; cycles repeated every 21 days;
- Protocol #2: irinotecan 20 mg/m\(^2\) D1–D5 and D8–D12 and temozolomide 100 mg/m\(^2\) D1–D5; cycles repeated every 21 days.

IT chemotherapy was delivered with a planned number of 6 cycles, or until disease progression (PD) or intolerable toxicity; whichever comes first.

Radiologic responses to IT were assessed by response evaluation criteria in solid tumors (RECIST v. 1.1) by an experienced radiologist. Progression free survival (PFS) following IT was defined as the time from initiation of the first cycle of IT chemotherapy until the first radiologic evidence of PD or death. We defined PFS following initiation of the VDC-IE as the time from initiation of the first cycles of the VDC-IE protocol until the first evidence of PD or death. OS was counted from time of diagnosis until the date of last follow up or death. This study was initiated following acquisition of institutional review board approval at the King Hussein Cancer Center.
**MGMT methylation testing**

Genomic DNA (gDNA) Extraction: tumor gDNA was isolated from 5 unstained 5 μm-thick precut non-decalcified FFPE tumor sections ideally with at least 40% tumor abundance using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer’s instructions. Similarly, gDNA was extracted from normal tissue to define the technical methylation cutoff in our cohort. Upon extraction, gDNA was quantified using the Qubit 3 Fluorometer (Invitrogen, OR, USA) according to instructions and then diluted to 4 ng/µL with low-EDTA TE buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0).

Primer Design: We used Primer3 tool to design a pair of primers targeting the clinically relevant CpG-rich methylation-specific site (MSP) –476 to –368 bp upstream from the transcription start site (TSS) of the MGMT gene [17, 18]. We tested the specificity of the designed primers in silico using the UCSC In-Silico PCR tool and hg19 genome assembly. The designed primers specifically generated the anticipated 155 bp MGMT promotor target [19]. Primer sequences are available upon request.

Methylation Sensitive Restriction Enzyme-Quantitative PCR (MSRE-qPCR): is a semi-quantitative method for methylation profiling [20]. The One-Step qMethyl™ Kit (Zymo Research Corp., CA, USA) was implemented to assess the MGMT promoter methylation status. Five microliters of the prepared gDNA (4 ng/µL) were used in the kit according to instructions. Enzymatic digestion, PCR, and detection steps were conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., CA, USA). Briefly, the OneStep qMethyl™ Kit detects methylation via discriminatory amplification of methylated and unmethylated CpG-rich targets. The gDNA sample was split into two duplicated reaction sets: a “test reaction” set and a “reference reaction” set. The test reaction set was digested using a cocktail of methylation sensitive restriction enzymes (MSRE’s) that cut at specific unmethylated restriction loci, while the reference set was not exposed to MSRE digestion. In contrast, methylated cytocines were protected from MSRE digestion leaving the gDNA target intact. Both reaction sets were then PCR amplified and fluorescence was detected in the presence of SYTO® 9 fluorescent dye to determine cycle thresholds (Ct’s). The test and reference reaction sets would have different Ct’s influenced by the methylation status, with unmethylated DNA samples having considerable Ct differences. After generating Ct values for both test and reference sets, the methylation percentage for each sample was calculated using the equation: 100 \times 2^{-\Delta Ct} where ΔCt is the test average minus the reference average of duplicate Ct’s.

**MGMT promoter methylation status was classified into two subgroups: unmethylated 0-39%, and methylated ≥ 40%. This was defined through comparison of the methylation status in neoplastic to normal tissue. In addition, the impact on patient outcome was evaluated using different methylation cutoffs: 30% and 40% [21]. A statistically significant clinical impact was only observed when implementing the 40% MGMT promoter methylation as cut-off.**

**Statistical analysis**

Descriptive statistics were utilized to describe the study sample utilizing means, medians, and standard deviations. The chi-square test was utilized to compare the proportion of responders by MGMT methylation status (methylated vs. unmethylated). The Kaplan-Meier method was used to estimate PFS, and OS. We planned to compare PFS from time of initiation of the IT protocol between the groups of methylated and unmethylated MGMT. In addition, we planned to compare PFS from time of initiation of the VDC-IE chemotherapy protocol and OS from time of diagnosis between the two groups. Survival comparisons were carried out by the log-rank test. All statistical analyses were performed by the SPSS software, version 17 (SPSS Inc., Chicago, IL).

**Results**

**Patients characteristics**

A total of the 21 ES patients underwent methylation testing. One patient had an invalid MGMT result and insufficient remaining tissue for retesting and was excluded. Thus, a total of 20 patients remained eligible for analysis. Patients were of a median age of 18 years (range: 5-34 years). All patients had documented unresectable PD after standard VDC-IE chemotherapy. Patients received palliative IT chemotherapy in a second (n = 15) or third-line setting (n = 5). Clinical characteristics of eligible patients were summarized (Tab. 1). Five patients (25%) had methylated MGMT promoters,
whereas the remaining ones had unmethylated (15; 75%) promoters.

**IT chemotherapy**

Sixteen patients (80%) received protocol #1 and 4 (20%) received protocol #2. A total of 70 cycles of IT were delivered; median = 3.5, standard deviation (SD) = 2.1 (range: 1–6 cycles).

Thirteen patients (65%) had hematologic toxicities following any of the IT cycles. Nine (45%) had grade ≥ 3 hematologic toxicities. Nevertheless, only one patient (5%) had febrile neutropenia, although none of the patients had received primary growth factors prophylaxis. Five patients (25%) had diarrhea of any grade as an adverse event.

**Response to IT chemotherapy by MGMT methylation status**

Five patients (25%) had partial response as the best observed response, 9 (45%) had primary progressive disease (PD), and 6 (30%) had stable disease (SD). IT was discontinued due to progression in 11 patients (55%), both toxicity and progression in 1 patients (5%), because patients completed the planned number of cycles in 7 patients (35%), and treatment of one patient (5%) was still ongoing at time of this analysis.

Partial response was observed in one patient (20%) with a methylated MGMT promoter compared to 4 (27%) with unmethylated MGMT; p = 0.76. Primary progression on IT chemotherapy occurred in one patient (20 %) with a methylated MGMT promoter compared to 8 (53 %) of patients in the unmethylated group; p = 0.18 (Tab. 2).

**Survival outcomes following IT**

The median PFS following IT chemotherapy was 2.2 months. PFS by MGMT promoter methylation status was 4.9 months for the methylated group and 1.2 months for the unmethylated group;
Table 2. Response to irinotecan and temozolomide (IT) by MGMT promoter methylation

|                  | Partial response | No response | p-value |
|------------------|------------------|-------------|---------|
| Methylated       | 1 (20%)          | 4 (80%)     | 0.76    |
| Unmethylated     | 4 (27%)          | 11 (73%)    |         |

|                  | Disease progression | No progression | p-value |
|------------------|---------------------|----------------|---------|
| Methylated       | 1 (20%)             | 4 (80%)        | 0.18    |
| Unmethylated     | 8 (53%)             | 7 (47%)        |         |

Survival outcomes following VDC-IE by MGMT methylation status

Patients had a median time to progression of 10.8 months from starting prior VDC-IE chemotherapy. MGMT promoter methylation status sig-

\[ p = 0.69 \] (Fig. 1). In a sub analysis for patients who received IT in a second-line setting, PFS was 4.9 vs. 2.9 months for the methylated and unmethylated group, respectively, \( p = 0.81 \). The median OS following initiation of IT was 21.1 months for the methylated vs. 7.4 months for unmethylated group; \( p = 0.32 \). In an exploratory analysis to examine for any correlation between absolute methylation values for each patient with PFS outcome after IT chemotherapy, we did not observe a statistically significant correlation (Fig. 2).
nificantly correlated with PFS after initiation of the VDC-IE protocol; patients with methylated MGMT promoters had significantly superior PFS compared to patients with unmethylated MGMT promoters; 27.8 and 8.6 months, respectively; p = 0.034 (Fig. 3).

The median OS from time of diagnosis was 49.3 months for the entire cohort. Median OS for patients with methylated MGMT was 67.3 months and for those with unmethylated MGMT was 35 months, respectively; p = 0.27.

Discussion

According to our results, MGMT promoter methylation did not significantly correlate with response rate nor PFS with the IT regimen. Nevertheless, we observed a significantly superior PFS following initiation of the VDC-IE protocol in patients with a methylated compared to unmethylated MGMT promoter. The magnitude of improvement of PFS following initiation of the primary VDC-IE protocol in the methylated group was both statistically significant and clinically meaningful.

There was a reasonable rational for assessing oncologic outcomes following IT chemotherapy in this study. Firstly, methylation of the MGMT promoter causes gene silencing, thus making tumor cells more susceptible to the effect of alkylating agents [12–14]. Secondly, many studies and meta-analysis established MGMT promoter methylation as a prognostic biomarker in patients with glioblastoma multiforme treated with temozolomide [12, 14, 22]. Of note, some studies showed no association between MGMT promoter methylation and OS in GBM patients, likely due to small sample size or differences in methodology of MGMT testing [23]. The impact of the methylation status on outcomes has been shown to vary for other solid tumors [15, 24, 25].

Palmerini E, et al. [16] assessed efficacy of the IT regimen in 59 patients with advanced ES. Eight high-risk patients received the IT regimen upfront and 51 patients after relapse. Responses were observed in 50% of patients who received IT upfront and in 31% who received it at relapse. MGMT status was assessed in 30 patients, and did not correlate with outcomes. Important differences from our study include methodology of MGMT testing, study populations, and outcomes assessed. In the study reported by Palmerini, MGMT expression was assessed by immunohistochemistry. This expression did not significantly correlate with outcomes following the IT regimen. Further, survival times from the initiation of the primary chemotherapy regimen were not compared by MGMT status [16].

In regard to the method of MGMT assessment, Sahara et al. assessed the diagnostic accuracy of immunohistochemistry (IHC) in detecting the methylation status [26]. MGMT methylation status was investigated using the IHC and PCR techniques. Diagnostic value of IHC was analyzed, with PCR considered as the gold standard reference method. In their study, IHC detected MGMT methylation with sensitivity of 86.2%, specificity of 63.0%, positive predictive value of 59.5%, negative predictive value of 87.9% and accuracy of 72.0%. The researchers concluded that IHC examination can be used to detect the MGMT methylation status of glioma patients in limited resources setting, where the PCR technique is not available. Wang et al. reported a low concordance rate between IHC and methylation-specific PCR of 30.8%. Although sensitivity of the IHC in detecting the MGMT status was 84.4%, the specificity was just 45.7% [27]. Similarly, Rodriguez et al. reported that there is no significant correlation between MGMT expression and methylation as detected by methylation-specific PCR in human glioblastoma [28].

In our study, we utilized MSRE-qPCR on non-decalcified FFPE tumor blocks to ensure ac-

Figure 3. Kaplan-Meier progression free survival estimation after starting vincristine, doxorubicin, and cyclophosphamide alternating with ifosfamide and etoposide (VDC-IE) protocol by MGMT methylation status
accurate assessment of methylation. Interestingly, we identified differential outcomes by \textit{MGMT} methylation status from time of initiation of the standard primary chemotherapy regimen (VDC-IE protocol), where PFS difference in favor of the methylated group was both clinically and statistically significant. In contrary, the methylation status was not predictive of outcomes following the IT regimen in our study.

In the last decade, therapeutic options for relapsed ES have expanded [25]. Many active and tolerable regimens are available including IT, cyclophosphamide and topotecan, etoposide with carboplatin or cisplatin, ifosfamide, and gemcitabine and docetaxel [8–11, 30–34]. There is also a growing body of evidence suggesting activity of small molecules tyrosine kinase inhibitors (VEGF-TKI), such as regorafenib, pazopanib, and caboctanzinitib [29, 35]. Many other phase 2 studies assessing VEGF-TKI, such as regorafenib in ES, are currently ongoing (e.g. NCT02389244). The treatment paradigm for progressive ES is likely to evolve dramatically following announcement of the final results of the rEECur and many other ongoing clinical trials. Nevertheless, with expansion of therapeutic options, more studies should focus on assessing molecular biomarkers and their potential utility to inform the design of future personalized therapeutic trails.

We acknowledge limitations for our study. Having patients with advanced disease treated with the IT regimen was a key eligibility criterion. As such, any possible prognostic value of \textit{MGMT} methylation following primary therapy may not be representative for the entire population presenting with localized disease. In fact, those with the best outcomes (who did not have relapse) were already excluded by our study design. In addition, the small sample size is an important limitation. Multicenter studies to recruit a large number of ES patients may be required to confirm our results.

Another important limitation is the differences among the two utilized IT protocols in regard to chemotherapy dosing, schedule and differences in line of therapy, which might be the reasons why we failed to observe a significant survival difference from time of initiation of IT protocol by methylation status. Finally, defining the appropriate \textit{MGMT} methylation cutoff is another limitation that we acknowledge. In our study, we utilized a cutoff of \textit{MGMT} methylation similar to what have been utilized in GBM. However, there is no data that identified a standard cutoff point for non GBM patients. For instance, a study in triple negative breast cancer has utilized a cutoff of >10% to define methylated \textit{MGMT} [36], which is lower than the cutoff point utilized in GBM and in our study.

\section*{Conclusion}

In this study, \textit{MGMT}-promoter methylation did not correlate with clinical activity or outcomes following the IT regimen for patients with advanced ES. However, the methylated \textit{MGMT}-promoter status predicted significantly superior PFS following initiation of the primary VDC-IE chemotherapy protocol.

\section*{Conflict of interest}

None to declare.

\section*{Funding}

This project received funding by the King Hussein Cancer Center intramural grants program.

\section*{Acknowledgment}

We are grateful to the King Hussein Cancer Center for support of this work by providing funding through the intramural grants program.

\section*{References}

1. Ozaki T. Diagnosis and treatment of Ewing sarcoma of the bone: a review article. J Orthop Sci. 2015; 20(2): 250–263, doi: 10.1007/s00776-014-0687-z, indexed in PubMed: 25691401.

2. Delattre O, Zucman J, Plougastel B, et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature. 1992; 359(6391): 162–165, doi: 10.1038/359162a0, indexed in PubMed: 1522903.

3. Salah S, Halalsheh H, Abuhijla F, et al. The impact of local control timing in Ewing sarcoma. Rep Pract Oncol Radiother. 2020; 25(2): 255–259, doi: 10.1016/j.rpor.2020.02.001, indexed in PubMed: 32140082.

4. Nakao T, Fukushima H, Fukushima T, et al. Interinstitutional patient transfers between rapid chemotherapy cycles were feasible to utilize proton beam therapy for pediatric Ewing sarcoma family of tumors. Rep Pract Oncol Radiother. 2018; 23(5): 442–450, doi: 10.1016/j.rpor.2018.08.006, indexed in PubMed: 30197580.

5. Brennen B, Kirton L, Marec-Beard P, et al. Comparison of two chemotherapy regimens in Ewing sarcoma (ES): Overall and subgroup results of the Euro Ewing 2012 ran-
domized trial (EE2012). J Clin Oncol. 2020; 38(15_suppl): 11500–11500, doi: 10.1200/jco.2020.38.15_suppl.11500.

6. McCabe M, Kirton L, Khan M, et al. Results of the second interim assessment of rECoR, an international random-ized controlled trial of chemotherapy for the treatment of recurrent and primary refractory Ewing sarcoma (RR-ES). J Clin Oncol. 2020; 38(15_suppl): 11502–11502, doi: 10.1200/jco.2020.38.15_suppl.11502.

7. McCabe M, Moroz V, Khan M, et al. Results of the first interim assessment of rECoR, an international random-ized controlled trial of chemotherapy for the treatment of recurrent and primary refractory Ewing sarcoma. J Clin Oncol. 2019; 37(15_suppl): 11007–11007, doi: 10.1200/jco.2019.37.15_suppl.11007.

8. Palmerini E, Jones RL, Setola E, et al. Irinotecan and te-mozolomide in recurrent Ewing sarcoma: an analysis in 51 adult and pediatric patients. Acta Oncol. 2018; 57(7): 958–964, doi: 10.1080/0284186X.2018.1449250, indexed in Pubmed: 29533113.

9. Salah S, To YH, Khourouz O, et al. Irinotecan and temozolomide chemotherapy in paediatric and adult populations with relapsed Ewing Sarcoma. Clin Transl Oncol. 2021; 23(4): 757–763, doi: 10.1007/s12094-020-02466-9, indexed in Pubmed: 32761317.

10. Wagner LM, McAllister N, Goldsby RE, et al. Temozolomide and intravenous irinotecan for treatment of advanced Ewing sarcoma. Pediatr Blood Cancer. 2007; 48(2): 132–139, doi: 10.1002/pbc.20697, indexed in Pubmed: 16317751.

11. Casey DA, Wexler LH, Merchant MS, et al. Irinotecan and temozolomide for Ewing sarcoma: the Memorial Sloan-Kettering experience. Pediatr Blood Cancer. 2009; 53(6): 1029–1034, doi: 10.1002/pbc.22206, indexed in Pubmed: 19637327.

12. Esteller M, Garcia-Foncillas J, Andion E, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med. 2000; 343(19): 1350–1354, doi: 10.1056/NEJM200011093431901, indexed in Pubmed: 11070098.

13. Hansen RJ, Nagasubramanian R, Delaney SM, et al. Role of O6-methylguanine-DNA methyltransferase in protecting from alkylating agent-induced toxicity and mutations in mice. Carcinogenesis. 2007; 28(5): 1111–1116, doi: 10.1093/carcin/bgl218, indexed in Pubmed: 17116724.

14. Bobola MS, Alnoor M, Chen JYS, et al. O-methylgua-nine-DNA methyltransferase activity is associated with response to alkylating agent therapy and with promoter methylation in glioblastoma and anaplastic glioma. BBA Clin. 2015; 3: 1–10, doi: 10.1016/j.bbac.2014.11.003, indexed in Pubmed: 25558448.

15. Qi Z, Tan H. Association between MGMT status and re-sponse to alkylating agents in patients with neuroendo-crine neoplasms: a systematic review and meta-analysis. Biosci Rep. 2020; 40(3), doi: 10.1042/BSR20191247, indexed in Pubmed: 32141507.

16. Palmerini E, Pasello M, Jones R, et al. Irinotecan and temozolomide upfront and in relapsed Ewing sarcoma: A translational study on MGMT (O6-methylguanine–DNA methyltransferase) and ABCG2 (MGMT-Liberati). J Clin Oncol. 2020; 38(15_suppl): e23564–e23564, doi: 10.1200/jco.2020.38.15_suppl.e23564.

17. Ye J, Couloures G, Zaretskaya Y, et al. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 2012; 13: 134, doi: 10.1186/1471-2105-13-134, indexed in Pubmed: 22708584.

18. van Nijefried KA, van den Berg J, van der Meide WF, et al. Absence of the MGMT protein as well as methylation of the MGMT promoter predict the sensitivity for temozolomide. Br J Cancer. 2010; 103(1): 29–35, doi: 10.1038/sj.bjc.6605712, indexed in Pubmed: 20517307.

19. Kent WJ, Sugnet CW, Furey TS, et al. The human genome browser at UCSC. Genome Res. 2002; 12(6): 996–1006, doi: 10.1101/gr.229102, indexed in Pubmed: 12045153.

20. Hashimoto Ko, Kokubun S, Itoi E, et al. Improved quantifi-cation of DNA methylation using methylation-sensitive restriction enzymes and real-time PCR. Epigeneics. 2007; 2(2): 86–91, doi: 10.4161/epi.2.2.4203, indexed in Pubmed: 17965602.

21. Brigliadori G, Foca F, Dall’Agata M, et al. Defining the cutoff value of MGMT gene promoter methylation and its predic-tive capacity in glioblastoma. J Neurooncol. 2016; 128(2): 333–339, doi: 10.1007/s11060-016-2116-y, indexed in Pubmed: 27029617.

22. Binabaj MM, Bahrami A, ShahidSales S, et al. The prog-nostic value of MGMT promoter methylation in glioblastoma: A meta-analysis of clinical trials. J Cell Physiol. 2018; 233(1): 378–386, doi: 10.1002/jcp.25896, indexed in Pubmed: 28266716.

23. Jovanović N, Mitrović T, Cvetković VJ, et al. The Impact of Promoter Methylation and Temozolomide Treatment in Serbian Patients with Primary Glioblastoma. Medicina (Kaunas). 2019; 55(2), doi: 10.3390/medicina55020034, indexed in Pubmed: 30717206.

24. Chen C, Hua H, Han C, et al. Prognosis value of MGMT promoter methylation for patients with lung cancer: a meta-analysis. Int J Clin Exp Pathol. 2015; 8(9): 11560–11564, indexed in Pubmed: 26617891.

25. Li Y, Lüy Z, Zhao L, et al. Prognostic value of MGMT methylation in colorectal cancer: a meta-analysis and literature review. Tumour Biol. 2015; 36(3): 1595–1601, doi: 10.1007/s13277-014-2752-9, indexed in Pubmed: 25596081.

26. Sahara N, Hartanto RA, Yoshuautani N, et al. Diagnostic Accuracy of Immunohistochemistry in Detecting MGMT Methylation Status in Patients with Glioma. Asian Pac J Cancer Prev. 2021; 22(12): 3803–3808, doi: 10.31557/APJC.2021.22.12.3803, indexed in Pubmed: 34967558.

27. Wang L, Li Z, Liu C, et al. Comparative assessment of three methods to analyze MGMT methylation status in a series of 350 gliomas and gangliogliomas. Pathol Res Pract. 2017; 213(12): 1489–1493, doi: 10.1016/j.prp.2017.10.007, indexed in Pubmed: 29103769.

28. Rodriguez FJ, Thibodeau SN, Jenkins RB, et al. MGMT immunohistochemical expression and promoter methylation in human glioblastoma. Appl Immunohistochem Mol Morphol. 2008; 16(1): 59–65, doi: 10.1097/PAL.0b013e-31802fac2f, indexed in Pubmed: 18091318.

29. Van Mader D, Wagner L. Management of recurrent Ewing sarcoma: challenges and approaches. Onco Targets Ther. 2019; 12: 2279–2288, doi: 10.2147/OTT.S170585, indexed in Pubmed: 30988632.

30. Hunold A, Weddeling N, Paulussen M, et al. Topotecan and cyclophosphamide in patients with refractory or
relapsed Ewing tumors. Pediatr Blood Cancer. 2006; 47(6): 795–800, doi: 10.1002/pbc.20719, indexed in Pubmed: 16411206.

31. Farhat R, Raad R, Khoury NJ, et al. Cyclophosphamide and topotecan as first-line salvage therapy in patients with relapsed ewing sarcoma at a single institution. J Pediatr Hematol Oncol. 2013; 35(5): 356–360, doi: 10.1097/MPH.0b013e318270a343, indexed in Pubmed: 23042020.

32. van Maldegem AM, Benson C, Rutkowski P, et al. Etoposide and carbo-or cisplatin combination therapy in refractory or relapsed Ewing sarcoma: a large retrospective study. Pediatr Blood Cancer. 2015; 62(1): 40–44, doi: 10.1002/pbc.25230, indexed in Pubmed: 25251256.

33. Ferrari S, del Prever AB, Palmerini E, et al. Response to high-dose ifosfamide in patients with advanced/recurrent Ewing sarcoma. Pediatr Blood Cancer. 2009; 52(5): 581–584, doi: 10.1002/pbc.21917, indexed in Pubmed: 19142994.

34. Mora J, Cruz CO, Parareda A, et al. Treatment of relapsed/refractory pediatric sarcomas with gemcitabine and docetaxel. J Pediatr Hematol Oncol. 2009; 31(10): 723–729, doi: 10.1097/MPH.0b013e3181b2598c, indexed in Pubmed: 19727011.

35. Italiano A, Mir O, Mathoulin-Pelissier S, et al. Cabozantinib in patients with advanced Ewing sarcoma or osteosarcoma (CABONE): a multicentre, single-arm, phase 2 trial. Lancet Oncol. 2020; 21(3): 446–455, doi: 10.1016/s1470-2045(19)30825-3, indexed in Pubmed: 32078813.

36. Jank P, Gehlhaar C, Lederer B, et al. MGMT promoter methylation in triple negative breast cancer of the GeparSixto trial. PLoS One. 2020; 15(8): e0238021, doi: 10.1371/journal.pone.0238021, indexed in Pubmed: 32841306.