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ORIGINAL ARTICLE

Clinical and in vitro studies of the correlation between MGMT and the effect of streptozocin in pancreatic NET

Susumu Hijioka[1,2] · Keiichiro Sakuma[3] · Masahiro Aoki[3,4] · Nobumasa Mizuno[1] · Takamichi Kuwahara[1] · Nozomi Okuno[1] · Kazuo Hara[1] · Yasushi Yatabe[5]

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Abstract

Purpose This study aimed to determine the correlation between DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) status and the response to streptozocin in advanced well-differentiated pancreatic neuroendocrine tumors (WD panNETs).

Methods To test the hypothesis that MGMT deficiency was required for an alkylating drug response, we retrospectively reviewed the response of 13 patients with WD panNETs to alkylating agents in relation to MGMT status. We also studied MGMT expression in streptozocin resistance using panNET cell lines.

Results The cohort included 54% of patients with and 46% without MGMT expression. Among these, 83.3% (5/6) of MGMT-negative cases showed a partial response to streptozocin. In contrast, only 14.2% (1/7) of MGMT-positive cases showed a partial response ($P=0.013$). Induced expression of MGMT in BON1 cells (a panNET cell line with undetectable endogenous MGMT) produced streptozocin resistance. Knockdown of MGMT in QGP1 cells, which express MGMT endogenously, did not alter the response to streptozocin.

Conclusions We observed a relationship between MGMT status and streptozocin response in both patients and cell culture. Despite limited cases examined, high concordance of negative expression of MGMT and response to streptozocin treatment suggest that MGMT expression can be a potential biomarker for this treatment.

Keywords MGMT · Streptozocin · Pancreatic neuroendocrine tumors · Methylation · BON1

Introduction

Neuroendocrine tumors (NETs) are uncommon malignancies but are becoming increasingly more recognized [1]. The management of patients with pancreatic NETs (panNETs) is not uniform due to the wide spectrum of clinical presentations stemming from morphological and genetic heterogeneity [2]. PanNETs are generally treated with surgical resection with a curative intent if localized. Treatment options in patients with metastatic panNET generally involve chemotherapy. Recently, several agents (somatostatin analogue, targeted therapies, cytotoxic chemotherapy) have become available for chemotherapy [3–6], and cytotoxic chemotherapy with alkylating agents is often used, particularly for high tumor burden and/or highly aggressive cases [7, 8].

However, no studies have evaluated the optimal way to use these drugs, and no promising predictive biomarkers for medical treatment have been established for well-differentiated (WD) panNETs. The alkylating agents

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✉ Susumu Hijioka
shijioka@ncc.go.jp

1 Department of Gastroenterology, Aichi Cancer Center Hospital, Nagoya, Japan

2 Department of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital, Tokyo, Japan

3 Division of Pathophysiology, Aichi Cancer Center Research Institute, Nagoya, Japan

4 Department of Cancer Genetics, Program in Function Construction Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan

5 Department of Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital, Nagoya, Japan

streptozocin (STZ), temozolomide (TMZ), and dacarbazine (DTIC) are used to treat advanced WD panNETs. Recent STZ-based chemotherapy studies have achieved objective response rates (RR) ranging from 28 to 43% [9–12]. More recently, the oral alkylating agent TMZ has become a treatment option and has been used as both a single agent and also in combination, such as with capecitabine. The RR to this regimen was approximately 70%, although the study involved a small number of patients and was retrospective [13].

The mechanism of action of alkylating agents is induction of cancer cell death due to DNA damage caused by alkylating the O6-guanine site of DNA; this forms a cross-link between adjacent strands of DNA and results in DNA mismatches [14, 15]. One mechanism of resistance to alkylating agents is an increase in the expression of the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT). *MGMT*, located on chromosome 10q26, encodes a DNA repair enzyme [16] that catalyzes dealkylation, such that the alkyl groups on O6-guanine sites modified by an alkylating agent are removed by transfer to a cysteine residue in the active site. Expression of the MGMT protein in cancer cells is regulated by epigenetic mechanisms; if expression of MGMT is decreased by methylation of the *MGMT* promotor region, DNA repair is decreased. A decrease in MGMT expression, which frequently occurs during carcinogenesis [17], may increase the sensitivity of tumor cells to alkylating agents that induce DNA damage, thus increasing the response to alkylating agents. In contrast, in cases in which the *MGMT* promotor region is not methylated, MGMT is expressed, DNA methylation is rapidly repaired, and resistance to alkylating agents may appear.

In a clinical setting, MGMT status can be evaluated by assessing MGMT protein expression using immunohistochemistry (IHC) or by determining *MGMT* promoter methylation using a methylation assay. The relationship between MGMT status and response to alkylating agents has been most extensively studied in patients with glioblastoma, in which MGMT deficiency is a biomarker for response to therapy and predicts improved survival during treatment with TMZ [18–21]. Investigations into the importance of MGMT status in WD panNETs have been carried out regarding the response to alkylating agents, especially TMZ [22–31]. However, these reports are limited by small sample sizes and have yielded conflicting results.

To clarify this issue, we retrospectively reviewed the response of patients with WD panNETs who were treated with TMZ at our institution. We assessed archived tumor tissues and used IHC to assess MGMT status. Only patients with WD panNETs were included in this study to maintain a homogeneous patient population. Our hypothesis was that MGMT deficiency, as determined by IHC, is required for a response to an alkylating drug.

Materials and methods

Study patients

Patients treated with STZ from February 2015 to December 2016 at Aichi Cancer Center Hospital and who satisfied the following inclusion criteria were enrolled retrospectively: (1) the primary lesion was in the pancreas; (2) the tumor was a morphologically well-differentiated NET; (3) the treatment response could be determined; (4) material from the tumor from a biopsy or surgery at our hospital was available and was suitable for the preparation of MGMT IHC slides.

Medical records were reviewed for patient demographics, pathology reports, and outcomes. Approval for data collection and analysis was obtained from the relevant Institutional Review Board.

Evaluation of response, progression-free survival (PFS), and overall survival (OS)

The overall response to STZ therapy was determined by a reference radiologist who was blinded to MGMT status using the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1. Responses were determined by identifying the baseline with the nearest pretreatment contrast computed tomography (CT) before STZ therapy and assessing the response on a subsequent contrast CT. The CT scan was repeated every 3 months (± 1 month; median duration of CT scan intervals was 2.4 months). PFS was defined as the time from the initiation of the STZ-based chemotherapy to the date of disease progression or death from any cause. OS was defined as the time from the initiation of STZ-based chemotherapy to the date of death due to any cause or the last date of follow-up. Surviving patients were censored on their last follow-up date.

Evaluation of tumor grade and MGMT expression status

Each tumor was classified according to the WHO 2017 classification with a combination of both the Ki67 labeling index (Ki57-LI) and mitosis index. If the grade given by the mitotic count differed from that given by Ki67-LI, the higher grade was applied. For FNA and biopsy specimens, if the tumor cell area did not exceed a size equivalent to 10 high-powered fields (HPFs) under microscopy, counting of the mitotic rate was not possible, and grading was carried out by Ki67-LI only. If the tumor grade was assigned to NEN-G3 (mitotic count > 20 per 10 HPFs and/

or Ki67-LI > 20%), each case was confirmed to meet the inclusion criteria (well-differentiated NET).

MGMT expression status was determined by IHC of the tumor tissues. The sections were stained with an anti-MGMT antibody (MT3.1; Abcam, Cambridge, UK). Several antibodies against MGMT are used for IHC assessment of this marker, and no universal standard has been adopted. Therefore, MT3.1 was used, as it is employed in many prior studies, including the Kulke et al. study [27]. A tumor sample known to exhibit intact MGMT expression was used as an external positive control, and non-neoplastic cells in the tumor sample served as internal positive controls. MGMT expression was considered positive when nuclear staining was present in ≥ 10% of tumor cells (Fig. 1a). Expression was considered negative when nuclear staining was present in < 10% of tumor cells and when positive expression was seen in adjacent non-neoplastic cell nuclei [28] (Fig. 1b).

Cell culture

BON1 cells [32], provided by Dr. Hironori Koga (Kurume University, Kurume, Japan), and QGP1 cells, obtained from the JCRB cell bank (Osaka, Japan), were maintained in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) medium, respectively, supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA). STZ (Sigma–Aldrich, St. Louis, MO) was freshly dissolved in distilled water at 50 mM and added to the culture medium to give the final concentrations indicated.

RT-PCR

Total RNA was extracted from cells using ISOGEN reagent (Nippon Gene, Tokyo, Japan) and subjected to first strand cDNA synthesis with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) using oligo(dT) primers. Conventional RT-PCR was performed using KOD Plus Neo (Toyobo, Osaka, Japan). The primers used were as follows: *MGMT* forward, GATTTCTTACCAGCAATTAGCAGCCCTGGC; *MGMT* reverse, CCTTCATGGGCCAGAAGCCATTCCTTCA; *GAPDH* forward, GAACCATGAGAAGTATGACAACAGCCTCAA; *GAPDH* reverse, GTAGAGGCAGGGATGATGTTCTGGA.

Western blotting

Cells were lysed in RIPA buffer (Thermo Fisher Scientific) containing a cocktail of protease inhibitors (Roche Life Science, Mannheim, Germany). The lysates were subjected to SDS-PAGE followed by transfer onto PVDF membranes (Bio-Rad, Hercules, CA). After blocking with Blocking-One (Nacalai Tesque, Kyoto, Japan), the membranes were incubated with primary antibodies at 4 °C overnight, followed by incubation with an HRP-conjugated secondary antibody (Southern Biotech, Birmingham, AL) at room temperature for 1 h. The signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA). Primary antibodies used included a rabbit polyclonal antibody for GAPDH (Santa Cruz Biotechnology, Dallas, TX) and a murine monoclonal antibody for MGMT (clone MT3.1, Thermo Fisher Scientific).

Plasmids, transfection, and lentiviral transduction

Lentiviral shRNA constructs targeting *MGMT* (TRCN0000022364-8) were obtained from Dharmacon (Lafayette, CO). Full-length *MGMT* cDNA was prepared from QGP1 cells by RT-PCR using primers including restriction enzyme-targeted sequences and was subcloned into the lentiviral vector pLEX-MCS (GE Healthcare, Buckinghamshire, UK). The plasmid was co-transfected with packaging

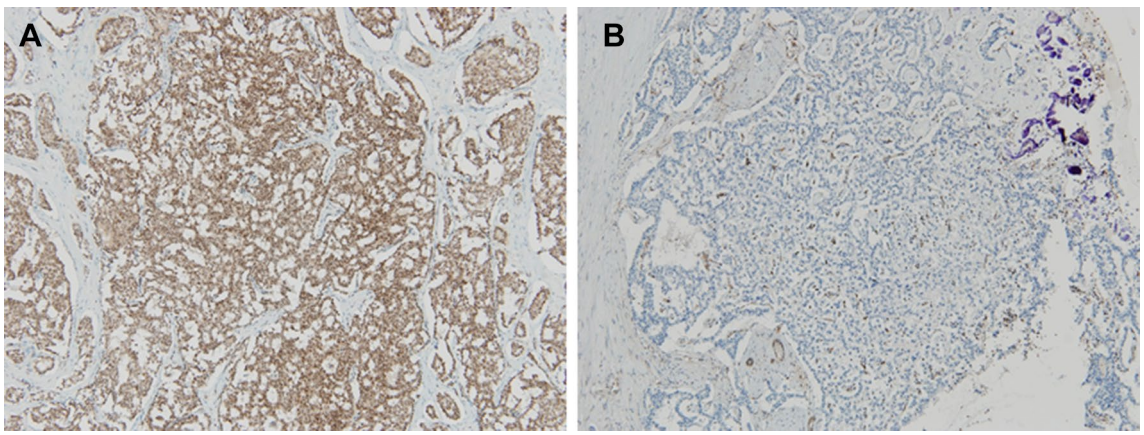

Fig. 1 Representative MGMT staining in pancreatic neuroendocrine tumors. **a** MGMT-positive (intact) IHC staining. Magnification: × 100. **b** MGMT-negative (deficient) IHC staining. Magnification: × 100

plasmids into HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific), and the supernatants were used for viral transduction with polybrene (Sigma–Aldrich) at a final concentration of 8 μg/ml.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay

MTT assay was performed using a CellQuanti-MTT Cell Viability Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. Briefly, 2 days after seeding cells in a 96-well plate at 3×10^3 cells/well, the cells were treated with STZ. After 24 h of treatment, the MTT substrate was added to the culture medium, and the cells were cultured for an additional 4 h, followed by addition of lysis buffer to solubilize the formazan dye. Absorbance at 595 nm was determined with a Genios microplate reader (Tecan, Männedorf, Switzerland).

Statistical analysis

PFS and OS were estimated using the Kaplan–Meier method and compared with the log-rank test. Differences in characteristics between groups were tested by Fisher's exact test or the Mann–Whitney *U* test, as appropriate. All statistical analyses were performed using SPSS version 22 software (IBM, Tokyo, Japan). Given the exploratory nature of the study, a *P* value less than 0.05 was defined as significant.

Results

Patient characteristics

We analyzed 13 patients with panNET; patient characteristics are detailed in Table 1. The panNET grade according to the WHO 2017 classification was G1 in three cases, G2 in eight cases, and NET-G3 in two cases. The Ki67-LIs of the two NET-G3 cases were 27% and 50%. No cases were NEC-G3. STZ was used as first-line therapy in three cases and as second-line or subsequent therapy in 10 cases. These 10 cases had previously received targeted therapy (everolimus and/or sunitinib). All 10 cases were confirmed to exhibit progression following previous treatment. STZ was prescribed as a daily regimen in six cases and a weekly regimen in seven cases. The objective RR to STZ was partial response (PR) in six cases (46.1%), stable disease (SD) in three cases (23%), and progressive disease (PD) in four cases (30.7%). In the three cases who received first-line treatment with an STZ-based regimen, the RR was 33% (1/3). The RRs by tumor grade (G1, G2, and G3) were 66% (2/3), 50% (4/8), and 0% (0/2), respectively. In terms of toxicity, one of the 13 cases presented with G3 toxicity in the form of liver dysfunction. Though an anti-tumor effect had been shown in this MGMT-negative case, treatment was discontinued within 4 months. For the other 12 cases, treatment was halted upon diagnosis of PD. MGMT expression, as determined by IHC, was positive in seven cases (54%) and negative in six cases (46%). There was no significant correlation between patient characteristics and MGMT status (Table 2).

Table 1 Patient characteristics

Sex, male/female	10/3
Median age in years (range)	64 (33–77)
Grade (WHO 2017)	NET-G1, $n=3$; NET-G2, $n=8$; NET-G3, $n=2$
Treatment line	First line, $n=3$; second line, $n=4$; third line $n=3$ Forth line and later, $n=3$
Regimen	Weekly, $n=7$; daily, $n=6$
5-FU doublet	Yes, $n=3$ (all in weekly regimen); no, $n=10$
RR to STZ-based regimen (all lines)	Partial response, $n=6$ (46.1%) Tumor stability (PR + SD), $n=9$ (36%)
RR to STZ-based regimen (1st -line)	33% (1/3)
RR to STZ-based regimen (by grade)	G1, 66% (2/3); G2, 50% (4/8); G3, 0% (0/2)
Toxicity (≥ grade 3)	1/13 (7.7%)
Median PFS in months (range)	141 (61–1246)
MGMT status	MGMT positive ($n=7$) 54% MGMT negative ($n=6$) 46%

RR response rate

Table 2 Correlation between MGMT status and patient characteristics

Patient characteristic	Number of patients	MGMT positive ($n=7$)	MGMT negative ($n=6$)	P value
Sex				
Female	3	2	1	0.56
Male	10	5	5	
Age (years)				
< 60	4	2	2	0.66
> 60	9	5	4	
Grade				
G1 (Ki67 < 3%)	3	2	1	0.56
G2 (3 < Ki67 < 20%)	8	3	5	
G3 (Ki67 > 20%)	2	2	0	

Relationship between MGMT IHC and response to treatment

The treatment response was evaluated in terms of anti-tumor response (PR vs. SD + PD) and tumor stability (PR + SD vs. PD).

Relationship between MGMT IHC and anti-tumor response (PR vs. SD + PD)

Of the six MGMT-negative cases, 83.3% (5/6) exhibited a PR. By contrast, 85.7% (6/7) MGMT-positive cases did not show an anti-tumor response (SD + PD) ($P=0.013$). The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of MGMT IHC were 83.3% (5/6), 85.7% (6/7), 83.3% (5/6), 85.7% (6/7), and 84.6% (11/13), respectively (Supplementary Table 1).

Relationship between MGMT IHC and tumor stability (PR + SD vs. PD)

All six MGMT-negative cases (100%) demonstrated tumor stability (PR + SD), while 57.1% (4/7) of MGMT-positive cases did not show tumor stability (PD) ($P=0.026$). The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of MGMT IHC were 66.6% (6/9), 100% (4/4), 100% (6/6), 57.1% (4/7), and 76.9% (10/13), respectively (Supplementary Table 1).

Relationship between MGMT IHC and PFS

The median PFS of the MGMT-positive and MGMT-negative groups were 140 days (range 61–643 days) and 172 days (range130–1099 days), respectively (Fig. 2). This difference was not statistically significant ($P=0.496$).

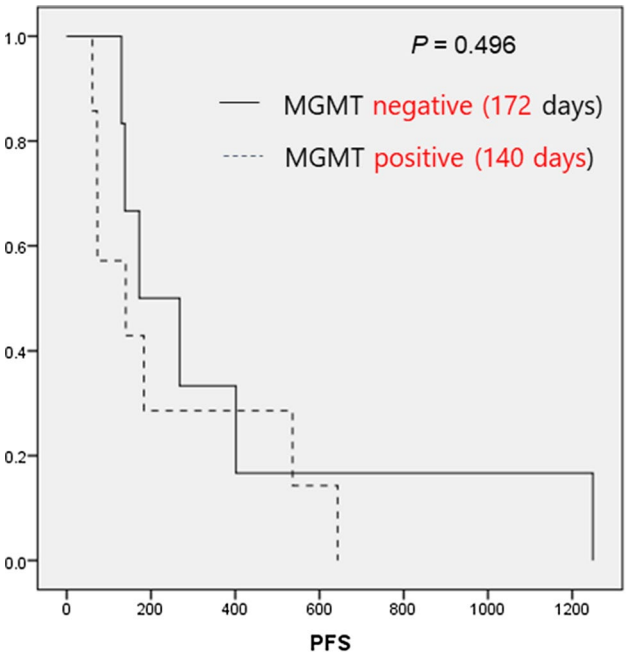

Fig. 2 PFS for pancreatic neuroendocrine tumor patients treated with streptozocin therapy. The solid line is MGMT- negative (deficient) as determined by IHC, and the dotted line is MGMT- positive (intact) as determined by IHC. The median PFS of the MGMT- negative and MGMT- positive groups was 172 days (range 130–1099 days) and 140 days (range 61–643 days), respectively ($P=0.496$)

Relationship between MGMT IHC and OS

The median OS of the MGMT-positive and MGMT-negative groups were 447 days (range 81–1218 days) and 827 days (range163–1296), respectively (Online Resource 1). Although there was a tendency for OS to be longer among MGMT-negative cases, no statistically significant difference was observed ($P=0.339$).

Overexpression of *MGMT* cDNA induces STZ resistance in BON1 cells

We next examined the association between MGMT expression and STZ resistance in vitro using panNET cell lines. We first performed RT-PCR to assess *MGMT* expression in BON1 and QGP1 cells, the only panNET cell lines available to us (Fig. 3a). After 30 cycles of PCR, MGMT expression was only observed in QGP1 cells (Fig. 3b). Western blotting analysis also indicated MGMT expression in QGP1 cells but not in BON1 cells (Fig. 3c). Therefore, we decided to overexpress *MGMT* cDNA in BON1 cells and to knock down *MGMT* in QGP1 cells. Among five commercial *MGMT* shRNA constructs tested, the two constructs showing the best knockdown efficiency in QGP1 cells were selected for further experiments (Fig. 3d). We thus established subclones of BON1 and QGP1 cells transduced

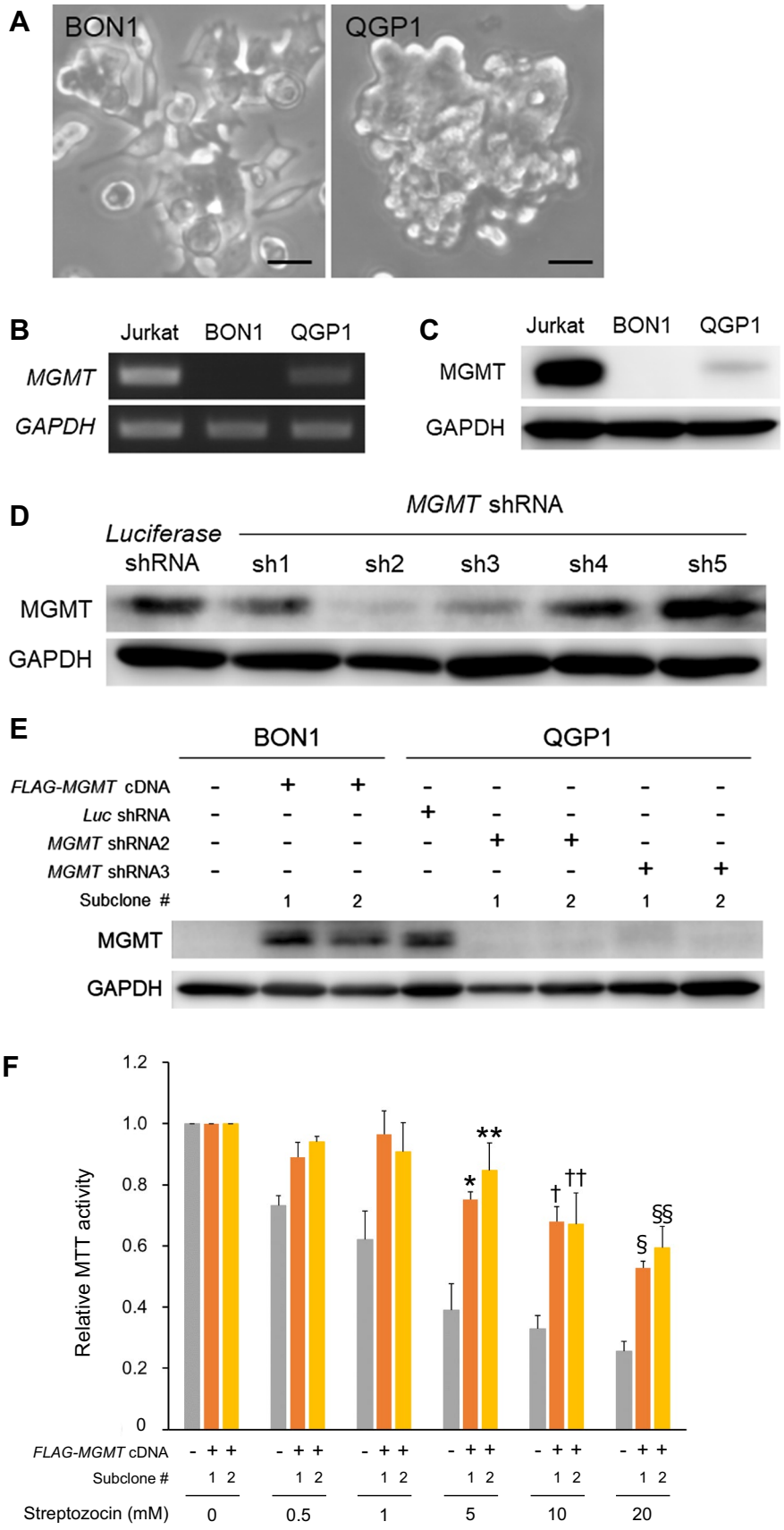

Fig. 3 MGMT overexpression induces streptozocin resistance in BON1 cells. **a** Morphology of BON1 and QGP1 cells observed under phase contrast microscopy. Scale bars, 20 µm. **b** Conventional RT-PCR analysis to assess *MGMT* expression in BON1 and QGP1 cells. Jurkat cells were used as a positive control. **c** Western blotting analysis to assess MGMT expression in BON1 and QGP1 cells. Jurkat cells were used as a positive control. **d** Western blotting analysis to assess the knockdown efficiencies of five *MGMT* shRNA constructs. **e** Western blotting analysis to assess MGMT expression in *MGMT*-overexpressing BON1 subclones and *MGMT* shRNA-transduced QGP1 subclones. **f** MTT activity of *MGMT*-overexpressing BON1 subclones treated with streptozocin. $P = 0.030$ (*), 0.036 (**), 0.016 (†), 0.050 (††), 0.011 (§), and 0.025 (§§), compared with parental BON1 cells treated with the same concentration of streptozocin

with lentiviral constructs harboring *MGMT* cDNA and *MGMT* shRNA, respectively, and selected subclones that showed suitable overexpression or knockdown efficiency as determined by western blotting analysis (Fig. 3e). We then tested the STZ resistance of these subclones using MTT assay. At 24 h after STZ treatment, MTT activity was significantly higher in *MGMT*-overexpressing BON1 subclones than in the parental BON1 cells, suggesting a correlation between MGMT expression and STZ resistance (Fig. 3f, see figure legend for individual *P* values). However, *MGMT*-knockdown QGP1 cells did not show an altered response to STZ treatment (Online Resource 2).

Discussion

The results of this study showed a strong correlation between STZ response and MGMT, in both patients and cell lines. Previous reports have shown inconsistent results regarding an association between the response of panNET to alkylating agents and MGMT (Table 3). This clinical study exhibits two differences compared with previous reports. First, most previous reports have concentrated on the alkylating agents TMZ and DTIC. By contrast, the relationship between STZ and MGMT has not been well studied; only one paper has reported the relationship between STZ response and MGMT [25]. In this previous article, the authors examined 20

Table 3 Previous studies examining the relationship between MGMT status and response to an alkylating agent

	Author/year	Number of cases	Method of determining MGMT expression	RR of only MGMT examined cases/total RR	Drug	Concomitant drug	Loss of MGMT expression	RR of MGMT Loss cases	Relationship between MGMT expression and RR or PFS
1	Ekevland et al. (2007) [28]	23 (panc. 8)	IHC	N.A./total 8% (1/12)	TMZ	None	43% (10/23)	40% (4/10)	Possible (no statistical analysis)
2	Kulke et al. (2009) [27]	101 (panc. 37)	IHC	N.A./total 34% (18/53)	TMZ	Total, doublet 96.0% (7/101)	51% (19/37)	80% (4/5)	Possible
3	Schimitt et al. (2014) [26]	10	9 (IHC) 10 (MSP)	30% (3/10)	TMZ	N.A.	40% (IHC) 30% (MSP)	75% (IHC) 100% (MSP)	$P=0.206$ (IHC) $P=0.033$ (MSP)
4	Walter et al. (2015) [25]	107 (panc. 62)	89 (IHC) 99 (MSP) 99 (pyrosequencing)	14/69 (20%)	STZ DTIC TMZ	Alone 9.2% (7/69) Doublet 89.8% (62/69)	32.5% (IHC) 12.1 (MSP) 24.2 (pyrosequencing)	50%	Possible (PFS) $P=0.002$
5	Cives et al. (2016) [24]	52	IHC	N.A.	TMZ	Capecitabine	38%× (IHC)	35% (IHC)	No statistical correlation $P=0.56$
6	Cros et al. (2016) [29]	43	IHC + pyrosequencing	39.5 (17/43)	TMZ	Capecitabine	69.7% (30/43)	15/30 (50%)	Possible (RR: $P=0.04$, PFS $P=0.001$)
7	Raj et al. (2017) [22]	36	36 (IHC only) 28 (IHC + PCR)	41.6% (15/36)	TMZ DTIC	N.A.	55% (IHC) 86% (PCR) 11% (IHC + PCR)	50% (IHC) 38% (PCR) 100% (IHC + PCR)	Interpreted as negative (no statistical analysis)
8	Girot et al. (2017) [23]	22	22 (IHC only) 20 (IHC + PCR)	13.6% (3/22)	TMZ	Capecitabine 19/22 (86.4%)	59% (IHC) 15% (PCR) 15% (IHC + PCR)	15% (IHC) 38% (PCR) 100% (IHC + PCR)	Interpreted as negative (no statistical analysis)
9	Dwight et al. (2017) [30]	20	IHC	30% (6/20)	TMZ	Capecitabine	40% (8/20)	63% (IHC)	Trend toward longer PFS
10	Davide et al. (2018) [31]	95 (panc 43)	42 (MSP) 53 (pyrosequencing)	27.4% (26/95)	TEM	Capecitabine	28.4% (27/95)	51.8% (PCR)	Possible (PFS) $P=0.001$

IHC immunohistochemistry, *STZ* streptozocin, *RR* response rate, *Panc.* pancreas, *TMZ* temozolomide, *DTIC* dacarbazine, *MSP* methylation-specific

patients treated with STZ-based chemotherapy and observed that PFS and overall survival were significantly improved in the methylated *MGMT* group compared with those in the non-methylated *MGMT* group [25]. However, this report included not only patients with panNET, but also NETs in other organs (including the gastrointestinal tract, lung, and others). Thus, the relationship between response to STZ in panNET and MGMT is unclear and was the focus of the current study. Second, in previous reports, the main therapeutic regimen was combination therapy consisting of an alkylating agent plus a doublet [9]; therefore, 5-fluorouracil (5-FU) may have affected the relationship between MGMT and the RR and/or PFS. For example, in MGMT-positive cases, alkylating agents should intrinsically have little effect; however, an effect may be noted in such cases because of the effect of 5-FU. In addition, concurrent capecitabine may counteract MGMT-associated resistance to TMZ [33]. Dihydropyrimidine dehydrogenase and thymidylate synthase are biomarkers that are associated with a therapeutic effect of 5-FU [34]. In our patient group, 76.9% (10/13) of patients were treated with STZ as a single agent, and thus, we were better able examine the relationship between STZ response and MGMT status.

Considerable controversy remains regarding the optimal method of MGMT detection in tumor samples. In panNETs, both methylation-specific PCR (MSP) and pyrosequencing have been used to evaluate *MGMT* promoter methylation status as a surrogate of MGMT activity [25, 26]. However, differences in the method for IHC [24] and a low rate of agreement between IHC and MSP are possible reasons for the heterogeneity of the results. The direct measurement of MGMT protein expression by IHC is the most convenient technique for measuring MGMT status in clinical settings, despite the pitfalls associated with the interpretation of staining [25, 35]. No formal recommendations have been established regarding the criteria for the interpretation of MGMT immunostaining [22–28]. Sample bias, sampling issues, inter-observer variability, and/or IHC technical differences (including the use of different antibodies against MGMT) may account for differences in results. Some studies have reported no significant correlation between MGMT protein expression as determined by IHC and *MGMT* promoter methylation as determined by MSP [26]. Thus, other mechanisms in addition to promoter methylation may control *MGMT* expression [26].

We assessed the correlation between MGMT protein expression and STZ response using panNET cell lines. Specifically, overexpression of *MGMT* in BON1 cells, in which MGMT protein expression was undetectable by western blotting analysis, significantly increased the number of STZ-resistant cells. Unexpectedly, knockdown of *MGMT* in QGP1 cells did not increase their response to STZ. We speculate that there may be two reasons for this result. First, *MGMT*-knockdown QGP1 cells may still exhibit residual MGMT protein expression that is sufficient to exert an effect on STZ resistance. Second, other MGMT-independent mechanism(s) may regulate STZ resistance in QGP1 cells. Further studies are needed to address these possibilities. To the best of our knowledge, this is the first in vitro study to examine the relationship between MGMT expression in panNET cells and their response to alkylating agents. In addition to our clinical data, these in vitro studies suggest that MGMT is a promising candidate for a predictive marker capable of determining the response of panNETs to alkylating agents.

Our study has some limitations. First, we evaluated MGMT status with IHC only. As mentioned above, some reports suggest that concordance is relatively low (around 30–60%) between IHC and MSP assessments of MGMT status. From a methodological point of view, several authors have claimed that in gliomas, MGMT IHC and MSP are not interchangeable [35–37]. Second, in other cancerous tumors, in addition to MGMT status, the status of the mismatch repair gene *hMLH1* is also predictive of the effectiveness of alkylating agents [38, 39]. Thus, the effectiveness of alkylating agents is not determined only by the presence or absence of MGMT expression. Lastly, this study includes a potential selection bias of patients due to its retrospective design and small number of patients. Thus, we performed an in vitro study in addition to the clinical study. However, a prospective multicenter study is desirable.

In conclusion, we evaluated the relationship between MGMT expression as determined by IHC and RR for STZ in WD panNET. Despite limited cases examined, high-concordance of negative expression of MGMT and response to streptozocin treatment suggest that MGMT expression can be a potential biomarker for this treatment. These results should be confirmed in a prospective study.

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Compliance with ethical standards

Conflict of interest Susumu Hijioka has received speaker honoraria from Novelphama, Novartis, and Teijin Pharma. Nobumasa Mizuno has received research funding from AstraZeneca, Zeria Pharmaceutical, Taiho Pharmaceutical, Merck Serono, Eisai, NanoCarrier, MSD, Dainippon Sumitomo Pharma, and Novartis. Keiichiro Sakuma, Masahiro Aoki, Takamichi Kuwahara, Nozomi Okuno, Kazuo Hara, and Yasushi Yatabe have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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