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Efficacy of temozolomide combined with capecitabine (CAPTEM) on refractory prolactinomas as assessed using an *ex vivo* 3D spheroid assay

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Abstract

Purpose Refractory prolactinomas resistant to dopamine agonists (DAs) pose a clinical challenge. Temozolomide (TMZ) is a recommended treatment option, but its effects are difficult to predict, and the alternatives are limited. Recent reports suggested that TMZ combined with capecitabine (CAPTEM) can be effective for the treatment of aggressive pituitary tumors. This study sought to evaluate the effect of TMZ in an *ex vivo* three-dimensional (3D) spheroid culture assay and determine if this assay could be used to predict the therapeutic effect of CAPTEM in actual refractory prolactinomas.

Methods Surgically resected tumor tissues from two patients with refractory prolactinoma were cultured as 3D spheroids. The effects of TMZ were assessed based on its suppression of cell viability and reduction of prolactin (PRL) levels.

Results In Case 1, the 3D culture assay showed no effect of TMZ on cell viability or PRL suppression. Clinically, TMZ treatment did not reduce PRL levels (8870→8274 ng/mL) and the tumor progression. However, CAPTEM partially reduced PRL levels (9070→4046 ng/mL) and suppressed the tumor growth. In Case 2, TMZ in the 3D culture assay showed a 50% reduction of cell viability and 40% reduction of PRL levels. Clinically, CAPTEM was highly effective, with a considerable reduction in PRL level (17,500→210 ng/mL), and MRI showed almost no residual tumor.

Conclusion This is the first report to describe the effects of CAPTEM treatment on refractory prolactinomas. The *ex vivo* 3D spheroid culture assay reliably predicted TMZ sensitivity and informed the selection between TMZ or CAPTEM treatment for refractory prolactinomas.

Introduction

Prolactinomas are the most commonly occurring pituitary tumors and are unique for their high responsiveness to dopamine agonists (DAs). Cases of refractory prolactinomas resistant to DAs are rare; however, they can be aggressive and often have a very high Ki-67 labeling index (LI) [1, 2]. Surgery is the treatment of choice for such cases, but the remission rate is low even when managed by experts because of its invasive nature [3]. Radiotherapy can be selected with consideration for tumor aggressiveness, taking into account side effects and treatment limitation due to tumor location. Multimodal treatments have to be discussed in case of aggressive pituitary tumors [4]. Temozolomide (TMZ), an alkylating agent, is the only recommended agent for treating refractory prolactinomas [5], but its effect is not always beneficial, particularly in cases with high O^6-methylguanine-DNA-methyltransferase (MGMT) expression [6-8].

Researchers have attempted to find treatment options other than TMZ but with limited success. Molecular-targeted drugs such as lapatinib and everolimus and immune checkpoint inhibitors (ICIs) have been used to treat refractory prolactinomas, but their effectiveness is limited. [9-11]. Thus, refractory prolactinomas resistant to TMZ are quite challenging. Recent reports suggested that TMZ combined with capecitabine (CAPTEM) have shown to be more effective than TMZ monotherapy for the treatment of advanced neuroendocrine neoplasms [12]. CAPTEM is rarely used to treat pituitary tumors, but reports have been used primarily for cases of aggressive corticotroph adenomas, with remarkable effects on reducing tumor size and decreasing plasma adrenocorticotropic hormone levels [13, 14]. To date, no report has been made of using CAPTEM for treating refractory prolactinomas, especially those resistant to TMZ. However, CAPTEM, if shown its highly effective accumulated data, may be offered as an alternative to first-line temozolomide in corticotroph pituitary tumors, which is the current standard for refractory pituitary adenomas. Furthermore, determining whether to use TMZ alone or in combination with capecitabine is difficult; thus, decision-making indicators are required.

In a previous study, since *in vitro* experiments using mouse corticotroph adenoma cell line AtT20 had shown that CAPTEM was more effective than TMZ alone, CAPTEM had been applied for clinical use in preference to TMZ for refractory corticotroph adenomas, leading to successful outcome [13].

Here we describe two cases of refractory prolactinomas treated with CAPTEM. Both patients had undergone transsphenoidal surgery (TSS), but had DA-resistant residual tumors. Thus, we considered the choice of either TMZ or CAPTEM treatment for these patients. Considering the adverse effects of capecitabine, TMZ alone or CAPTEM is an important treatment alternative. Moreover, recent reports suggested that MGMT is not the only factor that determines TMZ resistance [15, 16]. Therefore, we evaluated TMZ sensitivity using the patient-derived three-dimensional (3D) spheroid culture assay. The patient-derived 3D culture system is a promising preclinical model and is used to screen drugs for the treatment of refractory neoplasms [17]. This system mimics the tumor environment more realistically than two-dimensional (2D) cultures and is considered an ideal screening model to evaluate drug treatment effects [18]. Because of limited cell numbers, we could only test TMZ treatment in this evaluation. Taking into account the results of the 3D culture, it was determined whether each tumor showed TMZ resistance, and the results were used as a criterion for whether to administer CAPTEM as the next treatment.

Both TMZ and capecitabine are off-label drugs used for the treatment of aggressive and malignant pituitary adenomas in Japan. Therefore, we obtained permission to use these drugs from the Ethics Committee of Moriyama Memorial Hospital (MMH) (Permission No. 21003).

Methods

All clinical data were obtained at MMH, and *ex vivo* studies were conducted at Kobe University. Written informed consent for publication of the clinical details and clinical images was obtained from both patients, and the chart audit was approved by the Research Ethics Board, MMH, Tokyo.

***Ex vivo* 3D spheroid culture assay**

The resected tumor tissues were immediately stored in ice-cold phosphate-buffered saline. The cells were transferred to the bench and enzymatically digested using Dulbecco's modified Eagle's medium (DMEM) containing 0.3% bovine serum albumin, 0.35% collagenase, and 0.15% hyaluronidase, after which they were dispersed. Matrigel (growth-factor-reduced; phenol-red-free; BD Biosciences, San Jose, CA) was polymerized for 10 min at 37°C. The tumor cells were embedded into Matrigel and placed in 96-well plates (Corning) at a density of 10,000 cells per well. Then, 50 μL of DMEM culture medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and antibiotics was added to each well. For assessment cell viability, we used RealTime-Glo MT Cell Viability Assay (Promega). MT Cell Viability Substrate and NanoLuc® Enzyme were added into the culture medium according to the manufacturer's instructions. The cells were incubated at 37°C in a 5% CO_2 incubator. Twenty-four hours after the incubation, the cells were treated with TMZ (5, 25, or 50 μM) or vehicle (dimethyl sulfoxide) for 96 h. IC_{50} was used as a reference for setting drug concentration conditions [19]. The conditions were set at 5-50 μM with reference to the previous report. The maximum blood concentration of TMZ used clinically in subjects is 7.67 μg/mL, which is equivalent to 39.5 μM. In Case 1, the experiment was performed with triplicate, and in Case 2, the experiment was performed with quadruplicate. Culture medium was changed at 72 hours after treatment, and medium stored with cells for 24 hours were collected. Luminescence was measured by EnSpire™ Multimode Plate Reader (PerkinElmer). Prolactin (PRL) levels in the collected media were measured using an enzyme electrochemiluminescence assay kit (Roche, Tokyo, Japan). These experiments were conducted in compliance with the protocol that was reviewed and approved by the Research Ethics Committee of Kobe University Hospital (IRB#1363).

Immunocytochemistry

Tumor specimens were obtained during surgery and fixed in 10% buffered formaldehyde, dehydrated in graded ethanol, embedded in paraffin, and examined using routine histological methods. Immunocytochemical studies were performed using mouse monoclonal antibodies, PRL (INN-hPRL-1; GeneTex; 1:2000), MGMT (MT 3.1; Novus; 1:100), and MSH6 (EP49; DAKO; 1:50 dilution). Ki-67 labeling was performed using antibodies (MIB-1; DAKO 1:500) to assess tumor proliferation.

Case presentation

Case 1

A 57-year-old woman had undergone TSS at another hospital 7 years ago. The pathological findings of the resected tumors included diffusely positive PRL, and the Ki-67 LI was remarkably high at 10%–20%. She underwent CyberKnife radiosurgery for the residual tumor. After 5 years of stable disease (SD), her serum PRL levels started increasing again with tumor regrowth despite being on a high-dose cabergoline regimen (9 mg/week). She was admitted to our hospital to undergo a second TSS. The tumor was invasive; it destroyed the clivus and extended into the right cavernous sinus, encasing the right internal carotid artery (ICA). Although total resection was impossible (Fig. 1A), her serum PRL levels decreased from 4660 to 1470 ng/mL postoperatively but gradually increased again with tumor regrowth (Fig. 1B). Physical examination revealed that the right fifth and sixth cranial nerves were affected. Treatment with oral TMZ was started after 1 year instead of radiotherapy because she had already undergone radiosurgery after the first TSS.

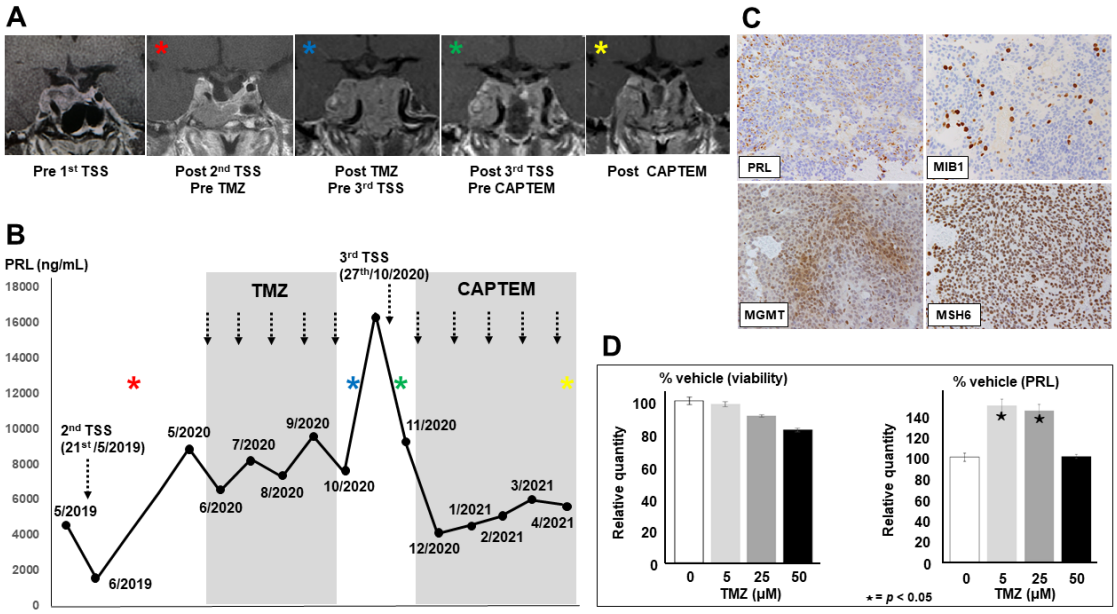
Unfortunately, TMZ was not effective; neither the PRL level (Fig. 1B) nor the tumor size reduction (Fig. 1A). TMZ monotherapy was discontinued after the fifth cycle (Fig. 1B). By then, the patient

presented with complete abducens palsy on the right side and required additional TSS to relieve the nerves from tumor compression. The tumor extended laterally from the right ICA and posteriorly to the brain stem. To avoid complications, we did not attempt complete resection but removed as much of the tumor mass as possible (Fig. 1A). Pathological analysis of the third operation specimen confirmed that the tumor was a prolactinoma with a remarkably high Ki-67 LI of 10%–15% (Fig. 1C). A bone specimen showed tumor invasion between the trabeculae. Immunohistochemistry revealed strong MGMT expression in approximately 50% of the tumor cells (Fig. 1C), which suggested that TMZ alone was not effective. Some studies suggested that MutS homolog 6 (MSH6) expression contributes to the effectiveness of TMZ in malignant pituitary neoplasms [20, 21]. MSH6 was strongly positive in Case 1 (Fig. 1C), indicating that the residual tumor may be sensitive to TMZ [21].

To confirm the direct effect of TMZ on PRL secretion in the tumor, we employed a 3D spheroid culture of resected prolactinomas, as described in the Methods section and in a previous report [22]. First, we conducted a viability assay using 3D culture experiments. In the TMZ-treated group, the cell viability decreased by 20% compared with that in the vehicle-treated group (Fig. 1D). The PRL levels in the culture media did not decrease in the TMZ-treated group but instead significantly increased by 40% ($p = 0.05$) with 5 and 25 μM TMZ treatment (Fig. 1D).

These results suggested that TMZ monotherapy is not adequate for this tumor, which was evident in her clinical course from the previous TMZ treatment. Therefore, capecitabine (750 mg/m^2 twice daily on days 1–14) and TMZ (200 mg/m^2 once daily on days 10–14) were administered in combination for 2 weeks, followed by 2 weeks off, as previously described [13]. The combination treatment showed higher efficacy than TMZ treatment alone (Fig. 1B), and its effect was considered a partial response (PR). The patient's PRL levels decreased by 50% (9070→4046 ng/mL) after the first cycle. However, no further decrease or increase in PRL level was noted, and it did not normalize thereafter (Fig. 1B). The tumor evolution even with the TMZ monotherapy ceased after CAPTEM

treatment, and the tumor size almost remains the same (Fig. 1A). Hand–foot syndrome (HFS), a known side effect of capecitabine [23], emerged and was ameliorated with the application of a urea/lactic acid-based topical keratolytic agent. By maintaining this protocol, the patient's clinical condition



remained stable as of the last follow-up after the 10th cycle of CAPTEM.

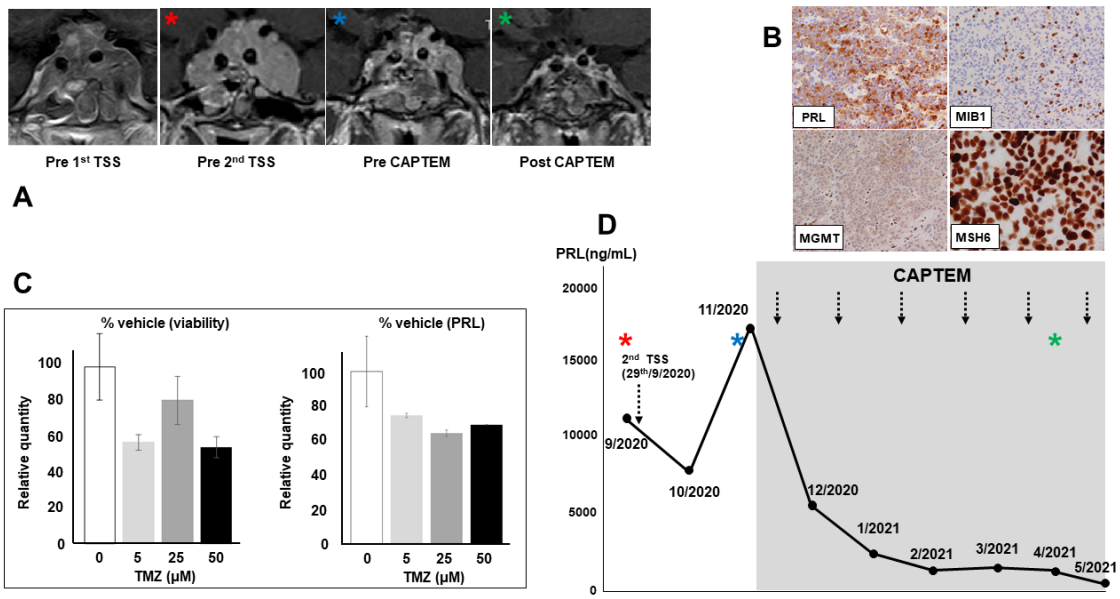
Case 2

A 53-year-old man with aggressive dopamine-resistant prolactinoma underwent TSS at another hospital 1 year previously. Most of the tumors were left untouched owing to the close proximity to the ICAs and an insufficient operative field (Fig. 2A). However, the tumors continued to proliferate, and the patient's serum PRL levels increased to 11,000 ng/mL despite a high-dose cabergoline regimen (9 mg/week). Thus, he was admitted to our hospital for a second surgery. He complained of severe headaches and dizziness. His visual examination revealed left-eye bitemporal hemianopsia. Optical coherence tomography revealed decreased retinal nerve fiber layer and ganglion cell layer thickness. Considering the extent of the tumor, he underwent extended TSS (Fig. 2A). Tumors in the suprasellar

area were firmly adhered to the surroundings, and unexpected arterial bleeding occurred during tumor debulking.

Hemostasis was achieved via meticulous compression using Gelfoam. The arterial damage became a pseudoaneurysm but was completely repaired using stent-assisted coiling [24]. Complete surgical removal was not possible, and alternative therapy was necessary. Radiation therapy was once discussed but was not chosen, because it might affect the pseudoaneurysm and cause rebleeding. The patient's serum PRL levels decreased after TSS but rapidly increased up to 17,500 ng/mL. Pathologically, the tumor was diagnosed as a prolactinoma, and its proliferative nature was confirmed with a very high Ki-67 LI (18%) (Fig. 2B). MGMT was positive in approximately 40% of the tumor cells but was weaker than that of Case 1 (Fig. 2B). As well as Case 1, MSH6 was strongly positive in Case 2 (Fig. 2B), indicating that the residual tumor may be sensitive to TMZ [21].

To ascertain whether TMZ treatment would be adequate for this patient, we used *ex vivo* 3D culture methods, as previously performed in Case 1. In Case 2, the number of viable cells seems to be reduced in the TMZ treatment culture than in the vehicle treatment (50%). Although the response was not dose dependent, the effect was more apparent than that in Case 1 (Fig. 2C). Moreover, the PRL levels in the



culture media tended to be decreased in the TMZ treatment culture, compared with that in the vehicle treatment (40%, Fig. 2C). Those responses in viability and PRL reduction were not statistically significant, likely due to the variations in vehicle treatments and small sample size of the assay. However, these responses were clearly different from Case 1. Based on these findings, we concluded that TMZ was more effective in this case but was still insufficient to normalize PRL levels with complete tumor shrinkage. Taken together with previous evidence, we discussed with patient whether to use TMZ or CAPTEM, which was thought to have a higher tumor shrink effect. Then, we selected CAPTEM therapy to expect a stronger treatment effect.

The patient was treated with a combination of capecitabine 750 mg/m^2 (twice daily on days 1–14) and TMZ 200 mg/m^2 (once daily on days 10–14), followed by 2 weeks off, the same protocol as that in Case 1. CAPTEM treatment successfully led to tumor shrinkage, with the tumor becoming almost undetectable (Fig. 2A). Moreover, his serum PRL levels substantially decreased and were maintained around 200 ng/mL, which was almost one-hundredth of the pretreatment value (Fig. 2D). His visual field deficit was restored, and his headaches disappeared. He complained of slight nausea during the TMZ cycle, but this was tolerated with antiemetics. CAPTEM treatment is ongoing for this patient and is being considered as complete response (CR). So far, 10 cycles of CAPTEM were accomplished and his serum PRL continues to decline.

Discussion

Aggressive prolactinomas are resistant to conventional therapy, exhibit high proliferation rates, and invade adjacent structures. No treatment except TMZ has been established for these tumors [5, 7]. CAPTEM has been used as another treatment option for aggressive pituitary tumors, although all cases were corticotroph tumors [13, 14]. Theranostic markers of TMZ are required for choosing TMZ monotherapy or CAPTEM. MGMT expression has been considered but is contentious, especially in

pituitary tumors. In this study, we showed for the first time two cases of refractory prolactinomas that were treated with CAPTEM. In this treatment selection, an *ex vivo* 3D culture assay was used to clearly confirm TMZ resistance in once case and to determine based on TMZ partial responsiveness results in the next case.

Multiple studies have suggested an association between low MGMT expression and better response to TMZ [7, 25, 26]. Although this association has not been observed in other studies [27-29], MGMT expression levels are the most reliable predictive marker for TMZ response so far. MGMT expression levels in tumors are thought to be defined by MGMT promoter methylation status, especially in gliomas, which is also associated with TMZ sensitivity [30]. However, few reports have shown that this methylation status provided a better prediction of TMZ sensitivity than IHC expression analysis in pituitary tumors. Therefore, we used MGMT expression analysis rather than its promoter methylation analysis. Another candidate biomarker is MSH6, a DNA mismatch repair protein. Mutations in *MSH6* in glioblastomas are associated with resistance to TMZ [31]. A study on TMZ showed that MSH6 expression was positively correlated with pituitary tumor regression but not MGMT [28]. However, subsequent reports failed to confirm this correlation [25, 32]. Because data are limited, the prognostic efficacy of MGMT and MSH6 expressions in the response to TMZ remains unclear.

Patient-derived 3D culture is a promising drug-screening tool that has been used for various refractory neoplasms [17]. These culture models have an environment that closely mimics various solid tumors, including cell structures, cell-to-cell interactions, extracellular matrix components, gradients for efficient diffusion of growth factors, and removal of metabolic waste, more so than 2D culture models [33]. To the best of our knowledge, this is the first report to evaluate drug effectiveness using a 3D spheroid culture assay for pituitary tumors and use the findings to select the most optimal treatment choice. In Case 1, TMZ had no effect on the reduction of PRL levels and had limited effect on tumor

244 growth in the 3D culture assay, which was consistent with the lack of *in vivo* efficacy of the previous
245 TMZ monotherapy. These findings, together with high MGMT expression, informed the selection of
246 CAPTEM for further treatment. CAPTEM was partially effective in reducing the PRL level, which
247 was reduced to approximately 50% of the pre-CAPTEM value, although the levels did not normalize
248 subsequently. In Case 2 however, TMZ reduced tumor viability and PRL levels more significantly than
249 in Case 1, although it was not statistically significant. Because the effect of TMZ alone for this tumor
250 was partial, we selected CAPTEM treatment. The effect of CAPTEM was greater in Case 2 than in
251 Case 1 and was comparable to the results obtained from the 3D culture of TMZ. We have not been
252 able to model CAPTEM treatment in 3D culture, but the results of the 3D culture assay for TMZ
253 monotherapy showed some predictable clinical effects of CAPTEM.
254 CAPTEM treatment followed by TMZ alone for aggressive pituitary tumors has been reported in
255 several cases [7, 25, 29, 34]. The outcomes were mostly unfavorable, and only one case exhibited
256 partial tumor regression [35]. Theoretically, CAPTEM should be more effective in TMZ-sensitive
257 cases than in TMZ-resistant ones. Capecitabine is an antimetabolite and attenuates MGMT repair by
258 inhibiting thymidylate synthase activity and reducing thymidine levels, thereby enhancing the
259 antitumor effect of TMZ [36, 37]. A recent meta-analysis described the safety and efficacy of
260 CAPTEM in the treatment of advanced neuroendocrine tumors [12]. Logically, CAPTEM should be
261 selected for MGMT-positive tumors. Nonetheless, how the MGMT expression level affects the
262 response to CAPTEM remains unknown. Among aggressive corticotroph tumors treated with
263 CAPTEM reported previously, most of the patients exhibited very low or no MGMT expression,
264 whereas only one patient showed positive expression [13, 14, 35]. The outcomes of patients with low
265 MGMT levels varied from CR to SD. The MGMT-positive case showed PR with CAPTEM treatment
266 [35]. In the present study, TMZ monotherapy was not effective in Case 1, but CAPTEM was partially
267 effective even with the strong MGMT expression. On the other hand, CAPTEM was markedly

effective in Case 2 despite of MGMT expression. In addition to MGMT assessment, a 3D culture assay can help predict the effects of TMZ and CAPTEM treatment in these tumors. Further studies are required in establishing the efficacy of using the assay in selecting between TMZ and CAPTEM therapy.

The efficacies of all second-line medical therapies after TMZ have not been proven thus far because the data are largely limited to case reports and small case series. Other than TMZ, the limited experience in using cytotoxic chemotherapy to manage aggressive pituitary tumors has led to unfavorable results [7]. Molecular-targeted therapies are increasingly being considered because their clinical data have been accumulated in other neoplasms. Several targeted therapies and immunotherapies for DA-resistant refractory aggressive pituitary tumors have been investigated, including lapatinib, everolimus, bevacizumab, and ICIs. The effects of these drugs on clinically challenging pituitary tumors are currently under investigation [9-11]. Pasireotide, a second-generation somatostatin receptor ligand mainly targeting somatostatin receptor subtype 2 (SSTR2) and SSTR5, has been reported to inhibit prolactinoma cell proliferation [38]. However, the tumors in both our cases showed negative SSTR2 and SSTR5 immunostaining (data not shown). Drug screening for other drug selection using this 3D culture system was not possible this time but could be a future useful tool to determine if some more drugs are susceptible.

In conclusion, we describe the cases of two patients with refractory prolactinomas who were treated with CAPTEM therapy with partial and complete responses, the latter showing a notable reduction in PRL level and tumor shrinkage. These effects corroborated the results of the *ex vivo* 3D spheroid culture assay of TMZ treatment. These results suggest that CAPTEM is a promising treatment option for aggressive prolactinomas, even those with positive MGMT expression. Furthermore, 3D spheroid culture assays could be useful in predicting drug efficacy and could inform the selection of drug treatments. Nevertheless, further case studies are required to demonstrate the effectiveness of

CAPTEM and usefulness of this experimental assay.

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Declarations

Conflict of interest The authors have no multiplicity of interests to disclose.

Ethical approval Study protocols were approved by the Moriyama Memorial Hospital Institutional Review Board. Informed consent was obtained from the patients. 3D spheroid culture experiments were conducted in compliance with the protocol that was reviewed and approved by the Research Ethics Committee of Kobe University Hospital (IRB#1363).

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Figure legend

Fig. 1. Clinical course of Case 1 **A.** Pituitary MRI appearances after transsphenoidal surgery (TSS) showing that temozolomide (TMZ) and TMZ combined with capecitabine (CAPTEM) had little effect on tumor size reduction. (T1WI gadolinium-enhanced coronal images). Colored asterisks attached with the MR images correspond to the time points shown in Fig. 1B. **B.** Serum prolactin (PRL) levels were plotted in the line graph. Vertical axis represents the PRL level (ng/mL) along the horizontal time axis. TMZ did not reduce the level of PRL but CAPTEM had partial effect. **C.** Immunohistochemical staining for PRL, MIB1, O^6-methylguanine-DNA-methyltransferase (MGMT) and MutS Homolog 6 (MSH6). **D.** Patient-derived tumor spheroid assay. Compared with the vehicle treated control groups, TMZ has little reduction effect on cell viability (left) and did not reduce the level of tumor-secreting

412 PRL into culture media (right).

413

414 Fig. 2 Clinical course of Case 2. **A.** Pituitary MRI appearance before and after transsphenoidal
415 surgery (TSS) showing the dramatic tumor shrink by temozolomide (TMZ) combined with
416 capecitabine (CAPTEM) (T1WI gadolinium-enhanced coronal images). Colored asterisks attached
417 with the MR images correspond to the time points shown in Fig. 2D. **B.** Immunohistochemical staining
418 for prolactin (PRL), MIB1, O^6-methylguanine-DNA-methyltransferase (MGMT) and MutS Homolog
419 6 (MSH6). **C.** Patient-derived tumor spheroid assay. Compared with the vehicle treated control groups,
420 TMZ had partial reduction effect on the cell viability (left) and decreased the level of tumor-secreting
421 PRL into the culture media (right). **D.** Serum prolactin (PRL) levels were plotted in the line graph.
422 Vertical axis represents the PRL levels (ng/mL) along the horizontal time axis. CAPTEM almost
423 completely suppressed the level of tumor-secreting PRL.