

Kobe University Repository : Kernel

PDF issue: 2025-07-06

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

Ishida, Atsushi ; Shichi, Hiroki ; Fukuoka, Hidenori ; Inoshita, Naoko ; Ogawa, Wataru ; Yamada, Shozo

(Citation) Pituitary, 25(2):238-245

(Issue Date) 2022-04

(Resource Type) journal article

(Version) Accepted Manuscript

(Rights)

This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature's AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at:...

(URL)

https://hdl.handle.net/20.500.14094/90009082



1	Efficacy of temozolomide combined with capecitabine (CAPTEM) on
2	refractory prolactinomas as assessed using an <i>ex vivo</i> 3D spheroid assay
3	
4	Atsushi Ishida ¹ , Hiroki Shichi ² , Hidenori Fukuoka ³ , Naoko Inoshita ⁴ , Wataru Ogawa ² , Shozo Yamada ⁵
5	
6	1. Department of Neurosurgery, Moriyama Memorial Hospital, Tokyo 134-0081, Japan
7	2. Division of Diabetes and Endocrinology, Kobe University Graduate School of Medicine, Kobe
8	650-0017, Japan
9	3. Division of Diabetes and Endocrinology, Kobe University Hospital, Kobe 650-0017, Japan
10	4. Department of Pathology, Tokyo Metropolitan Geriatric Hospital, Tokyo 173-0015, Japan
11	5. Hypothalamic & Pituitary Center, Moriyama Neurological Center Hospital, Tokyo 134-0088,
12	Japan
13	
14	Address Correspondence to:
15	Hidenori Fukuoka MD, PhD.
16	Division of Diabetes and Endocrinology, Kobe University Hospital, 7-5-2 Kusunoki-cho, Chuo-ku,
17	Kobe 650-0017, Japan.
18	E-mail: fukuokah@med.kobe-u.ac.jp
19	
20	Keywords Refractory prolactinoma, temozolomide, capecitabine, CAPTEM, MGMT, 3D spheroid
21	culture
22	
23	

- 24 Abstract
- 25

26 Purpose Refractory prolactinomas resistant to dopamine agonists (DAs) pose a clinical challenge. 27 Temozolomide (TMZ) is a recommended treatment option, but its effects are difficult to predict, and 28 the alternatives are limited. Recent reports suggested that TMZ combined with capecitabine 29 (CAPTEM) can be effective for the treatment of aggressive pituitary tumors. This study sought to 30 evaluate the effect of TMZ in an ex vivo three-dimensional (3D) spheroid culture assay and determine 31 if this assay could be used to predict the therapeutic effect of CAPTEM in actual refractory 32 prolactinomas. 33 Methods Surgically resected tumor tissues from two patients with refractory prolactinoma were 34 cultured as 3D spheroids. The effects of TMZ were assessed based on its suppression of cell viability 35 and reduction of prolactin (PRL) levels. 36 Results In Case 1, the 3D culture assay showed no effect of TMZ on cell viability or PRL suppression. 37 Clinically, TMZ treatment did not reduce PRL levels (8870→8274 ng/mL) and the tumor progression. 38 However, CAPTEM partially reduced PRL levels (9070→4046 ng/mL) and suppressed the tumor 39 growth. In Case 2, TMZ in the 3D culture assay showed a 50% reduction of cell viability and 40% 40 reduction of PRL levels. Clinically, CAPTEM was highly effective, with a considerable reduction in 41 PRL level (17,500 \rightarrow 210 ng/mL), and MRI showed almost no residual tumor. 42 Conclusion This is the first report to describe the effects of CAPTEM treatment on refractory 43 prolactinomas. The ex vivo 3D spheroid culture assay reliably predicted TMZ sensitivity and informed 44 the selection between TMZ or CAPTEM treatment for refractory prolactinomas.

46 Introduction

47 Prolactinomas are the most commonly occurring pituitary tumors and are unique for their high 48 responsiveness to dopamine agonists (DAs). Cases of refractory prolactinomas resistant to DAs are 49 rare; however, they can be aggressive and often have a very high Ki-67 labeling index (LI) [1, 2]. 50 Surgery is the treatment of choice for such cases, but the remission rate is low even when managed by 51 experts because of its invasive nature [3]. Radiotherapy can be selected with consideration for tumor 52 aggressiveness, taking into account side effects and treatment limitation due to tumor location. 53 Multimodal treatments have to be discussed in case of aggressive pituitary tumors [4]. Temozolomide 54 (TMZ), an alkylating agent, is the only recommended agent for treating refractory prolactinomas [5], 55 but its effect is not always beneficial, particularly in cases with high O⁶-methylguanine-DNA-56 methyltransferase (MGMT) expression [6-8].

57 Researchers have attempted to find treatment options other than TMZ but with limited success. 58 Molecular-targeted drugs such as lapatinib and everolimus and immune checkpoint inhibitors (ICIs) 59 have been used to treat refractory prolactinomas, but their effectiveness is limited. [9-11]. Thus, 60 refractory prolactinomas resistant to TMZ are quite challenging. Recent reports suggested that TMZ 61 combined with capecitabine (CAPTEM) have shown to be more effective than TMZ monotherapy for 62 the treatment of advanced neuroendocrine neoplasms [12]. CAPTEM is rarely used to treat pituitary 63 tumors, but reports have been used primarily for cases of aggressive corticotroph adenomas, with 64 remarkable effects on reducing tumor size and decreasing plasma adrenocorticotropic hormone levels 65 [13, 14]. To date, no report has been made of using CAPTEM for treating refractory prolactinomas, 66 especially those resistant to TMZ. However, CAPTEM, if shown its highly effective accumulated data, 67 may be offered as an alternative to first-line temozolomide in corticotroph pituitary tumors, which is 68 the current standard for refractory pituitary adenomas. Furthermore, determining whether to use TMZ 69 alone or in combination with capecitabine is difficult; thus, decision-making indicators are required.

71 shown that CAPTEM was more effective than TMZ alone, CAPTEM had been applied for clinical use 72 in preference to TMZ for refractory corticotroph adenomas, leading to successful outcome [13]. 73 Here we describe two cases of refractory prolactinomas treated with CAPTEM. Both patients had 74 undergone transsphenoidal surgery (TSS), but had DA-resistant residual tumors. Thus, we considered 75 the choice of either TMZ or CAPTEM treatment for these patients. Considering the adverse effects of 76 capecitabine, TMZ alone or CAPTEM is an important treatment alternative. Moreover, recent reports 77 suggested that MGMT is not the only factor that determines TMZ resistance [15, 16]. Therefore, we 78 evaluated TMZ sensitivity using the patient-derived three-dimensional (3D) spheroid culture assay. 79 The patient-derived 3D culture system is a promising preclinical model and is used to screen drugs for 80 the treatment of refractory neoplasms [17]. This system mimics the tumor environment more 81 realistically than two-dimensional (2D) cultures and is considered an ideal screening model to evaluate 82 drug treatment effects [18]. Because of limited cell numbers, we could only test TMZ treatment in this 83 evaluation. Taking into account the results of the 3D culture, it was determined whether each tumor 84 showed TMZ resistance, and the results were used as a criterion for whether to administer CAPTEM 85 as the next treatment.

In a previous study, since in vitro experiments using mouse corticotroph adenoma cell line AtT20 had

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).

89

70

90 Methods

91 All clinical data were obtained at MMH, and ex vivo studies were conducted at Kobe University.

- 92 Written informed consent for publication of the clinical details and clinical images was obtained from
- 93 both patients, and the chart audit was approved by the Research Ethics Board, MMH, Tokyo.

95 Ex vivo 3D spheroid culture assay

96 The resected tumor tissues were immediately stored in ice-cold phosphate-buffered saline. The cells 97 were transferred to the bench and enzymatically digested using Dulbecco's modified Eagle's medium 98 (DMEM) containing 0.3% bovine serum albumin, 0.35% collagenase, and 0.15% hyaluronidase, after 99 which they were dispersed. Matrigel (growth-factor-reduced; phenol-red-free; BD Biosciences, San 100 Jose, CA) was polymerized for 10 min at 37°C. The tumor cells were embedded into Matrigel and 101 placed in 96-well plates (Corning) at a density of 10,000 cells per well. Then, 50 µL of DMEM culture 102 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and antibiotics was added to 103 each well. For assessment cell viability, we used RealTime-Glo MT Cell Viability Assay (Promega). 104 MT Cell Viability Substrate and NanoLuc® Enzyme were added into the culture medium according 105 to the manufacturer's instructions. The cells were incubated at 37°C in a 5% CO₂ incubator. Twenty-106 four hours after the incubation, the cells were treated with TMZ (5, 25, or 50 μ M) or vehicle (dimethyl 107 sulfoxide) for 96 h. IC₅₀ was used as a reference for setting drug concentration conditions [19]. The 108 conditions were set at 5-50 μ M with reference to the previous report. The maximum blood 109 concentration of TMZ used clinically in subjects is 7.67 µg/mL, which is equivalent to 39.5 µM. In 110 Case 1, the experiment was performed with triplicate, and in Case 2, the experiment was performed 111 with quadruplicate. Culture medium was changed at 72 hours after treatment, and medium stored with 112 cells for 24 hours were collected. Luminescence was measured by EnSpire[™] Multimode Plate 113 Reader (PerkinElmer). Prolactin (PRL) levels in the collected media were measured using an enzyme 114 electrochemiluminescence assay kit (Roche, Tokyo, Japan). These experiments were conducted in 115 compliance with the protocol that was reviewed and approved by the Research Ethics Committee of 116 Kobe University Hospital (IRB#1363).

118 Immunocytochemistry

119

120 in graded ethanol, embedded in paraffin, and examined using routine histological methods. 121 Immunocytochemical studies were performed using mouse monoclonal antibodies, PRL (INN-hPRL-122 1; GeneTex; 1:2000), MGMT (MT 3.1; Novus; 1:100), and MSH6 (EP49; DAKO; 1:50 dilution). Ki-123 67 labeling was performed using antibodies (MIB-1; DAKO 1:500) to assess tumor proliferation. 124 125 **Case presentation** 126 127 Case 1 128 129 A 57-year-old woman had undergone TSS at another hospital 7 years ago. The pathological findings 130 of the resected tumors included diffusely positive PRL, and the Ki-67 LI was remarkably high at 10%-131 20%. She underwent CyberKnife radiosurgery for the residual tumor. After 5 years of stable disease 132 (SD), her serum PRL levels started increasing again with tumor regrowth despite being on a high-dose 133 cabergoline regimen (9 mg/week). She was admitted to our hospital to undergo a second TSS. The 134 tumor was invasive; it destroyed the clivus and extended into the right cavernous sinus, encasing the 135 right internal carotid artery (ICA). Although total resection was impossible (Fig. 1A), her serum PRL 136 levels decreased from 4660 to 1470 ng/mL postoperatively but gradually increased again with tumor 137 regrowth (Fig. 1B). Physical examination revealed that the right fifth and sixth cranial nerves were 138 affected. Treatment with oral TMZ was started after 1 year instead of radiotherapy because she had 139 already undergone radiosurgery after the first TSS. 140 Unfortunately, TMZ was not effective; neither the PRL level (Fig. 1B) nor the tumor size reduction

Tumor specimens were obtained during surgery and fixed in 10% buffered formaldehyde, dehydrated

141 (Fig. 1A). TMZ monotherapy was discontinued after the fifth cycle (Fig. 1B). By then, the patient

142 presented with complete abducens palsy on the right side and required additional TSS to relieve the 143 nerves from tumor compression. The tumor extended laterally from the right ICA and posteriorly to 144 the brain stem. To avoid complications, we did not attempt complete resection but removed as much 145 of the tumor mass as possible (Fig. 1A). Pathological analysis of the third operation specimen 146 confirmed that the tumor was a prolactinoma with a remarkably high Ki-67 LI of 10%–15% (Fig. 1C). 147 A bone specimen showed tumor invasion between the trabeculae. Immunohistochemistry revealed 148 strong MGMT expression in approximately 50% of the tumor cells (Fig. 1C), which suggested that 149 TMZ alone was not effective. Some studies suggested that MutS homolog 6 (MSH6) expression 150 contributes to the effectiveness of TMZ in malignant pituitary neoplasms [20, 21]. MSH6 was strongly 151

positive in Case 1 (Fig. 1C), indicating that the residual tumor may be sensitive to TMZ [21].

152 To confirm the direct effect of TMZ on PRL secretion in the tumor, we employed a 3D spheroid 153 culture of resected prolactinomas, as described in the Methods section and in a previous report [22]. 154 First, we conducted a viability assay using 3D culture experiments. In the TMZ-treated group, the cell 155 viability decreased by 20% compared with that in the vehicle-treated group (Fig. 1D). The PRL levels 156 in the culture media did not decrease in the TMZ-treated group but instead significantly increased by 157 40% (p = 0.05) with 5 and 25 μ M TMZ treatment (Fig. 1D).

158 These results suggested that TMZ monotherapy is not adequate for this tumor, which was evident 159 in her clinical course from the previous TMZ treatment. Therefore, capecitabine (750 mg/m² twice 160 daily on days 1-14) and TMZ (200 mg/m² once daily on days 10-14) were administered in 161 combination for 2 weeks, followed by 2 weeks off, as previously described [13]. The combination 162 treatment showed higher efficacy than TMZ treatment alone (Fig. 1B), and its effect was considered a 163 partial response (PR). The patient's PRL levels decreased by 50% (9070-4046 ng/mL) after the first 164 cycle. However, no further decrease or increase in PRL level was noted, and it did not normalize 165 thereafter (Fig. 1B). The tumor evolution even with the TMZ monotherapy ceased after CAPTEM

166 treatment, and the tumor size almost remains the same (Fig. 1A). Hand–foot syndrome (HFS), a known 167 side effect of capecitabine [23], emerged and was ameliorated with the application of a urea/lactic 168 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.

170

171 Case 2

173 A 53-year-old man with aggressive dopamine-resistant prolactinoma underwent TSS at another 174 hospital 1 year previously. Most of the tumors were left untouched owing to the close proximity to the 175 ICAs and an insufficient operative field (Fig. 2A). However, the tumors continued to proliferate, and 176 the patient's serum PRL levels increased to 11,000 ng/mL despite a high-dose cabergoline regimen (9 177 mg/week). Thus, he was admitted to our hospital for a second surgery. He complained of severe 178 headaches and dizziness. His visual examination revealed left-eye bitemporal hemianopsia. Optical 179 coherence tomography revealed decreased retinal nerve fiber layer and ganglion cell layer thickness. 180 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 tumordebulking.

183 Hemostasis was achieved via meticulous compression using Gelfoam. The arterial damage became 184 a pseudoaneurysm but was completely repaired using stent-assisted coiling [24]. Complete surgical 185 removal was not possible, and alternative therapy was necessary. Radiation therapy was once 186 discussed but was not chosen, because it might affect the pseudoaneurysm and cause rebleeding. The 187 patient's serum PRL levels decreased after TSS but rapidly increased up to 17,500 ng/mL. 188 Pathologically, the tumor was diagnosed as a prolactinoma, and its proliferative nature was confirmed 189 with a very high Ki-67 LI (18%) (Fig. 2B). MGMT was positive in approximately 40% of the tumor 190 cells but was weaker than that of Case 1 (Fig. 2B). As well as Case 1, MSH6 was strongly positive in 191 Case 2 (Fig. 2B), indicating that the residual tumor may be sensitive to TMZ [21]. 192 To ascertain whether TMZ treatment would be adequate for this patient, we used ex vivo 3D culture

193 methods, as previously performed in Case 1. In Case 2, the number of viable cells seems to be reduced 194 in the TMZ treatment culture than in the vehicle treatment (50%). Although the response was not dose 195 dependent, the effect was more apparent than that in Case 1 (Fig. 2C). Moreover, the PRL levels in the



196 culture media tended to be decreased in the TMZ treatment culture, compared with that in the vehicle 197 treatment (40%, Fig. 2C). Those responses in viability and PRL reduction were not statistically 198 significant, likely due to the variations in vehicle treatments and small sample size of the assay. 199 However, these responses were clearly different from Case 1. Based on these findings, we concluded 200 that TMZ was more effective in this case but was still insufficient to normalize PRL levels with 201 complete tumor shrinkage. Taken together with previous evidence, we discussed with patient whether 202 to use TMZ or CAPTEM, which was thought to have a higher tumor shrink effect. Then, we selected 203 CAPTEM therapy to expect a stronger treatment effect.

204 The patient was treated with a combination of capecitabine 750 mg/m² (twice daily on days 1-14) 205 and TMZ 200 mg/m² (once daily on days 10-14), followed by 2 weeks off, the same protocol as that 206 in Case 1. CAPTEM treatment successfully led to tumor shrinkage, with the tumor becoming almost 207 undetectable (Fig. 2A). Moreover, his serum PRL levels substantially decreased and were maintained 208 around 200 ng/mL, which was almost one-hundredth of the pretreatment value (Fig. 2D). His visual 209 field deficit was restored, and his headaches disappeared. He complained of slight nausea during the 210 TMZ cycle, but this was tolerated with antiemetics. CAPTEM treatment is ongoing for this patient 211 and is being considered as complete response (CR). So far, 10 cycles of CAPTEM were accomplished 212 and his serum PRL continues to decline.

213

214 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.

224 Multiple studies have suggested an association between low MGMT expression and better response 225 to TMZ [7, 25, 26]. Although this association has not been observed in other studies [27-29], MGMT 226 expression levels are the most reliable predictive marker for TMZ response so far. MGMT expression 227 levels in tumors are thought to be defined by MGMT promoter methylation status, especially in 228 gliomas, which is also associated with TMZ sensitivity [30]. However, few reports have shown that 229 this methylation status provided a better prediction of TMZ sensitivity than IHC expression analysis 230 in pituitary tumors. Therefore, we used MGMT expression analysis rather than its promoter 231 methylation analysis. Another candidate biomarker is MSH6, a DNA mismatch repair protein. 232 Mutations in MSH6 in glioblastomas are associated with resistance to TMZ [31]. A study on TMZ 233 showed that MSH6 expression was positively correlated with pituitary tumor regression but not 234 MGMT [28]. However, subsequent reports failed to confirm this correlation [25, 32]. Because data are 235 limited, the prognostic efficacy of MGMT and MSH6 expressions in the response to TMZ remains 236 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.

272 The efficacies of all second-line medical therapies after TMZ have not been proven thus far because 273 the data are largely limited to case reports and small case series. Other than TMZ, the limited 274experience in using cytotoxic chemotherapy to manage aggressive pituitary tumors has led to 275 unfavorable results [7]. Molecular-targeted therapies are increasingly being considered because their 276 clinical data have been accumulated in other neoplasms. Several targeted therapies and 277 immunotherapies for DA-resistant refractory aggressive pituitary tumors have been investigated, 278 including lapatinib, everolimus, bevacizumab, and ICIs. The effects of these drugs on clinically 279 challenging pituitary tumors are currently under investigation [9-11]. Pasireotide, a second-generation 280 somatostatin receptor ligand mainly targeting somatostatin receptor subtype 2 (SSTR2) and SSTR5, 281 has been reported to inhibit prolactinoma cell proliferation [38]. However, the tumors in both our cases 282 showed negative SSTR2 and SSTR5 immunostaining (data not shown). Drug screening for other drug 283 selection using this 3D culture system was not possible this time but could be a future useful tool to 284 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 292 CAPTEM and usefulness of this experimental assay.

Nobuhiro Miki, Masami Ono for discussion.

293

- Acknowledgement We would like to thank Drs. Hideki Shiramizu, Haruko Yoshimoto, Masataka Kato,
- 295
- 296
- 297 Declarations
- 298
- 299 **Conflict of interest** The authors have no multiplicity of interests to disclose.
- 300

301 Ethical approval Study protocols were approved by the Moriyama Memorial Hospital Institutional Review

302 Board. Informed consent was obtained from the patients. 3D spheroid culture experiments were

303 conducted in compliance with the protocol that was reviewed and approved by the Research Ethics

- 304 Committee of Kobe University Hospital (IRB#1363).
- 305
- 306 **Reference**
- Di Sarno A, Landi ML, Cappabianca P, et al (2001) Resistance to cabergoline as compared with
 bromocriptine in hyperprolactinemia: prevalence, clinical definition, and therapeutic strategy. J Clin
- 309 Endocrinol Metab 86(11):5256-5261.
- Delgrange E, Daems T, Verhelst J, Abs R, Maiter D (2009) Characterization of resistance to the
 prolactin-lowering effects of cabergoline in macroprolactinomas: A study in 122 patients. Eur J
- 312 Endocrinol 160:747-752.
- Lasolle H, Ilie MD, Raverot G (2020) Aggressive prolactinomas: How to manage? Pituitary 23(1):70 77.
- 4. Liu W, Zahr RS, McCartney S, Cetas JS, Dogan A, Fleseriu M (2018) Clinical outcomes in male

- patients with lactotroph adenomas who required pituitary surgery: A retrospective single center study.
 Pituitary 21(5):454-462.
- 318 5. Tang H, Cheng Y, Huang J, Li J, Zhang B, Wu ZB (2021) Case Report: Temozolomide treatment of
- 319 refractory prolactinoma resistant to dopamine agonists. Front Endocrinol (Lausanne) 12:616339.
- Almalki MH, Aljoaib NN, Alotaibi MJ et al (2017) Temozolomide therapy for resistant prolactin secreting pituitary adenomas and carcinomas: A systematic review. Hormones (Athens) 16(2):139-
- 322 149.
- 323 7. Nakano-Tateno T, Lau KJ, Wang J et al (2021) Multimodal non-surgical treatments of aggressive
 324 pituitary tumors. Front Endocrinol (Lausanne) 12:624686.
- McCormack A, Dekkers OM, Petersenn S et al (2018) Treatment of aggressive pituitary tumours and
 carcinomas: Results of a European Society of Endocrinology (ESE) survey 2016. Eur JEndocrinol
 178:265–276.
- 328 9. Zhang D, Way JS, Zhang X et al (2019) Effect of everolimus in treatment of aggressive prolactin329 secreting pituitary adenomas. J Clin Endocrinol Metab 104(6):1929-1936.
- Cooper O, Bonert VS, Rudnick J et al (2021) EGFR/ErbB2-targeting lapatinib therapy for aggressive
 prolactinomas. J Clin Endocrinol Metab 106(2): e917-e925.
- 11. Duhamel C, Ilie MD, Salle H et al (2020) Immunotherapy in corticotroph and lactotroph aggressive
 tumors and carcinomas: Two case reports and a review of the literature. J Pers Med 10(3):88.
- 12. Lu Y, Zhao Z, Wang J et al (2018) Safety and efficacy of combining capecitabine and temozolomide
- 335 (CAPTEM) to treat advanced neuroendocrine neoplasms: A meta-analysis. Medicine (Baltimore)
 336 97(41): e12784.
- 13. Nakano-Tateno T, Satou M, Inoshita N et al (2020) Effects of CAPTEM (capecitabine and
 temozolomide) on a corticotroph carcinoma and an aggressive corticotroph tumor. Endocr Pathol Aug
 24.

341

342

14. Zacharia BE, Gulati AP, Bruce JN et al (2014) High response rates and prolonged survival in patients with corticotroph pituitary tumors and refractory Cushing disease from capecitabine and temozolomide (CAPTEM): A case series. Neurosurgery 74(4): E447-455.

- 343 15. Syro LV, Rotondo F, Camargo M, Ortiz LD, Serna CA, Kovacs K (2018) Temozolomide and pituitary
- tumors: Current understanding, unresolved issues, and future directions. Front Endocrinol (Lausanne)
 9:318.
- Burman P, Lamb L, McCormack A (2020) Temozolomide therapy for aggressive pituitary tumours current understanding and future perspectives. Rev Endocr Metab Disord 21(2):263-276.
- 17. Vlachogiannis G, Hedayat S, Vatsiou A et al (2018) Patient-derived organoids model treatment
 response of metastatic gastrointestinal cancers. Science 359(6378):920-926.
- 350 18. Tuveson D, Clevers H (2019) Cancer modeling meets human organoid technology. Science
 351 364(6444):952-955.
- 352 19. Lee SY (2016) Temozolomide resistance in glioblastoma multiforme. Genes Dis 3(3):198-210.
- 353 20. Murakami M, Mizutani A, Asano S et al (2011) A mechanism of acquiring temozolomide resistance
- during transformation of atypical prolactinoma into prolactin-producing pituitary carcinoma: case
 report. Neurosurgery 68(6): E1761-1767.
- 356 21. Matsuno A, Murakami M, Hoya K et al (2014) Molecular status of pituitary carcinoma and atypical
- adenoma that contributes the effectiveness of temozolomide. Med Mol Morphol 47(1):1-7.
- 22. Tsujimoto Y, Shichi H, Fukuoka H et al (2021) Tumor shrinkage by metyrapone in cushing disease
- 359 exhibiting glucocorticoid-induced positive feedback. J Endocr Soc. 5(6) 143664.
- 360 23. Janssen JM, Jacobs BAW, Roosendaal J et al (2021) Population pharmacokinetics of intracellular 5-
- Fluorouridine 5'-Triphosphate and its relationship with hand-and-foot syndrome in patients treated
 with capecitabine. AAPS J 23(1):23.
- 363 24. Ishida A, Asakuno K, Kato M et al (2021) Treatment of an anterior cerebral artery pseudoaneurysm

secondary to a transsphenoidal surgery using stent-assisted coiling. Surg Neurol Int 12:20.

- 365 25. Bengtsson D, Schrøder HD, Andersen M et al (2015) Long-term outcome and MGMT as a predictive
- 366 marker in 24 patients with atypical pituitary adenomas and pituitary carcinomas given treatment with
- 367 temozolomide. J Clin Endocrinol Metab 100(4):1689-1698.
- 368 26. Raverot G, Burman P, McCormack A et al (2018) European Society of Endocrinology. European
- 369 Society of Endocrinology Clinical Practice Guidelines for the management of aggressive pituitary
 370 tumours and carcinomas. Eur J Endocrinol 178(1): G1-G24.
- 27. Lasolle H, Cortet C, Castinetti F et al (2017) Temozolomide treatment can improve overall survival
 in aggressive pituitary tumors and pituitary carcinomas. Eur J Endocrinol 176(6):769-777.
- 28. Hirohata T, Asano K, Ogawa Y et al (2013) DNA mismatch repair protein (MSH6) correlated with the
- responses of atypical pituitary adenomas and pituitary carcinomas to temozolomide: the national
- 375 cooperative study by the Japan Society for Hypothalamic and Pituitary Tumors. J Clin Endocrinol
 376 Metab 98(3):1130-1136.
- 29. Losa M, Bogazzi F, Cannavo S et al (2016) Temozolomide therapy in patients with aggressive pituitary
 adenomas or carcinomas. J Neurooncol 126(3):519-525.
- 379 30. Bush ZM, Longtine JA, Cunningham T et al (2010) Temozolomide treatment for aggressive pituitary
- 380 tumors: correlation of clinical outcome with O(6)-methylguanine methyltransferase (MGMT)
- 381 promoter methylation and expression. J Clin Endocrinol Metab 95(11):E280-90.
- 382 31. Yip S, Miao J, Cahill DP et al (2009) MSH6 mutations arise in glioblastomas during temozolomide
- therapy and mediate temozolomide resistance. Clin Cancer Res 15(14):4622-4629.
- 384 32. Micko ASG, Wöhrer A, Höftberger R et al (2017) MGMT and MSH6 immunoexpression for
 385 functioning pituitary macroadenomas. Pituitary 20(6):643-653.
- 386 33. Breslin S, O'Driscoll L (2013) Three-dimensional cell culture: the missing link in drug discovery.
- 387 Drug Discov Today 18(5-6):240-249.

- 388 34. Campderá M, Palacios N, Aller J et al (2016) Temozolomide for aggressive ACTH pituitary tumors:
 389 failure of a second course of treatment. Pituitary 19(2):158–166.
- 390 35. Lin AL, Jonsson P, Tabar V et al (2018) Marked Response of a Hypermutated ACTH-Secreting
- 391 Pituitary Carcinoma to Ipilimumab and Nivolumab. J Clin Endocrinol Metab 103(10):3925-3930.
- 392 36. Kulke MH, Hornick JL, Frauenhoffer C et al (2009) O6-methylguanine DNA methyltransferase
- deficiency and response to temozolomide-based therapy in patients with neuroendocrine tumors. Clin
 Cancer Res 15(1):338–345.
- 395 37. Murakami J, Lee YJ, Kokeguchi S et al (2007) Depletion of O6-methylguanine-DNA
 396 methyltransferase by O6-benzylguanine enhances 5-FU cytotoxicity in colon and oral cancer cell lines.
 397 Oncol Rep 17(6):1461-1467.
- 38. Lasolle H, Vasiljevic A, Borson-Chazot F, Raverot G (2019) Pasireotide: a potential therapeutic
 alternative for resistant prolactinoma. Ann Endocrinol 80:84–88.
- 400

```
401 Figure legend
```

403 Fig. 1. Clinical course of Case 1 A. Pituitary MRI appearances after transphenoidal surgery (TSS) 404 showing that temozolomide (TMZ) and TMZ combined with capecitabine (CAPTEM) had little effect 405 on tumor size reduction. (T1WI gadolinium-enhanced coronal images). Colored asterisks attached 406 with the MR images correspond to the time points shown in Fig. 1B. B. Serum prolactin (PRL) levels 407 were plotted in the line graph. Vertical axis represents the PRL level (ng/mL) along the horizontal time 408 axis. TMZ did not reduce the level of PRL but CAPTEM had partial effect. C. Immunohistochemical 409 staining for PRL, MIB1, O6-methylguanine-DNA-methyltransferase (MGMT) and MutS Homolog 6 410 (MSH6). D. Patient-derived tumor spheroid assay. Compared with the vehicle treated control groups, 411 TMZ has little reduction effect on cell viability (left) and did not reduce the level of tumor-secreting 412 PRL into culture media (right).

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, O6-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.