and Taylor 2002; Brueggemann et al. 2005) have reported that AVP does not inhibit CCE in all A7r5 cell lines, and neither do all cell lines show activation of NCCE by AVP (Broad et al. 1999; Moneer and Taylor 2002; Dyer et al. 2005). These features were originally found in only a minority, including lacrimal glands, but they have become more common in cells from different sources. Our current data are consistent with above results and the results of our previous study (Miura et al. 2011). Thus, we suggest that PAR2-AP-evoked NCCE is dominant over CCE.

2-APB was originally introduced as a membrane-permeable inhibitor of IP_3 receptors (Maruyama et al. 1997) and has since been widely used to examine the functions of these receptors and the functions of other Ca^{2+} signaling mechanisms such as store-operated Ca^{2+} entry (Bootman et al. 2002). Certain TRPC channels (TRPC1, TRPC3, TRPC5 and TRPC6) have been reported to be inhibited by 2-APB (Ma et al. 2000; Delmas et al. 2002; Hu et al. 2004; Xu et al. 2005). In contrast, some TRPV channels (TRPV1, TRPV2 and TRPV3) are known to be activated by 2-APB (Chung et al. 2004; Hu et al. 2004). In our 2-APB experiments, PAR2-induced Ca^{2+} entry was almost completely inhibited in the presence of 2-APB. From our PCR analysis of RNA isolated from cells, TRPC1, -3 and -6 were expressed (Fig. 10). Our data are consistent with these previous studies. Moneer et al. (2005) showed that almost 90 % of TRPC expressed in A7r5 cells is TRPC1, whereas cells in which AVP stimulates NCCE and inhibits CCE express a more balanced mixture of the major TRPC subtypes: 35 % TRPC1, 35 % TRPC6, 16 % TRPC2, and 7 %TRPC3. They suggest that these cells are perhaps more likely to express hetero-oligomeric TRPC channels (Clapham et al. 2001). Our data are similar to above data, with the exception of TRPC2. It is noteworthy that, in other cells, a change in the expression of a single TRPC protein has been reported to change the properties of both CCE and NCCE pathways, suggesting that the different channels may share some TRPC subunits. In HEK-293 cells, for example, overexpression of TRPC3 does not increase the amplitude of CCE, yet it both modifies its behavior by suppressing its sensitivity to Gd^{3+}, NO, and mitochondrial uncouplers and increases receptor-regulated Ca^{2+} entry via NCCE (Zhu et al. 1998; Thyagarajan et al. 2001). In DT40 cells, TRPC3 can also modulate the behavior of endogenous CCE and contribute to NCCE (Putney 2004). Thus, we suggest that TRPC1 interacts with CCE and NCCE, and that both TRPC3 and -6 interact with NCCE. Therefore, hetero-oligomers of TRPC proteins may contribute to the formation of both CCE and NCCE in lacrimal gland acinar cells, and the composition of the channel may determine its susceptibility to inhibition by PKG (for CCE) and activation by NO (for NCCE).

Recent studies indicated that TRPV1 receptor increased secretion of submandibular gland (Zhang et al. 2010), and that PAR2 regulates TRPV1 to induce hyperalgesia and sensitize TRPV1 by PKC in HEK 293 cell (Amadesi et al. 2006). Consistently, our PCR analysis showed expression of TRPV1 in lacrimal glands. TRPV1 can play a role in PAR2 activation-induced $[Ca^{2+}]_i$ dynamics in the lacrimal gland, although further experiments will be necessary to completely clarify the relationship between CCE/NCCE and TRPV1 receptors in the lacrimal gland.

Based on the results of Ca^{2+} influx from extracellular spaces after stimulation with PAR2-AP, it was concluded that acinar cells express CCE and NCCE. Further experiments will be necessary to completely clarify the relationship between CCE and NCCE with respect to PAR2 in the lacrimal gland. Regardless of the lack of details of the intracellular signaling systems, we can conclude that exocrine cells possess redundant mechanisms to maintain secretion at a certain functional level.

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References

Amadesi S, Cottrell GS, Divino L, Chapman K, Grady EF, Bautista F, Karanjia R, Barajas-Lopez C, Vanner S, Vergnolle N, Bunnett NW (2006) Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cepsilon- and A-dependent mechanisms in rats and mice. J Physiol 575:555–571

Berridge MJ (2009) Inositol trisphosphate and calcium signalling mechanisms. Biochim Biophys Acta 1793:933–940

Birnbaumer L, Yildirim E, Abramowitz J (2003) A comparison of the genes coding for canonical TRP channels and their M, V and P relatives. Cell Calcium 33:419–432

Böhm SK, Kong W, Bromme D, Smeekens SP, Anderson DC, Connolly A, Kahn M, Nelken NA, Coughlin SR, Payan DG, Bunnett NW (1996) Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. Biochem J 314:1009–1016

Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM (2002) 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2 +entry but an inconsistent inhibitor of InsP3-induced Ca2+ release. FASEB J 16:1145–1150

Broad LM, Cannon TR, Taylor CW (1999) A non-capacitative pathway activated by arachidonic acid is the major Ca2+ entry mechanism in rat A7r5 smooth muscle cells stimulated with low concentrations of vasopressin. J Physiol 517:121–134

Brueggemann LI, Markun DR, Barakat JA, Chen H, ByronK L (2005) Evidence against reciprocal regulation of Ca2+ entry by vasopressin in A7r5 aortic smooth muscle cells. Biochem J 388:237–244

Camerer E, Huang W, Coughlin SR (2000) Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc Natl Acad Sci U S A 97:5225–5260

Cheng KT, Liu X, Ong HL, Ambudkar IS (2008) Functional requirement for Orai1 in store-operated TRPC1–STIM1 channels. J Biol Chem 283:12935–12940

Chung MK, Lee H, Mizuno A, Suzuki M, Caterina MJ (2004) TRPV3 and TRPV4 mediate warmth-evoked currents in primary mouse keratinocytes. J Biol Chem 279:21569–21575

Clapham DE, Runnels LW, Strübing C (2001) The TRP ion channel family. Nat Rev Neurosci 2:387–396

Cocks TM, Moffatt JD (2000) Protease-activated receptors: sentries for inflammation? Trends Pharmacol Sci 21:103–108

Coughlin SR (2000) Thrombin signaling and protease-activated receptors. Nature 407:258–264

Dartt DA (1994) Regulation of tear secretion. Adv Exp Med Biol 350:1–9

Dartt DA (2009) Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. Prog Retin Eye Res 28:155–177

Delmas P, Wanaverbecq N, Abogadie FC, Mistry M, Brown DA (2002) Signaling microdomains define the specificity of receptor-mediated InsP3 pathways in neurons. Neuron 34:209–220

Déry O, Corvera CU, Steinhoff M, Bunnett NW (1998) Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. Am J Physiol 274:C1429–C1452

Dyer JL, Liu Y, Pino de la Huerga I, Taylor CW (2005) Long-lasting inhibition of adenylyl cyclase selectively mediated by inositol 1,4,5-trisphosphate-evoked calcium release. J Biol Chem 280:8936–8944

Garrido R, Segura B, Zhang W, Mulholland M (2002) Presence of functionally active protease-activated receptors 1 and 2 in myenteric glia. J Neurochem 83:556–564

Gromada J, Jørgensen TD, Dissing S (1995) The release of intracellular Ca2 + in lacrimal acinar cells by a-, b-adrenergic and muscarinic cholinergic stimulation: the roles of inositol triphosphate and cyclic ADP-ribose. Pflügers Arch 429:751–756

Hirano K, Kanaide H (2003) Role of protease-activated receptors in the vascular system. J Atheroscler Thromb 10:211–225

Hodges RR, Dartt DA (2003) Regulatory pathways in lacrimal gland epithelium. Int Rev Cytol 231:129–196

Hollenberg MD (1999) Protease-activated receptors: PAR-4 and counting: how long is the course. Trends Pharmacol Sci 20:271–273

Hu HZ, Gu Q, Wang C, Colton CK, Tang J, Kinoshita-Kawada M, Lee LY, Wood JD, Zhu MX (2004) 2-aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3. J Biol Chem 279:35741–35748

Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, Tram T, Coughlin SR (1997) Protease-activated receptor 3 is a second thrombin receptor in humans. Nature 386:502–506

Jardin I, Redondo PC, Salido GM, Rosado JA (2008) Phosphatidylinositol 4,5-bisphosphate enhances store-operated calcium entry through hTRPC6 channel in human platelets. Biochim Biophys Acta 1783:84–97

Kahn ML, Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, Farese RV Jr, Tam C, Coughlin SR (1998) A dual thrombin receptor system for platelet activation. Nature 394:690–694

Kamada Y, Saino T, Oikawa M, Kurosaka D, Satoh Y (2012) P2Y purinoceptors induce intra- cellular calcium dynamics of acinar cells in rat lacrimal glands. Histochem Cell Biol 137:97–106

Kawabata A, Kuroda R (2000) Protease-activated receptor (PAR), a novel family of G protein-coupled seven trans-membrane domain receptors: activation mechanisms and physiological roles. Jpn J Pharmacol 82:171–174

Kawabata A, Kuroda R, Minami T, Kataoka K, Taneda M (1998) Increased vascular permeability by a specific agonist of protease-activated receptor-2 in rat hindpaw. Br J Pharmacol 125: 419–422

Kawabata A, Morimoto N, Nishikawa H, Kuroda R, Oda Y, Kakehi K (2000a) Activation of protease-activated receptor-2 triggers mucin secretion in the rat sublingual gland. Biochem Biophys Res Commun 270:298–302

Kawabata A, Nishikawa H, Kuroda R, Kawai K, Hollenberg MD (2000b) Proteinase-activated receptor-2 (PAR-2): regulation of salivary and pancreatic exocrine secretion in vivo in rats and mice. Br J Pharmacol 129:1808–1814

Kawabata A, Kuroda R, Morimoto N, Kawao N, Masuko T, Kakehi K (2001) Lipopolysaccharide-induced subsensitivity of protease-activated receptor-2 in the mouse salivary glands in vivo. Naunyn Schmiedebergs Arch Pharmacol 364:281–284

Kawabata A, Kuroda R, Nishida M, Nagata N, Sakaguchi Y, Kawao N, Nishikawa H, Arizono N, Kawai K (2002) Protease-activated receptor-2 (PAR-2) in the pancreas and parotid gland: immunolocalization and involvement of nitric oxide in the evoked amylase secretion. Life Sci 71:2435–2446

Liao Y, Erxleben C, Abramowitz J, Flockerzi V, Zhu MX, Armstrong DL, Birnbaumer L (2008) Functional interactions among Orai1, TRPCs, and STIM1 suggest a STIM-regulated heteromeric Orai/TRPC model for SOCE/Icrac channels. Proc Natl Acad Sci USA 105:2895–2900

Linley JE, Rose K, Patil M, Robertson B, Akopian AN, Gamper N (2008) Inhibition of M current in sensory neurons by exogenous proteases: a signaling pathway mediating inflammatory nociception. J Neurosci 28:11240–11249

Looms DK, Tritsaris K, Nauntofte B, Dissing S (2001) Nitric oxide and cGMP activate Ca2+-release processes in rat parotid acinar cells. Biochem J 355:87–95

Ma HT, Patterson RL, van Rossum DB, Birnbaumer L, Mikoshiba K, Gill DL (2000) Requirement of the inositol trisphosphate receptor for activation of store-operated Ca2+ channels. Science 287:1647–1651

Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R (2001) Proteinase-activated receptors. Pharmacol Rev 53:245–282

Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K (1997) 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P3-induced Ca2+ release. J Biochem (Tokyo) 122:498–505

Matsuoka H, Harada K, Ikeda T, Uetsuki K, Sata T, Warashina A, Inoue M (2009) Ca2 + pathway involved in the refilling of store sites in rat adrenal medullary cells. Am J Physiol Cell Physiol 296:C889–C899

Miura H, Saino T, Sato M, Satoh Y (2011) Role of protease activated receptors in intracellular calcium dynamics of neurons and satellite cells in rat superior cervical ganglia. Bioimages 19:17–27

Moneer Z, Taylor CW (2002) Reciprocal regulation of capacitative and non-capacitative Ca2 + entry in A7r5 vascular smooth muscle cells: only the latter operates during receptor activation. Biochem J 362:13–21

Moneer Z, Dyer JL, Taylor CW (2003) Nitric oxide co-ordinates the activities of the capacitative and non-capacitative Ca2+-entry pathways regulated by vasopressin. Biochem J 370:439–448

Moneer Z, Pino I, Taylor EJ, Broad LM, Liu Y, Tovey SC, Staali L, Taylor CW (2005) Different phospholipase-C-coupled receptors differentially regulate capacitative and non-capacitative Ca2+ entry in A7r5 cells. Biochem J 389:821–829

Montell C (2003) The venerable inveterate invertebrate TRP channels. Cell Calcium 33:409–417

Nguyen TD, Moody MW, Steinhoff M, Okolo C, Koh D-S, Bunnett NW (1999) Trypsin activates pancreatic duct epithelial cell ion channels. J Clin Invest 103:261–269

Nishikawa H, Kawai K, Tanaka M, Ohtani H, Tanaka S, Kitagawa C, Nishida M, Abe T, Araki H, Kawabata A (2005) Protease-

activated receptor-2 (PAR-2)-related peptides induce tear secretion in rats: involvement of PAR-2 and non-PAR-2 mechanisms. J Pharmacol Exp Ther 312:324–331

Nishiyama T, Nakamura T, Obara K, Inoue H, Mishima K, Matsumoto N, Matsui M, Manabe T, Mikoshiba K, Saito I (2007) Up-regulated PAR-2-mediated salivary secretion in mice deficient in muscarinic acetylcholine receptor subtypes. J Pharmacol Exp Ther 320:516–524

Nystedt S, Emilsson K, Wahlestedt C, Sundelin J (1994) Molecular cloning of a potential proteinase activated receptor. Proc Natl Acad Sci USA 91:9208–9212

Ossovskaya VS, Bunnett NW (2004) Protease-activated receptors: contribution to physiology and disease. Physiol Rev 84:579–621

Putney JW Jr (2004) The enigmatic TRPCs: multifunctional cation channels. Trends Cell Biol 14:282–286

Putney JW Jr, Broad LM, Braun FJ, Lievremont JP, Bird GS (2001) Mechanisms of capacitative calcium entry. J Cell Sci 114:2223–2229

Rohatgi T, Sedehizade F, Sabel BA, Reiser G (2003) Protease-activated receptor subtype expression in developing eye and adult retina of the rat after optic nerve crush. J Neurosci Res 73:246–254

Satoh Y, Oomori Y, Ishikawa K, Ono K (1994) Configuration of myoepithelial cells in various exocrine glands of guinea pigs. Anat Embryol 189:227–236

Satoh Y, Sano K, Habara Y, Kanno T (1997) Effects of carbachol and catecholamines on ultrastructure and intracellular calcium-ion dynamics of acinar and myoepitjelial cells of lacrimal glands. Cell Tissue Res 289:473–485

Shuttleworth TJ (2004) Receptor-activated calcium entry channels—who does what, and when? Sci STKE 243:pe40

Taylor CW (2002) Controlling calcium entry. Cell 111:767–769

Thyagarajan B, Poteser M, Romanin C, Kahr H, Zhu MX, Groschner K (2001) Expression of Trp3 determines sensitivity of capacitative Ca2+ entry to nitric oxide and as a subunit of capacitative Ca2+ entry channels. J Biol Chem 276:48149–48158

Vazquez G, Wedel BJ, Kawasaki BT, Bird GS, Putney JW Jr (2004) Obligatory role of Src kinase in the signaling mechanism for TRPC3 cation channels. J Biol Chem 279:40521–40528

Vu TK, Wheaton VI, Hung DT, Charo I, Coughlin SR (1991) Domains specifying thrombin-receptor interaction. Nature 6345:674–677

Wang H, Wu X, Li JY, Chai BX, Wang J, Mulholland MW, Zhang W (2010) Functional protease-activated receptors in the dorsal motor nucleus of the vagus. Neurogastroenterol Motil 22(431–438):e105

Xu WF, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, Gilbert T, Davie EW, Foster DC (1998) Cloning and characterization of human protease-activated receptor 4. Proc Natl Acad Sci USA 95:6642–6646

Xu SZ, Zeng F, Boulay G, Grimm C, Harteneck C, Beech DJ (2005) Block of TRPC5 channels by 2-aminoethoxydiphenyl borate: a differential, extracellular and voltage-dependent effect. Br J Pharmacol 145:405–414

Yoo HY, Park SJ, Seo EY, Park KS, Han JA, Kim KS, Shin DH, Earm YE, Zhang YH, Kim SJ (2012) Role of thromboxane A_2-activated nonselective cation channels in hypoxic pulmonary vasoconstriction of rat. Am J Physiol Cell Physiol 302:C307–C317

Zhang Y, Cong X, Shi L, Xiang B, Li YM, Ding QW, Ding C, Wu LL, Yu GY (2010) Activation of transient receptor potential vanilloid subtype 1 increases secretion of the hypofunctional, transplanted submandibular gland. Am J Physiol Gastrointest Liver Physiol 299:G54–G62

Zhu X, Jiang M, Birnbaumer L (1998) Receptor-activated Ca^{2+} influx via human Trp3 stably expressed in human embryonic kidney (HEK)293 cells: evidence for a non-capacitative Ca^{2+} entry. J Biol Chem 273:133–142

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ORIGINAL ARTICLE

Randomized trial of chemoradiotherapy and adjuvant chemotherapy with nimustine (ACNU) versus nimustine plus procarbazine for newly diagnosed anaplastic astrocytoma and glioblastoma (JCOG0305)

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Abstract

Purpose Glioblastoma (GBM) is one of the worst cancers in terms of prognosis. Standard therapy consists of resection with concomitant chemoradiotherapy. Resistance to nimustine hydrochloride (ACNU), an alkylating agent, has been linked to methylguanine DNA methyltransferase (MGMT). Daily administration of procarbazine (PCZ) has been reported to decrease MGMT activity. This study investigated the efficacy of ACNU + PCZ compared to ACNU alone for GBM and anaplastic astrocytoma (AA).

Methods Patients (20–69 years) who had newly diagnosed AA and GBM were randomly assigned to receive radiotherapy with ACNU alone or with ACNU + PCZ. The primary endpoint was overall survival (OS). This was designed as a phase II/III trial with a total sample size of 310 patients and was registered as UMIN-CTR C000000108.

Results After 111 patients from 19 centers in Japan were enrolled, this study was terminated early because temozolomide was newly approved in Japan. The median OS and median progression-free survival (PFS) with ACNU alone ($n = 55$) or ACNU + PCZ ($n = 56$) in the

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intention-to-treat population were 27.4 and 22.4 months ($p = 0.75$), and 8.6 and 6.9 months, respectively. The median OS and median PFS of the GBM subgroup treated with ACNU alone ($n = 40$) or ACNU + PCZ ($n = 41$) were 19.0 and 19.5 months, and 6.2 and 6.3 months, respectively. Grade 3/4 hematologic adverse events occurred in more than 40 % of patients in both arms, and 27 % of patients discontinued treatment because of adverse events.

Conclusions The addition of PCZ to ACNU was not beneficial, in comparison with ACNU alone, for patients with newly diagnosed AA and GBM.

Keywords Glioblastoma · Anaplastic astrocytoma · Nimustine · ACNU · Procarbazine · MGMT

Abbreviations

GBM	Glioblastoma
AA	Anaplastic astrocytoma
ACNU	Nimustine hydrochloride
BCNU	Carmustine
TMZ	Temozolomide
MGMT	Methylguanine DNA methyltransferase
WHO	World Health Organization
PFS	Progression-free survival
OS	Overall survival
RT	Radiotherapy
HR	Hazard ratio
AE	Adverse event
ND	Not determined
CR	Complete response
PR	Partial response
SD	Stable disease
PD	Progressive disease
WBC	White blood cell
3D-CRT	Three-dimensional conformal radiotherapy
CT	Computed tomography
IMRT	Intensity-modulated radiation therapy
BEV	Beam's eye views
DVH	Dose–volume histograms
GTV	Gross tumor volume
CTV	Clinical target volume
PTV	Planning target volume
ICRU	International Commission on Radiation Units
FLAIR	Fluid-attenuated inversion recovery
OAR	Organ-at-risk

Introduction

Glioblastoma (GBM) is one of the worst cancers in terms of prognosis, with almost all patients experiencing progression without cure. According to the report of the Brain Tumor Registry of Japan, the %5-year survival of World Health Organization (WHO) grade IV GBM is 6.9 % and that of WHO grade III anaplastic astrocytoma (AA) is 33.9 % [1].

Standard therapy against GBM consists of the maximal resection that is safely possible, with concomitant chemo-radiotherapy. Currently, temozolomide (TMZ) is the

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standard agent used in the treatment of GBM. However, before the TMZ era, nitrosourea had been widely used for GBM and AA. The Glioma Meta-analysis Trialists Group described that chemotherapy including nitrosourea showed significant prolongation of survival, with a hazard ratio of 0.85 ($p < 0.0001$) [2].

Nimustine hydrochloride (ACNU) was developed in Japan, and for more than 20 years since 1980, it has been the standard chemotherapeutic agent against gliomas [3]. Wolff et al. [4] analyzed 364 studies, including a total of 24,193 patients with high-grade glioma, and reported that the survival gain in the 15 ACNU-treated cohorts was 8.9 months, compared to those who received different drugs or no chemotherapy. Takakura et al. [5] reported that the overall survival (OS) of AA and GBM treated by radiotherapy (RT) and concomitant ACNU were 36 and 12 months, respectively. Furthermore, the response rate of a more than 50 % reduction in tumor size was 46.2 % in both AA and GBM. Alkylating agents, including ACNU and procarbazine (PCZ), confer cytotoxic effects on glioma cells by alkylation at the O^6-position of guanine in DNA. This results in the formation of DNA cross-links [6]. Methylguanine DNA methyltransferase (MGMT) removes methylation damage induced by nitrosourea from the O^6-position of DNA guanines before cell injury, and this enzyme was detectable in 76 % of glioma tissues [7]. MGMT in glioma cells is a primary defense against nitrosourea, but the cellular methyltransferase activity of MGMT is exhausted after MGMT takes effect. Daily administration of PCZ for 10 days was reported to cause the accumulation of O^6-methylguanine; it also decreased MGMT activity in rat liver [8] and lymphocytes in lymphoma patients [9]. Inhibition of MGMT by O^6-benzylguanine increased the cytotoxicity of TMZ and carmustine (BCNU) to tumor cells [10]. From these results, it can be predicted that daily administration of PCZ, by depleting MGMT activity, will increase the efficacy of ACNU against AA and GBM.

To prove this hypothesis and establish a more potent standard therapy for AA and GBM, the Brain Tumor Study Group of the Japan Clinical Oncology Group (JCOG) conducted this clinical trial. The study was terminated at the end of the phase II part. The current report describes the final outcome of the study.

Subjects and methods

Patient eligibility criteria

Patients aged 20 to less than 70 years of age who had newly diagnosed and histologically proven supratentorial GBM or AA were eligible for this study. Patients were enrolled between 3 and 14 days after their operation. To be eligible, a patient's preoperative MRI had to show that more than 50 % of the tumor was located in supratentorial areas, except the optic nerve, olfactory nerve, or pituitary gland. Eligible patients had Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2 or 3 (only in cases with neurologic symptoms caused by a tumor) and adequate hematologic, pulmonary, renal, and hepatic function, defined as follows: white blood cell (WBC) count $\geq 3.0 \times 10^3$/mL, hemoglobin level ≥ 8.0 g/dL, platelets count $\geq 1.0 \times 10^6$/mL, aspartate transaminase (AST) level ≤ 100 IU/L, alanine transaminase (ALT) level ≤ 100 IU/L, serum creatinine level ≤ 1.0 mg/dL. Additionally, written informed consent was obtained from all the participating patients. We excluded patients with multiple or disseminated tumors or large tumors in which the planned target volume for irradiation exceeded 1/3 of the whole-brain volume. Additionally, we also classified as ineligible any patient who was pregnant, had meningitis, pneumonia, diabetes mellitus with insulin injection, myocardial infarction, or unstable angina pectoris within the last 3 months, mental disorders, a history of pulmonary fibrosis or interstitial pneumonia, or other forms of active cancer occurring within 5 years of treatment. The study protocol was approved by JCOG Protocol Review Committee and institutional review board at each center.

Treatment

After the confirmation of the eligibility criteria, registration was made by telephone or fax to the JCOG Data Center. Patients were randomized within 14 days of surgery to either ACNU with RT (the control arm, A) or to ACNU + PCZ with RT (the experimental arm, B) (Fig. 1a) by a minimization method with adjustment factors consisting of histology (GBM vs. AA), age (younger than 60 vs. 60 years or older), residual tumor (presence vs. absence), and institution. Residual tumor was assessed using a gadolinium-enhanced MRI obtained within 72 h of the surgery.

Radiotherapy with concomitant chemotherapy was started within 3 weeks after the surgery. Patient positioning and immobilization with an individual head mask and computed tomography (CT)-based planning were required. Treatment was delivered using linear accelerators with nominal energies ≥ 4 MV. Intensity-modulated radiation therapy (IMRT) technique was not permitted. All fields were to be treated every day. Three-dimensional conformal radiotherapy (3D-CRT) planning including the use of beam's eye views (BEV) and dose–volume histograms (DVH) were recommended for volumetric dose evaluation. Quality assurance reviews were done at the Radiotherapy Support Centre in Tokyo, Japan, with feedback sent to each

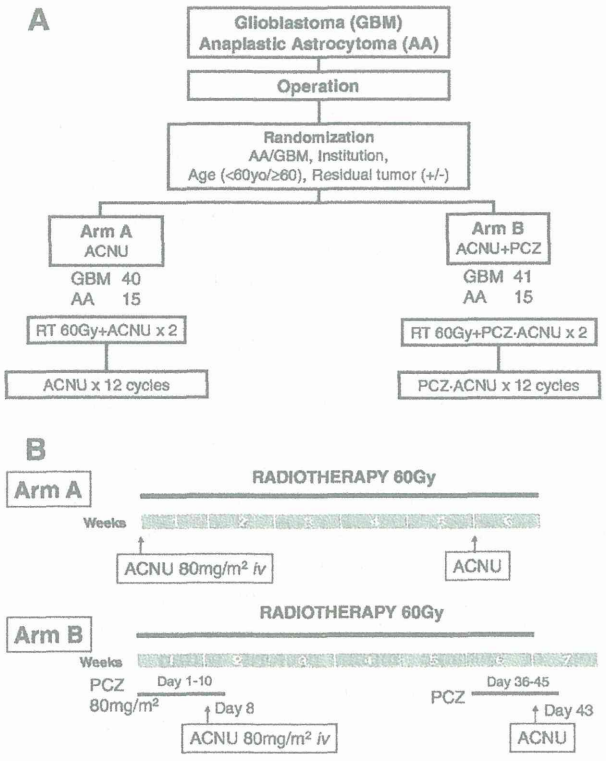

Fig. 1 **a** Study design of JCOG 0305: RT + ACNU versus RT + ACNU + PCZ; 40 patients with GBM and 15 patients with AA were assigned to *arm A*, and 41 patients with GBM and 15 patients with AA were assigned to *arm B*. **b** Treatment schedule of RT + ACNU (*Arm A*) and RT + ACNU + PCZ (*Arm B*)

institution by the radiotherapy study coordinator (Minako Sumi). The minimum and maximum dose to the PTV should be comprised between 95 and 107 % of the International Commission on Radiation Units (ICRU) reference point dose. The gross tumor volume (GTV) was defined as the primary tumor with or without enhancement on CT or magnetic resonance imaging (MRI). The clinical target volume 1(CTV1) included GTV, the resection cavity and surrounding edema (high-intensity area on T2-weighted or fluid-attenuated inversion recovery (FLAIR) image) plus a 1.5-cm margin. The CTV2 included GTV and the resection cavity plus a 1.5-cm margin. Planning target volume (PTV) was defined as CTV plus a margin of 0.5 cm or more. The doses for PTV1 and PTV2 were 50 and 10 Gy, respectively. The protocol required contouring organ-at-risk (OAR), including optic chiasm, brain stem, and retina. Cumulative doses to the optic chiasm and brainstem were limited to a maximum dose of 50 and 45 Gy for the retina.

In the control arm A, 80 mg/m^2 of ACNU was administered intravenously on days 1 and 36 during RT (Fig. 1b). In the experimental arm B, 80 mg/m^2 of oral PCZ was administered daily from days 1 to 10 and days 36 to 45, and given together with intravenous ACNU (80 mg/m^2) on days 8 and 43. Adjuvant therapy consisting of 80 mg/m^2 of ACNU alone in arm A or ACNU plus PCZ (PCZ: 80 mg/m^2 orally on days 1–10, ACNU: 80 mg/m^2 intravenously on day 8) in arm B started 56 days from the final administration of ACNU and was given every 8 weeks, for up to 12 cycles. Doses of ACNU and PCZ were calculated using actual body surface area, reduced for toxicity, and were not escalated.

Evaluations and follow-up

Baseline and follow-up examinations included vital signs, subjective symptoms, neurologic examination, MRI scan, and blood and serum laboratory examinations. For each patient, these examinations were performed weekly, with the exception of MRI scans, which were performed between the end of the initial chemoradiotherapy and the beginning of adjuvant therapy. All examinations were performed before each cycle of adjuvant chemotherapy, at a frequency of nearly every 2 months. After completion of the treatment protocol, patients were assessed every 3 months until progression. Toxicity was graded using the National Cancer Institute Common Toxicity Criteria (version 2). Findings of radiation necrosis were also assessed on MRI. Each patient was required to undergo a follow-up examination for at least 2 years from the date of randomization.

Tumor progression on MRI was defined according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0 [11]. Progression of disease was defined as a 20 % increase in tumor size, as shown by contrast-enhanced imaging, or the development of new lesions, neurologic deterioration, or death by any cause. Further treatment at recurrence or progression was discretionary, but recorded.

A central pathology review by 3 independent pathologists (Yoichi Nakazato, a member of the Working Group for WHO 2007 classification; Nobuaki Funata; and Toru Iwaki) was performed and determinations given. A central review of radiological response was also performed.

Statistical analysis

When we planned this study, TMZ had been widely approved and was used worldwide. However, TMZ was not available in Japan. ACNU remained the standard therapy in Japan, but there was no sufficient data regarding this treatment. We planned a phase II/III clinical trial, with the phase II part designed to confirm the feasibility of ACNU and ACNU + PCZ.

The primary and secondary endpoints for the phase II part were %6-month survival and adverse events (AEs) in ACNU + PCZ arm. The primary endpoint of the phase III part was OS, while the secondary endpoints were PFS, response rate, complete response rate, and AEs.

Overall survival was calculated from the date of random assignment to the date of death from any cause and censored at the last follow-up for event-free patients. PFS was calculated from the date of randomization to the date of progression or death from any cause and censored at the last verifiable progression-free date for event-free patients. OS and PFS were estimated by the Kaplan–Meier method. OS was analyzed by the stratified log-rank test for eligible patients with adjustment factors, excluding institution. Unstratified log-rank tests were used for the analysis of PFS and subgroup analyses of OS and PFS. Fisher's exact test was used for categorical data. All p values are two-sided, except for primary analysis of OS.

We assumed %2-year survivals in AA and GBM for arm A were 50 and 20 %, and the ratio of those patients with AA or GBM enrolled in this study was expected to be 2:3. The phase III study was designed to enroll 155 patients per arm with 5 years of accrual and 2 years of follow-up, including those for the phase II part and about 10 % of ineligible patients, to achieve at least 75 % power to detect a hazard ratio (HR) of 0.74, with a one-sided alpha of 0.05 [12].

Three interim analyses were planned. The first was planned during phase II to test whether %6-month survival in arm A was superior to the predefined threshold (80 %), with a one-sided alpha of 0.1 and beta of 0.2, when 56 patients were included in ACNU + PCZ arm. The second and third interim analyses of OS were planned during phase III. For analyses of phase III part, multiplicity was adjusted by the Lan and DeMets alpha-spending function with the O'Brien and Fleming stopping boundary to control the type I error for primary endpoint.

In March 2007, protocol was amended to stop patient accrual after 111 patients had enrolled and to carry out the final analysis without planned interim analyses for both of phase II and phase III part. This was done because toxicity of both arms was unexpectedly high in phase II and because TMZ became available in Japan.

All statistical analyses were performed using SAS software, release 9.1 (SAS Institute, Cary, NC).

This trial is registered with UMIN-CTR (www.umin.ac.jp/ctr/), number C000000108.

Results

Patient characteristics

A total of 111 patients from 19 centers were randomly assigned to arm A ($n = 55$) or arm B ($n = 56$) from March 2004 to September 2006. Primary analyses were performed in September 2007, and the updated analyses were completed in July 2009. All patients were eligible for this study. Baseline characteristics were well balanced between the arms (Table 1). The median ages for arms A and B were 56 and 54 years, respectively. Total numbers for AA and GBM patients were 15 (27.3 %) and 40 (72.7 %) in arm A and 15 (26.8 %) and 41 (73.2 %) in arm B. PS 0 to 1 in arms A and B were 45 (81.8 %) and 41 (73.2 %), respectively. Eighteen (32.7 %) patients in arm A and 21 (37.5 %) patients in arm B underwent gross total removal, and no residual tumor was confirmed on MRI scans. The median duration of follow-up was 20.2 (range 0–48.0 months) for all eligible patients.

Treatment

Patient compliance with the treatment regimen is depicted in Table 2. Among the 111 total patients, 1 patient in arm A died from pulmonary embolism before the beginning of initial chemoradiotherapy. Fifty-three (96.3 %) patients in arm A completed initial chemoradiotherapy and received ACNU twice. In arm B, 48 out of 56 (85.7 %) patients received 2 cycles of PCZ + ACNU, and 8 patients (14.3 %) received 1 cycle of PCZ + ACNU in initial chemoradiotherapy. Eighteen (32.7 %) patients in arm A and 20 (35.7 %) patients in arm B failed to start adjuvant chemotherapy. Furthermore, 14 (25.5 %) patients in arm A and 23 (41.1 %) patients in arm B discontinued protocol therapy by the fourth cycle of adjuvant chemotherapy. The numbers of patients who received 4 cycles of chemotherapy or more were 23 (41.8 %) and 13 (23.2 %) in arm A and B, respectively. Only 5 (9.1 %) patients in arm A and 2 (3.6 %) patients in arm B completed the full protocol therapy. Nineteen (34.5 %) patients in arm A and 22 (39.3 %) patients in arm B discontinued the protocol for reasons other than completion of protocol or disease progression [arm A: 31 (56.4 %), arm B: 32 (57.1 %)]. Reasons for discontinuation were as follows: AEs [arm A: 6 (10.9 %), arm B: 13 (23.2 %)]; patient refusal related to AE

Table 1 Baseline characteristics in the ITT population

	Arm A ($n = 55$) (RT + ACNU)	Arm B ($n = 56$) (RT + PCZ + ACNU)
Age	56 (24–69)	54 (24–69)
Sex		
Male	32 (58.2 %)	33 (58.9 %)
Female	23 (41.8 %)	23 (41.1 %)
PS		
0, 1	45 (81.8 %)	41 (73.2 %)
2, 3	10 (18.2 %)	15 (26.8 %)
Histology		
Grade 3 (AA)	15 (27.3 %)	15 (26.8 %)
Grade 4 (GBM)	40 (72.7 %)	41 (73.2 %)
Surgery		
Gross total removal	18 (32.7 %)	21 (37.5 %)
Partial removal	30 (54.5 %)	26 (46.4 %)
Biopsy only	7 (12.7′ %)	9 (16.1 %)

Table 2 Compliance

	Arm A ($n = 55$) (RT + ACNU)	Arm B ($n = 56$) (RT + PCZ + ACNU)
RT (completion)	54 (98.2 %)	56 (100 %)
Initial chemotherapy		
1 cycle	1 (1.8 %)	8 (14.3 %)
2 cycles	53 (96.4 %)	48 (85.7 %)
Adjuvant chemotherapy		
None	18 (32.7 %)	20 (35.7 %)
1–3 cycles	14 (25.5 %)	23 (41.1 %)
4–6 cycles	9 (16.4 %)	7 (12.5 %)
7–11 cycles	9 (16.4 %)	4 (7.1 %)
12 cycles (completion)	5 (9.1 %)	2 (3.6 %)

[arm A: 3 (5.5 %), arm B: 8 (14.3 %)]; and patient refusal not related to AE [arm A: 7 (12.7 %), arm B: 1 (1.8 %)].

After discontinuation of the protocol, 28 (50.9 %) patients in arm A and 29 (51.8 %) patients in arm B received TMZ as further treatment.

Central review of histology

The central pathology review diagnosis of all cases was performed according to the WHO 2007 classification (Table 3). Among 81 GBM in the intention-to-treat (ITT) population, 69 (85.2 %), 4 (4.9 %), 3 (3.7 %), 2 (2.5 %), and 3 (3.7 %) were diagnosed as GBM, anaplastic oligoastrocytoma (AOA), anaplastic oligodendroglioma (AO), AA, and others, respectively. Only 10 cases (33.3 %) were diagnosed as AA among the 30 AA in the ITT population, while 8 (26.7 %), 5 (16.7 %), 3 (10.0 %), 2 (6.7 %), and 2 (6.7 %) were confirmed as GBM, AOA, diffuse astrocytoma, pilocytic astrocytoma, and others, respectively. Among all 111 cases, phenotype change of astrocytic to oligodendroglial tumor occurred in 14 cases (12.6 %). Finally, 77 and 12 patients were diagnosed with GBM and AA, respectively, by central pathology review.

Response rate

The overall radiographic response rate for 66 measurable diseases after surgery, based on RECIST guideline, was assessed by Diagnostic Radiology Committee. The response rate was 21.2 % (7/33) in arm A and 6.1 % (2/33) in arm B. In GBM patients from the ITT population, response rates were 25.0 % in arm A [3 CR (complete response), 2 PR (partial response), 1 SD (stable disease), 14 PD (progressive disease)] and 9.1 % in arm B [1 CR, 1 PR, 1 SD, 19 PD] ($p = 0.23$). In AA patients from the ITT population, response rates were 15.4 % in arm A (2 PR, 1 SD, 10 PD) and 0 % in arm B (11 PD) ($p = 0.48$).

Progression-free survival

In the entire ITT population, PFS was 8.6 months [95 % confidence interval (CI); 5.1–20.5] in arm A ($n = 55$, 44 events), compared with 6.9 months (95 % CI 5.1–9.0, $p = 0.36$) in arm B ($n = 56$, 47 events) (Fig. 2a). According to grades in the ITT population, PFS of GBM in arm A ($n = 40$, 33 events) and B ($n = 41$, 35 events) was 6.2 (95 % CI 4.2–13.2) and 6.3 months (95 % CI 3.0–8.9), respectively ($p = 0.35$) (Fig. 2b). PFS of AA in arm A ($n = 15$, 11 PD) and B ($n = 15$, 12 events) was 8.6 (95 % CI 5.1–35.4) and 9.0 months (95 % CI 5.8–21.8), respectively ($p = 0.83$) (Fig. 2c). No difference was observed between the arms in any subgroup defined by histology, presence of remaining tumor, or age under or over 60.

In the subgroup defined by central pathology review, the PFS of GBM in arms A ($n = 37$, 33 events) and B ($n = 40$, 36 events) was 5.1 (95 % CI 4.2–10.3) and 5.7 months (95 % CI 2.7–8.4), respectively ($p = 0.49$) (Fig. 2d). The PFS of AA and AOA in arm A ($n = 9$, 4 events) and B ($n = 12$, 9 events) was ND (not determined) and 7.9 months (95 % CI 5.2–22.5), respectively ($p = 0.21$).

Table 3 Local diagnosis and central pathology review

Grade	Histology	Local diagnosis	Central pathology review		
			Total	Arm A	Arm B
IV	*Glioblastoma*	83	77	37	40
III	*Anaplastic astrocytoma*	30	12	6	6
III	Anaplastic oligoastrocytoma		9	3	6
III	Anaplastic oligodendroglioma		3	2	1
III	Anaplastic ependymoma		1	1	0
II	Diffuse astrocytoma		4	1	3
II	Oligoastrocytoma		1	1	0
II	Oligodendroglioma		1	1	0
I	Pilocytic astrocytoma		2	2	0
	Sarcoma		1	1	0
	Total	111	111	55	56

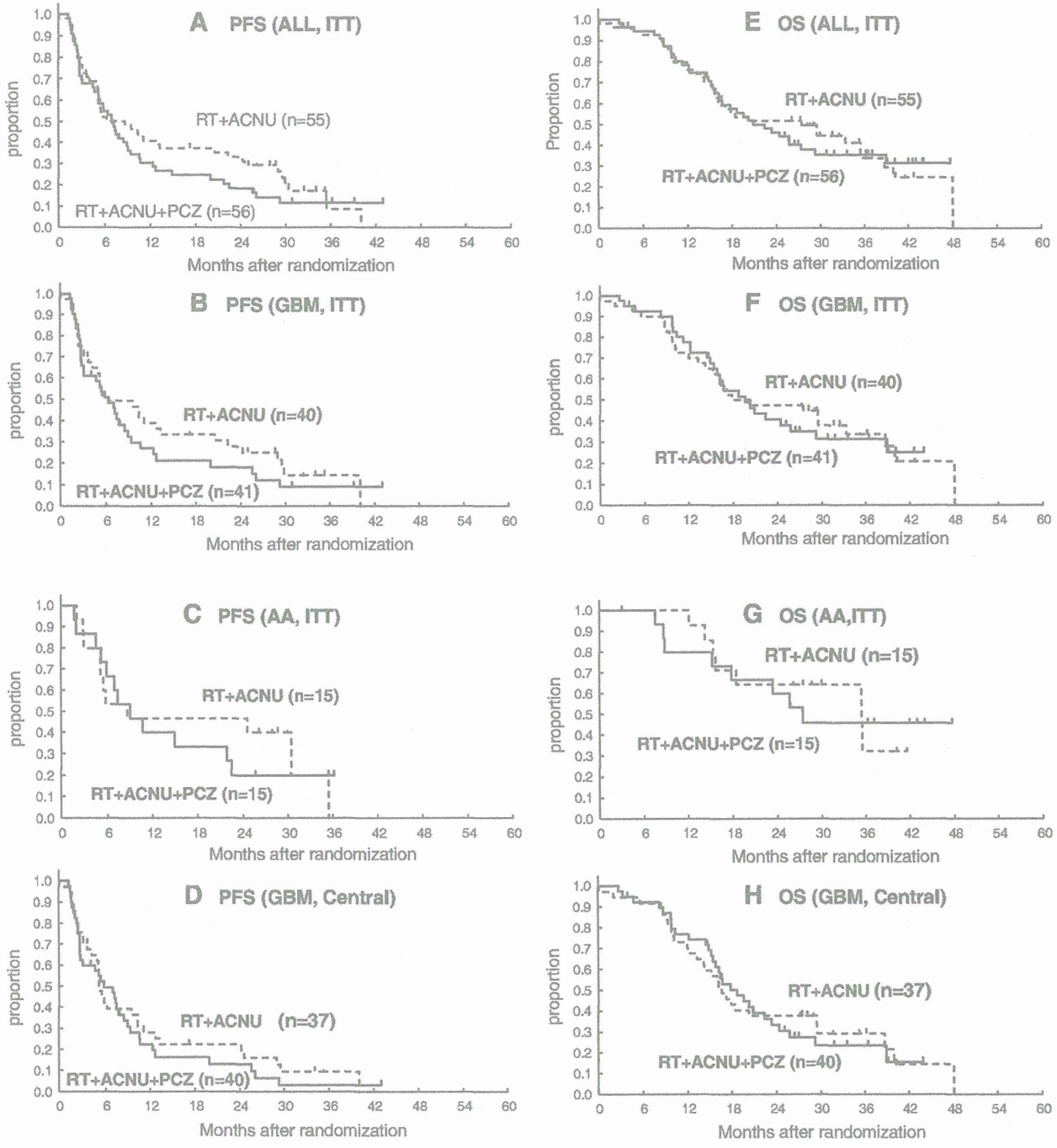

Fig. 2 Progression-free survival in the intention-to-treat (ITT) population (**a**), the GBM subgroup (**b**), the AA subgroup (**c**), and the GBM subgroup with central pathology review (**d**). Overall survival in the ITT population (**e**), the GBM subgroup (**f**), the AA subgroup (**g**), and the GBM subgroup with central pathology review (**h**). RT + ACNU alone (*solid line*), RT + ACNU + PCZ (*dotted line*)

Overall survival and cause of death

From the entire ITT population, 35 patients died in each group. In arm A ($n = 55$), OS was 27.4 months (95 % CI 16.2–35.4), compared with 22.4 months (95 % CI 16.4–29.2) in arm B ($n = 56$) (Fig. 2e). The %2-year survival in arms A and B was 51.9 % and 46.2 %, respectively. There was no difference between the 2 arms ($p = 0.75$ and pre-planned, one-sided $p = 0.62$, by stratified log-rank test).