Original Article

The clinicopathological evaluation of cyclooxygenase-2 expression in meningioma: immunohistochemical analysis of 76 cases of low- and high-grade meningioma

Yasutaka Kato[1], Hiroshi Nishihara[2], Hiromi Mohri[3], Hiromi Kanno[1], Hiroyuki Kobayashi[4], Taichi Kimura[1], Mishie Tanino[1], Shunsuke Terasaka[4] and Shinya Tanaka[1].

[1]Department of Cancer Pathology, [2]Department of Translational Pathology, and [4]Department of Neurosurgery, Hokkaido University, Graduate School of Medicine, Sapporo, Japan

[3]Laboratory of Oncology, Hokuto Hospital, Obihiro, Japan

Correspondence: Hiroshi Nishihara, MD., PhD.
Laboratory of Translational Pathology, Hokkaido University School of Medicine, North 15, West 7, Kita-ku, Sapporo, 060-8638, Japan.
Tel. +81-11-706-5053
Fax +81-11-706-5902
E-mail: hnishihara@med.hokudai.ac.jp

Key words: Cyclooxygenase-2, Meningioma, immunohistochemistry, clinicopathological analysis.

Abstract

The tumorigenic role of cyclooxygenase-2 (COX-2), a rate-limiting enzyme for the production of prostaglandins (PGs), has been proved in various types of cancer including brain tumors. In this analysis, we evaluated the expression of COX-2 in meningioma, which is one of the most common intracranial tumors in adults and accounts for 24 to 30 % of total intracranial tumors. We performed immunostaining for COX-2 in 76 cases of meningioma consisting of 44 cases of low-grade (WHO Grade I), and 32 cases of high-grade meningioma (29 cases of Grade II and 3 cases of Grade III), and evaluated COX-2 expression levels by staining intensity and the proportion in tumor cells. The expression level of COX-2 in meningioma cells was significantly correlated with WHO grade (P = 0.0314). In addition, the COX-2 expression was significantly correlated with MIB-1 labeling index in 76 cases of meningioma (P = 0.0001), suggesting the tumor promotive role of COX-2 in meningioma progression. Our results may indicate the therapeutic value of non-steroidal anti-inflammatory drugs against meningioma patients, especially with elevated proliferation rate, to regulate the tumorigenic role of COX-2 in meningioma cells.

Introduction

Meningioma is one of the most common intracranial tumors in adults and accounts for 24 to 30% of brain tumors[1,2]. According to the World Health Organization (WHO) classification 2007, meningiomas are histologically classified into Grade I, II and III[3], and about 90% of meningiomas belong to low-grade (Grade I), benign tumor; however, approximately 10% are classified as high-grade (Grade II or III), exhibiting an unfavorable clinical course[2]. Grade II and III meningiomas represent a risk of recurrence of 30-40% and 50-80%, respectively, after surgical resection[4], and the five-year survival rates of Grade II and III were 67.5% and 60%, respectively[5]. In addition, even low-grade meningiomas of Grade I sometimes show recurrence and/or malignant progression[4,6]. The therapeutic modality to high-grade meningioma includes surgical resection, preoperative embolization, adjuvant radiotherapy, and multidrug chemotherapy. However, the treatment efficacy, especially of chemotherapy, was limited and there is currently no standardized treatment for recurrent high-grade meningiomas after surgery and radiation therapy. Thus the proper diagnosis of high-grade meningioma based on reliable molecular markers is required of neuropathologists as well as neurosurgeons to treat the patients with high-grade meningioma and recurrent benign meningioma.

Cyclooxygenase-2 (COX-2), a rate-limiting enzyme for the production of prostaglandins (PGs), is induced in response to mitogens and pro-inflammatory

cytokines[7,8]. One of the key events in the development of cancer is the overexpression of COX-2. In particular, the tumorigenic role of COX-2 and also its products such as PGE_2 and $PGF_2\alpha$ have been well described during colorectal cancer development[9-15]. The correlation between COX-2 expression and MIB-1 labeling index was also previously shown in breast, renal and gastric cancers[16-18]. Moreover, long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) to inhibit COX, such as sulindac or aspirin, is associated with a 40-50% reduction in the incidence of colon adenomas and carcinomas[19-21]. Although the expression of COX-2 was also discussed in brain tumors including glioma and meningioma[22,23], the number of studies was limited and the clinicopathological value of COX-2 expression is still controversial, especially for meningioma. In this analysis, we performed immunohistochemical analysis of COX-2 expression in 76 cases of meningioma including 32 high-grade cases to explore the clinicopathological and prognostic value of COX-2 expression in meningioma.

Materials and methods

Patients

This study was performed with the approval of the Internal Review Board on Ethical issues of Hokkaido University Graduate School of Medicine, Sapporo, Japan. The samples and the patients' information were obtained under a blanket written informed consent. The subjects of this study were 76 patients who underwent surgery for meningioma between 2005 and 2012 at Hokkaido Neurosurgical Memorial Hospital, Sapporo Azabu Neurosurgical Hospital, Kashiwaba Neurosurgical Hospital, Nakamura Memorial Hospital, Sapporo, Japan, and Hokuto Hospital, Obihiro, Japan.

Outcomes of patients were analyzed as overall survival (OS) and progression-free survival (PFS). OS was defined as the time from surgery to death from any cause and PFS as the time from surgery to first evidence of progression of disease or death from any cause. The mean post-operative time was 19.4 months (range 1.4 - 108.8 months). The mean follow-up time was 23.7 months (range 1.4 - 108.8 months). The detail of each patient was showed in Supplemental Table1.

Classification methods

All patients were firstly classified according to the WHO Classification of Tumors of the

Central Nervous System (4th edition). Routinely formalin-fixed, paraffin-embedded tissue sections of tumors were stained with hematoxylin and eosin (H&E) and used for pathological diagnosis.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections of meningioma specimens. After deparaffinization and dehydration, specimens were brought to the boil in EDTA buffer (pH 9.0). Anti-COX2 antibody (Cayman Chemical, Michigan, USA; polyclonal, 1:100 dilution) and anti-Ki67 (Dako, Tokyo, Japan, Clone MIB-1, M7240, 1:100 dilution) were incubated at 4°C overnight, and reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision/HRP; Dako). Each slide was evaluated independently by three pathologists (Y. K., H. M., and H. N.). Immunostaining of COX-2 was evaluated in terms of the proportion and staining intensity of tumor cells. The proportion was assessed according to the percentage of immunopositive cells as follows: 0, 0 %; +1, less than 10 %; +2, 10 to 50 %; and +3, greater than 50 %. The staining intensity was evaluated as weak (+1), moderate (+2) and strong (+3). When the sum of the proportion score and intensity score was more than 4 (range 0 to 6), we evaluated it as "high-level" of COX-2 in tumor cells. The MIB-1 labeling index was calculated in a representative maximal activity and evaluated by counting the percentage of positive nuclei

in a high-powered field of the whole neoplastic lesion; all degrees of nuclear staining intensity were taken into consideration.

Statistical analysis

All calculations were performed by the statistical software Ekuseru-Toukei 2012 software for Windows (Social Survey Research Information Co., Ltd., Tokyo, Japan). The correlations between COX-2 scores and WHO grade and MIB-1 index were analyzed by Pearson's chi-square test in addition to Spearman's rank correlation coefficient.

Results

Patients' characteristics

The clinicopathological characteristics of these cases are summarized in Table 1. Mean age of patients was 60.3 years (range, 17 to 90). Twenty-three patients (30.3 %) were males, and the remaining 53 patients (69.7 %) were females. The number of the patients with WHO Grade I meningioma was 44 (57.9 %), with Grade II meningioma was 29 (38.2 %) and with Grade III meningioma was 3 (3.9 %). The histological subtypes of meningioma were varied as shown in Table 1. The representative H&E sections are shown in Fig. 1 and Supplemental Figure 1.

COX-2 expression in meningioma cells according to the WHO grade

To confirm the specificity of COX-2 staining, we compared COX-2 immunoreactivity in meningioma cells with that in colon cancer cells in which COX-2 expression had been confirmed in previous study[24]. As a result, similar COX-2 staining was observed in meningioma cells as well as in colon cancer cells (Supplemental Figure2). We also observed distinct expression of COX-2 in macrophages which were identified in tumor stroma (Fig. 1 and Supplemental Figure 2). In addition, we performed Reverse Transcription Polymerase Chain Reaction (RT-PCR) and detected COX-2 mRNA in tissue section of meningioma (Supplemental Figure 2). We finally concluded that the COX-2

staining in meningioma was now suitable for the evaluation of COX-2 expression in meningioma cells.

For objective evaluation of COX-2 by immunohistochemistry, we employed the immunohistochemical scoring system as indicated in Materials and Methods, in which the total score was obtained by the sum of the proportion score and intensity score, and the result is shown in Table 2. COX-2 was identified in the cytoplasm of meningioma cells as well as of inflammatory cells (Fig. 1B, F, J). Among the 76 cases, 19 cases (25.0 %) had a total score of 0, meaning absence of COX-2 expression, and 37 cases (48.7 %) were categorized into high-level of COX-2 due to a total score of more than 4. We divided the 76 cases into low- (Grade I) and high-grade (Grade II, III), and compared the COX-2 expression level according to the immunohistochemical total score (Table 3 and Supplemental Figure 3). As a result, the COX-2 expression level was statistically correlated with the WHO grade in meningioma by Pearson's chi-square test ($P = 0.0153$); i,e., high-grade meningiomas express much higher levels of COX-2 in tumor cells.

Correlation of COX-2 expression with MIB-1 labeling index in meningioma

We performed immunostaining for the 76 cases with Ki-67 antibody to obtain the MIB-1 labeling index as a cell proliferation index and compare it with the COX-2 expression level. The representative pictures of immunohistochemistry are shown in Fig. 2 (A –D), and the

distribution of MIB-1 labeling index according to the total score of COX-2 is indicated in Fig. 2E and Table 3. The scatter plot analysis revealed the significant relation between COX-2 expression level and MIB-1 labeling index (R^2=1.0) (Fig. 2E). In addition, Spearman's rank correlation coefficient also confirmed this result (Table 3).

Discussion

The excessive expression of COX-2 and its prognostic impact were discussed in various types of cancer such as colon[24-26], breast[27,28] and lung cancer[29]. In meningeal tumor, only 4 previous studies reported immunohistochemical expression of COX-2[22,30-32], and only one report among them revealed the statistical correlation between COX-2 expression and WHO grade in meningioma[22]. However, this result was based on the meningioma grade by the previous version of WHO classification for brain tumor (2^{nd} edition; 1993), and the statistical value was diminished when the updated WHO classification (3^{rd} edition; 2000) was applied[22]. Our study is the first report for COX-2 expression according to the histological tumor grade of meningioma based on the recent version of WHO classification (4^{th} edition; 2007). In addition, we explored the evidence that COX-2 expression level was also correlated with the MIB-1 labeling index of meningioma, suggesting that COX-2 should play a pivotal role in meningioma progression. The fact that inhibition of COX-2 by NSAIDs in meningioma cell lines decreased MIB-1 labeling index in tumor xenografts[33] supports our result.

The prognostic value of COX-2 was still controversial even for colorectal cancer. Although some previous reports for colon cancer explored that COX-2 expression in cancer cells was associated with poor patients' prognosis[24,25], another report indicated that COX-2 expression could not become an independent prognostic factor[26]. For meningeal

tumor, there was no previous prognostic report associated with COX-2 expression; therefore, we examined the correlation of COX-2 expression level in tumor cells with overall survival and progression free survival in 32 cases of high-grade meningioma patients. We divided the 32 cases into low-level (total score: less than 3) and high-level (total score: more than 4) of COX-2 (Table 3), and analyzed them by the Kaplan-Meier method. As a result, there was no significant correlation between COX-2 expression level and patients' prognosis, even in progression free survival (Supplemental Figure 4), although we could not exclude the possibility that therapeutic differences might affect these results.

Currently there is no established standard chemotherapy for high-grade meningioma and recurrent benign meningioma. Molecular targeting therapy based on the various molecular alterations of meningioma tumorigenesis and progression is in high demand[34]. The expression of several growth factor receptors, including platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), and basic fibroblast growth factor receptor (BFGFR,) have been identified in meningioma so far[35-38]; however, the clinical trials with molecular targeting drugs against such growth factor receptors demonstrated minimal efficacy[39-43]. COX-2 and its product, eicosanoids, are the possible target molecules for cancer treatment[44]; in fact, the clinical benefit in administration of NSAIDs such as aspirin and sulindac to the patients with familial adenomatous polyposis was reported[45,46]. In addition, several cohort studies revealed

that use of NSAIDs including aspirin was significantly correlated with favorable patients' prognosis in colon cancer[47-49]. In terms of pharmacological target of NSAIDs, we should not ignore the involvement of inflammatory cells such as macrophages and lymphocytes in tumor stroma, because COX-2 is highly induced in macrophages and other cells under conditions of inflammatory stimuli [50]. In addition, the positive feedback loop of COX-2 expression through PGE_2 and cAMP was previously described in various types of non-cancerous cells such as macrophages and vascular endothelial cells [51]. Both atypical and anaplastic meningiomas often show necrosis, which could be accompanied by infiltrates of inflammatory cells, including neutrophils and macrophages, and a recent study showed the co-existence of CD45 (-) neoplastic cells and CD45(+) immune infiltrating cells including macrophages in all meningiomas[52]. In fact, we have recognized the infiltration of macrophages with COX-2 expression in stroma of meningioma during this analysis (Fig. 1). The clinical benefit of NSAIDs usage for patients with malignant tumor might be obtained by inhibition of the positive feedback loop of COX-2 expression among the tumor cells and inflammatory cells in stroma. Our results are giving us the hope to treat the patients with high-grade meningioma using NSAIDs in combination with another chemotherapeutic agents and/or radiation.

Acknowledgements

We thank Dr. Tamio Itoh (Nakamura Memorial Hospital), Dr. Masahito Kato (Hokkaido neurosurgical memorial hospital), Dr. Shin Fujimoto (Kashiwaba Neurosurgical Hospital) and Dr. Junichi Murata (Sapporo Azabu neurosurgical hospital) for providing clinical data for this analysis.

This research was supported by a Grant-in-Aid for Scientific Research (KAKENHI) and by the Japan Society for the Promotion of Science (JSPS) through the “Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program),” initiated by the Council for Science and Technology Policy (CSTP).

References

1. **Aghi MK, Carter BS, Cosgrove GR, Ojemann RG, Amin-Hanjani S, Martuza RL, et al. (2009) Long-term recurrence rates of atypical meningiomas after gross total resection with or without postoperative adjuvant radiation. Neurosurgery 64: 56-60; discussion 60.**
2. **Norden AD, Drappatz J, Wen PY (2009) Advances in meningioma therapy. Curr Neurol Neurosci Rep 9: 231-240.**
3. **Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, editors (2007) WHO Classification of Tumours of the Central Nervous System, 4th Edition: IARC Press, Lyons. p 164-172 p.**
4. **Lamszus K (2004) Meningioma pathology, genetics, and biology. J Neuropathol Exp Neurol 63: 275-286.**
5. **Pasquier D, Bijmolt S, Veninga T, Rezvoy N, Villa S, Krengli M, et al. (2008) Atypical and malignant meningioma: outcome and prognostic factors in 119 irradiated patients. A multicenter, retrospective study of the Rare Cancer Network. Int J Radiat Oncol Biol Phys 71: 1388-1393.**
6. **Al-Mefty O, Kadri PA, Pravdenkova S, Sawyer JR, Stangeby C, Husain M (2004) Malignant progression in meningioma: documentation of a series and analysis of cytogenetic findings. J Neurosurg 101: 210-218.**
7. **Wu KK (1995) Inducible cyclooxygenase and nitric oxide synthase. Adv Pharmacol 33: 179-207.**
8. **Smith WL, Garavito RM, DeWitt DL (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 271: 33157-33160.**
9. **Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107: 1183-1188.**

10. Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, et al. (1995) Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res 55: 3785-3789.

11. Kargman SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA, Jothy S (1995) Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. Cancer Res 55: 2556-2559.

12. Wendum D, Masliah J, Trugnan G, Flejou JF (2004) Cyclooxygenase-2 and its role in colorectal cancer development. Virchows Arch 445: 327-333.

13. Dekanty A, Giulianelli S, Coso OA, Rudland PS, Jimenez de Asua L (2006) Differential involvement of ERK1-2 and p38MAPK activation on Swiss 3T3 cell proliferation induced by prostaglandin F2alpha. FEBS Lett 580: 2512-2516.

14. Nishihara H, Kizaka-Kondoh S, Insel PA, Eckmann L (2003) Inhibition of apoptosis in normal and transformed intestinal epithelial cells by cAMP through induction of inhibitor of apoptosis protein (IAP)-2. Proc Natl Acad Sci U S A 100: 8921-8926.

15. Nishihara H, Hwang M, Kizaka-Kondoh S, Eckmann L, Insel PA (2004) Cyclic AMP promotes cAMP-responsive element-binding protein-dependent induction of cellular inhibitor of apoptosis protein-2 and suppresses apoptosis of colon cancer cells through ERK1/2 and p38 MAPK. J Biol Chem 279: 26176-26183.

16. Miyata Y, Koga S, Kanda S, Nishikido M, Hayashi T, Kanetake H (2003) Expression of cyclooxygenase-2 in renal cell carcinoma: correlation with tumor cell proliferation, apoptosis, angiogenesis, expression of matrix metalloproteinase-2, and survival. Clin Cancer Res 9: 1741-1749.

17. Boland GP, Butt IS, Prasad R, Knox WF, Bundred NJ (2004) COX-2 expression is associated with an aggressive phenotype in ductal carcinoma in situ. Br J Cancer 90: 423-429.

18. Joo YE, Chung IJ, Park YK, Koh YS, Lee JH, Park CH, et al. (2006) Expression of

cyclooxygenase-2, p53 and Ki-67 in gastric cancer. J Korean Med Sci 21: 871-876.

19. Thun MJ, Henley SJ, Patrono C (2002) Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J Natl Cancer Inst 94: 252-266.

20. Marnett LJ, DuBois RN (2002) COX-2: a target for colon cancer prevention. Annu Rev Pharmacol Toxicol 42: 55-80.

21. Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresalier R, et al. (2003) A randomized trial of aspirin to prevent colorectal adenomas. N Engl J Med 348: 891-899.

22. Lin CC, Kenyon L, Hyslop T, Hammond E, Andrews DW, Curran WJ, Jr., et al. (2003) Cyclooxygenase-2 (COX-2) expression in human meningioma as a function of tumor grade. Am J Clin Oncol 26: S98-102.

23. Nathoo N, Barnett GH, Golubic M (2004) The eicosanoid cascade: possible role in gliomas and meningiomas. J Clin Pathol 57: 6-13.

24. Al-Maghrabi J, Buhmeida A, Emam E, Syrjanen K, Sibiany A, Al-Qahtani M, et al. (2012) Cyclooxygenase-2 expression as a predictor of outcome in colorectal carcinoma. World J Gastroenterol 18: 1793-1799.

25. Masunaga R, Kohno H, Dhar DK, Ohno S, Shibakita M, Kinugasa S, et al. (2000) Cyclooxygenase-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients. Clin Cancer Res 6: 4064-4068.

26. Ogino S, Kirkner GJ, Nosho K, Irahara N, Kure S, Shima K, et al. (2008) Cyclooxygenase-2 expression is an independent predictor of poor prognosis in colon cancer. Clin Cancer Res 14: 8221-8227.

27. Costa C, Soares R, Reis-Filho JS, Leitao D, Amendoeira I, Schmitt FC (2002) Cyclo-oxygenase 2 expression is associated with angiogenesis and lymph node metastasis in human breast cancer. J Clin Pathol 55: 429-434.

28. Dhakal HP, Naume B, Synnestvedt M, Borgen E, Kaaresen R, Schlichting E, et al. (2012) Expression of cyclooxygenase-2 in invasive breast carcinomas and its prognostic impact. Histol Histopathol 27: 1315-1325.

29. Li F, Liu Y, Chen H, Liao D, Shen Y, Xu F, et al. (2011) EGFR and COX-2 protein expression in non-small cell lung cancer and the correlation with clinical features. J Exp Clin Cancer Res 30: 27.

30. Pfister C, Ritz R, Pfrommer H, Bornemann A, Tatagiba MS, Roser F (2007) Are there attacking points in the eicosanoid cascade for chemotherapeutic options in benign meningiomas? Neurosurg Focus 23: E8.

31. Ragel BT, Jensen RL, Gillespie DL, Prescott SM, Couldwell WT (2005) Ubiquitous expression of cyclooxygenase-2 in meningiomas and decrease in cell growth following in vitro treatment with the inhibitor celecoxib: potential therapeutic application. J Neurosurg 103: 508-517.

32. Buccoliero AM, Castiglione F, Rossi Degl'Innocenti D, Arganini L, Taddei A, Ammannati F, et al. (2007) Cyclooxygenase-2 (COX-2) overexpression in meningiomas: real time PCR and immunohistochemistry. Appl Immunohistochem Mol Morphol 15: 187-192.

33. Ragel BT, Jensen RL, Gillespie DL, Prescott SM, Couldwell WT (2007) Celecoxib inhibits meningioma tumor growth in a mouse xenograft model. Cancer 109: 588-597.

34. Pham MH, Zada G, Mosich GM, Chen TC, Giannotta SL, Wang K, et al. (2011) Molecular genetics of meningiomas: a systematic review of the current literature and potential basis for future treatment paradigms. Neurosurg Focus 30: E7.

35. Smith JS, Lal A, Harmon-Smith M, Bollen AW, McDermott MW (2007) Association between absence of epidermal growth factor receptor immunoreactivity and poor prognosis in patients with atypical meningioma. J Neurosurg 106: 1034-1040.

36. Yang SY, Xu GM (2001) Expression of PDGF and its receptor as well as their relationship to proliferating activity and apoptosis of meningiomas in human meningiomas. J Clin Neurosci 8 Suppl 1: 49-53.

37. Lusis EA, Chicoine MR, Perry A (2005) High throughput screening of meningioma biomarkers using a tissue microarray. J Neurooncol 73: 219-223.

38. Wernicke AG, Dicker AP, Whiton M, Ivanidze J, Hyslop T, Hammond EH, et al. (2010) Assessment of Epidermal Growth Factor Receptor (EGFR) expression in human meningioma. Radiat Oncol 5: 46.

39. Norden AD, Raizer JJ, Abrey LE, Lamborn KR, Lassman AB, Chang SM, et al. (2010) Phase II trials of erlotinib or gefitinib in patients with recurrent meningioma. J Neurooncol 96: 211-217.

40. Reardon DA, Norden AD, Desjardins A, Vredenburgh JJ, Herndon JE, 2nd, Coan A, et al. (2012) Phase II study of Gleevec(R) plus hydroxyurea (HU) in adults with progressive or recurrent meningioma. J Neurooncol 106: 409-415.

41. Wen PY, Yung WK, Lamborn KR, Norden AD, Cloughesy TF, Abrey LE, et al. (2009) Phase II study of imatinib mesylate for recurrent meningiomas (North American Brain Tumor Consortium study 01-08). Neuro Oncol 11: 853-860.

42. Nayak L, Iwamoto FM, Rudnick JD, Norden AD, Lee EQ, Drappatz J, et al. (2012) Atypical and anaplastic meningiomas treated with bevacizumab. J Neurooncol 109: 187-193.

43. Lou E, Sumrall AL, Turner S, Peters KB, Desjardins A, Vredenburgh JJ, et al. (2012) Bevacizumab therapy for adults with recurrent/progressive meningioma: a retrospective series. J Neurooncol 109: 63-70.

44. Wang D, Dubois RN (2010) Eicosanoids and cancer. Nat Rev Cancer 10: 181-193.

45. Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, et al. (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous

polyposis. N Engl J Med 342: 1946-1952.

46. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, et al. (1993) Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med 328: 1313-1316.

47. Chan AT, Ogino S, Fuchs CS (2009) Aspirin use and survival after diagnosis of colorectal cancer. JAMA 302: 649-658.

48. Burn J, Gerdes AM, Macrae F, Mecklin JP, Moeslein G, Olschwang S, et al. (2011) Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. Lancet 378: 2081-2087.

49. Chan AT, Ogino S, Fuchs CS (2007) Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. N Engl J Med 356: 2131-2142.

50. Barrios-Rodiles M, Chadee K (1998) Novel regulation of cyclooxygenase-2 expression and prostaglandin E2 production by IFN-gamma in human macrophages. J Immunol 161: 2441-2448.

51. Klein T, Shephard P, Kleinert H, Komhoff M (2007) Regulation of cyclooxygenase-2 expression by cyclic AMP. Biochim Biophys Acta 1773: 1605-1618.

52. Domingues PH, Teodosio C, Ortiz J, Sousa P, Otero A, Maillo A, et al. (2012) Immunophenotypic identification and characterization of tumor cells and infiltrating cell populations in meningiomas. Am J Pathol 181: 1749-1761.

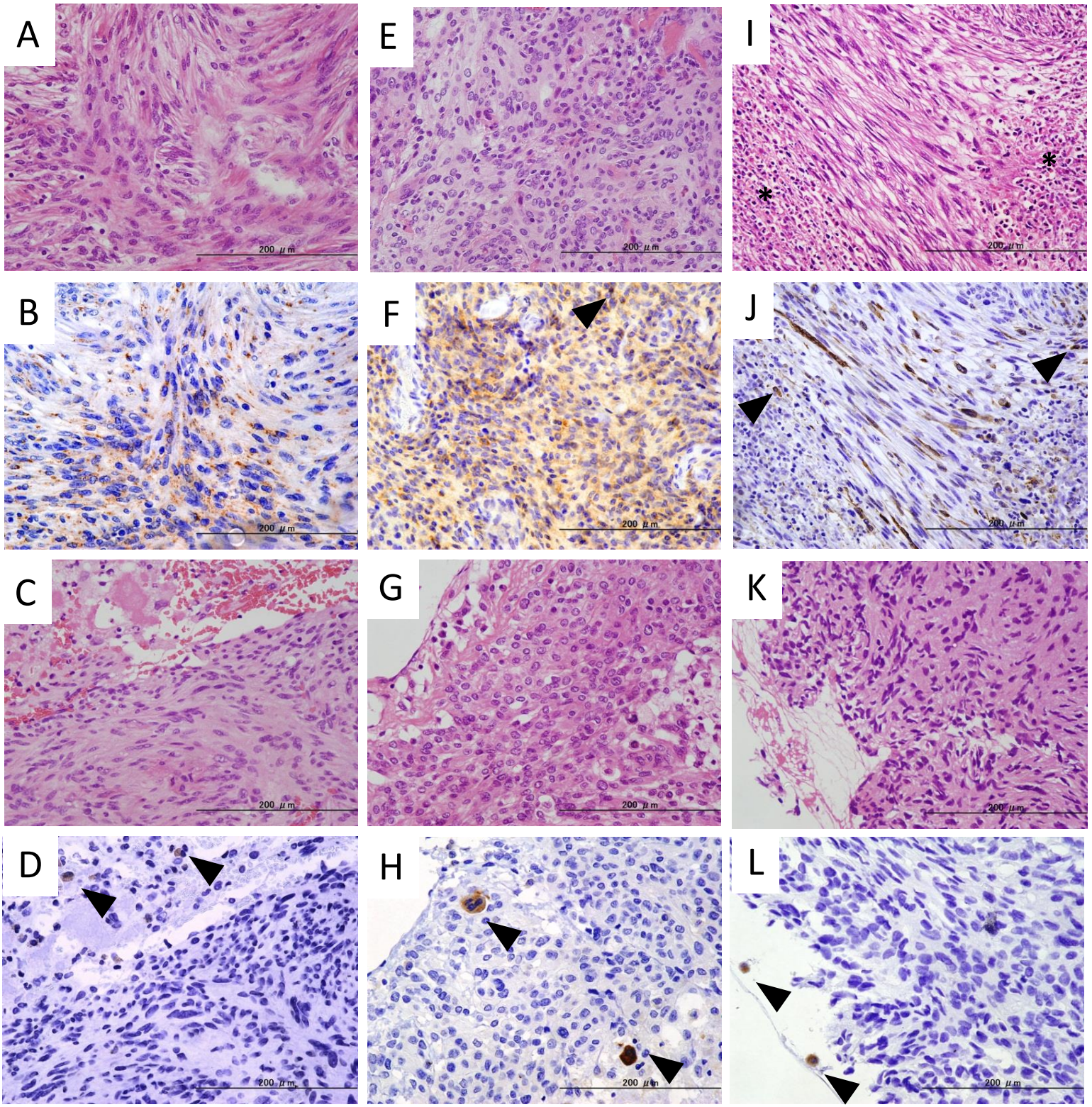
Figure legends

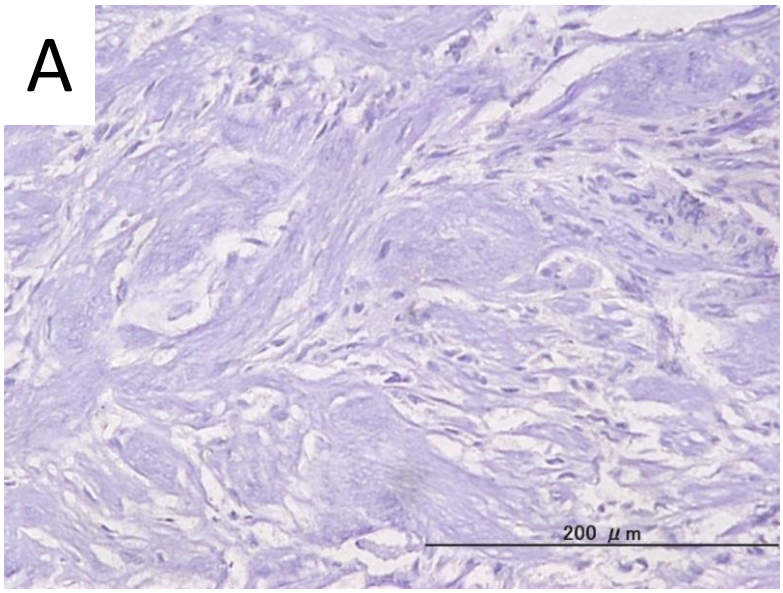
Fig. 1 Representative pictures of immunostaining for COX-2 in meningioma

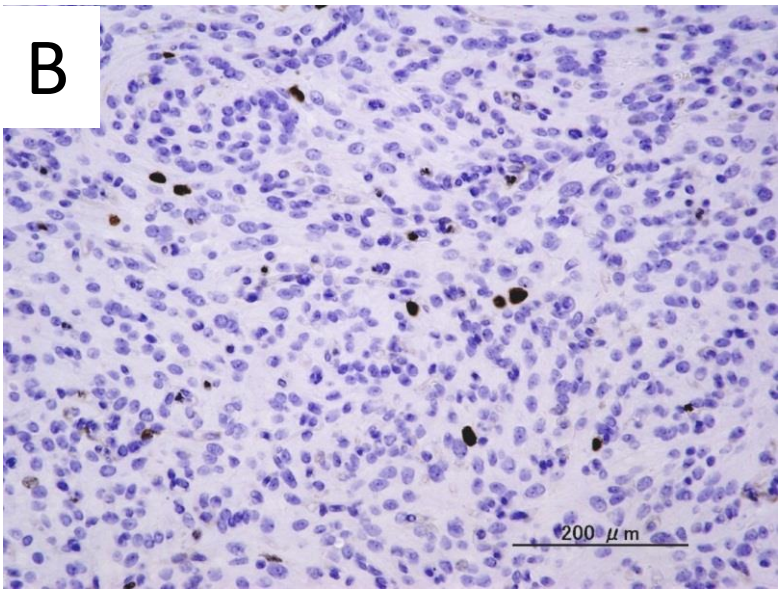
A, C, E, G, I, K: H&E stain, and B, D, F, H, J, L: COX-2 stain. A, B: WHO Grade I, fibrous meningioma. C, D: WHO Grade I, meningothelial meningioma. E, F, G, H: WHO Grade II, atypical meningioma. I, J, K, L: WHO Grade III, anaplastic meningioma. The intensity of COX-2 staining was as follows; B, J: score 2, F: Score 3. The arrowheads indicate distinct COX-2 expression in inflammatory cells including macrophages and plasma cells. The asterisks represent necrosis. All pictures are in x400. Scale bars: 200 μm.

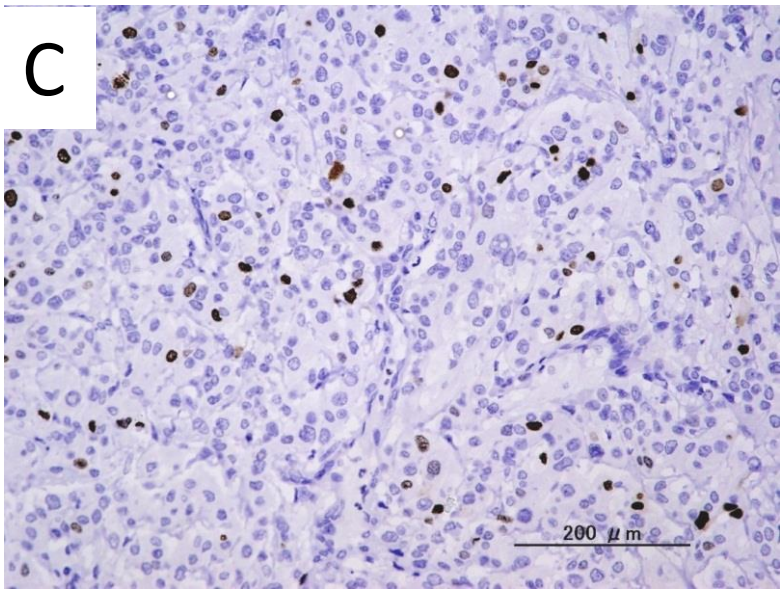
Fig. 2 Correlation of COX-2 expression with MIB-1 labeling index in meningioma

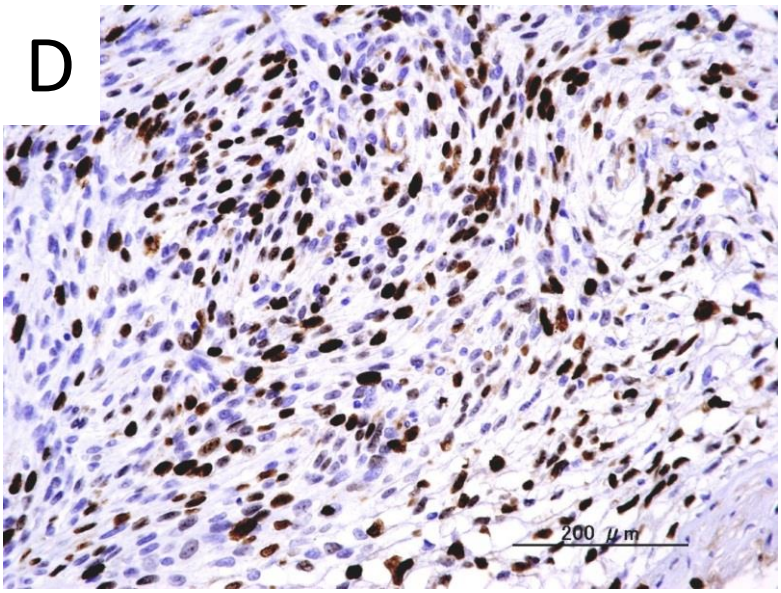
A-D: Representative staining of MIB-1. E: Scatter plot analysis of COX-2 expression level and MIB-1 index. A-E: Each picture represent MIB-1 index as follows: A: 0 %, B: low (1-5 %), C: middle (5-10 %), D: high (>10%). All pictures at the lower right are in x400 (Scale bars: 200 μm). E: Scatter plot analysis of two parameters; COX-2 expression level and MIB-1 index. There is a statistically strong relation between COX-2 expression level and MIB-1 index (R^2=1.0).


A
E
I
B
F
J
C
G
K
D
H
L
200 μm


A
200 μm


B
200 μm


C
200 μm


D
200 μm

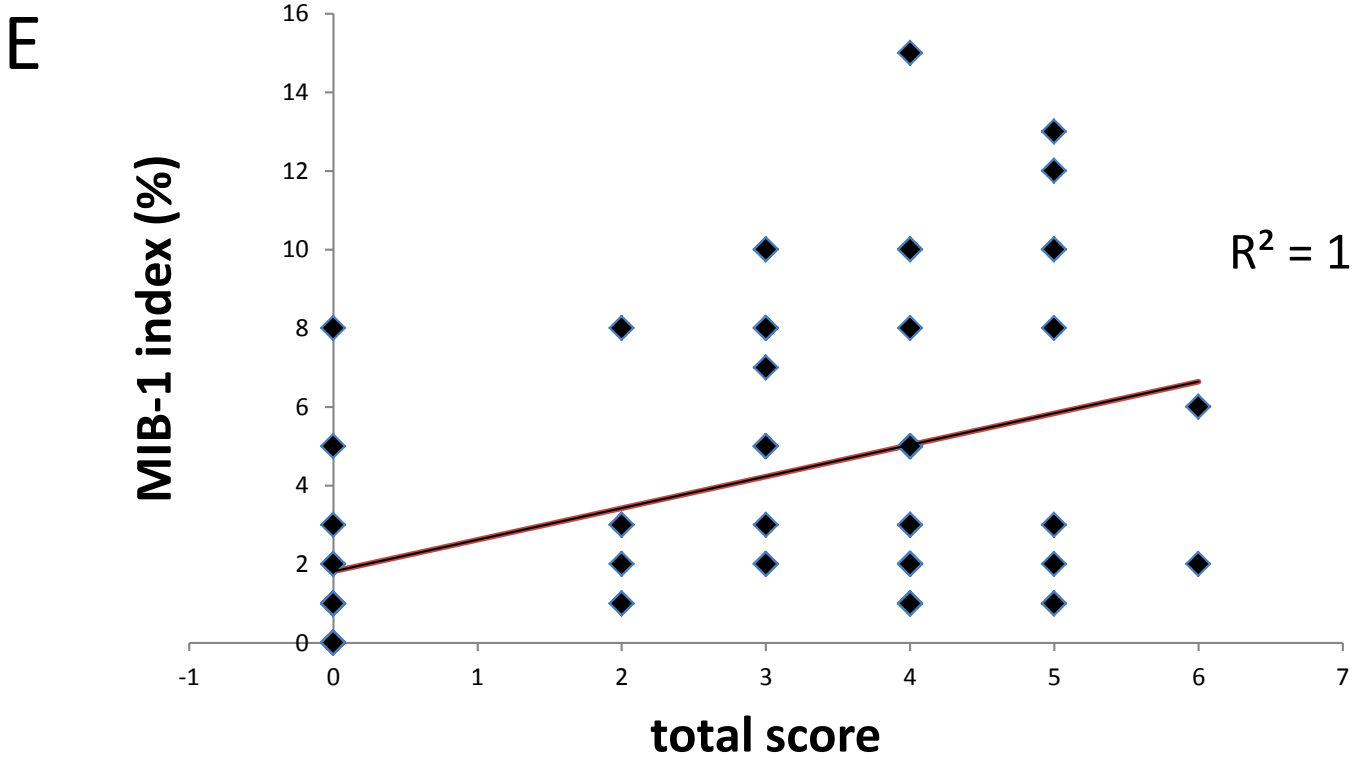

E
MIB-1 index (%)
16
14
12
10
8
6
4
2
0
-1
0
1
2
3
4
5
6
7
total score
R² = 1

Table 1. Histopathological features of meningioma

	No. (n=76)	%
Median age (range)	60.3 (17 to 90)	
Sex		
Male	23	30.3%
Female	53	69.7%
WHO grade		
I	44	57.9%
II	29	38.2%
III	3	3.9%
Histological type		
Fibrous meningioma	15	19.7%
Meningothelial meningioma	19	25.0%
Transitional meningioma	2	2.6%
Angiomatous meningioma	3	3.9%
Microcystic meningioma	3	3.9%
Psammomatous meningioma	1	1.3%
Secretory meningioma	1	1.3%
Atypical meningioma	27	35.5%
Transitional meningioma with brain invasion	1	1.3%
Anaplastic meningioma	3	3.9%
Unknown	1	1.3%

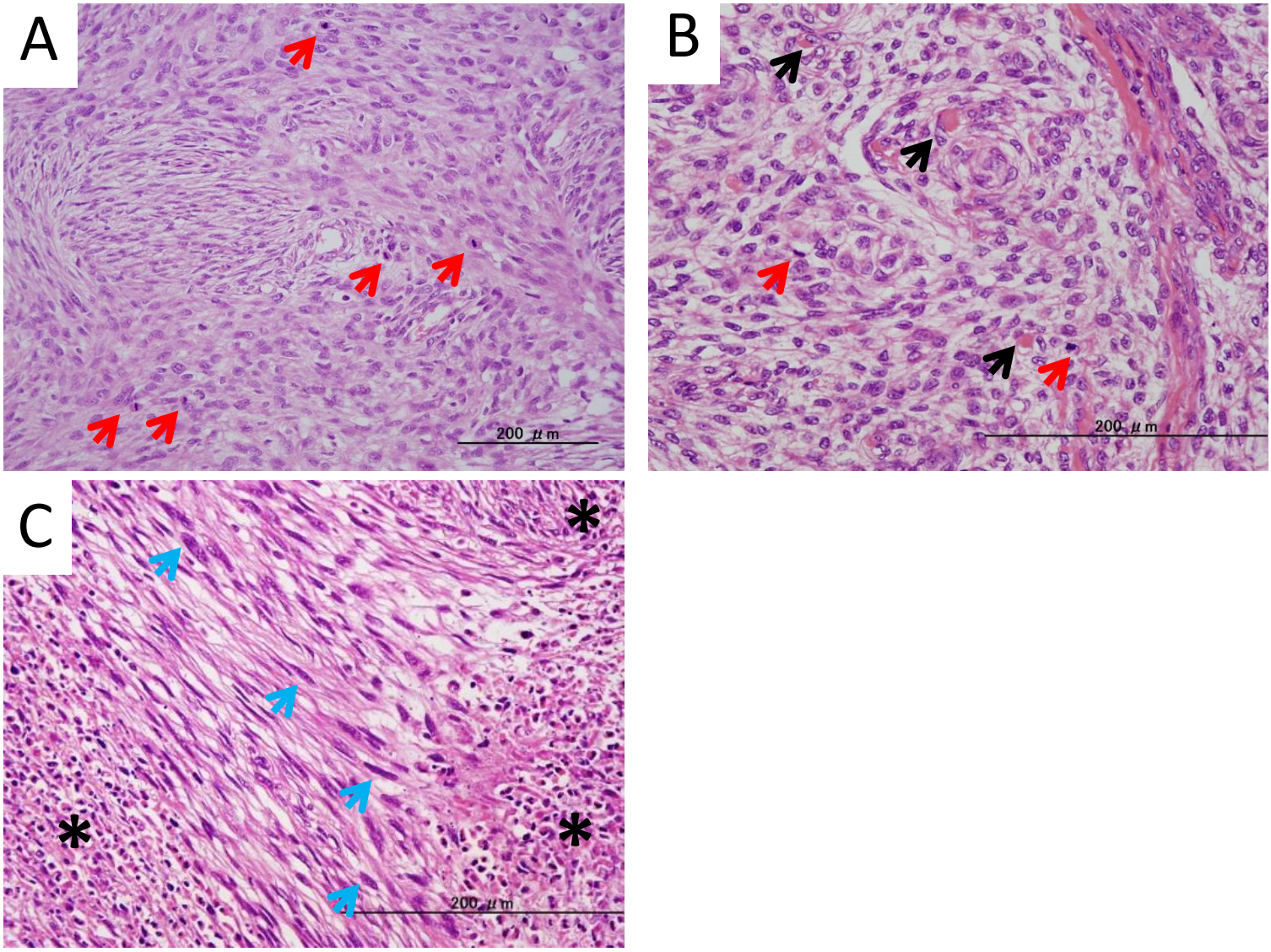
Table 2. Expression of COX-2 in meningioma

	Proportion score			
	0	1	2	3
	0%	1 %-10 %	10 %- 50 %	> 50 %
Staining intensity				
Score 0	19 (25.0 %)	0	0	0
Score 1	0	7 (9.2 %)	12 (15.8 %)	18 (23.7 %)
Score 2	0	1 (1.3 %)	6 (7.9 %)	10 (13.2 %)
Score 3	0	1 (1.3 %)	0	2 (2.6 %)

Table3. Corerelation between COX-2 expression and WHO grade / MIB-1 labeling index in meningioma

	Total		COX-2 expression												
			Low level						High level						
	No.	%	Total score<1		Total score 2		Total score 3		Total score 4		Total score 5		Total score 6		*p* value
1.WHO grade															
Grade I	44	55.7%	17	38.6%	5	11.4%	4	9.1%	14	31.8%	3	6.8%	1	2.3%	0.0153*
Grade II, III	32	40.5%	2	6.3%	2	6.3%	9	28.1%	11	34.4%	7	21.9%	1	3.1%	
2.MIB-1 index															
0%	5	6.6%	5	100.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0.0075*
1-4%	36	47.4%	11	30.6%	5	13.9%	3	8.3%	12	33.3%	4	11.1%	1	2.8%	
5-9%	23	30.3%	2	8.7%	1	4.3%	8	34.8%	9	39.1%	2	8.7%	1	4.3%	
>10%	12	15.8%	1	8.3%	1	8.3%	2	16.7%	4	33.3%	4	33.3%	0	0.0%	

Supplemental Figure1. Histological appearance of WHO GradeIII anaplastic meningioma.

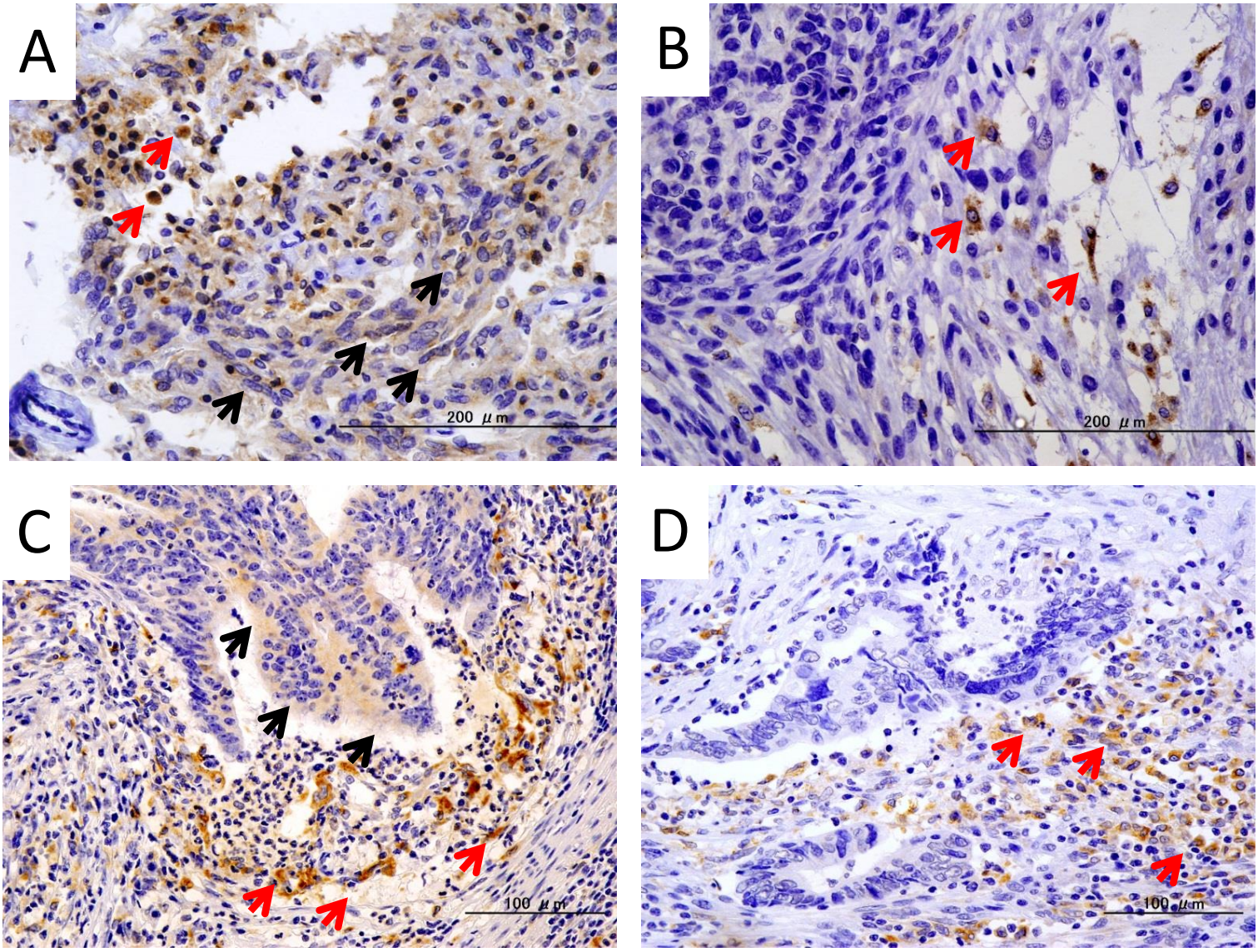


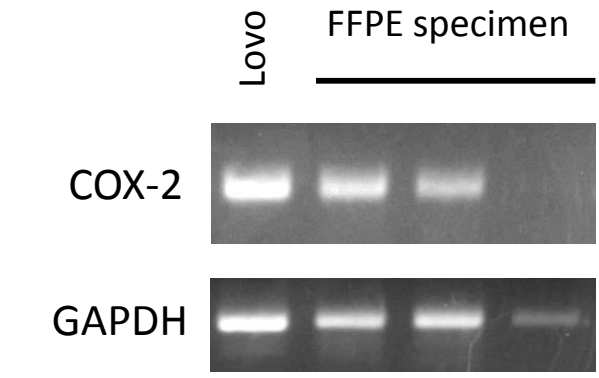
Supplemental Figure 1. Histological appearance of WHO Grade III anaplastic meningioma

The representative pictures of anaplastic meningioma are shown. A: This case (Case 5 in supplemental table 1) represented mitosis-rich lesion. B and C: Representative pictures showing rhabdoid and spindle shaped cells (Case 28 in supplemental table 1). The red arrow indicates mitosis, black arrow indicates rhabdoid cell with eccentrically placed nucleus and eosinophilic globular paranuclear inclusion. The blue arrow indicates spindle shaped, sarcomatoid cell. According to these histological features and MIB-1 index (Supplemental Table1), we diagnosed these cases as WHO Grade III meningioma.

Supplemental Figure2.

COX-2 expression in meningioma and colon cancer.





Supplemental Figure 2. COX-2 expression in meningioma and colon cancer.

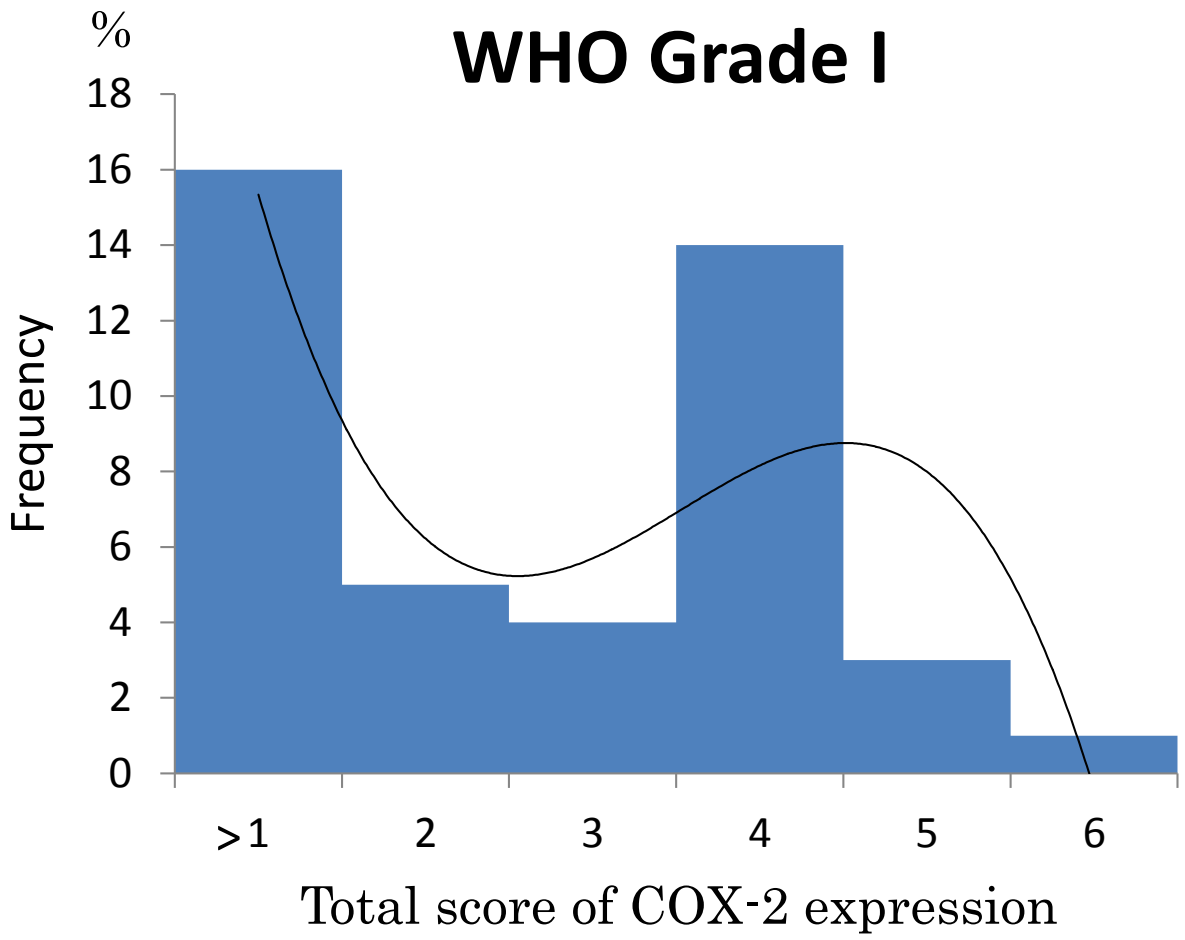
A, B; WHO grade 2, atypical meningioma. C, D; Colorectal adenocarcinoma. Black arrows indicate COX-2 expression in tumor cells (A; meningioma, C; colon cancer). Distinct COX-2 expression was observed in macrophages in all sections (red arrow). E: RT-PCR using FFPE specimen of atypical meningioma. Two of three samples expressed high-level of COX-2 mRNA, while Lovo, a colorectal adenocarcinoma cell line, expressed much higher level of COX-2 mRNA.

Supplemental material and method

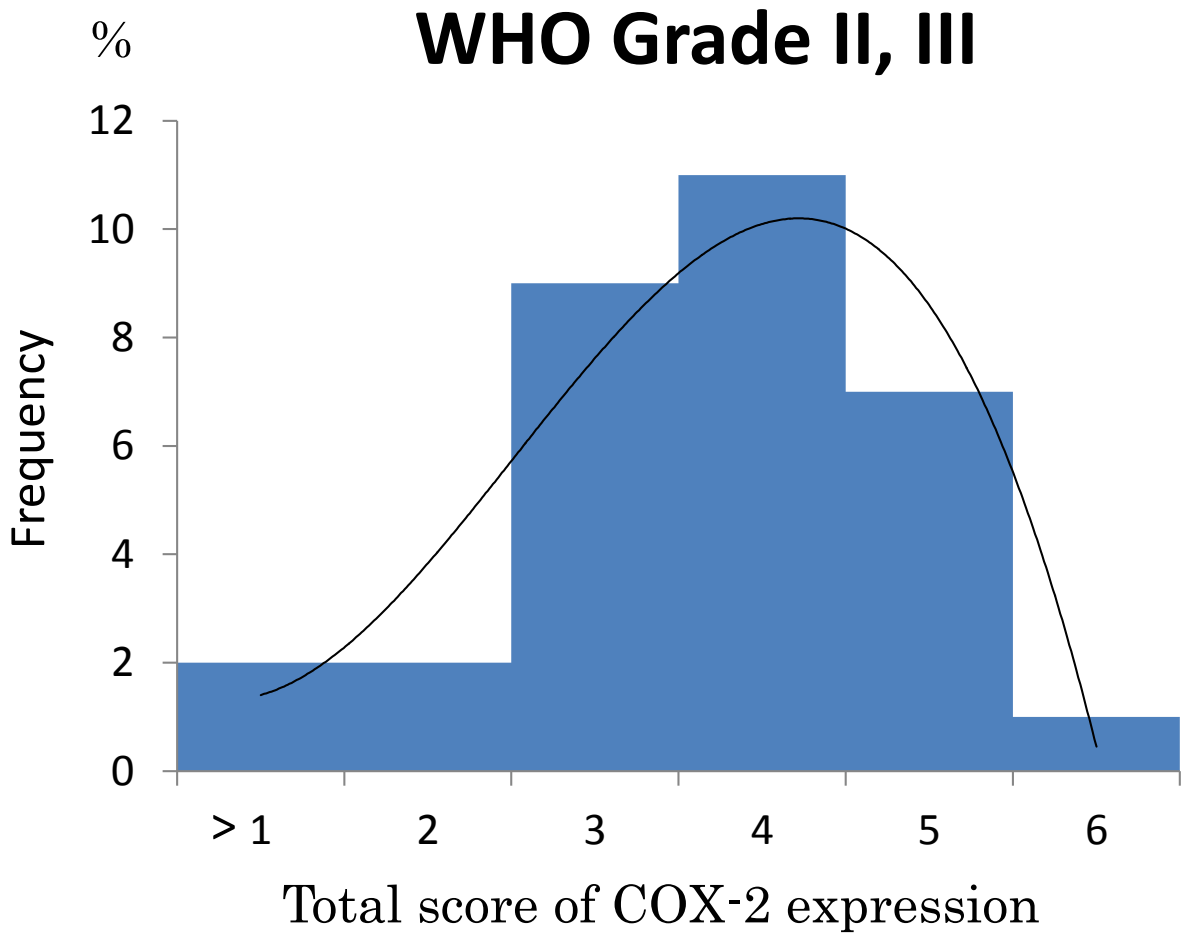
RNA Isolation and RT-PCR Analysis

Total RNA was isolated from formalin-fixed paraffin embedded (FFPE) specimens of three atypical meningioma following the manufacturer's instructions using the RNeasy FFPE Kit (Quiagen, Hilden, Germany). Complementary DNA was synthesized by using reverse transcriptase (Superscript II, Invitrogen, Karlsruhe, Germany) and oligo (dT) primers, and used as template for PCR reactions. PCR was carried out using COX-2 primers and GAPDH primers. . Primers for COX-2 were: forward- 5'-ATGCTCGCCCGCGCCCTGCTGCT-3', reverse- 5'-CCAGTATAAGTGCGATTGTACCCG-3' and GAPDH were: forward- 5'-CGGGTACAATCGCACTTATACTGG-3', reverse- 5'-GATGCAGGGATGATGTTC-3' . A PCR mixture (total volume 25 μL) was prepared that included 2 μL of the sample containing each nucleotide, each primer at final concentration of 200 nM, and 9.5 uL of Taq-polymerase (Go Taq Green Master Mix, Promega, Madison, WI, USA). As a positive control of RT-PCR, the colon cancer cell line (Lovo) was employed. Each PCR reaction was run for 33 cycles with a denaturation step for 30 sec at 95 °C, and an extension for 60 seconds at 72 °C, Annealing was for 30 seconds at 55 °C.

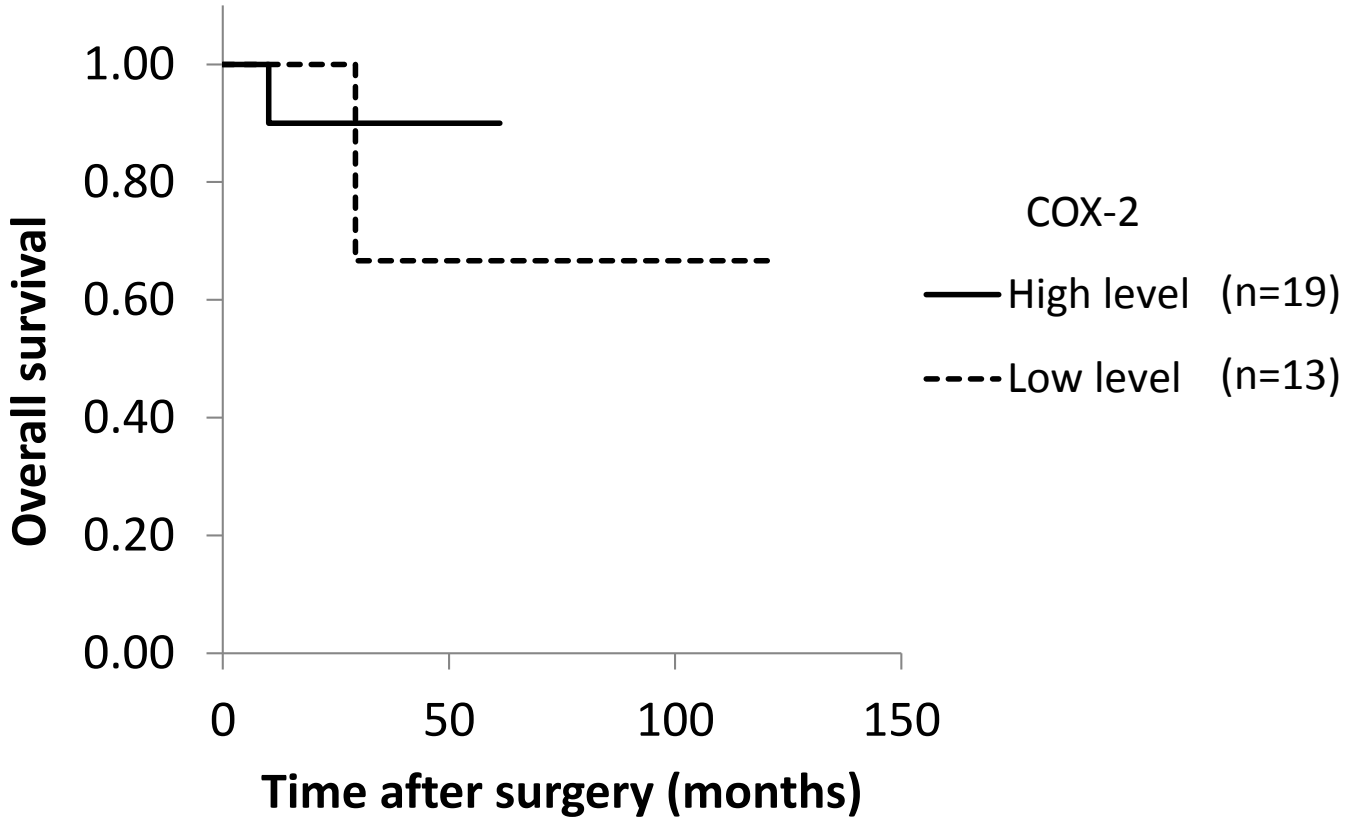
Supplemental Figure 3A. Frequency distribution graph of COX-2 expression in WHO Grade I meningioma.



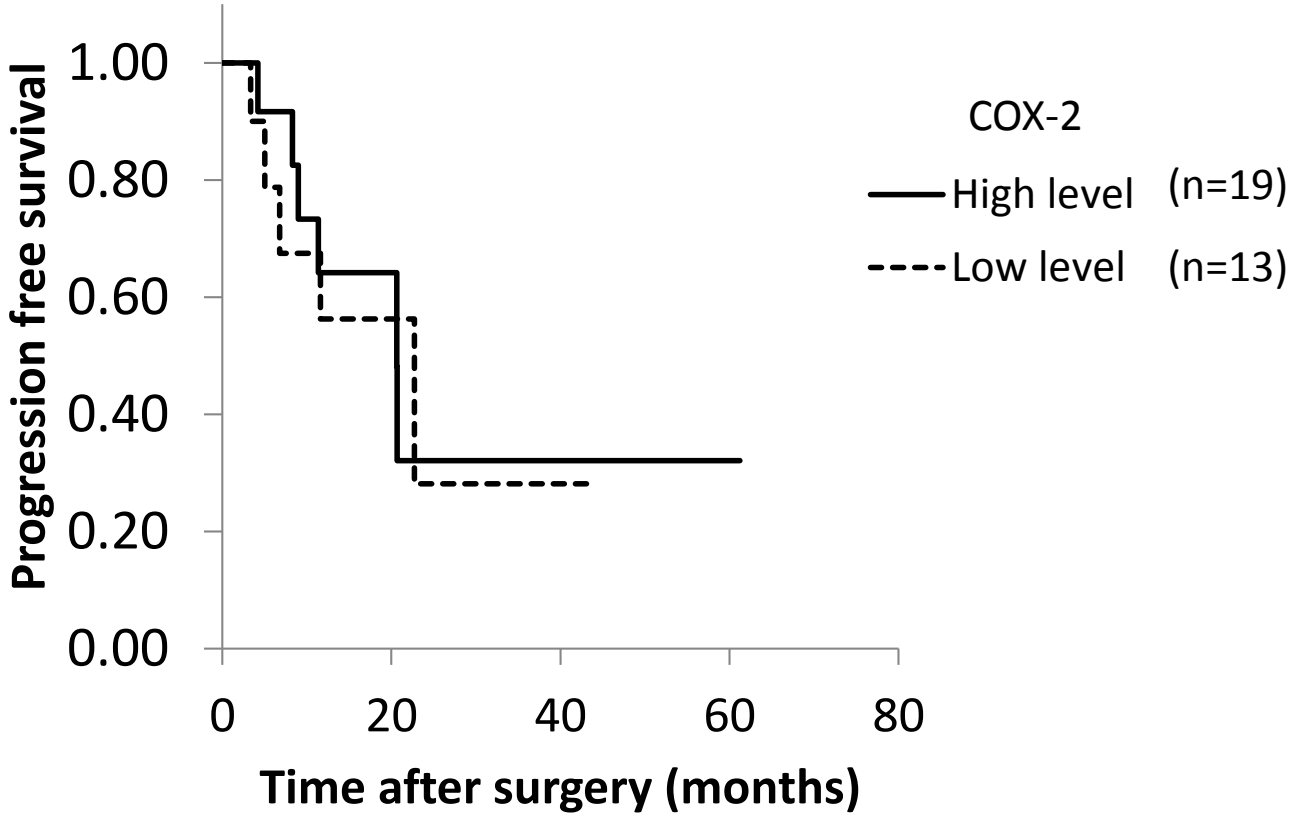
Supplemental Figure 3B. Frequency distribution graph of COX-2 expression in WHO Grade II and III meningioma.



Supplemental Figure4A. Overall survival (OS) analysis based on the expression level of COX-2



Supplemental Figure4B. Progression free survival (PFS) analysis according to expression level of COX-2.



Supplemental Figure 4. Survival analysis based on the expression level of COX-2 in WHO Grade II and III meningioma cells

Overall survival (OS; A) and progression free survival (PFS; B) according to the expression level of COX-2 in tumor cells. There is no statistically significance (OS; Log-rank 0.0014, P=0.9703, PFS; Log-rank 0.05, P=0.822). Time to survival, measured from the date of first surgical resection to disease progression and death, respectively, or the date of last follow-up visit was analyzed by the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival duration in the patient groups. Log-rank test was employed for comparing the curves.

Supplemental Table1. Clinicopathological data of 32 patients diagnosed as WHO GradeII and III meningioma

Case no.	Age	Sex	Histologic subtype	WHO Grade	COX-2 intensity score	COX-2 propotion score	COX-2 total score	MIB-1 index	Post-operative time (months)	Follow-up time (months)
1	64	M	Atypical meningioma	2	2	3	5	5	39.8	97.1
2	53	F	Atypical meningioma	2	2	3	5	8	18.1	18.1
3	70	F	Atypical meningioma	2	1	3	4	10	4.2	10.2
4	64	F	Atypical meningioma	2	1	3	4	10	2.5	5.8
5	79	F	Anaplastic meningioma	3	0	0	0	15	33.4	33.4
6	49	F	Anaplastic meningioma	3	1	1	2	20	20.7	21.6
7	61	F	Transitional meningioma with brain invasion	2	1	2	3	3	13.8	13.8
8	42	F	Atypical meningioma	2	1	2	3	15	11.6	14.2
9	82	M	Atypical meningioma	2	1	2	3	8	13.8	13.8
10	67	F	Atypical meningioma	2	1	3	4	8	11.2	11.5
11	71	M	Atypical meningioma	2	1	3	4	8	8.0	4.0
12	24	M	Atypical meningioma	2	1	3	4	5	1.4	1.4
13	63	M	Atypical meningioma	2	2	3	5	3	61.2	61.2
14	65	F	Atypical meningioma	2	1	2	3	5	43.6	43.6
15	35	M	Atypical meningioma	2	2	3	5	10	20.6	20.6
16	63	F	Atypical meningioma	2	1	2	3	5	18.2	18.2
17	66	F	Atypical meningioma	2	1	3	4	3	8.3	8.3
18	69	F	Atypical meningioma	2	1	3	4	5	18.7	18.7
19	64	F	Atypical meningioma	2	1	2	3	10	4.5	4.5
20	36	F	Atypical meningioma	2	1	2	3	7	5.0	5.0
21	55	M	Atypical meningioma	2	1	3	4	5	1.6	1.6
22	78	F	Atypical meningioma	2	2	3	5	13	2.0	2.0
23	42	F	Atypical meningioma	2	3	3	6	6	3.4	7.6
24	46	M	Atypical meningioma	2	1	2	3	8	22.7	22.7
25	59	F	Atypical meningioma	2	2	3	5	10	9.0	19.6
26	85	M	Atypical meningioma	2	1	2	3	10	17.4	17.4
27	60	M	Atypical meningioma	2	2	3	5	12	5.8	5.8
28	67	F	Anaplastic meningioma	3	3	1	4	20	11.3	34.0
29	62	M	Atypical meningioma	2	0	0	0	8	47.9	47.9
30	47	M	Atypical meningioma	2	1	3	4	5	108.8	108.8
31	81	M	Atypical meningioma	2	1	3	4	5	13.0	13.0
32	66	M	Atypical meningioma	2	1	1	2	8	19.6	52.2
Mean (range)	60.5 (24-85)							8.5 (3-20)	19.4 (1.4-108.8)	23.7 (1.4-108.8)

Supplemental Table2. COX-2 expression according to WHO Grade

A. Expression of COX-2 in WHO Grade I meningioma

	No. Cases (%)			
	Percentage of COX-2-positive tumor cells			
	0%	1 %-10 %	10 %- 50 %	> 50 %
Staining intensity				
Score 0	17 (38.6 %)	0	0	0
Score 1	0	5 (11.1 %)	3 (6.8 %)	8 (17.8 %)
Score 2	0	1 (2.2 %)	6 (13.3 %)	3 (6.7 %)
Score 3	0	0	0	1 (2.2 %)

B.Expression of COX-2 in WHO Grade II meningioma

	No. Cases (%)			
	Percentage of COX-2-positive tumor cells			
	0%	1 %-10 %	10 %- 50 %	> 50 %
Staining intensity				
Score 0	1 (3.4 %)	0	0	0
Score 1	0	1 (3.4 %)	9 (31.0 %)	10 (34.5 %)
Score 2	0	0	0	7 (24.1 %)
Score 3	0	0	0	1 (3.4 %)

C. Expression of COX-2 in WHO Grade III meningioma

	No. Cases (%)			
	Percentage of COX-2-positive tumor cells			
	0%	1 %-10 %	10 %- 50 %	> 50 %
Staining intensity				
Score 0	1 (33.3 %)	0	0	0
Score 1	0	1 (33.3 %)	0	0
Score 2	0	0	0	0
Score 3	0	1 (33.3 %)	0	0

≧15 %
5-15 %
≦5 %
0%