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LABORATORY INVESTIGATION

Overexpression of CD44 is associated with a poor prognosis in grade II/III gliomas

Chongxian Hou[1] · Yukitomo Ishi[1] · Hiroaki Motegi[1] · Michinari Okamoto[1] · Yafei Ou[2] · Jiawei Chen[3] · Shigeru Yamaguchi[1]

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Abstract

Purpose Overexpression of CD44 has been detected in many types of tumor tissues. Moreover, CD44 is recognized as a cancer stem cell marker for many cancers. However, the prognostic value of CD44 for glioma patients has not yet been clarified. The authors tried to explore the impact of *CD44* expression on grade II/III glioma patients.

Methods To assess the RNA expression levels of *CD44* in glioma tissues and normal brain tissues, meta-analyses were conducted in the online Oncomine database. The mRNA expression levels of *CD44*, *CD44s*, and *CD44v2–v10* in 112 grade II/III glioma patients in Hokkaido University Hospital (HUH) were detected by qPCR. The RNA-seq data and clinical data of grade II/III glioma patients were obtained from The Cancer Genome Atlas (TCGA) and the Chinese Glioma Genome Atlas (CGGA) databases.

Results Based on the Oncomine database, *CD44* has significantly high expression in glioma tissues as compared with normal tissues. We explored the clinical relevance of *CD44* mRNA expression based on the HUH cohorts, the TCGA cohorts, and the CGGA cohorts. In survival analysis, high mRNA expression of *CD44* was correlated with poor overall survival and poor progression-free survival in grade II/III glioma patients. Multivariate Cox regression analyses confirmed *CD44* as an independent prognostic factor for grade II/III glioma patients.

Conclusions The present study suggests that overexpression of *CD44* is associated with a poor prognosis for grade II/III glioma patients. Moreover, our findings suggest that *CD44* could serve as a prognostic biomarker in grade II/III glioma patients.

Keywords CD44 · Glioma · Prognostic factor · Biomarker

Introduction

Glioma accounts for about 80% of primary malignant brain tumors. Based on the World Health Organization (WHO) criteria, gliomas are classified into four grades (i.e., WHO grade I, II, III, and IV). Glioblastoma (GBM), is considered a grade IV tumor and accounts for 50% of all gliomas [1]. GBM is the most aggressive type of brain tumor in adults. Despite surgery and post-operative chemotherapy and radiotherapy, the median survival is only 14.6 months [2]. Glioma stem cells (GSCs) are considered to be largely responsible for the poor prognosis in GBM [3].

CD 44 is a major cell surface receptor for hyaluronan (HA) and many other extracellular matrix components, and is implicated in cell adhesion, cell migration, and signaling [4]. The concentrations of HA in malignant tumors are usually higher than that are seen in the corresponding benign or normal tissues, and the high expression levels of HA contribute to tumor proliferation, progression, and metastasis [5]. Overexpression of HA is correlated with poor prognosis in many cancers [6], suggesting that CD44 might be important in tumor progression, and migration. There are two families of CD44 isoforms: (1) the standard isoform

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✉ Shigeru Yamaguchi
yama-shu@med.hokudai.ac.jp

[1] Department of Neurosurgery, Faculty of Medicine, Hokkaido University, North 15 West 7, Kita-ku, Sapporo 060-8638, Japan

[2] Graduate School of Information Science and Technology, Hokkaido University, Sapporo 060-0814, Japan

[3] Shantou University Medical College, Shantou 515041, Guangdong, China

of CD44 (CD44s), and (2) the variant isoforms of CD44 (CD44v). CD44s is encoded by ten constant exons. CD44v is encoded by ten constant exons and any combination of the remaining nine exons [7]. Different isoforms of CD44 possess similar or distinct cellular functions [8].

Compared with normal tissues, CD44 is overexpressed in a variety of tumors, including glioma [9]. Some studies have reported that increased expression levels of CD44 are associated with a poor prognosis in GBM patients [10–15], while others found no correlation [16, 17]. Still, others have identified CD44 as a positive prognostic indicator of survival for GBM patients [18]. Klank et al., suggested a biphasic relationship between *CD44* expression levels and survival of glioma patients [19].

GSCs define a small subpopulation of tumor cells in GBM with the ability to self-renew, and to differentiate into tumor lineages and initiate tumors. GSCs in GBM are responsible for tumor progression, chemo-resistance, radio-resistance, recurrence, and metastasis [20, 21]. CD44 is recognized as a cancer stem cell marker in various cancers [22],however, whether or not CD44 is an applicable GSC marker remains controversial. Several studies support the suggestion that CD44 might be a GSC marker [23–25]. However, Wang et al., have found that CD44 low-expressing cells exhibit more GSC traits, and his group suggested that CD44 is not an appropriate GSC marker [26].

Compared to GBM, the prognostic value of *CD44* for grade II/III glioma patients has not been investigated. In the present study, we investigated in the gene expression patterns of total *CD44*, *CD44s*, and *CD44v2–v10* in grade II/III gliomas. We correlated gene expression of *CD44* to the clinical characteristics of glioma patients, and estimated its potential prognostic value for grade II/III glioma patients. Moreover, a gene set enrichment analysis (GSEA) was performed to explore the function of *CD44* and its related signaling pathways.

Methods and Materials

Oncomine analysis

To evaluate the mRNA expression of *CD44* in glioma tissues as compared to normal tissues, a meta-analysis was conducted using previously published and publicly available microarray data in the online Oncomine database (www.oncomine.com; Oncomine™, Compendia Bioscience, Ann Arbor, MI, USA). RNA expression levels are reported as Log_2 median-centered intensity in the Oncomine database. *CD44* mRNA expression levels in tumor specimens were compared with that in normal controls by the Student's t-test to generate a P value.

Patients in Hokkaido University Hospital

A cohort of 112 patients from the department of Neurosurgery in Hokkaido University Hospital (HUH) between January 2003 and March 2019 were evaluated. All the patients were diagnosed as grade II or III gliomas based on WHO 2000 criteria, WHO 2007 criteria, or WHO 2016 criteria. Patient who was younger than 16 years old at the time of diagnosis was excluded from the present study. Both clinical data and detailed follow-up data were obtained for all patients. The isocitrate dehydrogenase (IDH) mutation status was investigated using Sanger sequencing. In addition, we also investigated in the 1p/19q loss of heterozygosity status of the tumors using a multiplex ligation-dependent probe amplification procedure.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR) analysis

The total RNA was extracted from the frozen specimens stored in − 80 °C using a RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was synthesized using the PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) with 1 mg of total RNA. The primer sequences of *CD44, CD44s, CD44v2, CD44v3, CD44v4, CD44v5, CD44v6, CD44v7, CD44v8, CD44v9, CD44v10*, and *β-actin* are listed in Supplementary Table 1. Reverse transcription-qPCR analysis was performed using FastStart Essential DNA Green Master with LightCycler 96 (Roche Diagnostics, Basel, Switzerland). The PCR product specificities were confirmed by melt curve analysis. All PCR experiments were done in triplicates, and the means of three values are presented. The relative target gene mRNA expression levels compared to β-actin were measured by qPCR using the $2^{-\Delta\Delta CT}$ method [27].

Data mining in TCGA and CGGA

Clinical information, gene expression, and gene mutation status were obtained from the Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) database and the Chinese Glioma Genome Atlas (CGGA, https://cgga.org.cn/) database for grade II/III glioma patients. Patient who was less than 16 years old at the time of diagnosis was excluded. Patients without survival data were also excluded from the present study. The raw count data of RNA-seq was obtained from TCGA and then normalized using the edgeR package (version 3.26.1) in R (version 3.5.3). The RNA-seq data from the CGGA database

was presented directly as the value of fragments per kilobase per million mapped reads (FPKM).

Gene set enrichment analysis

GSEA is a method to identify groups of genes or proteins that are over-represented in a large set of genes or proteins. These groups of genes or proteins may have an association with biological functions or phenotypes. In the present study, the phenotype was determined by the expression level of *CD44* (high versus low) based on the TCGA database. We selected annotated gene sets (c2.cp.kegg. v6.2 symbols) as the reference gene sets. The normalized enrichment score (NES), nominal p-value, and false discovery rate (FDR) q-value were used to indicate the significance of association between the gene sets and the pathways.

Statistical analysis

The mRNA expression levels of *CD44* were compared by the Mann–Whitney U-test between groups. Categorical variables were expressed as frequency and were compared using the Chi-square ($\chi 2$) test. The value of *CD44* mRNA expression higher than the median value was considered as *CD44* high expression, while the value of *CD44* mRNA expression level lower than the median value was considered as *CD44* low expression. OS and PFS were presented as Kaplan–Meier curves. The Kaplan–Meier survival curves with the log-rank test were calculated and then plotted using Graphpad Prism version 8. Univariate and multivariate Cox proportional hazard regression analyses were conducted using SPSS version 22.0. Factors that were significant at the 0.1 level on univariate analysis were selected for multivariate analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The mRNA expression levels of CD44 in glioma tissues and normal brain tissues

Based on the data in the Oncomine database, we further confirmed that *CD44* mRNA expression was significantly higher in glioma tissues as compared to normal brain tissues (Supplementary Fig. 1).

The mRNA expression levels of CD44, CD44s, CD44v2, CD44v3, CD44v4, CD44v5, CD44v6, CD44v7, CD44v8, CD44v9, and CD44v10 in grade II/III gliomas

Compared with mRNA expression level of CD44s, the mRNA expression levels of *CD44v3, CD44v4, CD44v5, CD44v6, CD44v7, CD44v8, CD44v9,* and *CD44v10* were much lower (Fig. 1a). Further, extremely low mRNA expression level of CD44v2 was detected in grade II/III gliomas. We even failed to detect the mRNA expression of *CD44v2* in 30 specimens. Besides, tumor specimens which belonged to *CD44* high group also belonged to *CD44s* high group (data not shown). Thus, in grade II/III gliomas, the mRNA expression level of *CD44s* could practically represent the mRNA expression level of total *CD44.*

The relationship between the mRNA expression levels of CD44 and the clinicopathological characteristics seen in glioma patients

Based on the RNA-seq data in the TCGA and CGGA databases, and the qPCR analysis of the glioma cases in HUH, we next explored the relationship between *CD44* mRNA expression levels and various clinicopathological characteristics seen in grade II/III glioma patients (Table 1, Supplementary Table 2 and 3, Fig. 1b–n). In HUH cohorts, compared with patients younger than 60 years old, *CD44* expression was significantly higher in patients over 60 years old (Fig. 1b). The *CD44* expression levels in IDH wild type tumors were significantly higher than that in IDH mutant tumors (Fig. 1h). In the TCGA cohorts, the *CD44* expression levels in grade III gliomas and IDH wild type gliomas were significantly higher than that in grade II gliomas and IDH mutant gliomas, respectively (Fig. 1f, i). Besides, high *CD44* expression level was significantly associated with high recurrent probability (Fig. 1n). In the CGGA cohorts, the *CD44* expression levels in 1p/19q non co-deleted gliomas were significantly higher than that in 1p/19q co-deleted gliomas (Fig. 1l). To some extent, the majority of the results showed the similar tendency among the HUH cohorts, the TCGA cohorts, and the CGGA cohorts, although some of the results did not reach statistical significance. The above results indicated that *CD44* expression was associated with various clinicopathological characteristics and might serve as a potential prognostic biomarker for glioma patients.

The correlation between CD44 expression and overall survival of glioma patients

The Kaplan–Meier survival curves and log-rank test analyses illustrated that high expression of *CD44* was significantly associated with poor OS of grade II/III gliomas in the HUH cohorts, the TCGA cohorts and the CGGA cohorts (Fig. 2). We then performed survival analysis towards *CD44* mRNA expression in subgroups of grade II/III gliomas (Supplementary Fig. 2). The results suggested a similar tendency that high *CD44* expression was associated with a poor OS of each subgroup, although inconsistencies exist among the

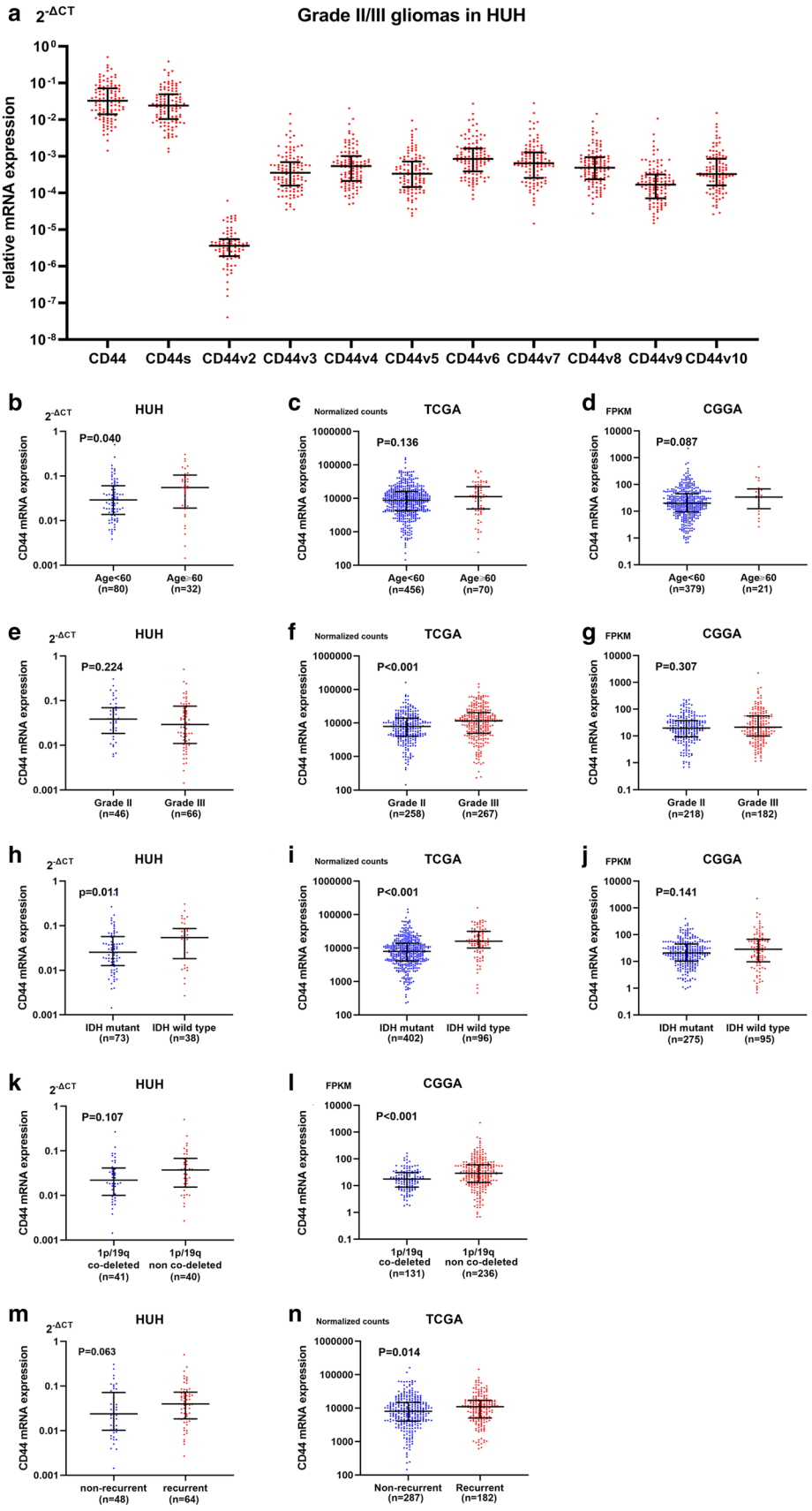

Fig. 1 The mRNA expression levels of *CD44* in the HUH cohorts, the TCGA cohorts, and the CGGA cohorts. **a** The mRNA expression levels of *CD44, CD44s*, and *CD44v2–v10* in grade II/III gliomas in the HUH cohorts. **b–n** The relationship between *CD44* mRNA expression levels and the clinicopathological characteristics in the HUH cohorts, the TCGA cohorts, and the CGGA cohorts. Data are presented as median with the first and third quartiles: **b–d** age < 60 years old and age ≥ 60 years old; **e–g** grade of gliomas; **h–j** IDH status of gliomas; **k, l** 1p/19q status of gliomas; **m, n** recurrent status

Table 1 Patient characteristics and the relationship between CD44 mRNA expression and clinicopathological characteristics in grade II/III gliomas in Hokkaido University Hospital cohorts

Parameters	No. of patients (%)	Total CD 44 expression		P value
		High (n=56)	Low (n=56)	
Age (years)				0.02
< 60	81	35	46	
≥ 60	31	21	10	
Gender				0.449
Male	58	27	31	
Female	54	29	25	
Grade				0.249
II	46	26	20	
III	66	30	36	
KPS				0.675
≥ 70	106	52	54	
< 70	6	4	2	
IDH status				0.014
IDH wild type	38	25	13	
IDH mutant	73	30	43	
Unknown	1	1	0	
1p/19q status				0.095
1p/19q co-deleted	41	14	27	
1p/19q non co-deleted	40	21	19	
Unknown	31	21	10	
Surgery				0.522
Biopsy	18	9	9	
Partial resection	67	36	31	
Total resection	27	11	16	
Adjuvant chemotherapy				0.321
Yes	73	34	39	
No	39	22	17	
Adjuvant radiotherapy				1
Yes	70	35	35	
No	42	21	21	
Recurrence status				0.056
Yes	64	37	27	
No	48	19	29	

KPS Karnofsky performance score, *IDH* isocitrate dehydrogenase

three cohorts. Thus, we suggest that *CD44* could serve as a potential prognostic factor for grade II/III glioma patients.

The correlation between CD44 expression and progression-free survival of glioma patients

The results of the survival analyses demonstrated that high *CD44* mRNA expression was significantly associated with a poor PFS of grade II/III glioma patients (Fig. 2d). Moreover, high CD44 expression was significantly associated with a poor PFS of each subgroup (Fig. 2e–h).

CD44 was an independent prognostic marker for grade II/III glioma patients

Based on the above findings, we used univariate and multivariate Cox regression analyses to evaluate the utility of *CD44* expression as an independent prognostic factor (Table 2, Supplementary Tables 4, 5). Multivariate Cox regression analyses found that *CD44* expression was significantly associated with OS as a prognostic factor in the HUH cohorts (P = 0.027), the TCGA cohorts (P = 0.029), and the CGGA cohorts (P = 0.003).

Gene set enrichment analysis

We performed GSEA to explore the function of *CD44* and its related signaling pathways base on the TCGA database. The significantly enriched signaling pathways were picked out according to the NES, FDR q-value, and nominal p-value. In the present study, gene sets of Toll-like receptors (TLRs) signaling pathway, cell adhesion molecules, regulation of actin cytoskeleton, and chemokine signaling pathway are differentially enriched in *CD44* high expression phenotype (Supplementary Table 6, Fig. 3).

Discussion

Overexpression of HA is correlated with poor prognosis in many cancer types [6]. CD44, which is a major cell surface receptor for HA and many other extracellular matrix components, is implicated in cell adhesion, migration, and signaling [4].

There are two families of CD44 isoforms: (1) the standard isoform of CD44; and (2) the variant isoforms of CD44 [8]. Miwa et al., found that CD44s cells were associated with increased chemotaxis, invasiveness, and decreased tumorigenicity in gallbladder cancer, while CD44v cells were associated with decreased chemotaxis, invasiveness, and increased tumorigenicity [28]. In human gliomas, Ranuncolo et al., found that overexpression of CD44s is associated with a highly invasive behavior [17]. CD44s appears to be the dominant form of CD44 in primary brain tumors [29, 30]. Expression levels of CD44s do not seem to correlate with the grading range of gliomas [29, 31]. Several studies have demonstrated that absent or low expression of CD44v was found in primary brain tumors, although high expression levels of CD44v were detected in metastatic brain tumors [17, 30, 32–34]. Thus, the lack of CD44v expression might be one of the explanations for the lack of metastatic potential of gliomas. In other words, CD44v might play a role in

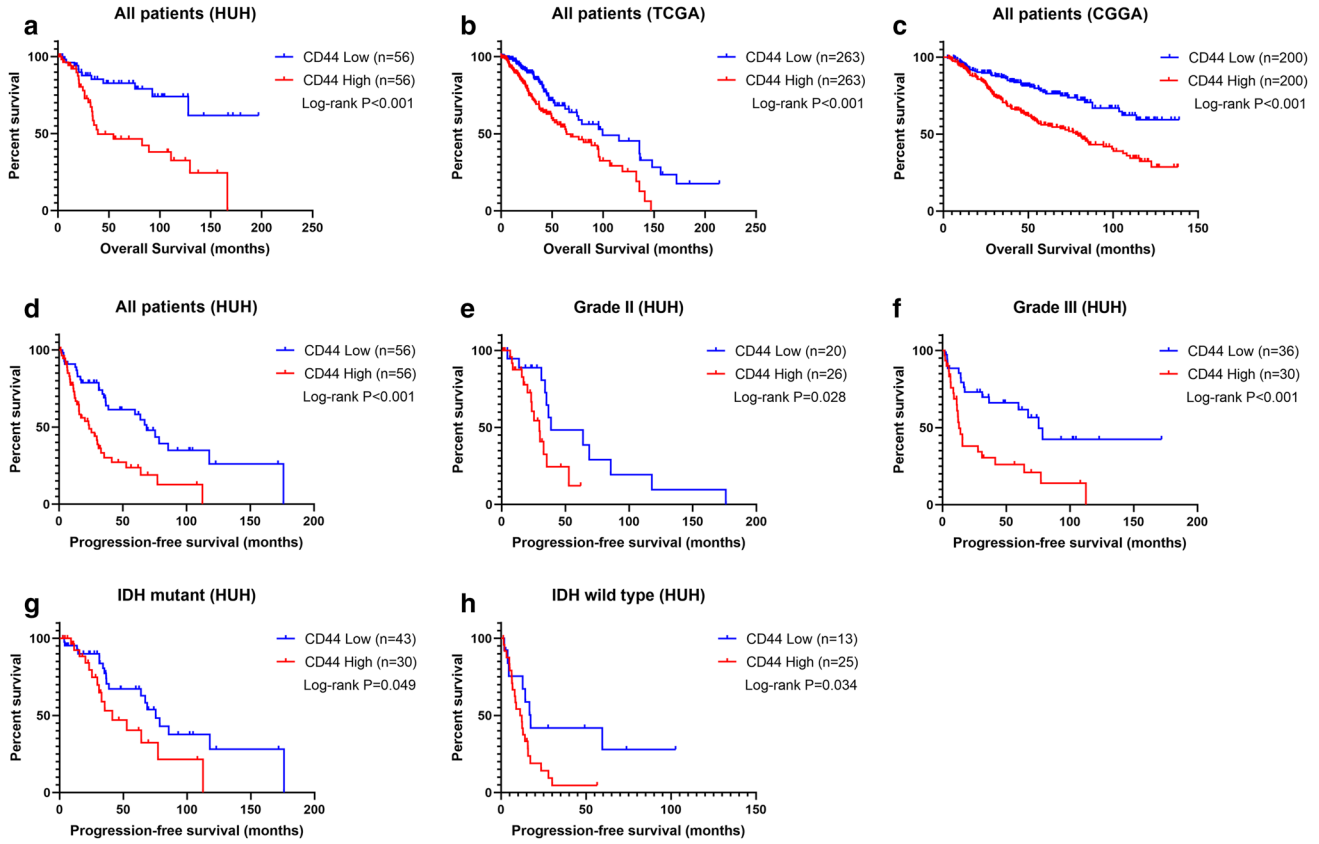

Fig. 2 Correlation between *CD44* mRNA expression and OS as well as PFS of glioma patients. **a** The association between *CD44* mRNA expression and OS in all grade II/III glioma patients in HUH cohorts. **b** The association between *CD44* mRNA expression and OS in all grade II/III glioma patients in the TCGA cohorts. **c** The association between *CD44* mRNA expression and OS in all grade II/III glioma patients in the CGGA cohorts. **d** The association between *CD44* mRNA expression and PFS in all grade II/III glioma patients in HUH cohorts. **e** The association between *CD44* mRNA expression and PFS in grade II gliomas. **f** The association between *CD44* mRNA expression and PFS in grade III gliomas. **g** The association between *CD44* mRNA expression and PFS in IDH mutant gliomas. **h** The association between *CD44* mRNA expression and PFS in IDH wild type gliomas

the intracranial spread of metastatic brain tumors. However, Kaaijk et al. suggest a strong focal expression of CD44v5 in highly malignant gliomas [29].

GSCs are considered to be largely responsible for the poor prognosis of GBMs [3], however, whether or not CD44 could be a useful GSC marker remains controversial, although it has been considered as a cancer stem cell marker for many types of tumors [22–26]. Several studies have explored the prognostic significance of CD44 for GBM patients, but the results are inconsistent [10–19]. Moreover, the prognostic value of CD44 for grade II/III glioma patients has not been studied.

In the present study, we confirmed that *CD44* expression was significantly up-regulated in glioma tissues. Further, we confirmed that *CD44s* is the dominant form of *CD44* in grade II/III gliomas. *CD44* expression was associated with the age of patients, IDH status, tumor WHO grade, and recurrent probability of grade II/III gliomas. We then performed survival analyses. The Kaplan–Meier survival curve analysis and log-rank test revealed that high *CD44* expression was significantly associated with poor OS and PFS in grade II/III glioma patients, which suggested that *CD44* mRNA expression might serve as a prognostic factor for grade II/III gliomas. Multivariate cox regression analyses further confirmed that *CD44* expression might serve as an independent prognostic factor for grade II/III glioma patients. Next, we found that gene sets of Toll-like receptors (TLRs) signaling pathway, cell adhesion molecules, regulation of actin cytoskeleton, and chemokine signaling pathway are differentially enriched in *CD44* high expression phenotype.

Toll like receptor signaling pathway

TLRs are expressed by various immune cells, endothelial cells, epithelial cells, and tumor cells. Modulation of TLR signaling can have anti- and pro-tumor effects depending on the TLR, the tumor subtype, and the immune cells

Table 2 Univariate and multivariate Cox proportional hazard regression analyses of overall survival in grade II/III gliomas in Hokkaido University Hospital cohorts

Variables	Univariate cox regression		Multivariate cox regression	
	HR (95% CI)	P value	HR (95% CI)	P value
Age				
< 60	Reference		Reference	
≥ 60	3.595 (1.855–6.967)	< 0.001	0.680 (0.261–1.775)	0.431
Grade				
II	Reference		Reference	
III	1.986 (0.957–4.123)	0.065	1.188 (0.519–2.721)	0.684
KPS				
≥ 70	Reference		Reference	
< 70	2.636 (0.924–7.522)	0.07	0.638 (0.200–2.032)	0.447
IDH status				
IDH wild type	Reference		Reference	
IDH mutant	0.102 (0.049–0.214)	< 0.001	0.131 (0.053–0.322)	< 0.001
Surgery				
Biopsy	Reference		Reference	
Partial resection	0.107 (0.048–0.241)	< 0.001	0.143 (0.055–0.372)	< 0.001
Total resection	0.122 (0.047–0.317)	< 0.001	0.164 (0.054–0.500)	0.001
CD44 expression				
Low	Reference		Reference	
High	3.344 (1.643–6.807)	0.001	2.632 (1.115–6.216)	0.027

KPS Karnofsky performance score, *IDH* isocitrate dehydrogenase, *HR* hazard ration; *CI* conficence interval

infiltrating the tumor. The pro-tumor effect is mainly driven by TLR expressed by tumor cells [35]. The stimulation of TLR in tumor cells could result in increased cell survival and proliferation, or resistance to chemotherapy [35]. It has been reported that high expression TLR2 and TLR9 is associated with poor prognosis of glioma patients. The contribution of TLR2, TLR4, and TLR9 to glioma progression has been mostly described as tumor promoting [36–38]. Qadri et al. suggested that TLR2 activation could be regulated by CD44 [39].

Regulation of actin cytoskeleton

The actin cytoskeleton is essential for whole cell migration and cell interaction with the environment [40]. Infiltration of glioma cells is largely regulated by reshaping the cytoskeleton. The regulation and organization of the cytoskeleton in glioma cells differs strongly from that of the normal glia cells [41]. Compared with normal glia cells, several actin skeleton associated proteins and signaling molecules high expressed in glioma cells, such as Arp2/3, Rac1, RhoG, FAK, etc. Increased Cdc42 and Rac1 activity was observed in invading glioma cells [41]. Besides, invading glioma cells also exhibit increased expression of FAK [42]. Kwiatkowska et al. suggest RhoG plays an important role in the invasive behavior of glioblastoma cells [43]. CD44 could interact with various GTPases (e.g. RhoA, Rac1, and Cdc42) during tumor progression [44].

Chemokine signaling pathway

Chemokines are a group of secreted chemotactic cytokines which play a fundamental role in immune cell migration, tumor growth, tumor angiogenesis, and tumor metastasis [45]. Chemokines have been considered as the central components of cancer-related inflammation [46]. The chemokine superfamily consists of about 50 chemokine ligands and 20 G protein–coupled receptors, including the CC, CXC, CX3C, and XC subfamilies [45]. Various sets of chemokine and its corresponding chemokine receptor, including CX3CL1/CX3CR1, CXCL12/CXCR4, and CXCL16/CXCR6, contribute to tumor proliferation, migration, and invasion [47–49]. Further, accumulating evidence indicates that CXCL12/CXCR4 axis plays an important role in glioma cell invasion [48, 49]. Tang et al. suggest that CXCL12/CXCR4 expression is associated with glioma recurrence [50]. Thus, chemokine signaling might have an important effect on regulation of glioma cell functions and immune cell infiltration in CD44 high expression gliomas.

TLR signaling pathway, regulation of actin cytoskeleton, and chemokine signaling pathway are closely related with cell adhesion molecules, focal adhesion, and tumor microenvironment. The results of GSEA suggested that the poor prognosis of *CD44* high phenotype might be due, at least in part, to the distinct functions of adhesion molecules and the distinct components of the tumor microenvironment.

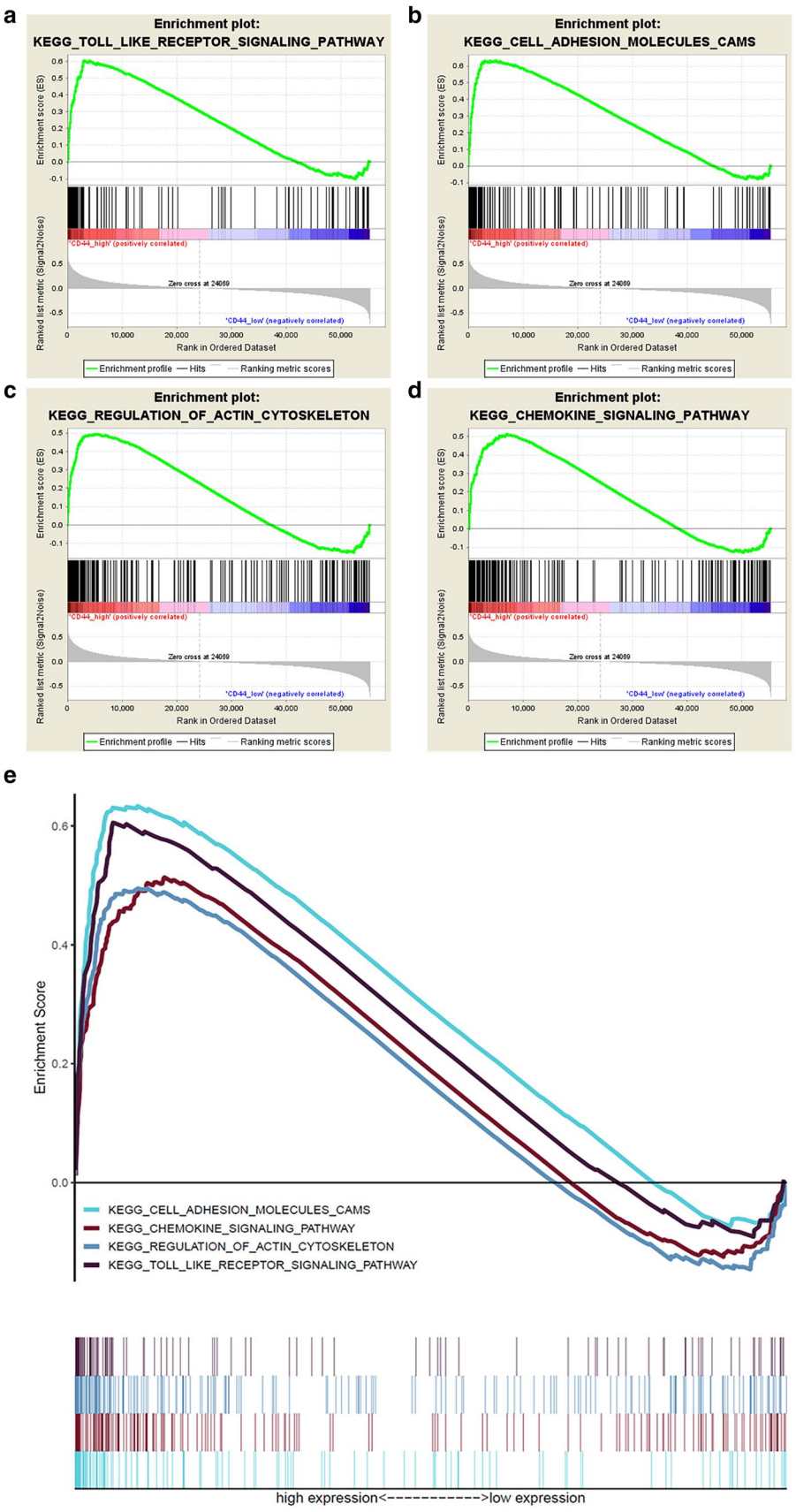

Fig. 3 Enrichment plots from Gene Set Enrichment Analysis. **a** Toll-like receptor signaling pathway enriched in *CD44* high expression phenotype. **b** Cell adhesion molecules enriched in *CD44* high expression phenotype. **c** Gene set of regulation of actin cytoskeleton enriched in *CD44* high expression phenotype. **d** Chemokine signaling pathway enriched in *CD44* high expression phenotype. **e** A merged plot showing the pathways mentioned above

Conclusions

In conclusion, the present study demonstrated that overexpression of *CD44* is correlated with a poor prognosis for grade II/III glioma patients. Our findings suggest that *CD44* could play an important role as a useful prognostic biomarker for grade II/III glioma patients.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval The present study was approved by the local Ethics Committee at Hokkaido University Hospital (Sapporo, Japan; 015–0154). As this study was retrospective, informed consent was waived by the IRB. All procedures performed in the present study were in accordance with 1964 Helsinki Declaration and its later amendments.

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