Short communications

Title

The expression of histone lysine demethylase 2B in canine hemangiosarcoma is associated with disease progression

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Abstract

Canine hemangiosarcoma (HSA), a highly fatal mesenchymal tumor of dogs, originates from the endothelial cells lining of blood vessels. It is characterized by a short survival time with a mean survival time of only 4 months. Recently, one study showed that histone lysine demethylase 2B (KDM2B) was highly expressed in canine HSA and was important in HSA tumor cell survival by positively regulating DNA repair mechanisms. KDM2B has been reported to be related to disease progression and patient survival in several human cancers. Thus, in this study, we studied the relationship of KDM2B expression levels with several patient clinical profiles to investigate the role of KDM2B in clinical HSA tumors. We analyzed 37 canine HSA cases and found that KDM2B is highly expressed in stage 3 HSA compared to stage 1 HSA. High KDM2B expression was also found in male dogs compared to female dogs. No correlation

was observed between KDM2B expression and age. Classifying HSA patients into high and low KDM2B expression groups revealed that the high KDM2B group showed shorter overall survival than the low KDM2B group. Based on these results, we suggest that KDM2B expression is associated with disease progression in HSA.

Keywords

Canine hemangiosarcoma, disease progression, epigenetics, KDM2B, metastasis, patient profile

Text

Canine hemangiosarcoma (HSA) is the most common mesenchymal tumor in dogs.[1] It arises from vascular endothelial cells (EC) and is characterized by high rates of recurrence and metastasis.[2] Patients with HSA have short survival times with a mean survival time of only 4 months and fewer than 10% of dogs survive a year after initial diagnosis.[3,4] It usually develops from the spleen, liver, heart, and skin.[5] It has been documented in several species including mice, horses, cows, and humans.[6,7] At present, due to lack of a viable prognostic marker, clinical staging is used to assess patient survival. A recent study found that lysine demethylase 2B (KDM2B) highly expressed in canine HSA and played an important role in HSA tumor cell survival by positively regulating DNA repair mechanisms.[8] KDM2B has been reported to be an oncogene in a variety of human cancers.[9–17] It promotes disease progression in pancreatic cancer and ovarian cancer, and its expression levels were positively correlated to poor patient prognosis in gliomas and breast cancers.[18–21] Based on these findings, we speculated that KDM2B was also corelated to HSA malignancy like other human cancers. Therefore, in this study, we aimed to

44 investigate the relationships between KDM2B expression levels and disease progression or
45 patients' prognosis in HSA.

46 Clinical information on HSA cases was obtained from the archives of two veterinary
47 pathology laboratories. Only HSA patients from 2010 to 2019, which were histologically
48 diagnosed by at least 3 certified veterinary pathologists, were included. Samples, which
49 consisted of tumor biopsies after splenectomy or the tumor with major organs at necropsy, were
50 collected from patients and fixed in 10% neutral buffered formalin. The samples were then cut,
51 embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to standard
52 protocols. Immunohistochemistry with anti-KDM2B antibody (sc-293279, Santa Cruz
53 Biotechnology, Dallas, TX) was performed as previously described.[8] Briefly, paraffin-embedded
54 tissue sections were deparaffinized, rehydrated, and then heated in 10 mM EDTA pH 8.0 buffer
55 for 15 mins in a microwave to retrieve antigens. Endogenous peroxidase activities were
56 quenched with 0.3% H_2O_2 in methanol. Tissue sections were blocked with 10% normal rabbit
57 serum (Nichirei biosciences, Tokyo, Japan) for an hour at room temperature (RT). The tissue
58 sections were then washed and incubated with anti-KDM2B antibody (1:50) or PBS (for
59 negative control) overnight at 4°C. Chromogenic detection was performed by incubating with
60 simple stain mouse MAX-PO (Nichirei biosciences, Tokyo, Japan) for an hour at RT, followed
61 by addition of HRP-conjugated streptavidin solution and 3-3'-diaminobenzidine (DAB; Dojindo,
62 Kumamoto, Japan). The slides were scanned with Nano Zoomer 2.0-RS (Hamamatsu Photonics,
63 Hamamatsu, Japan) and then KDM2B DAB Max intensity of tumor cells and EC was measured
64 with QuPath ver. 0.2.1.[22] KDM2B expression levels in tumor cells were normalized by that in
65 EC on the same slide. Normalized KDM2B expression levels were used for further analysis as
66 the KDM2B ratio. The R software (version 3.6.3) was used to perform statistical analysis.

Shapiro-Wilk test was used to check the normality of variables distribution. When the values are normally distributed, Student's *t*-test or Tukey's test were used. When the values are not normally distributed, Mann-Whitney U test was performed. Disease-free survival (DFS) was defined as the time between surgery and detection of tumor metastases or death. Overall survival (OS) was defined as the time between surgery and death. DFS and OS were analyzed with the Cox regression analysis or the Log-rank test.

A total of 37 cases were included in this study. Detail information is summarized in Table 1. The median and average age of the patients at the time of diagnosis were 10 and 10.4 years, respectively (range: 5-14 years). The study population consisted of twenty male and seventeen female dogs, some of which were neutered. The most common breed was miniature dachshund, followed by retriever breeds and miniature schnauzer. HSA developed in the spleen in almost 80% cases (29/37 cases), while there were only one or two cases each in other sites. Most metastases were observed in the liver (8/37 cases) and the lung (6/37 cases). The medians of DFS and OS were 87.5 and 125 days, respectively (both DFS and OS ranges were 4-926 days). The average KDM2B ratio was 1.49 while the median KDM2B ratio was 1.31 (range: 0.55-3.10).

We first performed the Cox regression analysis to evaluate the relationship between KDM2B ratio and DFS or OS (Table 2). There was no correlation between KDM2B ratio and DFS or OS ($P = 0.2443$ and $P = 0.2692$, respectively). KDM2B ratio was also not correlated with age ($r = -0.0529$) (Fig. 1). Next, to investigate the correlation of KDM2B ratio with disease progression, we classified HSA patients into three clinical stages. HSA patients with tumors confined to the primary organ were classified under clinical stage 1, patients with ruptured primary HSA or patients with tumor spread to draining and regional lymph nodes were classified

under clinical stage 2, and HSA patients with distant metastases were classified under clinical stage 3.[23] Since only one case was classified into stage 2, we compared KDM2B ratio between stage 1 and stage 3. The results indicated that KDM2B ratio was significantly high in patients under clinical stage 3 compared to patients in stage 1 (Fig. 2A). The number of metastatic sites was not correlated with KDM2B ratio (Fig. 2B). We also compared KDM2B ratio with sex and found that it was significantly higher in males than in females (Fig. 2C). These results suggest that the KDM2B expression is correlated with disease progression and may be influenced by gender.

Although KDM2B ratio was higher in clinical stage 3, the Cox regression analysis did not detect correlation between KDM2B ratio and DFS or OS. To further investigate the association of KDM2B expression with clinical outcomes, each dog was classified into high KDM2B or low KDM2B group based on the optimal threshold (KDM2B ratio = 1.16) determined by using X-tile software ver. 3.6.1 (Yale School of Medicine, CT, USA).[24] The DFS of the high KDM2B group (median DFS: 50 days) was likely to be shorter than that of the low KDM2B group (median DFS: 176.5 days) ($P = 0.06$; Fig. 3A). The OS of the high KDM2B group (median OS: 52 days) was significantly shorter than that of the low KDM2B group (median OS: 188 days) ($P = 0.02$; Fig. 3B). These results suggest that KDM2B expression might be related to the patients' prognosis.

In the present study, we found that KDM2B expression was correlated to disease progression in HSA. Furthermore, although the sample size was small, the patients classified in high KDM2B group showed the shorter overall survival time than in low KDM2B group. These results suggest that KDM2B is associated with HSA malignancy. KDM2B has been reported to promote disease progression in cooperation with the histone methyltransferase EZH2.[13,25] A

previous study showed that KDM2B and EZH2 were both upregulated in HSA cell lines compared to the normal canine endothelial cells,[8] suggesting that KDM2B and EZH2 might work together in HSA. Since the role of EZH2 in HSA has not yet been studied, investigating KDM2B and EZH2 functions would be beneficial to understand HSA pathogenesis.

Given that the clinical staging that we used in this study was mainly based on metastatic status, KDM2B might be able to make HSA tumor cells more metastatic like in other cancers.[12,26] Metastasis is one of the major factors causing hypovolemia leading to death in HSA patients.[27] However, in this study, the Cox regression analysis did not find correlation between KDM2B ratio and DFS or OS even though classifying patients based on KDM2B ratio revealed statistically significant difference in OS. This is probably because of the small sample size. To prove whether KDM2B promotes metastatic ability and contributes to patient survival in HSA, further research with an increased sample size is necessary.

We also found a difference in KDM2B expression in male and female patients. In a previous case-control study in HSA, males were found to be more likely to develop HSA than females.[28] However, another research reported that there was no sex predisposition in HSA.[3,7] Thus, it is still unclear that the high KDM2B expression in males observed in this study is related to HSA tumorigenesis. To address this question, further studies are required that focuses on KDM2B and sex prediction.

In conclusion, we showed the KDM2B expression is associated with HSA disease progression. To further elaborate how KDM2B contributes to HSA malignancy and whether its expression is associated with the prognosis of patients, additional research with the increased number of patients must be conducted.

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Table legends

Table 1. Signalment and clinical information of HSA patients

Table 2. The Cox regression analysis for KDM2B ratio

Figure legends

Fig. 1 Comparisons of KDM2B ratio and the age of patients *r* = Pearson correlation coefficient.

Fig. 2 Comparisons of KDM2B ratio and clinical stage, number of metastasis or sex. A, Comparison of KDM2B ratio between stage 1 and stage 3 patients. Student's *t*-test. **B**, Comparion of KDM2B ratio among the number of metastatic sites. Tukey's test. **C**, Comparison of KDM2B ratio between male and female patients. Mann–Whitney U test.

Fig. 3 Kaplan-Meier survival curves of DFS and OS according to KDM2B expression. A, Representative KDM2B staining images of the high KDM2B group (Top) and the low KDM2B group (Bottom). **B and C,** The DFS (**B**) and OS (**C**) of dogs in the high KDM2B group (black) and in the low KDM2B group (grey). The cross marks represent censored patients. Log-rank test.

Patient No.	Sex	Age (years)	Breed	Primary site	Metastasis	Metastasis site	Clinical stage	Surgery to metastasis	Surgery to date of death
1	Male (castrated)	7	Miniature Dachschund	Spleen	No	†	1	†	†
2	Female (spayed)	7	Golden Retriever	Spleen	Yes	Intraperitoneal	3	120	139
3	Male	14	Miniature Schnauzer	Spleen	Yes	Liver	3	997	†
4	Female (spayed)	10	Mixed	Pelvic cavity	Yes	Spleen, Liver	3	514	†
5	Male (castrated)	12	Beagle	Pelvic cavity	Yes	Recurrence+	3	150	188
6	Male	8	Labrador Retriever	Spleen	Yes	Intraperitoneal	3	10	35
7	Female (spayed)	10	Miniature Dachschund	Spleen	Yes	Lung	3	166	167
8	Female (spayed)	14	Miniature Dachschund	Skin	Yes	Lung	3	50	85
9	Female (spayed)	14	Miniature Schnauzer	Spleen	No	-	1	†	99
10	Male (castrated)	12	Miniature Schnauzer	Spleen	No	-	1	†	645
11	Male	13	French Bulldog	Spleen	Yes	Brain, skin	3	41	246
12	Male (castrated)	14	Miniature Dachschund	Spleen	No	†	1	†	703
13	Female (spayed)	12	Labrador Retriever	Femur	No	†	1	†	†
14	Male (castrated)	14	Lhasa Apso	Liver	No	†	1	†	†
15	Female (spayed)	12	Beagle	Spleen	Yes	Liver	3	387	†
16	Female (spayed)	14	Miniature Dachschund	Spleen	Yes	Thymus, muscle, subcutaneous, liver, lungs	3	52	52
17	Male (castrated)	14	Miniature Dachschund	Spleen	Yes	Lung	3	0	†
18	Female	8	Flat-coated Retriever	Spleen	†	†	1	†	250
19	Female (spayed)	11	Scottish Terrier	Spleen	Yes	Thoracic cavity	3	†	38
20	Female	5	Golden Retriever	Heart	Yes	Liver, lung, esophagus, mediastinum	3	†	†
21	Male	5	Beagle	Heart	No	†	1	†	†
22	Female	9	Golden Retriever	Spleen	Yes	superficial cervical lymph node	2	†	†
23	Male	10	Miniature Schnauzer	Spleen	Yes	Omentum, pancreas, gastric ligament	3	†	†
24	Female	12	Mixed	Cervical mass	Yes	Lymph nodes	3	†	28
25	Male	9	Great Pyrenese	Spleen	Yes	Omentum	3	†	†
26	Male	7	Jack Russell Terrier	Spleen	Yes	Liver	3	†	153
27	Female (spayed)	9	Miniature Dachschund	Spleen	†	†	1	†	†
28	Male (castrated)	8	Labrador Retriever	Spleen	Yes	Omentum	1	†	†
29	Female (spayed)	10	Miniature Dachschund	Spleen	Yes	Liver, neoplastic emboli in blood vessels	1	†	4
30	Male (castrated)	10	Maltese	Spleen	Yes	Liver, lung	3	352	†
31	Male	10	Golden retriever	Spleen	No	-	1	†	50
32	Male (castrated)	8	French Bulldog	Spleen	No	-	1	†	†
33	Female (spayed)	12	Mix	Spleen	No	-	1	†	†
34	Female (spayed)	8	Jack Russell Terrier	Spleen	No	-	1	†	149

35	Male (castrated)	5	Golden retriever	Spleen	Yes	Liver	3	76	82
36	Male	13	Labrador Retriever	Spleen	No	-	1	†	†
37	Male (castrated)	14	Labrador Retriever	Spleen	No	-	1	†	14

† No data available

	KDM2B Ratio		
	Hazard ratio	***p*-value**	**Confidence interval**
DFS	1.4844	0.2443	0.7735-2.849
OS	1.5093	0.2692	0.7341-3.103

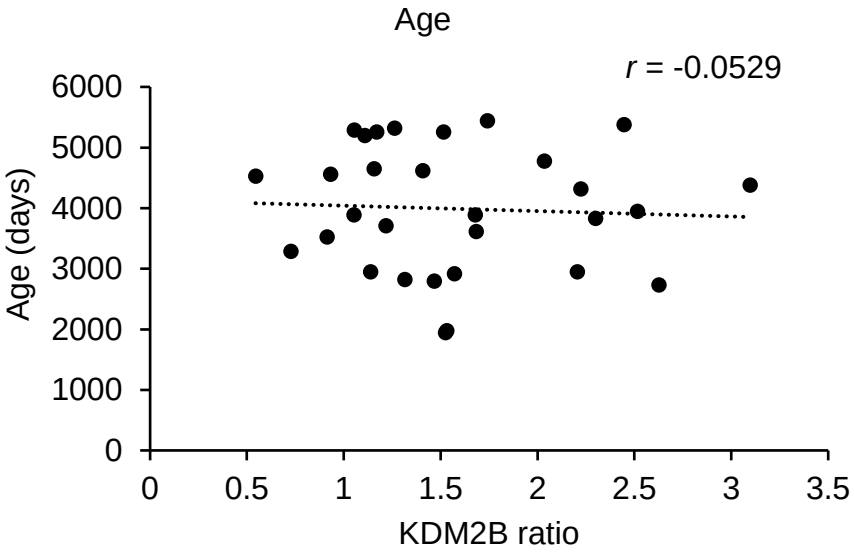
Age
r = -0.0529
Age (days)
6000
5000
4000
3000
2000
1000
0
0
0.5
1
1.5
2
2.5
3
3.5
KDM2B ratio

Fig. 1

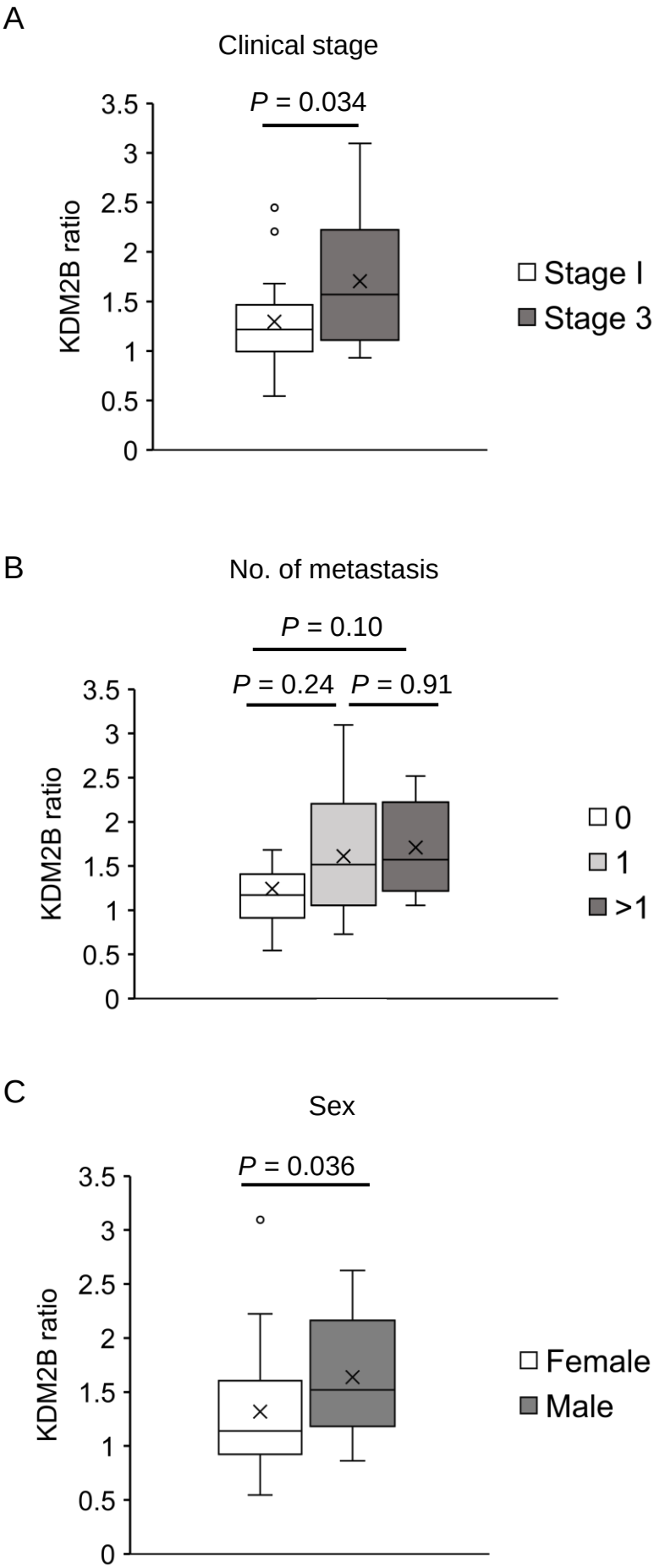
A
Clinical stage
P = 0.034
KDM2B ratio
3.5
3
2.5
2
1.5
1
0.5
0
Stage I
Stage 3
B
No. of metastasis
P = 0.10
P = 0.24
P = 0.91
KDM2B ratio
0
1
>1
C
Sex
P = 0.036
KDM2B ratio
Female
Male

Fig. 2

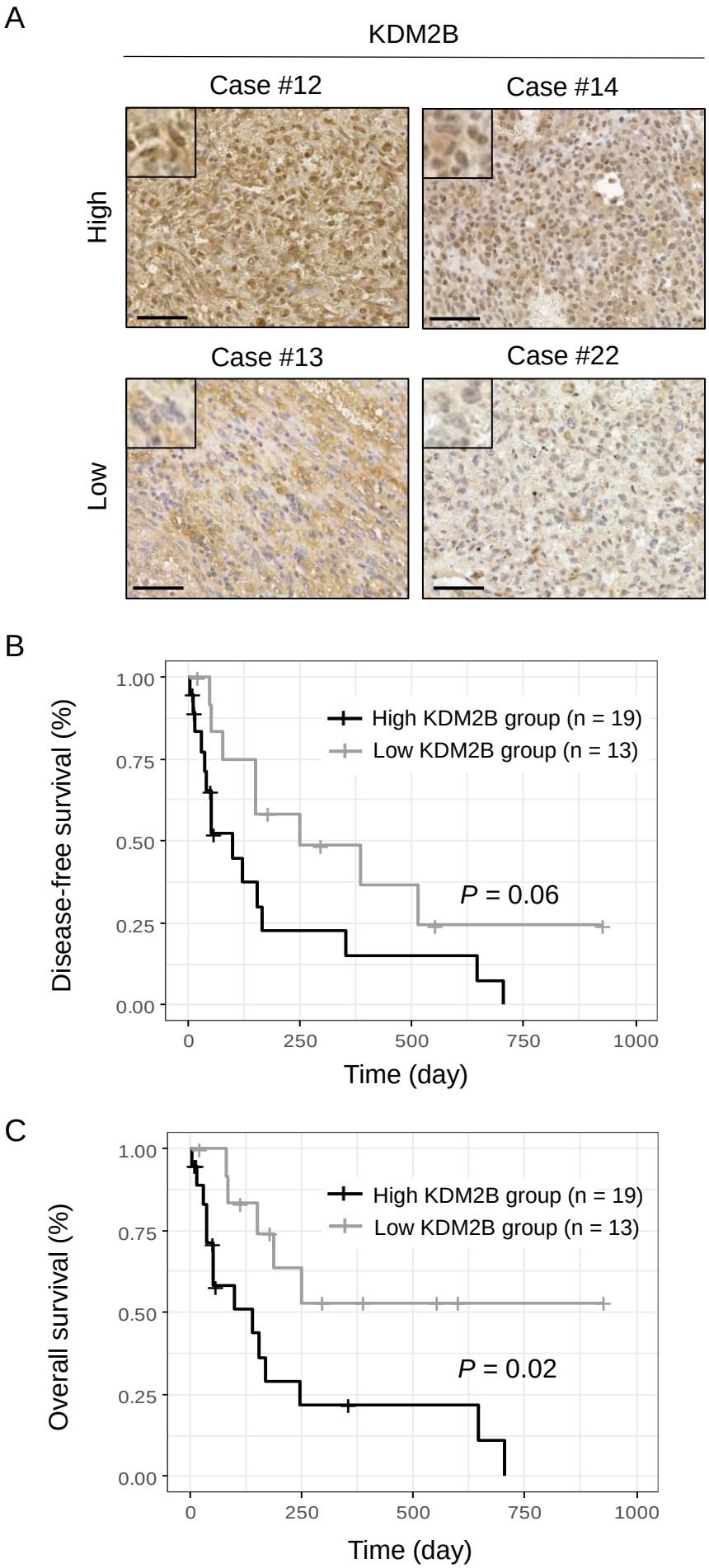
A
KDM2B
Case #12
Case #14
High
Case #13
Case #22
Low
B
Disease-free survival (%)
1.00
0.75
0.50
0.25
0.00
High KDM2B group (n = 19)
Low KDM2B group (n = 13)
P = 0.06
0
250
500
750
1000
Time (day)
C
Overall survival (%)
1.00
0.75
0.50
0.25
0.00
High KDM2B group (n = 19)
Low KDM2B group (n = 13)
P = 0.02
0
250
500
750
1000
Time (day)

Fig. 3