**Short communications**

**Title**

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

**Word count:** 1751

**Number of figures:** 3

**Number of Tables:** 2


**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

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

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

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

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

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

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

131 In conclusion, we showed the KDM2B expression is associated with HSA disease
132 progression. To further elaborate how KDM2B contributes to HSA malignancy and whether its
133 expression is associated with the prognosis of patients, additional research with the increased
134 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 |

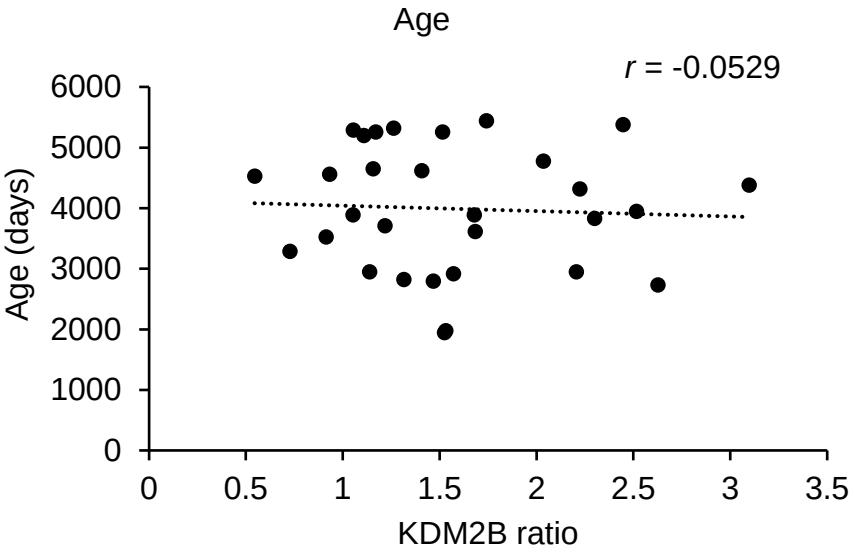

Age
r = -0.0529
6000
5000
4000
3000
2000
1000
0
Age (days)
0
0.5
1
1.5
2
2.5
3
3.5
KDM2B ratio

Fig. 1

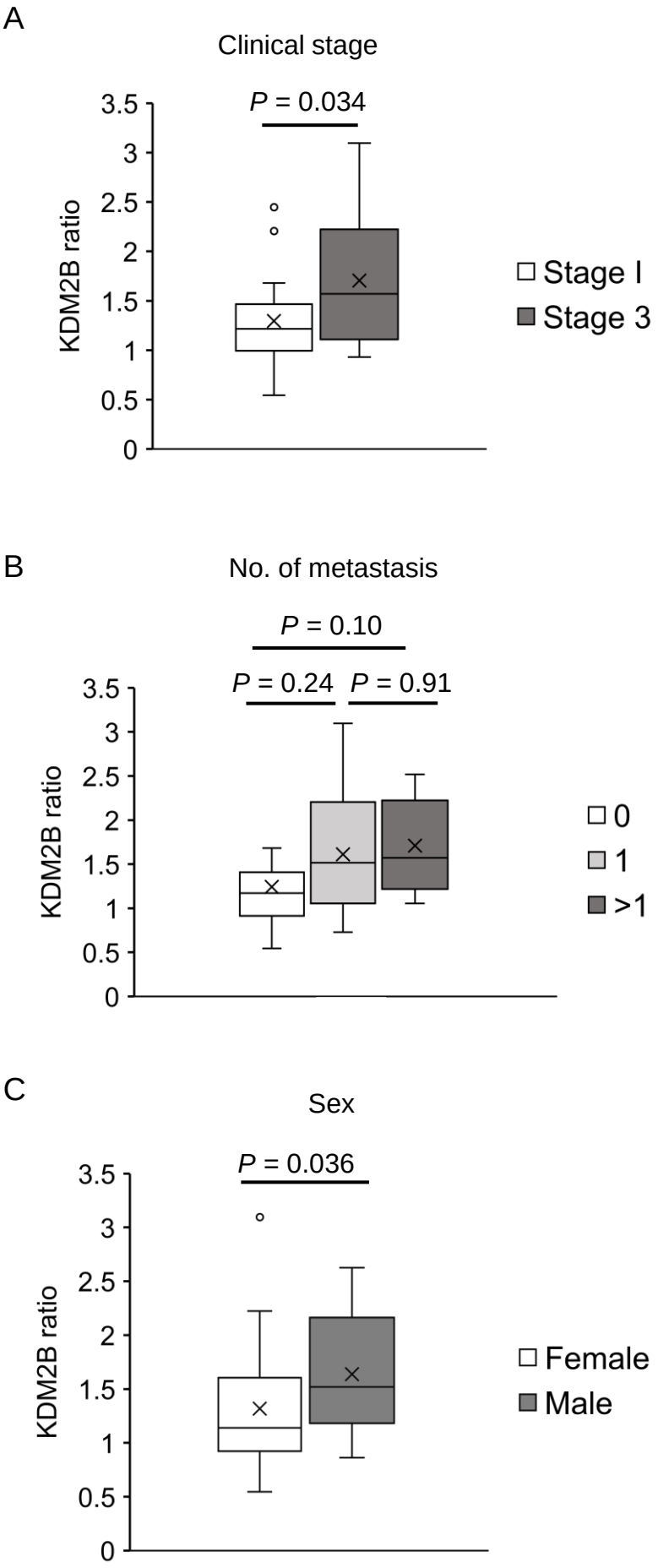
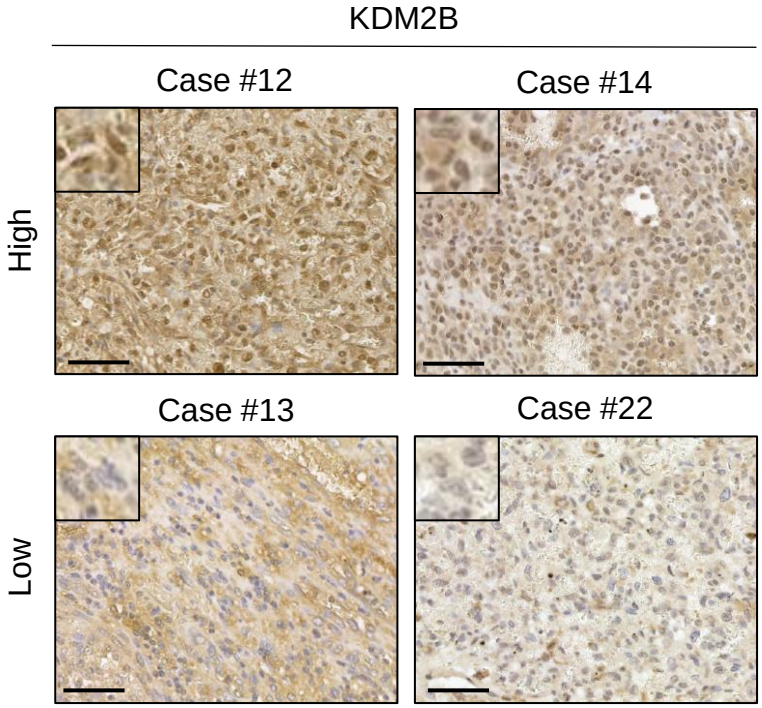
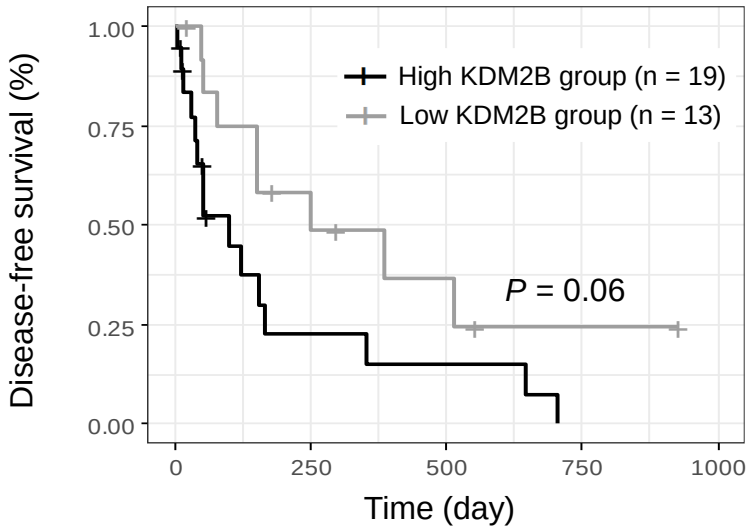

A
Clinical stage
P = 0.034
KDM2B ratio
3.5
3
2.5
2
1.5
1
0.5
0
Stage I
Stage 3
B
No. of metastasis
P = 0.10
P = 0.24
P = 0.91
KDM2B ratio
3.5
3
2.5
2
1.5
1
0.5
0
0
1
>1
C
Sex
P = 0.036
KDM2B ratio
3.5
3
2.5
2
1.5
1
0.5
0
Female
Male

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

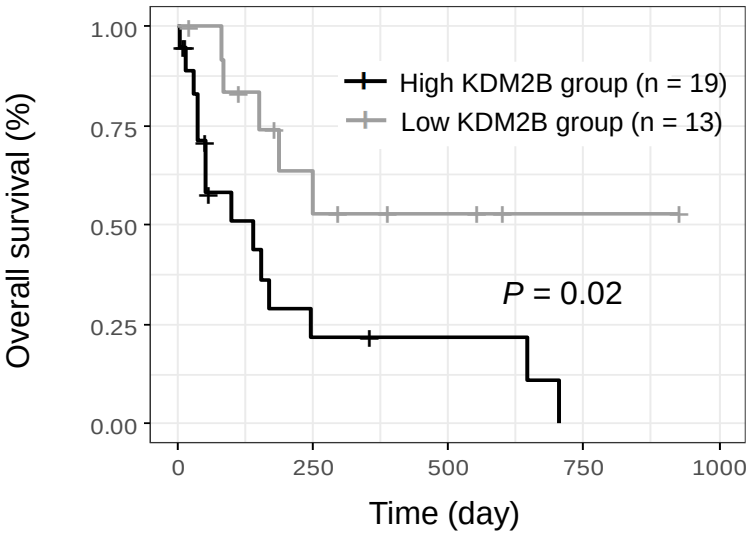

Overall survival (%)
1.00
0.75
0.50
0.25
0.00
High KDM2B group (n = 19)
Low KDM2B group (n = 13)
P = 0.02
0
250
500
750
1000
Time (day)

Fig. 3