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1 **Short communications**

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3 **Title**

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5 disease progression

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10

11 **Abstract**

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

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

26

27 **Keywords**

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

29

30 **Text**

31 Canine hemangiosarcoma (HSA) is the most common mesenchymal tumor in dogs.¹ It
32 arises from vascular endothelial cells (EC) and is characterized by high rates of recurrence and
33 metastasis.² Patients with HSA have short survival times with a mean survival time of only 4
34 months and fewer than 10% of dogs survive a year after initial diagnosis.^{3,4} It usually develops
35 from the spleen, liver, heart, and skin.⁵ It has been documented in several species including mice,
36 horses, cows, and humans.^{6,7} At present, due to lack of a viable prognostic marker, clinical
37 staging is used to assess patient survival. A recent study found that lysine demethylase 2B
38 (KDM2B) highly expressed in canine HSA and played an important role in HSA tumor cell
39 survival by positively regulating DNA repair mechanisms.⁸ KDM2B has been reported to be an
40 oncogene in a variety of human cancers.⁹⁻¹⁷ It promotes disease progression in pancreatic cancer
41 and ovarian cancer, and its expression levels were positively correlated to poor patient prognosis
42 in gliomas and breast cancers.¹⁸⁻²¹ Based on these findings, we speculated that KDM2B was also
43 correlated 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% H₂O₂ 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.

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

73 A total of 37 cases were included in this study. Detail information is summarized in Table
74 1. The median and average age of the patients at the time of diagnosis were 10 and 10.4 years,
75 respectively (range: 5-14 years). The study population consisted of twenty male and seventeen
76 female dogs, some of which were neutered. The most common breed was miniature dachshund,
77 followed by retriever breeds and miniature schnauzer. HSA developed in the spleen in almost
78 80% cases (29/37 cases), while there were only one or two cases each in other sites. Most
79 metastases were observed in the liver (8/37 cases) and the lung (6/37 cases). The medians of
80 DFS and OS were 87.5 and 125 days, respectively (both DFS and OS ranges were 4-926 days).
81 The average KDM2B ratio was 1.49 while the median KDM2B ratio was 1.31 (range: 0.55-
82 3.10).

83 We first performed the Cox regression analysis to evaluate the relationship between
84 KDM2B ratio and DFS or OS (Table 2). There was no correlation between KDM2B ratio and
85 DFS or OS ($P = 0.2443$ and $P = 0.2692$, respectively). KDM2B ratio was also not correlated
86 with age ($r = -0.0529$) (Fig. 1). Next, to investigate the correlation of KDM2B ratio with disease
87 progression, we classified HSA patients into three clinical stages. HSA patients with tumors
88 confined to the primary organ were classified under clinical stage 1, patients with ruptured
89 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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211

212

213 **Table legends**

214 **Table 1. Signalment and clinical information of HSA patients**

215 **Table 2. The Cox regression analysis for KDM2B ratio**

216

217 **Figure legends**

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

220 **Fig. 2 Comparisons of KDM2B ratio and clinical stage, number of metastasis or sex. A,**

221 Comparison of KDM2B ratio between stage 1 and stage 3 patients. Student's t -test. **B,**

222 Comparison of KDM2B ratio among the number of metastatic sites. Tukey's test. **C,** Comparison

223 of KDM2B ratio between male and female patients. Mann–Whitney U test.

224 **Fig. 3 Kaplan-Meier survival curves of DFS and OS according to KDM2B expression. A,**

225 Representative KDM2B staining images of the high KDM2B group (Top) and the low KDM2B

226 group (Bottom). **B and C,** The DFS (**B**) and OS (**C**) of dogs in the high KDM2B group (black)

227 and in the low KDM2B group (grey). The cross marks represent censored patients. Log-rank test.

228

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 Dachs Hund	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 Dachs Hund	Spleen	Yes	Lung	3	166	167
8	Female (spayed)	14	Miniature Dachs Hund	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 Dachs Hund	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 Dachs Hund	Spleen	Yes	Thymus, muscle, subcutaneous, liver, lungs	3	52	52
17	Male (castrated)	14	Miniature Dachs Hund	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 Pyreneese	Spleen	Yes	Omentum	3	†	†
26	Male	7	Jack Russell Terrier	Spleen	Yes	Liver	3	†	153
27	Female (spayed)	9	Miniature Dachs Hund	Spleen	†	†	1	†	†
28	Male (castrated)	8	Labrador Retriever	Spleen	Yes	Omentum	1	†	†
29	Female (spayed)	10	Miniature Dachs Hund	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	<i>p</i>-value	Confidence interval
DFS	1.4844	0.2443	0.7735-2.849
OS	1.5093	0.2692	0.7341-3.103

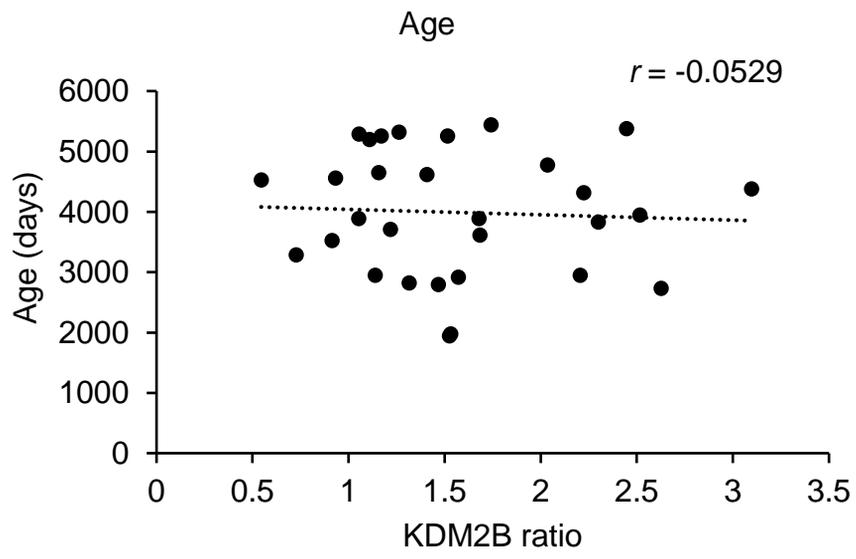


Fig. 1

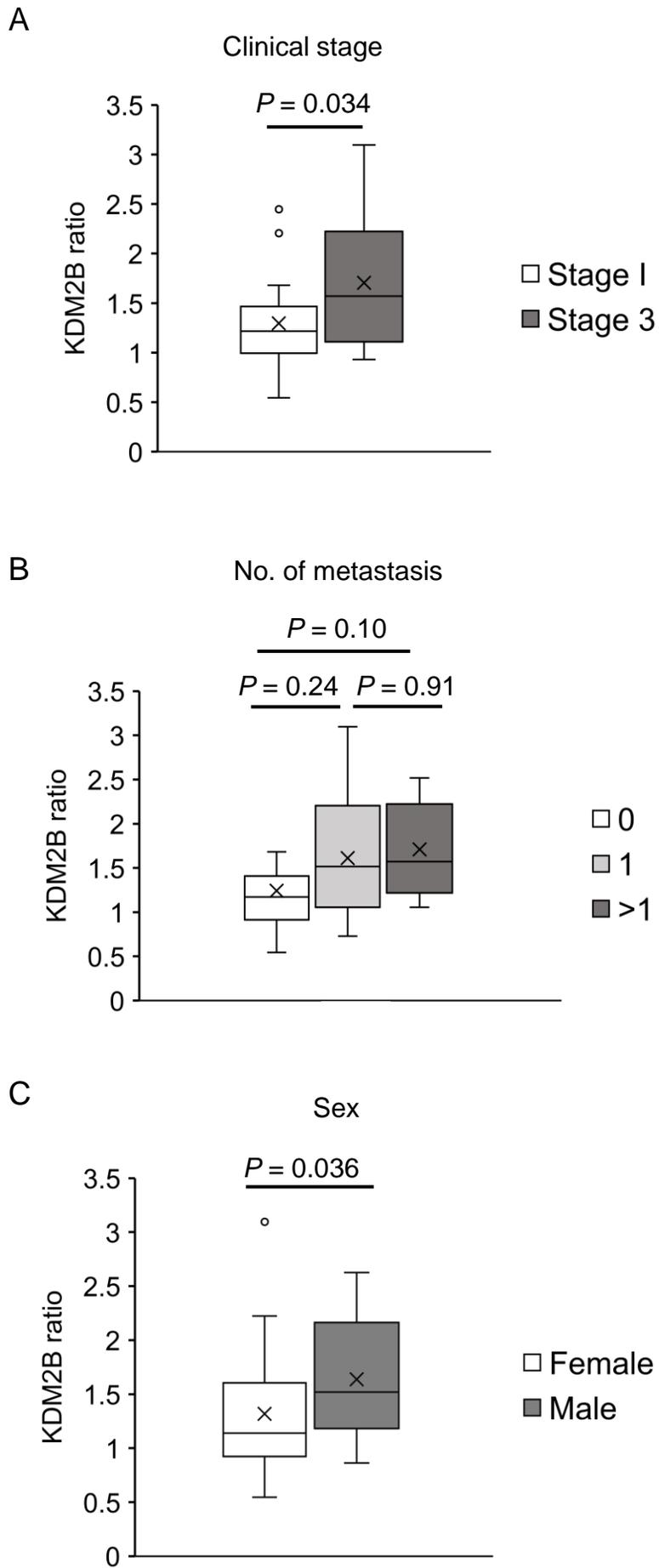
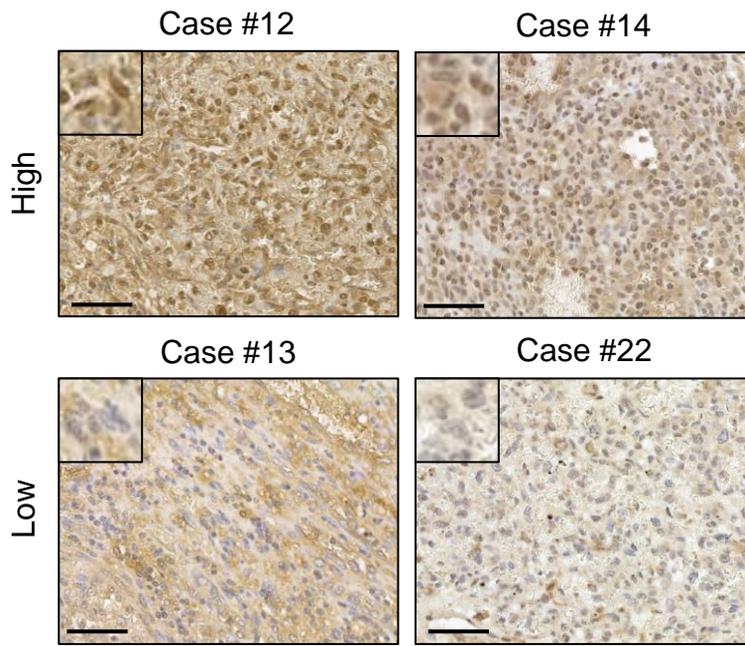
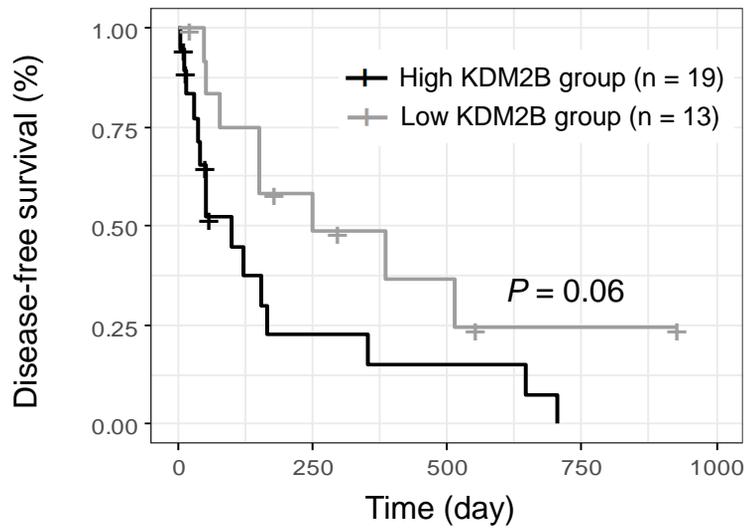
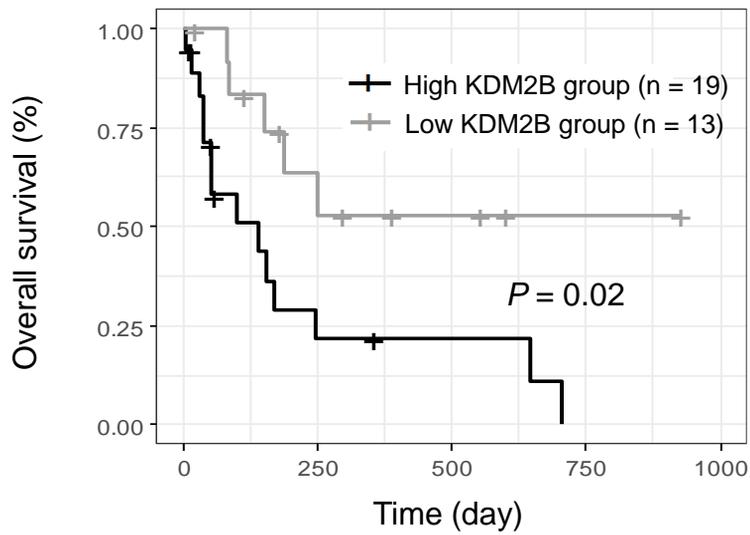


Fig. 2

A**KDM2B****B****C****Fig. 3**