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High drug efflux pump capacity and low DNA damage response induce doxorubicin resistance in canine hemangiosarcoma cell lines

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Abstract

Canine hemangiosarcoma (HSA) is an aggressive malignant endothelial tumor in dogs and characterized by poor prognosis because of its high invasiveness, high metastatic potential, and poor responsiveness to anti-cancer drugs. Although doxorubicin-based chemotherapy is regularly conducted after surgical treatment, its effects on survival rates are limited. Acquisition of drug resistance is one of the causes of this problem, but the underlying mechanisms remain unclear. In the present study, we aimed to identify the drug-resistance mechanism in canine HSA by establishing doxorubicin-resistant (DR) HSA cell lines. HSA cell lines were exposed to doxorubicin at gradually increasing concentrations. When the cells were able to grow in the presence of a 16-fold higher doxorubicin concentration compared with the initial culture, they were designated DR-HSA cell lines. Characterization of these DR-HSA cell lines revealed higher drug efflux pump capacity compared with the parental cell lines. Furthermore,
the DR-HSA cell lines did not show activation of the DNA damage response despite
carrying high DNA damage burdens, meaning that apoptosis was not strongly induced.
In conclusion, canine HSA cell lines acquired doxorubicin resistance by increasing their
drug efflux pump capacity and decreasing the DNA damage response. This study
provides useful findings to promote further research on the drug-resistance mechanisms
in canine HSA.

Keywords
Apoptosis, Cancer, DNA damage response, Doxorubicin, Drug resistance,
Hemangiosarcoma.

Introduction
Hemangiosarcoma (HSA) is a malignant tumor in dogs (*Canis lupus familiaris*)
and composed of malignant endothelial cells that are characterized by invasive growth,
high metastatic rate, and poor prognosis (Withrow et al., 2013). Surgery and doxorubicin-
based chemotherapy are selected as standard treatments, but several studies have
described their limited efficacy (Ogilvie et al., 1996; Withrow et al., 2013). The mean
survival time is 2–3 months after surgical treatment alone, and less than 1 year even
when chemotherapy is conducted postoperatively (Batschinski et al., 2018; Brown et al., 1985). Although several improved doxorubicin-based chemotherapy protocols were recently developed, they provided limited improvement of prognosis and were unable to preclude tumor progression (Kahn et al., 2013; Matsuyama et al., 2017). Moreover, a dose-intensified doxorubicin protocol did not show significant effects on survival time, probably through acquisition of drug resistance by the tumor cells (Sorenmo et al., 2004). To overcome this issue, it is essential to understand the molecular mechanisms for drug resistance in HSA.

Chemotherapy resistance has been extensively investigated in many types of cancers, and is recognized as a major cause of tumor recurrence after chemotherapy (Holohan et al., 2013; Zandvliet and Teske, 2015). Several types of resistance mechanisms have been reported. For example, anti-cancer drugs can be pumped out by highly expressed drug efflux pumps, and genetic mutations in drug targets can prevent drugs from working properly (Holohan et al., 2013). Most tumors have heterogeneity and are composed of various types of tumor cells with different characteristics. This heterogeneity can contribute to acquisition of drug resistance, because a particular cell population with resistance to anti-cancer drugs will be selected and become dominant in tumors after treatment (Burrell and Swanton, 2014; Swanton, 2012). In HSA,
acquisition of drug resistance after doxorubicin treatment was reported and some populations had high drug efflux pump capacities (Khammanivong et al., 2016; Sorenmo et al., 2000). However, the underlying mechanisms are not fully understood.

Most chemotherapeutic agents, including cisplatin, 5-fluorouracil, etoposide, and doxorubicin, cause DNA damage, especially DNA double-strand breaks (DSBs), in tumor cells (Cheung-Ong et al., 2013; Yang et al., 2015). Once DSBs are present in a cell, PI3K-like serine/threonine kinases, such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), and DNA-dependent protein kinase (DNA-PK) are activated and become localized in DNA damage sites, leading to activation of the DNA damage response (DDR) pathway (Collis et al., 2005; Marechal and Zou, 2013). This pathway provokes cellular senescence, cell cycle arrest, and apoptosis, and eventually induces tumor cell death. DDR dysregulation was reported to contribute to drug resistance (Bouwman and Jonkers, 2012; Nowsheen and Yang, 2012). For example, human oral squamous cell carcinoma cells with suppressed DDR activity showed resistance to cisplatin (Wang et al., 2012). However, it remains unclear whether DDR dysregulation is involved in drug resistance in HSA.

Drug resistance is one of the major problems in HSA. To obtain better outcomes after chemotherapy, it is necessary to understand the underlying molecular mechanisms
for drug resistance in HSA. The aims of this study were to establish doxorubicin-resistant HSA cell lines and to characterize the drug-resistance mechanisms using the established cell lines.

**Materials and Methods**

**Cell culture**

Two canine HSA cell lines (JuB2, Re12) were kindly given by Dr. Sakai in Gifu University (Murai et al., 2012), and Human embryonic kidney 293 T cell line and HeLa cell line derived from human cervical cancer were purchased from RIKEN BioResource Center. They were cultured with Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich, MO, USA) containing 10% fetal bovine serum (FBS; Biowest, UT, USA), 100 unit/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, MA, USA) at 37°C with 5% CO₂. To establish doxorubicin-resistant (DR) HSA cell lines, five hundred thousand cells were seeded in 10 cm cell culture dish (Falcon, NC, USA) and cultured in medium containing 10 nM doxorubicin (Wako, Osaka, Japan) dissolved in DMSO (Kanto Chemical Co., Inc., Tokyo, Japan) based on our previous work (Aoshima et al., 2018). The medium was replaced to the fresh one every 3 days until the surviving cells restarted proliferating and became 80-90% confluent. Then, the cells were passaged.
(1:3) and cultured in medium containing 20 nM doxorubicin with replacing the medium to the fresh one every 3 days until the cells became 80-90% confluent. These processes were repeated with doubling doxorubicin concentration every passage timing until the cells were able to normally proliferate in a 16-fold higher doxorubicin concentration compared with the initial culture. Established cells were maintained in 160 nM doxorubicin containing medium. To inhibit drug efflux pump function, JUB2 and Re12 were exposed to 200 μM and 150 μM verapamil (Wako), a multi-pump inhibitor, respectively. These concentrations were confirmed to fully inhibit efflux of Dye Cycle Violet (DCV, Thermo Fisher Scientific) (Fig. S1).

**Cell viability assay**

Three thousand cells were seeded in 96 well cell culture plates (Corning, NY, USA) and were cultured with 50 μl culture medium. On the next day, they were treated with DMSO or five different concentrations (10 nM, 100 nM, 1 μM, 10 μM and 100 μM) of doxorubicin. Survival rates were analyzed using Cell Counting Kit-8 (CCK-8: Dojindo, Kumamoto, Japan) according to the manufacturer’s instruction with slight modifications. Briefly, 5 μl of CCK-8 solution was added to each well 72 hours after adding DMSO or doxorubicin. After 2 hours incubation, absorbance at 450 nm was measured by Nano
Drop 2000 (Thermo Fisher Scientific). Survival rates were calculated by setting that of DMSO treated samples as 100%. Ky Plot 5.0 software (KyensLab, Inc., Tokyo, Japan) was used to draw survival curves.

Colony formation assay

Three hundred cells were seeded in 35 mm cell culture dish (FALCON) and were cultured with 160 nM doxorubicin containing medium for 10 days at 37°C with 5% CO2. Then, cells were fixed with 4% paraformaldehyde (Merck-Millipore, MA, USA) for 15 mins at room temperature (RT). After washing with distilled water three times, cells were stained with 0.01% Crystal Violet (Sigma-Aldrich) for 30 mins at RT. After washing with phosphate buffered saline (PBS) and drying at RT, colonies composed of more than 50 cells were counted manually using an inverted microscope (Eclipse TS100; Nikon, Tokyo, Japan).

Flow cytometry

Cells were detached from the cell culture dishes by 0.05% trypsin-EDTA (Wako) and then filtered with 70 μm mesh filter (Grainer Bio-One, Kremsmunster, Austria). One million cells were stained with 15 μM Dye Cycle Violet (DCV, Thermo Fisher Scientific)
and incubated for 60 mins at 37°C with mixing the cells every 15 mins. JuB2 and Re12 were exposed to 200 μM and 150 μM verapamil when DCV was added to the cells. Afterwards, cells were analyzed by FACS Aria II (Becton Dickinson, NJ, USA). DCV efflux was measured by detecting DCV signals excited by the violet laser using 616/23 nm band-pass filters after excluding dead cells which were positive for 7-aminoactinomycin D (Thermo Fisher Scientific) (Erdei et al., 2018).

Alkaline Comet assay

Five hundred thousand DS- and DR-HSA cells were seeded in 6 cm culture dishes and cultured without doxorubicin. On the next day, cells were treated with verapamil alone, or doxorubicin and verapamil. JuB2 was treated with 200 μM verapamil with/without 4.6 μM doxorubicin (= the 50% growth inhibition: IC50) for 30 mins. Re12 was treated with 150 μM verapamil with/without 0.77 μM doxorubicin (= IC50) for 60 mins. DMSO was used in the control. Then, the medium was replaced to the fresh one without both doxorubicin and verapamil, and the cells were cultured for six hours. Afterwards, the cells were detached from the dishes with 0.05% Trypsin-EDTA and suspended in 1% low-melting point agarose (NIPPON GENE, Tokyo, Japan). Cell suspensions containing five thousand cells were applied to MAS-coated slide glasses
(MATSUNAMI, Osaka, Japan) which were pre-coated with 1% agarose (Takara bio, Kusatsu, Japan). Slides were immersed in comet lysis buffer {1.2 M NaCl (Wako), 100 mM EDTA (Dojindo), 0.1% sodium lauryl sarcosinate (Wako), 0.26 M NaOH (Wako)} and incubated at 4°C for overnight. Then, slides were washed with alkaline rinse buffer (0.03 M NaOH, 2 mM EDTA). After DNA was separated in alkaline rinse buffer at 9 V for 15 mins, slides were washed with distilled water twice and stained with 10 μg/ml propidium iodide (Dojindo) at 4°C for 30 min. Fluorescent images were captured using an inverted fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan), and comet tails of at least 30 cells per slide were analyzed with Open Comet plugin for ImageJ software (NIH, MD, USA) (Ding et al., 2016; Gyori et al., 2014; Olive and Banath, 2006; Rasband, 1997-2018; Schneider et al., 2012).

**Gamma H2A.X stain**

Glass bottom dishes (MATSUNAMI, Osaka, Japan) were sterilized by exposing UV for 30 mins and coated with 0.5% bovine gelatin (Wako) for 30 mins at 37°C. Eighty thousand DS- and DR-HSA cells were seeded in the sterilized coated dishes and cultured without doxorubicin for 24 hours. Then, the cells were treated with doxorubicin/verapamil in the same way as alkaline comet assay. DMSO was used in the
control. Then, the cells were washed with PBS twice and incubated in the fresh medium without doxorubicin and verapamil at 37°C with 5% CO2. After six hours culture, the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 15 mins at RT. After washing with PBS twice, cells were permeabilized with ice-cold methanol (Wako) for 10 mins at -30°C. After washing with PBS three times and blocking with 0.3% Triton X-100 in PBS containing 1% bovine serum albumin (Sigma-Aldrich) at RT for 1 hour, the cells were incubated with gamma-H2A.X antibody (#A300-081A, 1:250, Bethyl Laboratories, TX, USA) in 0.05% Triton X-100 in PBS (PBST) overnight at 4°C. After washing with PBST twice, the cells were incubated with anti-Rabbit IgG (H+L) secondary antibody Alexa Fluor 555 (#A27039, 1:500, Thermo Fisher Scientific) and Hoechst 33342 (Thermo Fisher Scientific) at RT for 1 hour. After washing with PBST, fluorescent images were captured with a confocal microscopy (LSM-700, Carl Zeiss AG, Jena, Germany) and were processed with ImageJ software (Nikolova et al., 2014; Rasband, 1997-2018; Schneider et al., 2012).

Protein extraction and western blotting

Samples were prepared, separated and transferred to PVDF membranes as described previously (Aoshima et al., 2018). Membranes were either incubated with anti-
ABCB1 antibody (#ab170904, 1:2,500, abcam, Cambridge, UK), anti-ABCG2 antibody (#ab3380, 1:1,000, abcam), anti-ATM antibody (#NB100-104, 1:1,000, Novus biologicals, CO, USA), anti-cleaved caspase-3 (Asp175) antibody (#9661, 1:1,000, Cell signaling, MA, USA) (Penzo-Méndez et al. 2015) or anti-actin antibody clone C4 (#MAB1501, 1:10,000, Merck Millipore) for overnight at 4°C. After washing with Tris-buffered saline containing 0.05% Tween 20 (TBST), membranes were incubated with ECL rabbit IgG HRP-linked whole Ab (#NA934-1ML, 1:10,000, GE Healthcare, IL, USA) or ECL mouse IgG HRP-linked whole Ab (#NA931-1ML, 1:10,000, GE Healthcare) for 1 hour at RT. After washing with TBST, signals were developed with Immobilon Western Chemiluminescent HRP substrate (Merck Millipore) and detected by ImageQuant LAS 4000 mini (GE Healthcare). Signal intensities were obtained by ImageJ software (Rasband, 1997-2018; Schneider et al., 2012). Expression levels of ATM and cleaved caspase-3 were normalized with actin expression levels.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR) Primers and samples were prepared as described previously (Aoshima et al., 2018). qPCR was performed using the primers listed in Table 1. Results were normalized.
based on the geometric mean of reference genes (ACTB, HMBS, RPL3A and TBP),
which were selected from 9 potential internal controls (GAPDH, ACTB, B2M, HMBS,
HPRT1, RPL3A, RPL32, TBP and YWHAZ) by geNorm software (Fig. S2) (Peters et al.,
2007; Vandesompele et al., 2002). Relative expression levels were calculated by setting
the DS-HSA cell lines as the control.

Statistical Analysis

Levels of significance were determined by Mann-Whitney U test, Student’s t
test or Dunnet’s test. p values lower than 0.05 were considered statistically significant.

Results

Establishment of doxorubicin-resistant canine hemangiosarcoma cell lines

To confirm that the established DR-HSA cells had truly attained resistance to
doxorubicin, they were treated with various concentrations of doxorubicin for 72 h and
analyzed for their survival rates to create survival curves. Comparisons of these survival
curves with those of parental doxorubicin-sensitive (DS) HSA cells revealed that DR-
HSA cells were able to survive under higher doxorubicin concentrations than DS-HSA
cells (Fig. 1a top). IC\textsubscript{50} values of DR-HSA cells were higher than those of DS-HSA cells
(Fig. 1a bottom). Colony formation assay was performed to investigate the proliferation in the presence of high concentrations of doxorubicin (160 nM). DR-HSA cells were able to form larger numbers of colonies than DS-HSA cells (Fig. 1b). These results indicated that DR-HSA cell lines were successfully generated.

DR-HSA cells have higher efflux pump capacity than DS-HSA cells.

High drug efflux pump capacity was previously determined to be an important factor for acquisition of drug resistance, because it enables tumor cells to pump out anti-cancer drugs (Gottesman et al. 2002). To measure the drug efflux pump activities in DR-HSA cells, cells were stained with DCV, a cell-permeable DNA dye that can be excreted by drug efflux pumps, with or without verapamil, a multi-drug pump inhibitor. Comparing the percentages of DCV negative population of DR-HSA and DS-HSA cells clearly indicated that DR-HSA cells had higher drug efflux pump capacity than DS-HSA cells in both JuB2 and Re12 cells, even though the percentages of DCV negative population were different between JuB2 and Re12 (Fig. 2a). Next, we analyzed gene and protein expression levels of major ATP-binding cassette (ABC) transporters to determine which drug pump is responsible for this phenotype. Although no ABC transporter was upregulated in DR-HSA cells at the mRNA level (Fig. 2b), ABCB1 and ABCG2 were
significantly upregulated in DR-HSA cells at the protein level (Fig. 2c). To further confirm that the high efflux pump capacity was responsible for doxorubicin resistance in HSA cells, we analyzed the survival rates of DR- and DS-HSA cells after 24h treatment with ABC transporter inhibitor, verapamil, with or without 160 nM doxorubicin. The viability of cells treated with verapamil alone for 24h was quite low because verapamil per se exerts toxic effects on cell viability by disrupting the calcium balance in cells (Zhao et al., 2016). Cell viability of DR-JuB2 cells, however, was the same as that of DS-JuB2 cells under doxorubicin and verapamil treatments, suggesting that drug efflux pump inhibition by verapamil treatment may reverse doxorubicin sensitivity in DR-JuB2 cells (Fig. 2d). These results suggest that high efflux pump capacity contributes to doxorubicin resistance, especially in JuB2 cells.

DNA damage response in DR-HSA cells is weaker than that in DS-HSA cells

Doxorubicin could be pumped out of some DR-HSA cells based on the above results, while other cells remained DCV-positive despite their normal proliferation in the presence of doxorubicin (Fig. 1a, 2a). In DR-Re12 cells in particular, approximately 90% of cells were not defined as the DCV negative population, suggesting that other functions are involved in the acquisition of doxorubicin resistance in HSA cells. Because
doxorubicin-induced cell death is mainly provoked by DNA damage through DSBs (Yang et al., 2015; Yang et al., 2014), two hypotheses were conceived. The first was that DNA in DR-HSA cells received less damage by doxorubicin than that in DS-HSA cells, and the second was that the DDR did not work effectively in DR-HSA cells and they were unable to induce apoptosis.

To address the first hypothesis, we evaluated DNA damage by alkaline comet assays (Ding et al., 2016; Olive and Banath, 2006). To compare the effects of doxorubicin on DNA damage between DS-HSA and DR-HSA cells, verapamil was added with doxorubicin to eliminate any discrepancy caused by the drug efflux pump capacities. After treatment with the drugs, JuB2 and Re12 cells were cultured with fresh medium without drugs for more 6 h, and their tail percentages were calculated as indicators of DNA damage at specific time points. DNA damage was significantly accumulated in the doxorubicin-treated groups compared with the control groups in all cells except for DR-Re12 cells, and the DNA damage was not affected by verapamil treatment alone (Fig. 3a and c). Interestingly, accumulation of DNA damage did not differ significantly between DR-JuB2 and DS-JuB2 cells (Fig. 3b), while DR-Re12 cells accumulated DNA damage even before addition of doxorubicin, the degree of which was close to that in DS-Re12 cells after 6 h of doxorubicin treatment (Fig. 3c and d). In both cell lines, sooner or later,
DR-HSA cells accumulated DNA damage to a similar or greater extent than DS-HSA cells after doxorubicin treatment.

Next, we addressed the second hypothesis. To examine whether the DDR was induced by doxorubicin, γH2A.X signals were investigated. γH2A.X is widely used as a biomarker for DSBs because it accumulates at DSB sites to promote repair of the damage by the DDR (Nikolova et al., 2014). We used an anti-human γH2A.X antibody, which was confirmed to cross-react to canine γH2A.X (Fig. S3a). Verapamil was added with doxorubicin to eliminate the differences in high efflux pump capacity between DS-HSA and DR-HSA cells. After γH2A.X staining, the mean number of γH2A.X foci/cell in DR-HSA cells was significantly lower than that in DS-HSA cells (Fig. 4), suggesting that the DDR was repressed in DR-HSA cells compared with DS-HSA cells. DDR-related genes were also downregulated in DR-HSA cells (Fig. 5a). Protein expression of ATM, a major DDR factor protein, was analyzed using an anti-human ATM antibody. The antibody was confirmed to cross-react canine ATM (Fig. S3b) and showed that ATM protein expression was significantly downregulated in DR-HSA cells (Fig. 5b).

To further validate that the DDR was repressed in DR-HSA cells, we investigated the apoptotic activity in the cell lines by analyzing cleaved caspase-3 expression after verapamil treatment with or without doxorubicin. Cleaved caspase-3
was detected after treatment in both DR-HSA and DS-HSA cells, but DR-HSA cells showed much lower expression than DS-HSA cells (Fig. 5c). These results suggested that apoptosis was less activated in DR-HSA cells compared with DS-HSA cells.

The above findings indicated that the DDR was repressed in DR-HSA cells despite their accumulation of DNA damage, suggesting that DR-HSA cells avoided apoptosis and acquired resistance to doxorubicin.

Discussion

High drug efflux pump capacity has been recognized as one of the major drug-resistance mechanisms in tumors (Zandvliet and Teske, 2015). Likewise, in the present study, DR-HSA cells exhibited higher DCV negative population percentages than DS-HSA cells. Interestingly, the DCV negative population percentages also differed between JuB2 and Re12 cells. Specifically, approximately 65% of DR-JuB2 cells were identified as the DCV negative population, compared with only 9% of DR-Re12 cells. Furthermore, verapamil treatment reversed doxorubicin sensitivity in JuB2 but not in Re12 cells. This difference implies that high drug efflux pump capacity is related to doxorubicin resistance but is not always a major drug resistance mechanism in HSA. The IC_{50} value of JuB2 cells was approximately 5–6 times higher than that of Re12 cells. This
discrepancy probably arose by the different drug efflux pump capacities, because a much
chigher percentage of JuB2 cells were identified in the DCV negative population
compared with Re12 cells. ABCB1 and ABCG2 protein expression was upregulated in
DR-HSA cells compared with DS-HSA cells. The mRNA expression of these genes and
some other ABC transporters, however, was not enhanced in DR-HSA cells. This
discrepancy could be explained by the suppression of the ubiquitin-proteasome
degradation pathway (Katayama et al., 2016), translational efficiency, or a difference in
the half lives of mRNA and protein (Day and Tuite, 1998; Haenisch et al., 2007; Petriz et
al., 2004). Another possibility for the differences between JuB2 and Re12 may be related
to tumor heterogeneity. Some specific populations could be selected by long-term cell
culture to establish the cell lines, which may have endowed different features on each
cell line. The tumor origins may also be responsible because JuB2 cells originated from
a hepatic HSA, while Re12 cells originated from a cardiac HSA. It was reported that
endothelial cells are heterogeneous and possess organ-specific phenotypes (Marcu et al.,
2018; Rafii et al., 2016). Thus, it is possible that canine HSA cells have different
phenotypes based on their origins, and further research is required to address this issue.

We also found that the DDR was repressed in DR-HSA cells despite their high
DNA damage burdens by doxorubicin, leading to doxorubicin resistance probably by
circumventing apoptosis. Apoptosis evasion is well known as a hallmark of cancer cells (Fouad and Aanei, 2017). It can be established by several mechanisms, including DNA damage unresponsiveness, mutations in anti-apoptotic genes, and posttranslational deregulation of pro-apoptotic genes (Fernald and Kurokawa, 2013). In the present study, γH2A.X signals were not significantly increased in both DR-HSA cell lines after doxorubicin treatment. Furthermore, the gene and protein expression levels of DDR factors were significantly decreased in the DR-HSA cell lines. It was reported that ATM expression can be repressed by micro RNA in human breast cancers and colorectal cancers (Song et al., 2011; Zhou et al., 2014), but the detailed mechanism in HSA remains unclear. Moreover, cleaved caspase 3 induction by verapamil treatment alone (V+/D−) was weak in DR-HSA cells compared with DS-HSA cells. Verapamil treatment did not affect the DNA damage burden, suggesting that other mechanisms unrelated to the DDR lead to apoptosis evasion in DR-HSA cells. However, doxorubicin has been reported to induce apoptosis in DNA damage-independent manner (Gewirtz, 1999; Swift et al., 2006), so further studies are required to reveal the mechanisms completely.

In this study, DR-JuB2 and DR-Re12 cells reacted differently to doxorubicin treatment. DR-JuB2 cells received DNA damage in the same way as DS-JuB2 cells, while DR-Re12 cells accumulated significantly more DNA damage than DS-Re12 cells even
before doxorubicin treatment. This difference probably arose through the number of cells with high drug efflux pump capacity in the population, i.e. JuB2 cells mainly relied on drug efflux to circumvent the harmful effects of doxorubicin. Meanwhile, in DR-Re12 cells, the weak DDR was probably more important than drug efflux, because most DR-Re12 cells did not have high drug efflux pump capacity and showed less ATM expression and distinctly lower apoptosis induction than DR-JuB2 cells. Verapamil treatment alone induced more cell death in DR-Re12 compared with DS-Re12, which might be explained by other verapamil toxicities such as autophagy-like processes in apoptosis-resistant cells (Pajak et al., 2012). There was no significant difference in the tail DNA percentages with and without doxorubicin in DR-Re12 cells. This may have arisen because the assay condition in the present study was unable to detect very high DNA damage, i.e. the signals were saturated. Tumor cells with high DNA damage burdens and apoptosis-evasion capability will accumulate DNA mutations in their genome followed by increasing genome instability (Lord and Ashworth, 2012). This can lead to genome amplification or deletion, which may result in cell cycle dysregulation, acquisition of invasiveness and metastatic ability as well as resistance against fatal DNA damage (Squatrito et al., 2010; Yao and Dai, 2014; Zhivotovsky and Kroemer, 2004). To greater or lesser extents, both cell lines in the present study acquired resistance to doxorubicin
by evading apoptosis. Further characterization of DR-HSA cells should be conducted to reveal whether doxorubicin resistance in HSA cells bestows more malignant features on the cells.

In conclusion, we have established doxorubicin-resistant canine HSA cell lines and indicated that high DNA efflux pump capacity and low DNA damage responses are part of their resistance mechanisms. Angiosarcoma, a human counterpart of HSA, has similar characteristics to canine HSA and also has no effective treatment (Young et al., 2010). Because human angiosarcoma is a very rare tumor, canine HSA has attracted attention as a good animal model for understanding its pathogenesis (Fosmire et al., 2004). Although further research using experimental animal models and clinical cases is required to fully elucidate the mechanism for chemotherapy resistance in HSA, the present findings provide a stepping stone to promote drug resistance studies in HSA as well as human angiosarcoma.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

References


Figure legends

Fig. 1 Establishment of DR-HSA cell lines.

(a) (Top) Survival curves of DR- and DS-HSA cells under doxorubicin treatment. $p$-value was calculated with Mann-Whitney U test. All samples were analyzed in triplicate. Survival curves are plotted as average percentages ± SD. Survival curves were calculated using the following equations by least-squares method using Ky Plot 5.0 software based on the results of cell viability assay. DS-JuB2: $y=4.4\times10^{-4}/(x+4.2\times10^{-6})$, DR-JuB2: $y=2.7\times10^{-3}/(x+2.9\times10^{-5})$, DS-Re12: $y=6.7\times10^{-5}/(x+5.8\times10^{-7})$, DR-Re12: $y=4.5\times10^{-4}/(x+3.9\times10^{-6})$. $y=$ survival rate (%), $x=$ doxorubicin concentration (M). (Bottom) $IC_{50s}$ of DR and DS-HSA cell lines. (b) Colony formation assay for DR- and DS-HSA cells. **$P$ < 0.01. Student's $t$ test. All samples were analyzed in triplicate and the scores are presented as means ±SD.

Fig. 2 DR-HSA cells have higher drug efflux pump capacity than DR-HSA cells.

(a) (Left) Flow cytometry analysis to analyze DCV efflux capacity. DCV− = DCV negative population. All samples were analyzed in triplicate. (Right) Quantitative
analysis of the DCV negative populations in each cell line. **$P<0.01$. Student’s $t$ test.

The percentages of the DCV negative populations are presented as average percentages ± SD. (b) Gene expression profiles of ABC transporters. All samples were analyzed in triplicate and the scores are presented as means ± SD. (c) Representative images of western blotting and quantitative data analysis for ABCB1 (top) and for ABCG2 (bottom).

293T (Dox 24h) is a protein sample from 293T cells treated with doxorubicin for 24h and was used as the positive control for both antibodies. *$P<0.05$. **$P<0.01$. Student’s $t$ test. All samples were analyzed in triplicate. The scores are presented as means ± SD. (d) Survival rate of DR- and DS-HSA cell lines after 24h treatment of verapamil with or without doxorubicin (V+/D− and V+/D+, respectively). *$P<0.05$. **$P<0.01$. Student’s $t$ test. All samples were analyzed in triplicate. The scores are presented as means ± SD.

Fig. 3 DR-HSA cells have similar amounts of DNA damage burden to DS-HSA cells after doxorubicin treatment.

(a, c) Alkaline comet assay for DR- and DS-HSA cells treated with verapamil (V) and with/without doxorubicin (D). V+/D− (0h) indicates the groups analyzed immediately after adding drugs. V+/D− (6h) and V+/D+ (6h) indicate the groups analyzed after drug treatments followed by 6h culture without doxorubicin and
verapamil. V−/D− (0h) indicates the control group cultured with DMSO. DNA damage burdens were analyzed by percentages of DNA in the tails (Tail DNA%) calculated using Open Comet plugin for imageJ software. \( *P < 0.05, \; **P < 0.01 \). Dunnet's test. All samples were analyzed in triplicate and Tail DNA% are present as means ± SD. (b, d) Combined graphs of (a) and (c), respectively, to compare DNA damage burdens between DS− and DR− HSA cells in each condition. \( *P < 0.05, \; **P < 0.01 \). Student's t test.

Fig. 4 DNA damage response is repressed in DR-HSA cells.

(a, c) Representative images of γH2A.X staining for DR− and DS− HSA cells treated with verapamil (V) and with/without doxorubicin (D). (b, d) The number of γH2A.X signal foci was calculated by imageJ software. \( **P < 0.01 \). Student's t test. Bars = 10 μm. All samples were analyzed in triplicate. Scores are presented as means ± SD.

Fig. 5 DDR is repressed in DR-HSA cells.

(a) Gene expression levels of DDR factors were evaluated by qPCR. The gene expression levels in DS− HSA cells were set to 1 in each cell line. \( **P < 0.01 \). Student's t test. All samples were analyzed in triplicate and the scores are presented as means ±
(b) Representative images of western blotting for ATM and quantitative data analysis for ATM protein expression levels. *P < 0.05. Student's t test. All samples were analyzed in triplicate. The scores are presented as means ± SD.

(c) Representative images of western blotting and quantitative data analysis for cleaved-caspase3 after 12h (JuB2) and 24h (Re12) culture with verapamil (V) and with/without doxorubicin (D). The concentrations of verapamil and doxorubicin were the same as those in alkaline comet assay. *p < 0.05. Student's t test. All samples were analyzed in triplicates. The scores are presented as means ± SD.

Fig. S1

(Top) Flow cytometry analysis to confirm that verapamil functions to inhibit DCV efflux in HSA cell lines. These graphs compared the DCV negative population between DR- and DS-cells in each cell line under verapamil treatment conditions. Original data are shown in Figure 2a. DCV− = DCV negative population. All samples were analyzed in triplicate. (Bottom) Quantitative analysis of the DCV negative populations in each cell line. Student's t test. The percentages of the DCV negative populations are presented as average percentages ± SD.

Fig. S2
Results of geNorm analysis for reference gene candidates. To determine optimal number of reference genes, 0.15 V value was used as the cut-off value (Vandesompele et al. 2002).

Fig. S3

Western blot analysis for (a) γH2A.X and (b) ATM. 293T cells and HeLa cells were used as the positive controls.
Author contributions

AM and KA designed and conceived the experiments. AM, KG, YS and HY performed the experiments and analyzed the corresponding results. AM and KA wrote the paper. HY, AK and TK revised the paper critically for important intellectual content. All authors read and approved the final manuscript.

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a

**JuB2**

![Graph showing survival rate vs. doxorubicin concentration for JuB2.]

**Re12**

![Graph showing survival rate vs. doxorubicin concentration for Re12.]

<table>
<thead>
<tr>
<th></th>
<th>DS-JuB2</th>
<th>DR-JuB2</th>
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<tbody>
<tr>
<td>IC50</td>
<td>4.6 (± 0.23) μM</td>
<td>25.6 (± 5.5) μM</td>
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<table>
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<tr>
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<th>DS-Re12</th>
<th>DR-Re12</th>
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<tbody>
<tr>
<td>IC50</td>
<td>0.77 (± 0.04) μM</td>
<td>5.1 (± 0.27) μM</td>
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b

![Images showing colony growth for JuB2 and Re12.]

- DS-JuB2: 
- DR-JuB2: 
- DS-Re12: 
  - # of survival colony: DS-Re12 < DR-Re12
- DR-Re12: 
  - # of survival colony: DS-Re12 < DR-Re12

**Fig. 1 Morita et al.**
Fig. 2 Morita et al.
Fig. 4 Morita et al.
**Fig. 5 Morita et al.**

(a) Graphs showing relative gene expression for ATM, ATR, PRKDC (DNA-PK), and CHEK1.

(b) Western blots for ATM and Actin comparing DS and DR conditions for JuB2 and Re12.

(c) Western blots showing cleaved caspase-3 and Actin levels with the addition of Verapamil and Doxorubicin in DS and DR conditions for JuB2 and Re12.
Fig. S1 Morita et al.
Determination of the optimal number of reference targets

Average expression stability of remaining reference targets

Fig. S2 Morita et al.
Fig. S3 Morita et al.