Functional analysis of CD44 variants and xCT in canine tumours

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Abstract
The cell surface glycoprotein CD44 has various types of splicing variants, which contribute to its multiple distinct cellular functions. Recently, it was reported that the CD44v8-10 isoform interacts with the system Xc(-) transporter-related protein (xCT), and inhibits the accumulation of reactive oxygen species by promoting the synthesis of the antioxidant glutathione in human tumour cells. In this study, we investigated the expression and function of CD44 variants and xCT in canine tumours. From semi-quantitative reverse transcription polymerase chain reaction analysis, the mRNA expression of the CD44v8-10 isoform was observed in canine tumour tissues as well as human cases. The overexpression of CD44v8-10 may promote the synthesis of glutathione and enhance the resistance to radiation of canine breast tumour cells. Furthermore, canine xCT mRNA expression was significantly upregulated in the canine breast tumour tissues as compared to the normal tissues surrounding the tumours. To investigate the function of canine xCT, we treated canine tumour cells with the xCT inhibitor sulfasalazine. Consequently, the sulfasalazine-treated cells were more sensitive to oxidative stress than the non-treated cells. Taken together, these results suggested that CD44v8-10 and xCT play important roles in the therapy resistance of canine tumours as well as human tumours.

KEYWORDS
canine, CD44, oxidative stress, radiation, xCT

1 | INTRODUCTION

CD44 is a cell surface molecule that mediates cell adhesion and communication with the extracellular matrix. CD44 has various biological functions, including leukocyte homing, wound healing, cell proliferation and migration, as well as tumour cell growth, invasion and metastasis (Goodison et al., 1999; Ponta et al., 2003). Moreover, CD44 is considered to be a major marker for stem-like cells in several types of tumours (Zöller, 2011).
et al., 2019; Wada et al., 2018; Wu et al., 2015; Yoshikawa et al., 2013). CD44v9 is associated with the system Xc(−) transporter-related protein (xCT), which is a component of the plasma membrane antiporter system xCT (Hirata et al., 2013; Horibe et al., 2018; Ishimoto et al., 2011; Ogihara et al., 2019; Wada et al., 2018; Yoshikawa et al., 2013). This system comprises xCT and CD98hc subunits, and it regulates the uptake of cysteine (Huang et al., 2005; Lo et al., 2008). Cysteine is an essential amino acid for the synthesis of glutathione (GSH), which is required for the reduction of intracellular oxidants. Thus, the association between xCT and CD44v9 in the plasma membrane is considered to be an important factor for the intracellular redox system in tumour cells. On the other hand, CD44 standard isoform (CD44s), which contains no variant exons, was revealed to be upregulated in several tumours, such as breast and liver tumours (Asai et al., 2019; Brown et al., 2011; Mima et al., 2012). CD44s-positive tumour cells show high metastatic ability, as breast and liver tumours (Asai et al., 2019; Brown et al., 2011; Mima et al., 2012). CD44s-positive tumour cells show high metastatic ability, whereas their tumourigenicity appears to be dependent on the tumour type (Miwa et al., 2017; Preca et al., 2015).

CD44 is recognized as a cancer stem cell marker in canine breast tumours, (Barbieri et al., 2015; Du et al., 2017; Zhou et al., 2018) and its expression is also increased in canine leukaemia, melanoma and osteosarcoma (Gelain et al., 2014; Guth et al., 2014; Milovancev et al., 2013). Although it is known that the canine CD44 gene encodes 20 exons, including 10 variant exons, as observed with the human CD44 gene, the expression pattern and role of CD44 isoforms in canine tumours remain unclear (Miide et al., 1994; Motegi et al., 2018). Here, we investigated the expression and function of CD44s and CD44v in canine tumours.

2 | MATERIALS AND METHODS

2.1 | Cell lines and culture conditions

Canine melanoma cell line MCM-N1 was purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Canine lung adenocarcinoma cell line CLAC and canine osteosarcoma cell line OS730 were previously established and characterized by a primary canine lung adenocarcinoma (Nemoto et al., 2011) and a primary canine osteosarcoma, (Tanabe et al., 2016) respectively. Canine breast tumour cell line CIMC-A was provided by Dr. Kikumi Ogihara (Azabu University). CIMC-A was previously established and characterized by a primary canine inflammatory breast tumour (unpublished data). Canine melanoma cell line CMeC and canine osteosarcoma cell line PoS were provided by Dr. Takayuki Nakagawa (University of Tokyo). CMeC was previously established and characterized by a primary canine skin melanoma, (Inoue et al., 2004) whereas PoS was established from a primary canine osteosarcoma (Kadosawa et al., 1994). Canine osteosarcoma cell line HMPoS and canine mastocytoma cell line CoMS were provided by Dr. Masahiro Okumura (Hokkaido University). HMPoS was previously established and characterized by lung metastatic PoS cells, (Barroga et al., 1999) whereas CoMS were established and characterized by a mast cell tumour of the oral mucosa (Ishiguro et al., 2001).

MCM-N1, OS730, CIMC-A and CLAC were cultured in DMEM medium (Sigma-Aldrich, St Louis, MO, USA), and CMeC, PoS, HMPoS and CoMS were cultured in RPMI1640 medium (Sigma-Aldrich). Both media were supplemented with 10% foetal bovine serum, 200 unit/ml penicillin (Thermo Fisher Scientific, Waltham, MA, USA), 200 μg/ml streptomycin (Thermo Fisher Scientific) and 2 mM L-glutamine (Thermo Fisher Scientific), and the cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. The canine cancer cell lines used in this study were treated with the mycoplasma remover MC-210 (Wakenbtech, Kyoto, Japan) before experiments. The canine cancer cell lines were passaged less than 10 times after thawing.

2.2 | Canine tumour and normal tissue samples

Canine tumour tissues and the normal tissues surrounding the tumours were collected from 39 canine tumour patients who underwent surgery at Taguchi Animal Hospital (Saitama, Japan) between 2010 and 2011. This study was approved by the appropriate ethics committee of Azabu University Veterinary Teaching Hospital, and written informed consent was obtained from all dog owners. The sampling of all tumour tissues and normal tissues surrounding each tumour was performed according to standard surgical resection procedures. The final diagnosis of tumours and normal tissues was determined by histopathological examination, and the tumours were classified based on the pathological diagnosis. Resected tissues were immediately immersed in RNAlater (QIAGEN Inc., Hilden, Germany) and stored at 4°C.

2.3 | RNA extraction and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared using an RNeasy Mini Kit (QIAGEN Inc.) according to the manufacturer’s instructions, and then reverse-transcribed to cDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). For the analysis of CD44 splice variants in the canine breast tumour tissues, the normal tissues surrounding the tumours and the canine tumour cell lines, semi-quantitative RT-PCR analysis was performed with the following primer sets: CD44-F 5′- CAG TGA AAG GAG CAC TTC GG - 3′ and CD44-R 5′- GAT CCA TGA GTG GTA TGG GAC -3′; and GAPDH-F 5′- AAC GGG AAG CTC ACT GCC AT -3′ and GAPDH-R 5′- CCT GAC AAA GTG GTC ATT GAG GG -3′.

2.4 | Quantitative real-time PCR analysis

Quantitative real-time PCR analysis of the GAPDH and xCT (SLC7A11) genes was performed using the LightCycler FastStart DNA MasterPLUS SYBR Green I system (Roche Applied Science). The quantitative real-time PCR was performed with the following primer sets: xCT-F 5′- CCA GGT TAT TCT ATG TTG CG -3′ and xCT-R 5′- CAC CTG
the copy number of the target by the copy number of between samples, each target gene was first normalized by dividing the absorbance at 260 nm with a spectrophotometer, and the copy numbers were calculated from the concentration of the samples. A standard curve was created by plotting the threshold cycle versus the known copy number for each plasmid template in the dilutions. The copy numbers for all unknown samples were determined according to the standard curve using LightCycler version 3.5.3 (Roche Applied Science). To correct for differences in both RNA quality and quantity, two housekeeping genes, GAPDH and β-actin, were used for normalization and calculation of the absolute copy numbers of the target transcripts. The fragments of the target gene amplified by PCR using the primer sets were ligated into the pGEM-T-easy cloning vector (Promega, Madison, WI, USA). The concentrations of these purified plasmids were determined by measuring the absorbance at 260 nm with a spectrophotometer, and the copy numbers were calculated from the concentration of the samples. The copy numbers of the target by the copy number of GAPDH.

2.5 | Plasmid vector construction and transfection

Using the first-strand cDNA derived from CIMC-A as a PCR template, CD44s and CD44v8-10 plasmid vector were generated by PCR using the following CD44 primer set: CD44-F 5′- ATG GAC AAG TTG GGG TGG CA-3′ and CD44-R 5′- CAC CCC AAT CTT CAT ATC CA-3′. These PCR-generated DNA fragments containing the coding region of CD44s and CD44v8-10 were cloned into pCR™ 2.1-TOPO vector (Thermo Fisher Scientific). The pCR™ 2.1-TOPO vectors containing CD44s or CD44v8-10 were digested by Hind III/Not I (CD44s) or Kpn I/Not I (CD44v8-10). Subsequently, the samples were ligated into the pcDNA3.1 expression vector (Thermo Fisher Scientific). For transient transfection, 3 × 10^6 CIMC-A cells were seeded in a 100-mm dish, and transfected with 3 µg of CD44s, CD44v8-10 or control empty vector with the use of the Polyethylenimine “Max” (Polyscience Inc., Warrington, PA, USA).

2.6 | H_2O_2 and sulfasalazine (SSZ) treatment and MTT assay

Cell survival after H_2O_2 treatment was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. CIMC-A cells (3 × 10^5) were cultured in a 100-mm dish, and transfected with 3 µg of CD44s, CD44v8-10 or control empty vector. At 48 hr after the transfection, the cells were trypsinized and counted. Then, 1 × 10^5 cells were plated in a 96-well plate, and 1 to 3 mM H_2O_2 was added 16 hr later. After 24 hr, 4 mg/ml MTT (Sigma-Aldrich) in PBS was added (10 µl/well), and the plates were incubated at 37°C for 4 hr. After the incubation, the formazan crystals were dissolved in 100 µl of 0.04 M hydrochloride-2-propanol solution, and the absorbance was measured at 570 nm. The cell survival with SSZ treatment was also measured by the MTT assay. CIMC-A cells (1 × 10^5) were plated in a 96-well plate, and 100 µM SSZ was added 16 hr later. After 24 hr, the cells were treated with or without 100 µM H_2O_2 for 24 hr, and then the cell viability was measured by the MTT assay.

2.7 | Exposure to radiation

CIMC-A cells (3 × 10^5) were cultured in a 100-mm dish, and transfected with 3 µg of CD44s, CD44v8-10 or control empty vector. At 48 hr after the transfection, ionizing radiation at a dose of 0, 1 or 5 Gy was irradiated to the transfected CIMC-A cells. Radiation was delivered with a linear accelerator radiation machine (Hitachi Medical, Tokyo, Japan). After radiation exposure, the cells were cultured for 1, 3 or 5 days, harvested by trypsinization. Cell viability was measured by the MTT assay and viability rate was calculated as the optical density (OD) value of radiation-exposed cells/OD value of non-exposed control cells ×100%.

2.8 | Measurement of GSH

The intracellular level of GSH was measured with the GSH-Glo™ Glutathione Assay kit (Promega) according to the manufacturer’s instructions. Cells (5 × 10^5) were suspended in passive lysis buffer, and incubated for 30 min at room temperature. Then, 100 µl of luciferin detection reagent was added. After 15 min of incubation at room temperature, the luminescence signal generated in a coupled reaction with firefly luciferase was measured by a Glomax 20/20 Luminometer (Promega).

2.9 | Western blotting

Anti-myc antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-β-actin antibody was purchased from Sigma-Aldrich. CIMC-A cells (3 × 10^5) were cultured in a 100-mm dish, and transfected with CD44s, CD44v8-10 or control empty expression vector. At 48 hr after the transfection, 1 × 10^5 cells were lysed in 100 µl of lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5% glycerol and protease inhibitor cocktail [Roche Applied Science]) for 30 min on ice. Then, 50 µl of 3x sodium dodecyl sulphate sample buffer was added, and the samples were boiled at 100°C for 5 min. Next, the samples were subjected to 8% to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, then transferred onto a Hybon-ECL nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA). The transferred antigens on the membrane were detected by Western blotting with anti-myc or β-actin antibody.

2.10 | Statistical analysis

The results represent the mean ± standard deviation (SD) values. The Statcel 3 add-in (OMS Publishing, Saitama, Japan) for Microsoft Excel was used for statistical analysis. The Statcel 3 add-in (OMS Publishing, Saitama, Japan) for Microsoft Excel was used for statistical analysis. The Statcel 3 add-in (OMS Publishing, Saitama, Japan) for Microsoft Excel was used for statistical analysis. The Statcel 3 add-in (OMS Publishing, Saitama, Japan) for Microsoft Excel was used for statistical analysis.
Excel was used for the statistical analyses. Yates chi-squared ($\chi^2$) test was used to analyse the differences in the expression of CD44 variants between the canine tumours and the normal tissues surrounding the tumours. Statistical analysis of the cytotoxic effects of $H_2O_2$ or radiation treatment was performed using the Student’s t-test or two-way analysis of variance followed by the Tukey–Kramer post hoc test. The xCT mRNA expression was compared between the breast tumour tissues and the normal tissues surrounding the tumours using the Wilcoxon rank-sum test. $p$ values <0.05 or <0.01 were considered to be statistically significant.

3 | RESULTS

3.1 | Semi-quantitative RT-PCR analysis of CD44 mRNA in canine tumours and normal tissues surrounding the tumours

We first examined the CD44 mRNA expression in the canine tumours and the normal tissues surrounding the tumours by semi-quantitative RT-PCR. As shown in Figure 1, CD44s was expressed in all canine tissues. In contrast, although CD44v was expressed in several tumour tissues, it was hardly detected in the normal tissues (Figure 1). The number of cases expressing CD44 variants was significantly different between the breast tumour tissues and the normal tissues surrounding the tumours ($\chi^2 = 10.8, p = 0.001$; Table 1).

Furthermore, we examined the expression of CD44s and CD44v mRNA in several canine tumour cell lines, including breast tumour (CIMC-A), melanomas (MCM-N1 and CMeC), osteosarcomas (OS730, PoS and HMPOs), mastocytoma (CoMS) and lung adenocarcinoma (CLAC) (Figure 2a). The expression of CD44v mRNA was observed in CIMC-A and CLAC cells (Figure 2a). These results indicated that the expression of CD44 variant isoforms is upregulated in several tumour tissues and cell lines. In addition, we obtained the PCR products from the DNA bands in agarose gels, and examined the nucleotide sequence of the PCR product derived from CIMC-A by Sanger sequencing. We found that the PCR product derived from CIMC-A included variant exons 8 to 10, and the nucleotide sequence was consistent with that in the GenBank database (XM_025452466.1) and a previous report on canine CD44 variant exons (Figure 2b) (Milde et al., 1994).

3.2 | Overexpression of CD44v8-10 increases the amount of cellular GSH and prevents canine tumour cell death by $H_2O_2$ or radiation treatment

To investigate the roles of CD44s and CD44v8-10 in the ROS-resistance of canine breast tumour cells, CIMC-A cells were transfected with CD44s or CD44v8-10 isoforms, and the resistance to $H_2O_2$ treatment was examined (Figure 3a and b). As shown in Figure 3b, a significant increase in cell viability was observed in the CD44v8-10 transfectants, but not in the CD44s transfectants. In addition, the intracellular GSH content was increased in the CD44v8-10 transfectants when compared with the control and the CD44s transfectants (Figure 3c). To further investigate the role of CD44v8-10 in canine breast tumours, we examined the resistance of these transfectants to radiation treatment. After 3 and 5 days of radiation treatment, the cell viability was significantly higher in the CD44v8-10 transfectants than in the control and the CD44s transfectants (Figure 3d).

3.3 | Quantitative RT-PCR analysis of xCT mRNA in canine tumours and the effect of SSZ on $H_2O_2$-resistance in canine breast tumour cells

We next investigated the mRNA expression of xCT in the canine breast tumours, the normal tissues surrounding the tumours and the canine tumour cell lines by quantitative real-time PCR. As shown in
Figure 4a and b, the mRNA expression of xCT was significantly upregulated in the canine breast tumour tissues as compared to the normal tissues. To investigate whether the inhibition of xCT enhances the cytotoxic effect of $\text{H}_2\text{O}_2$, we applied SSZ, which is an xCT inhibitor, to CIMC-A cells. As shown in Figure 4c, the $\text{H}_2\text{O}_2$ sensitivity of CIMC-A cells was increased by SSZ treatment (Figure 4c).

**DISCUSSION**

Semi-quantitative RT-PCR was used to demonstrate that CD44s mRNA expression is observed in both normal and tumour canine tissues. CD44s is generally present on the membranes of most hematopoietic and normal epithelial cells, and it binds extracellular matrix ligands, such as hyaluronic acid and fibronectin (Goodison et al., 1999; Ponta et al., 2003; Zöller, 2011). Several studies have reported that CD44s modulates cellular signalling by interacting with various receptor tyrosine kinases, and it induces epithelial-to-mesenchymal transition and stem-like properties in tumour cells (Brown et al., 2011; Mima et al., 2012; Ponta et al., 2003; Zöller, 2011). In contrast, CD44v mRNA was expressed only in a subset of canine tumours. Alternative mRNA splicing of CD44 is mediated by epithelial splicing regulatory protein 1 (ESRP1), (Brown et al., 2011; Jeong et al., 2017; Preca et al., 2015; Yae et al., 2012) and several reports have suggested that the upregulation of ESRP1 is associated with poor prognosis of tumours (Jeong et al., 2017; Yae et al., 2012). A variety of CD44v isoforms generated by ESRP1, such as CD44v6 and CD44v8-10, have been shown to be involved in multiple cellular functions, including proliferation, adhesion and metastasis (Hirata et al., 2013; Horibe et al., 2018; Ishinoto et al., 2011; Li et al., 2014; Nagano et al., 2013; Ogihara et al., 2019; Patel et al., 2018; Wada et al., 2018; Wu et al., 2015; Yoshikawa et al., 2013). In canine, it has been reported that CD44v3, v6 and v7 mRNAs are expressed in B-cell lymphoma, and the expression of these mRNAs is associated with chemo-resistance and a poor prognosis (Motegei et al., 2018). However, the relative importance of CD44v isoforms in comparison to the CD44s isoform as a factor for tumour progression remains controversial. Although there have been conflicting reports about the role of CD44 isoforms on tumour phenotypes, we demonstrated that CD44v8-10, but not CD44s, facilitated the resistance of canine breast tumour cells to $\text{H}_2\text{O}_2$ and radiation in this study. Namely, CD44v8-10 was shown to be associated with the xCT transporter, and it contributes to the production of GSH, a major cellular antioxidant (Huang et al., 2005; Lo et al., 2008). Tumour cells are continuously exposed to ROS stress derived from intracellular or extracellular sources; thus, a robust antioxidant system is required for survival and efficient growth (Nagano et al., 2013; Vučetić et al., 2017). Furthermore, upregulation of CD44v has been observed in chemotherapy-resistant and recurrent tumours, (Hirata et al., 2013; Horibe et al., 2018; Wu et al., 2015; Yoshikawa et al., 2013) suggesting that CD44v plays important roles in tumour progression via the regulation of the cellular antioxidant system.

**Table 1** The differences of CD44 variants expression between in the canine tumours and the normal tissues surrounding the tumours

| Neoplasm                                | CD44v | Normal | Tumour | $\chi^2$ | p     |
|-----------------------------------------|-------|--------|--------|----------|-------|
| Negative                                |       |        |        |          |       |
| Positive                                |       |        |        |          |       |
| Benign breast tumour (adenoma + mixed adenoma) | Negative | 11 | 6 | 6.5 | 0.042 |
|                                         | Positive | 0 | 5 |     |       |
| Malignant breast tumour (carcinoma + mixed tumour) | Negative | 9 | 4 | 6.9 | 0.035 |
|                                         | Positive | 0 | 5 |     |       |
| Total breast tumour (benign + malignant) | Negative | 20 | 10 | 10.8 | 0.001 |
|                                         | Positive | 0 | 10 |     |       |
| Oral squamous cell carcinoma            | Negative | 2 | 2 | 0.317 | 0.167 |
|                                         | Positive | 0 | 2 |     |       |
| Melanoma                                | Negative | 2 | 2 | 0.750 | 0.386 |
|                                         | Positive | 1 | 1 |     |       |
| Mastocytoma                             | Negative | 3 | 2 | 0.000 | 1.000 |
|                                         | Positive | 1 | 2 |     |       |
| Soft tissue sarcoma                     | Negative | 2 | 2 |         |       |
|                                         | Positive | 0 | 0 |     |       |
| Angiosarcoma                            | Negative | 5 | 3 | 0.625 | 0.429 |
|                                         | Positive | 0 | 2 |     |       |
| Lymphoma                                | Negative | 3 | 3 |         |       |
|                                         | Positive | 0 | 0 |     |       |
| Total                                   | Negative | 37 | 22 | 13.6 | 0.0002 |
|                                         | Positive | 2 | 17 |     |       |

Note: (−) indicates incalculable.
Moreover, we found that xCT mRNA expression was significantly upregulated in the canine breast tumour tissues as compared to the normal mammary gland tissues surrounding the tumour. The upregulation of xCT was observed regardless of CD44v expression, suggesting that the transcriptional machinery for xCT could be activated independently of CD44v in canine breast tumours. In human, several studies have reported that the transcription of the xCT gene is regulated by transcription factors Nrf2 and ATF4 (Lewerenz & Maher, 2009; Lewerenz et al., 2012; Sasaki et al., 2002). Translation of the ATF4 protein is enhanced by multiple types of stresses, including oxidative stress; thus, ATF4 is thought to be involved in the resistance of tumours to chemotherapy via the regulation of xCT expression (Lewerenz & Maher, 2009; Lewerenz et al., 2012). To investigate the function of xCT in the resistance of tumours to chemotherapy, several studies have employed SSZ as an inhibitor of xCT (Gout et al., 2001; Ishimoto et al., 2011; Ma et al., 2015; Oghihara et al., 2019; Wada et al., 2018; Yoshikawa et al., 2013). Indeed, previous studies showed that SSZ treatment enhanced the effect of several chemotherapies (Gout et al., 2001; Ishimoto et al., 2011; Oghihara et al., 2019; Wada et al., 2018; Yoshikawa et al., 2013). Consistent with the previous reports, we showed that SSZ treatment enhanced the cytotoxic effect of H2O2 in canine breast tumour cells, suggesting that xCT plays an important role in the therapy resistance of canine tumours as well as human tumours.

In conclusion, our present findings suggested that CD44v and xCT are expressed in several canine tumours, and they contribute to the resistance of tumour cells to oxidative stress and radiation. CD44v8-10-overexpressing canine breast tumour cells were shown to produce GSH at a higher level than CD44s-overexpressing cells. Thus, tumour-specific antioxidant systems may be a potential target for the chemotherapy and radiotherapy of canine tumours.

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The authors declare no conflicts of interest for this article.

CONFLICT OF INTEREST

The authors declare no conflicts of interest for this article.

AUTHOR CONTRIBUTION

Atsushi Tanabe: Investigation; Writing-original draft. Kento Kimura: Investigation. Hana Tazawa: Investigation. Takuya Maruo: Formal analysis; Investigation. Masayuki Taguchi: Investigation; Resources. Hiroeki Sahara: Conceptualization; Funding acquisition; Project administration; Writing-review & editing.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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