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Original Article

Canine squamous cell carcinoma cell lines with high expression of survivin are sensitive to survivin inhibitor YM155

R. Miyamoto, S. Kurita, H. Tani, T. Ikeda, M. Ishizaka, H. Saima, M. Kobayashi, K. Tamura, M. Bonkobara*

Department of Veterinary Clinical Pathology, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180-8602, Japan

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ABSTRACT

Treatment of unresectable canine squamous cell carcinoma (SCC) remains challenging and new therapeutic strategies are needed. Survivin is a member of the inhibitor of apoptosis protein family and its inhibitor, YM155, is a potential anti-tumour agent. In the present study, 10 canine tumour cell lines (representing eight different tumour types) were screened for sensitivity to YM155; the drug potently inhibited the growth of the HAPPY SCC cell line. The growth inhibitory properties of YM155 were then examined in more detail using a panel of seven SCC cell lines. YM155 inhibited the growth of the cell lines HAPPY and SQ4; in contrast to the other lines in the panel, these two cell lines had high levels of expression of survivin. In HAPPY cells, YM155 inhibited expression of the *survivin* gene at the transcriptional level. In contrast, YM155 down-regulated survivin at the post-transcriptional level in SQ4 cells. YM155 suppressed cell growth in HAPPY cells, mostly via induction of apoptosis, but this was not the case in SQ4 cells. Two canine SCC cell lines with high cellular expression of survivin were sensitive to YM155. The possible underlying mechanisms of the cytotoxic effect of YM155 in these cell lines were different. One cell line had down-regulation of survivin mRNA and protein expression, associated with induction of apoptotic cell death. The other cell line had post-transcriptional down-regulation of survivin expression and subsequent induction of non-apoptotic cell death. Targeting survivin with YM155 is a potential approach for the treatment of canine SCCs with high expression of survivin.

Introduction

Squamous cell carcinoma (SCC) is a common canine tumour that arises from the squamous epithelium in a variety of locations, such as the skin, oral cavity, nasal cavity, tonsils and lung (Webb et al., 2009). With the exception of certain locations (e.g. tonsillar SCC), SCCs are not often metastatic, but rather locally invasive. Aggressive surgery, or surgery combined with adjuvant radiotherapy, may thus be effective (Webb et al., 2009). However, if the tumour reaches a certain size, is in a location where removal is not technically possible or feasible, or has already metastasised, treatment is challenging. Under these circumstances, chemotherapy seems logical. However, no effective chemotherapeutic approaches have yet been established for canine SCC and new therapeutic strategies are required.

Survivin, a member of the inhibitor of apoptosis protein family, is expressed in a large number of human malignancies and has been shown to function as an inhibitor of caspase activation, thereby leading to negative regulation of apoptosis in various neoplastic cells (Chen et al., 2016). Survivin is therefore an attractive target for tumour therapy and its inhibitors are a potential new class of anti-tumour agents. YM155, an imidazolium-based small molecule, is a potent survivin inhibitor that functions as a transcriptional suppressor of the survivin promoter (Rauch et al., 2014). YM155 has growth inhibitory activity in a variety of human tumours (Rauch et al., 2014), as well as in canine histiocytic sarcoma (Yamazaki et al., 2015a,b), both in vitro and in xenograft models. Early results from phase I and II clinical trials have shown safety and some efficacy of YM155 in human patients with various tumour types, including lung cancer, malignant melanoma, prostate cancer and lymphosarcoma/lymphoma (Rauch et al., 2014); further clinical trials are ongoing (Khan et al., 2017).

Similar to human tumours, survivin is widely-expressed in a variety of malignancies in dogs, including various cutaneous and subcutaneous tumours (Kavya et al., 2017), osteosarcoma (Shoeneman et al., 2012), haemangiosarcoma (Murakami et al., 2008), lymphosarcoma/lymphoma (Wimmershoff et al., 2010),

* Corresponding author.
E-mail address: bonkobara@nvlu.ac.jp (M. Bonkobara).

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transitional cell carcinoma (Rankin et al., 2008), histiocytic sarcoma (Yamazaki et al., 2015a) and malignant melanoma (Bongiovanni et al., 2015). This makes YM155 an attractive agent for canine tumour therapy. In the present study, we screened canine tumour cell lines for sensitivity to YM155. On the basis of these results, we focussed on SCC as a target for YM155 treatment, and investigated the growth inhibitory properties and mechanisms of action of this drug in SCC cell lines.

Materials and methods

Screening of canine tumour cell lines for sensitivity to the survivin inhibitor YM155

Nineteen canine cell lines, including 16 tumour cell lines, two non-neoplastic squamous epithelial cell lines and one non-neoplastic renal epithelial cell line, were used in this study (Table 1). The tumour cell lines used for sensitivity to YM155 were malignant melanoma (LMeC and NML), osteosarcoma (RIAM and RION), SCC (HAPPY), histiocytic sarcoma (CHS-6), mammary gland carcinoma (EMC-1), transitional cell carcinoma (CTCJ), mast cell tumour (VI-MC) and prostate cancer (CHP-1) canine cell lines. The screening was conducted using 10 nM YM155, since the steady-state plasma concentration of YM155 is 10–20 nM in humans given YM155 at a dose of 4.8 mg/m^2 (Tolcher et al., 2008), which has been used in phase II trials (Giaccone et al., 2009).

All cell lines, except for VI-MC, were cultured in 96-well tissue-culture treated plates (1.0×10^3/well) for 24 h in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (Thermo Fisher Scientific), 50 U/mL penicillin (Thermo Fisher Scientific) and 50 μg/mL streptomycin (Thermo Fisher Scientific) (cDMEM), then treated with 10 nM YM155 (Cayman Chemical) for 48 h. VI-MC cells were suspended in RPMI-1640 (Life Technologies) supplemented with the same additives as cDMEM (cRPMI), seeded at 1.2×10^4 cells/well in 96-well tissue-culture treated plates and treated with 10 nM YM155 (Cayman Chemical) for 48 h. As controls, all cell lines were treated with vehicle (0.1% dimethyl sulphoxide, DMSO) for 48 h. Cell viability was then measured with a WST-1 cell proliferation assay kit (Takara).

Squamous cell carcinoma growth inhibition assay

Seven canine SCC cell lines (HAPPY, SQ4, SYRUP, CSCC-R1, KUCKY, SCC 2/88 and POCO) and three non-neoplastic canine cell lines, including two squamous epithelial cell lines (CPEK and COPK) and one renal epithelial cell line (MDCK), were cultured in 96-well tissue-culture treated plates (1.0×10^3 cells/well) for 24 h in cDMEM. Subsequently, cells were treated with decreasing concentrations of YM155 (10^4 nM to 0 nM) for 48 h. Cell viability was measured using a WST-1 cell proliferation assay kit (Takara). The half-maximal inhibitory concentration (IC_{50}) of YM155 for each cell line was calculated using GraphPad Prism.

Semi-quantitative reverse transcription PCR

Total RNA was extracted from HAPPY and SQ4 cells treated with YM155 (2.5 nM) for 0, 24, 48 or 72 h using RNASTAT 60 (Tel-TestB) and reverse-transcribed into cDNA using SuperScript III (Thermo Fisher Scientific). Aliquots of cDNA were subjected to PCR using a primer set designed using Lasergene (DNA Star) on the basis of the nucleotide sequence of canine *survivin* (GenBank NM_001003348): 5′ primer: 5′-GCTTCATCCACTGTCCCACT-3′; 3′ primer 5′-TTGTTGGTTTCCTTTGC-3′ or canine *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*, GenBank NM_001003142): 5′ primer: 5′-ACTTGTCATCAACGGGAAGT-3′; 3′ primer 5′-CAATCTTGAGGGAGTTGTCA-3′. PCR reactions were performed using Tks Gflex DNA polymerase (Takara) in a reaction mixture (Gflex PCR Buffer, Takara) containing 400 nM of each primer on a T100 Thermal Cycler (Bio-Rad). The PCR cycling protocol consisted of denaturing for 10 s at 98 °C, annealing for 15 s at 58 °C for *survivin* and at 60 °C for *GAPDH*, and extension for 30 s at 68 °C. After 27 cycles of PCR amplification, products (5 μL aliquots) were size-fractionated on a 1.2% agarose gel and visualised with ethidium bromide using the LAS-500 (Fujifilm). Band intensities were semi-quantified using ImageQuant TL software (Fujifilm) and levels of expression of survivin mRNA were normalised to levels of expression of GAPDH mRNA.

Western blot analysis

HAPPY, SQ4, SYRUP, CSCC-R1, KUCKY, SCC 2/88 and POCO cells were lysed with cell lysis buffer (#9803, Cell Signaling Technology). Proteins in the cell lysate were separated by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad) using a Trans-Blot Turbo Blotting System (Bio-Rad). After blocking non-specific protein binding with 5% non-fat dry milk, the membrane was incubated with rabbit anti-human survivin (polyclonal, Novus), confirmed to cross-react with canine survivin (Yamazaki et al., 2013) or mouse anti-rabbit GAPDH (Abcam, clone 6C5), followed by horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG; GE Healthcare) or goat anti-mouse IgG (Jackson ImmunoResearch). Immunoreactive bands on the membranes were visualised using ECL Prime (GE Healthcare) and LAS-500 (Fujifilm). Band intensities were semi-quantified using ImageQuant TL software (Fujifilm) and levels of expression of survivin were normalised to levels of expression of GAPDH.

Detection of apoptosis

Apoptosis was detected using flow cytometer-based annexin V/propidium iodide (PI) dual staining. HAPPY, SQ4 and CSCC-R1 cells treated with YM155 (0, 2.5 or 5.0 nM) for 48 h were stained with fluorescein isothiocyanate-(FITC) conjugated annexin V and PI using the MEBCYTO apoptosis kit (MBL technologies) and subjected to flow cytometry (FACSCalibur, Becton-Dickinson) for the detection of apoptosis.

Statistical analysis

Statistical analysis was performed using unpaired two-tailed Student's *t* tests in Excel (Microsoft), with $P < 0.05$ considered to be significant.

Results

Identification of a cell line highly sensitive to YM155

Table 2 shows the results of screening for canine tumour cell lines sensitive to YM155. Amongst the 10 cell lines screened (including eight different tumour types), only the SCC cell line

Table 1
Canine cell lines used for this study.

Cell line	Tumour type	Origin	Source
LMeC	Malignant melanoma	Oral cavity	Dr Nakagawa, University of Tokyo
NML	Malignant melanoma	Nail bed	Established in our laboratory
RIAM	Osteosarcoma	Bone	Dr Ono, Japan Animal Referral Medical Centre
RION	Osteosarcoma	Bone	Dr Ono, Japan Animal Referral Medical Centre
CHS-6	Histiocytic sarcoma	Synovium	Established in our laboratory
EMC-1	Mammary gland carcinoma	Mammary gland	Established in our laboratory
CTCJ	Transitional cell carcinoma	Urinary bladder	Established in our laboratory
VI-MC	Mast cell tumour	Intestine	Dr Nakagawa, University of Tokyo
CHP-1	Prostate cancer	Prostate	Established in our laboratory
HAPPY	Squamous cell carcinoma	Oral cavity	Dr Ono, Japan Animal Referral Medical Centre
SQ4	Squamous cell carcinoma	Nasal cavity	Established in our laboratory
SYRUP	Squamous cell carcinoma	Oral cavity	Established in our laboratory
CSCC-R1	Squamous cell carcinoma	Oral cavity	Drs Endo, Hagiwara and Ishii, Rakuno Gakuen University
KUCKY	Squamous cell carcinoma	Muzzle	Dr Ono, Japan Animal Referral Medical Centre
SCC 2/88	Squamous cell carcinoma	Lip	CELLnTEC
POCO	Squamous cell carcinoma	Tonsil	Established in our laboratory
CPEK	Non-neoplastic squamous cell	Skin	CELLnTEC
COPK	Non-neoplastic squamous cell	Lip	CELLnTEC
MDCK	Non-neoplastic kidney epithelial cell	Kidney	RIKEN BioResource Centre