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Research paper

Comparison of the antitumor effects of an MDM2 inhibitor, nutlin-3, in feline lymphoma cell lines with or without *p53* mutation

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ABSTRACT

The P53 tumor suppressor protein is a multifunctional transcription factor that prevents the malignant transformation of normal cells. In human malignancies, *p53* is the most frequently altered gene and is mutated in approximately 50% of all malignancies. In contrast, *p53* gene mutation has been rarely detected in feline malignancies, and most feline malignancies conceivably retain the wild-type *p53* (wt-*p53*) gene. MDM2 negatively regulates the P53 protein by inhibiting its transcriptional activity and nuclear transport and by inducing its degradation. Inhibition of P53–MDM2 interaction stabilizes P53 protein and activates P53 pathway. Nutlin-3, a small molecule that inhibits P53–MDM2 interaction, was shown to have an antitumor effect in several human cancer cells retaining the wt-*p53* gene. In the present study, we evaluated and compared the antitumor effect of nutlin-3 in 5 different feline lymphoma cell lines, of which 3 harbored wt-*p53*, and 2, mutated *p53* (mt-*p53*). Treatment with nutlin-3 resulted in increased amounts of P53 protein in conjunction with augmented expression of P53-target genes in 3 feline lymphoma cell lines with the wt-*p53* gene, but not in 2 feline lymphoma cell lines with the mt-*p53* gene. Nutlin-3 treatment also induced G1-S and/or G2-M cell cycle arrest and apoptosis in lymphoma cell lines with wt-*p53*. Nutlin-3 treatment induced cell cycle arrest but not apoptosis in the cell lines with mt-*p53*. From these results, we concluded that nutlin-3 has an antitumor effect on feline lymphoma cell lines harboring the wt-*p53* gene through accumulation and activation of P53 leading to cell cycle arrest and apoptosis. The present study suggests that inhibition of P53–MDM2 interaction using nutlin-3 may be a new therapeutic strategy for treating feline lymphoma retaining the wt-*p53* gene.

1. Introduction

Lymphoma is the most common neoplastic disease in cats (Dorn et al., 1967). Multi-drug chemotherapy including vincristine, cyclophosphamide, and doxorubicin has been widely used for the treatment of cats with high-grade lymphoma. Although multi-drug chemotherapy is the mainstay for treating high-grade feline lymphoma, 20–60% of patients do not respond to the therapy (Mooney et al., 1989; Teske et al., 2002; Hadden et al., 2008; Simon et al., 2008). Therefore, new therapies are needed to allow a better prognosis for these patients.

The tumor suppressor protein P53 regulates the signal transduction associated with cell proliferation, DNA repair, cell cycle checkpoints, and apoptosis, and is recognized as a guardian to protect cells from malignant transformation (Vogelstein et al., 2000). There are several strategies for reactivating P53 in cancer cells, such as adenovirus vector-mediated P53 transduction, P53 activation by regulating proteins that modify P53, and reactivation

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of mutant P53 (Chen et al., 2010). Another strategy of P53 activation targets the interaction of P53 with its negative regulator MDM2. MDM2 is an E3 ubiquitin ligase that represses P53 function by inhibiting its transcriptional activity and nuclear transport and by inducing its degradation (Michael and Oren, 2003). A growing number of recent reports suggest that small molecule inhibitors targeting the P53–MDM2 interaction may represent promising, specific, and novel therapeutics against various types of human cancer cells (Vassilev et al., 2004; Tovar et al., 2006; Shangary et al., 2008). Nutlin-3 is a representative MDM2 inhibitor that can potently displace P53 from MDM2 and nestle into the P53-binding pocket of MDM2. By preventing MDM2 from binding to P53, nutlin-3 can stabilize the P53 protein and induce apoptosis and cell cycle arrest in the malignant cells retaining the wild-type *p53* (wt-*p53*) gene.

However, these MDM2 inhibitors have little effect on malignancies involving *p53* gene mutation, which has been found in half of the neoplasms in humans (Vassilev et al., 2004; Tovar et al., 2006; Shangary et al., 2008). Unlike in human malignancies, *p53* gene mutations are far less common (approximately 10%) in feline neoplasms, including lymphoma (Okuda et al., 1994; Mayr et al., 1998a,b, 2000), indicating that most of feline neoplasms retain the wt-*p53* gene. Therefore, interruption of the P53–MDM2 interaction using an MDM2 inhibitor may be a new therapeutic strategy for treating feline lymphoma. The aim of the current study is to examine the antitumor effects of an MDM2 inhibitor, nutlin-3, in 5 feline lymphoma cell lines with or without mutation of the *p53* gene.

2. Materials and methods

2.1. Cell lines and reagents

Five feline lymphoma cell lines were used in this study: 3201 (Snyder et al., 1978), MCC (Cheney et al., 1990), FT-1 (Miura et al., 1987), FT-G (Wu et al., 1995), and MS4 (Mochizuki et al., 2011). Crandell-Rees feline kidney (CRFK) (Crandell et al., 1973) was used to assess cytotoxicity of nutlin-3 in feline nonneoplastic cells. All cell lines were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Biowest, Nuaille, France), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Sigma–Aldrich, St. Louis, MO) at 37 °C in a humidified atmosphere containing 5% CO_2. Nutlin-3 was purchased from Sigma–Aldrich and was dissolved in 100% DMSO to prepare a 20 mM stock solution that was stored at −20 °C until use. Cells were exposed to 1–20 μM nutlin-3 for 4–48 h. To avoid the influence of DMSO, the final concentration of DMSO was adjusted to 0.1% (v/v) in all experiments.

2.2. Mutation analysis of p53 cDNA in feline lymphoma cells

Total RNA was extracted from each cell line by using the RNAqueous Kit (Ambion, Austin, TX). To synthesize cDNA, total RNA was reverse transcribed using the PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan) after treatment with DNase I (Invitrogen).

To amplify the entire open reading frame of the *p53* gene, the following primers were designed on the basis of the sequence of feline *p53* cDNA (GenBank accession number: NM_001009294.1) by using Primer3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi): 5′-GTGACACGCTCCCCTGAG-3′ [fp53-forward, nucleotide (nt) 75–92] and 5′-TTCCGAGGCATACCTGTACC-3′ (fp53-reverse, nt 1433–1414). After initial denaturation at 94 °C for 2 min, cDNA samples were subjected to PCR amplification by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2 min using Takara LA Taq (Takara Bio). PCR products were purified using SUPREC PCR (Takara Bio). Sequencing analyses of these PCR products was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and Genetic Analyzer 3130xl (Applied Biosystems) using 3 primers (fp53-forward, fp53-reverse, and fp53-inner: 5′-ATGCCAAGTACCTGGACGAC-3′, nt 711–730). All procedures were repeated 2 times in separate experiments. The *p53* cDNA sequences in the feline lymphoma cell lines were compared to the wt-*p53* feline cDNA sequence (NM_001009294.1).

2.3. Western blotting

Whole cell lysates were extracted with an ice-cold lysis buffer [50 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and Protease Inhibitor Cocktail (Complete Mini; Roche Diagnostics, Manheim, Germany)] from log-phase cells, and at 4 h after incubation with or without 10 μM nutlin-3. Protein concentrations were determined using a Lowry protein assay kit (DC Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA). Samples containing an equal amount of protein (10 μg) were separated by SDS–PAGE and transferred to a polyvinylidene fluoride membrane (Amersham Hybond-P; GE Healthcare, Buckinghamshire, England). Membranes were blocked in 5% skim milk/Tris-buffered saline with 0.1% Tween 20, followed by incubation with a primary rabbit polyclonal antibody against human P53 that cross-reacts with feline P53 (Morris et al., 2008; Favrot et al., 2009) (1:2000, NCL-p53-CM1; Novocastra, Newcastle, GB) or with a primary mouse monoclonal antibody against β-actin (1:2000, clone AC-15; Sigma Chemical, St. Louis, MO). After incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5000; GE Healthcare) or HRP-conjugated anti-mouse IgG (1:3000; Santa Cruz Biochemistry, Santa Cruz, CA), positive immunoreactivity was detected with the Luminata Forte Western HRP Substrate (Millipore, Bedford, MA) and visualized with ChemDoc XRS-J (Bio-Rad Laboratories). All procedures were repeated 2 times in separate experiments.

2.4. Cell proliferation assay

Exponentially growing cells were cultured in a 96-well plate with different concentrations of nutlin-3 (0, 1, 2, 5, 10, 15, or 20 μM) for 48 h. The viable cell count in the wells was measured using a Cell Counting Kit-8 (Dojindo,