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Localization of heat shock protein 110 in canine mammary gland tumors

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ABSTRACT

Heat shock proteins (HSPs) function as molecular chaperones in the regulation of protein folding, conformation, and assembly; in addition, they also protect cells from protein–protein aggregation resulting from cellular stress. Recently, HSPs were shown to be overexpressed in several human cancer cells compared with normal cells. HSPs are considered to be related to apoptosis-associated proteins, and inhibition of apoptosis promotes tumor growth.

Canine mammary gland tumors have received a great deal of attention from researchers due to the many common biological and histological characteristics that they share with human tumors. We previously confirmed that HSP110 is a canine mammary gland tumor antigen and reported that HSP110 mRNA expression significantly increased in tumor tissue. We have now created a functional recombinant canine HSP110 protein and a rabbit anti-HSP110 polyclonal antibody. This recombinant protein can refold heat-denatured firefly luciferase at 42 °C. Immunohistochemical analysis showed that HSP110 was mainly localized in the cytoplasm of epithelial and interstitial cells in canine mammary gland tumors.

Extensive genomic research has revealed genetic similarities between humans and dogs; comparative oncological studies between these species have made remarkable progress. The results reported here contribute valuable oncological knowledge for the development of novel therapeutic methods in both veterinary science and human medicine.

1. Introduction

Heat shock protein (HSP) expression is induced in both prokaryotic and eukaryotic cells when they are exposed to a variety of cellular stresses such as heat shock, hydrogen peroxide, or infection (Leach et al., 2012; Lindquist and Craig, 1988). HSPs are classified into a series of protein families on the basis of molecular weight: HSP27, HSP40, HSP60, HSP70, HSP90, and HSP110 (Kampinga et al., 2009). These molecules have essential functions in the regulation of protein folding, conformation, assembly, and sorting. HSPs are also molecular chaperones that help maintain protein native conformational states and prevent protein–protein aggregation (Akerfelt et al., 2010; Leach et al., 2012).

In recent years, some HSPs have been associated with tumorigenesis stemming from inhibition of apoptosis signaling cascades and/or promotion of cell proliferation by refolding associated proteins (Gabai et al., 1997; Ghosh et al., 2013). In particular, HSP90, which has been purified from cultured tumor cell extracts and plays a key role in the conformational maturation of oncogenic signaling proteins, including HER-2/ErbB2, Akt, Raf-1, and mutated p53 (Trepel et al., 2010). Over the last decade, some HSP90 inhibitors have been vigorously studied as possible antitumor drugs. 17-allylaminogeldanamycin (17-AAG) was the first HSP90 inhibitor to enter clinical trial, though acceptable results could not be produced. And then second and third generation HSP90 inhibitors are now in phase I and II human clinical trials (Iyer et al., 2012; Richardson et al., 2011; Tatokoro et al., 2015). In addition, the heat shock protein HSP70 can bind to tumor antigens and present them onto dendritic antigen-presenting cells (Calderwood et al., 2012). Therefore, several anticancer vaccines were created based on the

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HSP70 chaperone function, with some experiments demonstrating tumor suppressive effects through activation of both innate and adaptive immune responses *in vivo* in an animal model (Cho et al., 2009; Jiang et al., 2013). HSP110, is expressed at lower levels in many normal tissues and functions as an HSP70 nucleotide exchange factor (Dragovic et al., 2006). In recent years, researchers have reported that HSP110 is overexpressed in many tumor types, including colorectal, esophageal, mammary, and bladder carcinomas, as well as in hematopoietic tumors (Kai et al., 2003). Other studies have shown that HSP110 can induce dendritic cells to upregulate MHC class II protein expression and to secrete cytokines (Yokomine et al., 2006). Mutant HSP110 transfected into colorectal tumor cells increased cell sensitivity to anticancer agents by inhibiting wild-type HSP110 (Dorard et al., 2011). Taken together, HSP110 is regarded as an attractive therapeutic target molecule in multiple malignancies and its expression might serve as an important prognostic factor.

In veterinary science, neoplastic diseases are as common as they are in humans, accounting for almost half of all deaths. Mammary gland tumors (MGTs) are the most common neoplasm in female dogs and have a malignancy rate of 50%, with 25% of tumors showing metastatic behavior (Brody et al., 1983; Gobar et al., 1998). However, minimally-sized lesions and early distant metastases are often difficult to detect. There are also limited options for diagnosing early canine MGT, because, in contrast to humans, no diagnostic molecules for detecting early tumorigenesis or definitive prognostic factors have been developed. In canines, only one study has reported on MGT adoptive immunotherapy; however, no desirable target antigen has been reported (Visonneau et al., 1999). In a previous study, we identified novel canine MGT tumor antigens using serological immunoscreening. HSP110, one of these antigens, was overexpressed in tumor tissues at the mRNA level and may play some important roles in canine MGT (Furuya et al., 2013). However, its molecular role within mammary tumors was unclear, so the creation of a protein and an antibody of HSP110 were needed to reveal it.

In this study, we report the creation of a recombinant canine HSP110 protein and a polyclonal antibody and their use in determining HSP110 expression details and biological functions in canine MGT tissues.

2. Materials and methods

2.1. Plasmid construction

The full length canine HSP110 cDNA derived from testis was amplified by Phusion® taq polymerase (New England BioLabs, MA, USA) with specific primers (sense: 5′-ACC GAT ATC CAA AAA TGG TGG AGTT-3′; antisense: 5′-GCT CTC GAG TCA GTC CAA GTC CAT ATT AAT GGA-3′) and subcloned into pGEX6p-1 (GE healthcare, WI, USA). The constructed vector was sequenced to confirm insertion in the correct frame (Takara Bio, Shiga, Japan), and it was then transformed to BL21(DE3)pLysS Competent cells (Merck KGaA, Darmstadt, Germany). The cells were incubated at 37 °C to O.D. 0.4 and recombinant N-terminal-HSP110 fusion protein expression was induced by shaking the culture overnight at 20 °C with 10 mM isopropyl β-D-thiogalactopyranoside.

The cells were harvested by centrifugation and sonicated in lysis buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA). The soluble fraction was extracted, proteins analyzed by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and stained with Bio-safe TM Coomassie G-250 (Bio-Rad, CA, USA) for visualization. From the gel, soluble extracts were transferred onto a PVDF membrane, blocked with 1% BSA in 0.05% Tween20-PBS, and incubated with a goat polyclonal anti-GST primary antibody (1:1000; Amersham Biosciences, NJ, USA). The membrane was then reacted with a secondary HRP-conjugated rabbit anti-goat IgG (1:5000; Sigma–Aldrich, MO, USA), and detected by 0.02% 3.3′-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich) staining. Recombinant GST-tagged HSP110 protein was excised from a stained gel and prepared for mass spectrometry analysis. The protein identification was performed by Mascot search engine (www.matrixscience.co.uk).

2.2. Purification of recombinant canine HSP 110

The protein was purified with affinity chromatography using GSTrap 4B columns (GE Healthcare). Briefly, the soluble extract was applied to a GSTrap 4B column, extracted in elution buffer (50 mM Tris–HCl, 20 mM glutathione pH 8.0), and then digested from the GST-tag by PreScission Protease (GE Healthcare). The GST-tag and the protease were eliminated by applying the sample to a GSTrap 4B column again, and then the flow-through fraction containing the recombinant protein was collected. Finally, the protein was concentrated by ultrafiltration (Vivaspin 20; GE healthcare) and analyzed by SDS-PAGE and Western blotting.

2.3. Refolding assay

Luciferase refolding assays were performed according to the previous references (Cashikar et al., 2005; Rosano et al., 2011). Firefly luciferase (Promega, WI, USA) diluted in TKM buffer (10 mM Tris–HCl, pH 7.5; 10 mM KCl; 1 mM $MgCl_2$) was heat-denatured at 42 °C for 10 min to eliminate its original activity. Inactivated luciferase (1 nM) was mixed with 3 μg of recombinant canine HSP110 protein (intact, preheated at 42 °C or 60 °C) and ATP (2 mM), and then the mixture was incubated at 25 °C for 10 min. Afterward, the aliquots were finally mixed with Luciferase assay reagent (Promega). The luciferase activity was analyzed by ARVO X5 (Perkin Elmer, MA, USA).

2.4. Statistical analysis

In the luciferase refolding assay, the experiments were repeated 3 times and the mean values are reported. The bioluminescence intensity was statistically analyzed using the Kruskal–Wallis test. Statistical significance was defined as $p < 0.05$.

2.5. Rabbit polyclonal antibody

The animal study was approved by the Experimental Animal Committee of Osaka Prefecture University (OPU). Two female rabbits (Kbl: JW, Oriental Yeast Co., Ltd. Tokyo, Japan) were housed in the Education and Research Center for Experimental Animal Science of OPU, according to institutional guidelines. The rabbits were immunized with 150 μg recombinant protein that was dissolved in 250 μl PBS and mixed with 250 μl complete Freund's adjuvant (Sigma–Aldrich). The rabbits received a total of four booster injections (one every 2 weeks for 8 weeks) of 150 μg recombinant protein dissolved in 250 μl PBS mixed with 250 μl incomplete Freund's adjuvant for each immunization. Pre- and post-immune sera were collected and tested by ELISA method. Specifically, recombinant HSP110 was diluted to 10 μg/ml in carbonate buffer ($NaHCO_3$, 100 mM; Na_2CO_3, 34 mM, pH 9.5) and 100 μl samples were incubated in an ELISA plate for 2 h at room temperature. After washing with 0.05% Tween 20-PBS, the plate was blocked with 3% BSA-PBS for 1 h at room temperature. After washing with 0.05% Tween 20-PBS, the plate was incubated with the sera (serial dilution at 1:1000–128,000) for 2 h at room temperature. After washing with 0.05% Tween 20-PBS, the plate was incubated with HRP-conjugated goat anti rabbit IgG for 1 h at room temperature. After washing with