Gene 638 (2018) 1–6

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Autoubiquitination of feline E3 ubiquitin ligase BCA2

Weiran Wang[a,1], Meng Qu[a,1], Jiawen Wang[a,c], Xin Zhang[a], Haihong Zhang[a], Jiaxin Wu[a], Bin Yu[a], Hui Wu[a], Wei Kong[a,b,*], Xianghui Yu[a,b,*]

[a] National Engineering Laboratory for AIDS Vaccine, School of Life Sciences, Jilin University, Changchun, Jilin Province, People's Republic of China
[b] Key Laboratory for Molecular Enzymology and Engineering of the Ministry of Education, School of Life Sciences, Jilin University, Changchun, Jilin Province, People's Republic of China
[c] Center for New Medicine Research, Changchun University of Chinese Medicine, Changchun, Jilin Province, People's Republic of China

ARTICLE INFO

Keywords:
Feline BCA2
E3 ubiquitin ligase
Autoubiquitination
RING finger domain

ABSTRACT

BCA2/RNF115/Rabring7 is a RING type E3 ubiquitin ligase that is overexpressed in human breast tumors and is important for regulating breast cancer cell migration. In the present investigation, feline BCA2 (fBCA2) was identified and characterized. Compared with its human counterpart, the *fBCA2* cDNA was confirmed to be 918 base pairs in length showing 92.6% consensus and identity positions, encoding a protein of 305 amino acids with 96.7% consensus and 93.1% identity positions. The fBCA2 protein contains a RING domain at the C-terminus, which was found to be essential for its autoubiquitination.

1. Introduction

Ubiquitin and ubiquitin-like pathways are important for the normal functioning of eukaryotic cells (Joazeiro and Weissman, 2000; Tanaka et al., 2001; Dohmen, 2004; Pan et al., 2004; Pickart and Eddins, 2004; Aragon, 2005; Mani and Gelmann, 2005). Ubiquitination promotes protein turnover, trafficking and regulation of protein function (Sawasdikosol et al., 2000; Marmor and Yarden, 2004). The lack of ubiquitination procedures has been described in the pathogenesis of several human diseases, including cancer. The addition of many ubiquitin moieties leads to degradation of the protein by the 26S proteasome. The E3 enzymes with RING finger domain, such as ring finger proteins BCA2, Mdm2 and BRCA1, have been shown to play an important biological role in breast cancer and other cancers (Joazeiro and Weissman, 2000; Burger and Seth, 2004; Burger et al., 2005).

Breast cancer associated gene 2 (BCA2), also known as ring finger protein 115 (RNF115) or Rabring7, belongs to the E3 ubiquitin ligase family with a ring finger domain, which is overexpressed in more than 50% of infiltrating breast cancers compared with normal tissues and can be associated with proliferation of breast cancer cells *in vitro* (Burger et al., 1998; Burger et al., 2005). Overexpression of BCA2 increases the proliferation of NIH3T3 fibroblasts, while siRNA inhibits the growth of BCA2-expressing breast cancer cells (Burger et al., 2005). The BCA2 protein has autoubiquitination activity, which depends on its RING domain, and mutation at two lysines (K26 and K32) in the BCA2 zinc finger (BZF) domain also can eliminate autoubiquitination activity. Meanwhile, BCA2 E3 ligase has been shown to be an important factor in regulating the migration of breast cancer cells (Amemiya et al., 2008). Although it has been widely accepted that E3 enzyme can play an active role in the carcinogenesis process (Lipkowitz, 2003; Ohta and Fukuda, 2004; Burger et al., 2005), how deregulation of an E3 may occur is less well understood.

Breast cancer is the most common neoplasm in women, with approximately 231,840 new cases of invasive breast cancer, 60,290 additional cases of *in situ* breast cancer and 40,290 deaths occurring in 2015 in the United States. Meanwhile, about 2350 men will be diagnosed with breast cancer, and 440 men will die from the disease. Metastasis is the most devastating and life-threatening problem in women with breast cancer and therefore has become an important focus for research (Werbeck et al., 2014). Feline mammary carcinoma (FMC) has been proposed as a model for human breast cancer based on age of incidence, risk factors, histopathology, prognostic aspects, metastatic pattern and response to therapy (Hahn et al., 1994). The annual incidence of feline mammary neoplasia was estimated at 13 to 25 per 100,000 female cats (Hassan et al., 2017), and the feline mammary tumors are the most frequent neoplasm causing death in cats (Jemal

Abbreviations: BCA2, breast cancer associated gene 2; RNF115, ring finger protein 115; BZF, BCA2 zinc finger; Mdm2, mouse double minute 2; BRCA1, breast cancer 1; FMC, feline mammary carcinoma; WHO, World Health Organization; ATCC, American Type Culture Collection; BCIP, 5-bromo-4-chloro-3indolyl phosphate; NBT, nitro blue tetrazolium; IPTG, isopropyl-β-d-thiogalactoside; BLAST, Basic Local Alignment Search Tool; PBS, phosphate-buffered saline; EDTA, ethylene diamine tetraacetic acid

* Corresponding authors at: National Engineering Laboratory for AIDS Vaccine, School of Life Sciences, Jilin University, Changchun, Jilin Province, People's Republic of China.
E-mail addresses: weikong@jlu.edu.cn (W. Kong), xianghui@jlu.edu.cn (X. Yu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.gene.2017.09.059
Received 8 July 2017; Received in revised form 13 September 2017; Accepted 26 September 2017
Available online 28 September 2017

et al., 2003), which occur predominantly in middle-aged to older female cats with a mean age of 10 to 12 years (Zappulli et al., 2005). Like human breast cancer, FMCs usually present as *in situ* or invasive carcinomas (Soares et al., 2016), typically classified according to criteria of the World Health Organization (WHO). Changes in the expression of genes at the mRNA and protein levels have been described in both human breast cancer and FMCs (Zappulli et al., 2005). Since FMC has a high rate of malignancy, it will be useful to investigate the pathogenesis of metastasis and expression of tumor-related genes.

In the current study, we described the isolation of feline BCA2 (fBCA2), analyzed the autoubiquitination ability of fBCA2 and compared it with that of human BCA2 (hBCA2). Full-length *fBCA2* cDNA was isolated from CrFK cells in culture and cloned into a mammalian or prokaryotic expression vector. We then showed evidence that fBCA2 has E3 ubiquitin ligase activity, and its RING domain was found to be essential for its autoubiquitination. Our findings showed that fBCA2 may be a potential biomarker for FMC, which will help to contribute to an animal model for human breast cancer.

2. Materials and methods

2.1. Cell cultures and transfections

HEK293T cells, Hela cells and cat kidney cortex epithelial cells (CrFK cells) were purchased from the American Type Culture Collection (ATCC). The cells were cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco) at 37 °C and 5% CO_2. Primary antibodies for Western blotting were as follows: anti-BCA2 goat polyclonal antibody (Sigma), anti-HA mouse monoclonal antibody (Covance), anti-cMyc mouse monoclonal antibody (Millipore), anti-Flag mouse monoclonal antibody (Sigma), anti-tubulin mouse monoclonal antibody (Covance), anti-GST antibody monoclonal antibody (Abcam) and anti-Ub biotin antibody (Abcam). Secondary antibodies with alkaline phosphatase conjugated were purchased from Jackson ImmunoResearch Laboratories (West Grove). Transient plasmid transfections into 293T cells were performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.2. Plasmids

Total RNA was extracted from feline CrFK cells or Hela cells using TRIzol (Invitrogen) separately. The BCA2 sequences, including hBCA2, hBCA2 CA, hBCA2 KR, fBCA2, fBCA2 CA and fBCA2 KR, were cloned into the mammalian expression VR1012 vector and pGEX-6P-1 vector. The E2 plasmid was generated by subcloning UbcH5b-HA into the VR1012 vector, and all the sequences were confirmed. All primers were synthesized by the solid phase phosphoramidite triester method (Comate Bioscience). The Ub-cMyc and VR1012 plasmids were described previously (Yu et al., 2003; Wang et al., 2015).

2.3. Co-immunoprecipitation assay

293 T cells were harvested at 48 h after transfection, then washed twice with cold PBS and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100) supplemented with cOmplete Mini Protease Inhibitor Cocktail Tablets (EDTA Free, Roche) at 4 °C for 45 min. The cell lysates were then centrifuged at 10,000 × *g* for 30 min, incubated with anti-Flag mAb at 4 °C for 1.5 h and incubated with protein G (Roche) at 4 °C for 3 h or anti-HA Affinity Matrix (Roche) at 4 °C for 1.5 h. Subsequently, the beads were washed three times with washing buffer (20 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween-20), boiled in SDS sample buffer and then subjected to SDS-PAGE. The proteins were transferred onto nitrocellulose membranes by semidry transfer (Bio-Rad). After blocking in 5% non-fat milk, the membranes were probed with various primary antibodies against proteins of interest. Secondary antibodies were alkaline phosphatase-conjugated anti-goat or anti-mouse IgG, and staining was carried out with 5-bromo-4-chloro-3indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) solutions.

2.4. Proteins expression and purification

The BCA2-pGex-6p-1 plasmid series were transformed into *Escherichia coli* BL21, and the expressed proteins were found to be water soluble after the induction of isopropyl-β-d-thiogalactoside (IPTG). Approximately 5 h after induction by IPTG, cells were harvested in PBS, centrifuged at 6000 × *g* for 20 min and then resuspended with cold PBS, followed by ultrasonication for 20 min. The cell lysates were centrifuged at 10,000 × *g* for 30 min, and the supernatant was passed through a 0.45 μm filter before purification. Lysed samples were loaded onto a column containing glutathione-Sepharose beads (GE Healthcare Dharmacon) pre-equilibrated with PBS, and the column was washed with PBS. The BCA2 protein was eluted with elution buffer containing 10 mM reduced glutathione in 50 mM Tris-HCL (pH 8.0) and concentrated using an ultrafiltration tube (Millipore) to the final concentration of approximately 1 mg/ml.

2.5. In vitro protein ubiquitination assay

Ubiquitination assays were performed at 37 °C for 2 h. The reaction system contained E1 (200 nM), E2 (UbcH5b, 4 μM), ubiquitin (1 μM), Mg-ATP (10 mM) and BCA2 (1 μM), according to the instructions of the Ubiquitinylation Kit (Enzo). The ubiquitination reactions were then quenched by the addition of 2 × SDS loading buffer and analyzed by Western blotting.

3. Results

3.1. Cloning and characterization of fBCA2

BCA2 is a cellular factor that is highly conserved among diverse mammalian species, including human, chimpanzee, dog, cow, mouse and zebrafish. Here, we predicted the fBCA2 genes from the available genomic sequences, with the use of sequence BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and automated computational analysis with Genescan. We designed oligonucleotide primers based on the predicted sequences and performed RT-PCR on RNA isolated from CrFK cells for fBCA2 (MF327275) or Hela cells for hBCA2 as a control (Fig. 1A). The amplified cDNA bands were purified and inserted into the VR1012 vector. The base composition that we obtained did not completely match the predicted genomic sequences (XM_006935057.2), probably due to differences between feline individuals but the amino acid compositions were all identical. As previously demonstrated, the fBCA2 amino acid sequence showed 96.7% consensus positions and 93.1% identity positions compared with hBCA2 (Fig. 1B). The expression of the fBCA2 protein in 293T cells could be detected with the hBCA2 polyclonal antibody (Fig. 1C).

3.2. fBCA2 interacts with UbcH5b and ubiquitin

Autoubiquitination and ubiquitination of the target protein are described as the general function of most proteins containing the RING domain (Yang et al., 2000; Chen et al., 2002; Glickman and Ciechanover, 2002; Lai et al., 2002). hBCA2 was demonstrated to have intrinsic autoubiquitination activity only in the presence of the E2 ubiquitin–conjugating enzyme UbcH5b (Burger et al., 2005). In order to verify whether fBCA2 has the function of autoubiquitination, we examined the interaction between fBCA2 and UbcH5b, and the modification of fBCA2 by ubiquitin. Here we performed an immunoprecipitation assay with 293T cells transfected with UbcH5b-HA and hBCA2-flag, fBCA2-flag or VR1012 vector. Cell lysates were