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Identification of a polymorphism in the RING finger of human Bmi-1 that causes its degradation by the ubiquitin–proteasome system

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ABSTRACT

Bmi-1 is a polycomb protein that plays an important role in tumor cell development and maintaining stem cell populations of many cell lineages. Here we identify a polymorphism in human Bmi-1 that changes a cysteine within its RING domain to tyrosine. This C18Y polymorphism is associated with a significant decrease in Bmi-1 level and its elevated ubiquitination, suggesting that it is being destroyed by the ubiquitin–proteasome system. Consistent with this, treating cells with the proteasome inhibitor MG-132 significantly increases C18Y Bmi-1 levels. This is the first example of a polymorphism in Bmi-1 that reduces levels of this important protein.

Structured summary:
MINT-6948574: *Bmi-1* (uniprotkb:P35226) *physically interacts* (MI:0218) with *Ubiquitin* (uniprotkb:P62988) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

Bmi-1 is a member of the polycomb group family of proteins, which function as negative regulators of the transcription of a number of important target genes [1–6]. Bmi-1 was first cloned as a c-myc cooperating oncogene in murine lymphomas [7,8], and found to have significant sequence relatedness to *Drosophila* polycomb proteins [9,10]. Previous studies have identified several ways in which Bmi-1 mediates its effects on cell proliferation, including inhibiting expression of the Ink4A/ARF locus [11,12], modulating the p21-Rb pathway [13], and inducing telomerase activity [14].

In addition to its function as an oncogene, Bmi-1 also plays important roles in determination of cell fate and stem cell renewal of the neural, hematopoietic, and other cell lineages [15–23]. Bmi-1 contains a conserved RING-finger domain near the NH_2 terminus, which is important for the function of this protein [7,14,24–27]. Bmi-1 has been found to be predominantly localized to the nucleus, which is mediated by a nuclear localization sequence (NLS) located in the C-terminal region of this protein [14,24,28].

In this paper we characterize a polymorphism in the human Bmi-1 protein that changes a cysteine in the RING-finger domain (cysteine 18) to tyrosine. The results show that the C18Y polymorphism results in a significant reduction in levels of the Bmi-1 protein by leading to its ubiquitination and destruction by the proteasome. In light of the important functions of Bmi-1 in stem cell renewal and determination of cellular identity, these results suggest that this C18Y polymorphism could have deleterious effects in the people that have it.

2. Materials and methods

2.1. SNP database searching

To identify potential polymorphisms within the Bmi-1 gene, we searched the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html). This search identified a SNP polymorphism (rs1042059) that is predicted to change the cysteine at amino acid 18 of Bmi-1 to tyrosine. This Bmi-1 C18Y polymorphism was found in both the CEU (ancestry from northern and western Europe) and YRI (Yoruba in Ibadan, Nigeria) populations that were analyzed by the International HapMap Project [29–31].

2.2. Cell culture and plasmids

HEK 293T cells were grown at 37 °C in DMEM supplemented with 10% FBS and 100× antibiotic–antimycotic (Invitrogen) in 5% CO_2. pEGFP-Bmi-1 plasmid was generated by using PCR to amplify

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from the plasmid pOTB7-hBmi-1 cDNA (Accession number BC011652, Open Biosystems) a coding fragment of Bmi-1 having KpnI and BamHI sites at the ends using the following primers: 5′-GCG GGT ACC ATG CAT CGA ACA ACG AGA-3′ and 5′-CGC GGA TCC TCA ACC AGA AGA AGT TGC TGA-3′. This PCR product was then cloned into pEGFP-C1 vector at the KpnI and BamHI sites to make the pEGFP-Bmi-1 plasmid. This plasmid was confirmed by DNA sequencing. pEGFP-C18Y-Bmi-1 plasmid was generated using the QuickChange mutagenesis method (Stratagene) according to the manufacturer's protocol. The mutation was confirmed by DNA sequencing.

2.3. Fluorescence microscopy

HEK293 cells were seeded onto coverslips that were acid-washed and flamed, and then coated with laminin (5 μg/ml) (Sigma–Aldrich). Forty-eight hours after transfection with the plasmids encoding the wild-type or C18Y GFP-Bmi-1 fusion proteins, cells were washed twice in ice-cold 1× PBS, followed by fixation in 3.7% paraformaldehyde for 20 min at room temperature. After a final wash with PBS, coverslips were mounted on a slide with Vectashield mounting medium plus 1.5 μg/ml DAPI (4′,6 diamidino-2-phenylindole) (Vector Laboratories). Fluorescence of the GFP-Bmi-1 proteins was visualized using a Nikon fluorescent microscope with a 100× oil immersion objective and a Nikon Spotcam digital-imaging camera.

2.4. Extract preparation and Western blot assay

HEK293 cells were transfected with the wild-type or C18Y GFP-Bmi-1 expression plasmids using Jet-PEI reagent according to the manufacturer's instructions. At 48 h post-transfection, cells were extracted on ice with NP-40 lysis buffer (1% NP-40, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, complete protease inhibitor cocktail (Roche Applied Science) and 20 mM N-ethylmaleimide (added fresh)) for 20 min. After centrifugation at 13 000 rpm at 4 °C for 10 min, the supernatant was transferred to a fresh tube, and then the whole cell lysate was used for the following assays. SDS–PAGE and Western blot were performed according to standard protocols. Goat anti-GFP antibody (Bethyl Laboratories Inc.) was used for the Western blots at 1:2000. For the immunoprecipitation assay of ubiquitinated Bmi-1, mouse monoclonal anti-ubiquitin antibody (gift of Dr. Haining Zhu, University of Kentucky, Lexington, KY) was used at 1:1000.

2.5. Proteasome inhibition assay

HEK293T cells were transfected with the wild-type or C18Y GFP-Bmi-1 expression plasmids as described above, except that at 44 h post-transfection 10 μM MG-132 proteasome inhibitor (Calbiochem, gift of Dr. Dan Noonan, University of Kentucky, Lexington, KY) was added to the media and the cells incubated for 4 more hours.

2.6. Bmi-1 ubiquitination assay

HEK293T cells were transfected with pEGFP-Bmi-1 or pEGFP-C18Y-Bmi-1 as described above. At 48 h post-transfection, cells were extracted on ice with NP-40 lysis buffer (1% NP-40, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, complete protease inhibitor cocktail (Roche Applied Science) and 20 mM N-ethylmaleimide (added fresh)) for 20 min. Lysates were then cleared by centrifugation at 13 000 rpm for 10 min at 4 °C. Supernatants were precleared by incubation with goat (control) IgG and protein G-sepharose beads for 2 h at 4 °C with gentle rotation. Precleared extracts were then incubated with primary goat polyclonal anti-GFP antibody or control IgG and 50% slurry of protein G-sepharose for 4 h at 4 °C with rotation. After washing beads 6 times for 5 min each at 4 °C with NP-40 buffer, bound proteins were released by boiling in SDS–PAGE sample dye and analyzed by Western blot assay using the anti-ubiquitin mouse monoclonal antibody or anti-GFP goat polyclonal antibody.

3. Results

As depicted in Fig. 1A, the Bmi-1 protein contains a RING-finger domain in its N-terminal region [7,14,24–27]. Examination of the dbSNP database revealed the existence of a polymorphism in human Bmi-1 that changes amino acid 18, a cysteine in the RING-finger domain, to tyrosine (Fig. 1B). Further analysis revealed that individuals that are heterozygous for this polymorphism are found in both the CEU (Utah residents with ancestry from northern and western Europe) and YRI (Yoruba in Ibadan, Nigeria) populations that were studied as part of the International HapMap Project [29–31].

Because the cysteine that is changed to tyrosine in this polymorphism is a residue of the RING-finger domain, we hypothesized that this alteration could lead to alteration in the functional properties of the Bmi-1 protein. Bmi-1 has been found to be a predominantly nuclear-localized protein [14,24,28], mediated by a nuclear localization sequence (NLS) in the C-terminal region (Fig. 1A) [14,24]. Therefore, one functional property of Bmi-1 we hypothesized could be affected by the C18Y polymorphism is its pattern of localization within the cell. To test this hypothesis we transfected wild-type and C18Y GFP-Bmi-1 expression plasmids into HEK293 cells and then examined the sub-cellular localization of the transfected proteins using fluorescence microscopy analysis. The results of this experiment, shown in Fig. 2, revealed that the wild-type and C18Y Bmi-1 proteins both exhibit predominant nuclear localization, indicating that the C18Y polymorphism does not appear to significantly alter the sub-cellular localization of the Bmi-1 protein.

Although the results of the fluorescence microscopy experiment shown in Fig. 2 did not indicate a difference in localization, they did show that the intensity of the signal from the C18Y GFP-Bmi-1 was markedly less than that exhibited by wild-type Bmi-1. This suggested that the C18Y polymorphism may be associated with a decrease in levels of the Bmi-1 protein. To test this we subjected extracts of HEK293 cells transfected with the wild-type or C18Y GFP-Bmi-1 expression plasmids to Western blot assay with anti-GFP antibodies to detect the transfected Bmi-1 proteins, or with β-actin antibodies as a loading control. The results of this experiment show that the levels of the C18Y GFP-Bmi-1 protein are

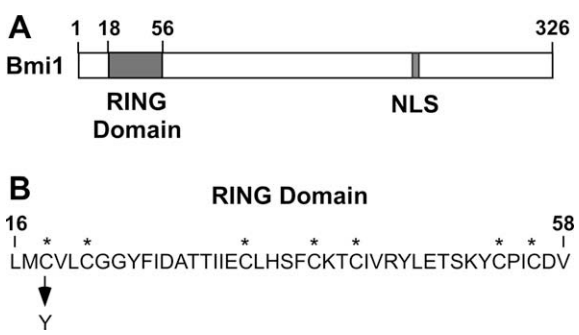

Fig. 1. Identification of a SNP resulting in a C18Y polymorphism of Bmi-1. (A) Schematic showing the location of the RING-finger and NLS in the Bmi-1 protein, as well as its nuclear localization sequence (NLS). (B) Schematic showing the amino acid sequence of the RING-finger domain and location of the C18Y polymorphism of Bmi-1.