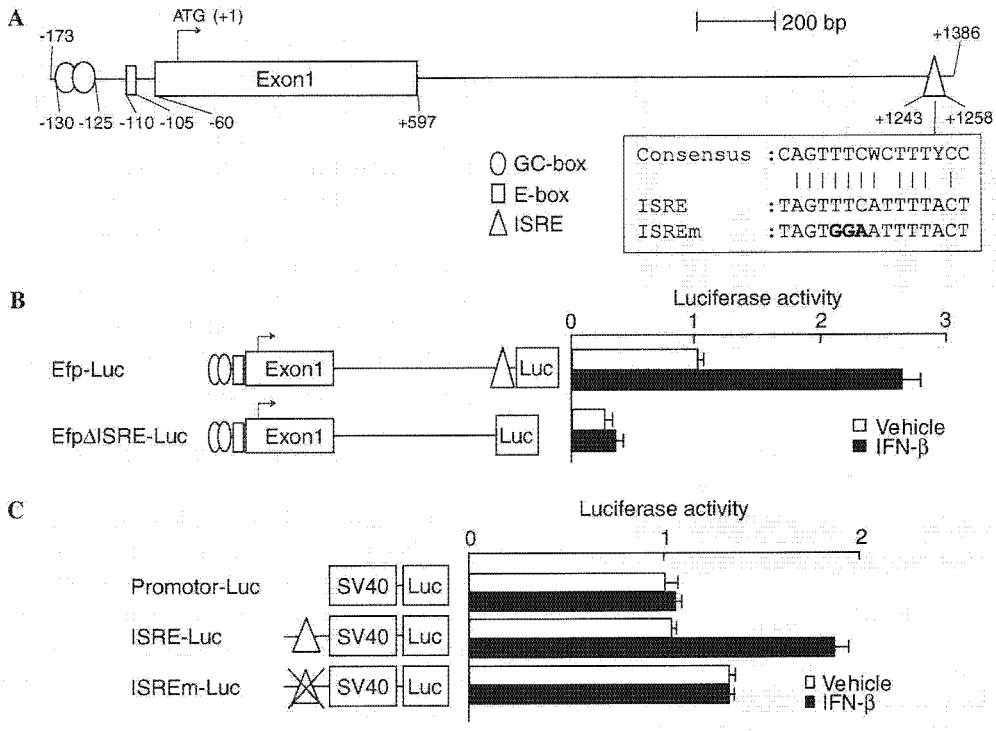

Fig. 2. Functional IFN-stimulated response element (ISRE) in human Efp intron 1. (A) Schematic representation of 5′-flanking region, exon 1, and intron 1 of human Efp gene, and comparison of ISRE sequences with consensus element. E-box, GC-box [19], and ISRE sequences are shown by the box, circles, and a triangle on the map, respectively. In consensus ISRE sequences, W and Y stand for A/T and C/T, respectively. (B,C) Promoter analysis of human Efp gene. The structures of the Efp-promoter luciferase reporter of plasmids are shown schematically (left) and their relative luciferase activities are shown to the right of each construct. The cross represents the mutation in ISRE (ISREm). HeLa cells were plated at a density of 1×10^4 cells per well on 24-well plates and transfected with 0.1 μg of Efp-Luc, EfpΔISRE-Luc, Promoter-Luc, ISRE-Luc or ISREm-Luc together with 0.02 μg of pRL-TK. Plasmids in (C) were generated using a luciferase reporter vector containing a SV40 promoter. Cells were treated with 500 U/ml IFN-β or vehicle for 24 h, and luciferase assay was performed. Data are expressed as means ± SD of three independent experiments performed in triplicate.

IFNs or vehicle for 6 and 24 h (Fig. 4A). ISG15 was only responsive to type I IFN, whereas UBE1L and UBCH8 were markedly induced by both type I and type II IFNs in HeLa cells. Together with the results of Efp (Fig. 1A) that is assumed as an E3 ligase for ISGylation [12], the expression of ISGylation components could be up-regulated by type I IFN, and to a lesser extent by type II IFN. Then, we analyzed Efp protein modification with ISG15 and ubiquitin as E3 ligases, which often exhibit self-conjugation [22,23]. As shown in Fig. 4B, a broad signal with smeared, retarded migration corresponding to poly-ubiquitinated Efp was detected. In addition, a band consistent with the molecular weight of Efp conjugated with a single ISG15 molecule was detected. These results suggest that Efp is an E3 ligase for both ISGylation and ubiquitination, which also exerts its own protein modification.

Discussion

In the present study, we demonstrated that IFN stimulates Efp mRNA and protein expression in human culture cells. Promoter analysis revealed that an ISRE located in the first intron activated the Efp transcriptional activity in an IFN-dependent manner. *In vitro* and *in vivo* binding of STAT1 to this intronic ISRE was shown by EMSA and ChIP analysis, respectively, in an IFN-dependent manner. These results strongly suggest that Efp expression is transcriptionally regulated by STAT1, which could be activated by IFN stimulation. We have previously revealed that the proximal promoter of Efp is activated by estrogen receptor that binds to the estrogen-responsive element in 3′-untranslated region [19,24]. Thus, the gene regulatory region of Efp is capable of responding to both IFN and estrogen stimuli.

Efp is a member of TRIM family proteins which are particularly implicated in antiviral responses [17]. For example, TRIM19/PML has been considered to be involved in resistance to virus infections such as HIV, vesicular stomatitis virus, and influenza A virus [13,14]. Staf50/TRIM22 down-regulates HIV-1 long-terminal repeat-directed transcription, indicating that TRIM22 may have a negative effect on HIV replication cycle [25]. Moreover, TRIM5 proteins from rhesus and African green monkey restrict HIV-1 replication at an early step in reverse transcription prior to nuclear import [16]. Human, rhesus, and African green monkey TRIM5 has been shown to restrict N-tropic murine leukemia virus (N-MLV) [15]. Among these TRIMs, IFN-regulated expression of TRIM5, TRIM19, and TRIM22 has been shown [13,20,25]. Some other TRIMs including TRIM21/SSA1,

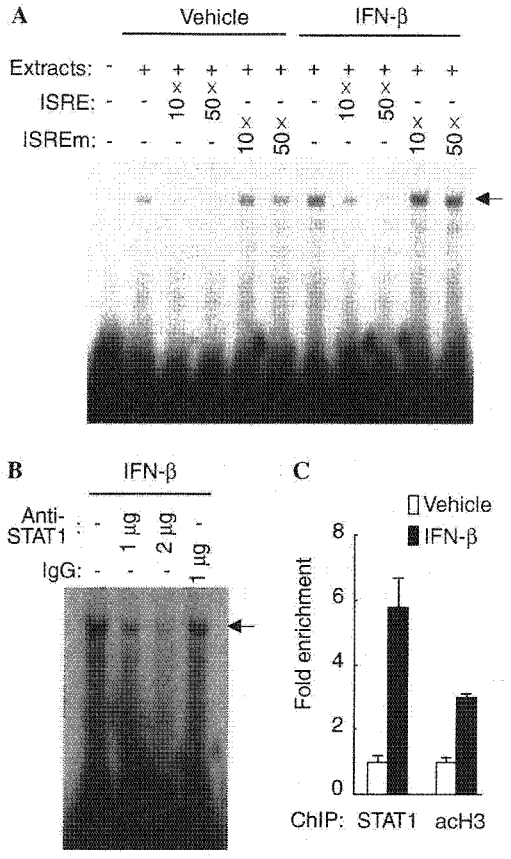

Fig. 3. Recruitment of STAT1 onto the intronic ISRE of human Efp gene. (A) EMSA of the ISRE sequence of Efp gene. ^{32}P-labeled ISRE oligonucleotides were incubated with 5 μg of HeLa cell nuclear extracts treated with or without 500 U/ml IFN-β for 24 h. A 10- or 50-fold excess of unlabeled ISRE/ISREm oligonucleotides was added for competition. (B) Antibody interference with the complex bound to the ISRE oligonucleotide. Anti-STAT1 antibody or non-immune IgG was used in the EMSA prior to addition of ^{32}P-labeled ISRE. Arrows in panels A and B indicate the identical protein–DNA complex containing STAT1. (C) ChIP assay was performed using HeLa cells treated with 500 U/ml IFN-β or vehicle for 24 h using STAT1 or acetylated histone H3 specific antibodies. Protein-crosslinked genomic DNAs were isolated from the cells, and ChIP-enriched or control input DNAs were used as templates for quantitative PCR specific for the ISRE sequence of Efp intron 1 or the coding region of GAPDH as a negative control. Results were normalized by input DNAs and are represented as fold enrichment in IFN-β-treated cells relative to the values in vehicle-treated cells.

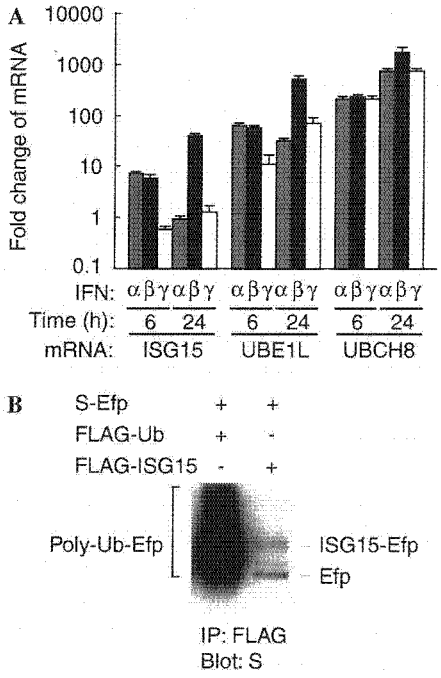

Fig. 4. ISG15-conjugation of Efp protein by IFN-induced ISGylation components. (A) IFN-induced mRNA expression of ISG15, UBE1L (E1), and UBCH8 (E2). HeLa cells were treated with the indicated IFNs (500 U/ml each) or the vehicle for 6 or 24 h. qPCR was performed using cDNAs generated from total RNA. Experiments were repeated three times and the results are represented as means ± SD. (B) ISGylation and ubiquitination of Efp protein. 293T cell extracts transiently transfected with Flag-tagged Efp (Flag-Efp), Flag-tagged ubiquitin (Flag-Ub), and Flag-tagged ISG15 (Flag-ISG15) were subjected to immunoprecipitation with anti-Flag antibody and, subsequently, to Western blot analysis probed with anti-S antibody. Signals for poly-Ub-Efp, ISG15-Efp, and Efp are indicated.

TRIM6/long-formed IFP, and TRIM34/short-formed IFP were also found to be regulated by IFNs [26]. Thus, the TRIM family may play a widespread and evolutionarily conserved role in innate immunity against viral infection.

Coimmunoprecipitation experiments showed that Efp protein could be modulated by both ISG15 and ubiquitin, suggesting that Efp might function as an E3 ligase for self-ISGylation and self-ubiquitination. Dual E3 ligase activities of Efp in ISG15- and ubiquitin-conjugation have been recently revealed [11,12]. ISGylation is carried out through sequential enzyme reactions resembling ubiquitination. Regarding ISGylation, E1 (UBE1L), E2 (UBCH8 and UBCH6), and E3 (Herc5 and Efp) enzymes are identified so far [4,27–30]. We also showed that other ISGylation components including ISG15, UBE1L (E1), and UBCH8 (E2) were among the IFN response genes in HeLa cells, in consistency with recent evidence [31,32]. With regard to the interaction between ISGylation and ubiquitination, there are reports that ISGylation competes with ubiquitination. For example, ISGylation of UBC13 disrupts its ability to form thioester bond with ubiquitin [33], and ISG15 conjugation to UBCH6 and UBCH8 suppresses their ubiquitin E2 enzyme activities [27,28]. Such interference is a possible mechanism for the inhibition of HIV-1 virion release because IFN-induced ISG15 inhibits the ubiquitination of HIV-1 Gag and vacuolar protein-sorting protein Tsg101 and disrupts the interaction between them [7]. Moreover, ISG15 expression may protect mice from Sindbis virus infection through an unclear mechanism [8]. It is tempting to speculate that IFN-stimulated Efp could modulate protein ISGylation and ubiquitination contributing together to antiviral response.

In addition, ISG15 may play a role in reproduction system since expression of ISG15 and ISG15-conjugated proteins are found in uterus during pregnancy and ISG15 expression is induced by type I IFN-τ secreted from conceptus in ruminants [34,35]. Elevation of ISG15 expression is

also detected in some tumors where an antagonistic role of ISG15 is suggested in regulating the ubiquitin-mediated protein turnover [36]. Efp is expressed predominantly in reproductive tissues and is demonstrated to be necessary for estrogen-dependent growth in uterus [37,38]. Furthermore, Efp is up-regulated in some breast cancer tissues [24,39] and plays a crucial role in tumor growth where the ubiquitin E3 ligase activity of Efp is involved in the ubiquitin–proteasomal degradation of 14-3-3σ protein [11]. Thus, Efp may modulate target protein functions as a dual ISG15 and ubiquitin E3 ligase to mediate integrated signals between IFN and estrogen in reproductive tissues and cancer.

Acknowledgments

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Identification and Herc5-mediated ISGylation of novel target proteins ☆

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Abstract

ISG15, a protein containing two ubiquitin-like domains, is an interferon-stimulated gene product that functions in antiviral response and is conjugated to various cellular proteins (ISGylation) upon interferon stimulation. ISGylation occurs via a pathway similar to the pathway for ubiquitination that requires the sequential action of E1/E2/E3: the E1 (UBE1L), E2 (UbcH8), and E3 (Efp/Herc5) enzymes for ISGylation have been hitherto identified. In this study, we identified six novel candidate target proteins for ISGylation by a proteomic approach. Four candidate target proteins were demonstrated to be ISGylated in UBE1L- and UbcH8-dependent manners, and ISGylation of the respective target proteins was stimulated by Herc5. In addition, Herc5 was capable of binding with the respective target proteins. Thus, these results suggest that Herc5 functions as a general E3 ligase for protein ISGylation.

Keywords: ISG15; Interferon; Ubiquitin; Herc5

Type I interferon functions in cellular antiviral response via induction of genes called interferon-stimulated genes (ISGs) [1]. Recently, ISG15, one of the ISGs, was found to function as an antiviral protein against Sindbis virus and HIV-1 [2,3] although the molecular mechanism remains unknown. ISG15 contains two ubiquitin-like domains and belongs to the ubiquitin-like protein family. The expression of ISG15 is induced by interferon stimulation and ISG15 is conjugated to various cellular proteins (ISGylation) in a manner similar to ubiquitination that is catalyzed by the sequential action of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) [4,5]. Target proteins modified with ISG15 [6–8], the E1 (UBE1L) and E2 (UbcH8) enzymes functioning in ISGylation [9–11], and a de-ISGylating enzyme (UBP43) [12] have been identified, but biological consequences of ISGylation have been studied in only a few cases [13–15]. Recently, Efp and Herc5 have been reported to function as E3 ligases for ISGylation [16,17], but there seems to be a difference in function between Efp and Herc5 because Herc5, but not Efp, influences the ISGylation status of whole cellular proteins [16,17].

In this study, we identified six novel candidate target proteins for ISGylation and confirmed that four identified proteins, XPD (ERCC2), STK38, RGS3 isoform 1, and α-tubulin, are actually ISGylated in UBE1L- and UbcH8-dependent manners. In addition, we found that Herc5 is capable of binding with and stimulating ISGylation of the respective novel target proteins.

Materials and methods

Cell culture and transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated calf serum (Hyclone). Transfection was performed according to the standard calcium precipitation protocol.

☆ *Abbreviations:* ISG, interferon-stimulated gene; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; XP, xeroderma pigmentosum; ERCC, excision-repair, complementing defective, in Chinese hamster; STK, serine/threonine kinase; RGS, regulator of G protein signaling; PP, protein phosphatase; Efp, estrogen-responsive finger protein; Herc, HECT domain and RCC1-like domain containing protein.

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Plasmid construction. The mammalian expression plasmids of ISG15, UBE1L, and UbcH8 were generated as described previously [15]. The open-reading frames of human XPD (ERCC2), STK38, RGS3 isoform 1, α-tubulin, and Herc5 were amplified by PCR. All constructs were verified by DNA sequencing. To generate the expression plasmids, the PCR fragments were subcloned into pCI-neo-3Flag and pCI-neo-2S vectors that had been generated by inserting oligonucleotides encoding three repeats of Flag-tag sequence and two repeats of S-peptide sequence, respectively, into the pCI-neo mammalian expression vector (Promega). To generate the expression plasmid of Efp, the open-reading frame of human Efp was cut from the Myc-Efp plasmid that had been constructed as described previously [18] and was subcloned into the pCI-neo-2S vector.

Isolation of ISGylated proteins. HeLa cells that had been transiently transfected with Flag-tagged ISG15, S-tagged UBE1L, and S-tagged UbcH8 expression plasmids and cultured for 36 h were washed with ice-cold phosphate-buffered saline and lysed with RIPA buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM DTT, and 5 mM *N*-ethylmaleimide. The cell lysate was sonicated for 3 s and the debris was removed by centrifugation. The resulting supernatant was incubated with anti-Flag M2 antibody-immobilized agarose (Sigma), and the resulting immunoprecipitate was washed three times with RIPA buffer, followed by washing three times with a buffer containing 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.2% Nonidet P-40, and 10% glycerol. The materials bound to the beads were eluted with 3× Flag peptide (Sigma) (200 μg/ml) and subjected to acetone precipitation.

SDS–PAGE and Western blotting. Isolated ISGylated proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue. Western blotting was performed as described previously [13].

Peptide mass fingerprinting. Peptide mass fingerprinting was performed as described previously [19] except for the use of MS-fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) for analysis of peptide mass fingerprints. Protein classification was made by searching the OMIM™ database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM).

Immunoprecipitation and affinity purification. To analyze ISGylation, HeLa cells that had been transiently transfected with indicated plasmids and cultured for 24 h were lysed with buffer A containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 5 mM *N*-ethylmaleimide, and the supernatant of the cell lysate was subjected to immunoprecipitation using anti-Flag tag antibody-immobilized agarose beads. The resulting immunoprecipitate was washed five times with buffer A and subjected to Western blotting. To analyze the binding capacity of Herc5 or Efp, HeLa cells that had been transiently transfected with indicated plasmids and cultured for 24 h were lysed with buffer B containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride, and the supernatant of the cell lysate was subjected to affinity purification using S-protein-immobilized agarose beads (Novagen). The resulting precipitate was washed three times with buffer B and subjected to Western blotting.

Results and discussion

Identification of proteins modified with ISG15

To determine the physiological meanings of ISGylation, it is necessary to identify proteins that are modified with ISG15. We carried out a proteomic analysis of ISGylated proteins. Flag-tagged ISG15, S-tagged UBE1L, and S-tagged UbcH8 were expressed in HeLa cells, and ISGylated proteins were isolated from the cell extract by immunoprecipitation with anti-Flag tag antibody-immobilized agarose beads and subsequent elution with 3× Flag peptide. The isolated proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue

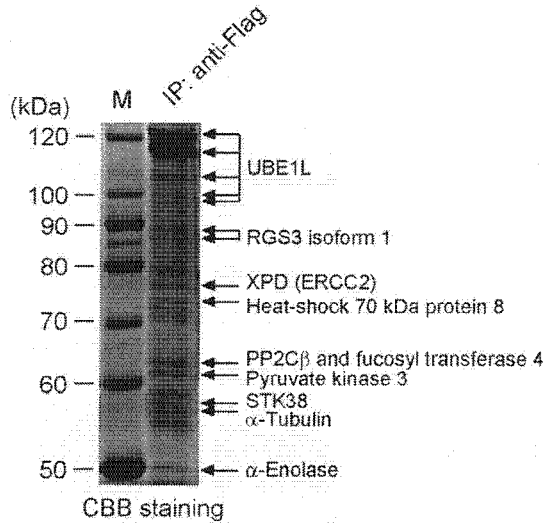

Fig. 1. Identification of proteins ISGylated. The extract of HeLa cells that had expressed Flag-tagged ISG15 together with S-tagged UBE1L and S-tagged UbcH8 was subjected to immunoprecipitation (IP) with anti-Flag tag antibody-immobilized agarose beads, and isolated ISGylated proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue (CBB). The bands identified by peptide mass fingerprinting are indicated by arrows. M, molecular mass markers.

(Fig. 1). The separated protein bands were subjected to peptide mass fingerprinting and 10 candidate target proteins for ISGylation were identified (Table 1). Among them, six proteins are novel candidate target proteins that have not been reported.

To confirm the above results of peptide mass fingerprinting, we cloned four novel candidate target proteins for ISGylation, XPD (ERCC2), STK38, RGS3 isoform 1, and α-tubulin. The respective Flag-tagged candidate target proteins were expressed together with T7-tagged ISG15, S-tagged UBE1L, and S-tagged UbcH8 in HeLa cells, and the extracts of transfected cells were subjected to immunoprecipitation using anti-Flag tag antibody-immobilized agarose beads and then to Western blotting with anti-Flag tag and anti-T7 tag antibodies (Fig. 2). In either case, two bands with slower mobilities (open arrowheads) than that of the original one (a closed arrowhead) were

Table 1
Candidate target proteins for ISGylation identified in this study

Name	Function
XPD (ERCC2)[a]	DNA repair helicase
STK38[a]	Serine/threonine kinase
RGS3 isoform 1[a]	GTPase-activating protein
α-Tubulin[a]	Cytoskeleton
PP2Cβ[a]	Serine/threonine phosphatase
Fucosyl transferase 4[a]	Fucosyl transferase
UBE1L[b]	E1 for ISGylation
Heat-shock 70 kDa protein 8[b]	Chaperone
Pyruvate kinase 3[b]	Phosphotransferase
α-Enolase[b]	Phosphopyruvate hydratase

[a] Newly identified in this study.
[b] Previously reported [7,8].

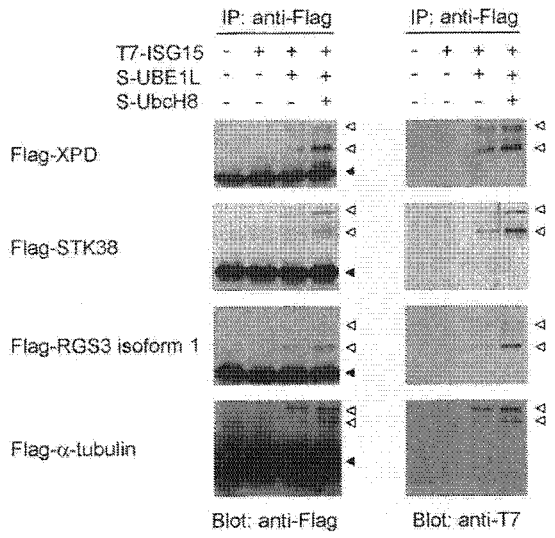

Fig. 2. Four candidate target proteins, XPD (ERCC2), STK38, RGS3 isoform 1, and α-tubulin, are ISGylated in UBE1L- and UbcH8-dependent manners. The extract of HeLa cells that had expressed the respective Flag-tagged candidate proteins together with T7-tagged ISG15, S-tagged UBE1L, and S-tagged UbcH8 as indicated was subjected to immunoprecipitation (IP) with anti-Flag tag antibody-immobilized agarose beads. The resulting immunoprecipitate was subjected to Western blotting with anti-Flag tag and anti-T7 tag antibodies. ISGylated forms of the candidate proteins are indicated by open arrowheads and the original forms are indicated by closed arrowheads.

detected in the presence of both UBE1L and UbcH8 by immunoblotting with anti-Flag tag antibody (left panel), and the bands with the same slower mobilities were also detected by immunoblotting with anti-T7 tag antibody (right panel). These results suggest that XPD (ERCC2), STK38, RGS3 isoform 1, and α-tubulin are actually ISGylated.

Herc5 stimulates ISGylation of novel target proteins

Efp and Herc5 have been reported to be E3 ligases for ISGylation [16,17], although they also function as ubiquitin E3 ligases [18,20]. We carried out experiments to determine which E3 ligase stimulates ISGylation of four target proteins newly identified in this study, XPD (ERCC2), STK38, RGS3 isoform 1, and α-tubulin. First, we constructed mammalian expression plasmids of Efp and Herc5 and expressed them together with T7-tagged ISG15, S-tagged UBE1L, and S-tagged UbcH8 in HeLa cells. The extracts of transfected cells were subjected to Western blotting with anti-T7 tag and anti-S peptide antibodies (Fig. 3). Consistent with previous reports [16,17], Herc5, but not Efp, stimulated ISGylation of the whole cellular proteins. Next, the above four Flag-tagged target proteins were expressed together with T7-tagged ISG15, S-tagged UBE1L, S-tagged UbcH8, and S-tagged Herc5 or Efp in HeLa cells, and the extracts of transfected cells were subjected to immunoprecipitation using anti-Flag tag antibody-immobilized agarose beads and then to Western blotting with anti-Flag tag and anti-T7 tag antibodies

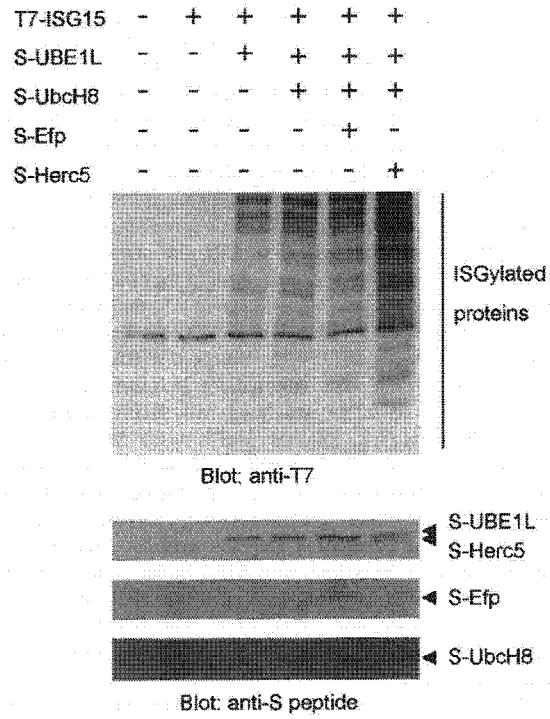

Fig. 3. Herc5 stimulates ISGylation of the whole cellular proteins. The extract of HeLa cells that had been transiently transfected with indicated plasmids was subjected to Western blotting with anti-T7 tag and anti-S peptide antibodies.

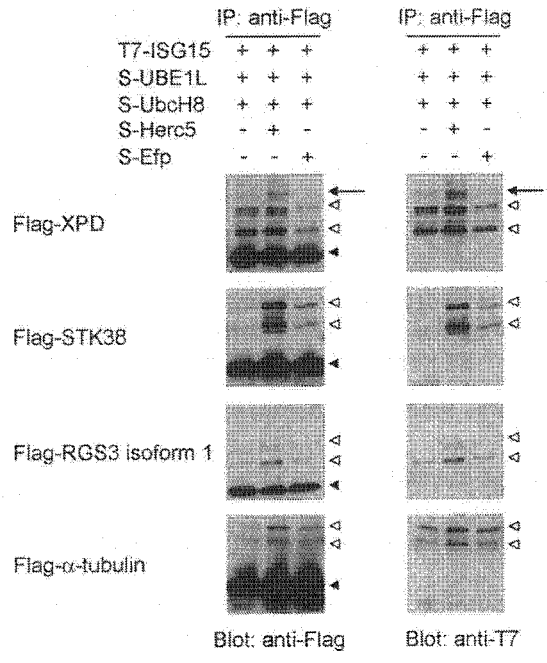

Fig. 4. Effects of co-expressions of Herc5 and Efp on ISGylation of four target proteins, XPD (ERCC2), STK38, RGS3 isoform 1, and α-tubulin. The extracts of HeLa cells that had been transiently transfected with indicated plasmids were subjected to immunoprecipitation (IP) with anti-Flag tag antibody-immobilized beads and then to Western blotting with anti-Flag tag and anti-T7 tag antibodies. ISGylated forms of the target proteins are indicated by open arrowheads and the original forms are indicated by closed arrowheads. An additional band due to ISGylated XPD (ERCC2) is indicated by an arrow.

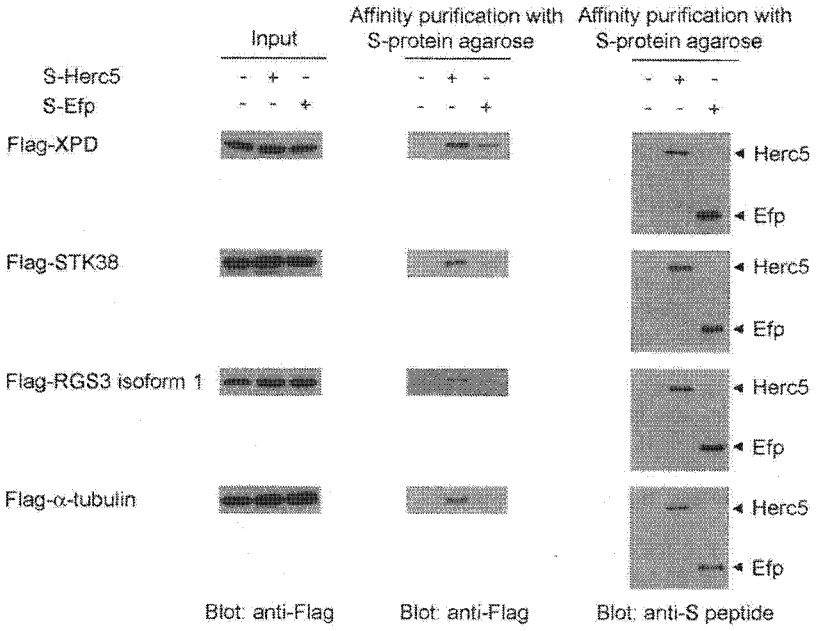

Fig. 5. Herc5 is capable of binding with four target proteins, XPD (ERCC2), STK38, RGS3 isoform 1, and α-tubulin. The extract of HeLa cells that had expressed the respective Flag-tagged target proteins together with S-tagged Herc5 or Efp as indicated was subjected to affinity purification using S-protein-immobilized agarose beads and then to Western blotting with anti-Flag tag and anti-S peptide antibodies.

(Fig. 4). ISGylation of STK38 or RGS3 isoform 1 was strongly stimulated by co-expression of Herc5 and very weakly by Efp (see two bands indicated by open arrowheads in either case). In the case of XPD (ERCC2), an additional band due to an ISGylated form was detected when Herc5, but not Efp, was co-expressed (see one band indicated by an arrow). On the other hand, ISGylation of α-tubulin was stimulated by Efp as well as by Herc5 (see two bands indicated by open arrowheads). These results suggest that Herc5 functions as an E3 ligase for ISGylation of all four target proteins.

Herc5 is capable of binding with novel target proteins for ISGylation

It is well known that the ubiquitin E3 ligase recognizes or binds with target proteins for ubiquitination [4]. We next determined whether Herc5 is capable of binding with four target proteins, XPD (ERCC2), STK38, RGS3 isoform 1, and α-tubulin. The respective Flag-tagged target proteins were expressed together with S-tagged Herc5 or Efp in HeLa cells, and the extracts of transfected cells were subjected to affinity purification using S-protein-immobilized agarose beads and then to Western blotting with anti-Flag tag and anti-S peptide antibodies (Fig. 5). Herc5 was found to bind with each of the four target proteins, while Efp was found to bind weakly only with XPD (ERCC2).

In conclusion, we identified six novel target proteins for ISGylation and found that ISGylation of XPD (ERCC2), STK38, RGS3 isoform 1, and α-tubulin is catalyzed by three enzymes, UBE1L, UbcH8, and Herc5. These results agree well with results of a previous study showing that Herc5 is an accelerator of a broad range of ISG15 conjugation [17]. The construction of an *in vitro* ISGylation system containing recombinant Herc5 together with ISG15, UBE1L, and UbcH8 will verify the speculation that Herc5 functions as a general E3 ligase for protein ISGylation.

Acknowledgments

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Review

Epigenetic and proteolytic inactivation of 14-3-3σ in breast and prostate cancers

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Abstract

14-3-3σ is an epithelial marker whose expression is induced by DNA damage through a p53-dependent pathway. 14-3-3σ functions sequesters cyclin B1-CDC2 complexes outside the nucleus and thereby contributes to a G2 arrest. Down-regulation or lack of 14-3-3σ is a frequent event in breast and prostate cancers. Epigenetic silencing by CpG methylation, p53 inactivation, and proteasome-dependent proteolysis leads to loss of 14-3-3σ. Hypermethylation of the *14-3-3σ* gene is often observed in precancerous lesions and likely to be causally linked to the onset of cancer. Proteolytic inactivation of 14-3-3σ has been recently found in breast and prostate cancers. In breast cancer, the estrogen-responsive E3 ubiquitin ligase Efp specifically targets 14-3-3σ for degradation. The E2 ubiquitin conjugating enzyme UBC8 and Efp also mediates ISG15 modification of 14-3-3σ. Detection of 14-3-3σ inactivation on the protein or DNA methylation level may be used for cancer prognosis. Furthermore, 14-3-3σ may be a potential therapeutic target in breast and prostate cancer.

Keywords: 14-3-3σ; Breast cancer; Prostate cancer; CpG methylation; Proteasome-dependent proteolysis; ISG15 modification

Contents

1. Introduction

Breast and prostate cancer are common malignancies diagnosed in Western countries as well as in Japan, and the incidence of these endocrine tumors is steadily increasing. Despite recent advances of steroid hormone research in endocrine-dependent tumors, the molecular basis of these neoplastic transformations is still largely unknown. Loss of function in tumor suppressor genes as well as gain of function in proto-oncogenes accounts for the genetic defects in tumor development. *p53* is one of the key tumor suppressor genes which is frequently mutated in cancers. The p53 protein is a transcription factor that inhibits cell growth and stimulates cell death when induced by cellular stressors, as for example DNA damage [1]. Various biological effects of p53 are mediated through p53 target genes. After DNA damage *14-3-3σ*, or *stratifin*, is induced by p53 [2]. 14-3-3σ expression is lost or reduced in a number of carcinomas. This review focuses on the contribution of proteolysis of 14-3-3σ protein and CpG-methylation its promoter to tumor formation and progression, particularly in breast and prostate cancers.

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2. Genetic and epigenetic inactivation of 14-3-3σ in malignancies

14-3-3σ is a member of a family of highly conserved, acidic dimeric 14-3-3 proteins. Among seven distinct *14-3-3* genes, *14-3-3σ* is the only gene that is induced by p53 and has been directly implicated in the etiology of human cancer [3]. 14-3-3σ sequesters cyclin B1-CDC2 complexes outside the nucleus and thereby helps to maintain a stable G2 block after DNA damage [4,5]. *14-3-3σ* has been originally cloned from human mammary epithelial cells [6] as well as from keratinocytes [7] and its expression seems to be restricted in epithelial cells. The *14-3-3σ* gene localizes to chromosome 1p36.11, which is involved in the 1p32–36 region where loss of heterozygosity (LOH) has been often observed in breast cancer [2,8]. LOH, however, seems to be not a primary mechanism for loss of 14-3-3σ expression as LOH was observed only for 5% (1/20) of primary breast cancer samples from patients with heterozygosity in their normal DNAs [9]. Genetic alterations within the coding region of *14-3-3σ* have not been observed even in breast cancer cells [9].

Epigenetic silencing of the *14-3-3σ* gene by CpG methylation has been detected at a high frequency in breast carcinoma [9], particularly in invasive tumors during early stages [10]. The *14-3-3σ* gene has a CpG-rich region within its first and only exon that begins near the transcription initiation site and ends ~800 bp downstream, including 27 CpG dinucleotides [9]. Epigenetic silencing of *14-3-3σ* has been also detected in carcinomas of the prostate [11–15], endometrium [15], ovary [15–17], lung [18], liver [19], oral epithelia [20], and skin [21]. Silencing of tumor suppressive genes by CpG methylation is presumably of equal importance for tumor development as functional inactivation by point mutation or allelic loss [22].

Inactivation of p53 is another mechanism for the cancer-specific loss of *14-3-3σ* expression, as mutations of p53 are frequent events in various types of carcinoma. It is unclear at present whether *p53* mutation and *14-3-3σ* methylation are mutually exclusive in human cancers [3]. Nevertheless, mutations in p53 may be late events during tumor formation and not a direct cause for *14-3-3σ* methylation, as several different *p53* mutations as well as CpG methylation of *14-3-3σ* have been observed in normal epidermis adjacent to basal cell carcinoma [23]. As keratinocytes may be immortalized by inactivation of *14-3-3σ* [24], *14-3-3σ* methylation may play a significant role in the escape from replicative senescence of precancerous epithelial cells. Cells with silenced *14-3-3σ* present in premalignant lesions may subsequently acquire p53 inactivation and then progress to full-blown malignancy.

3. Proteasome-mediated proteolysis of 14-3-3σ by Efp

In addition to epigenetic silencing and p53 inactivation, the expression of 14-3-3σ is also down-regulated by ubiquitin-dependent proteolysis. Using a yeast two-hybrid approach and the estrogen-responsive RING finger protein Efp as a bait, we identified an interaction between 14-3-3σ and Efp [25]. The RING finger features a set of cysteine and histidine residues that have a distinct spacing owing to their roles as the ligands of two zinc ions that stabilize a characteristic globular conformation [26]. RING finger proteins often function as E3 ubiquitin ligases, acting as scaffolds to promote ubiquitin transfer form E2 ubiquitin-conjugating enzymes to substrates [27,28]. Efp is indeed a specific E3 ligase for 14-3-3σ, preferentially recruiting UbcH8/UBC8 as the E2 conjugating enzyme [25]. Recently, proteins that contains three zinc-binding domains, a RING finger, types 1 and 2 B-boxes, and a coiled-coil region have been designated as members of the tripartite motif (TRIM) family [29]. Efp is also referred as TRIM25. Transfection experiments showed that the B-box-coiled-coil domain in Efp was essential for the binding to 14-3-3σ, while the RING finger in Efp was critical for the interaction with UbcH8/UBC8 [25] (Fig. 1).

Efp is predominantly expressed in estrogen responsive tissues including mammary glands, uteri, and osteoblasts [30,31]. Efp is robustly expressed in breast cancers and its transcription is positively regulated by estrogen in human breast cancer cell MCF7 [32]. The estrogen-response element situated in the 3′-untranslated region of Efp was identified to be responsible for the transcription of the gene [32]. The Efp gene is localized on chromosome 17q23.2, a region commonly amplified in breast tumors, especially those with poor prognosis [33]. Efp is essential for growth of female organs, as mice deficient in Efp gene show a defect in development of uteri [34]. Loss-of-function studies of Efp in MCF7 cells inoculated in nude mice revealed that Efp was a critical gene for tumor growth [25]. Whereas MCF7 cells form tumors in mice in an estrogen-dependent manner, MCF7 cells with overexpression of Efp (Efp-Mcf7) form tumors in ovariectomized mice [25]. In Efp-MCF7 cells, the expression of 14-3-3σ protein was markedly reduced and cell proliferation was significantly increased when compared with control MCF7 cells transfected with vector alone.

Efp immunoreactivity is a significant prognostic factor in breast cancer patients as shown by multivariate analyses of disease-free survival and overall survival for 151 Japanese patients [35]. Despite of previous reports regarding high frequency of hypermethylation at 14-3-3σ locus in breast cancer (91%) [9], a relatively high percentage of breast tumors that express 14-3-3σ immunoreactivity (38%) was detected in another study [35]. This discrepancy may suggest that CpG methylation is not the only determinant of 14-3-3σ expression.

4. Efp as an ISG15 E3 ligase for 14-3-3σ

Recently, it has been shown that Efp also functions as an ISG15 (15 kDa protein encoded by an interferon-stimulated gene) E3 ligase for 14-3-3σ [36]. Efp is up-regulated by type I interferon (IFN) treatment and the promoter region of Efp contains an IFN-stimulated response element (ISRE) like other genes related to ISG15 modification. ISG15 is a ubiquitin-like modification and it forms covalent conjugates with cellular proteins upon IFN treatment. Similar to ubiquitinylation, there are a series of enzymes including E1, E2, and E3 involved in the process of protein modification by ISG15 (ISGylation). The E1 enzyme of the ISG-conjugating system is the E1-like protein UBE1L [37]. UBE1L shows high homology to the ubiquitin-activating enzyme UBE1. Interestingly, the ubiquitin

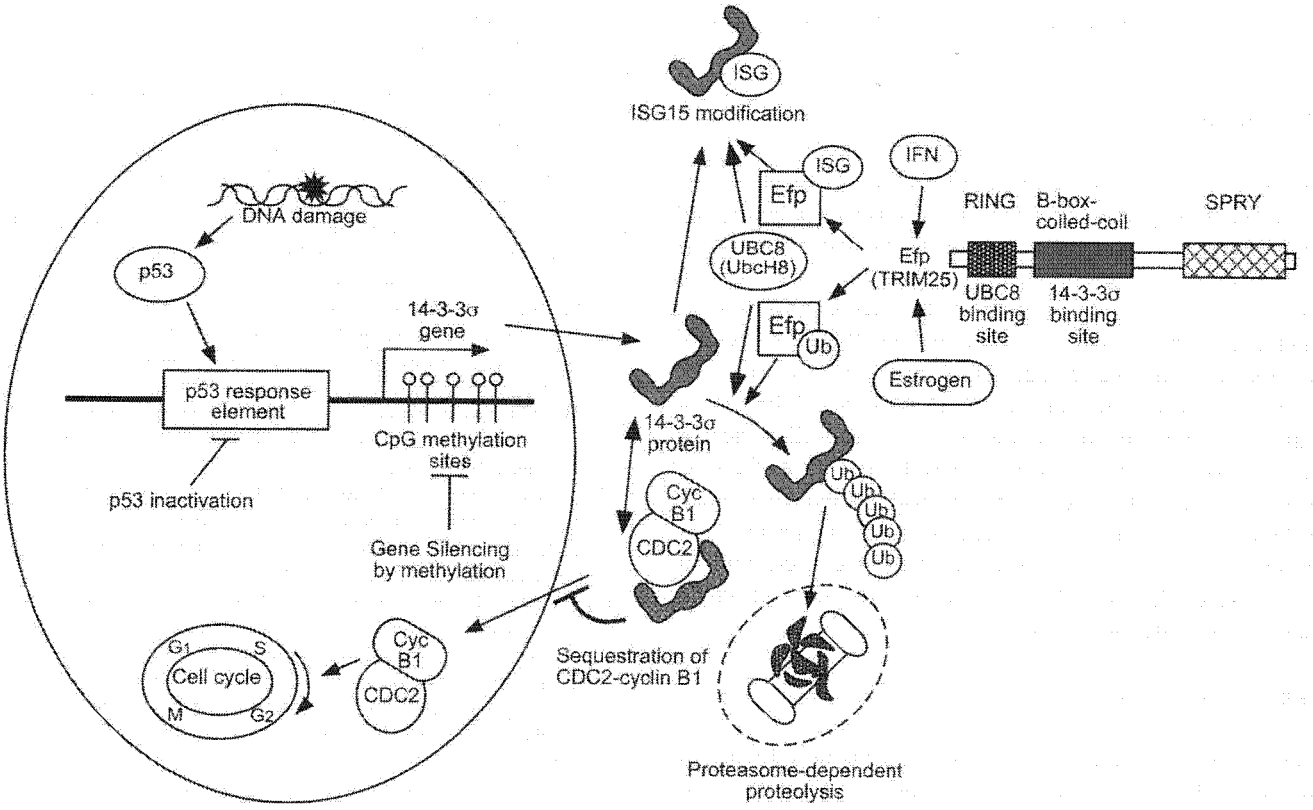

Fig. 1. 14-3-3σ regulation and function in cancer cells. *14-3-3σ* is a p53 target gene that has a p53 response element in the promoter region. DNA damage generated by such as telomere attrition and genotoxic stress by ROS production in mitogenic signaling pathways in cancer cells activate the p53 damage response. *14-3-3σ* gene expression is down-regulated by CpG methylation or by p53 inactivation. 14-3-3σ associates with cyclin B/CDC2 complexes and inhibits their nuclear translocation, which is required for cell cycle progression into G2 phase. 14-3-3σ expression is also regulated through proteasome-dependent proteolysis, which is mediated by the E3 ubiquitin ligase Efp (TRIM25) and the E2 ubiquitin conjugating enzyme UBC8 (UbcH8). Efp and UBC8 are also common components for ISG15 modification of 14-3-3σ, which can be activated by type I interferon stimulation. Efp has a structure of TRIM protein, containing RING finger, B-box-coiled-coil, and SPRY domains. UBC8 interacts with the RING finger whereas 14-3-3σ interacts with the B-box-coiled-coil domain of Efp. Repression or lack of 14-3-3σ expression through cancer-associated mechanisms leads to deregulated cell cycle progression and promotes tumor growth.

E2 UBC8 also functions as the ISG15 E2 conjugating enzyme [38,39]. So far, Efp is the only UBC8-interacting enzyme that functions as a dual E3 ligase for both ISG15 and ubiquitin [36].

Unlike ubiquitin modification, the role of ISG modifiation has not been well defined although ISG15 has been identified more than 25 years ago. ISG modification has been implicated in reproduction and innate immunity [37] and it has been found exclusively in vertebrates. However, it has been reported that generation and analysis of ISG15-deficient mice [40] and ISG15-conjugation deficient Ube 1L knockout mice [36] did not reveal any significant developmental abnormalities. Thus, the physiological relevance of ISG15 modification of 14-3-3σ and the spatio-temporal availability of ubiquitination or ISG15 modification of 14-3-3σ by Efp and UBC8 remain to be elucidated.

5. Regulation of 14-3-3σ expression in prostate cancer

In prostate cancer, epigenetic inactivation and proteolytic regulation of 14-3-3σ may be involved in tumorigenesis but the specificity of either mechanism may vary according to androgen responsiveness or tumor stages. Immunohistochemical studies showed high expression of 14-3-3σ in normal prostate epithelial and benign prostate hyperplasia cells, whereas prostate cancer cells displayed low expression or lack of 14-3-3σ [11,13]. Methylation-specific PCR analysis showed CpG methylation of *14-3-3σ* in all 41 primary prostate cancer samples [11]. A prototypical androgen receptor (AR)-positive prostate cancer cell line LNCaP also exhibited CpG methylation of *14-3-3σ* [11,13], whereas AR-negative human prostate cancer PC3 and DU145 cells did not exhibit CpG methylation of *14-3-3σ* [11,13]. In PC3 and DU145 cells, however, protein expression of 14-3-3σ was reduced compared with primary prostate epithelial cells and the protein expression was increased by the proteasome inhibitor MG132, indicating that proteasome-mediated proteolysis is responsible for the down-regulation of 14-3-3σ protein in these cell lines [13]. Interestingly, 14-3-3σ expression in PC3 and DU145 cells was also induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [13], a member of the TNFα superfamily that induces apoptosis in a variety of transformed cells including PC3 and DU145 cells by engaging and activating death receptors [41]. TRAIL induces apoptosis in cancer cells with minimal cytotoxicity to normal cells. TRAIL also causes cell cycle arrest through the up-regulation of 14-3-3σ levels in TRAIL-sensitive cancer cells. TRAIL may therefore

have a therapeutic potential against advanced cancers regardless their p53 status.

6. Conclusions

14-3-3σ plays a role as a tumor suppressor gene whose expression is frequently reduced or diminished in breast and prostate cancers. Epigenetic silencing by CpG methylation, p53 inactivation, and proteasome-dependent proteolysis are responsible for loss of 14-3-3σ expression. The attenuation of checkpoint functions by loss of 14-3-3σ promotes cell cycle into G2/M transition and stimulates cell growth. Cancer cells lacking 14-3-3σ are further susceptible for genotoxic stress and exhibit genetic instability. The methylation status of *14-3-3σ* as well as the protein expression levels of 14-3-3σ may be useful markers for the determination of cancer prognosis, and reagents that induce the demethylation of *14-3-3σ* gene or inhibit of proteasome-dependent 14-3-3σ proteolysis and thereby lead to elevation of 14-3-3σ protein levels could be potential therapeutic options for cancers, especially those in advanced stages.

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Q89R Polymorphism in the LDL Receptor-Related Protein 5 Gene Is Associated With Spinal Osteoarthritis in Postmenopausal Japanese Women

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Study Design. An association study investigating the genetic etiology for spinal osteoarthritis.

Objective. To determine the association of single-nucleotide polymorphism (SNP) causing an amino-acid change (Q89R) in the low-density lipoprotein receptor-related protein 5 (LRP5) coding region with spinal osteoarthritis.

Summary of Background Data. Wnt/β-catenin signaling pathway regulates bone density through a Wnt coreceptor LRP5. This pathway is also involved in cartilage development and homeostasis, suggesting that genetic variation in LRP5 gene may affect the pathogenesis of cartilage-related diseases, such as osteoarthritis.

Methods. We evaluated the presence of osteophytes, endplate sclerosis, and narrowing of disc spaces in 357 Japanese postmenopausal women. Missense coding SNP for Q89R of LRP5 gene was determined using TaqMan polymerase chain reaction (PCR) method.

Results. We found that subjects without the R allele (QQ; n = 321) had a significantly lower osteophyte formation score than did subjects bearing at least one R allele (QR + RR; n = 36) (7.80 *vs.* 10.89, $P = 0.0019$ by analysis of covariance).

Conclusions. We suggest that a genetic variation at the LRP5 gene locus is associated with spinal osteoarthritis, in line with the involvement of the LRP5 gene in the bone and cartilage metabolism.

Key words: single-nucleotide polymorphism (SNP), low-density lipoprotein receptor-related protein 5 (LRP5), spinal osteoarthritis, osteophytosis. **Spine 2007;32:25–29**

Osteoarthritis of the spine is a very common condition in the axial skeletons of aged people.[1] Vertebral osteophytes, endplate sclerosis, and intervertebral disc narrowing are recognized as characteristic features of spinal degeneration. Recent studies indicate that the appearance of these radiographic features is influenced by genetic factors, physical loading, and other environmental factors.[2,3] Association studies in using various definitions of osteoarthritis have been performed, mainly investigating genes encoding structural proteins of the extracellular matrix of cartilage (*e.g.*, collagen Type II α1, cartilage matrix protein, and aminoguanidine) or genes playing a role in the regulation of bone density and mass (*e.g.*, vitamin D receptor, insulin-like growth factor-I, and estrogen receptor α).[4,5]

The Wnt (wingless-type MMTV integration site family) represents a large group of secreted signaling proteins that are involved in cell proliferation, differentiation, and morphogenesis.[6] The name of "Wnt" is derived from wingless gene in *Drosophila melanogaster*[7] and murine int-1 oncogene identified in tumors induced by mouse mammary tumor virus.[8] It is also known that Wnt and bone morphogenetic protein (BMP) signals control apical ectodermal ridge formation and dorsal-ventral patterning during limb development.[9,10] Wnt proteins activate signal transduction through Frizzled, which act as receptors for Wnt proteins[11] and induce stabilization of cytoplasmic β-catenin protein, which also regulates target gene expression as a transcriptional coactivator. The physiologic role of Wnt in the regulation of osteoblastogenesis has been studied in experimental models, in embryonic mesenchymal progenitor cells expressing Wnt3a[12] or in mice expressing Wnt10b transgene in bone marrow.[13] It is also shown that activated β-catenin modulate osteoblast and chondrocyte differentiation.[14,15] Meanwhile, LDL receptor-related protein 5 and 6 (LRP5/6) were also found to be required for Wnt coreceptors.[16,17] Recent reports demonstrated that the Wnt/β-catenin signaling pathway regulates bone density through LRP5.[18–21] These findings indicate that Wnt-β-catenin signaling pathway plays important roles in the skeletal biology.

In addition to the regulation of limb development and bone metabolism, Wnt/β-catenin signaling may be involved in maintenance and pathophysiology of cartilage. This possibility is indirectly supported by the observation that several Wnt proteins and Frizzled receptors are expressed in synovial tissue of arthritic cartilage.[22] In

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addition, a secreted Frizzled-related protein (FrzB-2) that act as an antagonist for Frizzled receptor is strongly expressed in osteoarthritic cartilage and may regulate chondrocyte apoptosis.[23] It is also reported that chondrocytes express β-catenin at a low level and accumulation of β-catenin is sufficient to cause dedifferentiation of chondrocytes, suggesting that Wnt signaling is involved in cartilage metabolism.[24] Thus, it is assumed that LRP5 modulates Wnt/β-catenin signaling pathway in the bone and cartilage homeostasis. In the present study, we examine an association between a polymorphism in LRP5 gene and radiographic features of spinal osteoarthritis, including osteophyte formation, endplate sclerosis, and disc space narrowing number to investigate a possible contribution of LRP5 to human bone and cartilage metabolism.

Materials and Methods

Subjects. Genotypes were analyzed in DNA sample obtained from 357 healthy postmenopausal Japanese women (mean age ± SD; 65.22 ± 8.20 years) living in central area of Japan. Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (*e.g.*, corticosteroids, anticonvulsants, heparin sodium), or unusual gynecologic history. Patients with severe hip and knee arthritis were excluded from the present study. The eligibility of subjects was determined by taking history-physical examination. All were nonrelated volunteers and provided informed consent before this study. Ethical approval for the study was obtained from appropriate ethics committees.

Radiographic Grading of Osteoarthritis of the Spine. Conventional thoracic and lumbar spinal plain roentgenograms in lateral and anteroposterior projection were obtained from all participants. The severities of spinal degeneration, including osteophyte formation, endplate sclerosis, and disc space narrowing, were assessed semiquantitatively from T4–T5 to L4–L5 disc level or from T4 to L5 vertebrae by using the grading scale of Yu *et al.*[25] Briefly, osteophyte formation at a given disc was graded 0° to 3°, endplate sclerosis at given vertebra was graded 0° to 2°, and disc space narrowing was graded 0° to 1°. Then we defined sum of each degree from T4–T5 to L4–L5 disc level for osteophyte formation on anteroposterior radiographs as a score of osteophyte formation. We also defined sum of each degree from T4 to L4 vertebra for endplate sclerosis and that from T4–T5 to L4–L5 disc level for disc space narrowing on lateral radiographs as a score of endplate sclerosis and disc narrowing, respectively. Then we defined sum of each 13 grade for osteophyte formation on anteroposterior radiographs as a score of osteophyte formation. We also defined sum of 13 grade for endplate sclerosis and disc space narrowing on lateral radiographs as a score of endplate sclerosis and disc narrowing, respectively.

Measurement of Bone Mineral Density (BMD) and Biochemical Markers. The lumbar spine BMD and total body BMD (in g/cm^2) of each participant were measured by dual-energy radiograph absorptiometry using fast-scan mode (DPX-L; Lunar, Madison, WI). The BMD data were recorded as "Z scores," that is, deviation from the weight-adjusted average BMD for each age. Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20,000 Japanese women.

We measured serum concentration of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), intact-osteocalcin (I-OC, ELISA; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin (CT), and 1, 25(OH)2D3. We also measured urinary ratios of urinary deoxypyridinoline (DPD, HPLC method) to creatinine.

Determination of a Single Nucleotide Polymorphism in the LRP5 Gene. DNA was extracted from peripheral leukocytes by standard techniques. Missense coding SNP for Q89R (c. 266A>G) of the LRP5 gene was determined using Assays by Design SNP Genotyping Products (Applied BioSystems) that based on the TaqMan PCR method.[26] Missense coding means that the alteration of a codon (an array of three consecutive bases in mRNA) that encodes a different amino acid. TaqMan PCR method uses two kinds of TaqMan probes that correspond to a DNA fragment including the target SNP site with different alleles and the 5′–3′ nuclease activity of Taq polymerase that is essential for PCR. TaqMan probes include fluorescence dyes at their 5′ ends and a quencher at their 3′ ends. During PCR cycles, TaqMan probes will anneal to target DNA and will be excised by the 5′–3′ nuclease activity of Taq polymerase if there is no mismatch between the probes and target sequences. Then the fluorescence dyes will be released from the probes and the intensity of fluorescence can be monitored by using ABI PRISM 7000 (Applied Biosystems) as a fluorescence detector. The allele frequencies of Q89R polymorphism were confirmed as they were not significantly deviated from Hardy-Weinberg equilibrium. Since Hardy-Weinberg equilibrium is based on the following assumptions including no genetic drift, no gene flow, no natural selection, negligible mutations, and random mating, the population under the equilibrium is not evolving and its genotype and allele frequencies are predicted to remain unchanged over successive generations. Thus, we considered that our subjects were eligible for the correlation study.

Statistical Analysis. We divided subjects into those having one or two chromosomes of the minor G-allele (QR + RR) and those with only the major A-allele (QQ) encoded at the same locus. Comparisons of Z scores of lumbar spine and biochemical markers between these two groups were subjected to statistical analysis (unpaired *t* test; StatView-J 4.5, SAS Institute Inc.). The association between these two groups and osteoarthritis parameters (number of osteophyte, endoplate sclerosis, and disc narrowing) was assessed by unpaired *t* test and by analysis of covariance (ANCOVA) with adjustment of confounding clinical variables (age, body weight, and height). A *P* value less than 0.05 was considered statistically significant.

Results

We analyzed the genotypes for the SNP of LRP5 at Q89R (c.266 A>G) in subjects, using TaqMan methods. Among 357 postmenopausal Japanese women, 321 were QQ homozygotes, 35 were QR heterozygotes, and 1 was RR homozygote. The allelic frequencies of this SNP in the present study were in Hardy-Weinberg equilibrium.

Because only 1 of these subjects carried the RR genotype of the Q89R polymorphism, we compared those who carried the R allele (QR or RR) with those who did

Table 1. Comparison of Background, Clinical Characteristics Between Subjects Bearing at Least 1 R allele (QR + RR) and Subjects With No R allele (QQ) in the *LRP5 Gene Coding Region* (Q89R)

Item	Genotype (mean ± SD) QQ	QR + RR	P
No. of subjects	321	36	
Age (yr)	65.0 ± 8.2	67.3 ± 8.0	NS
Height (cm)	150.7 ± 5.7	151.1 ± 7.1	NS
Body weight (kg)	50.5 ± 7.6	51.3 ± 7.9	NS
Lumber spine BMD (Z score)	−0.28 ± 1.40	−0.17 ± 1.89	NS
ALP (IU/L)	190.8 ± 61.3	194.8 ± 81.1	NS
I-OC (ng/mL)	8.2 ± 4.0	7.4 ± 3.0	NS
DPD (pmol/μmol of Cr)	7.6 ± 4.0	7.6 ± 2.3	NS
Intact PTH (pg/mL)	35.6 ± 16.7	34.6 ± 14.1	NS
1,25 $(OH)_2D_3$ (pg/mL)	36.1 ± 10.8	37.3 ± 14.6	NS
BMI	22.1 ± 3.0	22.8 ± 3.1	NS

BMD indicates bone mineral density; ALP, alkaline phosphatase; I-OC, intact-osteocalcin; DPD, deoxypyridinoline; PTH, parathyroid hormone; BMI, body mass index; NS, not significant. Statistical analysis was performed according to the method described in the text.

not (QQ). The lumbar BMD was not statistically different between these groups (Table 1). The background and biochemical data were not statistically different between these groups (Table 1). On ANCOVA analysis, we found significant associations between LRP5 Q89R genotype and osteophyte formation score after controlling for age, weight, and height. Women without the R allele (QQ; n = 321) had a significantly lower osteophyte formation score than did subjects bearing at least one R allele (QR + RR; n = 36) (7.80 ± 6.51 *vs.* 10.89 ± 7.6, $P = 0.0019$, Figure 1A; Table 2). We also found significant association between them on unpaired *t* test ($P = 0.0083$, Table 1). On the other hand, the occurrence of disc narrowing and endplate sclerosis did not significantly differ in those with and without at least one R allele (Figure 1B, C; Table 2).

Table 2. Association of the *LRP5* SNP Genotype

Item	Genotype (mean ± SD) QQ	QR + RR	P (unpaired *t* test)	P (ANCOVA)
No. of subjects	321	36		
Osteophyte formation score	7.80 ± 6.51	10.89 ± 7.6	0.0083	0.0019
Endoplate sclerosis score	0.368 ± 0.845	0.389 ± 0.994	NS	NS
Disc space narrowing score	2.03 ± 1.88	2.06 ± 1.84	NS	NS

ANCOVA indicates analysis of covariance; NS, not significant.

Discussion

The present study is the first report that shows the influence of a single-nucleotide polymorphism of LRP5 gene on spinal osteoarthritis as far as we know. Targeting the pathogenesis of low back pain, we have previously investigated associations of genetic factors with osteoporosis. LRP5 has been shown as one of the correlated genes in Japanese postmenopausal women.[27] Because spinal osteoarthritis is another major reason for low back pain, we have extended our association study of LRP5 polymorphism with spinal osteoarthritis. We demonstrated that the Japanese postmenopausal women who had one or two allele(s) of a nonsynonymous change (Q89R) in LRP5 gene showed significantly higher osteophyte formation score of spine. Our finding may also be supported by genome-wide scan for osteoarthritis-susceptibility loci that showed a linkage to chromosome 11q12-13,[28,29] which includes the LRP5 gene locus on 11q13.4. It has been recently shown that single-nucleotide polymorphisms in LRP5 gene provided no correlation with knee osteoarthritis status while haplotype analysis revealed that there was a common haplotype that provided a 1.6-fold increased risk,[30] suggesting that LRP5 might be involved in the pathogenesis of osteoarthritis also in

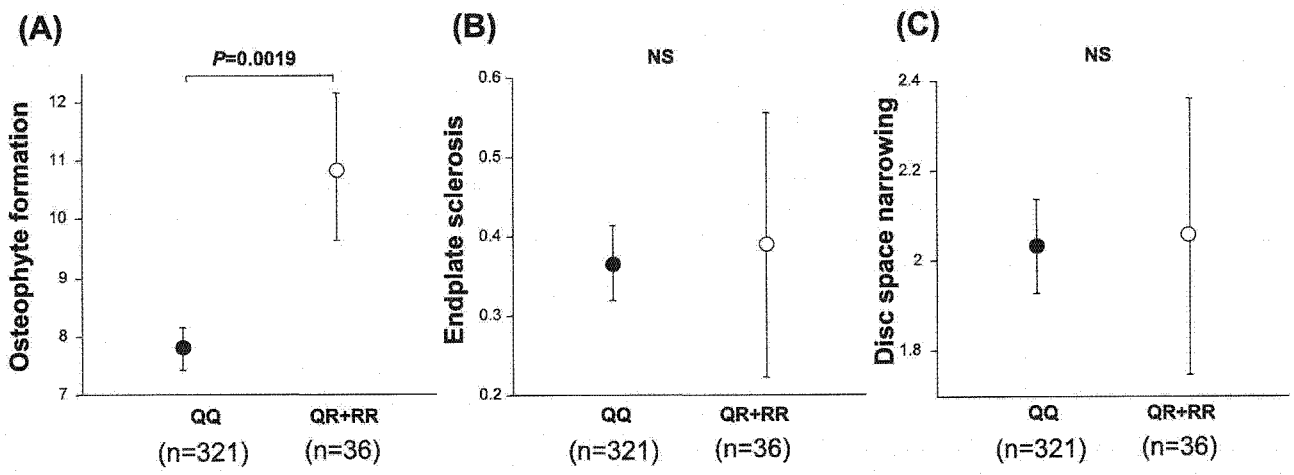

Figure 1. Scores of spinal osteoarthritis between the genotypes of polymorphism at Q89R (QQ *vs.* QR + RR). **A**, Scores of osteophyte formation are shown for genotype QQ and for genotype QR + RR. Scores are expressed as mean ± SE. Numbers of subjects are shown in parentheses. **B**, Scores of endplate sclerosis. **C**, Disc space narrowing scores. The association of the two genotype groups with osteoarthritis parameters was determined by ANCOVA, a type of multifactorial analysis, with adjustment of confounding clinical variables (age, body weight, and height).

other joints. It is also reported that there was a significant association of a functional gene variant of secreted frizzled-related protein 3 (sFRP3), which antagonizes Wnt signaling, with hip osteoarthritis in women.[31] Taken together, our results and the recent evidence suggest that the canonical Wnt signaling pathway including LRP5 is critical in the pathogenesis of skeletal abnormality, including osteoarthritis and osteoporosis.

Recently, mutations of the LRP5 gene have been described to be associated with both osteoporosis-pseudoglioma syndrome and the high bone mass phenotype.[18–21] It was found that loss-of-function of LRP5 in both human[18] and mice[19] yielded a decrease in bone formation, or an active mutation of LRP5 that cannot bind to a Wnt inhibitor Dickkopf-1 resulted in a high bone mass trait.[20,21] Moreover, our group and several other groups have reported that single-nucleotide polymorphisms in LRP5 gene predicted the bone mass.[27,32–36] These SNPs included three of different missense variations; Q89R,[33,34] V667 M,[35] and A1330V.[36] In the present study, we investigated a possible contribution of Q89R LRP5 polymorphism to spinal osteoarthritis in Japanese women. V667 M polymorphism was not detected in our Japanese population. Regarding A1330V polymorphism, we could not detect an association of the SNP with spinal osteoarthritis (data not shown).

Two groups reported consistent association of Q89R with Ward's triangle BMD but not with lumbar BMD in Korean young men[33] and Chinese premenopausal women.[34] In our Japanese population, we did not find an association of Q89R polymorphism with lumbar spine. The present data together with published data related to osteoporosis suggest that Q89R polymorphism may be involved in the pathogenesis of both osteoporosis and spinal osteoarthritis and QQ genotype in LRP5 might be preventive for both diseases. Meanwhile, there are other cases in which genetic factors contribute to the pathogenesis of osteoporosis and osteoarthritis in an opposite way. For example, it has been reported that transforming growth factor-β1 (TGF-β1) gene polymorphism T869C, which gives Leu>Pro substitution contributes differentially to osteoporosis and osteoarthritis; people with CC genotype had significantly higher BMD than those with TC or TT, whereas this CC genotype was related to significantly greater osteophytes than TT or TC.[37]

Osteoarthritis occurs as result of both mechanical and biologic events that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix as well as subchondral bone.[1,38] Cartilage destruction during osteoarthritis involves the loss of differentiated phenotype and apoptotic death of chondrocytes,[39] Wnt proteins were shown to regulate dedifferentiation of apoptosis of chondrocytes.[40] It is also demonstrated the interaction of β-catenin with SOX9, a transcriptional factor that is required in successive steps of chondrogenesis, controls chondrocyte differentiation.[41] These data suggest Wnt/β-catenin may participate in the pathogenesis of cartilage diseases, such as osteoarthritis. Further studies will be required to clarify the role of Q89R missense variant of the LRP5 in the pathogenesis of osteophyte formation and osteoporosis.

Conclusion

We have shown an association of the Q89R polymorphism in the LRP5 gene with a radiographic feature of spinal osteophytosis in postmenopausal Japanese women. The women with QQ genotypes had significantly lower osteophyte formation scores. The LRP5 genotyping might be beneficial in the prevention and management of spinal osteophytosis as well as osteoporosis. The present findings regarding the correlation of LRP5 polymorphism with spinal osteoarthritis provide a new promising direction for the clinical medicine of the spine disease, which leads us to the development of new diagnostic markers as well as therapeutic options based on the molecular target.

Key Points

- Wnt/β-catenin signaling pathway regulates bone and cartilage metabolism.
- The single-nucleotide polymorphism, causing an amino-acid change (Q89R) in LRP5 gene that encodes a Wnt coreceptor, was associated with spinal osteophytosis in Japanese postmenopausal women.
- We suggest that a genetic variation at the LRP5 gene locus is associated with spinal osteoarthritis.

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A functional single nucleotide polymorphism in the vitamin-K-dependent gamma-glutamyl carboxylase gene (Arg325Gln) is associated with bone mineral density in elderly Japanese women

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Abstract

The vitamin-K-dependent gamma-glutamyl carboxylase (GGCX) carboxylates vitamin-K-dependent proteins including bone Gla protein (osteocalcin) and matrix Gla protein, which play important roles in bone metabolism. Therefore, GGCX polymorphism might explain in part individual susceptibility to osteoporosis. In the present study, polymorphisms in the exons of this gene were screened in Japanese elderly women and a non-synonymous single nucleotide polymorphisms (SNP) were found; c.8762 G>A; (Arg325Gln). When the kinetic parameters of GGCX325-Gln and GGCX325-Arg were compared *in vitro*, Vmax/Km was significantly higher for GGCX325-Gln (944.4±9.21 pmol/30 min/mg/mM FLEEL) than for GGCX325-Arg (671.9+10.79 pmol/30 min/mg/mM FLEEL) ($p=0.018$). Then, association study of this polymorphism with forearm bone mineral density (BMD) of Japanese postmenopausal women ($n=500$, age 73.6±5.74) was conducted. As a result, the body mass index (BMI)-adjusted Z score in the subpopulation older than 75 years ($n=207$) was higher in those with 325-Gln (0.650±0.883, mean±SD) than those with 325-Arg/Gln or 325-Arg (0.133±0.650) ($p=0.0383$). This is the first report to demonstrate the different activities of GGCX between the common genotypes and their association with BMD.

Keywords: Vitamin-K-dependent gamma-glutamyl carboxylase; Single nucleotide polymorphism; Osteoporosis; Bone mineral density; Bone metabolism

Introduction

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture [1]. Because osteoporosis consequently leads to deterioration in activities of daily living (ADL) and quality of life (QOL), prevention and treatment are becoming more important in the current aging society.

Several risk factors for osteoporotic fractures have been established [2], which include the family history of fractures. Epidemiological studies also support the heritability of BMD [3], indicating the contribution of the genetic factors to the pathogenesis of osteoporosis. Therefore, the elucidation of genetic factors for this disease has been awaited. Recently, genetic factors for osteoporosis have been investigated with

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