Abstract. The X-linked inhibitor of apoptosis (XIAP) confers the resistance of various types of cancer to standard chemotherapeutic agents such as anthracycline and taxane. In breast cancer, XIAP is known to be overexpressed. However, the mechanisms underlying the overexpression of XIAP remain currently unclear. In order to elucidate the mechanisms responsible for the overexpression of the XIAP protein in breast cancer, we attempted to clarify the mechanisms by which the natural compound curcumin downregulates XIAP in breast cancer cells. In that process, we identified the ribosomal protein S3 (RPS3) as a curcumin-binding protein using curcumin-fixed magnetic FG beads. The knockdown of RPS3 inhibited cell growth and induced apoptosis as well as the downregulation of XIAP in breast cancer cells. Although RPS3 is known to directly bind to and activate the nuclear factor-κB (NF-κB), which induces several anti-apoptotic genes such as XIAP, the knockdown of RPS3 unexpectedly reduced the levels of the XIAP protein, but not the mRNA level of XIAP and the transcription factor NF-κB activity. These results reveal that RPS3 upregulates XIAP independently of the NF-κB pathway in human breast cancer cells.

Introduction

Breast cancer is the most common type of cancer and a leading cause of mortality among women (1). Various molecular-targeted therapies of breast cancer have been developed in the past decade (2-4) and treatments have been diversified. However, development of the resistance of cancer cells to chemotherapeutic agents remains an important clinical issue. Since the major cause of recurrence is chemoresistance to systemic therapies (5,6), the mechanisms underlying chemoresistance need to be elucidated in more detail.

The X-linked inhibitor of apoptosis (XIAP) is a potent inhibitor of apoptosis proteins (7). XIAP binds to and inhibits caspases, which are the principal inducers of apoptosis (8-11). In addition, XIAP has been associated with drug resistance in various types of cancer such as breast (12,13), bladder (14) and ovarian cancer (15). In breast cancer cells, XIAP is significantly upregulated in cells that are resistant to anthracycline or taxane (12), which are mainly used in adjuvant chemotherapies for breast cancer. Previous studies have revealed that XIAP was overexpressed at the mRNA and immunohistochemical levels in the breast cancer specimens of patients (12,16,17). Although the nuclear factor-κB (NF-κB) upregulates the expression of XIAP (18), the mechanisms underlying its overexpression remain currently unclear.

On the other hand, ribosomes are composed of small and large subunits and synthesize proteins from transcribed mRNAs. However, several ribosomal proteins have been identified to have extraribosomal functions (19,20). For instance, the ribosomal protein S3 (RPS3) is a component of the small 40S ribosomal subunit, but it is also a subunit in the transcription factor NF-κB, which controls inflammatory gene regulation (21). RPS3 activates the NF-κB signaling by binding to the p65 subunit of NF-κB. Moreover, RPS3 is known to function as a DNA repair endonuclease, which is also an extraribosomal function (22).

In order to clarify the mechanisms responsible for the overexpression of XIAP in breast cancer, we initially examined the molecular actions of the natural product curcumin which downregulates XIAP (23,24), since we considered the possibility that curcumin may target the proteins related to the expression of XIAP. We fixed curcumin onto magnetic FG beads as previously described (25) and RPS3 was identified as one of the curcumin-binding proteins using curcumin-fixed beads. Moreover, the knockdown of RPS3 reduced the expression of XIAP independently of the NF-κB pathway. In the present study, we identified a novel function for RPS3, which regulates the expression of XIAP in breast cancer cells.

Materials and methods

Reagents. Curcumin (Nagara Science Co., Ltd., Gifu, Japan) was purchased and dissolved in dimethyl sulfoxide (DMSO).
**Cell culture.** Human breast cancer MCF-7 (estrogen and progesterone receptor-positive, luminal type), MDA-MB-231 (triple-negative, basal type) and human prostate cancer PC-3 cells were obtained from the NCI-60 cancer cell line panel of the National Cancer Institute Developmental Therapeutics Program (NCI DTP). MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 50 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂. The MDA-MB-231 and PC-3 cells were cultured in RPMI-1640 medium with 10% FBS, 2 mM glutamine, 50 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂. It has been identified that the activity of NF-κB in MDA-MB-231 cells is higher than that in MCF-7 cells (26).

**Cell viability assay.** The cell growth was evaluated using the CellCounting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The CCK-8 solution was added to the medium of curcumin-treated or siRNA-transfected MCF-7 and MDA-MB-231 cells. The absorbance (450 nm) of the medium was determined using Multiskan FC (Thermo Fisher Scientific, Waltham, MA, USA) after 4 h of incubation.

**Detection of apoptosis.** The MCF-7 and MDA-MB-231 breast cancer cells treated with curcumin or transfected with each siRNA for RPS3 were harvested by trypsinization. The cells were washed with phosphate-buffered saline (PBS) and suspended in PBS containing 0.1% Triton X-100, 150 µg/ml RNase A and 50 µg/ml propidium iodide to stain the nuclei. The DNA content in the stained nuclei was analyzed by FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ, USA) and the hypodiploid population (sub-G1) was detected and quantified.

**Western blot analysis.** The MCF-7 and MDA-MB-231 breast cancer cells were treated with curcumin or transfected with each siRNA for RPS3 and lysed with RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM DTT and 0.5 mM PMSF] at 4°C for 30 min and centrifuged. The supernatants were separated by 12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA) and a mouse monoclonal anti-β-Actin antibody (#9538; Cell Signaling Technology, Danvers, MA, USA), a rabbit monoclonal anti-RPS3 antibody (Hs02758991_g1). Subsequently these proteins were subjected to in-gel digestion with Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) and the resulting peptide fragments were analyzed by Autoflex II (Bruker Daltonics, Billerica, MA, USA).

**Quantitative real-time RT-PCR.** The total cellular RNA was extracted from the MCF-7 and MDA-MB-231 cells transfected with each siRNA using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and complementary DNA (cDNA) was synthesized from total RNA with High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR analysis was performed by an ABI 7300 Real-Time PCR system (Applied Biosystems) with the cDNA and TaqMan probes (Applied Biosystems) to XIAP (Hs00745222_s1) and GAPDH (Hs02758991_g1).

**RNAi.** The MCF-7 and MDA-MB-231 cells were transfected with each siRNA using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA). The following siRNAs (Life Technologies) were used: siRPS3 #1 (HSS184418), 5'-GAGCUGGCCGAGAUGGCUACUG-3'; siRPS3 #2 (HSS184419), 5'-GCCGAUUCGGGACAGUCUGCAUA-3'; siRPS3 #3 (HSS184420), 5'-UGAACCACCGCGAGCCCGUUAA-3'; and negative control siRNAs (cat. no. 12935-112 and no. 12935-115). Only RNA sequences of sense strands are shown.

**Quantification of the NF-κB activity.** The nuclear extracts were prepared from the MCF-7 and MDA-MB-231 cells transfected with each siRNA using Nuclear Extract kit (Active Motif, Carlsbad, CA, USA). Subsequently, the activity of the p65 subunit of NF-κB in the nuclear extracts was determined by the NF-κB p65 Transcription Factor Assay kit (TransAM NFκB p65; Active Motif) and Multiskan FC (Thermo Fisher Scientific).

**Statistical analysis.** The data from triplicate samples are represented as the mean ± standard deviation (SD). Statistical analysis was performed using non-repeated measures ANOVA followed by Bonferroni post hoc test due to comparisons of more than two groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Curcumin downregulates the XIAP protein in breast cancer cells. Previous studies have identified that XIAP is strongly...
expressed in breast cancer tissues (16,17). In order to elucidate the mechanisms responsible for the overexpression of XIAP, we searched for compounds that downregulate XIAP in breast cancer cells. Curcumin, one of the most common polyphenols, inhibited the growth of the MCF-7 breast cancer cells (Fig. 1A) and induced apoptosis (Fig. 1B). We then found that curcumin downregulated the XIAP protein (Fig. 1C). These results indicate that curcumin reduces the protein levels of XIAP in the MCF-7 breast cancer cells.

Identification of RPS3 as a curcumin-binding protein. In an attempt to clarify the mechanisms by which curcumin downregulates XIAP in MCF-7 cells, we examined the curcumin-binding proteins using magnetic FG beads. Curcumin was conjugated onto the beads with K2CO3 (Fig. 2A). We initially used PC-3 prostate cancer cells to identify the curcumin-binding proteins and identified RPS3 as one of the curcumin-binding proteins (data not shown). RPS3 was also purified from the whole cell extracts of the MCF-7 cells using curcumin-fixed beads (Fig. 2B). These results reveal that RPS3 is a target of curcumin.

Knockdown of RPS3 downregulates the XIAP protein independently of the NF-κB pathway. Several extraribosomal functions of the ribosomal proteins have recently been identified (21,27-29). RPS3 is known to be a subunit in NF-κB complexes and was found to promote the transcription of anti-apoptotic molecules, including XIAP in Jurkat leukemia cells (21). Therefore, we examined whether RPS3 also regulates XIAP at the mRNA level in breast cancer cells. The knockdown of RPS3 inhibited cell growth (Fig. 3A) and induced apoptosis (Fig. 3B). Although the knockdown of RPS3 downregulated the XIAP protein (Fig. 3C), unexpectedly the mRNA level of XIAP was not reduced (Fig. 3D). These findings reveal that RPS3 regulates the expression of XIAP independently of the NF-κB pathway in the MCF-7 breast cancer cells.

Additionally, in order to confirm that RPS3 regulates the expression of XIAP independently of the NF-κB signaling, we used another breast cancer cell line, MDA-MB-231. This cell line is classified as triple-negative breast cancer, which is the most aggressive subtype (30,31) and the NF-κB pathway is activated in this cell line (26). Similar to the MCF-7 breast cancer cells, the knockdown of RPS3 inhibited the cell growth (Fig. 4A) and induced apoptosis (Fig. 4B). The RPS3 depletion downregulated the XIAP protein (Fig. 4C), but not the mRNA level of XIAP in the MDA-MB-231 cells (Fig. 4D). Collectively, these results clearly reveal that RPS3 regulates the expression of XIAP independently of the NF-κB pathway in breast cancer cells, which is inconsistent with a previous study (21).

In addition, we investigated the activation of NF-κB by quantifying the activity of the p65 subunit of the NF-κB. As shown in Fig. 5A, the activity of NF-κB in the MDA-MB-231 breast cancer cells was higher than that in the MCF-7 breast cancer cells as previously reported (26). The knockdown of RPS3 did not reduce the activity of NF-κB in the MCF-7 and
Figure 3. Knockdown of RPS3 downregulates the XIAP protein independently of the NF-κB pathway in MCF-7 breast cancer cells. (A-D) The MCF-7 cells were transfected with siRPS3 #1, siRPS3 #2, siRPS3 #3, or a negative control siRNA (siCtrl). After 120 h, cell growth (A) and apoptosis (B) were evaluated. After 96 h, the protein (C) and mRNA (D) levels of XIAP were analyzed. Columns, means of triplicate data; bars, SD. *P<0.05. XIAP, X-linked inhibitor of apoptosis; RPS3, ribosomal protein S3; sub-G1, hypodiploid population.

Figure 4. Knockdown of RPS3 also downregulates the XIAP protein independently of NF-κB in the MDA-MB-231 breast cancer cells. (A-D) The MDA-MB-231 cells were transfected with siRPS3 or control siRNA (siCtrl). After 120 h, the cell growth (A) and apoptosis (B) were assessed. After 96 h, the protein (C) and mRNA (D) levels of XIAP were assessed. Columns, means of triplicate data; bars, SD. *P<0.05. XIAP, X-linked inhibitor of apoptosis; RPS3, ribosomal protein S3; sub-G1, hypodiploid population.

Figure 5. Knockdown of RPS3 does not suppress the NF-κB activity in the MCF-7 and MDA-MB-231 breast cancer cells. (A) The NF-κB activity in the MCF-7 and MDA-MB-231 cells was quantified. (B and C) The MCF-7 and MDA-MB-231 cells were transfected with siRPS3 or control siRNA (siCtrl). After 96 h, the activity of the NF-κB in MCF-7 (B) and MDA-MB-231 cells (C) was quantified. Columns, means of triplicate data; bars, SD. *P<0.05. NF-κB, nuclear factor-κB.
MDA-MB-231 cells (Fig. 5B and C), whereas the RPS3 knockdown downregulated the XIAP protein (Figs. 3C and 4C). These findings further reveal that RPS3 regulates the expression of XIAP independently of NF-κB.

**Discussion**

In the present study in order to elucidate the mechanisms underlying the overexpression of XIAP in breast cancer (16,17), we focused on the natural compound curcumin, which downregulated the expression of XIAP in breast cancer cells as shown in Fig. 1C. We identified, for the first time, RPS3 as a curcumin-binding protein and found that the knockdown of RPS3 downregulated the expression of XIAP with the induction of apoptosis in breast cancer cells, suggesting that RPS3 can increase the expression of XIAP. Accordingly to previous findings obtained using Jurkat leukemia cells, RPS3 directly binds to the p65 subunit of NF-κB complexes and plays a role in gene transcription as part of these complexes (21). In contrast to these findings, our study revealed that RPS3 did not regulate the expression of XIAP at the transcriptional level, suggesting that RPS3 upregulates XIAP independently of the NF-κB pathway. Since this discrepancy may be attributed to the low activity of NF-κB in the MCF-7 breast cancer cells, we analyzed the expression of XIAP regulated by RPS3 using the MDA-MB-231 breast cancer cells in which the NF-κB pathway is more activated as reported previously (26). However, the results obtained using the MCF-7 and MDA-MB-231 breast cancer cells were similar. Collectively, our results indicate that RPS3 regulates the expression of XIAP independently of the NF-κB pathway in breast cancer cells.

As shown in Figs. 3 and 4, the knockdown of RPS3 downregulated XIAP at the protein level, but not at the mRNA level. The expression of XIAP is known to be regulated at the protein level in various manners. For example, many microRNAs suppress the translation of XIAP (32-36) and the E3 ligase TRIM32 degrades the XIAP protein (37). In addition, the TRIP-Bril and TRIP-Br3 proteins inhibit the degradation of XIAP (38) and a novel mechanism for XIAP degradation through an autophagy pathway has been identified (39). Therefore, multifaceted analyses that consider these molecules are needed in order to clarify the regulation of the expression of XIAP by RPS3.

It is considered important to search for biomarkers that may be used to predict sensitivity to chemotherapy. Previous studies reported that the anti-apoptotic protein XIAP is overexpressed in breast cancer (16,17) and furthermore, that the upregulation of XIAP is associated with resistance to anthracycline or taxane, which are often used in adjuvant therapies for breast cancer (12). Since our results revealed that RPS3 upregulates the expression of XIAP, these findings indicate that it is possible to predict sensitivity to adjuvant chemotherapies based on the RPS3 levels.

At present, we have analyzed the molecular mechanisms by which various food factors and natural products exert antitumor activities (40-44). In the course of our studies, we identified several binding proteins of natural compounds using magnetic FG beads and discovered novel functions through which these binding proteins regulate tumor properties by referring to the antitumor activities of these compounds (25,45-47). In the present study, by using curcumin as a chemical probe, we identified RPS3 as a curcumin-binding protein and found that RPS3 regulates the expression of XIAP independently of the NF-κB pathway in breast cancer cells. As demonstrated it is useful for clarifying novel molecular mechanisms in cells to apply natural compounds exhibiting bioactivities as chemical probes.

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**References**

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