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RASSF1A suppresses the activated K-Ras-induced oxidative DNA damage

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ABSTRACT

The mutant K-Ras elevates intracellular reactive oxygen species (ROS) levels and leads to oxidative DNA damage, resulting in malignant cell transformation. Ras association domain family 1 isoform A (RASSF1A) is known to play a role as a Ras effector. However, the suppressive effect of RASSF1A on K-RasV12-induced ROS increase and DNA damage has not been identified. Here, we show that RASSF1A blocks K-RasV12-triggered ROS production. RASSF1A expression also inhibits oxidative DNA damage and chromosomal damage. From the results obtained in this study, we suggest that RASSF1A regulates the cellular ROS levels enhanced by the Ras signaling pathway, and that it may function as a tumor suppressor by suppressing DNA damage caused by activated Ras.

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1. Introduction

The Ras/Raf pathway plays a central role in cell proliferation, and many genes including cyclin D1 are up-regulated for entry to the S-phase [1,2]. The mutational activation of Ras conveys uncontrolled proliferative signals into the nucleus and contributes to carcinogenesis. An increase in ROS levels induced by oncogenic Ras also contributes to malignant transformation. There have been many reports demonstrating that constitutively active H-Ras increases the ROS levels in many cells [3–6]. K-Ras is also commonly mutated and activated in many cancers. Although wild-type K-Ras plays a role as a tumor suppressor, mutant K-Ras enhances ROS levels and induces DNA damage, triggering cellular transformation [7–9].

The tumor suppressor gene, RASSF1A, is commonly inactivated by loss of heterozygosity (LOH) mutation or by hypermethylation of its promoter region during carcinogenesis [10–12]. The role of RASSF1A as a tumor suppressor has begun to be understood in recent years. RASSF1A expression suppresses colony formation and tumorigenicity, and tumors develop in RASSF1A-knockout mice [10,11,13]. RASSF1A modulates the cell cycle by controlling G1/S transition through cyclin D1 down-regulation [14,15]. RASSF1A also negatively controls the downstream pathway of Ras signaling. Knockdown of RASSF1A by shRNA suppresses Ras-induced Bax, and RASSF1A expression prevents oncogenic H-Ras-induced c-Jun N-terminal kinase (JNK) activation [16,17]. It seems that the action of RASSF1A on Ras occurs via Ras-RASSF1A interaction. RASSF1A has a Ras association (RA) domain and interacts with the mutant K-Ras [18]. Other RASSF proteins have been also reported to associate with the Ras proteins [18,19]. However, the exact mechanism of RASSF1A function as a Ras effector is not well elucidated. Therefore, we investigated the involvement of RASSF1A in the control of ROS production and DNA damage triggered by activated K-Ras in the present study.

2. Materials and methods

2.1. Cell culture and reagents

NCI-H1299 human lung carcinoma cells were cultured in RPMI1640 medium (Gibco-Invitrogen, Grand Island, NY). WI-38 VA13 (immortalized human lung fibroblasts) were cultured in Eagle's minimal essential media (EMEM, Gibco-Invitrogen). NIH3T3 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium, Gibco-Invitrogen). All media contained 10% heat-inactivated fetal bovine serum (FBS), sodium bicarbonate (2 mg/ml; Sigma–Aldrich, St. Louis, MO), penicillin (100 units/ml), and streptomycin (100 μg/ml; Gibco-Invitrogen). A mifepristone (MFP)-inducible K-Ras expression system was generated by subcloning the K-RasV12 ORF into a pGene/V5-His A vector, as previously described [20]. Dihdroxyethidium (DHE), 2′,7′-dichlorofluorescein diacetate (DCF-DA), MFP, and N-acetyl-L-cysteine (NAC) were purchased from Sigma–Aldrich.

2.2. Western blotting

The Western blotting procedures with anti-p53 (Santa Cruz, CA), anti-K-Ras (Santa Cruz), anti-γ-H2AX (Millipore, Billerica,

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MA), anti-β-actin (Santa Cruz), anti-flag (Sigma–Aldrich), anti-p-p53 (S15; Cell Signaling Technology, Danvers, MA), and H-Ras (Santa Cruz) antibodies were described previously [21].

2.3. ROS assay

ROS levels were examined by fluorescence microscopy (Olympus LX71 microscope, Tokyo, Japan) or FACScan flow cytometry

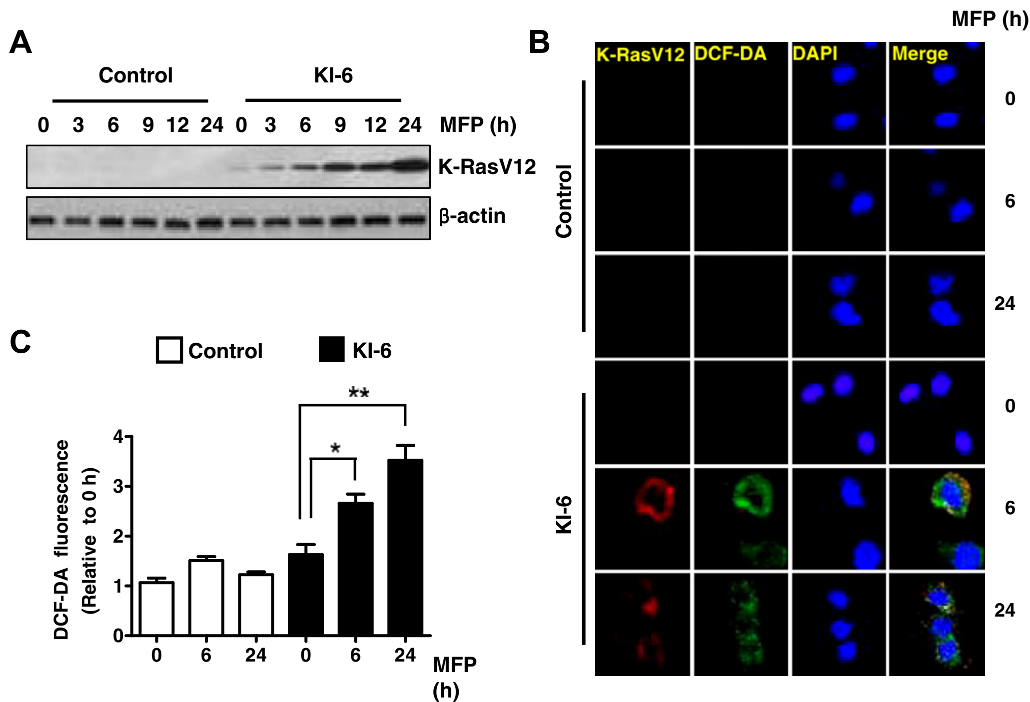

Fig. 1. K-RasV12-induced ROS production in stable transfectant cells expressing K-RasV12. (A) After NIH3T3 cells stably expressing K-RasV12 were treated with MFP, K-RasV12 expression was examined by Western blotting with anti-K-Ras antibody at the indicated times. β-Actin was used as internal loading control. (B) After the cells were treated with MFP, increased ROS levels were observed by fluorescence microscopy. (C) For quantification purposes, the images were overlaid, and DCF-DA fluorescence (green) was analyzed with MetaMorph software. Results represent the means (± S.E.) of three independent experiments performed in triplicate. *, $p < 0.05$; ***, $p < 0.005$ *versus* control by two-way ANOVA.

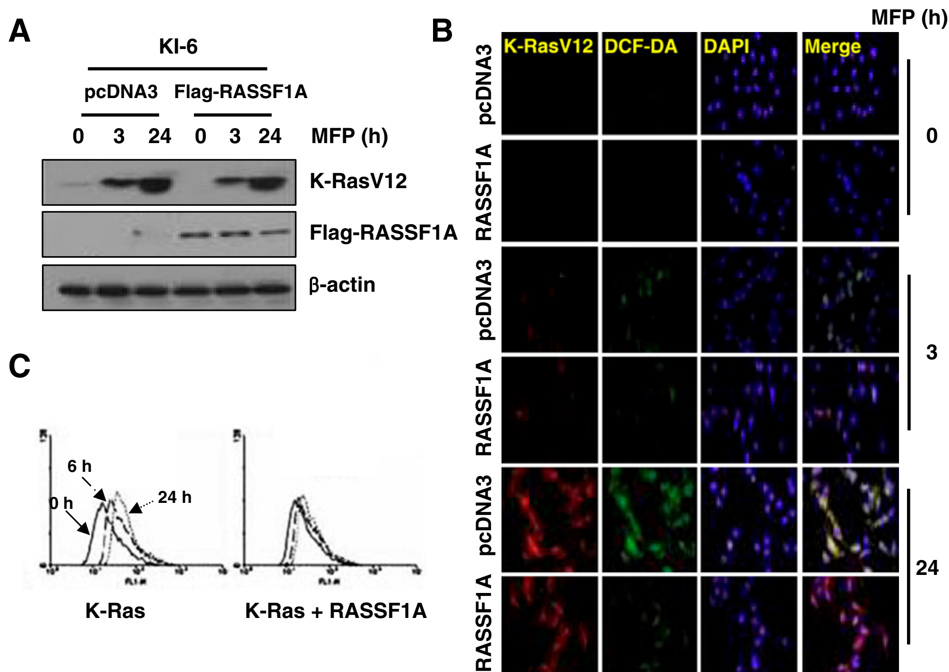

Fig. 2. RASSF1A inhibits K-RasV12-induced ROS production in NIH3T3 cells. (A) After KI-6 cells were transfected with RASSF1A, the cells were treated with MFP; then K-Ras and RASSF1A expressions were examined by Western blotting with anti-K-Ras and anti-Flag antibodies. β-Actin was used as an internal loading control. (B) After KI-6 cells were transfected with RASSF1A, the cells were treated with MFP and then ROS levels were observed by fluorescence microscopy or (C) analyzed by flow cytometry.

(Becton Dickson, San Jose, CA, USA) using DCF-DA (50 μM), as previously described [22]. To measure intracellular ROS production using a fluorescence microscope, the images were analyzed with Metamorph software (Universal Imaging, Westchester, PA). Fluorescent images from multiple fields of view were captured, and ROS fluorescence intensity was normalized based on cell number, which was determined by trypan blue staining.

2.4. Micronuclei formation assay

Micronucleus formation assay was performed using DAPI staining as previously described and cells were observed under a fluorescence microscope [23].

2.5. Statistical analysis

Each assay was repeated in triplicate and performed independently at least three times. Statistical significance was defined as $p < 0.05$. Means, S.E.s and *P*-values were calculated using GraphPad PRISM version 4.02 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1. The construction of a stable transfectant cell line expressing K-Ras using a MFP-inducible expression system

Several reports showed that mutant K-Ras increased cellular ROS levels [7–9]. To observe the same effect, we generated a stable

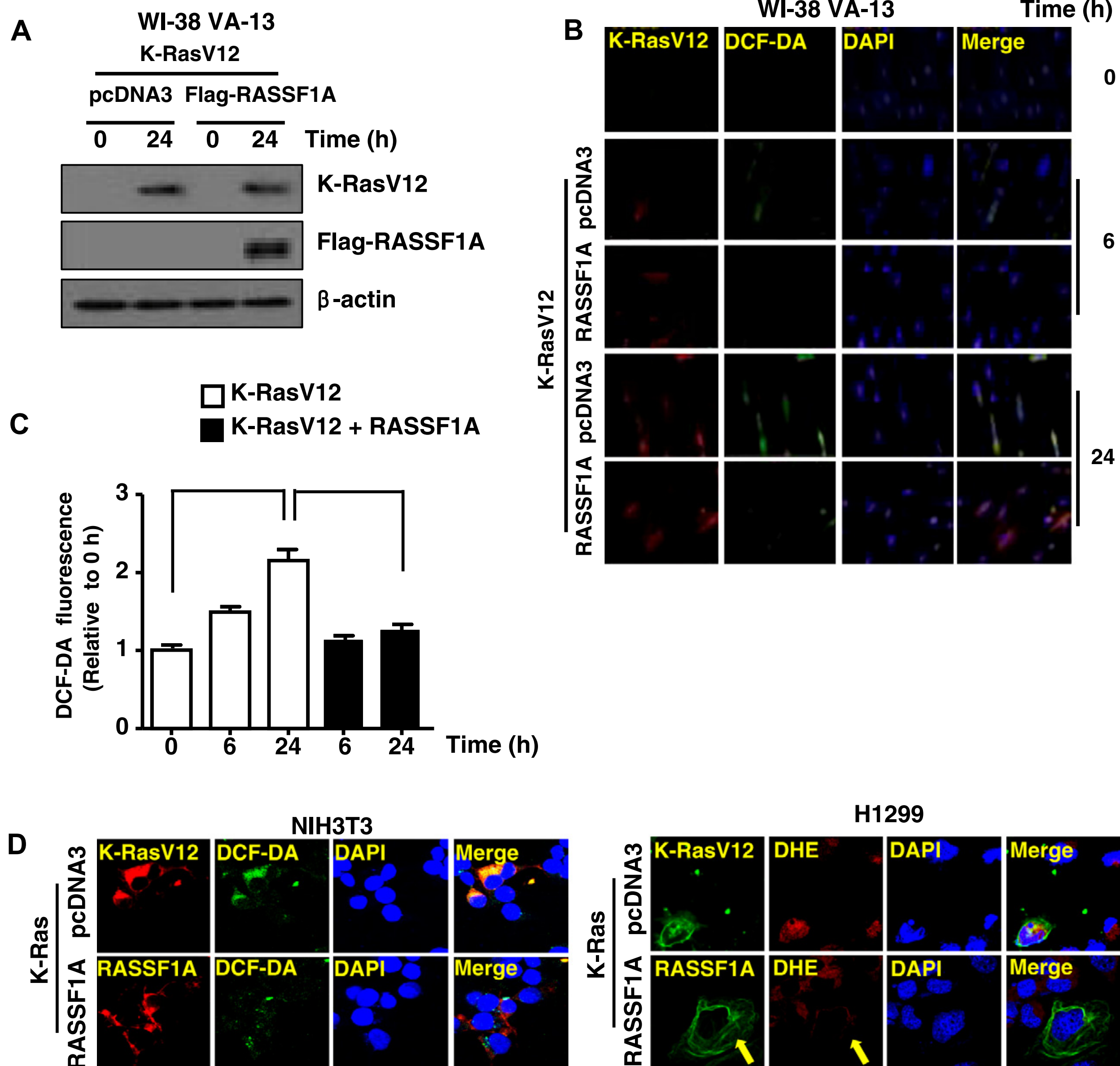

Fig. 3. RASSF1A prevents K-RasV12-induced ROS generation in various cell lines. (A) After WI-38 VA13 cells were transfected with K-RasV12 and Flag-RASSF1A, the expressions of K-RasV12 and RASSF1A were analyzed by Western blotting. β-actin was used as an internal loading control. (B) After WI-38 VA13 cells were transfected with K-Ras and Flag-RASSF1A, ROS change was analyzed by fluorescence microscopy. (C) For quantification purposes, the images were overlaid, and DCF-DA fluorescence (green) was analyzed with MetaMorph software. Results represent the means (± S.E.) of three independent experiments performed in triplicate. **, $p < 0.01$; ***, $p < 0.005$ *versus* control by two-way ANOVA. (D) After NIH3T3 and H1299 cells were transfected with K-RasV12 and Flag-RASSF1A, the cells were stained with DCF-DA or dihydroethidium (DHE) and the ROS levels were observed by fluorescence microscopy. The arrows in the right panel indicate the cells expressing RASSF1A (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

transfectant cell line expressing K-RasV12 (NIH3T3/pGene-K-Ras, KI-6) using a MFP-inducible expression system, in which K-RasV12 was induced by treating the cells with MFP. MFP-induced K-RasV12 expression was assessed by Western blotting with anti-K-Ras antibody. Among many NIH3T3 cell lines stably expressing K-RasV12, one cell line was chosen and named KI-6. After KI-6 was treated with MFP for various time periods, K-RasV12 expression levels were examined. As shown in Fig. 1A, K-RasV12 expression was increased according to the increase in MFP-treatment times. Since it was reported that mutant K-Ras increased intracellular ROS levels [7–9], ROS generation was measured after MFP treatment in KI-6 cells. K-RasV12 expression and ROS levels were simultaneously increased (Fig. 1B); the quantified results are shown Fig. 1C.

3.2. K-RasV12-induced ROS increase was attenuated by RASSF1A expression

To investigate the effect of RASSF1A on K-RasV12-induced ROS production, Flag-tagged RASSF1A was transfected into KI-6 cells prior to MFP treatment and then the expressions of K-RasV12 and RASSF1A were examined by Western blotting (Fig. 2A). Next, we measured ROS levels in KI-6 cells treated with MFP by fluorescence microscopy. As expected, K-RasV12 expression and increased ROS levels were observed in these cells (Fig. 2B). Interestingly, RASSF1A transfection inhibited K-RasV12-induced ROS increase. The same result was examined by FACS analysis (Fig. 2C). The suppressive effect of RASSF1A on K-RasV12-induced ROS increase was also examined in a transient expression experiment. K-RasV12 and Flag-tagged RASSF1A were co-transfected into WI-38 VA13 cells, and K-RasV12 and RASSF1A expressions were examined by Western blotting (Fig. 3A). Next, we measured ROS levels in the cells transfected with K-RasV12 and RASSF1A by fluorescence microscopy. As shown in Fig. 3B, K-RasV12 expression increased ROS levels and RASSF1A expression suppressed the K-RasV12-induced ROS increase. The changes of ROS levels by co-transfection were also quantified and are shown in Fig. 3C. These effects were further investigated in NIH3T3 and H1299 cells transiently transfected with K-RasV12 and RASSF1A and ROS levels were observed by fluorescence microscopy. As shown in Fig. 3D (left panel), RASSF1A expression decreased the K-RasV12-induced ROS levels. It has been reported that mutant K-Ras causes superoxide production [9]. Therefore, we examined whether RASSF1A expression inhibited K-RasV12-induced superoxide generation by fluorescence microscopy. As shown in Fig. 3D (right panel), RASSF1A expression blocked K-RasV12-induced superoxide generation.

3.3. RASSF1A suppressed K-RasV12-mediated oxidative DNA damage

Since phosphorylation of p53 (ser 15) and phosphorylation of H2AX were triggered by DNA damage [24,25], the levels of phospho-p53 (ser15) and the formation of γ-H2AX were assessed in NIH3T3 cells transfected with K-RasV12. As shown in Fig. 4A, K-RasV12 expression increased the phosphorylation of p53 and the formation of γ-H2AX. NAC treatment blocked the phosphorylation of p53 and formation of γ-H2AX, demonstrating that K-RasV12 expression induced oxidative DNA damage. RASSF1A expression also suppressed K-Ras-induced oxidative DNA damage. Next, the micronucleus formation assay was conducted to analyze the chromosomal damage by K-RasV12-induced ROS increase. As shown in Fig. 4B, RASSF1A expression significantly reduced micronuclei formation and the same result was observed in cells treated with NAC. These results suggest that RASSF1A suppressed K-Ras-induced oxidative DNA damage and the resulting genomic instability.

4. Discussion

Ras family members including H-Ras, K-Ras and N-Ras are often mutated during tumor development and they have distinct effects on malignant transformation [26]. Although it is well known that oncogenic Ras affects tumor cell proliferation, the mutated activation of Ras has also been reported to increase intracellular ROS levels, oxidative DNA damage and genomic instability, contributing to malignant transformation [3–9]. Due to the high levels of ROS in many types of cancer cells and the growing evidence for ROS as essential effectors in tumor progression, ROS may be one of many potential targets for anti-tumor therapy [27]. Indeed, antioxidant treatment attenuates activated Ras-induced cell transformation, demonstrating that ROS are also essential regulators in activated Ras-induced cell transformation [28,29].

Many studies have reported that RASSF1A is a potential tumor suppressor protein that regulates cell cycle arrest, apoptosis, microtubule stabilization, and migration [18]. RASSF1A attenuates tumor cell growth both *in vitro* and *in vivo* [12]. In the previous

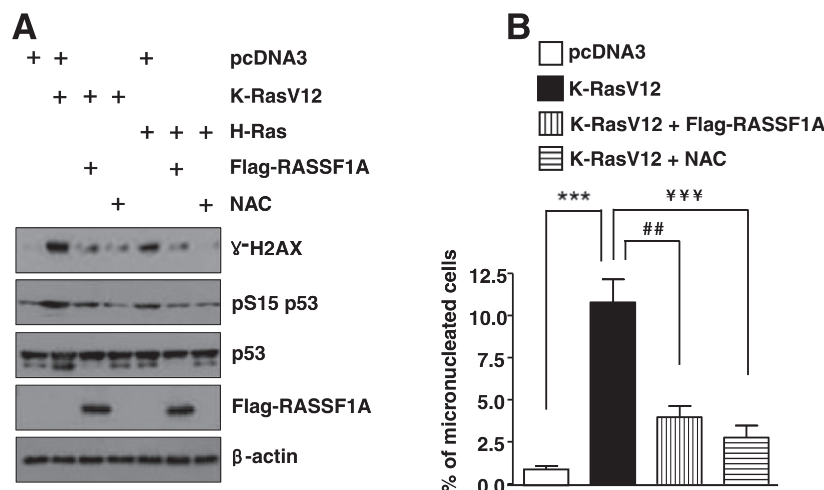

Fig. 4. RASSF1A expression reduces K-RasV12-induced oxidative DNA damage. (A) After NIH3T3 cells were transfected with K-RasV12, H-Ras and Flag-RASSF1A, Western blotting was performed with the indicated antibodies. The cells were treated with NAC (1 mM) as an antioxidant. β-Actin was used as an internal loading control. (B) After NIH3T3 cells were transfected with K-RasV12 and Flag-RASSF1A, the micronuclei formation assay was performed. Results represent the means (± S.E.) of three independent experiments performed in triplicate. ##, $p < 0.01$; ***, $p < 0.001$; ¥¥¥, $p < 0.001$ by two-way ANOVA.

report, we showed that RASSF1A inhibited the H-Ras-induced JNK activation [16]. However, the precise mechanism of RASSF1A function in cell transformation triggered by oncogenic Ras is still under investigation. In the present study, we demonstrate a suppressive effect of RASSF1A on ROS production triggered by activated K-Ras. RASSF1A also attenuated the K-Ras-triggered oxidative DNA damage and chromosomal damage. We also showed that RASSF1A reduced the H-Ras-triggered oxidative DNA damage (Fig. 4). ROS have been known to contribute to the initiation, promotion and progression of tumor formation [30]. Therefore, we suggest that RASSF1A plays a tumor suppressive role in malignant cell transformation by regulating ROS generation through Ras activity. Although the exact mechanism of the suppressive role of RASSF1A on K-Ras-induced oxidative DNA damage is still uncertain and more studies on RASSF1A action are required, the results obtained in this study provide important information about the role of RASSF1A in oncogenic K-Ras-induced oxidative DNA damage and the possibility of antioxidants as therapeutic agents preventing oncogenic K-Ras-mediated oxidative DNA damage. From the results described in this study, we suggest that RASSF1A has a tumor suppressive effect as a modulator for ROS production induced by oncogenic K-Ras.

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