

RESEARCH ARTICLE

RASSF1A Suppresses Proliferation of Cervical Cancer Cells

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Abstract

Background: This study aimed to explore the effects of ras association domain family 1 A (RASSF1A) on proliferation and apoptosis of human cervical cancer cell line HeLa cells. **Materials and Methods:** RASSF1A was cloned into the pcDNA3.1(+) vector to generate pcDNA3.1(+)-RASSF1A plasmid for transfection into HeLa cells. Changes in the proliferation and apoptosis of cultured HeLa cells were examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium chloride assay and flow cytometry. A protein array was used to analyze the expression of apoptotic factors. **Results:** Plasmid pcDNA3.1(+)-RASSF1A was generated and transfected into HeLa cells to stably express RASSF1A in HeLa cells. RASSF1A transfection was effective in inhibiting the proliferation of HeLa cells up to 52.4%, as compared to cells transfected with an empty plasmid. RASSF1A expression also successfully induced apoptosis in human cervical cells with an apoptosis rate of 20.5%. More importantly, protein array results showed that RASSF1A transfection induced overexpression of p21 and caspase 8, while decreasing the expression of survivin in HeLa cells. **Conclusions:** RASSF1A expression was effective in suppressing the proliferation and increasing apoptosis of HeLa cells, and may be a potential therapy for cervical cancer in clinic.

Keywords: RASSF1A - cervical cancer - HeLa cell - proliferation - apoptosis

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Introduction

Cervical cancer is one of the most severe malignant tumors since it is the second largest mortality rate that affect women's life worldwide (Parkin et al., 2005). There are multiple pathogenesis for cervical cancer, although the primary factor in cervical cancer is infection with human papillomavirus (HPV) (Walboomers et al., 1999). Previously, treatment for cervical cancer mainly focused on radiotherapy together with surgical excision and chemotherapy, however the recurrence rate is still high in many cases of cervical cancer (Jadon et al., 2014). Recent work mainly focused on different expression of oncogenes and tumor suppressor genes (Dammann et al., 2000) in cervical cancer, which are important for the regulation of cell cycle. Therefore, we studied Ras association protein to investigate for a cure of cervical cancer.

Ras protein belongs to a class of protein called small GTPase, which is involved in various cell signaling process (Goodsell, 1999). Activation of Ras signaling can result in gene expression that controls the growth, differentiation and survival of cells (Downward, 2003), thus lead to cell proliferation and cancer (Malumbres and Barbacid, 2003). Ras is one of the most common oncogenes (Bos, 1989), which was found in up to 90% cases in several types of cancer as a constitutively active form, including pancreatic

cancer (Blum et al., 2005). Therefore, Ras associated proteins were studied to investigate their relationship to various kinds of tumors.

Ras association domain family 1 A (RASSF1A) was found in the year 2000 by Dammann et al. as an important tumor-suppressor gene (Dammann et al., 2000), which located at chromosome 3p21. They also identified that there are three RASSF1 transcripts including RASSF1 A, B and C, all of which share common exons encoding a Ras-association domain (Sherwood et al., 2010). RASSF1A is a C-terminal RASSF proteins that represent potential Ras effectors and play important biological roles in cancer progress (Richter et al., 2009). Previous reports found that gene polymorphism in RASSF1A was associated with an early onset of breast tumor (Gao et al., 2008). In addition, RASSF1A could serve as an important diagnostic marker in cancer screening because that methylation of RASSF1A promoter was represented as an early and frequent event in tumorigenesis (Liu et al., 2013). However the role RASSF1A overexpression and mechanism in human cervical cancer were seldom reported.

In the current study, we used HeLa cells, which is the human cervical cancer cell line, to investigate the anti-tumor effect of RASSF1A expression. We found that RASSF1A transfection was effective in suppressing the proliferation and increasing cell apoptosis. Moreover,

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protein array showed that RASSF1A transfection can induce overexpression of p21 and caspase 8, while decreased the expression of survivin in HeLa cells, which could be a potential mechanism in this process.

Materials and Methods

Construction of pcDNA3.1(+)-RASSF1A plasmid

The plasmid pcDNA3.1(+)-RASSF1A was generated according to the cDNA sequence from GenBank using pET28(a)-RASSF1A as the template. RASSF1 A gene was generated by PCR amplification using primers listed below. The plasmid pcDNA3.1(+) was extracted through Max Preparation kit (Omega, USA). The PCR product was subcloned into the BamHI (Takara, USA) and Hind III (Takara, USA) sites of pcDNA3.1 plasmid by T4 ligase (Takara, USA). The pcDNA3.1(+)-RASSF1A construct was verified by DNA sequencing (Invitrogen). The primers used for RASSF1A were, forward, 5'-CGCGGATCCGCGATGTCGGGGGAGCC -3' (with protection bases for BamH I in the N-terminal), reverse, 5'-TGCTCTAGAGCATTATCACCCAAGGG -3' (with protection bases for XbaI in the N-terminal).

Plasmid transfection

HeLa cells were cultured in 12-well plates with 0.8x10⁵ cells in each well, in an incubator with constant supply of 5% CO₂ at 37°C. The medium was changed 24 h later with different concentrations of antibiotic G418 (0, 50, 100, 200, 400, 600, 800 and 1000 µg/ml) and replaced each three days. Medium with 800 µg/ml G418 was used for further experiments as it is the minimum concentration to induce total cell death 14 days after cell culture.

HeLa cells were transfected with the pcDNA3.1(+)-RASSF1A plasmids using lipofectamine 2000 according to the manufacturer's instructions. The density of cells was 2x10⁵ cells per well in 6-well plates. Monoclonal cell colony with G418 resistance was generated using limiting dilution method by culturing single cell in 100 µl medium in 96-wells plates for 24 h. Monoclonal HeLa cell colonies were digested 15 days later for further amplification to culture HeLa cells with stable RASSF1A expression in 24-well plates. Cells were transferred to cell culture flask until there was approximately 90% confluent.

Western blot

The expression of RASSF1A was examined by western blot according to standard protocol. Antibody used for RASSF1A was mouse anti human RASSF1A monoclonal antibody (Neo Marker Co.) with HRP labeled goat anti mouse IgG as the second antibody. Intensities of immune bands were measured using image analysis software.

Table 1. Suppression of Cell Proliferation in HeLa Cells after RASSF1A Transfection

Group	1d	2d	3d	4d	5d
HeLa-pcDNA3.1(+)-RASSF1A	19.64%	24.42%	45.95%	51.28%	52.38%
HeLa-pcDNA3.1(+)	7.14%	9.30%	13.51%	15.38%	16.67%

*(Data was shown as x ± s, n = 3)

Cell proliferation assay

The effect of RASSF1A on cell proliferation in the HeLa cells was tested using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium chloride (MTT) method (Abiramasundari et al., 2012). Briefly, the HeLa cells were added onto 96-well plates at the density of 1x10⁴ cells per well. At the time of 1 d, 2 d, 3 d, 4 d, 5 d after cell culture, 20 µl MTT (5 mg/mL) was administrated into each well. After 4 h incubation, culture medium was discarded, and then 150 µl DMSO was added into each well to dissolve the precipitate. Optical densities of each well were measured at 570 nm spectral wavelength to measure the suppression rate of cell proliferation. The suppression rate was calculated as $(1 - A_{570}^{RASSF1A} / A_{570}^{Control}) \times 100\%$.

Apoptosis assay

Apoptosis rate of HeLa cells transfected with RASSF1A was measured using Elite Esp flow cytometry according to the manufacturer's instruction. HeLa cells were randomly grouped and transfected with pcDNA3.1(+)-RASSF1A plasmids or empty plasmid. Cells were digested and then cultured in dark with propidium iodide for 15 min for examination of apoptosis rate.

Screening for apoptosis-related proteins

HeLa cells transfected with RASSF1A were digested for extraction of protein components. The expression of apoptosis-related proteins was analyzed by protein chip array kit (Raybiotech, USA) according to the manufacturer's instruction. Briefly, a mix of substrates and antibodies was incubated for 2 h, and then treated with Cy3 fluorescent dye. Dot blots were photographed by a Genepix 4000B scanner (Molecular Devices Corporation). Each experiment was repeated for three times.

Statistical analysis

Results were analyzed using x² test in the SPSS12.0 software (China). Data were shown as mean±standard deviation. p<0.05 was considered as significant difference.

Results

Stable expression of pcDNA3.1(+)-RASSF1A in HeLa cells

The plasmid pcDNA3.1(+)-RASSF1A was identified

Table 2. Map of Apoptosis Factors Array

1	Pos1	Pos2	Pos3	Neg	Neg	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	Caspase8
2	Pos1	Pos2	Pos3	Neg	Neg	bad	bax	bcl-2	bcl-w	BID	BIM	Caspaes3	Caspase8
3	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	neg	HSP27	HSP60	HSP70	HTRA	IGF-1
4	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	neg	HSP27	HSP60	HSP70	HTRA	IGF-1
5	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC
6	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC
7	Survivin	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Neg	Neg	Neg
8	Survivin	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Neg	Neg	Neg

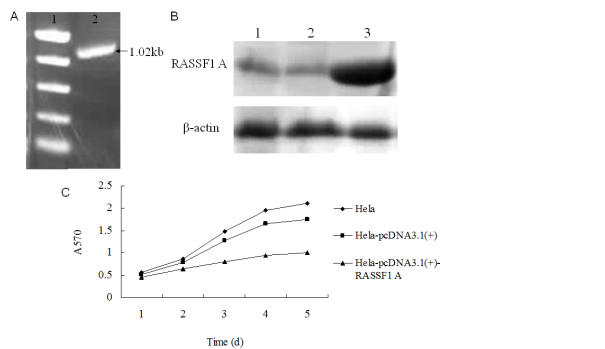


Figure 1. RASSF1A Expression Was Effective in Suppressing Cell Proliferation. (A), DNA gel for RASSF1A expression (1, DNA marker; 2, RASSF1A). (B), Identification of RASSF1A protein expression by Western-blot analysis (1, Control; 2, pcDNA3.1(+); 3, pcDNA3.1(+)-RASSF1A). (C), Growth curves of HeLa cells in different conditions, showing suppression of cell proliferation after RASSF1A transfection.

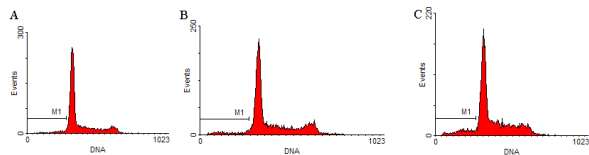


Figure 2. Histogram of DNA content in Cells in Different Conditions. (A), Histogram showing apoptosis of standard HeLa cells. (B), Histogram showing apoptosis of HeLa cells transfected with pcDNA3.1(+). (C), Histogram showing apoptosis of HeLa cells transfected with pcDNA3.1(+)-RASSF1A. (Red below line M1 indicated the DNA content of apoptotic cells)

with PCR and double digestion of Hind III and BamHI. The sequence of 1020 bp DNA of RASSF1A gene was confirmed as compared to GenBank (Figure 1A). The DNA band at 5.43 kb belonged to pcDNA3.1(+) (data not shown). Thus, the recombinant plasmid RASSF1A was successfully cloned.

Then the plasmid pcDNA3.1(+)-RASSF1A was transfected into HeLa cells to stably express RASSF1A. The expression of RASSF1A protein was confirmed by western blot. As compared to non-transfected cells or cells transfected with the control vector, RASSF1A was highly expressed in pcDNA3.1(+)-RASSF1A transfected cells (Figure 1B).

Effects of RASSF1A transfection on cell proliferation

We examined the cell proliferation of HeLa cells after RASSF1A transfection using MTT assay. The result showed that RASSF1A expression can significantly suppress the proliferation of HeLa cells ($p < 0.05$) (Table 1, Figure 1C). The suppression rate of cells transfected with pcDNA3.1(+)-RASSF1A at the fifth day was 52.38%, while that of cells transfected with HeLa-pcDNA3.1(+) was 16.67%. In summary, RASSF1A expression was effective in the inhibition of HeLa cell proliferation in a time-dependent manner ($p < 0.05$) (Table 1, Figure 1C).

RASSF1A expression increased apoptosis rate in HeLa cells.

By examining cell apoptosis after culturing for several

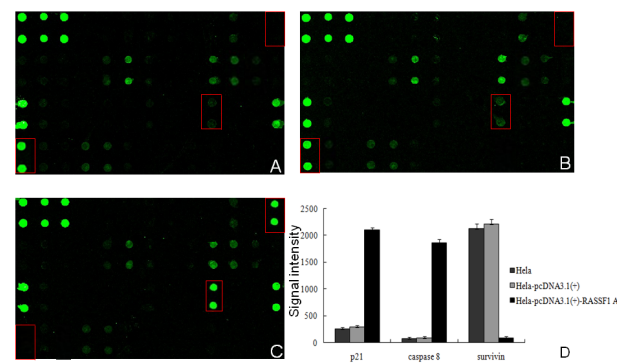


Figure 3. Protein Array Showing the Expression of Apoptosis Factors. (A), Protein array showing the expression of apoptosis factors in standard HeLa cells. (B), Protein array showing the expression of apoptosis factors in HeLa cells transfected with pcDNA3.1(+).

days using flow cytometry, we found that the apoptosis rate of HeLa cells was increased after RASSF1A transfection. The apoptosis rate of cells transfected with pcDNA3.1(+)-RASSF1A was 20.5%, while that of non-transfected cells and cells transfected with HeLa-pcDNA3.1(+) were 5.3% and 8.5% (Figure 2).

Moreover, using protein array to study apoptosis proteins, we found that results showed that RASSF1A transfection can significantly increase the expression of p21 and caspase 8 in three repeated experiments, while RASSF1A transfection decreased the expression of survivin (Table 2, Figure 3). These results indicate RASSF1A probably induce apoptosis through pathways involving p21, caspase 8 and survivin signaling.

Discussion

Cervical cancer is a malignant tumor that severely affect the health and life of women all over the world due to its high morbidity rate (JadonPembroke et al., 2014). Not much effective treatment for cervical cancer was established since it is a metastatic cancer. HeLa cell line is a well established cell line for scientific research, which is derived from cervical cancer cells (Rahbari et al., 2009). Thus, here in this paper, we studied the effect of RASSF1A expression in HeLa cells to investigate the role of RASSF1A in treatment of cervical cancer cells.

Previous reports showed that RASSF1A protein, which represented potential Ras effectors, played important biological roles in regulating gene transcription, cytoskeleton, cell signaling, cell cycle, cell proliferation and apoptosis (Agathangelou et al., 2003; Ahmed-Choudhury et al., 2005; Dallol et al., 2005). Overexpression of RASSF1A was also found to inhibit cell proliferation, induce apoptosis and decrease oncogenicity in tumor cell line (Agathangelou et al., 2005). Since RASSF1A is capable of interacting with microtubule during cell cycle to increase the stability of microtubules and therefore regulate mitosis, RASSF1A has been demonstrated to suppress the proliferation of tumor cells (Dallol et al., 2004; van der Weyden et al., 2005). However, the role RASSF1A in human cervical cancer, as well as its underlying mechanism, were unclear. In the present study,

we generated stable HeLa cell line which can express pcDNA3.1(+)-RASSF1A. We verified the expression of RASSF1A by PCR and western blot. By examining the cell proliferation in the MTT assay, we found that RASSF1A transfection was effective in decreasing the over-growth of cancer cells as compared to non-transfected HeLa cells, as well as cells transfected with control empty vector, indicating RASSF1A expression is capable of suppressing cervical cancer.

It has been reported that RASSF1A was an important component in death receptor induced Bax conformation and apoptotic process (Baksh et al., 2005). To further study the effect of RASSF1A on apoptosis of HeLa cells, we performed flow cytometry to address this question. We found that the apoptosis rate of HeLa cells was increased after RASSF1A transfection, suggesting RASSF1A expression can induce apoptosis in tumor cells.

Previous work showed that RASSF1A was involved in apoptosis through various apoptotic pathways, including activated Modulator of Apoptosis-1 (MOAP-1) and BCL2-associated X protein (Bax) (Vos et al., 2006), as well as through the pro-apoptotic kinase mammalian sterile 20-like kinase 1 (MST1) (Matallanas et al., 2007). Using protein array to study the expression of apoptosis factors in cells transfected with RASSF1A, we found that apoptosis factor caspase 8 and p21, a cyclin-dependent kinase inhibitor, were up-regulated after RASSF1A transfection. We also found that RASSF1A overexpression cells exhibited decreased expression of survivin, a member of the inhibitor of apoptosis family. Importantly, urinary survivin could be used as a marker for diagnosis of urinary bladder cancer (Srivastava et al., 2013).

Taken together, these results indicated that RASSF1A could regulate cell cycle and apoptosis to suppress tumor through other signaling pathways, including caspase 8, p21 and survivin. Our work identified a novel pathway for the treatment of cervical cancer in clinic through RASSF1A, as well as broadened the role of RASSF1A as a tumor suppressor gene.

In conclusion, RASSF1A transfection was effective in inhibiting the proliferation of HeLa cells. More importantly, RASSF1A expression induced increased apoptosis of HeLa cells, probably through a mechanism involving p21, caspase 8 and survivin signaling pathways.

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