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Expression of *RASSF1A* in epithelial ovarian cancers

L.-J. FU, S.-L. ZHANG

Department of Gynaecology and Obstetrics, Shengjing Hospital of China M[illegible]l University, Shenyang, Liaoning Province, China

Abstract. – OBJECTIVE: Ovarian cancer is the third most common cancer in female reproductive system. But ovarian cancer is hard to detect at early phase. It is very urgent to develop effective early diagnosis method for ovarian cancer. RASSF1A (Ras association domain family 1 isoform A) is a tumor suppressor, which modulates multiple apoptotic and cell cycle checkpoint pathways. We amed to find out the relationship between RASSF1A and ovarian cancer.

METHODS: We compared the expressions of RASSF1A gene in different ovarian cancer cell lines, and also in epithelial ovarian cancer tissues and normal ovarian tissues through RT-PCR (reverse transcription polymerase ch[illegible] action) technique.

RESULTS: RASSF1A was expressed in [illegible]10 and HO8910PM cells, while RASSF1A mRN[illegible]s absent in SKOV-3 and OVCAR-3 cells. RASS[illegible] was expressed in 10 normal ovarian tissue sa[illegible]ples (10/10, 100%), while RASSF[illegible]s only e[illegible] pressed in 2 ovarian cance[illegible] samples (2/47, 4.3%). The difference i[illegible]e fre[illegible]cy was significant in tissue samp[illegible]00% v[illegible]3%, $p < 0.05$).

CONCLUSIONS: RASSI[illegible]serve[illegible] a potential molecu[illegible]mari[illegible]diagnosing ovarian cancer at e[illegible]phase.

Key Words:
Ovarian can[illegible]RASS[illegible]PCR.

[illegible]roduction

Ma[illegible]varian [illegible]or is one of the most [illegible]lignan[illegible]rs in [illegible]ale reproductive system. [illegible]idden [illegible]rapid development as clinical [illegible]ogical b[illegible]ior features, 70% of ovarian can[illegible]ases were diagnosed as terminal types[1]. W[illegible]al rate around 20% within five [illegible]s, ovarian cancer is one of female malignant [illegible]rs with the highest lethality rate. There is no e[illegible]e diagnostic method at early phase for ovari[illegible] cancer so far[2]. Although the developing surgical techn[illegible]es could [illegible]ve[illegible]t cancer cells and th[illegible]nical applica[illegible] platinum, paclitaxel [illegible] secondary [illegible]mical drugs bring th[illegible]pes o[illegible]m to patients[3,4], a considerable part of the p[illegible] are still undergoing furth[illegible]erioration or [illegible]e after surgical and c[illegible]al treatments.

[illegible]ecently, with [illegible]e development of molecular [illegible]logical techn[illegible]es and methodologies, gene [illegible]py is emerg[illegible]as an effective new therapeuti[illegible]hod in [illegible]tic disease caused by single gene[illegible]d gaining worldwide attentions from experts of different fields[5]. In order to pro[illegible]ide much more precise and effective diagnostic, [illegible]ic, and precaution methods, medical [illegible]enti[illegible] are trying to reveal the relationship between expression level of a certain gene and clinical biological behaviors of ovarian cancer.

As a new member of *RAS* (rat sarcoma) gene family, tumor repressor gene *RASSF1A* is regarded to be involved in the regulation of cell proliferation and apoptosis[6]. Some reports showed that *RASSF1A* functions as a micro-tubule binding protein and regulates mitosis proceeding[6-10]. *RASSF1A* is localized on the cytoplasmic microtubule of interphase cells, especially on the spindle body and centrosome during mitosis. The bind of *RASSF1A* to microtubule is supposed to regulate mitosis by stabilizing the microtubule structure. Overexpression of *RASSF1A* could retain the cell at interphase. The mutation of *RASSF1A* could destabilize the microtubule structure, influence the spindle configuration, and impair the attachment of chromosome to spindle, which could easily lead to gene instability and cell transformation under influential factors. This could be further deteriorated into abnormal cell proliferation and tumor formation by the loss of cell cycle brake and reduced apoptosis by *RASSF1A*[6].

Some scientists also found that *RASSF1A* could regulate cell cycle directly, repress cell

Corresponding Author: Shulan Zhang, MD; e-mail: zsl0909@sina.com

growth *in vitro* and *in vivo*, and also induce apoptosis[11,12]. Shivakumai et al[13] found that cells transformed with *RASSF1A* could repress the accumulation of cyclinD1 and further prevent cell proliferation by hindering the cells at G1/S. STE20 (sterile twenty) is a serine/threonine kinase, which was firstly cloned from yeast. STE20 family members are the upstream kinases of MAPK (mitogen-activated protein kinase) cascade. Mst-1 (mammalian sterile 20-like kinase 1) is the homolog of STE20 in mammalian cells. Acting as the apoptosis inducer, Mst-1 induces the cell apoptosis through many pathways[14,15]. Many evidences have demonstrated that Mst-1 and RASSF1A can form a complex called RASSF1A/ Mst-1, which could maintain Mst-1 activity, help Mst-1 to be correctly localized inside cell, and further synergistically induce cell apoptosis[14,16]. After being successively cloned through yeast two hybrid screening in 2000[17], many reports showed that the *RASSF1* mRNA is absent or reduced in many types of cancer cells or tumor tissues, which is also accompanied by the abnormal hyper-methylation at the *RASSF1* promoter region[17-19]. All the results indicat[e] that *RASSF1A* plays an important role in the genesis of many tumor types.

In this study, we compared the expressio[n] of *RASSF1A* gene between ovarian cancer tiss[ue] and normal ovarian tissues, and also in differe[nt] ovarian cancer cell lines thr[ough] PCR (reverse transcription polyme[rase] cha[in] action) technique. In addition, w[e] cussed [the] significant role of *RASSF1A* g[ene] tum[or] ovarian cancer.

Mate[rials] and Method[s]

Ovarian T[u]mor Tissue[s] and Cell Lin[e] Collection

Fort[y-]en malignant ovarian epithelial tumor patie[nts] (forty-t[wo] serous cystadenocarcinoma ca[ses] and fo[ur] borderli[ne] cases), who received one ti[me] ent of [gy]cological operation at th[e] hosp[ital] om S[e]ptember 2005 to January were [chosen] for sample collection. Am[ong] these 47 [cases], there were 5 cases at clinical [stage] I, 4 cases at stage II, 37 cases at stage III [and] stage IV. Ovarian samples from patients who underwent ovarian anatomy or prophylactic ovariotomy were collected as control. All the ovarian samples were collected under sterile condition and pathological validated. After being excised from human body, the ovarian samples were quickly frozen in liquid nitrogen and transferred into –80°C refrigerator. The cell lines of ovarian epithelial t[umor] (HO8910, HO8910PM, SKOV-3, and OV[CAR]-3) were purchased from Cell Bank of Chine[se] Academy of Sciences. The ovarian sam[ples] were collected for this study upon the agre[ement] of all the enrolled patients.

Total RNA Extra[ction]

After being s[mashed] in Trizol (Invitrogen, Carlsbad, CA, USA) in 1.5 ml EP (Eppendorf) tube, 200 m[g] ovarian tissue was fractionated under 30 [W] sonication and centrifuged at 12000 g, 4°C fo[r] min. The supernatant was transferred into the 1.5 ml EP tube containing 200 µl chloroform and mixed [well] by vortex for 15 s. The mixture was placed under room temperature for 3 min. After centrifugation under 12000 g, 4°C for 15 min, colorless supernatant (about 500 µl) was transferred into a new EP tube containing 5[00] µl isopropanol and placed under room temperature for 10 min for RNA precipitation. After centrifugation at 12000 g, 4°C for 10 min, white RNA pellet was recovered and washed with 1 ml 75% ethanol. After centrifugation at 12000 g, 4°C for 5 min, supernatant was discarded and pellet was dried under room temperature for 10-20 min. The dry RNA sample was dissolved in 20 µl diethylpytocarbonate (DEPC) water. After diluting RNA samples 100 times, RNA concentration and purity were determined by measuring OD260 value and OD260/OD280 ratio. The RNA was stored under -80°C. For RNA concentration determination:

RNA concentration (µg/µl) = OD260×40×Dilution ratio/1000.

Reverse Transcription of cDNA

Total 2 µg RNA was used as template for cDNA reverse transcription. RT-PCR Kit (RNA PCR KitAMV ver3.00) was used in the process (Takara, Tokyo, Japan). The reaction was performed in a 10 µl reaction system containing 2 µl $MgCl_2$, 1 µl 10×RT buffer (reverse transcriptase buffer), 3.75 µl RNase free H_2O, 1 µl dNTP mixture (10 mM), 0.25 µl RNase inhibitor, 0.5 µl AMV (avian myeloblastosis virus) reverse transcriptase, 0.5 µl random 9-mers, and 1 µl RNA. The reaction mixture underwent the program of 10 min at 30°C, 30 min at 42°C, 5 min at 99°C, and 5 min at 5°C. The cDNA products were stored in –20°C freezer.

PCR Reaction

The PCR reaction was conducted in a 20 μl reaction system containing 4 μl cDNA, 4 μl 5×PCR buffer, 7.9 μl distilled water, 0.1 μl Ex Taq HS, 2 μl forward primer and reverse primer 2 μl. The reaction mixture underwent the program of 2 min 94°C, 30 sec at 94°C, 30 sec at 57°C, and 40 sec at 72°C for 30 cycles. The PCR products were stored at –20°C. Specific primers of *RASSF1A* was: forward, 5'-CTTCATCTGGGGCGTCGTG-3'; reverse, 5'-GCATCCTTGGGCAGGTAAAA-3'. Target fragment length for *RASSF1A* is 420 bp. Specific primers of β-actin was: forward, 5'-TGCGTGACATTAAGGAGAAGC-3'; reverse, 5'-GAAGGTGGACAGCGAGGC-3'. Primers were synthesized at Saibasheng Biotechology. Target fragment length for β-actin is 431 bp. Amplication was run on Biometra GmbH (Gottingen, Germany).

PCR Products Detection

Total 4 μl PCR products were mixed with 1 μl Loading Buffer and loaded onto 1.5% agarose gel. After running at 150 V, 100 mA for 3[illegible] in the DNA bands were detected via electro[illegible] gel imaging system (Chemizmager 5500, [illegible]ha Inno Tec Schweiz AG, Altishofen, Switzer[illegible]. The 100 bp DNA ladder markers were from [illegible] Biology Company, Dalian, China and GeneFin[illegible] erTM dye was purchased from[illegible]

Statistical Analysis

The statistical analys[illegible] car[illegible] SPSS12.0 software ([illegible]PSS[illegible]icago, IL, USA). When $p < 0$[illegible] data w[illegible]idered statistically significa[illegible]

Resu[illegible]

The E[illegible]ssion of RASSF1A in Ovarian Can[illegible] Cell L[illegible]

F1A [illegible] expr[illegible]ed in HO8910 and HO89[illegible]ls and [illegible]nt in SKOV-3 A and OVCAR-[illegible] (Fig[illegible]).

The [illegible]pressio[illegible] RASSF1A in Normal Ov[illegible]n and Ovarian Tumor Tissues

[illegible]n of *RASSF1A* was detected in [illegible]e 10 normal ovarian tissues (100%, 10/10), [illegible]e was detected in 2 cases among 47 ovarian t[illegible]amples (4.3%, 2/47). Figure 2 listed PCR resu[illegible]f some ovarian normal and cancer tissue

[illegible]re 1. The expres[illegible]on of RASSF1A in different ovarian [illegible]er cell lines. La[illegible] was the DNA marker; lane 2, 4, 6, [illegible]8 represent RAS[illegible]A band, while lane 3, 5, 7, 9 repre-[illegible]actin band.

samples. [illegible]ere was significant difference ($p <$ [illegible]05) for frequency (100% vs. 4.3%). The two [illegible]positive ovarian tissues were patholog-[illegible]ally [illegible]agnosed as serous cystadenocarcinoma cases, one of which was clinical stage I and the other was stage III, which indicated that the phenomenon is not connected with cancer staging.

Discussion

The anti-tumor roles of *RASSF1A* haven't been fully revealed so far. Some reports have shown that RASSF1A is a micro-tubule binding protein and involved in regulation of mitosis pro-

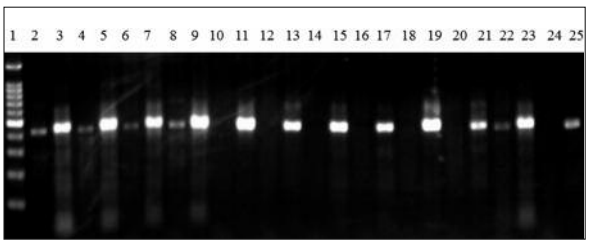

Figure 2. The expressionof RASSF1A in normal ovarian and ovarian tumor tissues. Lane 1 was the DNA marker; The even lanes represented RASSF1A band, while the odd lanes represented β-actin band. Lane 2-9 were samples from normal ovarian tissues, while lane 10-25 were samples from ovarian tumor tissues.

ceeding[6-10]. *RASSF1A* could regulate cell cycle directly, repress cell growth *in vitro* and *in vivo*, and also induce apoptosis[11,13,20]. Previous results showed that cells transformed with *RASSF1A* could repress the accumulation of cyclin D1 and further prevent cell proliferation by hindering the cells at G1/S[13]. Many evidences RASSF1A/Mst-1 complex could maintain high Mst-1 activity and assist Mst-1 to be correctly localized inside cell, and further synergistically induce cell apoptosis[21].

Previous results from breast cancer[22-26], lung cancer[17], liver cancer[27-30], gastric cancer[31-33] have discovered the absence of *RASSF1A* expression is connected with tumor development. In this study, we found that *RASSF1A* expression was absent from ovarian epithelial tumor tissues, which is inconsistent with previous report[34] which stated that *RASSF1A* was overexpressed in the ovarian tumor tissues. But our results are consistent with *RASSF1A* as tumor suppressor as previously reported[35,36]. However, small number of samples may be the reason why these differences appeared. Thus, large number of ovarian cancer tissues and normal samples is nee to confirm our results in the future and fu studies are needed to find out whether *RA IA* functions as a tumor suppressor through reg ing cell cycle and apoptosis in ovarian tumo in other cancer types.

Our findings also provide in clues fo developing new prognostic hod ovarian cancer. As we all know, th s no eff e diagnostic method at early for o so far because of its lden d rapid development as clini iologi havior features. For examp e serum BH eta human chorionic ropin) level uld be measured in mal hom pregnancy is a possibility. In addition, s lpha-fetoprotein (AFP) an ctate dehydroge (LDH) should be mea d in young girl and adolescents with susp d ovari umors because the younger the t, th eater t likelihood of a malignant g tumor these indicators are spec d the of them is little in diag- Our ow that *RASSF1A* was expres in all sted normal ovarian tissues, whi mong the 47 ovarian tumor tissues, only tw d *RASSF1A* expression. And the nce of *RASSF1A* mRNA happened at each cal stages. So *RASSF1A* could be served as o tential molecular marker for diagnosing ovar cancer at early phase.

Conclusions

The expressions of *RASSF1A* ge absen ovarian cancer tissues, while th s consider- able mRNA level of *RASSF1A* mal ovarian tissues. These findings suggest th *SF1A*, as a molecular marker, could instrum n de veloping new effective gnostic me ovarian cancer at early e.

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Conflict nter

The Authors declare that t no conflicts of interest.

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