

Original Article

Over-expression of REG3A gene can inhibit the proliferation, invasion and migration of human breast cancer

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Abstract: Breast cancer is now the most frequently diagnosed cancer and is the sixth leading cause of cancer-related death in Chinese women. The regenerating islet-derived (REG) gene family encodes a group of proteins highly expressed in several human pathologies, many of which are associated with oncogenesis. REG3A gene has been previously reported to be up-regulated in liver cancers. However, REG3A expression in breast cancer has not been described. Therefore, we have used real-time fluorescence quantification (RT-PCR) to demonstrate REG3A expression in breast cancer and pericarcinomatous tissue. The results suggest the expression of REG3A is significantly reduced in breast cancer. The screening of human breast cancer with RT-PCR reveals REG3A gene is low expressed in MCF-7 cell line. Moreover, over-expressed of REG3A gene with antiviral can effectively repress the cell proliferation and the cell cycle was blocked in G0/G1 phase. Over-expressed REG3A gene can affect cell invasion and migration, which is confirmed by cell invasion and migration related genes using west blot (snail, ICAM, RhoC, MTA1, MMP-2 and MMP-9). In addition, JAK2/STAT3 signaling pathway, which is related with oncogenesis, is retained by using west blot. Characterization of the role of REG3A in the development of tumors should lead to a better understanding of the changes occurring at the molecular level during the development and progression of primary human breast cancer.

Keywords: Breast cancer, RT-PCR, west blot, JAK2/STAT3, human

Introduction

As a result of recent rapid urbanization at an annual rate of 2-3%, 47% of the Chinese population live in urban areas and disease burden has concomitantly shifted from communicable to non-communicable diseases (including all cancers, but specifically breast cancer) [1]. Since the 1990s the incidence of breast cancer has increased more than twice as fast as have global rates, particularly in urban areas [2]. Breast cancer is now the most frequently diagnosed cancer and is the sixth leading cause of cancer-related death in Chinese women [3]. Annual diagnoses of breast cancer in China are now about half of those in the European Union (332 000 in 2008; population 498 million) [4], and are similar to the number of cases in the USA (182 000 cases in 2008; population 304 million) [5]. Meta-static breast cancer indicates it remains an incurable disease but is treatable

by means of serial administration of endocrine, cytotoxic, or biologic therapies. Prevention has been proved to be one of the most important ways to control disease burden. However, because of the national condition, currently the prevention of breast cancer in China is far away from being satisfactory or effective. On the other hand, treatment response is generally assessed with the use of serial imaging, but radiographic measurements often fail to detect changes in tumor burden. Therefore, there is an urgent need for biomarkers and target genes that measure can control tumor burden with high sensitivity and specificity.

Reg was found and defined as a regenerating and growth factor [6]. The Reg family belongs to the lectin superfamily and encodes five small secreted proteins [7, 8]. Members of the Reg family are grouped into four subtypes: types I, II, III, and IV [9]. In humans, the REG gene family

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currently consists in four well-established members: REG1A, REG1B, REG3A and REG4. All are found encoded in tandem on chromosome 2p12, except for REG4, which is found on 1p12. The intron-exon arrangement of all REGs is similar, again except for REG4, which has considerably longer intronic sequences [10, 11]. It has been demonstrated that they are highly expressed in a variety of inflammatory states and in tumor tissue when compared to normal tissue [12]. Parikh et al. suggested functions of the various REGs are many and surprisingly diverse, given the great similarity in the sequence of the members [13]. Of the human REGs, REG1A, REG3A and REG4 are the best studied. All REGs are expressed in the pancreas and small intestine. REG3A was originally identified as a pancreatitis-associated protein (PAP) released by the acini during acute pancreatitis, and is a secreted C-type lectin protein, which has been reported to be up-regulated in primary hepatocellular carcinomas [14, 15]. Several functional studies demonstrated that REG3A may be involved in cell recognition and adhesion, and also in the protection of cells from oxidative stress-induced apoptosis [16, 17]. An antimicrobial effect has been demonstrated for murine Reg3g and human REG3A and is possibly specific for membrane-bound peptidoglycans of Gram-positive bacteria [18]. REG1A, REG1B, REG3A and REG4 all show increased expression during inflammatory bowel disease (IBD)-related inflammation of the colon [19, 20]. Moreover, REG3A is dramatically down-regulated in primary human gastric cancers [21]. Present knowledge of the mRNA and protein expression patterns of the REG3A in human breast cancer is extremely limited, with only a few studies demonstrating the expression of REG3A in breast cancer [22, 23]. We hypothesized that REG3A expression is associated with breast cancer. In the present study, we have utilised real-time fluorescence quantification (RT-PCR) method enabling us to determine the expression level of human REG3A in normal and breast cancer. The expression pattern has been further investigated in six different cell types expressing REG3A with RT-PCR and western blot method. The conditions of cell proliferation, invasion and migration with over-expression of REG3A gene in MCF-7 cell line are examined with Flow Cytometer and transwell assay. Moreover, the proteins expression of genes (snail, ICAM,

RhoC, MTA1, MMP-2 and MMP-9) related to cell invasion and migration and the proteins expression of genes related to JAK2/STAT3 (JAK2 and STAT3) are tested with western blot method.

The current study represents a comprehensive evaluation of REG3A expression pattern in the breast cancer and will provide the necessary background information in further research on the regulation and function of these intriguing peptides.

Materials and methods

Clinical samples

Paired tumor and adjacent normal tissue samples were collected at the time of dissection from patient with breast cancer at the Sichuan Cancer Hospital. The biopsies were frozen and stored at -80°C until preparation of total RNA, as described below. Patients with breast cancer were included in the study. All tumor tissues were histologically confirmed to be from breast tumors.

Cell treatment

BT549 cell line, MCF-7 cell line, ZR-75-1 cell line, T-47D cell line, ZR-75-30 cell line, and MDA-MB-231 cell line were purchased from ATCC (Virginia, USA), and maintained in RPMI 1640 with 10% (v/v) FBS (Invitrogen, Carlsbad, CA). Cell lines were maintained in a humidified chamber at 5% CO₂, at 37°C. The ORF plasmid of REG3A was obtained from GeneCopoeia. pEZ-Lv201 Vector was used to build an over-expression system of REG3A. Negative control was pEZ-Lv201, control was the normal MCF-7 cells. All lentiviral particles were generated by following a standardized protocol using highly purified plasmids, Endo Fectin-Lenti™ and Titer Boost™ reagents (FulenGen, Guangzhou, China). The lentiviral transfer vector was co-transfected into MCF-7 cells with Lenti-Pac™ HIV packaging mix (FulenGen, Guangzhou, China). Lentivirus-containing supernatant was harvested 48 h after transfection, clarified, and stored at -80.

RT-PCR

Reverse transcription of mRNA from breast cancer, pericarcinomatous tissue, BT549 cell line, MCF-7 cell line, ZR-75-1 cell line, T-47D cell line, ZR-75-30 cell line, and MDA-MB-231 cell

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Table 1. Specific primers designed for the REG3A and GAPDH

Gene	Primers	Length (bp)	T _m (°C)
REG3A	5-CCAACCTGACCACCTCATTTC-3	107	59.97
	5-GCCTTAGGCCGTATGACAAA-3		59.97
GAPDH	5-CAAGTTCAACGGCACAGTCA-3	122	60.04
	5-CACCCCATTGTGTTAGCG-3		60.04

line was carried out in 100 µl final volume from 400 ng total-RNA using high capacity cDNA Archive kit (Applied Biosystems) according to manufacturer's instructions. REG3A and GAPDH mRNA levels were determined by RT-PCR, and primers were utilized as described in **Table 1**. Reactions were performed in 50 µl volumes containing, SYBR Green PCR master mix (Perkin-Elmer Biosystems). Real-time PCR was performed using a GeneAmp PCR System 9600 (Perkin-Elmer Biosystems) in 96-well optical plates. Thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min. Data were collected using the ABI analytical thermal cycler. RNA expression was calculated based on a relative standard curve with $\Delta\Delta Ct$ method, representing 10-fold dilutions of REG3A PCR products.

Western blot analysis

Total cellular protein in six different cell lines and the MCF-7 cell line with over-expressed REG3A was isolated by the addition of 1% PMSF and RIPA lysis buffer (50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS). After boiled with SDS-PAGE sample buffer for 5 min, the samples were performed for sodium dodecylsulfate-polyacrylamide gel electrophoresis. Then the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, USA). After being blocked for 1 h at room temperature, the membrane was incubated with a 1:1000 dilution of rabbit polyclonal anti-mouse REG3A, snail, ICAM, RhoC, MTA1, MMP-2 and MMP-9 pSTAT3, STAT3, pJAK2 and JAK2 antibody (ABGENT, USA) overnight. Before detected with an ECL chemiluminescence detection kit (Advansta, USA), proteins were incubated with the corresponding secondary antibody for 1 h at room temperature. The bands were obtained by GeneGnome 5 (Synoptics Ltd., UK).

Cell cycle analysis

Flow cytometric analyses were performed to define cell cycle distribution for transfected and

not transfected cells. After 6, 12, 24 and 48 h from transfection, cells were harvested by trypsinization and fixed with 70% ethanol. Cells were stained for total DNA content with a solution containing 20 µg/ml propidium iodide. Cell cycle distribution was then analyzed with a FACS calibur flow cytometer (BD Biosciences).

Migration and invasion assay

Cell migration assay was carried out using Transwell Permeable Support (Corning Incorporated, Corning, NY, USA). After transfection MCF-7 cells were carefully transferred on the top chamber of each transwell apparatus at a density of 1×10^6 per ml (100 µl per chamber). Cells were allowed to migrate for 24, 48 and 72 h at 37°C. Cells that had penetrated to the bottom side of the membrane were then fixed in methanol, stained using hematoxylin and counted at microscope. Cell invasion was analyzed by using Cultre x 24-well BME Cell Invasion Assay (Trevigen Inc., Gaithersburg, MD, USA) according to standard procedures. Briefly, after transfection, 10^3 MCF-7 cells were seeded in 100 µl serum-free media into the upper wells previously coated with Matrigel basement extract, and 500 µl of media were added in the bottom wells. After 24, 48 and 72 h of CO₂ incubation at 37°C, the non-invasive cells on the upper surface were removed and the cells migrated to the lower surface were fixed in 500 µl of Cell Dissociation Solution/Calcein-AM, incubated at 37°C in CO₂ incubator for 1 h and quantified by fluorimetric analysis (485 excitation, 520 nm emission).

Results

REG3A was down-regulated in primary human breast cancers and cell lines

In order to investigate the conditions of REG3A expression in breast tumor tissues, REG3A cDNA was selected for further verification of differential expression by RT-PCR. Using specific primers designed for the REG3A cDNA clone (**Table 1**), RT-PCR was performed on 15 paired breast cancer and pericarcinomatous tissues. The result of this RT-PCR analysis is shown in **Figure 1**, which shows that REG3A mRNA was detected in all of 30 samples. All of

REG3A gene in human breast cancer cell

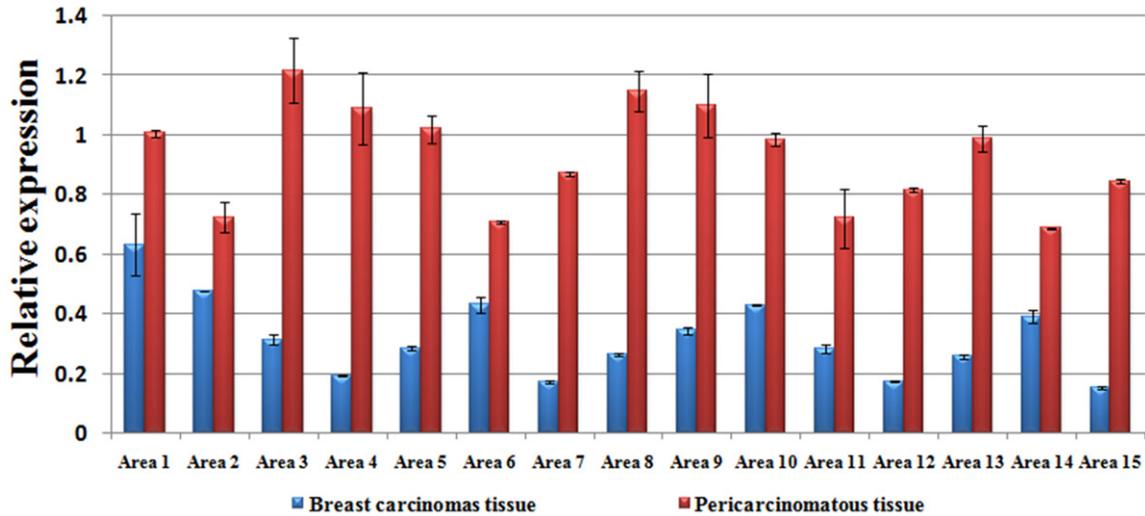


Figure 1. REG3A mRNA expression is down-regulated in primary human breast cancer RT-PCR was performed on 15 paired breast cancer and pericarcinomatous tissues. GAPDH was used as a control for cDNA synthesis. Red column and blue column indicate samples of pericarcinomatous tissues and breast cancer, respectively. REG3A mRNA expression was down-regulated in the breast cancer compared to pericarcinomatous tissues.

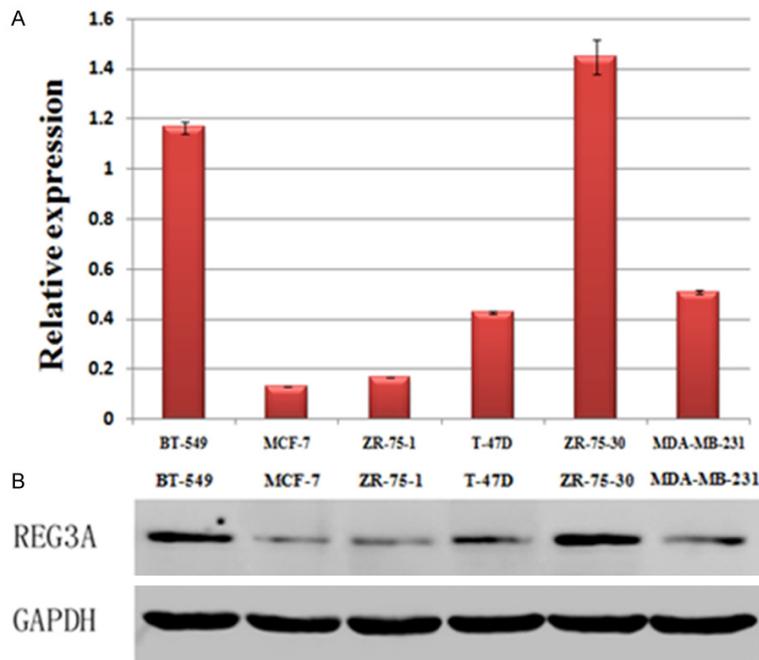


Figure 2. Expression of REG3A in six human breast cancer cell lines RT-PCR and western blot was performed on six human breast cancer lines. GAPDH was used as a control for cDNA synthesis. REG3A mRNA expression was significantly reduced in the human MCF-7 cell lines tested.

15 breast cancer samples analyzed, REG3A expression levels were markedly reduced with half of the expression levels of the reference GAPDH gene, except for Area 1. On contrary, all of 15 pericarcinomatous tissues samples analyzed, REG3A expression levels were similar to

the reference GAPDH gene, including Area 1, Area 3, Area 4, Area 5, Area 8, Area 9, Area 10, Area 12, Area 13 and Area 15. Collectively, our results indicate that abnormal reduction of REG3A is confirmed in human breast cancers, demonstrating that down-regulation of REG3A expression is common in primary breast cancer tissues. We then investigated REG3A mRNA expression in human breast cancer cell lines. Six breast cancer cell lines (BT549, MCF-7, ZR-75-1, T-47D, ZR-75-30, and MDA-MB-231) were assessed with regard to REG3A expression by RT-PCR, in which GAPDH was employed as a control for both cDNA quality and efficiency of the PCR amplification. As shown in **Figure 2**, the REG3A transcript was detectable in all breast cancer cell lines. However, the

highest expression level of REG3A gene appears on the ZR-75-30 cell line and the lowest expression level of REG3A gene appears on the MCF-7 cell line, indicating that REG3A mRNA expression in MCF-7 cell was more similar to the breast cancer in patients. Moreover,

REG3A gene in human breast cancer cell

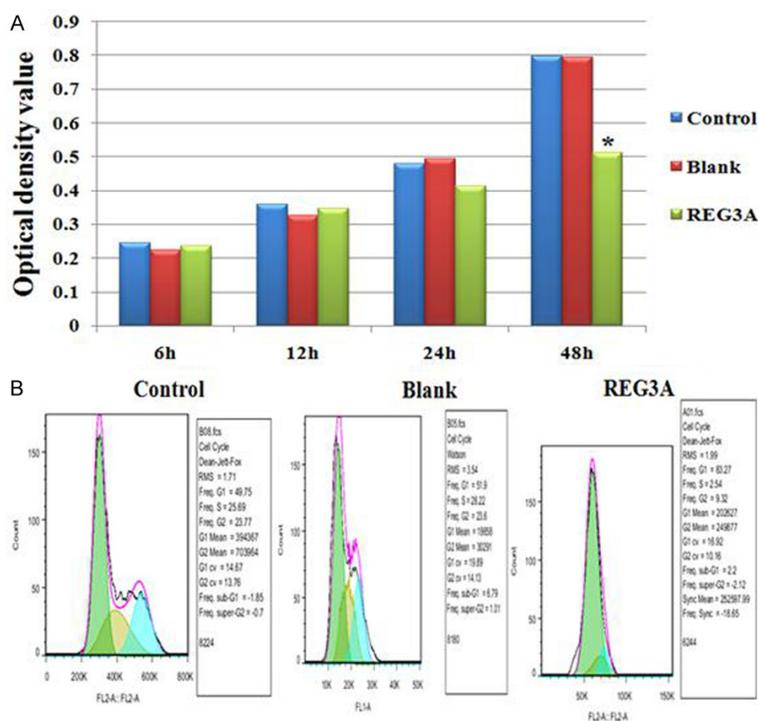


Figure 3. Cell proliferation and cycle of over-expressed REG3A gene in MCF-7 cell line. Control: normal MCF-7 cell, Blank: mock-vehicle of MCF-7 cell, REG3A: over-expressed REG3A gene of MCF-7. A. CCK8 method examines the cell proliferation of three different treatments in 6 h, 12 h, 24 h, and 48 h. B. Flow cytometry checks the cell cycle of MCF-7 with three different treatments up to 24 h.

protein expressions of REG3A were confirmed by western blot, which is consisted with the results of mRNA expressions in six different breast cancer cell lines (Figure 2). The lowest protein expression level of REG3A gene appears on the MCF-7 cell line.

Influence of over-expressed REG3A on MCF-7 cell behavior and cell cycle distribution

To test whether increased levels of REG3A might affect MCF-7 cell behavior, we introduced Control (Normal MCF-7 cell), Blank (Mock-vehicle) and REG3A (over-expressed REG3A). Transfection was validated by RT-PCR that assessed increased ΔC_t levels in transfected cells when compared with Control and Blank (Data not shown). We found that over-expressed REG3A progressively decreased cell proliferation up to 48 h of transfection (Figure 3A). Up to 48h, REG3A group was significant retained compared with other two groups ($P < 0.05$). Cell cycle distribution revealed that over-expressed REG3A gene in MCF-7 cells induced a transient cell accumulation in G0 and G1 phase (Figure

3B), while over-expressed REG3A gene resulted in accumulation of cells in G0 phase coincident with a cell decrease in G0 phase up to 48 h of transfection (Figure 3A), suggesting a block at G0/G1 check point. Cell proliferation of over-expressed REG3A gene in MCF-7 cells was significantly refrained compared with the Blank and Control groups up to 48 h. The results mentioned above suggested that over-expressed REG3A on MCF-7 cells can effectively repress the cell proliferation by preventing the cell cycling of G0/G1 phase. The invasion analysis by Boyden chamber and migration through polycarbonate membrane showed that over-expressed REG3A reduced cell invasiveness up to 24 h (Figure 4A) and cell migration (Figure 4B).

Western blot analysis of bio-markers related with invasion and migration

We evaluated the expressions of biomarkers related with invasion and migration (snail, ICAM, RhoC, MTA1, MMP-2 and MMP-9) (Figure 5) [24]. Of the Control group and Blank group (12 h), there is none of statistically significant different in the protein expression of biomarkers. However, the expressions of biomarkers in REG3A group were down-regulated compared with the other two groups. Interestingly, ICAM was up-regulated in REG3A group compared with the negative group. The results mentioned above suggested that over-expressed REG3A gene can retained the expression of key factors with invasion and migration. In summary, over-expressed REG3A gene can effective inhibit the expressions of key factors related with invasion and migration (*in vitro*).

Western blot analysis of JAK2/STAT3 signaling pathway

Two key factors of JAK2/STAT3 signaling pathway (JAK2 and STAT3) were selected to study the status of their expressions in Control, Blank and REG3A [25]. Cell extracts were analyzed

REG3A gene in human breast cancer cell

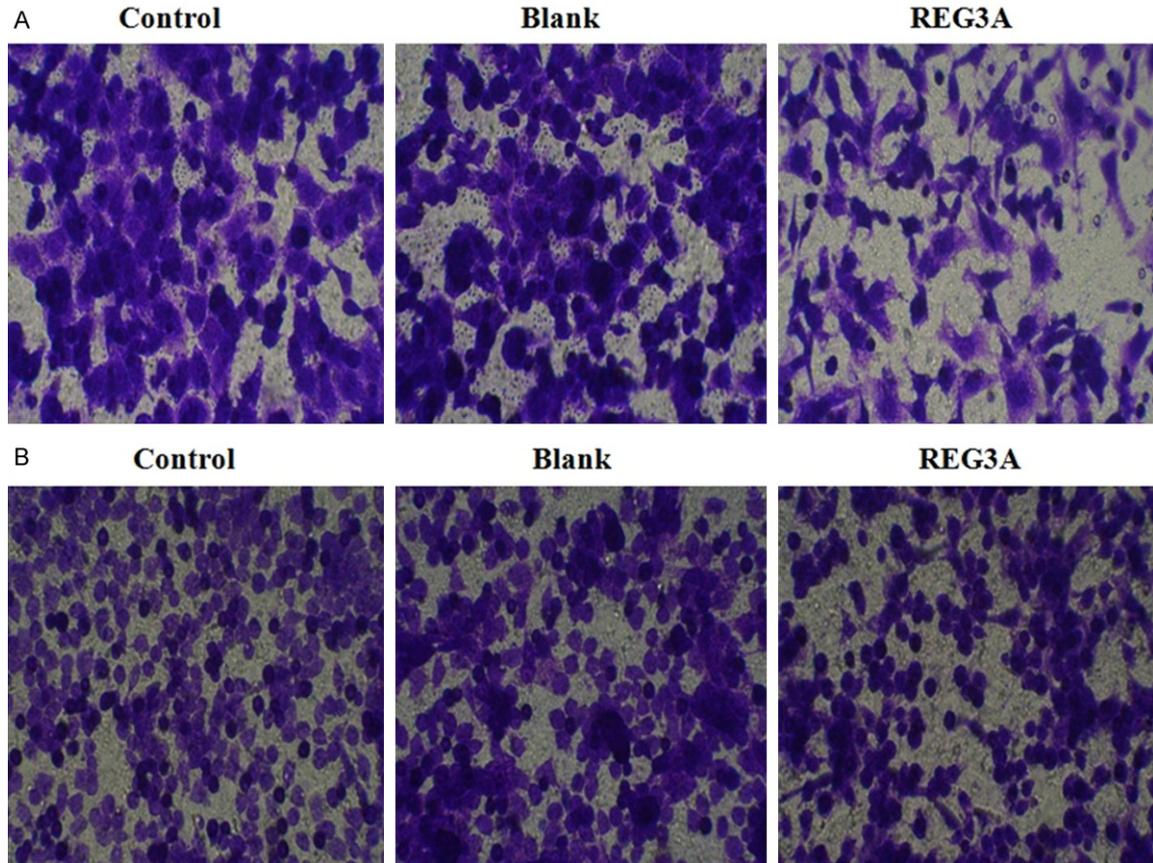


Figure 4. A: The cell motility of transfected MCF-7 cells was evaluated using the monolayer wound healing assay. MCF-7 cells transfected with REG3A were seeded in the upper chamber of transwell filters. After 24 h incubation, the top of the filters was scraped and cells that migrated through the filters were fixed and stained. Images are representative of migrated cells of one field. B: MCF-7 cells transfected with REG3A were seeded in the upper chamber of transwell filters. After 24 h incubation, the top of the filters were scraped and cells that Matrigel-invaded through the filters were fixed and stained. Images are representative of invaded cells of one field. Bar: 200 μ m.

with Western blotting using phosphorylated JAK2 (p-JAK2), total JAK2; phosphorylated STAT3 (p-STAT3), total STAT3. GAPDH served as the loading control. Western blot analysis showed that over-expressed REG3A caused a significant down-regulated protein expression of the activated key factors with JAK2/STAT3 signaling pathway (**Figure 6**). In summary, Mock-vehicle treatment had none of influence on the protein expression of the p-JAK2, total JAK2, p-STAT3 and total STAT3. However, over-expressed REG3A treatment could lead to the significantly down-regulated of the protein expression of p-JAK2, and p-STAT3, which were practically useful in biological function.

Discussion

As altered gene expression is a common feature of cancer cells, multiple genes have been

reported to be differentially expressed in tumor tissues [26, 27]. It can be postulated that such genes might be involved in the cellular transformation and/or phenotype of transformed cells. We were interested in the REG3A gene because it had been previously reported to be up-regulated in liver cancers [28]. In order to gain understanding into the molecular events of REG3A underlying the development of breast cancer, we isolated this gene which are dysregulated in primary human breast cancer tissues, using RT-PCR. In the present study, RT-PCR analysis clearly demonstrated that REG3A expression was dramatically reduced in the primary breast cancer tissues, i.e., all of the breast cancer samples, compared with the corresponding pericarcinomatous tissue. REG3A expression was different in six breast cancer cell lines (**Figure 2**) with the lowest expression level in MCF-7 cell line. Cell cycle distribution of

REG3A gene in human breast cancer cell

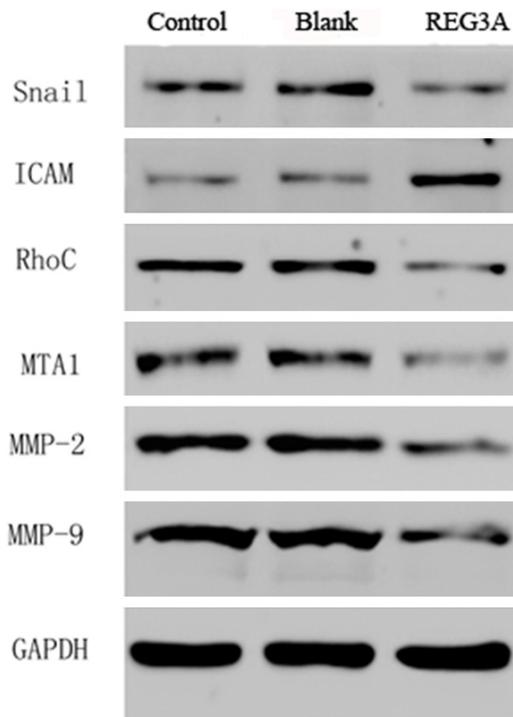


Figure 5. Western blot analysis of migration and invasion related genes with over-expressed of REG3A gene in MCF-7 cell. Control: normal MCF-7 cell, Blank: mock-vehicle of MCF-7 cell, REG3A: over-expressed REG3A gene of MCF-7.

Over-expressed REG3A gene in MCF-7 cell was retained in G0/G1 phase and cell proliferation was significantly refrained compared with the Blank and Control groups up to 48 h. In addition, the invasion analysis by Boyden chamber and migration through polycarbonate membrane showed that over-expressed REG3A reduced cell invasiveness up to 24 h and cell migration, which were confirmed by the western blot of biomarkers related with cell invasion and migration. The expressions of biomarkers in REG3A group were down-regulated compared with the Control and Blank groups. Moreover, we examined the protein expression level of JAK2/STAT3 signaling pathway, which had been proven to play a key role in promotion tumor growth in human breast cancer cells [29]. The results showed that over-expressed REG3A caused a significant down-regulated protein expression of the activated key factors with JAK2/STAT3 signaling pathway. REG3A is a member of Reg protein family and other members of Reg protein family are also related with human breast cancers. REG1 over-expression has been reported to be related with poor prog-

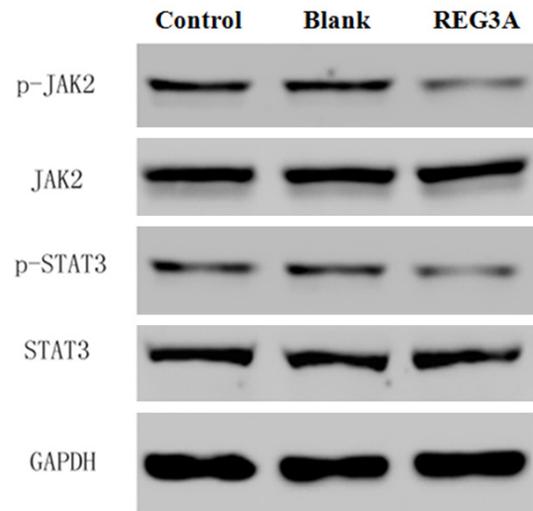


Figure 6. Western blotting was conducted to detect the expression levels of p-JAK, JAK, p-STAT3 and STAT3. Control: normal MCF-7 cell, Blank: mock-vehicle of MCF-7 cell, REG3A: over-expressed REG3A gene of MCF-7. Control: normal MCF-7 cell, Blank: mock-vehicle of MCF-7 cell, REG3A: over-expressed REG3A gene of MCF-7.

nosis of gastric cancers [30]. Over-expression of REGIV, another member of Reg protein family has been reported in certain types of human gastric cancers [31]. REG1 expression in gastric mucosa was mainly in enterochromaffin like cells [32] and REGIV over-expression was detected only in gastric cancers having neuroendocrine differentiation [33]. In contrast to REG1 and REGIV, REG3A is frequently down-regulated in human breast cancers according to our data and this discrepancy might suggest the distinct role of REG3A in mammary tissue. Several studies have shown that the methylation of the CpG island results in the inactivation of various genes, for example, cell adherence (E-cadherin), and cell cycle regulation (pRB, p15INK4b, p16INK4a) [34, 35]. An epigenetic mechanism involving DNA methylation has been shown to be responsible for the silencing of tumor associated genes in a variety of human cancers [36]. Indeed, Genes silenced by DNA methylation can be restored by treatment with 5-Aza-dC, a well-established DNA methyltransferase inhibitor [37]. REG3A gene expression was also restored by 5-Aza-dC in human stomach cancer cell lines, indicating that REG3A is transcriptionally silenced by DNA methylation. REG3A is a family in the calcium dependent (C-type) lectin superfamily consisting of a car-

bohydrate recognition domain (CRD) of 138 amino acids which is linked to a signal peptide [38]. The CRD of REG3A protein has been shown to have a specific lactose binding activity [16]. The C-type lectin family includes selectin cell adhesion molecules, endocytic receptors, and secreted molecules found in the extracellular matrix, and participates in cell surface recognition mediated by protein-carbohydrate interactions [38]. REG3A has been reported to bind to laminin-1 and fibronectin, and has also been implicated in cell to cell interaction, differentiation, and metastasis [16]. Functional studies have indicated that REG3A may play a role in the adhesion of cells to the extracellular matrix, and also in the protection of cells from oxidative stress-induced apoptosis [39]. Recently, it has been reported that REG3A stimulates liver regeneration after partial hepatectomy in transgenic mice [40]. REG3A increased hepatocyte DNA synthesis and protected these cells against TNF- α plus actino-mycin-D-induced apoptosis, implying that REG3A possesses mitogenic and anti-apoptotic abilities in hepatocytes, and consequently functions as a growth factor [40]. REG3A was previously reported to be up-regulated in 25% of primary liver cancers, although REG3A expression was not detected in normal liver tissue [15]. REG3A expression has been reported not only in liver cancers, but also in normal tissues such as the pancreas, small intestine, duodenal, jejunal, and ileal mucosa, displaying a number of positive cells in the bottom of the crypt. This pattern is consistent with that of paneth cells, suggesting that REG3A plays some role in these normal tissues [16]. REG3A gene expression was, however, undetectable in other adult normal tissues, such as the colon, brain, kidney, and lung. The present study demonstrated that REG3A was also expressed in the human breast, but down-regulated to a significant extent in the majority of primary human breast cancer (93%; 14 out of 15). In human breast cancer cell lines, REG3A promoter seems to be hyper-methylated, resulting in the transcriptional reducing of REG3A, although the CpG island hyper-methylation of REG3A has not yet been demonstrated by bisulfite modified sequencing analysis. The investigation of molecular and genetic alterations in breast cancers has provided useful insights into the pathogenesis of the disease and provided invaluable tools for the differential diagnosis of primary tumors.

For example, gene amplification and altered expression of c-erb B2 is selectively found in intestinal-type gastric cancer, and may serve as a prognostic marker for tumor invasion and lymph node metastasis [41]. Genetic mutations of E-cadherin have been reported to be responsible for a dominantly inherited form of gastric cancer, and this might improve conventional diagnosis [42]. Cyclin E over-expression can be strongly correlated with staging, invasiveness, and proliferation, and may serve as a marker for aggressiveness [43]. Although additional studies are required for the characterization of the biological significance of REG3A inactivation in breast tumorigenesis, our study suggests that the down-regulation of REG3A might be used as a molecular marker and target gene for the detection and treatment of human breast cancers. In the present study, we investigated the regulation of REG3A expression, and the mechanisms underlying its regulation in human breast cancers. REG3A is down-regulated in the majority of primary human breast cancers and REG3A expression was down-regulated in a subset of breast cancer cell lines. Characterization of the role of REG3A in the development of tumors should lead to a better understanding of the changes occurring at the molecular level during the development and progression of primary human breast cancer.

Disclosure of conflict of interest

None.

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