Claudin-4 controls the proliferation, apoptosis, migration and in vivo growth of MCF-7 breast cancer cells

XIAOTANG MA1, HUILAI MIAO2, BAOGUO JING3, QUNWEN PAN1, HUITING ZHANG1, YANFANG CHEN1, DAN ZHANG3, ZHONGZENG LIANG2, ZHILI WEN4 and MINGYI LI2

1Clinical Research Center, 2Department of Surgery; 3Cancer Center, Affiliated Hospital of Guangdong Medical College, Zhanjiang, Guangdong 524001; 4Affiliated Hospital of Infectious Diseases, Nanchang University, Nanchang, Jiangxi 330002, P.R. China

Received January 14, 2015; Accepted April 20, 2015

DOI: 10.3892/or.2015.4037

Abstract. Previous studies have shown that the expression of claudin-4 is upregulated in breast cancer. The aim of the present study was to investigate the role and the regulation of claudin-4 in MCF-7 breast cancer cells. For the in vitro experiments, MCF-7 cells were treated with recombinant vectors carrying cDNA for claudin-4 overexpression or short hairpin RNAs (shRNAs) for claudin-4 silencing. Cell proliferation was determined by an MTT assay and cell migration ability was measured by a wound-healing assay. The cell cycle profile and apoptotic rate were analyzed using flow cytometry. The effect of methylation status on claudin-4 expression was determined by PCR and western blotting. For the in vivo tumorigenesis analysis, MCF-7 cells with or without claudin-4 silencing were transplanted into nude mice. In vivo cell growth was evaluated 14 days after transplantation. We found that claudin-4 overexpression increased MCF-7 cell proliferation and migration, and reduced the rate of cell apoptosis. Silencing of claudin-4 induced the opposite effects in MCF-7 cells. In addition, claudin-4 expression was upregulated by demethylation. Moreover, the size of tumor formation was reduced in nude mice transplanted with claudin-4 silenced MCF-7 cells. These observations suggested that claudin-4, which was regulated by methylation status, plays an important role in breast cancer growth and malignancy via the control of cell proliferation, migration and apoptosis.

Introduction

Breast cancer is a common cancer and the second leading cause of cancer-related mortality in women. The limits in identifying patient subsets and the complexity of the disease presentation lead to major difficulties in current breast cancer diagnostic and therapeutic strategies (1). Investigations aiming to identify the genes responsible for promoting the growth and malignancy of breast cancer may provide insight into the nature of this disease.

The claudin family includes 24 related members which are integral transmembrane proteins (2). These members are the major components of the tight junction and are important in various cell activities through interaction with a variety of proteins in signaling pathways (3,4). The claudins are often tissue-specifically expressed (5,6), and a number of studies have described the abnormal expression of claudins in various types of cancer, such as breast cancer, hepatocellular carcinoma, colonic cancer, lung squamous cell and bladder carcinoma (7-14). Moreover, claudins are considered to participate in the pathology of these disorders (9-14). For example, claudin-5 has been reported to be downregulated and correlated with poor prognosis in patients with hepatocellular carcinoma (9). Additionally, claudin-1 is reduced in stage II colonic cancer and may be associated with recurrence and poor patient survival (14). These results suggest that the claudin family members are pivotal in tumorigenesis and cancer progression.

The relationship of TJs dysfunction with disease development in breast cancer has been shown. Claudin-1 has been suggested to function as a tumor suppressor and a tumor enhancer/facilitator in breast cancer (1,15,16). Osanai et al (17) found that claudin-6 knockout enhanced the migration and invasion of the human MCF-7 breast cancer cells. As an important member of the claudin family, claudin-4 has been investigated in breast cancer (7,8). In a case-controlled study of breast cancer, claudin-4 was found to be overexpressed and associated with high tumor grade and poor prognosis of the disease (18). Moreover, claudin-4 positivity has been associated with a shorter disease-free survival of patients with luminal breast cancer (19,20). Collectively, the abovementioned studies indicate that claudin-4 is a potential molecular marker for...
breast cancer. However, the functional role and regulation of claudin-4 in breast cancer remains unknown.

In the present study, we aimed to investigate the biological function and regulation of claudin-4 in breast cancer cells in vitro and in vivo experiments.

Materials and methods

Cells and animals. The human MCF-7 breast cancer cells were obtained from the Shanghai Bioleaf Biotech Co., Ltd. The cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics at 37°C in the presence of 95% air and 5% CO₂. Female BALB/c nude mice (4-6 weeks old) were purchased from the Experiment Animal Center of Guangdong, and maintained in a pathogen-free facility.

Vector constructs. The 659-bp claudin-4 cDNA was amplified by PCR and cloned into the EcoRI/BamHI sites of the green fluorescence protein expression vector pEGFP-C1 [Beijing Genomics Institute (BGI), Beijing, China], resulting in the claudin-4-expressing construct pEGFP-C1-Cldn4. Short hairpin RNAs (shRNAs) targeting the open reading frame of human claudin-4 was cloned into the Xhol/Hpal sites of the green fluorescence protein expressing vector PLL3.7 (BGI) to generate the PLL3.7-siCldn4 vector. shRNA was purchased from BGI and the primer sequences used were: Claudin-4 shRNA sense, TGTGATACCAACTGCTGGAGGTAGAATTCAAGAGATTCTCCTCCACGACGTGAGTACAC TTTC TTC and antisense, TCGAGAAAAAGTGTTACCACA ACTGCTGGAGGATGTACTCTTGAAATTCATCCTCC AGGCCAGTGTGACACA. Scrambled shRNAs were used to generate the PLL3.7-scramble control vector. The sequences used were: Scrambled shRNA sense, TGCCCTAGTGTAGATGGCTGCAAGAATCTCTTG and antisense, TCGAGAAAAAAGTGTACCA ACTGCCTGGAGGATGAATCTCTTGAATTCATCCTCC AGGCCAGTGTGACACA. Scrambled shRNAs were used to generate the PLL3.7-scramble control vector. The sequences used were: Scrambled shRNA sense, TGCCCTAGTGTAGATGGCTGCAAGAATCTCTTG and antisense, TCGAGAAAAAAGTGTACCA ACTGCCTGGAGGATGAATCTCTTGAATTCATCCTCC AGGCCAGTGTGACACA.

Generation of stable cell lines with claudin-4 overexpression or knockdown. Cells were cultured to 80% confluency in vitro and then washed in TBST. β-actin (1:1,000; EarthOx, San Francisco, CA, USA) was used to normalize protein loading. A secondary antibody (1:100,000; EarthOx) was added and cells were incubated overnight at 4°C and then washed in TBST. Cell proliferation assay. Cell migration assay. Cell cycle profile. Cell proliferation assay. The proliferative capabilities of cells with claudin-4 knockdown/overexpression and corresponding controls were measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (5 mg/ml; Sigma, St. Louis, MO, USA). Cells were seeded in 96-well plates at a concentration of 2x10^3 cells/well containing 200 µl of RPMI-1640 cell culture medium supplemented with 10% FBS. Every 24 h of culture in 5 days, 20 µl of MTT solution was added and cells were incubated for 4 h at 37°C. Then 150 µl DMSO was added to each well, and the cells were incubated for 20 min at 37°C. The optical density (OD) value of cells was read at 490 nm in a microplate reader (BioTek, Winooski, VT, USA). Cells in triplicate wells were counted at each time point, and the experiment was repeated in three independent experiments.

Cell migration assay. The migration rate of breast cancer cells was measured by a wound-healing assay. The cells were grown to confluence on 60-mm cell culture dishes. A scratch was made through the cell monolayer using a pipette tip. After washing with phosphate-buffered saline (PBS), 0.5% FBS maintenance medium was added. Images of the wounded area were captured immediately after making the scratch (0 h time-point) and the invasion of cells into the wounded area was monitored once every 8 h for 48 h using an inverted microscope (LEICA DFC500, Germany).

Apoptosis assay. MCF-7 cells with claudin-4 overexpression or knockdown and the corresponding controls were

RT-PCR. Total RNA was isolated from the cell lines using a TRIzol reagent kit (Invitrogen) according to the manufacturer's instructions. RNA samples were then reverse transcribed into cDNA using an MML-V reverse transcription kit (Invitrogen) in a total volume of 20 µl according to the manufacturer's instructions. Equal amounts of cDNA samples were used for RT-PCR to detect the level of claudin-4 expression under the following conditions: 3 min at 95°C, followed by a total of 40 cycles of two temperature cycles (15 sec at 90°C and 1 min at 60°C). The primers and annealing temperatures used for PCR are shown in Table I. GAPDH was used as an internal control (housekeeping gene). The relative quantification of claudin-4 was determined using the comparative CT method (2^ΔΔCt).
seeded into 60-mm cell culture dishes at a concentration of 4x10^5 cells/well. To induce apoptosis, the cells were treated with 0.1 µg/ml chemotherapeutic drug 5-fluoro-2,4(1 and 3H)pyrimidinedione (5-FU) (Sigma) for 24 h. Apoptotic cells were detected by the PE Annexin V apoptosis detection kit (BD Biosciences, San Diego, CA, USA). The cells were labeled with Annexin V-PE and 7-AAD following the manufacturer's instructions. The percentage of Annexin V-labeled cells was measured by flow cytometric analysis using FACSCalibur flow cytometry (BD Biosciences).

Methylation-specific PCR. The methylation status was detected using methylation-specific PCR (MSPCR) as previously described (23). The primers and annealing temperatures used for MSPCR are shown in Table I. Genomic DNA was extracted and purified from the cell lines using a QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Bisulfite modification was performed according to the manufacturer's instructions of the EpiTect® Plus DNA Bisulfite kit (Qiagen). The PCR mixture contained 10X Maxima Hot Start Taq buffer, 5 mmol/l MgCl2, 2 µl dNTP 10 mmol/l, 0.125 µl Maxima Hot Start Taq DNA Polymerase (all reagents from Thermo Scientific, UK), 1 µl each of forward and reverse primers, and 2 µl of DNA brought to a total volume of 25 µl by the addition of autoclaved deionized water. PCR reactions were hot started at 95°C for 5 min, followed by 35 cycles (30 sec at 95°C, 30 sec at the annealing temperature, 30 sec at 72°C) and a final 7-min elongation at 72°C. Each PCR product was loaded into a 1% agarose gel, stained with ethidium bromide and visualized under UV illumination.

To assess the effect of DNA methylation, the cell lines were treated with 10 µmol/l 5-AZA-2V-deoxycytidine (5-AZA; Sigma), an inhibitor of DNA methylation. Treatment of cell cultures started at 30-40% confluence for a total of 3 days. The medium and drug were changed every 24 h. RT-qPCR and western blotting were then performed to detect the level of claudin-4.

In vivo tumor growth. Animal experiments were carried out according to the protocol approved by the Animal Studies Committee at Guangdong Medical College. Six-week-old female BALB/c nude mice were housed in sterile microisolators, and were randomly divided into two groups (n=6) each for subcutaneous injection of 2x10^6/100 µl MCF-7 cells with claudin-4 knockdown or control MCF-7 cells into the breast fat. After 14 days, the mice were sacrificed for tumor dissection. The length and width were measured with metric calipers for tumor volume calculation using the equation: Volume = length x width x width/2.

Statistical analysis. Data are presented as the mean ± SD. Comparisons for two groups were performed using a Student's t-test (GraphPad Prism 5 software). Multiple comparisons were performed by one- or two-way ANOVA. P<0.05 was considered to indicate a statistically significant result.

Results

Overexpression of claudin-4 in MCF-7 cells. The MCF-7 cells with pEGFP-C1 and pEGFP-C1-Cldn4 stable transfection were observed by fluorescence microscope (Fig. 1A). In MCF-7 cells treated with vector pEGFP-C1-Cldn4 (claudin-4 overexpression), claudin-4 expression increased (7.34±1.38)-fold at the mRNA level and (2.41±0.69)-fold at the protein level in comparison to the pEGFP-C1-transfected cells (control) (P<0.01, Fig. 1B and C).

Knockdown of claudin-4 in MCF-7 cells. The green fluorescence of the PLL3.7-scramble and PLL3.7-siCldn4 stably transfected MCF-7 cells were observed using

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-4</td>
<td>5'-TTCCATCGCGCAACATTGTCACC-3'</td>
<td>60</td>
</tr>
<tr>
<td>F</td>
<td>5'-AGTGGTTACCTTTGCACTGCATCT-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GAAGGTTGAAGGTCGGAGTC-3'</td>
<td>60</td>
</tr>
<tr>
<td>F</td>
<td>5'-CAGAGTTCCGTAAGGCAG-3'</td>
<td></td>
</tr>
<tr>
<td>MS-PCR</td>
<td>Methylated</td>
<td>57</td>
</tr>
<tr>
<td>M-F</td>
<td>5'-CTACCGATAAAAACCGTCACG-3'</td>
<td></td>
</tr>
<tr>
<td>M-R</td>
<td>5'-GTGATTTTGCAACGTTAAGTTC-3'</td>
<td></td>
</tr>
<tr>
<td>Unmethylated</td>
<td>U-F</td>
<td>5'-ATATTACTACCAATAAAAACCATCACAC-3'</td>
</tr>
<tr>
<td>U-R</td>
<td>5'-TGTATTGTTGAAATGTTAAGTGT-3'</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
Figure 1. Overexpression of claudin-4 in MCF-7 cells. (A) Claudin-4 overexpression and control cells were observed by fluorescence microscope. (B and C) RT-PCR and western blot analysis of claudin-4 expression in claudin-4 overexpressed MCF-7 cells compared to the control cells. Claudin-4 overexpression, pEGFP-C1-Cldn4 stably transfected MCF-7 cells; control, pEGFP-C1 stably transfected cells. The results are presented as the mean ± SD (n=3). **P<0.01 vs. control.

Figure 2. Knockdown of claudin-4 in MCF-7 cells. (A) Claudin-4 silencing and control cells were observed by fluorescence microscope. (B and C) RT-PCR and western blot analysis of claudin-4 expression in claudin-4-silenced cells compared to the control cells. Claudin-4 silencing, PLL3.7-siCldn4 stably transfected MCF-7 cells; control, PLL3.7-scramble stably transfected MCF-7 cells. The results are presented as the mean ± SD (n=3). ##P<0.01 and ###P<0.001 vs. control.
fluorescence microscope (Leica, Germany) (Fig. 2A). Claudin-4 mRNA and protein levels were downregulated to (15.95±1.34) and (32.49±3.19)% in PLL 3.7-siCldn4 stably transfected MCF-7 cells (claudin-4 silencing) compared to the PLL3.7-scramble-transfected cells (control) (P<0.001 or P<0.01, Fig. 2B and C).

Claudin-4 is involved in MCF-7 cell proliferation. To determine whether claudin-4 protein influenced the proliferation of breast cancer cell lines, an MTT assay was carried out on the claudin-4-overexpressing MCF-7 cells and the corresponding negative control cells. The proliferation capability of claudin-4 overexpressed MCF-7 cells was significantly stronger (claudin-4 overexpression vs. control, P<0.05 or P<0.01, Fig. 3A). By contrast, when we knocked down the expression of claudin-4 in MCF-7 cells, the cell proliferation rate was significantly retarded as compared to the control cells (claudin-4-silence vs. control, P<0.05 or P<0.01, Fig. 3B). The results showed that claudin-4 significantly enhanced proliferation of the breast cancer cells. However, claudin-4 did not influence the cell cycle of the breast cancer cells according to our flow cytometric results (Fig. 4).
Claudin-4 regulates MCF-7 cell migration. A scrape injury assay and time-lapse analysis were carried out to assess the effect of claudin-4 on breast cancer cell migration. The result revealed a significant increase in the average migration distance of claudin-4 overexpression MCF-7 cells compared to the control cells (70.89±2.41 and 43.25±3.39 µm, claudin-4 overexpression vs. control, P<0.001, Fig. 5A and C). Conversely, the average migration distance of claudin-4 knockdown MCF-7 cells was significantly decreased compared to the control cells (20.35±2.70 and 43.98±1.89 µm, claudin-4 silenced vs. control, P<0.001, Fig. 5B and D). These results showed that claudin-4 inhibited migration of breast cancer cells.

Claudin-4 affects MCF-7 cell apoptosis. MCF-7 cell apoptosis was assessed using flow cytometry. 5-FU treatment for 24 h significantly increased the rate of cell apoptosis in claudin-4 overexpressed/silenced cells and corresponding control cells. However, 5-FU-induced apoptosis in claudin-4 overexpressed MCF-7 cells was significantly lower (6.00±0.72 and 16.60±2.79%, claudin-4 overexpression vs. control, P<0.001, Fig. 6A and C). By contrast, 5-FU-induced apoptosis of MCF-7 cells was increased after claudin-4 knockdown (34.90±2.14 and 18.07±3.65%, claudin-4 silence vs. control, P<0.01, Fig. 6B and D). These results showed that claudin-4 inhibited apoptosis of breast cancer cells.

Expression of claudin-4 is regulated by methylation in MCF-7 cells. The methylation status of claudin-4 was determined in MCF-7 cells by MSPCR. As shown in Fig. 7A, normal MCF-7 cells exhibited methylated and unmethylated PCR products. Following treatment with 5-AZA, an inhibitor of DNA methylation for 24 h, an unmethylated allele only was identified. 5-AZA treatment induced an increased claudin-4 expression in MCF-7 cells, as demonstrated by RT-PCR and western blotting. After 48 and 72 h of 5-AZA incubation, the expression of claudin-4 mRNA increased (1.53±0.36)- and (2.1±0.33)-fold in comparison to the untreated vehicle cells, respectively (P<0.05 or 0.01, Fig. 7B). Claudin-4 protein expression significantly increased (1.85±0.48)-, (2.86±0.18)- and (4.89±0.44)-fold after 24, 48 and 72 h following 5-AZA treatment (P<0.05, 0.01 or 0.001, Fig. 7C). This result indicated that the expression of claudin-4 expression was regulated by the methylation status.

Silencing of claudin-4 inhibits the tumor formation of MCF-7 cells in vivo. To explore the role of claudin-4 in vivo tumor formation, claudin-4-silenced MCF-7 and corresponding control MCF-7 cells (2x10⁶ cells) were injected subcutaneously into BALB/c nude mice. Palpable tumors were formed 7 days after injection in control and claudin-4-silenced MCF-7 groups. On day 14, the tumors derived from claudin-4-silenced MCF-7 were significantly smaller than those formed in the control cells (202.2±82.3 and 616.7±177.2 mm³, claudin-4-silenced MCF-7 vs. control MCF-7, P<0.01, Fig. 8A-C). The results suggested that knockdown of claudin-4 inhibited the oncogenicity of MCF-7 cells.
Discussion

Tight junctions exist in the junctional complexes of epithelial and endothelial cells, where they play important roles in various cell activities such as cell adhesion, permeability proliferation and differentiation (3,4,24). Claudins are the major components of tight junctions for backbone and barrier formation (3). The abnormality of claudins has been shown...
to contribute to tumor development (25). Previous studies have identified the overexpression of claudin-4 in breast cancer (18-20), suggesting that claudin-4 be a biomarker for the detection and diagnosis of breast cancer. In the present study, to the best of our knowledge we found for the first time that claudin-4 promoted the proliferation and migration of breast cancer cells, while inhibiting the apoptosis of these cells in vitro, resulting in a more aggressive phenotype. Claudin-4 inhibition repressed the tumorigenesis of MCF-7 breast cancer cells in nude mice. Moreover, the expression of claudin-4 may be regulated by the methylation status according to our preliminary study.

A high proliferative potential is one of the most important characteristics of malignant tumors (26). Members of the claudin family have been proven to regulate the proliferation of human cancer cells (27). Based on the findings that the expression of claudin-4 was increased in breast cancer (18-20), we hypothesized that claudin-4 overexpression contributes to the proliferative potential of cancer cells in breast cancer. As expected, we found that cell number expansion was promoted by claudin-4 in the breast cancer cell lines. The cell-cycle progression of breast cancer cells was not affected by the upregulation or downregulation of claudin-4 expression in the present study. Thus, the precise mechanisms by which claudin-4 regulates cell expansion should be studied. It has been shown that claudin proteins interact with various proteins through the PDZ domain-binding motif existing in the COOH-terminal of claudins (28). These interactions can serve as adapters for regulatory proteins such as Rab3b and Rab13, and transcription factors such as ZONAB (29,30), regulating cell proliferation.

Apoptosis is another essential factor for maintaining cell/tissue homeostasis. Dysregulation of cell apoptosis is one of the leading mechanisms in tumor formation (31). Recent studies have described the role of claudin proteins in cell apoptosis (32,33). In breast cancer, the anti-apoptotic effect of claudin-1 has been identified (34). In the present study, our results show that claudin-4 may also inhibit the apoptosis of breast cancer cells.

Most malignant tumors exhibit a highly invasive and migratory ability, which is closely associated with tumor metastasis. Previous studies have demonstrated the association between claudin dysregulation and cancer cell metastasis alteration (7). Claudin-4 overexpression has been identified to reduce the invasiveness of pancreatic cancer cells (35). In breast cancer cells, the upregulation of claudin-6 expression decreases cell invasiveness and migration (22). Claudin-7 downregulation contributes to the increased cellular discohesion and the ability of cancer cells to disseminate (21). Those results indicate that tight junction overexpression decreases cell invasion and motility. However, our results have shown that claudin-4 increased the migration of breast cancer cells. This discrepancy may be that different types of claudins function differently in different cells/tissues due to tissue-specific molecular mechanisms (7). Our finding is consistent with those of a previous study showing that knockdown of claudin-4 in ovarian cancer cell lines results in a decrease in the invasion of these cells, which is associated with increased matrix metalloproteinase-2 activity (36).
Based on our in vitro studies, claudin-4 overexpression promotes the aggressive behavior of human breast cancer cells, possibly by inhibiting apoptosis and promoting cell proliferation and migration. To confirm our results, we assessed the role of claudin-4 in tumorigenesis in nude mice. We found that claudin-4 silencing inhibited the tumorigenesis of MCF-7 cells, suggesting that claudin-4 overexpression promotes tumorigenesis in breast cancer. These results are consistent with our in vitro findings, and provides the preclinical evidence for claudin-4 to be a potential candidate for therapeutic target for breast cancer. It has been demonstrated that immunotoxin-mediated targeting of claudin-4 inhibited the proliferation of claudin-4-positive cancers (37).

All of the results suggest that claudin-4 is important in the development of breast cancer. However, the mechanisms of claudin-4 regulation in breast cancer remain to be elucidated. Overexpression of claudin-4 may be mediated through multiple mechanisms, one of which is gene epigenetic modification (23). Methylation of 5’-cytosines in CpG islands is an important epigenetic modulator of gene expression (38). Alterations in the methylation status have been proven to be associated with aberrant gene expression of claudin-4 in pancreatic carcinomas and ovarian cancer (23,39). In the present study, we found that claudin-4 expression was increased in MCF-7 breast cancer cells after unmethylated treatment. The results suggest that the increased expression of claudin-4 was associated with the hypomethylated status in breast cancer. As cultured cells may have an altered methylation pattern compared to their tissue counterparts (21), breast cancer tissue samples have to be examined to confirm our results.

In summary, the present study demonstrates a potential role of claudin-4 in the pathogenesis of breast cancer via the control of cancer cell proliferation, apoptosis and migration. In addition, our data reveal that methylation controls claudin-4 expression in breast cancer. However, further in-depth investigations on the role and the precise underlying mechanisms of claudin-4 regulation in breast cancer are necessary.

Acknowledgements

This study was supported by funding from the Scientific Research Foundation of Guangdong Medical College (no. XB1225), and the Affiliated Hospital of Guangdong Medical College (no. BK201210).

References


