Functional expression of TLR9 in esophageal cancer

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Abstract. The Toll-like receptor 9 (TLR9) plays a crucial role in both innate and adaptive immune responses against infection and danger signals. Stimulation of TLR9 has been linked to invasion in various cancer cells *in vitro*. The present study evaluated the expression of TLR9 in human esophageal cancer (EC) cells and normal and malignant esophageal squamous epithelium, and examined the association between TLR9 expression, clinicopathological variables, and EC patient outcome. We further characterized the direct effects of TLR9 agonist CpG oligonucleotides (CpG ODN) and inhibitor chloroquine (CQ), on the proliferation and invasion of EC cells *in vitro*. RT-PCR, western blot, flow cytometry and immunohistochemical analysis were used to determine the expression of TLR9 in EC cell line TE10, and 90 cases of esophageal squamous cell carcinoma, including 30 cases of adjacent esophageal epithelium. The TLR9 expression was compared with tumor size, location, grade, stage and proliferation. We found basal expression of TLR9 in TE10 cells. Esophageal carcinomas exhibited TLR9 expression that was positively associated with tumor size, location and TNM stage ($P<0.05$). CpG ODN significantly enhanced the invasion of TE10 cells, which could be abrogated by a TLR9 inhibitor CQ. CpG ODN led to activation of NF-κB and enhanced expression of matrix metalloproteinase (MMP)-2, MMP-7 and cyclooxygenase-2 (COX-2) mRNA. Expression of TLR9 in EC suggests a role of TLR9 related to cell proliferation and differentiation. Our findings indicate that TLR9 may represent a novel therapeutic target in this disease.

Introduction

Esophageal cancer (EC) is a highly malignant tumor and a major threat to health worldwide; it has a 5-year survival rate of <10% and 300,000 people succumb to the disease, half of whom are from China. In China, EC is characterized by its distinct geographic distribution and differences in ethnic prevalence (1). Ningxia, in Western China, has one of the highest prevalence and mortality rates of esophageal carcinoma in the world. Despite advances in clinical treatment, the complexity of cancer has posed a formidable challenge to both clinicians and researchers. Due to its relapse and metastasis characteristics, prognostic factors are essential to improve the classic risk classification in EC.

Chronic infection and inflammation can induce cancer formation via proinflammatory factors by promoting angiogenesis and metastasis contributing to cancer development and growth. Toll-like receptors (TLRs) are pathogen recognizing receptors that mediate innate immune responses including the secretion of cytokines as well as the release of chemokines, thus limiting microbe spreading and triggering inflammatory responses (2-6). The TLR family consists of >10 members in humans. TLR9 is one of the important members in this family. Ligand binding to TLR9 activates several different signaling factors, such as nuclear factor-κB (NF-κB) eventually characterized by increased production of inflammatory mediators (7). Recent studies found that high expression of TLR9 occurred not only in immune cells, but also in various cancer cells including breast, brain, ovarian, gastric, lung and prostate cancer cells (8). The role of TLR9 expressed by tumor cells in the evasion of immune surveillance was demonstrated in animal experiments showing that TLR9 stimulation may lead to tumor progression and inflammation and cell survival increasing (9). Furthermore, treatment of TLR9-expressing cancer cells with synthetic TLR9 ligands that are reminiscent of bacterial DNA increases the invasion of breast and prostate cancer cells *in vitro* (10). These findings may be useful in elucidating potential prognostic markers. However, currently, very little is known about the regulation of TLR9 expression and its actual role in EC cells.

The purpose of the present study was to investigate the expression of TLR9 in human EC cells and normal and malignant esophageal squamous epithelium and to analyze its possible association with EC invasion and prognosis. Therefore, we analyzed the protein levels of TLR9 by immunohistochemical techniques, western blotting, flow cytometric analysis. The mRNA levels of tumor progression and migration-related factors such as matrix metalloproteinase-2 (MMP-2), MMP-7 and cyclooxygenase-2 (COX-2) were examined by reverse transcription-polymerase chain reaction (RT-PCR).

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Furthermore, we performed MTT analysis and scratch assays to examine the direct effects of cytosine phosphate guanosine oligodinucleotide (CpG ODN), on tumor migration and the proliferation of tumor cells to further characterize the possible role of functional TLR9 expression in EC.

Materials and methods

Chemicals. The full phosphorothioated ODN 1816 (5'-TCCAT GACGTTCCTGACGTT-3') and primers were synthesized by SBS Genetech (Beijing, China). The mouse monoclonal antibodies against FITC-conjugated TLR9, TLR9, GAPDH, NF-κB and IgG2α isotype control were purchased from Abcam (Beijing, China). Annexin V-FITC/PI kit was obtained from Bender (Shenzhen, China). TRIzol, RPMI-1640 and FBS were from Gibco (Shanghai, China). Chloroquine was purchased from Sigma-Aldrich (Shanghai, China). Reverse transcription kit was bought from TransGen Biotechnology (Beijing, China). BCA and ECL kit were from Pierce (Shanghai, China).

Cell culture. TE10 cells were cultivated in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 g/ml). Cells were maintained at 37˚C in a humidified incubator gassed with 95% O_2 and 5% CO_2.

RNA extraction and RT-PCR analysis. Total RNA was extracted from TE10 cells by TRIzol. The purity and concentration of RNA was determined by spectrophotometry at 260 and 280 nm. Complementary DNA (cDNA) was synthesized using a reverse transcription kit. Quantitative PCR was performed as follows: 94˚C for 5 min, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 62˚C for 30 sec, and extension at 72˚C for 30 sec, and a single extension at 72˚C for 5 min. Primers used for the PCR were: TLR9: sense, 5'-GGACA CTCCCAGCTCTGAAG-3' and antisense, 5'-TTGGCTG TGGATGTTGTTGT-3'; MMP2: sense, 5'-CTTCCAAGT CTGGAGCGATGT-3' and antisense, 5'-TACCGTCAAAGG GGTATCCAT-3'; MMP7: sense, 5'-CGGGGTACCATAATG TCCTGAATGATACC-3' and antisense, 5'-CCCAAGCTTT GCCGTCCAGAGACAATTG-3'; COX-2: sense, 5'-GCCTGA ATGTGCCATAAGACTGAC-3' and antisense, 5'-AAA CCCACAGTGCTTGACACAGA-3'; β-actin: sense, 5'-TGG CACCCAGCACAATGAA-3' and antisense, 5'-CTAAGT CATAGTCCGCCTAGAAGCA-3'. PCR products were applied to 1.5% agarose gel electrophoresis. The gel was scanned and the electrophoresis image was input into Kodak gel image analysis system.

Western blotting. Cells were washed with PBS three times and lysed with RIPA buffer. The protein concentration was determined with BCA kit according to the manufacturer's instructions. Equal quantities of protein were loaded and ran on SDS/polyacrylamide gels and then transferred to a PVDF membrane. Membranes were blocked with 5% dried milk and incubated with primary antibody of TLR9 in TBST overnight at 4˚C. After rinsing in milk-TBST, blots were incubated in the horseradish peroxidase-conjugated secondary antibody. The expression of TLR9 was detected by using the enhanced chemiluminescence (ECL) detection system and X-ray films.

Flow cytometry. TE10 cells were detached with 0.05% trypsin/0.02% EDTA and washed with cold PBS. For detection of TLR9 expression, cells were fixed and permeabilized using the Cytofix/Cytoperm kit according to the manufacturer's instructions. Mouse anti-human TLR9 mAb or the appropriate isotypic IgG2a control mAb was performed at 0.5 mg/10^6 cells for 30 min on ice. After washing with cold PBS, cells were stained with fluorescein (FITC)-conjugated anti-mouse IgG. Cells were analyzed with a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and gated using forward vs. side scatter to exclude dead cells and debris. Fluorescence of 10^4 cells per sample was acquired in logarithmic mode for visual inspection of the distributions and in linear mode for quantifying the expression of the relevant molecules by calculating the mean fluorescence intensity with Cell Quest Pro software.

Cell viability assay. Cells were seeded and grown on sterile 96-well plates. The experimental cell concentration was $3x10^5$/l. Cells were pretreated with 10 μg/ml CpG ODN (TLR9 ligand) or CQ (TLR9 inhibitor) for 1 h. Then, 10 μg/ml CpG ODN were added to the cells and incubated for 12, 24 or 48 h. Cell growth was then quantified using the MTT-based *In Vitro* Toxicology Assay kit. A value was measured in a microplate reader at the wavelength of 450 nm at 37˚C incubated for 4 h.

Enzyme-linked immunosorbent assay (ELISA). TE10 cells were treated with 20 μg/ml CpG ODN for 12, 24 and 48 h. The supernatant was collected and the level of NF-κB p65 in culture medium was quantified according to the manufacturer's directions. Absorbance was determined at 450 nm using a microplate reader.

Scratch/(wound-healing) assay. TE10 cellular suspension at a concentration of $5x10^5$/ml was inoculated into a 6-well culture plate at 2 ml per well, and cultured for 24 h. The medium was removed and replaced by 20 μg/ml of CPG ODN 1816 culture medium or CQ with a concentration of 100 or 200 μg/ml after the cells reached 80% confluence. The supernatant was discarded and a wound (scratch) was created. The wound was created in the confluent layer of cells using a sterile scraping cutter and the ability of cells to heal the wound was analyzed. The assays were documented using digital photography.

Tissue samples and patients. The project was reviewed and approved by the Ethics Committee of Ningxia Medical University. A total of 60 sections resected by radical operation for esophageal carcinoma, which were filed and randomly selected in the Department of Pathology, the General Hospital of Ningxia Medical University from March 1992 to September 2011. The other 30 sections from normal esophageal tissues as control were from the same patients undergoing esophageal surgery for the tumors but without cancer cell infiltration. After fixation in 10% formalin and embedding in paraffin, 5 μm serial sections were made for each specimen.

Immunohistochemical analysis. Tissue sections were de-waxed, soaked in alcohol, and treated in antigen unmasking solution in the microwave for 10 min followed by incubation in 3% hydrogen peroxide for 10 min to inactivate endogenous

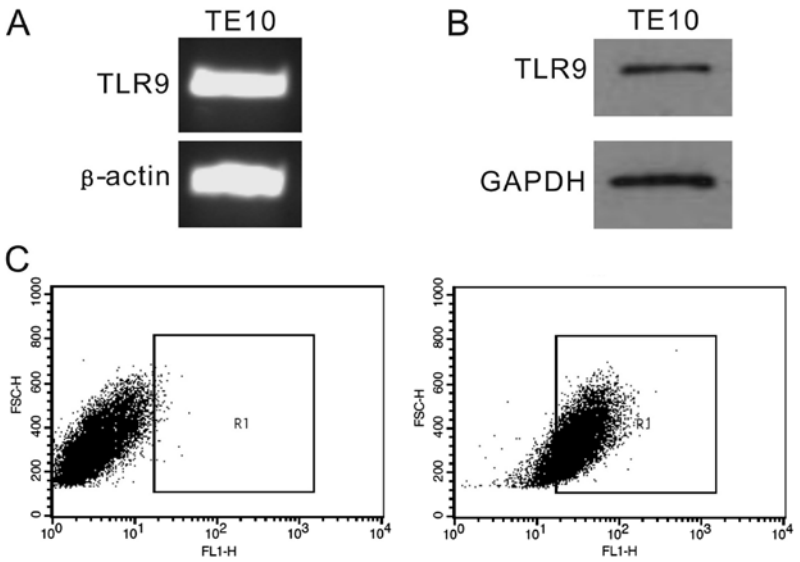

Figure 1. TLR9 expression in TE10 cells detected by RT-PCR, western blotting and flow cytometry experiments. (A) RT-PCR, (B) western blotting, (C) flow cytometry (left panel, IgG2a control; right panel, TLR9).

peroxidase activity. Sections were then incubated at 4˚C overnight with anti-TLR9 (1:100 dilution) primary antibody. Immunostaining was performed by using the Histostain™-Plus kit according to the manufacturer's instructions.

Statistical analysis. All experiments were replicated at least three times on TE10 cells. All data are expressed as the means ± SD. Data were analyzed by Student's t-test and one-way ANOVA, and statistical significance for comparison of means of different groups was calculated using LSD-*t* analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

TLR9 expression in TE10 cells. The expression of TLR9 mRNA in EC cell line TE10 was analyzed by RT-PCR (Fig. 1A). The results revealed that TLR9 mRNA expressed in TE10 cells. To confirm the RT-PCR findings, we further examined the protein level of TLR9 in TE10 cells by western blotting (Fig. 1B) and flow cytometric analysis (Fig. 1C). We found that TLR9 protein was expressed in TE10 cells.

CpG ODN promotes TE10 cell growth. MTT assay was performed to investigate the effect of CpG ODN and CQ on the proliferation of TE10 cells. When cells were pretreated with 10 μg/ml CpG ODN or 10 μg/ml CQ, and then treated with 10 μg/ml CpG ODN for 12, 24 or 48 h, the proliferation was significantly promoted compared with the control groups (Fig. 2). However, when cells were pretreated with TLR9 inhibitor CQ, the proliferation was not enhanced by CpG ODN.

CpG ODN promotes NF-κB p65 expression. Upon the recognition of CpG ODN, TLR9 recruits specific intracellular adaptor proteins such as NF-κB to initiate signaling pathways. The ELISA assay was performed to evaluate the activation of NF-κB. The results showed that NF-κB p65 expression

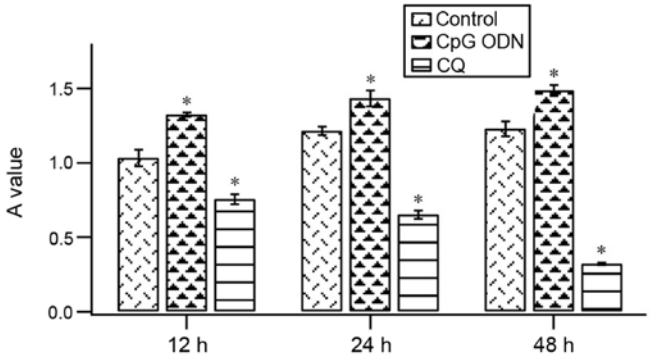

Figure 2. Effects of CpG ODN and CQ on cell viability of TE10 cells by MTT assay. Cells were pretreated with 10 μg/ml CpG ODN or CQ for 1 h, followed by treatment with 10 μg/ml CpG ODN for 12, 24 or 48 h. A value was measured in a microplate reader at the wavelength of 450 nm. Results were obtained from three independent experiments. *P<0.01 compared with control.

significantly increased at 12, 24 and 48 h of treatment of cells with 20 μg/ml CpG ODN by comparison with untreated cells indicating that CpG ODN activated NF-κB through the TLR9 signaling pathway (Fig. 3).

CpG ODN promotes MMP-2, MMP-7 and COX-2 mRNA expression. NF-κB activation leads to upregulation of the expression of many proinflammatory and apoptosis-related genes. Therefore, we investigated the effects of TLR9 on the gene expression of MMP-2, MMP-7 and COX-2 in TE10 cells treated with different concentrations of TLR9 ligand CpG ODN and inhibitor CQ. As shown in Fig. 4, the mRNA levels of MMP-2, MMP-7 and COX-2 were markedly increased in TE10 cells treated with CpG ODN, but were suppressed by CQ.

CpG ODN promotes TE10 cell migration. Cells scratch assay results showed that following CpG ODN treatment, cell

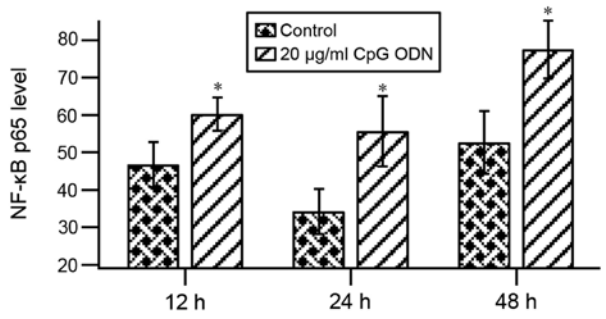

Figure 3. CpG ODN increases NF-κB p65 expression in TE10 cells measured by ELISA. Cells were treated with 20 μg/ml CpG ODN for 12, 24 or 48 h. Extracellular levels of NF-κB p65 were measured using an ELISA assay. Results are representative of three independent experiments. *P<0.05.

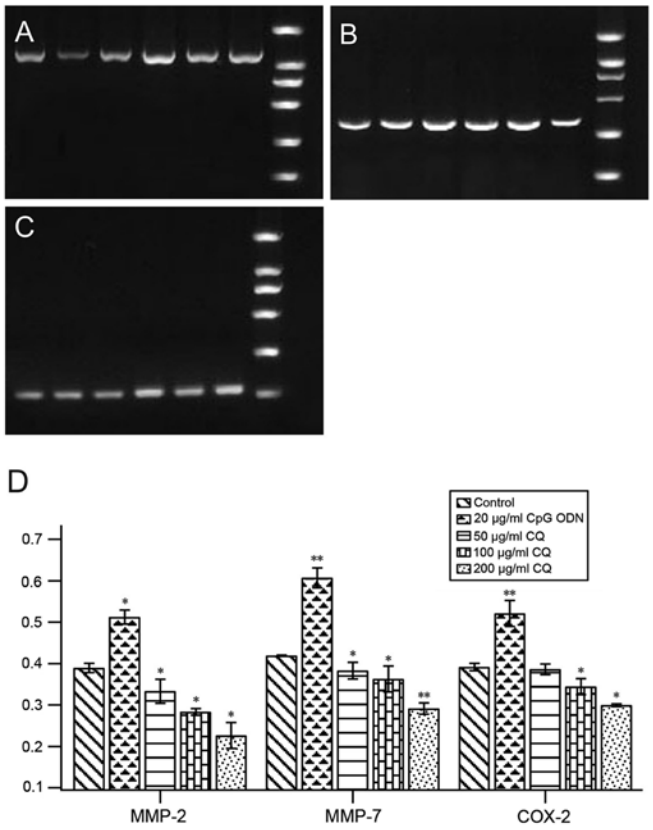

Figure 4. CpG ODN promotes mRNA levels of MMP-2, MMP-7 and COX-2 as detected by RT-PCR. (A) MMP-2, (B) MMP-7, (C) COX-2, (D) analyses of (A-C). From right: lane 1, marker; lane 2, 10 μg/ml CpG ODN; lane 3, 20 μg/ml CpG ODN; lane 4, 50 μg/ml CQ; lane 5, 100 μg/ml CQ; lane 6, 200 μg/ml CQ. *P<0.05, **P<0.01 compared with control.

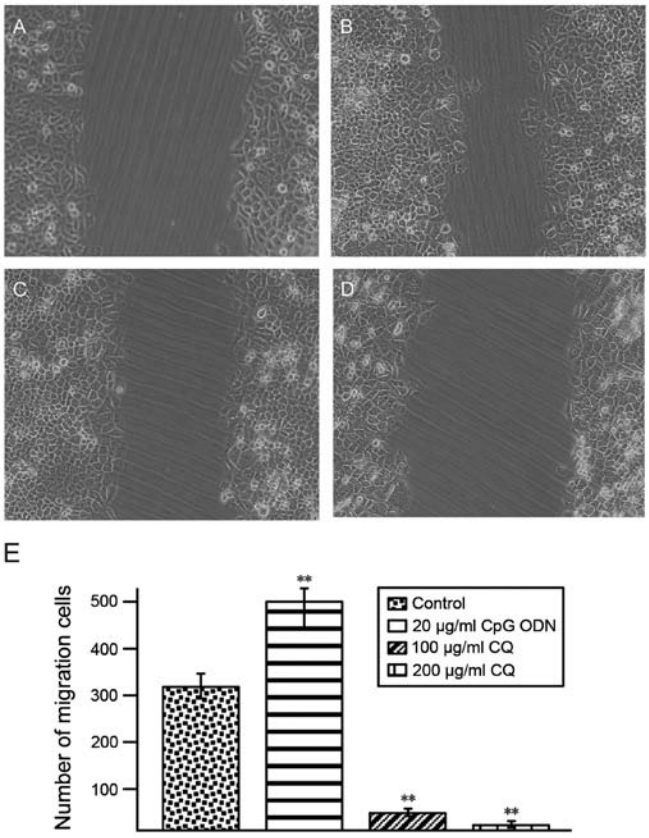

Figure 5. CpG ODN promotes TE10 cell migration by cell scratch assays. Cells were treated with 20 μg/ml CPG ODN or 100 μg/ml or 200 μg/ml CQ for 48 h and the supernatant was then abandoned and scratches were carried out in the center. A scratch was made in the confluent layer of cells using a sterile scraping cutter and the ability of cells to heal the scratch was analyzed. (A) Control, (B) 20 μg/ml CPG ODN, (C) 100 μg/ml CQ, (D) 200 μg/ml CQ, (E) analyses of (A-D). *P<0.05, **P<0.01 compared with control.

migration to the damage zone increased and the migrated cell numbers after 48 h of treatment were markedly increased (Fig. 5A). However, CQ treatment inhibited TE10 cell migration (Fig. 5B and C).

TLR9 expression in human EC and its association with EC clinicopathological characteristics. The expression of TLR9 in human EC tissues was examined using immunohistochemical method. To determine the prognostic value of TLR9 protein for EC patients, we assessed 60 esophageal carcinoma cases and 30 adjacent noncancerous tissues. Tissue sections were dewaxed and immunostained with anti-TLR9 primary antibody. The results in Fig. 6 show that TLR9 expressed in both carcinoma tissues and adjacent noncancerous tissues. However, among 60 esophageal carcinoma tissues, TLR9 expression was found in 48 cases and the positive expression rate was 80%. Among 30 adjacent noncancerous tissues, TLR9 expression was found in 17 cases and the positive expression rate was 56.7%. The positive rate in carcinoma tissues was significantly higher than that in noncancerous tissues (P<0.05). TLR9 protein located mainly in the cytoplasm and cell surface (yellow and brown).

The clinicopathological analyses indicated that TLR9 expression in esophageal carcinoma was closely related to the degree of tumor differentiation, tumor size, location, and Tumor Node Metastasis (TNM) stage (P<0.05), and there was no significant association with age, gender, lymph node metastasis of patients (P>0.05) (Table I).

Discussion

The present study analyzed the significance of TLR9 expression in cultured esophageal tumor cells as well as esophageal carcinoma tissues. We demonstrated that high basal level of

Table I. Statistical analysis of TLR9 expression and clinicopathological factors in esophageal carcinoma.

Characteristics	Patients	TLR9 (+) n (%)	χ^2	P-value
Adjacent tissue control	30	17 (56.7%)		
Esophageal carcinoma	60	48 (80%)	5.428	0.02
Age				
≥60 years	38	29 (76.3%)	1.103	0.294
<60 years	22	14 (63.6%)		
Gender				
Male	37	25 (67.6%)	0.799	0.371
Female	23	18 (78.3%)		
Position				
Upper esophagus	48	42 (87.5%)	6.257	0.012
Lower esophagus	12	6 (50.0%)		
Tumor size				
≥5 cm	27	14 (51.9%)	4.848	0.028
<5 cm	33	26 (78.8%)		
Tumor metastases				
Positive	19	14 (73.7%)	1.699	0.192
Negative	41	23 (56.1%)		
Tumor stage				
I/II	36	21 (58.3%)	4.159	0.041
III/IV	24	20 (83.3%)		
Tumor differentiation				
Well and moderately differentiated	38	20 (52.6%)	5.111	0.024
Poorly differentiated	22	18 (81.8%)		

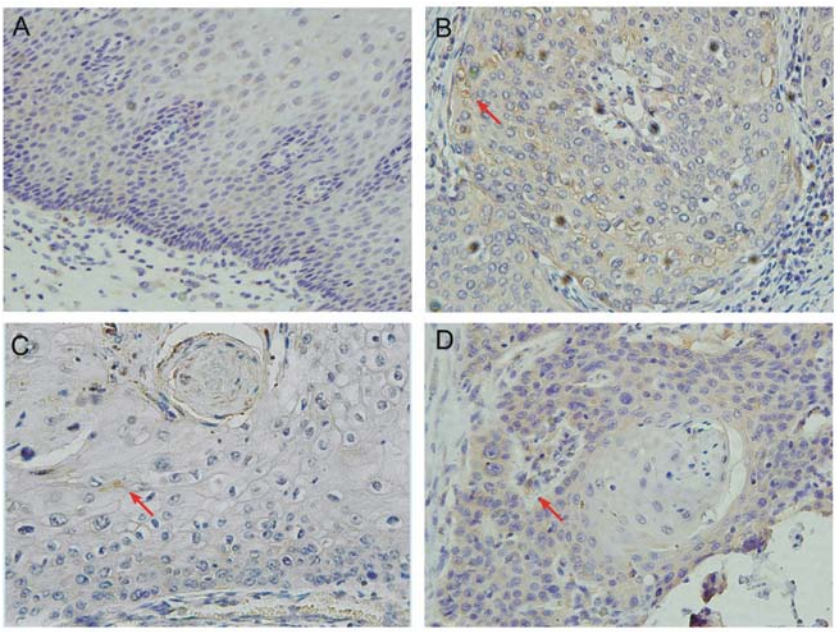

Figure 6. Immunohistochemical analyses of TLR9 expression in human EC tissues (10x40, yellow and brown, arrow). (A) Adjacent noncancerous tissues, (B) low differentiation EC tissue, (C) middle differentiation EC tissue, (D) high differentiation EC tissue.

TLR9 is present in the EC cell line, TE10, consistent with its overexpression and localization in esophageal carcinoma tissues, indicating an association of TLR9 expression with invasion and prognosis of EC.

TLR9 expression has been described in various cancer cell lines and different human tumors (8-11). To date, the association between TLR9 and clinicopathological parameters of cancer patients has not been widely evaluated. Tanaka *et al*

demonstrated that cell surface stimulation of TLR9 promotes cell proliferation and survival in human hepatocellular carcinoma (12). TLR9 expression has been shown to be significantly higher in high grade gliomas compared to low-grade gliomas (13). In breast cancer, it has been shown that the mRNA and protein levels of TLR9 significantly increased in recurrent breast carcinomas cells (14,15). It has been reported that the gene of TLR9 is correlated with the invasive and metastatic potential of human pancreatic carcinoma (16). In lung cancers, TLR9 was found to express in a selection of human lung cancer tissues and various lung tumor cell lines (17). These findings suggest that high TLR9 expression and TLR9 signaling promote tumor growth, survival and immune evasion (18). Thus, in this study, we investigated the possible role of TLR9 in EC. Firstly, we measured the TLR9 expression in EC TE10 cells by western blotting, RT-PCR and flow cytometric techniques. The results from different methods showed that TLR9 expressed in TE10 cells. The results were consistent in three experiments. The MTT assay showed that CpG ODN could promote EC cell proliferation and TLR9 inhibitor CQ could suppress this effect of CpG ODN. In a scratch assay, TE10 cell migration to heal a scratched area on the plate surface was measured. The data showed that CpG ODN treatment markedly enhanced cell migration, but CQ treatment inhibited cell migration. The data suggest a role of TLR9 in EC cell proliferation and migration.

TLR9 recognizes the ODN with CpG motif. Accumulating data indicated that TLR9 agonist CpG ODN can promote tumor development and metastasis (8-11,19). It has been proven that in TLR9 knockout mice, microbial DNA fragments cannot result in cancer invasion (20). It was also shown that the immunomodulating effect of natural and synthetic ODNs is mainly transmitted by TLR9 (21). After binding with CpG ODN, TLR9 signal pathway leads to the downstream activation of NF-κB and MAPK signaling pathways (22,23), which may upregulate the expression of proinflammatory and apoptosis-related genes. Thus, in this study, we investigated the effect of CpG ODN on NF-κB expression in EC TE10 cells. We found that CpG ODN could markedly activate NF-κB p65 expression by comparison with untreated cells. The results indicated that CpG ODN activates the NF-κB signaling pathway by binding to TLR9 in EC cells.

Next, we examined the mRNA levels of three downstream genes of NF-κB pathway upon CpG ODN treatment. MMPs are members of the neutral proteinase family. They were previously thought to be anti-fibrotic due to their ability to degrade and remodel of extracellular matrix. However, recent studies have shown that MMPs are implicated in tumor progression and migration (24-26). MMP-2 is a member of the metalloproteinase family, which degrades various components of the extracellular matrix (27). MMP-2 has been shown to be important in development and cell motility, and has an important role in cancer metastasis (28,29). MMP-7 is a highly active MMP family member, which can activate other family members, such as MMP-2 and MMP-9, and play a central role in the degradation of the extracellular matrix (30). It also inhibits apoptosis of cancer cells, reduces cell adhesion and induces angiogenesis, making it easier for the cancer cells to invade small blood vessels and lymphatic tube and metastasize (31,32). COX-2 has been extensively studied as an inducible expression protein and has been detected in various tumor tissues such as pancreatic cancer, colorectal carcinoma, non-small lung cancer positively correlates with tumor invasion and lymphatic metastasis (33,34). We then detected the effects of CpG ODN and CQ on mRNA expression of MMP-2, MMP-7 and COX-2. The results showed that CpG ODN increased MMP-2, MMP-7 and COX-2 mRNA expression and CQ inhibited their expression as expected indicating that CpG ODN may bind to TLR9, and then activates NF-κB, so to upregulate MMP-2, MMP-7 and COX-2 gene expression. However, the actual roles of these three factors in EC require further study.

Lastly, the expression of TLR9 in human EC tissues was examined and its association with EC clinicopathological characteristics was analyzed. In the present study, TLR9 highly expressed in carcinoma tissues but weakly expressed in adjacent noncancerous esophageal tissues. Similarly, Droemann *et al* found TLR9 highly expressed in lung cancer cells but weakly expressed in adjacent noncancerous lung tissues (17). In esophageal carcinomas, extensive TLR9 expression was associated with high tumor grade and TNM stage, but not with age, gender, lymph node metastasis of patients. Extensive TLR9 expression and association with poor differentiation has also been noted in breast and prostate cancer, which is consistent with our results (14,35). To date, there are very few reports on TLR9 expression in EC. Kauppila *et al* reported that high TLR9 expression was associated with poor differentiation, a high proliferation rate and increased TLR9 expression contributed to the growth and metastatic properties of esophageal adenocarcinoma (36). A study by Heikki *et al* showed that expression of TLR9 in the basal parts of normal esophageal epithelium related to cell proliferation and differentiation, and in dysplastic epithelium and in disseminated carcinomas indicated that TLR9 may serve as a novel marker for esophageal squamous dysplasia and carcinoma with metastatic potential (37).

In summary, we showed that TLR9 is highly expressed in cultured EC cells and in esophageal carcinoma tissues. CpG ODN could bind to TLR9 thus markedly activate NF-κB p65 expression and three cancer metastasis-related genes. In esophageal carcinomas, extensive TLR9 expression associated with high tumor grade and tumoral aggressiveness. Therefore, TLR9 might contribute to esophageal squamous cell carcinogenesis and may represent a suitable therapeutic target in EC. However, the mechanisms of the upregulation of TLR9 in esophageal malignancy require further investigation.

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