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ORIGINAL PAPER

TLR9 Signaling Promotes Tumor Progression of Human Lung Cancer Cell In Vivo

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Abstract Toll like receptor 9 (TLR9) was identified mainly in cells of the immune system, and CpG oligonucleotides (CpG ODN), which induces signaling through TLR9, are currently under investigation as adjuvants in clinical therapies against cancer. However, accumulating data suggested that functional TLR9 was also expressed in tumor cells and the effects of TLR9 signaling on the progression of tumor cells remain undefined. Our previous study demonstrated that the TLR9 signaling could significantly enhance the metastatic potential of human lung cancer cells in vitro. Here we carefully evaluated the direct effect of TLR9 signaling on tumor progression of human lung cancer cells in vitro and in vivo. We observed that TLR9 agonist CpG ODN could robustly enhance the tumor progression of 95D cells which expressed high level of TLR9 in nude mice. Furthermore, the CpG ODN could effectively induce the proliferation and IL-10 secretion of 95D cells in vitro. Finally, we demonstrated that CpG ODN could significantly elevate the tumor progression of TLR9 modifying 95C cells in vitro and in vivo, which could be dramatically abrogated by the inhibitory CpG ODN. Our findings indicated that the TLR9 signaling could promote the tumor progression of human tumor cells, which might provide novel insight into the implications for CpG based anti-tumor therapies.

Keywords CpG oligonucleotide · Lung cancer · TLR9 · Tumor progression · Tumor immunity

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Introduction

The discovery of a series of innate immune-specific receptors activated by pathogen-associated molecular patterns led to a new understanding of innate immunity mechanisms. Among the innate immune-specific receptors, the best characterized are the Toll-like receptors (TLR), which also play an essential role in adaptive immunity [1, 2]. TLR are evolutionarily well-conserved transmembrane proteins that are present in almost all multicellular organisms and recognize patterns specific of microbial components. In mammals, the TLR family is currently known to consist of 11 members, which exhibit specificity for pathogen-derived ligands. These receptors were identified mainly on cells of the immune system. The outcome of TLR activation is an immune reaction characterized by increased production of various proinflammatory cytokines and interleukins [3–7].

In humans, TLR9 has been described to be expressed in B-lymphocytes, monocytes and plasmacytoid dendritic

cells. This receptor species recognizes specific oligodeoxynucleotide sequences with unmethylated CpG-ODN, which are currently under investigation as adjuvant in therapy against infections and cancers [8–10]. Accumulating data suggested that CpG-ODN, which has been found to function as Th-1 adjuvant, is able to activate dendritic cells via TLR9 [11–13]. This led to the idea to utilize CpG-ODN for induction of anti-tumor immune response as an adjuvant therapeutic strategy based on TLR9 targeting [14–17]. However, human malignant tumor cells and the tumor cell lines could also express functional active TLR9 responses to CpG ODN, which may play a role in the tumor immunity [18]. In addition, accumulating data indicated that TLR9 agonist from pathogens can also promote tumor metastasis through inflammation dependent mechanisms [19, 20]. Our previous study demonstrated that the TLR9 signaling could significantly enhance the metastatic potential of human lung cancer cells in vitro [21]. However, whether TLR9 signaling could contribute to the progression of tumor in vivo remains undefined. To address this issue, here we carefully evaluate the effect of TLR9 signaling on tumor progression of human lung cancer cells, which is the major cause of death of the tumor-bearing patients.

In this study, we investigated the direct effects of TLR9 agonist CpG ODN on tumor progress of human lung cancer cell line 95C and 95D cells in vivo, which expressed lower and higher level of TLR9 respectively. We demonstrated that CpG ODN could effectively enhance the tumor growth and lung metastatic score of 95D cells in nude mice, which might be related to the elevated proliferation and IL-10 secretion of 95D cells. Most importantly, our data showed that CpG ODN also enhanced the tumor growth and metastasis of TLR9 modifying 95C cells in vivo and reduced the survival time of TLR9 modifying 95C cells bearing nude mice. Our finding suggested that TLR9 signaling might could enhance the progression of tumor cells and would facilitate our further understanding of the active role of tumor cells in tumor immunotherapy based on TLR9 targeting.

Materials and Methods

Reagents and Animals

The following ODNs were used and purchased from Integrated DNA Technologies (Coralville, IO): CpG ODN2216 5′-GGGGGACGATCGTCGGGGG-3′; control, ODN1612: 5′-GCTAGAGCTTAGGCT-3′; and inhibitory, ODN1502: 5′-GAGCAAGCTGGACCTTCCAT-3′. CpG ODN has a phosphorothioate backbone that provides a high degree of nuclease resistance. All other reagents were purchased from Sigma-Aldrich unless stated otherwise. Human lung cancer cell line 95C and 95D cells were cultured at 37°C under 5% CO_2 in completed RPMI 1640 (GIBICO, Grand land, NY, USA) medium containing 10% heat-inactivated fetal bovine serum and supplemented with 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate. BALB/c nude mice used in this work were purchased from the Center of Experimental Animal of Fudan University with permission of both Shanghai Municipal Government and Central Government. All animal experiments were performed according to the guides for the Care and Use of Laboratory Animals (Ministry of Health P.R. China, 1998) and the guidelines of the Shanghai Medical Laboratory Animal Care and Use Committee.

MTT Assay

95C or 95D cells were seeded at 3×10^3 cells each well and incubated in the presence or absence of CpG ODN in the dose of 10 μg/ml at 37°C in 5% CO_2 in 96 well plates for 72 h. Assessment of cell proliferation was measured in terms of optical absorbance (OD) per well by a semi-automated tetrazolium-based colorimetric assay using MTT. On Days 3 of the stimulation, 20 μl of MTT solution (5 mg/ml) was added to each well and further incubated at 37°C for an additional 4 hours. Supernatants were then removed from the wells and 120 μl of DMSO was added. Each well was mixed thoroughly for 10 min to dissolve the dark blue crystals of formazan. Proliferation was determined by a Bio-Rad EIA reader (Bio-Rad, USA) at the wave length of 570 nm.

Flow Cytometry

Flow cytometry was performed on a FACS Calibur (BD Biosciences) with Cell Quest Pro software using directly conjugated mAbs against TLR9-FITC (BD Biosciences). TLR9 staining was conducted using the intracellular staining kit (eBioscience) and run according to the manufacturer's protocol. In brief, cells (1×10^6 cells) were first incubated with fixation/Permeabilization buffers for 45 minutes. After washing twice, cells were incubated with the specific TLR9-FITC mAb for 30 minutes at 4°C in 0.1% bovine serum albumin/PBS. Following extensive washing, the cells were analyzed.

Plasmid Construction and TLR9 Gene Transfection

Total RNA was extracted from 95D cell line performed with Trizol (Invitrogen, Carlsbad, CA, USA) and TLR9 gene was amplified with RT-PCR subsequently. Primers were described as follows: TLR9 sense, 5′GCGAATTC

GACCCTCTGGAGAAGCCC-3′; TLR9 antisense, 5′ GCCTCGAGGGCAGGCAGAGGTGAGGT-3′. The TLR9 gene was then subcloned into the EcoRI and XhoI sites of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA, USA). 1 x 10^6 95C cells were transfected with the pcDNA3 or pcDNA3-TLR9 plasmids by electroporation with 280 V and 7 ms time in the total volume of 400 μl and screened in complete RPMI1640 medium containing 0.8 mg/ml G418 (Sigma, St Louis, USA). The individual G418 resistant clones were obtained by limiting dilution, then proliferated and expanded.

ELISA

95C or 95D cells were seeded at 3×10^3 cells each well and incubated in the presence or absence of CpG ODN in the dose of 10 μg/ml at 37°C in 5% CO_2 in 96 well plate for 72 h. Secretion of IL-10 from 95C or 95D cells with or without CpG ODN treatment was determined using commercial ELISA kits for human IL-10 (BD Biosciences). Samples (culture supernatants) were collected from replicated cultures and measured without dilution.

Evaluation of Tumor Growth In Vivo

Evaluation of tumor growth was performed as described previously with minor modifications [22]. Briefly, BALB/c nu/nu mice (6–8 weeks old) were injected subcutaneously with 0.2 ml of a single-cell suspension containing 2×10^6 tumor cells and kept in laminar flow cabinets under specific pathogen-free conditions. Tumors were measured every 3 or 4 days following tumor challenge using vernier calipers. Tumor volumes were obtained by multiplying the measured length by the measured width by the calculated mean of these measured values and were presented as the mean ± SEM. For detection of disease progression and metastases, cytospin preparations of single cell suspensions from spleen, lung, and draining lymph nodes were obtained, fixed with methanol, and stained with eosin and methylene blue (Fisher, Pittsburgh, PA). Although tumor cells appeared heterogeneous in size, they were easily differentiated as predominately larger cells with an elevated nuclear to cytoplasm ratio. Counts were performed on a total of 200–300 cells on coded slides.

Statistical Analyses

Statistical analyses of the data were performed with the aid of analysis programs in SPSS12.0 software. Statistical evaluation was performed using One-way analysis of variance (ANOVA; $p<0.05$) using the program PRISM 4.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

TLR9 Signaling Enhanced Tumor Progression of 95D Cells in Nude Mice

95C and 95D cell lines, which were of low and high metastatic potential respectively, were subcloned from a low differentiated human large cell lung carcinoma cell line PLA-801. Our previous study demonstrated that the CpG ODN could significantly elevate the metastatic potential of 95D cells but not 95C cells, which expressed higher and lower level of TLR9 respectively [21]. Here we investigated the direct effects of TLR9 agonist CpG ODN on the tumor progression of 95C and 95D cells in vivo. Groups of eight nude mice were challenged with 95C cells or 95D cells. Five days later, the tumor bearing mice were then injected in situ with 100 μg of the CpG ODN or the control CpG ODN at seven day intervals. As shown in Fig. 1a, the tumor progression of 95D cells in nude mice was significantly elevated in the CpG-ODN treated group compared to that in the control CpG-ODN treated group ($p<0.05$). However, we failed to observe a significant effect of CpG ODN on the tumor progression of 95C cells in nude mice (Fig. 1b). Furthermore, we found that the lung metastatic score of 95D cells but not 95C cells was significantly elevated in CpG ODN treated group compared with the control groups (Fig. 1c), which was consistent with our previous study in vitro [21]. These results showed that the CpG ODN could enhance the tumor progression of 95D cells which expressed high level of TLR9 in nude mice.

TLR9 Signaling Endowed Powerful Proliferation and IL-10 Secretion of 95D Cells

To elucidate the underlying mechanisms of the different effects of CpG ODN on 95C and 95D cells, 95C and 95D cells were stimulated with the CpG ODN and the proliferative response was determined. As shown in Fig. 2a, the CpG ODN robustly induced proliferative response of 95D cells compared with the control CpG ODN ($p<0.05$). However, CpG ODN failed to induce proliferative response of 95C cells (Fig. 2b). We further detected the IL-10 secretion of CpG ODN treated 95C or 95D cells. The production of IL-10 from 95D cells was also significantly elevated by the CpG ODN stimulation compared with the control CpG ODN stimulation ($p<0.05$). Similar to previous data, we also failed to observe the enhancement of IL-10 secretion from 95C cells induced by CpG ODN (Fig. 2c). These results may partially explain the different effects of CpG ODN on the tumor progression of 95C and 95D cells in vivo.

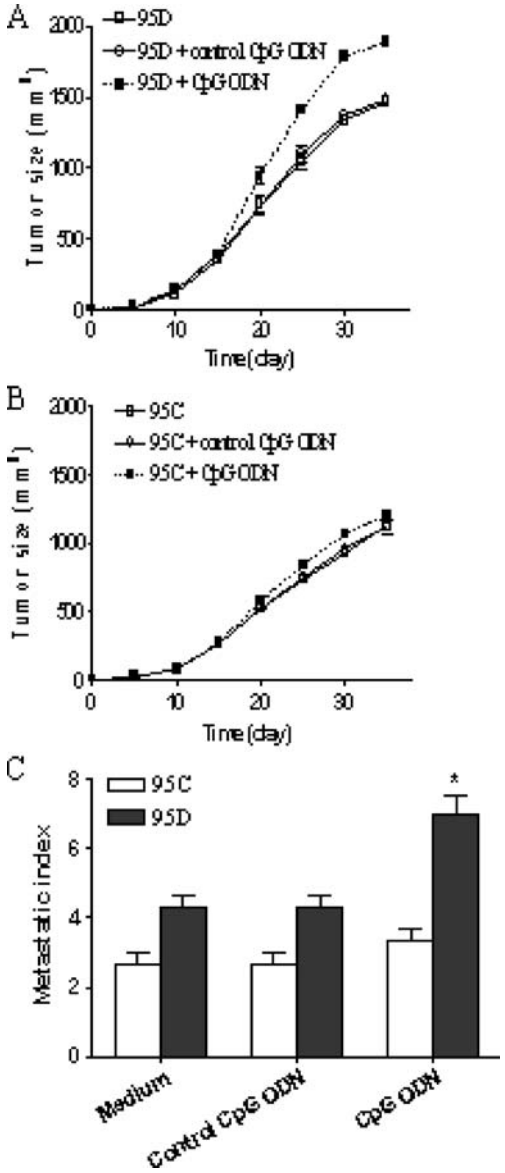

Fig. 1 CpG ODN enhanced tumor progression of 95D cells in nude mice. Groups of eight nude mice were challenged with 2×10^6 of 95C cells or 95D cells. 5 days later, the tumor bearing mice were injected in situ with 100 μg of CpG ODN at 7 day intervals. The control group received equal dose of the control CpG ODN or the equal volume of the medium. The tumor size of 95D cells (**a**) or 95C cells (**b**) and their metastatic index to lung (**c**) were performed. Each bar represents the means (±SD) from 8 nude mice in each group. *P<0.05

TLR9 Agonist Enhanced the Proliferation and IL-10 Secretion of TLR9 Modifying 95C Cells

To further confirm the effect of TLR9 signaling on progression of tumor cells, 95C cells were stably transfected with TLR9 (TLR9 modifying 95C cells, termed as 95C/TLR9) and the expression of TLR9 was firstly detected. As shown in Fig. 3Ad and 3Af, the TLR9 expression in 95C/TLR9 cells was comparable to that in 95D cells. Importantly, after treatment with CpG ODN, we found that the proliferation of 95C/TLR9 cells was significantly higher than that of 95C cells (p<0.05),

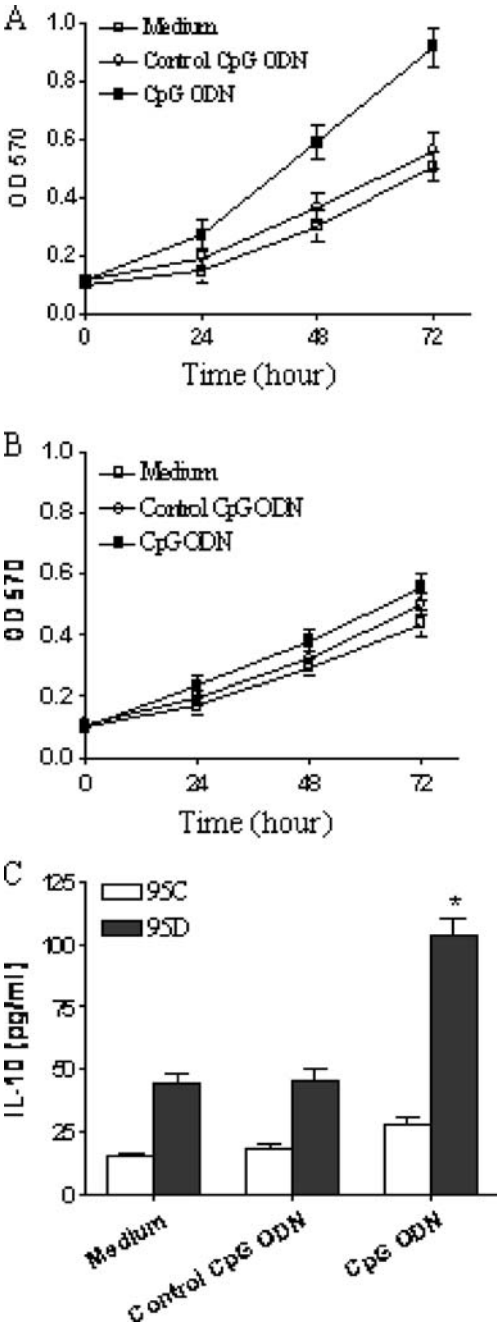

Fig. 2 CpG ODN induced proliferation and IL-10 secretion of 95D cells. 95C or 95D cells were seeded at 3×10^3 cells/well and incubated in the presence or absence of CpG ODN in the dose of 10 μg/ml in 96 well plates for 72 h. The proliferative responses of 95D cells (**a**) and 95C cells (**b**) were determined by a Bio-Rad EIA reader at the wave length of 570 nm using MTT assay. The concentration of IL-10 in cultured supernatants was determined by ELISA assay (**c**). Error bars indicate standard deviation of triplicate measurements. Data are representative of 3 independent experiments. *P<0.05

which was generally comparable to that of 95D cells (Fig. 3b). Furthermore, the IL-10 secretion of 95C/TLR9 cells was also significantly elevated ($p<0.05$) (Fig. 3c). To confirm that TLR9 was responsible for the effects of CpG ODN on TLR9 modifying 95C and 95D cells, the 95C/TLR9 and 95D cells were stimulated with CpG ODN in the presence of inhibitory CpG ODN. We found that both the proliferation and IL-10 secretion of 95C/TLR9 and 95D cells induced by CpG ODN were inhibited by the inhibitory CpG ODN in a dose dependent manner (Fig. 3d and e). These results suggested that the distinct expression level of TLR9 was responsible for the different effects of CpG ODN on the proliferation and IL-10 secretion of human lung cancer cells in vitro.

TLR9 Agonist Promoted the Tumor Progression of TLR9 Modifying 95C Cells In Vivo

To further confirm our previous data in vivo, the nude mice were challenged with 95C/TLR9 cells and were then injected in situ with the CpG ODN at seven day intervals as described previously. We found that the tumor progression and lung metastatic score in 95C/TLR9 cell bearing mice but not the 95C cell group were obviously elevated by the CpG ODN treatment, which was generally comparable to that in the 95D cell group (Fig. 4a and b). Furthermore, the survival time of 95C/TLR9 cell bearing mice was significantly reduced than that of the 95C cell bearing mice (Fig. 4c). In addition, the inhibitory CpG-ODN could obviously inhibit the tumor progression and lung metastatic

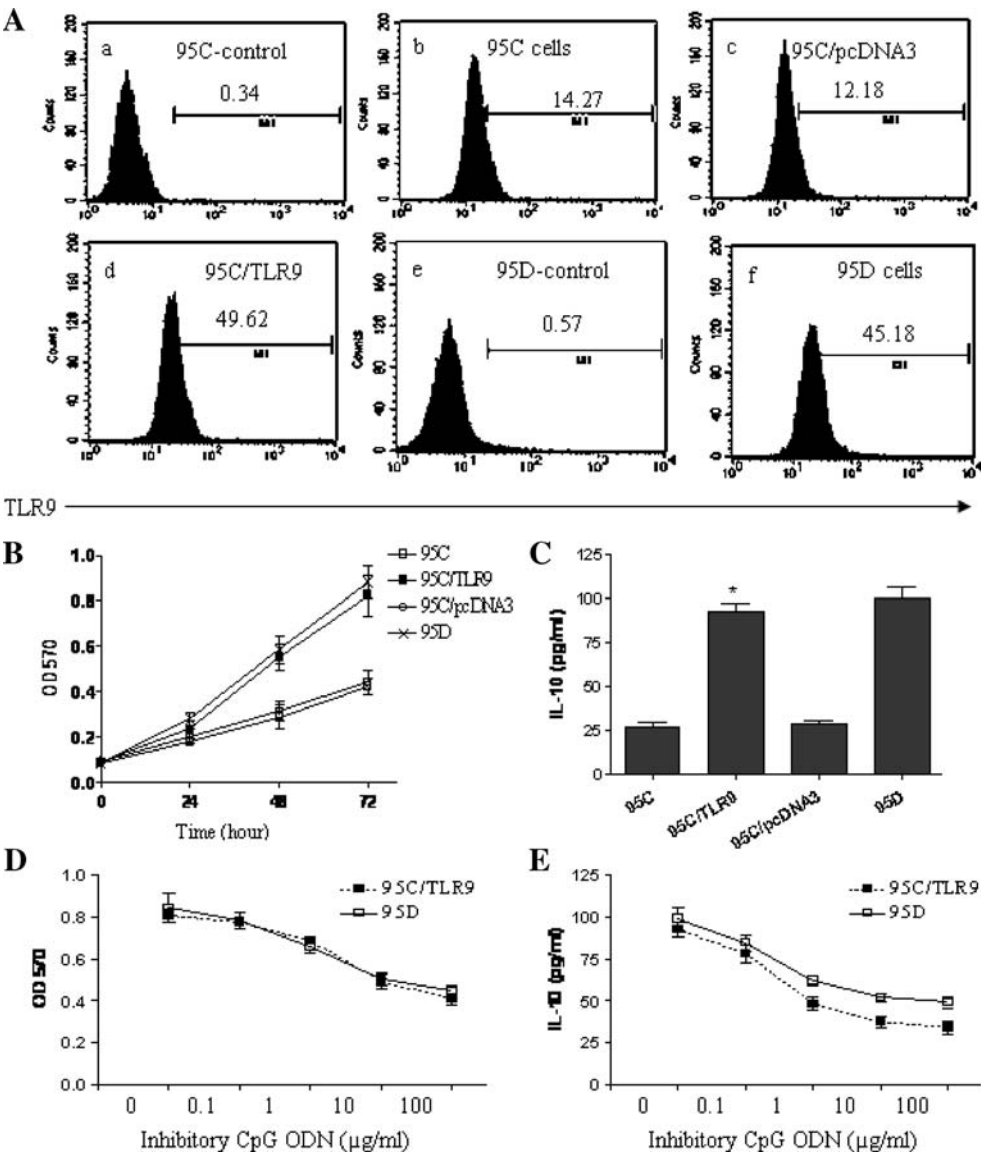

Fig. 3 CpG ODN enhanced the proliferation and IL-10 secretion of TLR9 modifying 95C cells. (**a**) Flow cytometric analysis was performed to determinate the expression of TLR9 in 95C cells, 95D cells, and 95C cells transfected with pcDNA3 (95C/pcDNA3) or TLR9 (95C/TLR9 cells). (**b**) The indicated cells were seeded at 3×10^3 cells/well and incubated with CpG ODN in the dose of 10 μg/ml for the indicated hours and the proliferative responses were determined. (**c**) The indicated cells were stimulated with 10 μg/ml of CpG ODN in vitro for 72 h, and then the IL-10 secretion was assayed. The indicated cells were stimulated with 10 μg/ml of CpG ODN in the presence of the indicated dose of inhibitory CpG ODN for 72 h. Then the proliferative response (**d**) and IL-10 secretion (**e**) were determined. Error bars indicate standard deviation of triplicate measurements. Data are representative of 3 independent experiments. *$P<0.05$

score of 95C/TLR9 cells in nude mice treated with CpG ODN (Fig. 4a and b), accompanied with the prolonged survival time of 95C/TLR9 cell bearing nude mice (Fig. 4c). These results strongly suggested that TLR9 signaling could enhance the tumor progression of human lung cancer cells in vivo.

Discussion

In the present study, we demonstrated for the first time that TLR9 signaling could enhance the tumor progression of human lung cancer cell line 95D cells in vivo. Furthermore, we found that TLR9 agonist CpG ODN has a potent stimulatory effect on the proliferation and the secretion of IL-10 of 95D cells which highly expressed TLR9. Finally, we demonstrated that the tumor growth and lung metastasis of TLR9 modifying 95C cells were also significantly enhanced by CpG ODN treatment in vivo, which could be abrogated by the inhibitory CpG ODN.

Tumorigenesis is a complex, multistep process that includes cellular neoplastic transformation, resistance to apoptosis, autonomous growth signaling, emergence of a vascular supply, evasion of immunologic surveillance, and the acquisition of invasive/metastatic properties. Up to now, more and more functional molecules involved in tumorigenesis were detected [23, 24]. Toll-like receptors (TLR), for recognition of the pathogen-associated molecular pattern, are widely explored in innate and adaptive

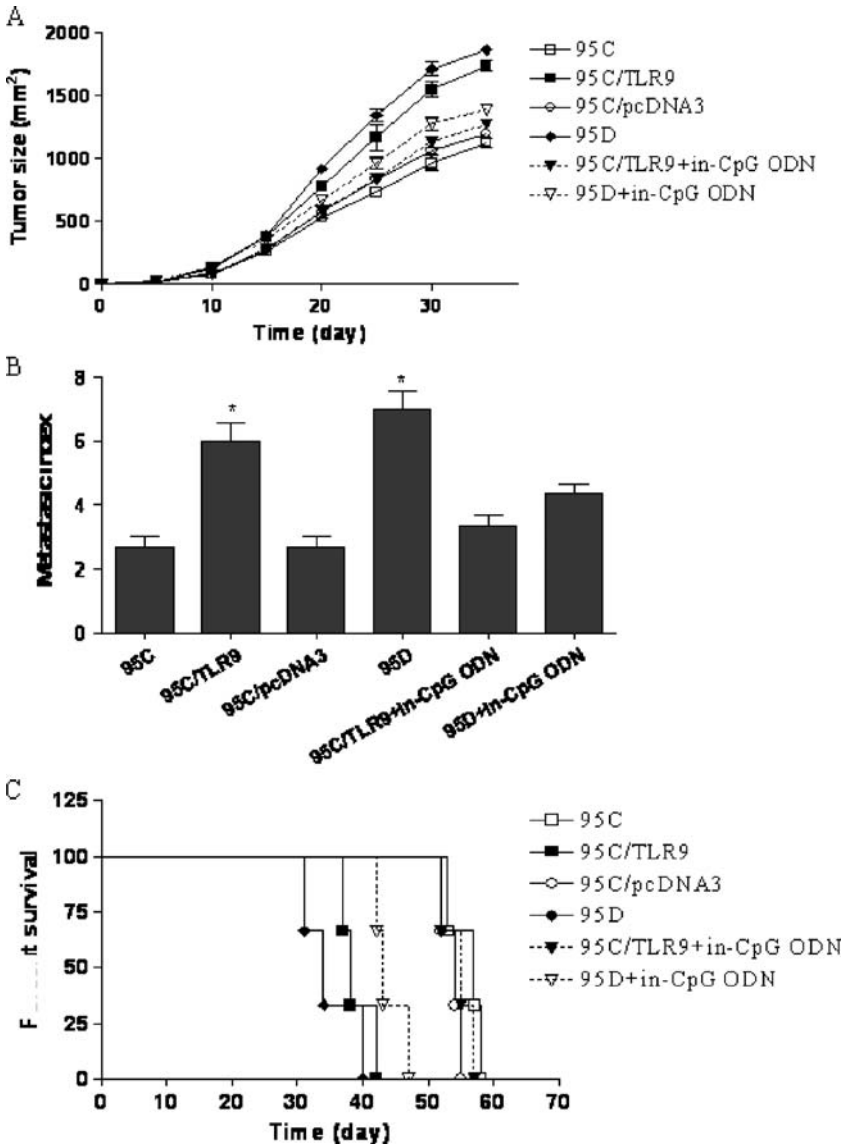

Fig. 4 CpG ODN promoted the tumor progression of TLR9 modifying 95C cells in vivo. Groups of eight nude mice were challenged with 2×10^6 of indicated tumor cells mentioned above. 5 days later, the tumor bearing mice were injected in situ with 100 μg of the CpG ODN combined with or without 100 μg of the inhibitory CpG ODN at 7 day intervals. The tumor size (**a**), metastatic score (**b**) and the survival time (**c**) were performed. Here the in-CpG ODN represents inhibitory CpG ODN. Each bar represents the means (±SD) from 8 nude mice in each group. *$P<0.05$

immune system [25, 26]. Recent studies reported that functional TLR including TLR9 were also detected on tumor cells [27–29]. Merrell et al reported that TLR9 agonist could promote the invasion of TLR9 expression tumor cells by increasing matrix metalloproteinase activity [30]. Our previous study also demonstrated the active role of TLR9 in the invasive potential of human lung cancer cells [21]. However, whether TLR9 signaling could contribute to the progression of tumor cells in vivo remains undefined. Here, we extended our previous work by demonstrating that TLR9 agonist could significantly enhance the tumor progression of human lung cancer cells determined by the tumor growth and metastatic score in nude mice. Similarly, Kelly et al have reported that TLR4 signaling could promote the growth of tumor cells [31]. All of these data suggested that functional expression of TLR were related to the tumor progression. However, the underlying mechanism of TLR signaling on the progression of tumor still remains to be elucidated.

Furthermore, we found that the TLR9 agonist could significantly enhance the proliferation and the secretion of IL-10 of 95D cells in vitro. This was worthwhile because IL-10 was found to be important factors related to tumor angiogenesis [32, 33]. The effect of TLR9 signaling on proliferation of 95D cells was consistent with previous findings which demonstrated that stimulation of the TLR9 expressing chronic lymphocytic leukemia B cells with CpG-ODN showed a marked proliferation effect [34]. These data suggested that TLR9 signaling could enhance tumor progression in vivo, which might be related to the enhanced proliferation and metastatic potential of tumor cells.

In this study, to further investigate the effect of TLR9 signaling on the progression of tumor cells, we transferred TLR9 into 95C cells which expressed low level of TLR9. Importantly, we observed a similar response of TLR9 modifying 95C cells (95C/TLR9 cells) to CpG ODN compared with that of 95D cells. Besides, the stimulatory effects of CpG ODN to 95D cells and 95C/TLR9 cells were dramatically inhibited by the inhibitory CpG ODN. All these data suggested that the TLR9 signaling could promote the progression of tumor cells.

In conclusion, we showed that the TLR9 agonist CpG ODN has a direct effect on the progression of human lung cancer cells by enhancing proliferation and invasive ability of tumor cells both in vitro and in vivo. These data suggested that TLR9 signaling might promote the progression of tumor. However, successively broad screening approaches on the effect of TLR9 signaling on other tumor cells will be worthwhile to further substantiate these initial results, which might provide a novel insight on the active role of tumor cells in the utility of CpG-ODN based anti-tumor therapies.

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