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Original Research

Methicillin-Resistant *Staphylococcus Aureus* infection exacerbates NSCLC cell metastasis by up-regulating TLR4/MyD88 pathway

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is a major public health problem worldwide, which brings to a more great threat for cancer patients. It's necessary to give attentions to lung cancer combined with MRSA. This study mainly focuses on the influences of MRSA on lung cancer cells (A549). We first found that MRSA infection can enhance metastasis ability of A549 cell and increase matrix metalloproteinase (MMP2 and MMP9) expressions in MRSA-infected A549 cell. Toll-like receptors (TLRs) have been reported to play an important role in tumor cell initiation and migration, and regulate the expression of MMPs in tumors. Our further research indicates that Toll-like receptor 4 (TLR4)/molecules myeloid differentiation factor 88 (MyD88) signaling was up-regulated in MRSA-infected A549 cell. After silencing TLR4 or MyD88 gene, the enhanced metastasis ability of A549 cell by MRSA was decreased significantly; Also, MMP2 and MMP9 expression increase was reversed. In conclusion, MRSA infection can enhance NSCLC cell metastasis by up-regulating TLR4/MyD88 signaling.

Key words: Methicillin-resistant *Staphylococcus aureus*, Non-small cell lung cancer, Matrix metalloproteinases, Toll-like receptor 4, Myeloid differentiation factor 88.

Introduction

Staphylococcus aureus is the most commonly isolated human bacterial pathogen. *S. aureus* isolates that are resistant to methicillin and generally to other β-lactam antimicrobial drugs are termed methicillin-resistant *Staphylococcus aureus* (MRSA) (1). MRSA infections can be commonly seen in hospitals, the community and livestock (2), which has been a major public health problem worldwide, being associated with high morbidity and mortality (3, 4).

Currently, vancomycin is the mainstay therapy for MRSA infection (5). Interestingly, a recent study has revealed that the use of vancomycin for treatment of MRSA bloodstream infections (BSIs) combined with cancer has a high failure rate, as well as a high mortality (6). This bespeaks that MRSA infections bring to a more great threat for cancer patients. We suspected that there may be some strategies adopted by MRSA to accelerate the advance of cancers in patients. Hence, a new survey focuses on the influences of MRSA on cancer should get more attentions.

Among all of the MRSA infections, pulmonary and bloodstream infections may be the most aggressive and difficult to treat, which are usually associated with significant morbidity and mortality (6). Lung cancer is one of the most common cancers also with high mortality worldwide (7). Approximately 80%–85% of the lung cancer are non-small cell lung cancer (NSCLC) (8, 9). The incidence of pathogenic bacterial infection was high in lung cancer (10). And the major pathogens responsible for pulmonary infection were Staphylococcus aureus including MRSA, Haemophilus influenzae, Klebsiella spp. and Pseudomonas aeruginosa (10). Therefore, it's quite important and meaningful to investigate the influences of MRSA on NSCLC.

As a myriad of studies have documented the ability of *S. aureus* to produce metastatic or secondary infections such as osteomyelitis, septic arthritis, and endocarditis (6), we speculated that MRSA also possesses the ability to promote metastasis. Based on this speculation, we detected the invasion and migration abilities of MRSA-infected A549 cell, which is one of the human NSCLC cell lines. We found that MRSA infection can enhance invasion ability of A549 cells, and induce A549 cell migration.

Toll-like receptors (TLRs), which comprise an evolutionarily conserved family of receptors, play a crucial role in the activation of innate immunity and inflammatory process (11-13). However, TLR expression is not restricted to immune cells but has also been demonstrated in many tumor cells from a wide variety of organs, such as lung cancer cells (14). This suggests that TLR signaling plays a key role in tumor development. Increasing evidence indicates that TLRs play an important role in tumor cell initiation and migration (15, 16), and can regulate the expression of MMPs (17). It was reported that MRSA infection can induce TLR 1, 2, and 6 expression in human mesenchymal stem cells (hMSCs) (18). We speculate that MRSA-induced A549 cell invasion and migration may be related to TLR members. Currently, there are 13 known types of mammalian TLR, of which TLR2 (19, 20), TLR3 (21), TLR4 (22, 23), TLR7 (24), TLR9 (25) are commonly associated with cancer metastasis. The role of these TLRs in MRSA-enhanced A549 cell metastasis thus therefore needs to be investigated.

In the present study, MRSA infection is found to promote NSCLC cell invasion and migration, which can

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be partly explained by increased MMP2 and MMP9 in MRSA-infected A549 cells. In addition, TLR4/MyD88 signaling can be up-regulated in MRSA-infected A549 cells. After silencing TLR4 or MyD88 gene, the invasion and migration abilities of MRSA-infected A549 cell were markedly decreased, and MRSA-induced MMP2 or MMP9 expression increase was reversed. Hence, we concluded that MRSA can enhance NSCLC cell metastasis by up-regulating TLR4/MyD88 signal pathway.

Materials and Methods

MRSA and Cell line

MRSA strain (Cat No. 33591™) and A549 cell line (Cat No. CCL-185™) used in this study were purchased from ATCC (Rockville, Maryland, USA). MRSA strain was grown in Tryptocase Soy Broth (TSB) (Sigma, St. Louis, MO) at 37°C in shaking conditions. A549 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Sigma), 50 U/ml of penicillin (Sigma), and 50 mg/ml of streptomycin (Sigma) under a humidified 5% CO_2 atmosphere at 37°C. During the study, cells were sub-cultured every 2-3 days.

In vitro Matrigel Invasion Assay

For the invasion assay, cell suspensions (2.5×10^5 A549 cells) in antibiotic-free DMEM were placed on the upper chamber (24-well) using inserts with 8 mm pore membranes pre-coated with Matrigel (BD Biosciences, MA, USA). Pelleted cells (MRSA) were washed twice in PBS and added to the upper chamber at the multiplicity of infection (MOI) of 1:100, 1:75, 1:50; 1:25 as MRSA infected groups. Appropriate TSB was added as control group. DMEM medium supplemented with 1% fetal bovine serum was added to the lower chamber. After incubation for 24 h, cells on the lower surface of the membrane were stained by H&E method (Sigma). The number of cells were counted under a light microscope (×400).

In vitro cell migration assay

A549 cells (1×10^6 per well) were seeded into 6-well plates and allowed to grow to post-confluence. Then, the medium was replaced with antibiotic-free and serum-free DMEM medium. Cell proliferation was suppressed by incubation with 25 μg/ml mitomycin C (Sigma) for 2 h. Two denuded areas (5 mm ×10 mm) were made in each well using a custom-made cell scraper. Simultaneously, MRSA were grown overnight in TSB at 37°C in shaking conditions until they reached an optical density of 1.0. Pelleted cells were washed twice in PBS and added to A549 cells at a MOI of 1:50 for a set time period (MRSA infection groups). TSB was added to A549 cells used as control group. After incubation for indicated time, cells were fixed with cold methanol (Sigma) and stained with Harris hematoxylin solution (Sigma). Migration was quantified by counting the number of cells migrated into the denuded area.

Quantitative RT-PCR

Total RNA was extracted from A549 cells using Trizol solution (Invitrogen, Carlsbad, CA, USA). A total of 4 μl RNA was used for the reverse transcription reaction and was transcribed using a RT kit (Roche Diagnostics, Mannheim, Germany) with oligo-dT (Amnion, Austin, TX) and random primers. Synthesized cDNA was measured on an ABI Prism 7700 Sequence Detection System using the SYBR Green Master Mix kit (Applied Biosystems, Foster City, USA). Primers are shown in Table 1. All the primers were synthesized by the Shanghai Sangon Biological Engineering and Technology Service (Shanghai, China). Data were analyzed using the ΔCt method and normalized to β-actin.

Western blot

A549 cells were lysed with RIPA buffer (Sigma). Protein concentration was detected by BCA assay (Sigma). Protein lysates were separated by SDS-PAGE (Sigma), then transferred onto PVDF membranes (Sigma). After blocking with 0.5% skimmed milk powder

Table 1. List of primers.

Gene name		Sequence from 5' to 3'
MMP2	Forward (F)	TGACATCAAGGGCATTCAGGAG
	Reverse (R)	TCTGAGCGATGCCATCAAATACA
MMP9	F	TCGAACTTTGACAGCGACAAGAA
	R	TCAGTGAAGCGGTACATAGGGTACA
TLR2	F	GGGTCATCATCAGCCTCTCC
	R	AGGTCACTGTTGCTAATGTAGGTG
TLR3	F	TGGTTGGGCCACCTAGAAGTA
	R	TCTCCATTCCTGGCCTGTG
TLR4	F	CAGAGTTGCTTTCAATGGCATC
	R	AGACTGTAATCAAGAACCTGGAGG
TLR7	F	GGCTAGATGGTTTCCTAAAACTCT
	R	GGTACACAGTTGCATCTGAAATCG
TLR9	F	GAAAGCATCAACCACACCAA
	R	ACAAGTCCACAAAGCGAAGG
MyD88	F	AGAGCTGCTGGCCTTGTTAGACC
	R	AGGCTTCTCGGACTCCTGGTT
TRIF	F	GGTTCACGATCCTGCTCCTGAC
	R	GCTGGGCCTGAGAACACTCAAG
β-actin	F	CTCCATCCTGGCCTCGCTGT
	R	GCTGTCACCTTCACCGTTCC

(Sigma) target proteins were probed by corresponding primary and second antibodies. The reactive bands were detected by enhanced chemiluminescence (ECL) and the relative levels of each protein to β-actin were analyzed. Rabbit anti-human MMP2 (ab37150), MMP9 (ab38898), TLR2 (ab108998), TLR3 (ab62566), TLR4 (ab13556), TLR7 (ab124928), TLR9 (ab37154), MyD88 (ab2068), TIR-domain-containing adapter-inducing interferon-β (TRIF; ab13810), and β-actin (ab119716) antibody were used as primer antibodies. HRP-conjugated goat anti-rabbit IgG (ab6721) used as the second antibody. All antibodies were obtained from Abcam (Cambridge, MA, USA).

siRNA transfection

A549 cells (5×10^5 cells/well) in 12-well plate at 70–80% confluence were transfected with TLR4 siRNA (20 pmol/well) or equivalent TLR4-scramble siRNA (Life Technologies, Grand Island, NY, USA) using lipofectamine 2000 (Sigma). Also, A549 cells (5×10^5 cells/well) in 12-well plate at 70%-80% confluence were transfected with MyD88 siRNA (20 pmol/well) or equivalent MyD88-scramble siRNA (Qiagen, Valencia, CA, USA) using lipofectamine 2000. Here, the scramble siRNAs matching for corresponding molecules were from different manufactures as described above. A549 cells only transfected with lipofectamine 2000 were used as Normal group. After 24 h of gene silencing, cells in different groups were washed with PBS and used for cell invasion and migration experiments with or without MRSA infection (MOI: 1:50) for 48 h.

Statistical analysis

The data obtained from three replicates were expressed as mean ± standard deviation (SD). Differences were analyzed by Student's-*t* test and considered statistically significant if $^{*}P<0.05$ or $^{\#}P<0.01$. Western blots were carried out for several times, and the results were similar for each detection. Ultimately, the best picture was chosen to present.

Results

MRSA promotes A549 cell metastasis

To explore the role of MRSA in NSCLC, we first detected the effect of MRSA infection on A549 cell invasion and migration *in vitro*. Results in Fig.1A shown that MRSA infection can obviously enhance A549 cell invasion with the increase of MOI ($^{*}P<0.05$ at MOI of 1:100 and 1:75; $^{\#}P<0.05$ at MOI of 1:50 and 1:25) when compared with control group. Based on the results of invasion assay, MOI of 1: 50 was chosen for following research. The results of the migration assay are presented in Fig.1B, which shows that the average number of migrated A549 cell in MRSA infection group was significantly higher than that in control group at time point of 24 h ($^{*}P<0.05$), 36 h and 48 h ($^{\#}P<0.05$). These results indicated that MRSA can indeed induce NSCLC (A549) cell metastasis, which is in keeping with our early assumption.

MMP2 and MMP9 were up-regulated after MRSA infection

Matrix metalloproteinases (MMPs) are major hydrolytic enzymes targeting extracellular matrix (ECM) during cell invasion and migration (26). Both MMP2 (27, 28) and MMP9 (29, 30) have been shown to play critical roles in cancer metastasis. Here, qRT-PCR and western blots were used to detect their expression. The results shown in Fig.2A and B suggest that the expression of MMP2 and MMP9 in A549 cells were significantly enhanced ($^{\#}P<0.01$ or $^{*}P<0.05$) after MRSA infection. Results in this section explain the enhanced invasion and migration ability of MRSA-infected A549 cells.

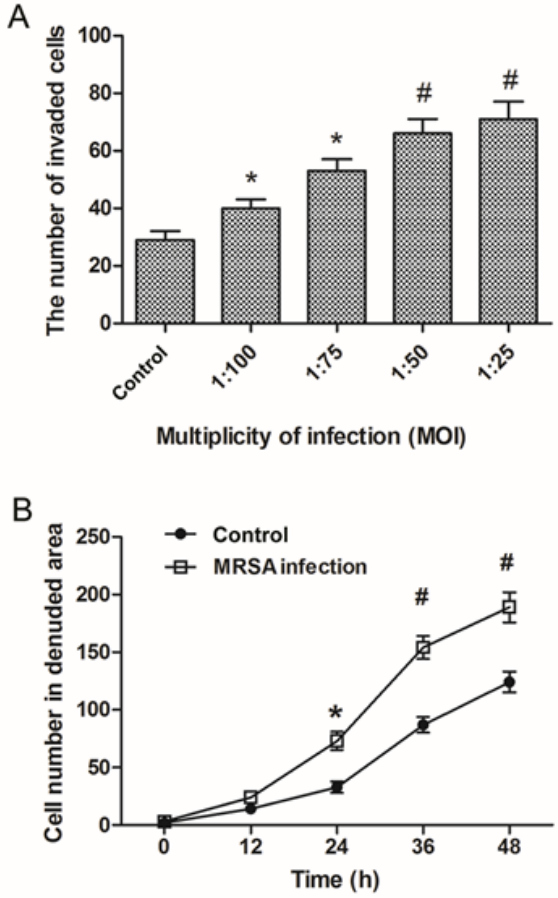

Figure 1. MRSA promotes A549 cell metastasis *in vitro*. The effect of MRSA on A549 cell invasion was evaluated by Matrigel invasion assay. A549 cell was infected with MRSA by different MOI (1:100, 1:75; 1:50; 1:25) for 24 h. (A) The average number of invaded A549 cells. The effect of MRSA on A549 cell migration was studied using cell migration assays. A549 cell was infected with MRSA at a MOI of 1:50 for indicated times. (B) The average number of A549 cells migrated into the denuded area over time. $^{*}P<0.05$ and $^{\#}P<0.01$ vs. Control group.

TLR4 was up-regulated in MRSA infected A549 cells

Toll-like receptor members (TLR2, 3, 4, 7, and 9) were detected by qRT-PCR and western blot in A549 cells with or without MRSA infection. Fig.3A and B indicate that only TLR4 expression was significantly up-regulated, while other TLRs such as TLR2, 3, 7 and 9 were unaffected. These results suggest that MRSA infection can dramatically increase TLR4 expression in A549 cells ($^{*}P<0.05$).

TLR4/MyD88 signaling was up-regulated by MRSA

The discovery of increased TLR4 expression in MRSA-infected A549 cell guided us further to measure its downstream MyD88 and TRIF. As shown in Fig.4A, the

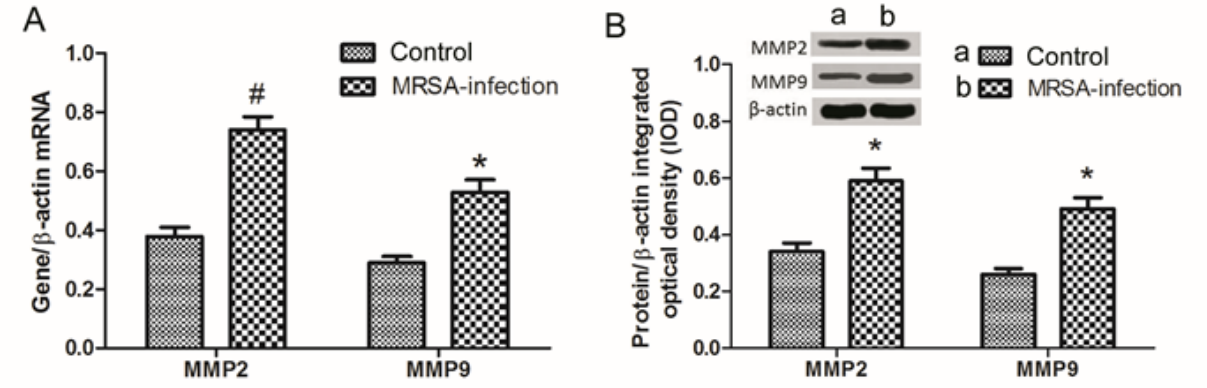

Figure 2. Expressions of MMP2 and MMP9 in A549 cells. A549 cell was infected by MRSA at a MOI of 1:50 for 48 h. (A) Levels of MMP2 and MMP9 mRNA were measured by qRT-PCR. (B) Expressions of MMP2 and MMP9 were detected by western blots. Integrated optical density (IOD) of each band was analyzed by Gel-Pro analyzer 4.0 software. Data were normalized to β-actin. $^{\#}P<0.01$ and $^{*}P<0.05$ v.s. Control group.

level of MyD88 mRNA was markedly increased in MRSA-infected A549 cells (*P<0.05), while TRIF mRNA level has no changes when compared with Control. In addition, similar results were detected by western blots shown in Fig.4B. These results indicate that MRSA infection induces the activitation of TLR4/MyD88 signal pathway in A549 cells, but not TLR4/TRIF signal pathway.

TLR4/MyD88 signaling contributes to MRSA-enhanced A549 cell metastasis

In order further to comfirm whether the TLR4/MyD88 signaling was necessary for MRSA to enhance A549 cell metastasis, TLR4 and MyD88 gene in A549 cells were silenced by corresponding siRNAs, respectively. TLR4-scrambe siRNA and MyD88-scramble siRNA were from different manufactures and used as negative controls. And the silence effectiveness was verified by qRT-PCR and western blots (Fig.5A and B). Then, cell invasion and migration abilities were measured again. The results shown in Fig.5C and D revealed that MRSA enhanced A549 cell invasion and migration were markedly inhibited (*P<0.05) after silencing TLR4. And similar results were also observed after silencing MyD88. These results indicate that MRSA infection exacerbates NSCLC cell metastasis by up-re-

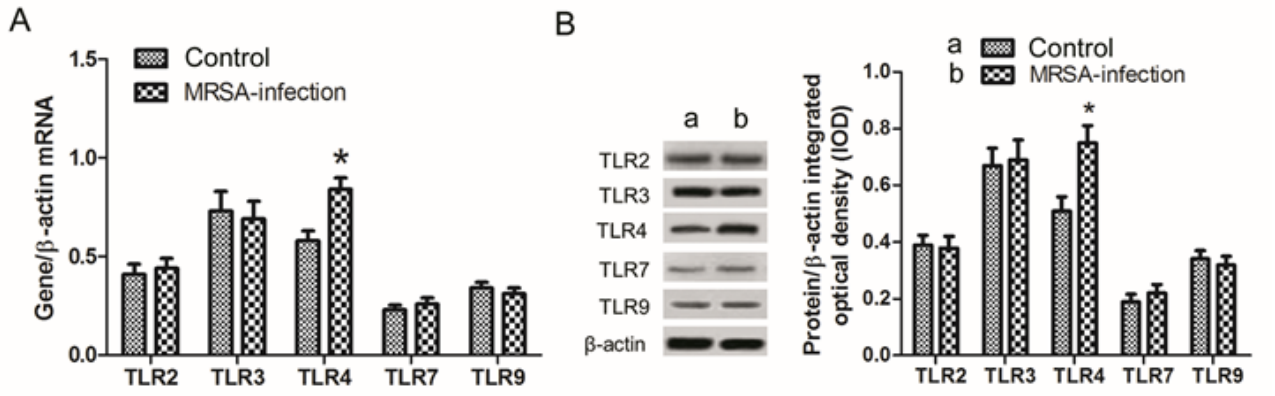

Figure 3. TLR4 is up-regulated in MRSA infected A549 cells. A549 cell was infected with MRSA at a MOI of 1:50 for 48 h. A549 cells without any treatment were used as Control. The expressions of TLRs listed in the methods were detected by qRT-PCR (A) and western blot (B). IOD of each band for TLRs and β-actin was analyzed by Gel-Pro analyzer 4.0 software. Data were normalized to β-actin. $^{*}P<0.05$ vs. Control group.

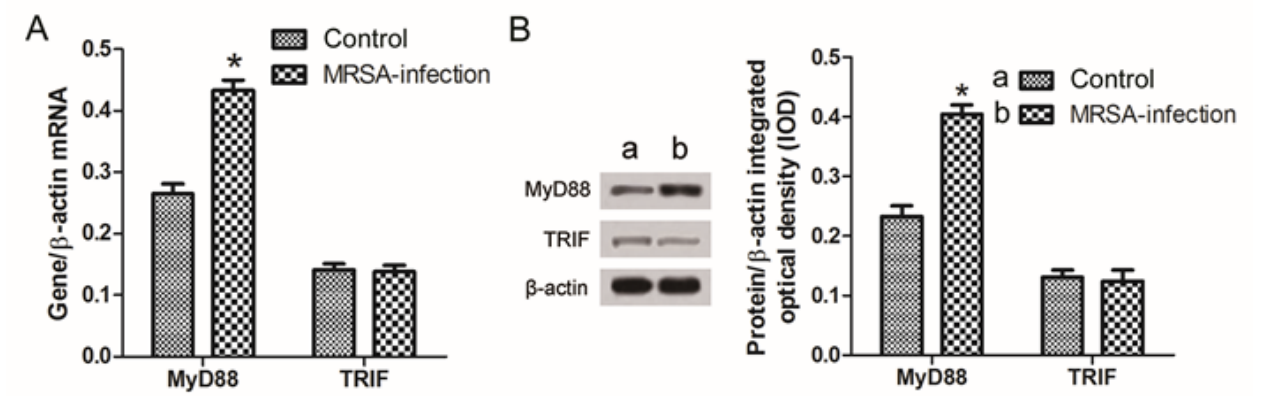

Figure 4. Expression of MyD88 and TRIF in A549 cells. A549 cell was infected by MRSA at a MOI of 1:50 for 48 h. (A) Levels of MyD88 and TRIF mRNA were measured by qRT-PCR. (B) Expressions of MyD88 and TRIF were detected by western blots. IOD of each band was analyzed by Gel-Pro analyzer 4.0 software. Data were normalized to β-actin. *P<0.05 v.s. Control group.

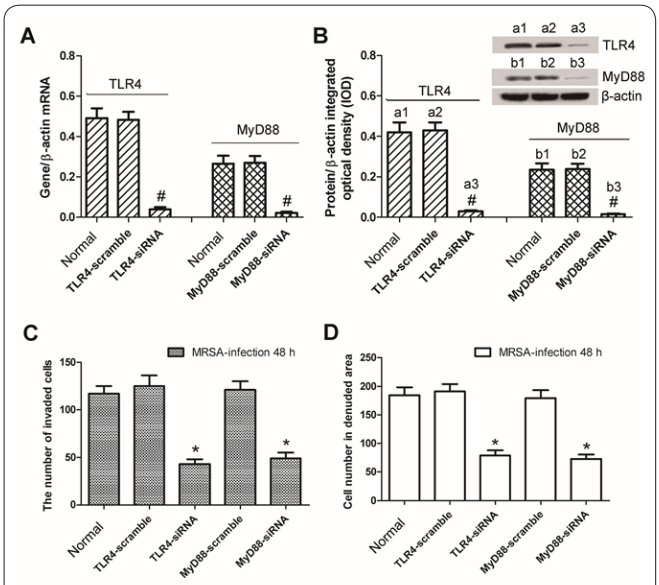

Figure 5. TLR4 or MyD88 deficiency inhibits MRSA-enhanced A549 cell metastasis. RNA interference was used to silence TLR4 or MyD88 gene in A549 cell. After siRNA transfection for 24 h, qRT-PCR (A) and western blot (B) were used to verify the silencing efficacy. IOD of each band was analyzed by Gel-Pro analyzer 4.0 software. Data were normalized to β-actin. $^{\#}P<0.01$ v.s. corresponding Normal or scramble siRNA group. After silencing TLR4 or MyD88, A549 cells in different groups were infected by MRSA at a MOI of 1:50 for 48 h. The invasion ability of A549 cell was measured by Matrigel Invasion Assay. (C) The average number of invaded A549 cells. The migration ability of A549 cell was measured by cell migration assay. (D) The average number of A549 cells migrated into the denuded area. $^{*}P<0.05$ vs. Normal or corresponding scramble siRNA group.

gulating TLR4/MyD88 signal pathway.

TLR4/MyD88 deficiency reduces MMP2 and MMP9 levels in MRSA-infected A549 cell

Accumulated evidences have demonstrated that activation of TLRs may induce MMPs expression, which can contribute to tumor invasion and metastasis (31) (32). After silencing TLR4/MyD88, cells were infected with or without MRSA, then, qRT-PCR was performed to remeasure MMP2 and MMP9 levels. As shown in Fig.6, we observed that TLR4 or MyD88 deficiency can significantly reduce the levels of MMP2 and MMP9 mRNA in MRSA-infected A549 cells. The above results indicate that up-regulated TLR4/MyD88 signal in MRSA-infected A549 cells led to up-regultaion of MMP2 and MMP9, which contribute to lung cancer metastasis.

Discussion

Previously, a myriad of researches have focused on the relationship between pathogenic bacteria and cancer (33). For instance, convincing evidence has linked helicobacter pylori with mucosa-associated lymphoid tissue (MALT) lymphoma (34), salmonella typhi with gallbladder cancer(35), streptococcus bovis with colon cancer(36), as well as chlamydia pneumoniae with lung cancer(37). The roles of bacterial species in particular cancers appear to differ among different individuals(33). Actually, bacteria not only can lead to a cancer(38, 39) but also resist a cancer(40-42) and possibly even play other roles.

MRSA infections are always difficult to treat due to multi-drug resistance (43). Among MRSA infections, MRSA pulmonary infection seems more aggressive (6). In the current study, we provide evidence that MRSA infection can exacerbate the metastasis of a major kind of lung cancer cell in *vitro*. First off, we found that MRSA infection can enhance the invasion and migration abilities of A549 cell. Also, increased MMP2 and MMP9 expression was detected in MRSA-infected A549 cell, which can be reduced when TLR4/MyD88 deficiency. Previous studies have proved that both MMP2 and MMP9 play critical roles in cancer metastasis(44), which are major hydrolytic enzymes targeting ECM during cell invasion and migration. Hence, the enhanced A549 cell invasion and migration by MRSA can be explained by the known activities of MMP2 and MMP9.

Currently, toll-like receptors (TLRs) expression and function in cancer and its association with tumorigenesis and tumor progression has become a very active research field (45). Our further research discovered that MRSA can up-regulate the TLR4/MyD88 signaling pathway in A549 cell, which actually mediated the invasion and migration of MRSA-infected A549 cell. A recent study by Maiti A et al. has reported that MRSA infection can induce TLR expression in hMSCs such as TLR1, 2, and 6 (18). They further proved that MRSA-mediated TLR ligands reduced osteoblast differentiation and increased hMSCs proliferation (18). While in our research, we discovered that MRSA infection can

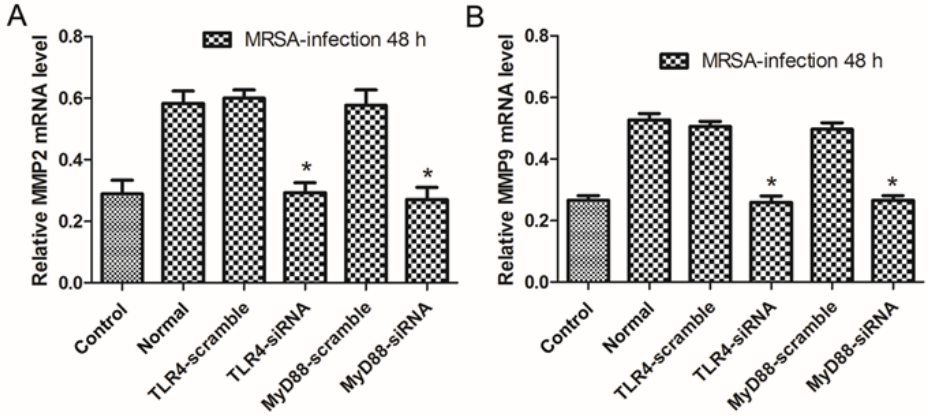

Figure 6. Levels of MMP2 and MMP9 mRNA in MRSA-infected A549 cells. After silencing TLR4 or MyD88, A549 cells in different groups were infected with or without MRSA at a MOI of 1:50 for 48 h. qRT-PCR was used to remeasure MMP2 and MMP9 mRNA levels. (A) Relative mRNA level of MMP2. (B) Relative mRNA level of MMP9. Data were normalized to β-actin. $^{*}P<0.05$ v.s. Normal group.

specifically induce TLR4 expression in NSCLC cell then result in enhanced cell invasion and migration, which supplies a new cognition on the role of TLRs in MRSA-infection.

In recent decades, TLR4/MyD88 signaling pathway has been reported to have oncogenic or cancer promoting effects in multiple kinds of cancer (46). Through TLR4/MyD88 signaling, lipopolysaccharide can increase the invasive ability of pancreatic cancer cell (47). And nickel can promote the invasive potential of human lung cancer cell (48). Besides, TLR4/MyD88 signaling can promote the migration of human melanoma cell (22), and facilitate the metastasis of hepatocellular carcinoma (23). In this study, we identified that MRSA infection can exacerbate NSCLC cell metastasis by up-regulaing TLR4/MyD88 signaling, which was in line with the cancer promoting effect of this signaling pathway.

In conclusion, MRSA infection can enhance NSCLC cell metastasis by up-regulating TLR4/MyD88 signaling pathway, which was due to the regulation of TLR4/MyD88 on MMP2 and MMP9 expressions. Hence, TLR4 may be a target molecule to weaken the threat of MRSA to NSCLC patients. In addition, previous studies have discovered that primary lung cancer patients are significantly more frequently colonized by MRSA (49). Based on our findings, it may be quite useful and important to resistant the MRSA existed in respiratory tracts of lung cancer patients. Our researchexposes the style and mechanism by which MRSA infection influences the progression of lung cancer, and supply a new strategy to control MRSA-infected lung cancer.

Acknowledgments

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