CpG Oligodeoxynucleotide1826 combined with radioresistant cancer cell vaccine confers significant antitumor effects

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Immunotherapy is a hot issue in cancer research over the years and tumor cell vaccine is one of the increasing number of studies. Although the whole tumor cell vaccine can provide the best source of immunizing antigens, there is still a limitation that most tumors are not naturally immunogenic. CpG Oligodeoxynucleotides (CpG ODNs), synthetic oligonucleotides containing a cytosine-phosphate-guanine(CpG) motif, was shown to enhance immune responses to a wide variety of antigens. In this study, we generated the radioresistant Lewis lung cancer cell by repeated X-ray radiation and inactivated it as a whole tumor cell vaccine to enhance the immunogenicity of tumor cell vaccine. Mice were subcutaneously immunized with this inactivated vaccine combined with CpG ODN1826 and then inoculated with autologous Lewis lung cancer (LLC) to estimate the antitumor efficacy. The results showed that the radioresistant tumor cell vaccine combined with CpG ODN1826 could significantly inhibit tumor growth, increased survival of the mice and with 20% of the mice surviving tumor free in vivo compared with the unimmunized mice bearing LLC tumor. A significant increase of apoptosis was also observed in the tumor prophylactically immunized with vaccine of inactivated radioresistant tumor cell plus CpG ODN1826. The potent antitumor effect correlated with higher secretion levels of tumor necrosis factor-alpha(TNF-α) and lower levels of interleukin-10(IL-10) concentration in serum. Furthermore, the results suggested that the antitumor mechanism was probably depended on the decreased level of programmed death ligand-1(PD-L1) which plays an important role in the negative regulation of immune response by the inhibition of tumor antigen-specific T cell activation. These findings clearly demonstrated that the radioresistant tumor cell vaccine combined with CpG ODN1826 as an appropriate adjuvant could induce effective antitumor immunity in vivo.

Key words: tumor cell vaccine, CpG oligodeoxyribonucleotides, lewis lung cancer, programmed death ligand-1

Lung cancer is a disease with one of the highest morbidity and mortality rates worldwide, and long-term survival remains poor. The conventional treatments for this carcinoma include surgery, radiotherapy and chemotherapy, but their efficacy are not satisfactory, not only because of the emergence of resistance, but also because of reduced immune function during treatment. The dysfunctions of the immune system and shortcomings of the tumor cells lead to the accelerated tumor proliferation.

Immunotherapy, an important part in tumor treatments, has a major advantage to specifically target tumor cell relative to normal cell. Last few decades cancer vaccines as the best choice of immunotherapy are available for clinical trials, ranging from single peptide and the recombinant viral vector vaccinations to whole cell therapies [1-4]. However, the immune system often fails to reject spontaneously arising tumors for the absence of sufficiently immunogenic tumor specific antigens (TSA) [5]. In this case, the whole tumor cell represents the best source of immunizing antigens without knowledge of any specific antigen targets. Unfortunately, studies aimed at dissecting antitumor immune responses have confirmed that most tumors are not naturally immunogenic and eventually escape from immune surveillance. Therefore, improving the immunogenic of tumor cell became the hotspot in the field of cancer immunotherapy.

Nonmethylated CpG dinucleotides are known to induce both innate and adaptive immunity [6],with the production of cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin(IL)-12, and interferon gamma, needed for development of antitumor immunity. In previous study, we have shown that CpG ODNs dramatically increased the radiosensitivity of Lewis lung cancer and enhance immune

function in mice in a dose related manner [7]. There is great potential for CpG ODNs as vaccine adjuvants. Meanwhile, studies have shown that administration of radiation has been utilized in vitro and in vivo to create an inflammatory setting, via induction of apoptosis, necrosis, cell surface molecules, and secretory molecules [8]. Radiation has been shown to increase the immunogenicity of tumor cells by amplifying the tumor-specific peptide repertoire, upregulating the cell surface expression of MHC molecules and VCAM-1 on the tumor vasculature [9-11].On the bases of current studies, we developed the radioresistant cancer cell by repeated X-ray irradiation, inactivated it as the tumor cell vaccine, then combined with CpG ODN1826 as the vaccine adjuvant to enhance the immunogenic and induce systemic antitumor immune responses in the mouse model of Lewis lung cancer. The findings from this study suggested that the radioresistant cancer cell vaccine plus CpG ODN1826 could offer new prophylactic potential strategy to induce an intense immune response in a synergetic manner, which was very promising for the in cancer treatment.

Materials and methods

Main instruments and reagents. The Lewis lung cancer cells used in this study were kindly supplied by the Life Science Institute of Academy in Shanghai and cultured in Dulbecco's Modified Eagle's Medium (Gibco BRL Co.Ltd. USA) supplemented with 10% fetal bovine serum. The cultures were maintained at 37.0°C in a humidified atmosphere containing 5% CO_2. The phosphorothioate-modified CpG ODNs were provided by Shanghai Sangon Biological Engineering Technology and Service Limited Company (Shanghai, China). The sequence of CpG ODN 1826 was 5'-TCCATGACGTTC-CTGACGTT-3'.ODNs were diluted with phosphate-buffered saline to a concentration of 1g/L and maintained at −20°C. The cell counting kit-8(CCK-8,Dojindo, Japan) was used to detect the cell proliferation and activity. The cell cycle test kit purchased from KeyGEN Biotechnological Co.,Ltd(Nanjing, China).Levels of TNF-α,IL-10 and IL-12 were measured using a standard Quantikine enzyme-linked immunosorbent assay kit from R&D Systems (Minneapolis, MN). A TdT-mediated dUTP nick end labeling (TUNEL) kit (Roche) was used to detect tumor cell apoptosis. Total RNA was extracted with the Trizol reagent(Invitrogen, USA). PCR amplification kit was purchased from TaKaRa Biotechnology (Takara, Dalian, China). Primary antibodies against PD-L1, α-tublin were purchased from Abcam Biotechnology (Abcam, UK).The rabbit anti-mouse and mouse anti-mouse secondary antibodies were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). A linear accelerator was used (Precise5839, Elekta, Stockholm, Sweden)to establish the radioresistant cell line.

Generation of the radioresistant cell line. LLC cells in an exponentially growing phase in a 25cm² culture flask were irradiated at a dose of 5Gy at room temperature (6Mv X-Ray,

exposure mode: source skin distance). When the irradiated cells reached the end of the exponential growth phase again, the cells were trypsinized and appropriate numbers of cells were plated in another flask. Reirradiation at a dose of 5Gy was administered after LLC cells reached the exponential growth phase again. A radioresistant variant cell line, designated R-LLC cell line was obtained by repeating these procedures for six times.

The detection of radiosensitivity via CCK-8 and clonogenic assays. Ninety six-well plates containing 8×10^3 LLC cells and R-LLC cells in 100μL of a medium per well were incubated for 24 hours, and then treated with a gradient of X-ray dose (0, 2,4, 6, and 8Gy). The CCK-8 solution was added to each well at the indicated time at 37°C and the absorbance of cells in each well was measured at 450 nm. The results were expressed as percentage of the absorbance present in LLC cells compared to R-LLC cells. LLC cells and R-LLC cells were cultured in six-well plates. After radiation with 0, 2, 4, 6, 8Gy of X-rays, the cells were immediately trypsinized and seeded in triplicate in 60mm dishes (the larger the radiation dose , the more the number of cells were inoculated per dish). Plates were incubated for two weeks, fixed in methanol, stained with methylene blue, and any colonies containing greater than 50 cells were counted using a low-power dissecting microscope. All plates were coded to prevent observer bias. Survival was calculated as the relative plating efficiencies (PE) of the irradiated to the control plates. A Single-hit Multi-Target model was used to further measure the survival of LLC cells and R-LLC cells by calculating the radiobiological parameters(D_0,N,Dq,SF_2). D_0 is the radiation dose required to reduce survival by a factor of l/e in the exponential region of the curve, N is the extrapolation number or zero-dose extrapolate, Dq is the quasithreshold dose, Dq= $D_0\times lnN$[12], and SF_2 is the survival fraction after 2Gy irradiation. The R-LLC cell line obtained by repeated exposure to radiation was used for the following experiments after routinely passaged more than ten generations.

Measurement of TNF-α and IL-12 release. Levels of TNF-α and IL-12 in cell culture supernatants were measured when the LLC cells and R-LLC cells were in exponential growth phase. The culture supernatants were separated by centrifugation and stored at -80°C. Levels of TNF-α and IL-12 were measured simultaneously using an ELISA kit (R&D, Minneapolis, MN).

Vaccine preparation. The culture supernatants were separated by centrifugation when the LLC and R-LLC cells were in an exponentially growing phase in a 75cm² culture flask. Then the tumor cells were extensively digested and washed three times by PBS, resuspended with the culture supernatants previously collected, inactivated by repeated freezing and thawing (37°C for 10 minutes, liquid nitrogen for 10 minutes, four times).The ultrasonic cell crusher and vortex oscillator were used to further release the ingredients in the tumor cells. Then the supernatants with tumor antigens were injected into mice as the tumor cell vaccine.

Animal vaccination and tumor challenge. 120 female C57BL/6J mice provided by the Shanghai Experimental Animal Center and maintained in a specific pathogen-free grade animal room until 6–8 weeks of age and weighing 18–22g were evenly randomly divided into six groups, with twenty mice each group. To evaluate the adjuvant activity, the mice were immunized subcutaneously into the right anterior leg with of vaccine containing about 1.0×10^6 inactivated tumor cells after the 0.15mL of CpG injected intraperitoneally, respectively, both three times at a five day interval, either alone or co-formulated with CpG ODN1826, LLC cell vaccine and R-LLC cell vaccine. Animals in the placebo group were immunized with serum free medium only(non-immune group). One week after the last vaccination(defined as day 1), each mouse was inoculated with 2×10^6 fresh LLC cells on the opposite anterior leg and 10 mice in each group were randomly selected to observe the tumor growth and survival. On day 18, the remaining tumor-bearing mice in each group were euthanized for other investigation. All protocols were carried out in accordance with the ethics committee of Jinshan Hospital, Shanghai, People's Republic of China, and approved by Jinshan District Health and Family Planning Commission (No.JSKJ-KTMS-2014-02).

Efficacy in transplanted tumors. Starting on day 5 after inoculation, we observed the tumor-bearing mice in each group every day, and recorded the tumorigenesis time when the tumors were 2mm in diameter. The tumor volume was measured when tumors were visible and the survival curve of these ten mice was surveyed during 60 days after inoculation. Tumor volume was determined by the following formula: tumor volume $(cm^3) = 1/2 \times length(cm) \times width^2(cm)$ [13]. On day 18, the remaining mice were sacrificed and we calculated the tumor inhibitory rate as: Tumor inhibitory rate = $1 - ($Mean tumor weight of immunized group/mean tumor weight of control group$) \times 100\%$ [14].

Cell apoptosis. A TUNEL kit was used to detect apoptosis in tumor cells, whereby apoptotic cell nuclei stain brown and nonapoptotic cells stain blue. Cell apoptosis was observed under a microscope by two pathologists working independently. About 1500 cells in total were counted in three high-power microscope fields of vision. The apoptosis rate was calculated as follows: (number of apoptosis cells/total number of cells) $\times 100\%$ [15].

The impact on immune function. On day 18, the serum was collected, all spleen and tumor tissue were removed from the mice. A enzyme-linked immunosorbent assay kit was used to measure serum TNF-α and IL-10 levels. Mean spleen exponents were calculated as the weight of the spleen divided by the weight of the mouse from which the tumor had been removed.

Evaluation of PD-L1 mRNA expression by RT-PCR. Total RNA was isolated from tumor tissue with the Trizol reagent, quantified, and used to create cDNA. Using both Oligo dT Primer and Random hexamers, efficient synthesis of cDNA from total RNA can be accomplished. The amplification of

cDNA was performed using Takara SYBR Premix Taq™II (Tli RNaseH Plus) kit (Takara Biotechnology Co., Ltd.). The sequences of primer pairs for the respective genes evaluated in this study were used as follows: β-actin sense primer: 5'-CATTGCCGACAGGATGCAG-3', β-actin anti-sense primer: 5'-CTCGTCATACTCCTGCTTGCTG-3'; PD-L1 sense primer: 5'-TATCACGGCTCCAAAGGACT-3', PD-L1 anti-sense primer: 5'-ACCACTAACGCAAGCAGGTC-3'. Gene amplification was performed as follows: 95°C for 30s (1 cycle), 95°C for 5s, 60°C for 32s(40 cycles), 95°C, for 15s, 60°C, for 1min (1 cycle).

Protein extraction and western blot analysis. The tumor tissue was homogenized in the cold lysis buffer with 1mM phenylmethanesulfonyl fluoride and lysed for at least 30 minutes on ice. Protein concentrations were measured using the Coomassie Blue Fast Staining Solution (Beyotime, Shanghai). Each of 50μg proteins from the tumor tissue was seprated on 10% SDS-PAGE and transferred to a PVDF membrane (Millipore) at 80V for 60min. Membranes were blocked by TBS/T containing 5% skim milk for 3h, and incubated with the PD-L1 antibody (1:300 dilution) overnight at 4°C, while the α-tublin antibody (1:5000 dilution) was used as an endogenous reference for quantification. Then the membranes were incubated with the secondary antibodies (1:5000 dilution) at room temperature for 1h after three times washes in TBS/T. After several washes with TBS/T, the blots were detected using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) and quantified using Tanon-4500 Gel Imaging System with GIS ID Analysis Software v4.1.5(Tanon Science & Technology Co., Ltd., Shanghai, China).

Statistical analysis. SPSS20.0 and GRAPHPAD™ PRISM 5.0 (GraphPad Software, San Diego, CA)software were used for statistical evaluation. All numeric data was expressed as the mean value ± standard error. The statistical analysis was performed by one-way ANOVA (when more than 3 groups) or Students t-test(between 2 groups). The survival rates were analyzed by the Kaplan–Meier method, and the log-rank test was used to compare the difference in survival rate between groups. Differences between the values were considered statistically significant if $p < 0.05$.

Results

Generation of R-LLC cells. The radioresistant Lewis lung cancer cells were maintained in normal culture after 6 times of repeated x-ray irradiation. As shown in Figure 1, the cell viability of two cell lines were inhibited by X-ray radiation while the R-LLC cells were less inhibited than the LLC cells. The cell viability of R-LLC cells was about 2.61 times higher than that of LLC cells at a dose of 8Gy ($p < 0.05$). As shown in Figure 2, the survival of LLC cells and R-LLC cells was measured by a Single-hit Multi-Target model. The survival fraction of R-LLC was significantly higher than that of LLC after 2, 4, 6, 8Gy radiation ($p < 0.05$). LLC cells and R-LLC cells presented an exponential curve with D_0 1.053±0.214Gy
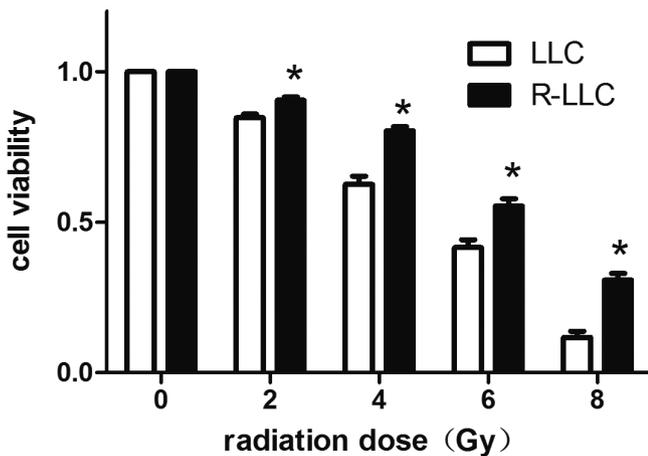
Figure 1. Generation of LLC and R-LLC cells
Cell Counting Kit-8 was used to detect the radiation sensitivity of LLC cells and R-LLC cells after 0, 2, 4, 6, 8Gy radiation, respectively. There was a significant difference in the cell viability between these two kinds of cells. Statistical significance was performed between these two cell lines (*denotes $p<0.05$, $t=3.090$).

Figure 3. Concentration of Tumor Necrosis Factor-a and Interleukin-12 in cell culture supernatants
Enzyme-linked immunosorbent assay kit was used to measure TNF-α and IL-12 levels in cell culture supernatants. The levels were significantly higher in R-LLC culture supernatants than that in LLC culture supernatants. Statistical significance was performed respectively (*denotes $p<0.05$, $t=2.867$ and 3.014).

versus 1.397±0.128Gy, N 2.68±1.179 versus 4.149±0.785, Dq 1.038 versus 1.988 and SF$_2$ 0.353versus0.682, respectively. These radio-biological parameters suggested that the radiation resistance had been obtained in R-LLC cells.

Release of TNF-a and IL-12. As shown in Figure3, TNF-α and IL-12 levels were significantly higher in R-LLC culture supernatants than that in LLC culture supernatants (23.12±0.648ng/ml versus 18.35±1.539 ng/ml, $p<0.05$; and 15.28±0.440ng/ml versus 12.10±0.969 ng/ml, $p<0.05$).

Protective antitumor effects of CpG ODN1826 combined with tumor cell vaccine. To evaluate the antitumor potential of a tumor cell vaccine combined with CpG ODN1826 as the adjuvant in Lewis lung cancer model, C57BL/6J mice were immunized and then challenged with live LLC tumor cells. The results showed that vaccination of R-LLC cell vaccine plus CpG ODN1826 not only resulted in a slower tumor growth ($p<0.05$), but also had a significant effect on overall survival of mice (Figure 4, $x^2=14.843$, $p<0.05$). As shown in Table 1, the tumorigenic time was 8.90±1.45 days in the CpG ODN1826 plus LLC vaccine group, 9.00±1.69 days in the CpG ODN1826 plus R-LLC vaccine group; There was no statistical difference in these two groups. But a significant statistical difference ($p<0.05$) was observed by comparing with the control group and the other immunized group. Interestingly, 2 among 10 mice could not develop tumor in CpG ODN1826 plus R-LLC vaccine group. Figure 5 demonstrated that the subcutaneous model mice in CpG ODN1826
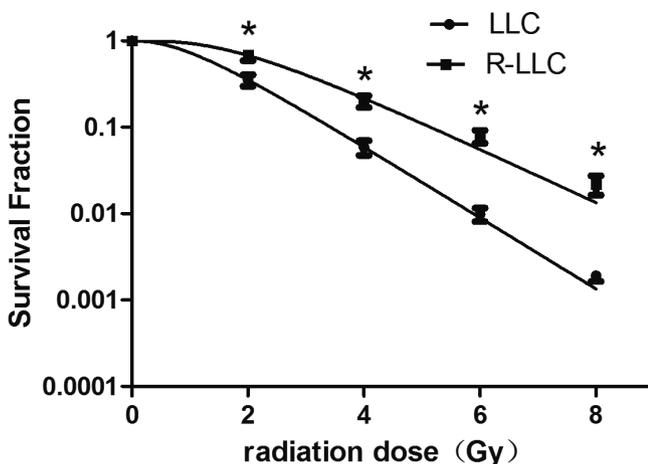

Figure 2. Generation of LLC and R-LLC cells
Clonogenic assays after radiation were performed and a single target multi-hit model was used to further measure the survival of LLC cells and R-LLC cells. The survival fraction of R-LLC was significantly higher than that of LLC after 2, 4, 6, 8Gy radiation. Statistical significance was performed at each dose tested (*denotes $p<0.05$, $t=4.379$, 6.615, 8.879 and 6.209).
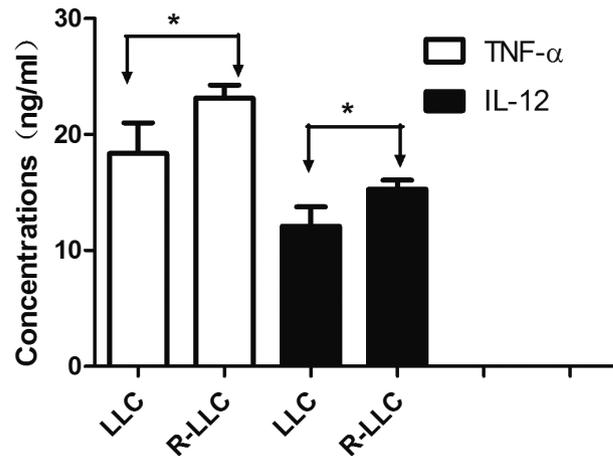
Table 1. The tumorigenesis time (x±s)

Group	Mice number	tumorigenesis rate	tumorigenesis time (day)
Control	10	100%	6.80±0.63
CpG	10	100%	6.90±0.88
LLC	10	100%	7.40±1.71
R-LLC	10	100%	7.50±1.27
CpG+ LLC	10	100%	8.90±1.45[abcd]
CpG+ R-LLC	8	80%	9.00±1.69[abcd]

Note: [a]Significantly different from control at $t=4.200$ and 3.491, $p<0.01$. [b]Significantly different from CpG at $t=3.735$ and 3.188, $p<0.01$. [c]Significantly different from LLC at $t=2.114$ and 2.211, $p<0.05$. [d]Significantly different from R-LLC at $t=2.298$ and 2.154, $p<0.05$.
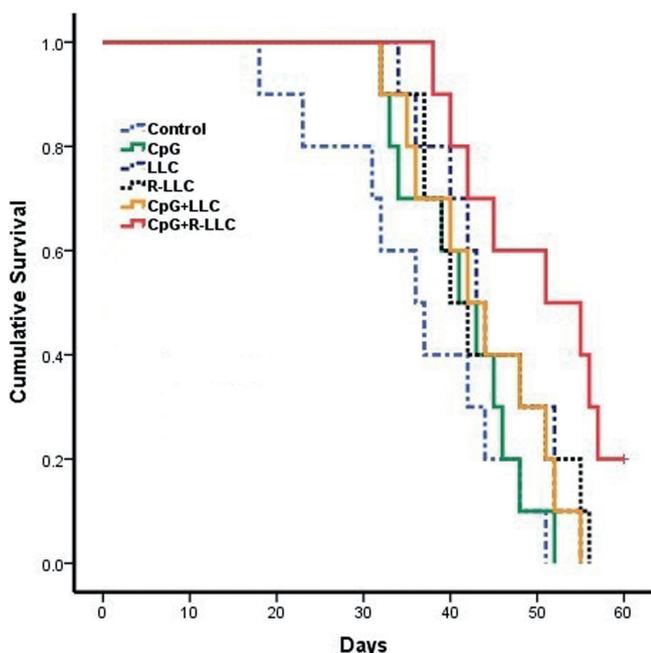
Figure 4. The survival of mice
Kaplan-Meier plot of the estimated survival functions for the six groups of mice. Survival in the CpG+R-LLC group was significantly different to the other five groups. Furthermore, two mice remained tumor-free survival in the CpG+R-LLC group (x^2=14.843, $p<0.05$).
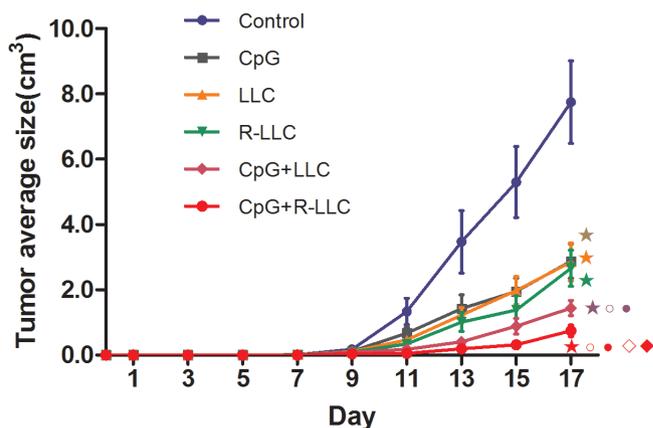

Figure 5. Tumor growth curve
Tumor volume (cm^3) is plotted as the mean ± SE. The mean tumor volumes in the immunized groups were smaller than that in the control group, with the lowest tumor volume recorded in the CpG+R-LLC group. On day 17, the mean tumor volume was 7.745±3.98 cm^3 in the control group, 2.877±1.66 cm^3 in the CpG group, 2.848±1.83 cm^3 in the LLC group, 2.662±1.75 cm^3 in the R-LLC group,1.441±0.74 cm^3 in the CpG+LLC group, 0.743±0.51 cm^3 in the CpG ODN+R-LLC group. The anti-tumor effect was most marked in the CpG+R-LLC group. On day 17,statistical significance was performed to control (★ denotes $p<0.05$, t=3.567, 3.532, 3.694, 4.919 and 5.500); a difference from CpG (○ denotes $p<0.05$, t=2.502 and 3.848); difference from LLC (● denotes $p<0.05$, t=2.256 and 3.474); difference from R-LLC (◇ denotes $p<0.01$, t=3.300); and difference from CpG+ LLC (♦ denotes $p<0.05$, t=2.364).Moreover, statistical significance was performed among six groups by one-way ANOVA (F=4.273, $p<0.05$).

plus R-LLC group developed significantly smaller tumor volumes compared to the other immunized groups (F=4.273, $p<0.01$). As shown in Table 2, the tumor weight was lower in the prophylactically immunized groups than in the control group, lowest was in the CpG ODN1826 joint R-LLC vaccine group, which was significantly different ($p<0.05$). The tumor inhibitory rate equaled 79.07% in the CpG ODN1826 joint R-LLC vaccine group, which was highest among each group. No adverse reactions such as death, allergies, dermatitis, induration and other toxic side effects were observed in each group.

Table 2. The tumor weight (x±s)

Group	Mice number	Tumor weight (g)	Tumor inhibitory rate (%)
Control	9	8.17±2.91	—
CpG	10	5.55±1.35[a]	32.07
LLC	10	5.22±1.34[a]	36.11
R-LLC	10	5.39±1.31[a]	34.03
CpG+ LLC	10	2.88±1.37[abcd]	64.75
CpG+ R-LLC	8	1.71±0.51[abcde]	79.07

Note: [a] Significantly different from control at t=2.466,2.792,2.638,4.983 and6.548, $p<0.05$.
[b] Significantly different from CpG at t=4.398 and 8.282, $p<0.05$. [c] Significantly different from LLC at t=3.861 and 6.981,$p<0.05$. [d] Significantly different from R-LLC at t=4.179 and 7.442, $p<0.05$. [e] Significantly different from CpG+ LLC at t=2.481, $p<0.05$.
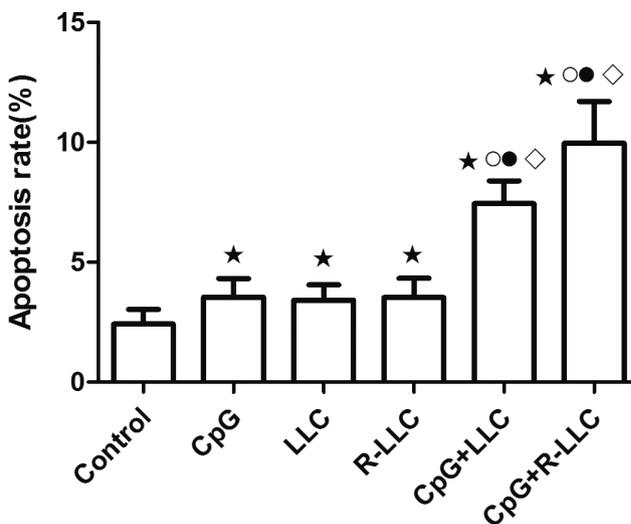

Figure 6. Apoptosis rate of cells in each experimental group
Apoptosis in Lewis lung cancer tumor cells in the control group and the groups immunized with different ways was assessed by TUNEL. The apoptosis rate was obviously highest in the CpG ODN1826 joint R-LLC vaccine group. Statistical significance was performed to control (★ denotes $p<0.01$, t=3.507,3.495,3.360, 13.476and12.286); a difference from CpG (○ denotes $p<0.01$, t=10.054and10.562); difference from LLC (● denotes $p<0.01$,t=11.078and11.121); difference from R-LLC (◇ denotes $p<0.01$, t=9.910and10.487); and difference from CpG+ LLC (♦ denotes $p<0.01$, t=3.952).
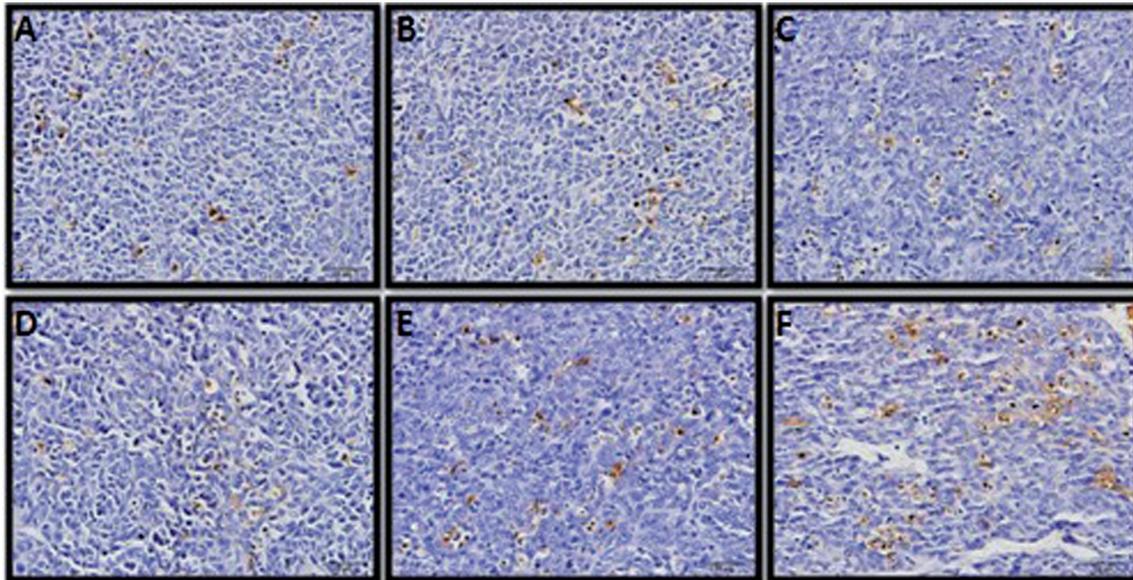
Figure 7. Apoptosis of cells in each experimental group (×200).
(A) Control group, (B) CpG group, (C) LLC group, (D) R-LLC group, (E) CpG+LLC group, (F) CpG+R-LLC group

Cell apoptosis. The apoptosis of LLC tumors were assessed by the method of TUNEL. As shown in Figure 6-7, the apoptosis rates were higher in the immunized groups than in the control group, obviously highest in the CpG ODN1826 joint R-LLC vaccine group. Furthermore, there was significant statistical difference between CpG ODN1826 joint LLC vaccine group and CpG ODN1826 joint R-LLC vaccine group($p<0.01$).
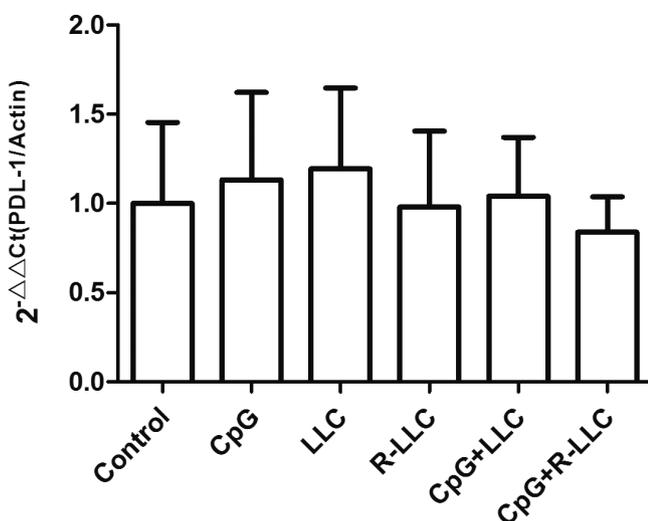

Figure 8. Expression of PD-L1 mRNA
RT-PCR showed the expression of PD-L1 mRNA in the transplanted tumor cells. Compared with the other groups, PD-L1 mRNA levels decreased in tendency in the CpG combined with R-LLC vaccine group, but the difference was not statistically significant ($F=0.8473$, $p>0.05$).

Impact on the immune function. As shown in Table 3, compared with the control group, the spleen volume of the immunized group was remarkably bigger than that of the control group ($p<0.05$), biggest in the CpG ODN1826 joint R-LLC vaccine group, but there was no significant difference in statistic among each immunized group($p>0.05$). In addition, the level of IL-10 decreased and the level of TNF-α increased when the mice were prophylactically immunized. This condition was most significant in the CpG ODN1826 joint R-LLC vaccine group ($p<0.05$).

Expression of PD-L1 in tumor tissue. The relative concentrations of PD-L1 mRNA in xenograft tissues were detected by RT-PCR. As shown in Figure 8, the relative gene expression

Table 3. Concentration of Tumor Necrosis Factor-α,Interleukin-10 and Spleen exponent (x±s)

Group	Mice number	TNF-α(μg/L)	IL-10(μg/L)	Spleen exponent (mg/g)
Control	9	33.69±12.51	63.77±19.28	6.78±2.25
CpG	10	50.51±10.92[a]	47.46±10.20[a]	9.75±2.37[a]
LLC	10	48.11±13.91[a]	43.85±9.09[a]	9.68±3.37[a]
R-LLC	10	50.45±13.87[a]	44.06±16.69[a]	9.90±3.06[a]
CpG+ LLC	10	63.11±13.78[abc]	34.88±7.33[abc]	9.61±2.42[a]
CpG+ R-LLC	8	83.08±13.84[abcde]	21.46±10.86[abcde]	10.30±3.90[a]

Note: [a] Significantly different from control at t=3.129, 2.365, 2.752, 4.852, 7.732, 2.342, 2.932, 2.389, 4.229, 5.472 and 2.877, $P<0.05$. [b] Significantly different from CpG at t=2.268, 5.593, 3.168, 5.224 and 2.280, $P<0.05$. [c] Significantly different from LLC at t=2.422, 5.311, 2.431,4.768 and 2.606, $P<0.05$. [d] Significantly different from R-LLC at t=4.965, 3.302 and 2.721, $P<0.05$. [e] Significantly different from CpG+ LLC at t=3.050, 3.128 and2.244, $P<0.05$.
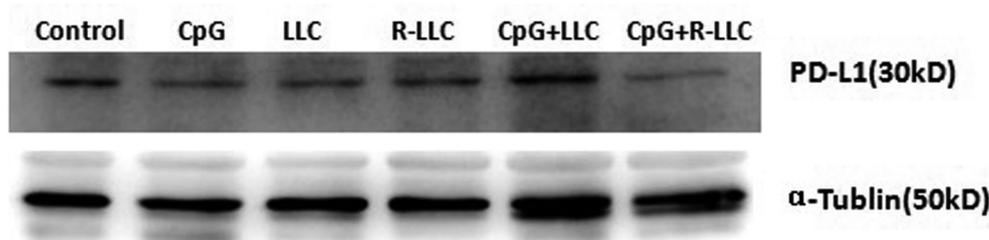
Figure 9. Expression of PD-L1 protein
Western blot demonstrated that PD-L1 was expressed at 30 kDa, while α-Tublin at 50 kDa. No significant difference was observed among each group.

of PD-L1 in CpG ODN1826 plus R-LLC vaccine group trend to decreased compared with the other groups (F=0.8473, p>0.05). Western-blotting analysis showed that the levels of PD-L1 protein expression were lower just in CpG ODN1826 combined with R-LLC vaccine group than in the other groups, but there was relatively no significant difference among all the groups (F=1.942, p>0.05) (Figure 9-10).

Discussion

Recurrence and metastasis of malignant tumors is the main difficulty in cancer treatment. New prophylactic strategies should be found to resolve this problem such as tumor cell vaccine. Classical immunological or molecular tumor vaccines, such as whole tumor cells or tumor cell lysate fragment vaccines may reduce tumor recurrence, peptide vaccines and genetically engineered vaccines may activate the humoral and cellular immunity, and can even reduce development of metastatic tumors , but its efficacy is not plausible [16,17]. However, due to the low immunogenicity of the tumor-associated antigens(TAAs), down regulation of MHC molecules, the lack of adequate costimulatory molecule expression, secretion of immunoinhibitory cytokines, etc, such expectations are rarely fulfilled [18]. Unmethylated CpG dinucleotides, nucleic acid segments present in a specific sequence context, are taken up in endosomes where they bind TLR9. This kind of binding induces changes in antigen presenting cells (making them more effective at antigen presentation), enhances the production and mobilization of signaling molecules that affect leukocytes and other immune cells, and increases the phagocytic activity of macrophages and neutrophils [19,20]. There have been considerable number of researches evaluating the role of CpG ODNs as an adjuvant and immune modulator in cancer treatment. Ohashi et al have demonstrated that the tumor cell vaccine combined with CpG ODN could enhance the antitumor efficacy after resecting the primary tumor [21]. In this study, we confirmed that the radioresistant cancer cell vaccine plus CpG ODN1826 could enhance the immune response and inhibit the tumor growth.

Our previous study has showed that TNF-α was reported to be upregulated in the human A549 cell line after combined treatment with CpG-ODN and X-rays [22].This study revealed

that there was an elevation of TNF-α and IL-12 levels in the cell culture supernatants after repeated exposure to X-ray. The increased secretion of TNF-α and IL-12 may enhance the immunogenicity of tumor cells.

In addition, the results of this study showed that prophylactically immunized using R-LLC cell vaccine combined with CpG ODN1826 remarkably inhibited the tumor growth to other immunized group. In contrast, immunization of CpG ODN1826 alone or combined with original LLC cell vaccine could not result in a significant anti-tumor effect. The synergy effect of R-LLC cell vaccine and CpG ODN1826 reflected in the enhancement of cytokine secretion, promotes of apoptosis in tumor cells and prolong survival in mice. Moreover, in view of the failed inoculation in the mice immunized by R-LLC cell vaccine plus CpG ODN1826, we hypothesized that CpG ODN1826 and R-LLC cell vaccine triggered the specific immune response to Lewis lung cancer.

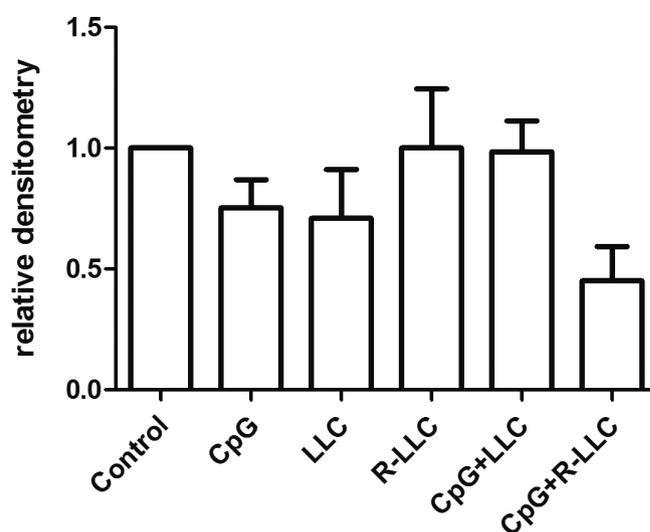PD-L1(programmed death ligand-1, PD-L1) is one member in co-stimulating molecules of B7 super family. PD-L1 binds

Figure 10. The densitometry of PD-L1 in Western blot
The densitometry represented that the expression level of PD-L1 was lower in tendency in the CpG combined with R-LLC vaccine group, but the difference was not statistically significant (F=1.942, p> 0.05).

to PD-1, which mainly expresses on the activated T cells and tumor cells, mediates the negative modulating signals. Interfering the PD-1/PD-L1 signal pathway regulates the immunological responses, which could be used as a therapeutic strategy for cancer, viral infection and auto-immune diseases. Some researchers have found that patients with higher expression of PD-L1 had a significantly poorer prognosis than patients with lower expression [23].There are a few studies also indicate that the tumor cells over expressing of PD-L1 in chronic lymphocytic leukemia, stomach cancer, esophageal cancer, kidney cancer, breast cancer and bladder cancer related to the immune escape mechanism and lead to the disability of antitumor T cells, blockade of PD-1 can enhance tumor-specific T cell response and inhibit the tumor cell proliferation [24,25]. In this study, a downward trend of PD-L1 mRNA expression in tumor cells was observed in the mice immunized by R-LLC cell vaccine and CpG ODN1826. A similar pattern of change in Western Blot was seen. There was no significant difference in the group immunized by CpG ODN1826 alone compared to the control group, which revealed that CpG might have no direct effect on PD-L1 expression. But the synergistic immunization could interfere PD-L1 and the down-regulation of PD-L1 expression was observed only in the CpG ODN1826 plus R-LLC vaccine group. The specific mechanism needs to be further studied.

In summary, we have obtained the antigen-enhanced tumor cell vaccine by repeated exposure to X-ray irradiation. Immunization of CpG ODN1826 combined with the antigen-enhanced tumor cell vaccine resulted in a powerful anti-tumor effect, such as the inhibition of tumor growth , enhancement of immune function in mice, promotion of apoptosis in tumor cells and improvement of the survival in mice and so on.

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