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Induction of tumor inhibition and apoptosis by a candidate tumor suppressor gene DRR1 on 3p21.1

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Abstract. Down-regulated in renal cell carcinoma gene (DRR1) is one of the candidate tumor suppressor genes (TSGs) on human 3p21.1. This study was performed to validate the expression status of DRR1 gene in cancer cells and the expression pattern of the protein in clinical specimens of human lung cancer and to examine its potential as a molecular target for treatment of lung cancer *in vivo*. DRR1 expression was analyzed in 7 human lung cancer cell lines. DRR1 protein expression was also examined in clinical non-small cell lung cancer (NSCLC) specimens. Furthermore, effects of DRR1 re-expression on A549 cells *in vitro* and A549 xenograft tumors in nude mice were evaluated. Loss of DRR1 mRNA expression was detected in 6 of the 7 human cancer cell lines, the exception was the renal cancer cell line OS-RC-2. DRR1 protein expression was absent in 15 of 20 (75%) human NSCLC specimens by immunostaining. Transfection of DRR1 gene into DRR1-negative-expressing A549 cells resulted in significant cell growth suppression and apoptosis. Plasmids containing DRR1 cDNA complexed with DOTAP:Chol liposomes were administered intravenously via tail vein to nude mice bearing A549 xenograft tumors resulting in tumor growth inhibition and elevation of apoptosis compared with the controls. DRR1 is a potent growth suppressor of NSCLC, acting through apoptosis pathway *in vivo* and it may be a potential therapeutic gene for human lung cancer.

Introduction

Lung cancer is one of the most common cancers and the leading cause of cancer-related death in men and women. The underlying causes for lung cancer have been attributed to various factors that include alteration and mutation in the tumor suppressor genes (TSGs). Allelic deletion at human short arm of chromosome 3 occurs frequently in >90% small cell lung cancers (SCLCs) and in 50-80% non-small cell lung cancers (NSCLCs) (1,2). Frequent and early loss of heterozygosity and the presence of homozygous deletions indicate that human chromosome 3p harbors multiple TSGs.

Down-regulated in renal cell carcinoma gene (DRR1), also called Tohoku University cDNA clone A on chromosome 3 (TU3A), from human chromosome 3p21.1 spans ~10 kb of genomic DNA with a 3.5 kb mature mRNA encoding a 144-amino acid protein (3,4). This gene has been characterized as a candidate TSG and its expression becomes low or even absent in a number of tumor cells and primary tumors. Re-expression of DRR1 in some DRR1-negative tumor cell lines can suppress cell growth (4). Loss of the DRR1 expression may play a crucial role in the development of lung, kidney, prostate and other epithelial cancers (1,5). These results indicate that DRR1 has a potential role in tumorigenesis. The present study was performed to identify the anti-tumor activity of DRR1 in NSCLC xenografts *in vivo*.

Materials and methods

Plasmids construction. A DNA fragment encoding human DRR1 was obtained from recombinant plasmid pQE30-DRR1 constructed by us previously (6). For the generation of DRR1-expressing plasmid (pVAX1-DRR1), DRR1 DNA was amplified using two specific primers. Primer sequences were designed to include either a *Bam*HI or an *Xho*I site (underlined): 5'-TATGGATCCATGTACTCGGAGATCCAGA-3' and 5'-TATCTCGAGTACAGCTCTCTCTCTTC-3'. PCR amplification was performed for 30 sec at 94˚C, 30 sec at 55˚C and 30 sec at 72˚C. An additional extension step was performed for 5 min at 72˚C. The product was the cloned into the pVAX1 expression vector (Invitrogen). DNA sequencing was performed to confirm that the pVAX1-DRR1 construct had the desired sequence and open reading frame. Parental vector (pVAX1) and pVAX1-DRR1 were respectively transformed into DH5α competent *Escherichia coli* (Takara). Plasmid DNA copies were amplified in liquid LB culture and purified using an EndoFree Plasmid Giga Kit (Qiagen). DNA used for treatment had an A260/A280 ratio of ~1.9.

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RNA preparation and RT-PCR analysis. To determine the expression level of DRR1 in human cancer cells, total RNA was extracted by TRIzol (Invitrogen) according to the manufacturer's instructions. These cancer cells included human lung cancer cell lines A549 cells and SPC-A1, nasopharyngeal carcinoma cell line HNE-1, hepatocellular carcinoma cell line HepG2, chronic myelogenous leukaemia cell line K562, cervical cancer HeLa cells and renal cancer cell line OS-RC-2. RT-PCR for DRR1 was performed using a One Step RT-PCR kit (Takara) with primers specific for a portion of DRR1. Product was normalized to human housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The DRR1 primers are 5'-GCTCATC AAGCCCAAGAAGC-3' (forward) and 5'-GCTCTCTCTC TTCGCTGGTC-3' (reverse). The GAPDH primers are 5'-TCATCTCTGCCCCCTCTG-3' (forward) and 5'-CCTGCTT CACCACCTTCTTG-3' (reverse). RT-PCR amplification was performed in a reaction volume of 25 μl using standard conditions.

Effect of re-expression of DRR1 gene on tumor cell growth. Human lung adenocarcinoma A549 cells were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS). Effects of DRR1 treatment on A549 cell growth was measured by CellTiter Aqueous One Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer's recommended protocol. The assay depends on the reduction of a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) to formazan. Briefly, cells were seeded at a density of $1x10^4$ per well in 100 μl culture medium into a 96-well plate. When cultured to 70% confluence, cells were transfected with pVAX1-DRR1 (0.2 μg/well) or pVAX1 (0.2 μg/well) complexed with Lipofectamine 2000 reagent (Invitrogen) respectively. After culturing for 48 h, 20 μl of MTS was added and then incubated at 37˚C for an additional 1-2 h. The absorption at 490 nm was measured using a microplate reader. Each treatment was performed five times.

For flow cytometric (FCM) analysis, A549 cells were stained at 4˚C for 30 min with propidium iodide (PI) at a final concentration of 5 μg/ml in the dark prior to analysis.

Human cancer xenograft model and plasmid treatment. A549 cells in log phase were harvested by trypsinization, centrifuged at 1500 rpm for 3 min. After washing with PBS twice, cells were resuspended in RPMI-1640 medium without fetal bovine serum (FBS) at a concentration of $1x10^7$ cells/ml prior to injection into mice. Female athymic nude mice nu/nu (4-6 weeks old; 20±2 g) were subcutaneously (s.c.) implanted with $2x10^6$ cells into the right flank. Mice were randomly assigned to treatment groups [phosphate-buffered saline (PBS), liposomes alone, pVAX1 plus liposomes, or pVAX1-DRR1 plus liposomes] ~14-21 days after tumor implantation or when the tumors were palpable (~2x2 mm). Treatment was initiated when the tumor volume reached 70-100 mm^3 (day 0). Plasmids (50 μg) complexed with cationic liposomes (50 nmol) in a volume of 300 μl were injected into the mouse tail vein three times a week (on Monday, Wednesday and Friday) for 25 days. The cationic liposomes consisting of N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) and cholesterol (Chol) were prepared for *in vivo* delivery of plasmid DNA by our laboratory as described (7). DOTAP:Chol liposomes proved to be a good non-viral vehicle for the delivery of plasmids efficiently *in vivo* and were shown to achieve effective levels of trans-gene expression in highly vascularized tissues, such as tumors or the lung (8,9).

There were 5 to 7 mice in each treatment group in an individual experiment. Experiments were repeated twice to insure reproducibility. Tumors were measured using calipers prior to each injection and tumor volumes were calculated (tumor volume = 0.52 x length x $width^2$).

Immunohistochemistry analysis. Twenty clinical cases of human primary lung cancers were stained on paraffin section. Briefly, tissue sections were deparaffinized, dehydrated and subjected to microwaving in citrate buffer (10 mmol/l) with pH 6.0 in an oven for 10 min to induce epitope retrieval. The slides were allowed to cool at room temperature for 30 min. Immunohistochemical staining for DRR1 was performed with rabbit polyclonal antibodies specific to human DRR1 prepared previously by our lab (6). The primary antibody was used at a dilution of 1:1000 to the slides and incubated at 37˚C for 40 min. Labeling was detected by sequentially adding biotinylated secondary antibody and streptavidin-peroxidase and development was performed using 3, 3'-diaminobenzidine (DAB) reaction. The sections were counterstained with hematoxylin. Substitution of the primary antibody with PBS served as a control.

The animal experiments were performed according to institutional guidelines. Observations ceased after 4 weeks from the day of DNA injection, when in the control group the tumor volume became large compared with animal size. Animals were sacrificed and tumors were immediately frozen in liquid nitrogen or fixed in formalin. Tumor tissues of xenograft model were harvested 72 h after the last treatment. Tumors were divided in two halves, one half of the dissected tumor for paraffin sections fixed in 10% neutral-buffered formalin and embedded in paraffin and the other half frozen at -80˚C. The paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E) and examined by an experienced pathologist by light microscopy to confirm histology and immunohistochemistry.

TUNEL analysis. Tumor species were prepared as described above. Paraffin-embedded tissues were cut into sections of 5 μm and mounted on slides, deparaffinized and rehydrated through xylene, graded ethanol to distilled water. Then the tissue sections were pretreated with 20 mg/l proteinase K for 30 min and analyzed with a DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's guidelines. It is based on the enzymatic addition of digoxigenin-nucleotide to the nicked DNA by terminal deoxynucleotidyl transferase.

Statistical analysis. The results were presented with means ± SD. Statistical significance was determined by one-way analysis of variance followed by the Student's t-test for tumor growth. Other data were analyzed using the t-test. $P<0.05$ was accepted as statistically significant.

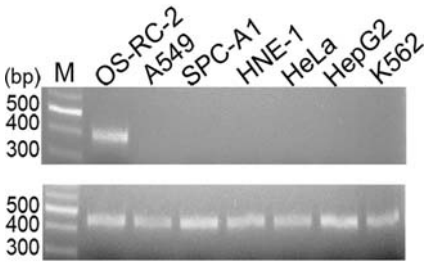

Figure 1. RT-PCR analysis of mRNA expression of DRR1 in human tumor cells. RT-PCR analysis of DRR1 mRNA expression in a panel of cancer cell lines (top). RT-PCR for GAPDH was performed to ensure integrity of isolated RNAs (bottom). M, DNA marker.

Results

DRR1 expression in tumor cells and tissues. To determine the expression level of DRR1 gene in tumor cell lines, we performed RT-PCR analysis on 7 human tumor cell lines including lung cancer cell lines A549 and SPC-A1, renal cancer cell line OS-RC-2, nasopharyngeal carcinoma cell line HNE-1, cervical cancer HeLa cells, hepatocellular carcinoma cell line HepG2 and chronic myelogenous leukaemia cell line K562 cells. The result showed loss of DRR1 expression in all the other cancer cells except the renal cancer cell line OS-RC-2 (Fig. 1).

We then analyzed whether DRR1 gene also showed altered expression in human lung cancers. The rabbit anti-human DRR1 polyclonal antibodies prepared previously were used. The polyclonal antibodies have proven to be useful in detecting endogenous DRR1 and exogenous recombinant DRR1 protein effectively (6). Immunohistochemical analyses were performed on paraffin-embedded sections of 20 primary lung cancers and two paracancerous normal lung tissues. Immunohistochemistry demonstrated that expression level of DRR1 gene was dramatically reduced or even undetectable in 15 of 20 lung cancers, whereas it showed a moderate level in 2 of 2 normal lung tissues (Fig. 2).

Induction of tumor cell growth suppression and apoptosis by re-expression of DRR1. The suppression of cell growth by DRR1 gene was identified in two DRR1-negative renal cell carcinoma cell lines (4). To assess the effect of this gene on DRR1-negative lung adenocarcinoma cell proliferation, A549 cells were transiently transfected with plasmids containing DRR1 gene or parental plasmids. The proliferation activity of A549 cells was reduced by 46% compared with the vector control (Fig. 3; left). Analysis of PI-stained DNA by flow cytometry showed the presence of apoptosis in A549 cells transfected with pVAX1-DRR1 (Fig. 3; right). These results indicated that DRR1 gene may play an important role in regulating cell proliferation.

Anti-tumor response induced by DRR1 expression in tumor cells. Suppression of cell growth by re-expression of DRR1 in several tumor cells indicates that this gene has a potential role in carcinogenesis (4). To determine whether treatment of

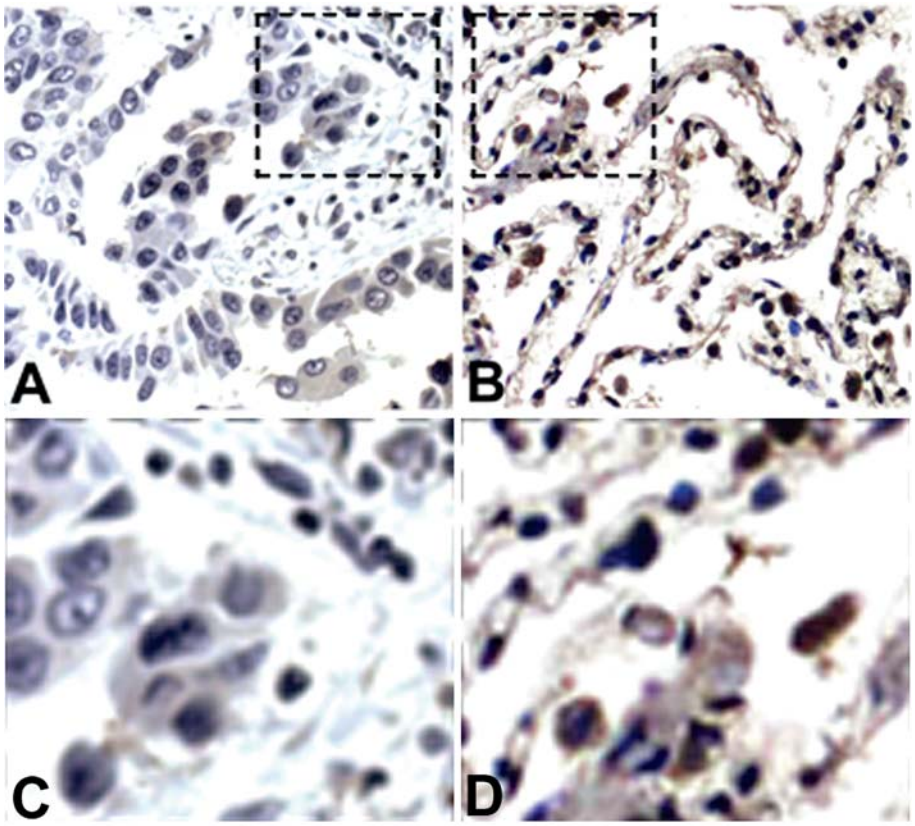

Figure 2. DRR1 immunohistochemical staining in human non-small cell lung cancer. Immunostaing of formalin-fixed paraffin-embedded sections of lung tissues revealed loss of DRR1 expression in lung cancer specimen (A) and moderate expression in paracancerous tissue (B). (C and D) Local sub-images from A and B, respectively.

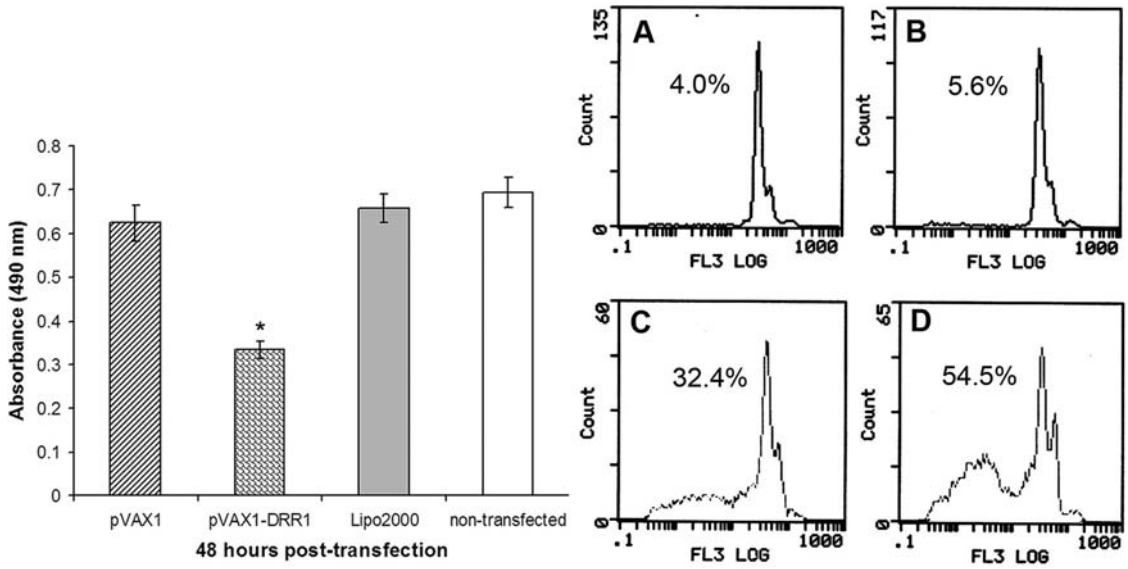

Figure 3. Effects of the DRR1 construct on A549 cells. Left, cell viability was suppressed markedly after 48 h transfection with pVAX1-DRR1 (0.2 μg/well) compared with the cell treated with pVAX1 (0.2 μg/well). Asterisk denotes statistical significance (P<0.05). Right, DNA fluroscence histograms of PI-stained A549 cells by FCM analysis. A549 cells transfected with parental plasmids (C) and DRR1 construct (D) were characterized by red fluorescence for PI (675 nm). Non-transfected cells (A) and liposome along treated cells (B) were used as negative controls.

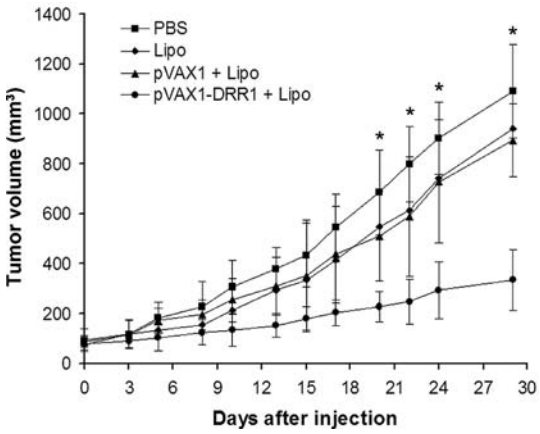

Figure 4. Induction of anti-tumor response by DRR1 gene in lung adenocarcinoma xenografts. Representative results from two independent experiments are shown. Injection was carried out from days 0. Mice received intravenous treatments (3x/week) with the DRR1 construct (50 μg) plus DOTAP:Chol liposomes (●), the parental plasmids plus DOTAP:Chol liposomes (▲), DOTAP:Chol liposomes alone (◆) or PBS (■) following 14-21 days tumor implantation. All cases received twelve treatments. Each point represents the mean value for 5 to 7 tumors from an individual experiment that was replicated two times. Statistical analysis was performed comparing fractional tumor volumes in the DRR1 construct plus DOTAP: Chol liposomes group (●) with the parental plasmid-treated group (▲) at each time point and significant values (*) were obtained (two-sided; P<0.05).

established tumors with DRR1 gene expression vector resulted in suppression of tumor growth, we developed a xenograft model using A549 cells inoculated subcutaneously in nude mice. We tested the tumor suppressor activity of DRR1 gene *in vivo* by intravenous injection of 50 μg of pVAX1-DRR1. Mice were treated three times a week and sacrificed 28 days later. Tumor volumes were significantly smaller in the mice that received DRR1 construct plus DOTAP:Chol liposomes than in the mice that received the corresponding parental plasmids plus DOTAP:Chol liposomes (Fig. 4).

Immunohistochemical and TUNEL analyses of lung tumors. Previous studies have demonstrated that human DRR1 protein and its homologous gene from *Xenopus laevis* are nuclear proteins (4,10). To investigate whether the DRR1 gene was re-expressed in xenograft tumors, immunohistochemisty was performed to assay the relationship between DRR1 expression and tumor growth suppression. The polyclonal antibodies against DRR1 revealed the presence of nuclear staining in DRR1-treated tumors and its absence in all control tumors. A detectable DRR1 expression was demonstrated in mice subjected to intravenous injection of pVAX1-DRR1 plasmids compared with treatment of parental plasmids (Fig. 5).

To determine the mechanism of the anti-tumor effect induced by treatment with the DRR1 construct plus liposomes, both necrotic and non-necrotic regions of the tumor xenografts were examined by hematoxylin-eosin staining. No clear difference in tumor necrosis between treatment groups was found (data not shown). To evaluate whether the observed growth retardation was associated with an increased rate of programmed cell death, tumors were harvested from each treatment group when the mice were sacrificed and stained for DNA fragmentation. Results demonstrated ~2-fold elevation in the rate of apoptosis in tumors treated with the DRR1 construct plus liposomes compared with tumors treated with the parental plasmids plus liposomes or liposomes along (Fig. 6).

Discussion

Chromosome 3p allelic losses are frequent and earliest events in many types of cancer including lung cancer suggesting the presence of multiple tumor suppressor genes on 3p. A number of TSGs have been located on chromosome 3p, particularly in the region between 3p11 and 3p25 (3). The candidate TSGs that have been identified include RASSF1A at 3p21.3, HYAL-1 at 3p21.3, SEMA3B at 3p21.3, VHL3 at 3p25,

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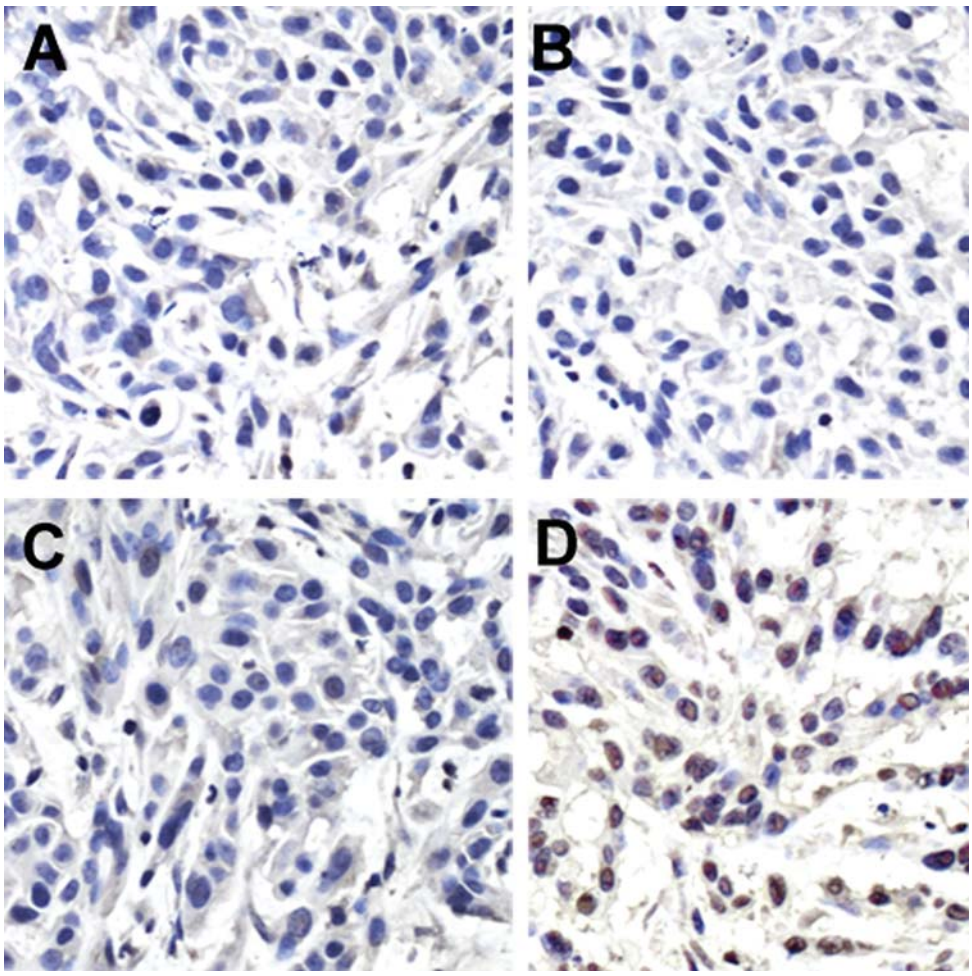

Figure 5. Immunohistochemical staining of tumor tissues. DRR1 staining was performed using anti-DRR1 polyclonal antibodies in the representative tissues of lung tumors treated with PBS (A), DOTAP:Chol liposomes along (B), parental plasmid pVAX1 (C) or pVAX1-DRR1 (D) plus DOTAP:Chol liposomes. Positive staining primarily in nuclei was observed in mice treated with pVAX1-DRR1 (D), while control groups showed negative results (A-C). DAB (3,3'-diaminobenzidine) was used for color development. Nuclear staining was performed with Mayer's hematoxylin.

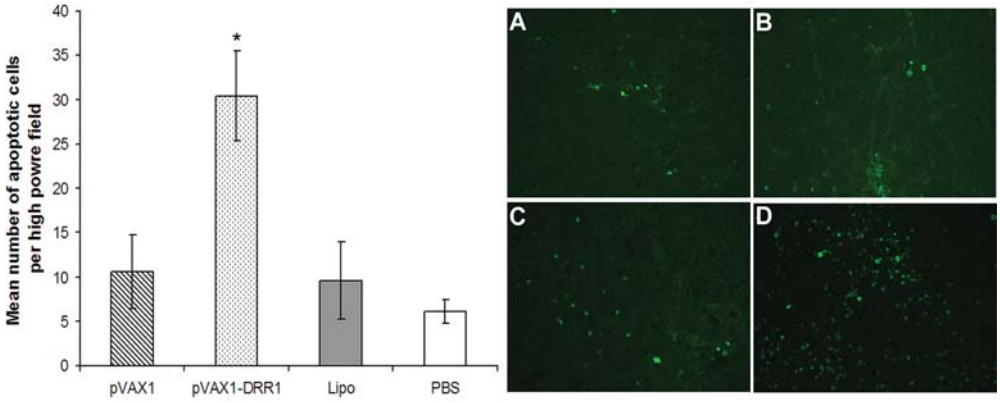

Figure 6. TUNEL analysis of xenograft tumors. Elevated apoptosis rates in DRR1-treated tumors (left). Mean rates of apoptosis (number of apoptotic cells per 5 to 7 high power fields) in the DRR1-treated tumors compared with that of plasmid-control tumors from an individual experiment that was replicated two times. *Significantly different from plasmid-control tumors (two-sided; $P<0.05$). Tumors treated with DRR1 construct (right; D) show a much higher TUNEL staining compared with plasmid-control group (C) besides DOTAP:Chol liposomes-treated tumors (B) and the negative controls (A).

RAR-ß at 3p24, and FHIT at 3p14.2 (2,11-15). As other TSGs on 3p, the expression of DRR1 gene is commonly down-regulated or even lost in lung cancer and other types of cancer (4,5,16-18). In this study, Immunostaining of DRR1 protein was only observed in 5 of 20 clinical non-small cell lung cancer specimens and thus DRR1 may play an important role in lung carcinogenesis. DRR1 mRNA was only detected in 1 of 6 human cancer cell lines, which is similar to the result from another study. The authors demonstrated by RT-PCR no TU3A (DRR1) expression in 11 of the 14 cancer cell lines

and slight expression in 2 bladder cancer cell lines (UMUC3 and 293J) and a testicular cancer cell line (NEC8) (19). We transfected DRR1 gene into lung cancer A549 cells, which do not express DRR1 gene, growth suppression of the tumor cells was also observed. Recently, a functional approach designated as 'Elimination Test' has provided evidence for the tumor-suppressive activity of DRR1 (20). In the present study, we first proved the tumor-suppressive effect of DRR1 in human lung tumor xenografts with induction of apoptosis *in vivo*. Our results further demonstrated that DRR1 is involved in carcinogenesis.

Promoter hypermethylation is an alternative way to inactivate TSGs in cancer. Alterations of human 3p frequently occur in many types of cancer, including lung carcinoma. Several studies have proven tumor-acquired promoter hypermethylation as a mechanism of inactivation of mRNA expression of some TSGs on 3p during pathogenesis of human lung cancer and several other cancers (5,21-30). Given the fact that no genetic alterations were identified in DRR1, it was suggested that epigenetic alteration such as promoter methylation may account for the inactivity of allelic loss (3,4). This hypothesis has also been demonstrated recently. Awakura *et al* confirmed that hypermethylation of TU3A/DRR1 promoter resulted in its inactivation and the methylation status is significantly related to the prognosis of renal cell carcinoma (RCC). At the same time, hypermethylation of TU3A/DRR1 promoter was shown to be associated with its inactivation in other types of cancer (19). In particular, hypermethylation of TU3A/DRR1 promoter was implicated in several types of cancer cell lines and primary cancers of the bladder and testis (4,19,31).

Coiled coil domain has been identified in many nuclear proteins including a series of transcription factors and the leucine zipper-containing proteins (32). DRR1 was proven to be a nuclear localization signal and contains a coiled domain (4), suggesting that it has a potential role in the regulation of gene transcription through interacting with other proteins and/or DNA. The mechanism of DRR1-induced apoptosis is still unknown. We are presently utilizing tandem affinity purification (TAP) and mass spectrometry technology platform to probe the components of interacting proteins associated with DRR1. TAP method is a useful tool that allows rapid purification of native protein complexes under their natural level (33-37). Such investigation will be informative to elucidate the function of DRR1 in regulating cell cycle.

In summary, we have demonstrated that DRR1 is a potent growth suppressor of NSCLC, acting through apoptosis pathway *in vivo*. The signaling pathway(s) mediating lung cancer cell growth suppression and apoptosis after the re-expression of DRR1 is still unclear and indirect mechanisms should be considered. Further studies will be necessary to elucidate the mechanism of suppressive effect of DRR1 in human tumors *in vivo*.

Acknowledgements

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