Genetics and Molecular Research

Inhibition of subcutaneously implanted human pituitary tumor cells in nude mice by *LRIG1*

X. Wang[1], X.J. He[2], H.Q. Xu[1], Z.W. Chen[3] and H.H. Fan[3]

[1]Department of Neurosurgery, The People's Hospital of Anyang City, Anyang, China
[2]Department of Neurology, The First People's Hospital of Shangqiu, Shangqiu, China
[3]ICU, Tangyin County People's Hospital, Anyang, China

Corresponding author: X. Wang
E-mail: kingecn@yeah.net

Genet. Mol. Res. 15 (2): gmr.15027681
Received October 18, 2015
Accepted December 8, 2015
Published May 6, 2016
DOI http://dx.doi.org/10.4238/gmr.15027681

ABSTRACT. The aim of this study was to explore the inhibition of subcutaneously implanted human pituitary tumor cells in nude mice by *LRIG1* and its mechanism. For this study, athymic nude mice were injected with either normal pituitary tumor RC-4B/C cells or *LRIG1*-transfected RC-4B/C cells. We then calculated the volume inhibition rate of the tumors, as well as the apoptosis index of tumor cells and the expression of Ras, Raf, AKt, and ERK mRNA in tumor cells. Tumor cell morphological and structural changes were also observed under electron microscope. Our data showed that subcutaneous tumor growth was slowed or even halted in *LRIG1*-transfected tumors. The tumor volumes were significantly different between the two groups of mice ($\chi^2 = 2.14$, $P < 0.05$). The tumor apoptosis index was found to be 8.72% in the control group and 39.7% in *LRIG1*-transfected mice ($\chi^2 = 7.59$, $P < 0.05$). The levels of Ras, Raf, and AKt mRNA in *LRIG1*-transfected RC-4B/C cells were significantly reduced after transfection ($P < 0.01$). Transfected subcutaneous tumor cells appeared to be in early or late

apoptosis under an electron microscope, while only a few subcutaneous tumor cells appeared to be undergoing apoptosis in the control group. In conclusion, the *LRIG1* gene is able to inhibit proliferation and promote apoptosis in subcutaneously implanted human pituitary tumors in nude mice. The mechanism of *LRIG1* may involve the inhibition of the PI3K/Akt and Ras/Raf/ERK signal transduction pathways.

Key words: Pituitary tumor; *LRIG1*; Nude mice; Proliferation; Apoptosis

INTRODUCTION

Pituitary tumors, also called pituitary adenomas, are common neuroendocrine tumors, accounting for 10-15% of central nervous system tumors (Liu et al., 2015). The majority of pituitary tumors are benign. Treatment primarily includes operative, drug, and radiation therapies. No treatment method, however, is able to achieve a complete cure.

In recent years, with the development of molecular biology and genetic research, targeted gene therapy for pituitary tumors has been rapidly developed (Kitano and Taneda, 2001). The gene *LRIG1* (leucine-rich repeat and immunoglobulin domain-containing protein-1) has been found to be a tumor suppressor gene (Rafidi et al., 2013). Research has shown that the mRNA and protein expression of this gene were reduced in a variety of tumor cells, including pituitary tumor cell lines and pituitary tumor tissue, compared with normal tissue. Therefore, these studies suggest that *LRIG1* expression could inhibit the growth of pituitary tumor cells (Qi et al., 2013).

In order to further clarify the role of the *LRIG1* gene and its mechanism in pituitary tumors, a plasmid containing *LRIG1* was successfully transfected into the subcutaneous area of nude mice to establish a pituitary tumor animal model *in vivo*. The effect of *LRIG1* in inhibiting the growth of pituitary tumor cells *in vivo* was investigated, as was the mechanism of this inhibition.

MATERIAL AND METHODS

Cell culture

Pituitary tumor cell line RC-4B/C cells were provided by Sichuan University Huaxi Medical College Cell Library. *In vitro* subcultures were maintained in RPMI1720 culture medium containing 10% fetal bovine serum. Routine culture was performed at 38°C in a 5%-CO_2 cell culture box. The culture medium was changed every 2 days until cells overgrew. For digestion and passage, 0.3% trypsin was added to the cultures. Half the RC-4B/C cells were then removed. Specific siRNA expression plasmids were designed according to the *LRIG1* gene sequence. The *LRIG1* plasmid was successfully transfected into the pituitary tumor cell line RC-4B/C cells.

Establishment of subcutaneously implanted tumor model in nude mice

Normal RC-4B/C cells were transfected with the *LRIG1* plasmid (Invitrogen, Carlsbad, CA, USA), and the transfected RC-4B/C cells were made into a cell suspension. The cell

concentration was maintained at 3 x 10^5/mL. Forty athymic nude mice at 4-5 weeks old (weight 18-20 g) were purchased from the animal experimental center in Medical College of Fudan University. Adaptive feeding was performed under the condition of SPF class for 1 week. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA), Eighth Edition, 2010. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the People's Hospital of Anyang City.

Mice were randomly divided into two groups: mice injected with normal pituitary tumor RC-4B/C cells (control group) and mice injected with *LRIG1*-transfected RC-4B/C cells (experimental group). There were 20 mice in each group. For injection of tumor cells, the mice were fixed and then 75% alcohol was used to disinfect the right inguinal region of the nude mice. A syringe needle was inserted 1.0-1.5 cm into the inguinal area. The cell suspension liquid was injected slowly. The injection was performed once a day for 4 weeks. The 40 nude mice were incubated and subcutaneous tumors formed 10 days after inoculation. Tumor diameter was 0.4-0.7 cm. We selected 32 nude mice with similar tumor diameters for the experiments. The diet, defecation, urine, and mental status of the mice were observed and recorded. The mice were sacrificed after continuous administration for 4 weeks, and the tumor body was peeled off.

Calculation of tumor volume inhibition rate

The long diameter (a) and short diameter (b) of the subcutaneous tumors of the mice in the two groups were measured by precise vernier caliper. The tumor volume was calculated with the equation V = 0.5Xal/. The tumor volume inhibition rate was calculated according to the following formula: tumor volume inhibition rate = (1 - average tumor volume in experimental group / average tumor volume in control group) x 100.

Expression of *LRIG1* and related protein in pituitary tumor tissue

A sample (l x 2 mm) of tumor body was removed from each mouse. RNA and protein were extracted, embedded in paraffin, and stored at -80°C. LRIG1 expression was detected in subcutaneous pituitary tumors by western blot, real-time PCR, and immunohistochemical methods according to manufacturer instructions, respectively. Downstream molecules in the EGFR signaling pathway (Ras, Raf, AKt, ERK, cell cycle protein) and their corresponding mRNA expressions were detected.

Morphological observation

A considerable proportion of the subcutaneous tumor cells from the experimental group occurred to be in early or late apoptosis in the experimental group under transmission electron microscope.

Statistical analysis

The experimental data were analyzed by the statistical SPSS14.0 software (SPSS Inc., Chicago, IL, USA). Measurement data are reported as means ± standard deviation. A single-

factor analysis of variance (ANOVA) was used for comparisons between multiple groups. The SNK-q test was used for two-by-two comparisons. Results were considered statistically significant at $P < 0.05$.

RESULTS

Tumor volume inhibition rate

After injection of the pituitary tumor cells into the two groups of mice, subcutaneous tumor size in the control group gradually increased over time. The subcutaneous tumors in the experimental group grew more slowly or stopped growing. There was a significant difference in tumor volume between the two groups ($\chi^2 = 2.14$, $P < 0.05$) (Table 1 and Figure 1).

Table 1. Comparison of implanted tumor volume and tumor inhibition rate after treatment.

Groups	Number of nude mice (N)	Tumor volume (mm^3)	Volume inhibition rate (%)
Control group	20	848.3 ± 58.2	-
Experimental group	20	517.1 ± 49.2	39.05

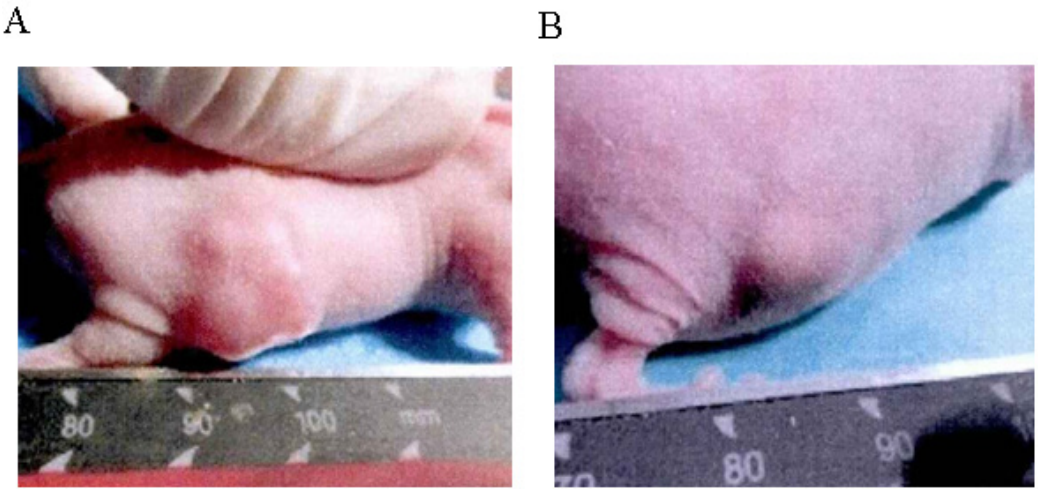

Figure 1. Subcutaneous pituitary tumor in the two groups of mice. **A.** Subcutaneous tumor sized increased gradually in the control group. **B.** Subcutaneous tumor size grew more slowly in the experimental group.

Detection of tumor cell apoptosis index

The tumor cell apoptosis index was 8.72% in the control group and 39.7% in the experimental group. Statistical analysis showed that there was a significant difference between the two groups ($\chi^2 = 7.59$, $P < 0.05$; Figure 2).

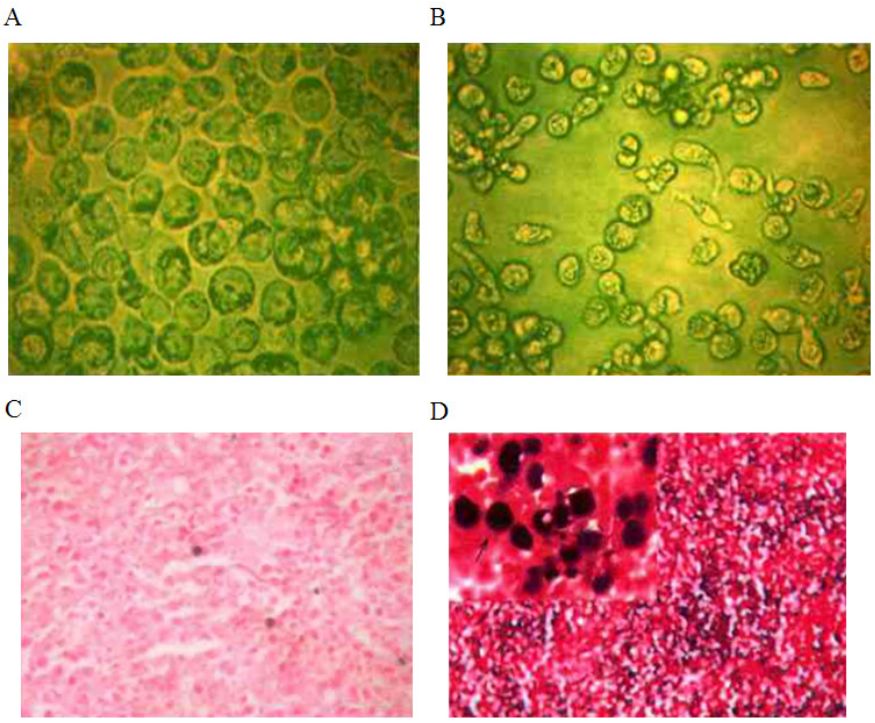

Figure 2. Survival of subcutaneous pituitary cells in the two groups of mice. **A.** Pituitary tumor cell survival in the control group was high. **B.** Pituitary tumor cell apoptosis was significantly increased in the experimental group. Giemsa-Wright staining. Inverted microscope (magnification 400X). **C.** Pituitary tumor cell survival in the control group was high. **D.** Pituitary tumor cell apoptosis was significantly increased in the experimental group. HE staining. Ordinary optical microscope (magnification 100X, except for top left corner of Panel B, where magnification is 400X).

Effect of *LRIG1* on tumorigenic ability of RC-4B/C pituitary tumor cells in nude mice

The mRNA levels of downstream molecules in the EGFR signaling pathway (Ras, Raf, AKt, and ERK) in the tumor cells were quantitatively detected by real-time PCR. The results showed that Ras, Raf, and AKt mRNA levels in RC-4B/C cells were reduced, suggesting that activation of Akt and ERK was inhibited. The transfection of RC-4B/C with *LRIG1* inhibited the activities of the PI3K/Akt and Ras/Raf/ERK signaling pathways in the EGFR signal transduction pathway ($P < 0.01$; Table 2).

Table 2. Effect of the *LRIG1* gene on the expressions of Ras, Raf, and AKt mRNA in RC-4B/C cells (means ± SD).

Items/groups	Control group	Experimental group
Ras mRNA	1.59 ± 0.29*	0.92 ± 0.21
Raf mRNA	1.51 ± 0.27*	0.78 ± 0.19
AKt mRNA	1.46 ± 0.28*	0.73 ± 0.22

*Compared with the control group, Ras, Raf, and AKt density values were significantly decreased in experimental group ($P < 0.01$).

Morphological observations

Early apoptotic cells exhibited fluorescence hyperfunction in that the cell staining with yellow green-yellow fluorescence was enhanced. In late apoptosis, cell volume was increased and edges were blurred. Apoptotic bodies and cells were swollen. The necrotic cells were stained orange-red. The electron microscope showed that the apoptotic cell membrane was blurred. Condensed heterochromatin was observed in the nuclei. The outer membrane of mitochondria in the cytoplasm appeared to be degraded. Nucleoli were also degraded into many small osmic acid granules distributed in the central nucleus. Different sizes of apoptotic cells, apoptotic bodies, and scattered cell debris surrounded by plasma membrane were observed after being cultured for 48 h. The pituitary tumor cells suffered severe damage. Many vacuoles appeared in the cytoplasm, and the nuclei were highly concentrated. The cell membrane was not complete. Organelles in the cytoplasm had dissolved into debris. In contrast, subcutaneous tumor cells of mice in the control group were mainly normal. The cell size was uniform, and the nuclear chromatin was uniformly green. Few early apoptotic cells were evident (Figure 3).

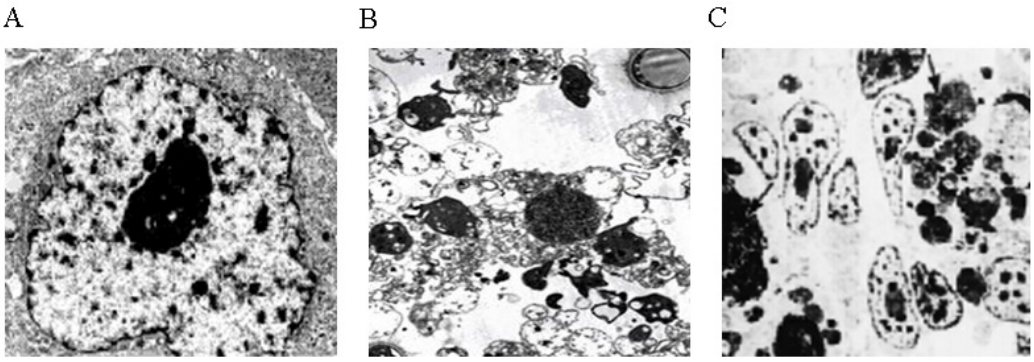

Figure 3. Apoptosis of subcutaneous tumor cells under electron microscopy. **A. B.** Cells are inflated, cell membrane is disrupted, cell contents have been released. **C.** Cell nuclei are condensed and fragmented. Necrosis has occurred. Transmission electron microscope (magnification 10,000X).

DISCUSSION

Pituitary adenomas are one of the most common intracranial tumors. The standard transsphenoidal treatment can safely resect most pituitary tumors, and the patient's prognosis is usually good. If, however, the tumor demonstrates invasive growth, where the parasellar and clival regions are widely invaded, serious clinical symptoms can occur. Because exposure is relatively limited through the standard transsphenoidal approach, it is difficult to completely remove the tumor (Mao et al., 2013). In addition, invasive pituitary adenomas have unique biological behaviors, and the pathogenesis is not yet clear. These factors lead to poor treatment results of invasive pituitary adenomas. Long-term recurrence also exists. Therefore, studies into the genes involved in invasive pituitary tumors are the basis of efficient diagnostic and treatment strategies (Lu et al., 2013).

The latest research indicates that the target genes of ErbB receptor family molecules may represent new therapeutic targets for the treatment of pituitary tumors. The *LRIG1* gene

was discovered and cloned in recent studies on the human EGFR-negative feedback regulation process (Krig et al., 2011). The expression of LRIG1 mRNA and protein was reduced in a variety of tumor cells, including pituitary tumors, compared with those of normal tissue. Recent studies have shown that this results in an inhibitory effect on liver cancer, breast cancer, and other malignant tumors cells. Little research exists, however, on whether the *LRIG1* gene inhibits pituitary tumor cell growth and induces pituitary tumor cell apoptosis *in vivo* (Yan et al., 2012). Therefore, we sought to discover whether *LRIG1* could inhibit the growth of pituitary tumor cells.

In order to further clarify the effect of *LRIG1* in pituitary tumors and the related mechanism, an animal model of the human pituitary tumor RC-4B/C cell line via subcutaneously implanted tumors was established in nude mice. The RC-4B/C cell morphology and biological activity were assessed *in vivo* following *LRIG1* gene transcription (Bai et al., 2012). The experimental results showed that *LRIG1* had a significant inhibitory effect on human pituitary tumor RC-4B/C cells implanted in nude mice.

In our study, mice were randomly divided into two groups. The mice in the control group were injected with normal pituitary tumor RC-4B/C cells, while the mice in the experimental group were injected with *LRIG1*-transfected RC-4B/C cells. The mice were sacrificed after continuous administration for 4 weeks, and the tumor body was peeled off. Our results showed that subcutaneous tumor growth of mice in the experimental group slowed or even halted over time. Tumor volumes between the two groups were significantly different. We also found that the apoptosis index was significantly higher in *LRIG1*-transfected tumors than in control tumors. In addition, we detected the levels of Ras, Raf, AKt, and ERK mRNA in the tumorigenic cells quantitatively by real-time PCR. Levels of Ras, Raf, and Akt mRNA were reduced in *LRIG1*-transfected RC-4B/C cells, suggesting an inhibitory effect of *LRIG1* in pituitary tumors that is likely to inhibit the activities of the PI3K/Akt and Ras/Raf/ERK EGFR molecular pathways ($P < 0.05$).

Research on tumor apoptosis has shown that protooncogenes and tumor suppressor genes associated with cell proliferation are involved in the regulation of cell apoptosis (Muller et al., 2013). There are 3 primary apoptosis signaling pathways: the FAS signal pathway, the mitochondrial pathway, and the endoplasmic reticulum pathway. At present, the mitochondrial pathway is thought to play a central role in the process of apoptosis (Mao et al., 2012; Wang et al., 2013). In this study, in the experimental group, a considerable proportion of subcutaneous tumor cells were found to be in early apoptosis or late apoptosis under transmission electron microscope. In contrast, only a few early apoptotic cells existed in the control cells, suggesting that *LRIG1* plays an important role in promoting pituitary tumor cell apoptosis. This provides an important experimental basis for the clinical application of *LRIG1* in the treatment of pituitary tumors (Xie et al., 2013).

This study confirmed that *LRIG1* prepared *in vitro* can inhibit proliferation and promote apoptosis of RC-4B/C cells. There is little known about the specific pathway *LRIG1* uses to exert its effect on RC-4B/C cell anti-proliferation and apoptosis (Li et al., 2011; Thomasson et al., 2011). The mechanism may involve the reduction of expression of PI3K/Akt and Ras/Raf/ERK in the RC-4B/C cell line via a negative regulatory pathway. Because the regulatory mechanisms of Ras, Raf, and AKt in cell proliferation are highly complex, additional research should focus on which signaling pathway of *LRIG1* is responsible for the inhibition of cell proliferation observed in this study.

Conflicts of interests

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We would be grateful for all the participants in this study.

REFERENCES

Bai L, McEachern D, Yang CY, Lu J, et al. (2012). LRIG1 modulates cancer cell sensitivity to Smac mimetics by regulating TNFα expression and receptor tyrosine kinase signaling. *Cancer Res.* 72: 1229-1238. http://dx.doi.org/10.1158/0008-5472.CAN-11-2428

Kitano M and Taneda M (2001). Extended transsphenoidal approach with submucosal posterior ethmoidectomy for parasellar tumors. Technical note. *J. Neurosurg.* 94: 999-1004. http://dx.doi.org/10.3171/jns.2001.94.6.0999

Krig SR, Frietze S, Simion C, Miller JK, et al. (2011). Lrig1 is an estrogen-regulated growth suppressor and correlates with longer relapse-free survival in ERα-positive breast cancer. *Mol. Cancer Res.* 9: 1406-1417. http://dx.doi.org/10.1158/1541-7786.MCR-11-0227

Li F, Ye ZQ, Guo DS and Yang WM (2011). Suppression of bladder cancer cell tumorigenicity in an athymic mouse model by adenoviral vector-mediated transfer of LRIG1. *Oncol. Rep.* 26: 439-446.

Liu JK, Patel J and Eloy JA (2015). The role of temozolomide in the treatment of aggressive pituitary tumors. *J. Clin. Neurosci.* 22: 923-929. http://dx.doi.org/10.1016/j.jocn.2014.12.007

Lu L, Teixeira VH, Yuan Z, Graham TA, et al. (2013). LRIG1 regulates cadherin-dependent contact inhibition directing epithelial homeostasis and pre-invasive squamous cell carcinoma development. *J. Pathol.* 229: 608-620. http://dx.doi.org/10.1002/path.4148

Mao F, Wang B, Xi G, Sun W, et al. (2012). Effects of RNAi-mediated gene silencing of LRIG1 on proliferation and invasion of glioma cells. *J. Huazhong Univ. Sci. Technolog. Med. Sci.* 32: 227-232. http://dx.doi.org/10.1007/s11596-012-0040-8

Mao F, Wang B, Xiao Q, Xi G, et al. (2013). A role for LRIG1 in the regulation of malignant glioma aggressiveness. *Int. J. Oncol.* 42: 1081-1087.

Muller S, Lindquist D, Kanter L, Flores-Staino C, et al. (2013). Expression of LRIG1 and LRIG3 correlates with human papillomavirus status and patient survival in cervical adenocarcinoma. *Int. J. Oncol.* 42: 247-252.

Qi XC, Xie DJ, Yan QF, Wang YR, et al. (2013). LRIG1 dictates the chemo-sensitivity of temozolomide (TMZ) in U251 glioblastoma cells via down-regulation of EGFR/topoisomerase-2/Bcl-2. *Biochem. Biophys. Res. Commun.* 437: 565-572. http://dx.doi.org/10.1016/j.bbrc.2013.06.116

Rafidi H, Mercado F, 3rd, Astudillo M, Fry WH, et al. (2013). Leucine-rich repeat and immunoglobulin domain-containing protein-1 (Lrig1) negative regulatory action toward ErbB receptor tyrosine kinases is opposed by leucine-rich repeat and immunoglobulin domain-containing protein 3 (Lrig3). *J. Biol. Chem.* 288: 21593-21605. http://dx.doi.org/10.1074/jbc.M113.486050

Thomasson M, Wang B, Hammarsten P, Dahlman A, et al. (2011). LRIG1 and the liar paradox in prostate cancer: a study of the expression and clinical significance of LRIG1 in prostate cancer. *Int. J. Cancer* 128: 2843-2852. http://dx.doi.org/10.1002/ijc.25820

Wang Y, Poulin EJ and Coffey RJ (2013). LRIG1 is a triple threat: ERBB negative regulator, intestinal stem cell marker and tumour suppressor. *Br. J. Cancer* 108: 1765-1770. http://dx.doi.org/10.1038/bjc.2013.138

Xie R, Yang H, Xiao Q, Mao F, et al. (2013). Downregulation of LRIG1 expression by RNA interference promotes the aggressive properties of glioma cells via EGFR/Akt/c-Myc activation. *Oncol. Rep.* 29: 177-184.

Yan ZJ, Cheng Y, Jiang JH, Hu JS, et al. (2012). Gene therapy study on bladder cancer with recombinant adenoviral vector carrying LRIG1 gene driven by Survivin promoter. *Zhonghua Wai Ke Za Zhi* 50: 732-736.