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Effects of Ezrin on the Proliferation and Apoptosis of Renal Cell Carcinoma*

YANG Xu-kai[1], *GUO Xiu-quan*[2], *WANG Yang-min*[1△], *ZHOU Feng-hai*[1], *LAN tian*[1], *QIAO Gou-mei*[1]

(1 Department of Urology, Lanzhou General Hospital of Lanzhou Command, GanSu, Lanzhou, 730050, China;

2 Department of Urology, Lanzhou University Second Hospital, Gansu, Lanzhou,730000, China)

ABSTRACT Objective: To construct the short hairpin RAN which is a specificity expression vector of membrane-cell skeleton join Ezrin protein gene, and to investigat its roles in proliferation and apoptosis of RCC (Renal Cell Carcinoma). **Methods:** Ezrin was designed, chemically synthesized and inserted into plasmid pGenesil-shRNA, which was transformed into DH5α. The recombinant plasmid was extracted in middle quantity and transferred into renal carcinoma cell line 786-0 by Lipofectamine 2000. Ezrin expressing in 786-0 cells transfected by shRNA recombinant plasmid was detected by qRT-PCR. 786-0 cells were transferred by effective shRNA plasmid and cultured in 1640 media containing G418 (800 μg/ml). The blocking effect of shRNA-Ezrin was detected by qRT-PCR; The proliferation and apoptosis were assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and Flow cytometry. Protein expression was assayed by western blotting. Autophagy was evaluated by transmission electron microscopy. **Results:** After transfected with pGeneSil-l-Ezrin, the proliferation capability of 786-0 cells decreased dramatically; Compared with that in the cells treated with shRNA-Ezrin1, the percentage of cells in G_0/G_1 stage were significantly higher than those in untransfected cells and 786-0cells transferred with shRNA-HK（P<0.01）and PI(proliferation index) decreased in the cells treated with shRNA-Ezrin1 compared with those in untransferred cells and 786-0 cells transferred with shRNA-HK （P<0.01）. **Conclusions:** Ezrin plays an important role in cell proliferation and apoptosis of human renal carcinoma cells, which may be regarded as a promising target for tumor gene therapy.

Key words: Ezrin; Renal cell carcinoma; RNA interference

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Ezrin protein is a kind of specific protein linking the cytoskeleton and cell membrane, it is documented that Ezrin expressed in the physiological process, such as cell movement, migration, mitosis, cell apoptosis, moreover, it plays an important role in the process of the tumor invasion [1,2]. Research on Ezrin's function in RCC cells is not frequently documented in literature. This report investigated its roles in proliferation and apoptosis of kidney cancer.

1 Materials and methods

1.1 Chemicals and Reagents

Plasmid vector pGenesil-1 and general negative contrast plasmid HK were purchased from Wuhan Genesil Biotechnology Co. Ltd . RCC 786-0 cell line was purchased from experimental center of South hospital. lipofectamine 2000 was purchased from Invitrogen, and SYBR Premix Ex Taq Fluorescence quantitative PCR reverse transcription, Transcription kit, the design and synthesis of primer were from Dalian Takala company (a China company). Ezrin was purchased from rabbit against human clone antibody from American GenScript company; RPMI1640 medium was purchased from Gibco. Fetal calf serum was purchased from Hangzhou sijiqing, a China company, and other reagents were domestic or imported analysis and purification.

1.2 Cell culture

The human RCC 786-0 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco) contained 10% heat- inactivated fetal bovine serum (FBS), penicillin (100 U/ mL) and streptomycin (100 mg/mL) at 37 ℃ under an atmosphere of 95% air and 5% CO_2. RCC cell line 786-0, cells cultured without antibiotic medium (RPMI1640 + 10% fetal bovine serum), was cultured at 37 ℃ , 5% CO_2 incubator training, while water was regularly changed, passage, then transfected the cells in three generations.

1.3 Hairpin type RNA (shRNA) plasmid construction and transfection

NCBI's gene pool (GeneBank) and computer aided design software were used to design two different sequences of specific oligonucleotide interference fragment (shRNA) contained Ezrin gene, the target gene (NM_003379.4GI:161702984)constructed and synthesized by Wuhan Genesil Biotechnology Co.Ltd.(a Chinese company), and cloned into pGenSilH1 plasmid, converted to *Escherichia coli* DH5α, amplificated and sequencing appraisal by Shanghai Yingjun biotechnology company (a Chinese company).

1.4 Screening the highest efficiency oligonucleotide interference fragments (shRNA)

48 hours after plasmid was transfected, Ezrin mRNA expression with SYBRPrimeScriptTM RT kit was detected, according to the instructions for real-time fluorescent quantitative polymerase chain reaction (PCR), this experiment followed[3] the relative quan-

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Author introduction: YANG Xu-kai, (1971-), male, medical doctor, Mainly engaged in urology cancer research, E-mail:yangxk125@126.com

△Corresponding author: WANG Yang-min, E-mail: wangymmr@163.com

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titative to detect Ezrin mRNA expression difference between the experimental group and the control group, β-actin as internal reference gene, Ct value obtained by the amplification curve for each sample, screening out interference efficiency higher fragment.

1.5 RNA interference influence on 786−0 cell proliferation, apoptosis

Proliferation activity were tested by MTT assay: cells were devided into untransfected group (blank), negative plasmid transfected group (HK), shRNA - Ezrin1 transfected group, shRNA-Ezrin2 transfected group, a blank control(experimental hole parallel without cells only add nutrient solution, other experimental procedure remains the same). In the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th day after being transfected cells were Collected to detected the light absorption value (A value)for each group. Cell apoptosis and proliferation cycle detected by Flow cytometry (groups as above), (1 ~ 5)× 10^6 cells were collected, centrifugaled (500 ~ 1000r/min, 5 min), nutrient solution abandoned. apoptosis cell detection index according to the G1 subpeak, cell proliferation index PI (proliferation index) = [S + G_2 / M] / [G_0 / G_1 + S + G_2 / M]. The ultrastructure of 786-0 cells was contrasted before and after transfection by Projection electron microscope (sem) departly.

1.6 Statistical analysis

The date were compiled with the software package SPSS13.0. Date were presented as mean ± SD and analyzed with one-way analysis of variance, p Value <0.05 was regarded as significant .

2 Results

2.1 Transfection efficiency measurement

786-0 cells transfected with pGeneSil-l-ezrin were cultured successfully. 24 hour after incubation, GFP(green fluorescent protein) expression cells was assessed with a fluorescence microscopy (Fig 2), and number of GFP positive cells per field of view at magnification of 200 was counted. The GFP-positive cells was no difference between the lipofectamine-shRNA- treated cells (P>0.05). In each group the number of GFP-positive cells accounted for 80-83%, this result demonstrated that lipofectamine was able to delivery of shRNA into cells.

Table 1 Determination of transfection efficiency of 786-0 cell mediated by shRNA

Group	n	Transfection efficiency(%)
shRNA-HK	6	82.758± 0.505
shRNA-Ezrin1	6	80.567± 0.090*
shRNA-Ezrin2	6	82.600± 0.078*

F=0.160, P=0.853; *P>0.05 vs shRNA-HK; P>0.05 shRNA- ezrin2 vs shRNA-ezrin1

2.2 Screening highest interference efficiency fragment

Fluorescence quantitative PCR results showed that transfected cells in all groups grown for 48 hours have some degrees of ezrin gene amplification, and the relative expression levels of ezrin mRNA were 100%, 94.61% , 33.45%, 47.63%, respectively, in the group of p-shRNA-0, p-shRNA-hk, p-shRNA- ezrin1 and p-shRNA- ezrin2, inhibition efficiency of shRNA-ezrin1 group was up to 66.55%.

2.3 Effects of different shRNA on renal carcinoma line 786−0cell proliferating activity by MTT

From the Fig 3, the proliferation rate of 786-0 cells expressing shRNA-ezrin reduced with the decreasing of OD values in a time-dependent manner, and there was significant differences in viability after 3-7 days, compared with the untransfected 786-0 cells.

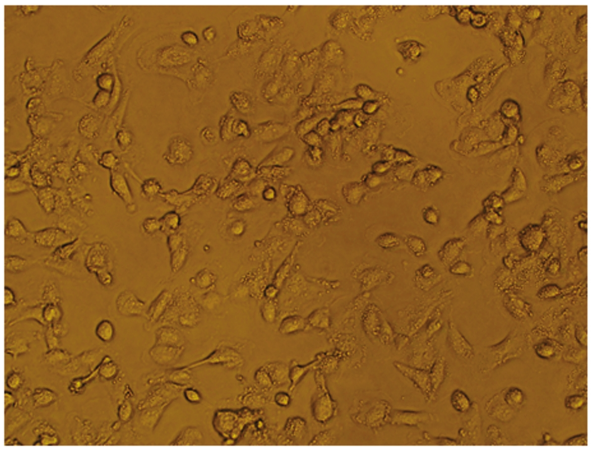

Fig.1 786-0 cells transfected with pGeneSil-l-ezrin were observed by light microscopy(× 300)

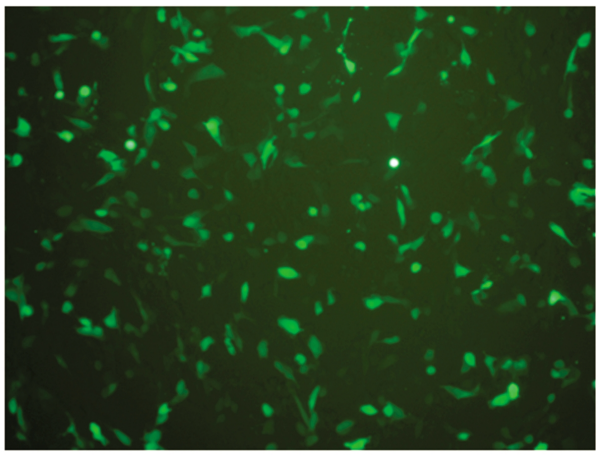

Fig.2 786-0 cells transfected with pGeneSil-l-ezrin were observed by fluorescence microscopy(× 200)

2.4 The influence of RNA interference on 786−0, apoptosis and cell proliferation cycle

Apoptosis of 786-0cells after 48h treatment of shRNA-ezrin was detected by flow cytometry. Apoptotic rate of cells treated with shRNA-ezrin1 for 48 hour was significantly higher than those

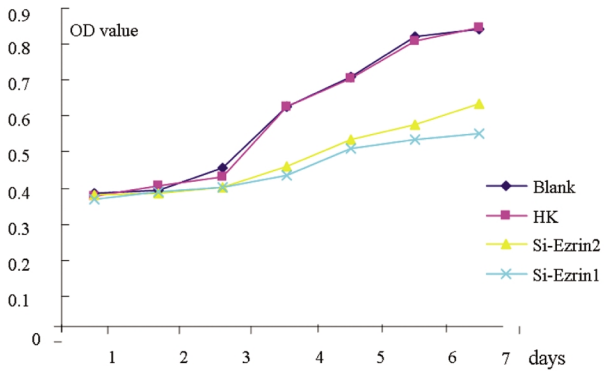

Fig.3 Growth curve of human renal carcinoma line cell 786-0 affected by RNAi

of untransfected cells and 786-0cells transfected with shRNA-hk (P<0.01); Compared with the cells treated with shRNA-ezrin, the percentage of G0/G1 cell was significantly higher than those of untransfected cells and 786-0cells transfected with shRNA-hk (P<0.01). PI (proliferation index) decreased in the cells treated with shRNA-ezrin compared with those of untransfected cells and 786-0cells transfected with shRNA-hk(P<0.01).

2.5 The changes of ultrastructure in 786−0 line cell were oberseved by Projection electron microscopy

Margination and fragmentation were found in unclear zone, and nuclear concentration appeared, which presented typical apoptotic cells formation (Fig 4); 786-0 cells untransfected were found nuclear membrane integrity, chromatin dispersion, without obvious nuclear apoptosis change phenomenon(Fig 4).

Table 2 Effect of RNAi on human renal cell carcinoma line 786-0 cell apoptosis and proliferation

Group	G_0/G_1(%)	S(%)	G_2/M(%)	Apoptosis(%)	PI(%)
Blank	55.330± 4.376	25.997± 6.619	8.551± 1.183	2.203± 0.758	32.017± 4.405
HK	57.047± 4.204	25.445± 8.000	7.337± 1.375	1.765± 0.680	30.637± 4.782
Ezrin2	83.317± 3.473#	12.575± 3.712#	5.270± 1.878	11.334± 5.387#	18.068± 1.622#
Ezrin1	76.753± 5.372*	14.488± 6.086*	6.989± 1.701	16.321± 7.618*	20.279± 4.821*

G_0/G_1:F=101.469, P<0.001;S: F=12.690, P<0.001; G_2/M:F=5.588, P=0.003; apoptosis: F=9.707, P<0.001; PI: F=29.712, P<0.001; *P<0.01 vs blank and HK; #P<0.01 vs blank and HK; G_0/G_1: Ezrin2 vs Ezrin1 P<0.01; proptosis and PI: Ezrin2 vs Ezrin1 P> 0.05; (G_0/G_1,S,G_2/M): blank vs HK P> 0.05.

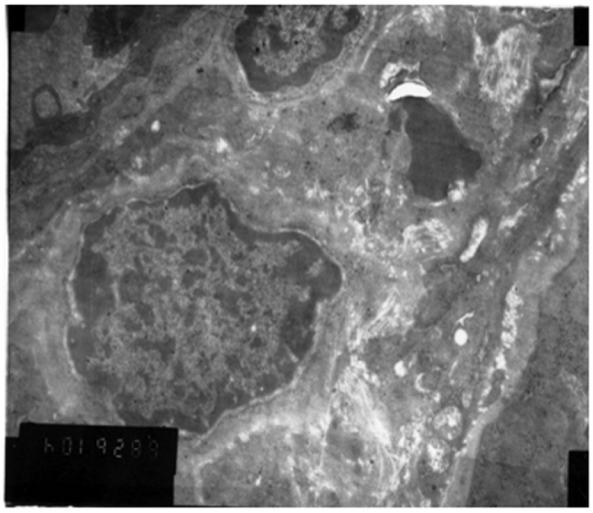

Fig.4 The changes of ultrastructure in 786-0 cells transfected

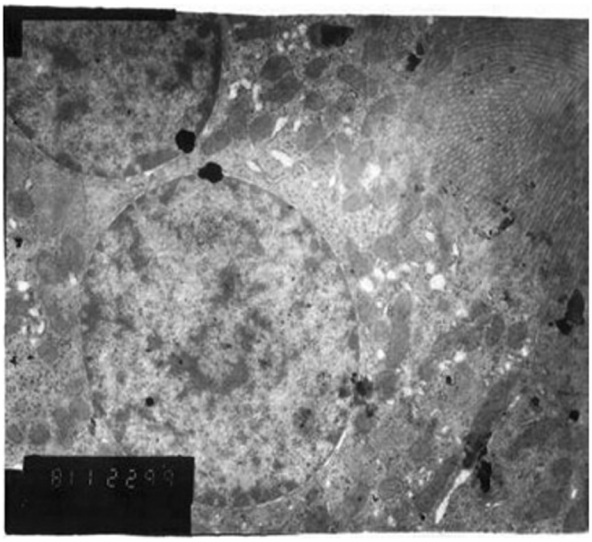

Fig.5 The changes of ultrastructure in 786-0 cells untransfected

3 Discussion

The metastasis in patients with malignant tumor is the main causes to death, about 90% of the malignant tumor patients died of tumor metastasis, and the way to prevent and control tumor metastasis is the key to improve the survival rate of patients with tumor. Ezrin is a kind of connection film-cytoskeletal protein, composition of membrane cells related complex and formed special membrane structure on cell to induce activity regulation[20], such as cell morphology, adhesion and movement[19], which have important relationships with tumor occurrence, development and metastasis[3]. The changes of the cytoskeleton in cell transformation and canceration process is a very common phenomenon, many activated proto-oncogenes can cause damages of transformed cell actin filament and local adhesivity decline [4]. The arrangement of cytoskeleton is the key factor to determine cell morphology and athletic ability, if changes in the tumor cells, it will lead tabular pseudopods and filamentous pseudopods increase the formation of tumor, enhance its movement and transformation ability. Ezrin, as a ERM protein family member, is a kind of membrane and cytoskeleton connec-

tion protein, transmissing extracellular signal to the skeleton, which cause skeleton protein to configuration, change the cell's ability to transport [5].

Ezrin is high expression in many tumor tissues[6,7], Akisawa et al [8] detection 16 kinds of pancreatic cancer cell lines, the results showed that the metastasis of pancreatic cancer have correlation with high expression of Ezrin. Wan et al [9] reported that a high level of Ezrin can induce transformation of abnormal growths in a wide variety of cell lines and the tumor cells with strong metastasis ability often accompanied by high expression of Ezrin, in esophageal cancer, lung cancer and prostate cancer within the tumor cells and Ezrin increased their expression has been proved, and increased expression of Ezrin has relationship with tumor infiltration and metastasis[10].

At present, researches on siRNA for RCC gene therapy is still rare documented, we use Ezrin gene shRNA plasmid vector transfection human RCC cell lines 786-0 cells, and the results show that the transfection efficiency shRNA in kidney cancer cell lines 786-0 cells is higher about 80%, through Real-time fluorescent quantitative PCR detection interference effect, Ezrin mRNA inhibitory rate of shRNA - Ezrin1, shRNA - Ezrin2, shRNA - HK were 66.55%, 52.37%, 5.39%, and the shRNA - Ezrin1 interference efficiency is the highest. With shRNA - Ezrin1 recombinant plasmid transfection of human RCC cell line 786-0 cells and changes of RCC cell proliferation, apoptosis ability were observed [11]. Proliferation, apoptosis ability were found changed significantly. Proliferation activity of shRNA - Ezrin1 interference after 786-0 cell significantly suppressed comparing 786-0 cell not interfered by shRNA - Ezrin1 in MTT test. 786-0 cells transfected by shRNA - Ezrin1 were detected in Flow cytometry instrument, whose G1 phase cell number ratio increased, cell proliferation index is reduced, apoptosis rate increased, transfected 786-0 cells appear nuclear mass concentrated, presented typical apoptotic cells morphological changeed, such as Nicholson in Transmission electron microscope [12], RNA interference can slowed down the cell growth rate, declined in the split proliferation issue of cell proportion, obviously inhibited the proliferation of tumor cell malignant and siRNA expression vector can effectively silent the Ezrin expression of tumor cells and inhibit the invasion and proliferation ability of tumor cell.

It were also found that the protein expression of Ezrin was inhibited by shRNA, the apoptosis of RCC 786-0 cell was increased, indicated the decline of Ezrin protein expression can promote tumor cell apoptosis. Apoptosis is restrained in the process of tumorigenesis, including CD95 (APO - 1 / Fas) mediated apoptosis way [13-15]. It is found that Ezrin protein is an important factor involving in CD95 mediated $CD4^+$ T lymphocyte apoptosis in vitro study. Hebert et al [16,17] reported Ezrin peotein activated phosphorylation can make specific connections with CD95, connected in the N FERM area of 149 to 168 amino acid residue, the root protein and membrane process protein of ERM family does not participate in CD95 connection. The connection between CD95 and Ezrin protein make CD95 and actin connect, polarity of cell membrane change, the sensitivity to CD95 mediated apoptosis change. PI3/PKB signal transduction pathways is very important to cell survival, the 353th tyrosine phosphorylation residue in Ezrin combine with PI3 kinase p85 subunit of the SH2 domain structure, activating PI3 kinase B protein, and Ezrin mutant (353th tyrosine be phenylalanine replaced) can lead to cell apoptosis. Men with high expression of Ezrin can survival longer than those with low expression in osteosarcoma cells accompany with lung tissue metastasis, moreover, tumor cell with Ezrin expression repressed will go to apoptosis [18]. We speculate that Ezrin may prevent the apoptosis of tumor cells in metastasis, by means of activating PI3K, so as to promote the metastasis of tumor cells.

To sum up, film cytoskeletal protein - Ezrin is closely with renal carcinoma proliferation, apoptosis, Ezrin - siRNA can effectively inhibit Ezrin gene expression in human kidney cancer cell, inhibiting the proliferation of the kidney cancer and play an important role in inducing tumor cell apoptosis, indicating that the Ezrin - siRNA is expected to become an effective means of gene therapy for RCC.

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RNA 干扰 Ezrin 体外对肾癌细胞株 786-0 增殖、凋亡的影响 *

杨旭凯[1] 郭秀全[2] 王养民[1△] 周逢海[1] 蓝 天[1] 乔够梅[1]

(1 兰州军区兰州总医院全军泌尿外科中心 甘肃 兰州 730050;2 兰州大学第二临床医学院泌尿外科 甘肃 兰州 730000)

摘要 目的:构建膜 - 细胞骨架联接蛋白 Ezrin 基因特异性短发卡 RAN 表达载体(small hairpin RNA,shRNA),探讨其对肾癌细胞株 786-0 细胞凋亡、增殖的影响。**方法:**以 Ezrin 为靶基因,以 Pgenesil-1 质粒为载体,设计和构建重组体,设计 2 条发夹式 RNA(shRNA),合成后克隆入载体 Pgenesil-1,扩增并中量提取质粒 ,应用脂质体 Lipofectamine 2000 转染进 786-0 细胞,重组质粒转染 786-0 肾癌细胞株,用运实时荧光定量 PCR 进行筛选鉴定,筛选出抑制率较高的重组质粒载体 shRNA-Ezrin1,用 shRNA-Ezrin1 转染 786-0 细胞,采用 MTT、流式细胞仪、电镜检测,观察 RNA 干扰 Ezrin 后肾癌细胞株 786-0 细胞增殖能力的改变。**结果:**shRNA 干扰后 786-0 细胞增殖活性减弱,G_0/G_1 时段明显延长(P<0.01),PI 缩短(P<0.01),细胞凋亡率增加(P<0.01)。**结论:**Ezrin 与肾癌细胞凋亡、增殖有关,有望成为肾癌基因治疗的一个新靶点。

关键词: Ezrin 蛋白;肾癌;RNA 干扰

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作者简介:杨旭凯(1971-),男,博士,副主任医师,主要从事泌尿系肿瘤研究,E-mail:yangxk125@126.com

△通讯作者:王养民,兰州军区兰州总医院全军泌尿外科中心主任,教授,硕士研究生导师,E-mail:wangymmr@163.com,Tel:0931-8994291

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