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Inhibitory Effects of PEI-RGD/^{125}I-(α_v) ASODN on Growth and Invasion of HepG2 Cells

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: To investigate the *in vitro* inhibitory effects of PEI-RGD/^{125}I-(α_v)ASODN (PEI, polyethylenimine; RGD, Arg-Gly-Asp; ASODN, antisense oligodeoxynucleotide) on the growth and invasion of HepG2 cells.

Material/Methods: ASODN of the integrin α_v-subunit was marked with ^{125}I and underwent complexation with PEI-RGD, a PEI derivative. Next, PEI-RGD/^{125}I-(α_v) ASODN was introduced into HepG2 cells via receptor-mediated transfection, and its inhibition rate on HepG2 cell growth was tested using the methyl thiazolyl tetrazolium (MTT) method. The effects of PEI-RGD/^{125}I-(α_v) ASODN on HepG2 cell invasion ability were evaluated using the Boyden chamber assay.

Results: 1) The ^{125}I marking rate of (α_v) ASODN was 73.78±4.09%, and the radiochemical purity was 96.68±1.38% (greater than 90% even after a 48-h incubation period at 37°C), indicating high stability. 2) The cytotoxicity assays showed that the cell inhibition rates did not differ significantly between the PEI-RGD/^{125}I-(α_v)ASODN group and the PEI-RGD/(α_v) ASODN group, but they were both significantly higher than in the other groups and were positively correlated (r=0.879) with the dosage within a certain range. 3) The invasion assays showed that the inhibition rate was significantly greater in the PEI-RGD/^{125}I-(α_v) ASODN group compared to the other groups.

Conclusions: PEI-RGD/^{125}I-(α_v) ASODN can efficiently inhibit the growth and proliferation of HepG2 cells and can also weaken their invasive ability.

MeSH Keywords: Integrin alphaV • Liver Neoplasms • Neoplasm Invasiveness

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Background

Integrins are a type of cell membrane receptor protein composed of various α and β subunits, among which the α_V subunit is highly expressed on the surface of hepatic cell carcinoma (HCC) [1]. The α_V subunit is critically important during the infiltration, metastasis, and neovascularization of tumor cells. It is reported that targeting of alpha-v integrins reduces malignancy of bladder carcinoma [2]. Currently, research on targeted therapies aiming at integrins include monoclonal antibodies against integrins, integrin antagonists, and targeted modulation of integrin genes, among which integrin antagonists are mainly express in the artificially synthesized polypeptides containing Poly (RGD) structures, such as cilengitide [3–5]. Gene therapy studies have reported on the antisense technologies and RNA interfering technologies of oligonucleotides [6–11].

Use of radionuclides in internal radiation therapies has been a research emphasis of tumor therapies, among which some radionuclides decayed through electron capture, like ^{125}I, emitting many low-energetic Auger electrons and Coster-Kroning (CK) electrons during its decay process. ^{125}I was often marked in nucleic acids and its analogues during targeted therapies. However, there is little published data on ^{125}I marked antisense oligodeoxynucleotide (ASODN) and small interfering RNA (siRNA). siRNA is easily cleared by the kidneys, it is degraded by enzymes, the cell uptake rate is low, and the markers on it are easy to drop, which limit its wide usage [12]. Thus, ^{125}I was marked on ASODN in this study.

As a PEI derivative, polyethylenimine-Arg-Gly-Asp (PEI-RGD) is a receptor-dependent nonviral vector. After PEI undergoes complexation with nucleic acids and its analogues and RGD binds to the α_V subunit, PEI-RGD together with nucleic acids and its analogues enter cells through pinocytosis. In this study, the α_V subunit of antisense oligodeoxynucleotide (ASODN) was marked by radionuclide ^{125}I. After complexation with PEI-RGD, the PEI-RGD/^{125}I-(α_V) ASODN compound was introduced into HepG2 cells via receptor-mediated transfected. We analyzed inhibitory effects of the compound on the growth and invasion of HepG2 cells and discussed the integration of antisense technology, RGD inhibitors, and nuclide internal irradiation in HCC treatment.

Material and Methods

Materials and reagent

The integrin α_V subunit ASODN (5'-GCCCTCCTTCCACAATCCCA-3') sequence was examined using BLAST and was found to be nonhomologous with other human gene coding sequences. Purification through polyacrylamide gel electrophoresis (PAGE) and the generation of phosphorothioates at the 5' and 3' ends were performed by Shanghai Sangon Biotech Co., Ltd (Shanghai, China). PEI-RGD was purchased from PolyPlus-transfection. The InnoCyte™ Cell Invasion Assay Kit (QIA129) was provided by Merck. TlcI3 was purchased from Sigma. Na^{125}I was purchased from ChengDu GaoTong Isotope CO., Ltd (CNNC).

(α_V) ASODN marking by ^{125}I, purification and identification

Approximately 20 μg of (α_V) ASODN was dissolved in 0.1 M ammonium acetate solution (pH 5.0) and heated at 60°C for 30 min. The mixture was then added successively to 10 μl of 0.25 mM KI, 10 μl of 5 mCi Na^{125}I, and 100 μl of 0.03 M TlcI3, mixed evenly and heated at 60°C for 45 min. After cooling at room temperature, 5 μl of 0.1 M Na_2SO_3 and 20 μl of 1 M ammonium acetate solution were added successively; then, the mixture was adjusted to pH 7.0 and heated at 60°C for 60 min [13]. Using acetone: acetic ether (1:1, V/V) as a developer, the marking ratio was computed using paper chromatography. Using 0.01 M hydroxyethyl piperazine ethanesulphonic acid (HEPES, pH 7.4) as an eluent, the reaction mixture was purified using a Sephadex G25 column. Then, the radiochemical purity was calculated. The radiochemical purity of the purified reactants was separately measured at 24, 48, 72, and 96 h, and the stability was observed *in vitro*.

Cultivation and transfection of HepG2 cells

The HCC strain HepG2 was kindly provided by the Model Organism Open Laboratory at the College of Life Science and Technology, Tongji University. The strain was cultured in Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum in an incubator with 95% humidity and 5% CO_2 at 37°C. The cells were routinely passaged using 0.25% pancreatic enzyme. Cells were grown to logarithmic phase, and drugs were delivered when 50–60% of the cells adhered to the walls of the culture flask. The cells were transfected with PEI-RGD (μl): ^{125}I-(α_V) ASODN (μg, 2:1).

Measurement of the cell intake rate of PEI-RGD/^{125}I-(α_V) ASODN

Log-phase HepG2 cells were adjusted with DMEM culture solution to 100 000 cells/mL and then inoculated into 96-well plates, with each mesh containing 500 μl. We established 5 study groups (2/1, 4/2, 8/4, 16/8, and 32/16 μl/μg PEI-RGD/^{125}I-[α_V] ASODN) and used a ^{125}I-(α_V) ASODN group and a ^{125}I group as the controls. Each experiment was performed in triplicate. The cells were cultured in a CO_2 incubator for 48 h, and when 50–60% of the cells adhered to the bottom of the plates, the drugs were delivered. After 24 h, the cells were digested with 0.25% pancreatic enzyme. The cell suspensions were collected into centrifuge tubes to measure the total counts per minute

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CPM(A_0). Then, the cells were centrifuged at 1500 rpm and washed 3 times. The CPM was then measured again (A). The cell intake rate (%) was defined as $A/A_0 \times 100\%$.

Cytotoxicity test of PEI-RGD/^{125}I-(α_V)ASODN

The cytotoxicity tests consisted of dose-effect and control tests. The dose-effect tests included 5 PEI-RGD/^{125}I-(α_V)ASODN groups: 2/1, 4/2, 8/4, 16/8, and 32/16 μl/μg. Seven groups were used for the control tests: a PEI-RGD/^{125}I-(α_V)ASODN group (2 μl/1 μg), a ^{125}I-(α_V)ASODN group, a (α_V)ASODN group, a PEI-RGD/(α_V)ASODN group, a PEI-RGD group, a ^{125}I group, and a blank group. After transfection (in the same manner described above), the cell inhibition rate was computed using the methyl thiazolyl tetrazolium (MTT) method.

Effects of PEI-RGD/^{125}I-(α_V)ASODN on cell invasion

Using the cytotoxicity test as a comparison, cell invasion was tested using the InnoCyte™ Cell Invasion Assay Kit (QIA129). Approximately 300–400 μl of serum-free culture solution was added to the upper chamber. After incubation for 30–60 min, approximately 500 μl of 10% foetal bovine serum medium was added to the lower chamber. Then, 300–350 μl of the transfected cell suspension was added to the upper chamber and placed into a CO_2 incubator for 48 h. Five hundred microliters of cell staining solution was added to the lower chamber. The cells that adhered to the bottom side of the upper chamber were knocked into the lower chamber and cultured for another 30 min. Approximately 200 μl of the cells in the staining solution was used for fluorescence detection, photographing, and calculation of the inhibition rates.

Statistical processing

All data were processed with SPSS, version 19.0 (IBM Corporation, Armonk, NY, USA) using analysis of variance (ANOVA) and multi-sample paired-comparisons. The results are expressed as means ± standard deviation.

Results

Marking and identification of ^{125}I-(α_V)ASODN

The marking rate of (α_V)ASODN by ^{125}I was 73.78±4.09%. After marking, ^{125}I-(α_V)ASODN was purified and placed at 37°C, and the radiochemical purity at 0, 24, 48, 72, and 96 h was 96.68±1.38%, 95.61±1.56%, 90.57±1.95%, 87.38±1.67%, and 77.79±1.86%, respectively. The radiochemical purity after 48 h was still greater than 90%, but it obviously decreased after 96 h. Thus, we suggest that further experiments with this marker should be finished within 48 h after marking.

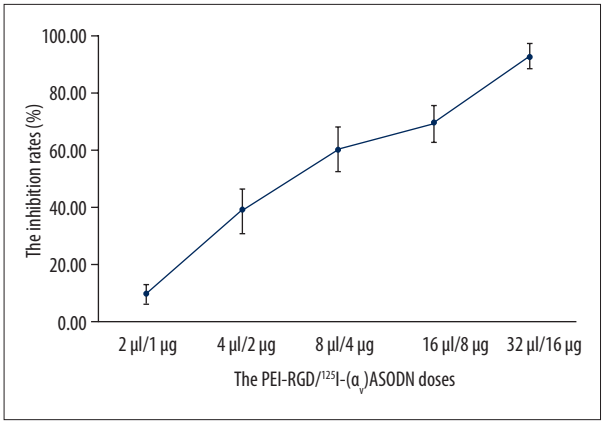

Figure 1. The dose-effect tests, with a correlation coefficient of r=0.879.

Cell intake rate of PEI-RGD/^{125}I-(α_V)ASODN

The cell intake rates of the PEI-RGD/^{125}I-(α_V)ASODN groups were 10.92±1.20% (2 μl/1 μg), 12.77±0.85% (4 μl/2 μg), 7.78±1.37% (8 μl/4 μg), 7.11±1.76% (16 μl/8 μg), and 1.42±1.03% (32 μl/16 μg), 2.19±1.35% (^{125}I-[α_V]ASODN) and 0.31±0.28% (^{125}I).

Toxic effect of PEI-RGD/^{125}I-(α_V)ASODN on the HepG2 cells

For the dose-effect tests, the inhibition rates of the PEI-RGD/^{125}I-(α_V)ASODN doses were 9.63±3.15% (2 μl/1 μg), 38.83±7.64% (4 μl/2 μg), 60.29±7.65% (8 μl/4 μg), 69.40±6.47% (16 μl/8 μg), and 92.64±4.41% (32 μl/16 μg) (Figure 1), with a correlation coefficient of r=0.879. For the control tests, the inhibition rates are shown in Table 1 and Figure 2.

Inhibitory effect of PEI-RGD/^{125}I-(α_V)ASODN on cell invasion

The invasion test fluorescence fields for all groups are shown in Figure 3, and the inhibition rates are listed in Table 2 and Figure 4. The inhibition rate in the PEI-RGD/^{125}I-(α_V) ASODN group was significantly greater than those in the other groups ($P<0.01$), indicating the greater inhibitory effect of PEI-RGD/^{125}I-(α_V) ASODN on HepG2 cell invasion.

Discussion

The integrin α_V subunit is highly expressed in several types of malignant tumour cells (e.g., HCC and melanoma) [14–16], and it is involved in the recognition of ligands, mainly through the RGD in its homologous repeated sequence identification ligands [17,18]. The integrin α_V subunit interacts with several ingredients in the extracellular matrix (ECM), such as vitronectin, fibronectin, and fibrinogen. It is excited by extracellular growth hormone and/or

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Table 1. The cell inhibition rates of the different groups, as assessed by MTT assay (n=3).

Group	Inhibition rate (%)
PEI-RGD/^{125}I-(α_v)ASODN	9.63±3.15*
PEI-RGD/(α_v)ASODN	7.95±1.63
PEI-RGD	0.97±1.00
^{125}I-(α_v)ASODN	1.85±0.72
(α_v)ASODN	2.32±1.03
^{125}I	1.70±0.64

* Compared with the PEI-RGD/(α_v)ASODN group, P>0.05; compared with other groups, P<0.001.

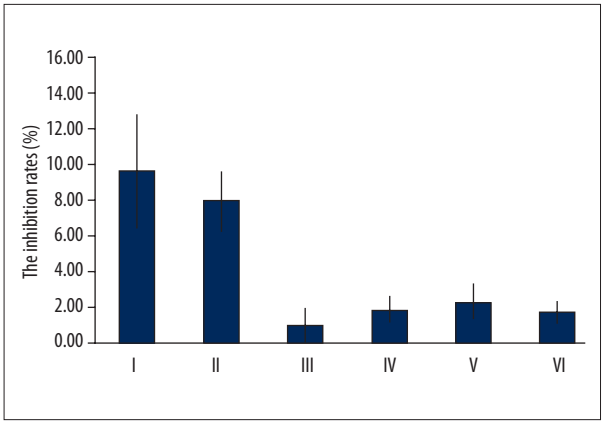

Figure 2. The cell inhibition rates of the different groups, as assessed by MTT assay (n=3). Group I: PEI-RGD/^{125}I-(α_v) ASODN; II: PEI-RGD/(α_v)ASODN; III: PEI-RGD; IV: ^{125}I-(α_v) ASODN; V: (α_v)ASODN; VI: ^{125}I.

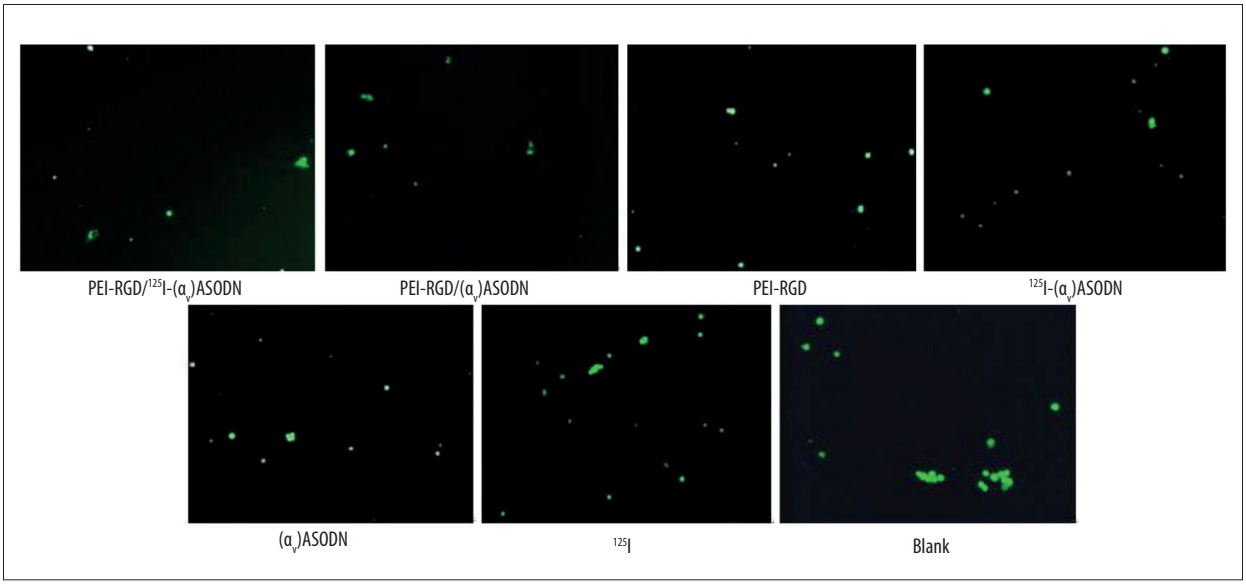

Figure 3. The invasion test fluorescence fields for all groups.

Table 2. The Boyden chamber invasion assay fluorescence values and inhibition rates for the study groups and the control group (n=3).

Group	Fluorescence	Inhibition rate (%)
PEI-RGD/^{125}I-(α_v)ASODN	32.53±3.54	52.60±4.11*
PEI-RGD/(α_v)ASODN	41.33±2.99	39.73±3.40*
PEI-RGD	47.91±2.76	30.05±5.19*
^{125}I-(α_v)ASODN	58.88±3.18	14.14±2.94**
(α_v)ASODN	59.80±3.13	12.79±2.68
^{125}I	67.94±3.21	0.91±2.91***
Blank	68.55±1.52	0

* Compared with the control group, P<0.01; ** Compared with the (α_v)ASODN group, P>0.05; *** Compared with the control group, P>0.05.

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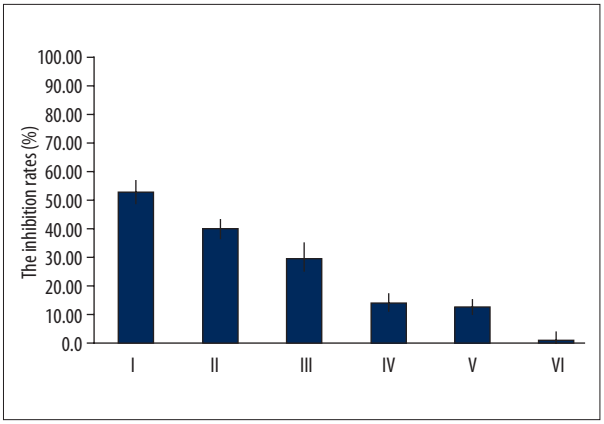

Figure 4. The Boyden chamber invasion assay fluorescence values and inhibition rates for the study groups and the control group (n=3). Group I: PEI-RGD/^{125}I-(α_V) ASODN; II: PEI-RGD/(α_V)ASODN; III: PEI-RGD; IV: ^{125}I-(α_V) ASODN; V: (α_V)ASODN; VI: ^{125}I.

ECM signals, and it alters the proliferation, differentiation, and migrating abilities of tumor cells by activating Ras/MAP and other signal pathways [19]. The integrin α_V subunit promotes the growth of tumor cells, inhibits their apoptosis, and controls their invasion and metastasis. Eberlein et al. [20] reported a human monoclonal antibody 264RAD targeting αvβ6 integrin reduced tumor growth and metastasis, and modulated key biomarkers *in vivo*.

Existing research concerning the treatment of HCC by the integrin α_V subunit focuses on immunotherapy and gene therapy; these methods have partially entered the stage of clinical drug trials [21]. As previously reported, LM609 [22,23] and Vitaxin [24,25], the monoclonal antibodies of integrin $\alpha_V\beta3$, both inhibited the invasion of tumors *in vivo*. In gene therapy, tumor-specific ASODN or nuclear enzymes are primarily used to reduce or alter the expression configuration of the integrin α_V subunit and interfere with α_V-subunit-mediated adhesion, movement, and transfer. Because most of the selected transfecting vectors are viruses or liposomes, problems will likely emerge, such as insecurity and poor specificity of target cells *in vivo*, as well as the use of time-consuming and complex steps (e.g., vector cloning, sequencing, and validation). Thus, these vectors have not been shown to be favorable for use *in vivo* experiments and clinical applications. Therefore, in the present study, we used receptor-mediated gene transfer technology; namely, the ASODN was introduced into cells through selective combination between the vector PEI-RGD and HCC membrane surface. PEI-RGD is a linear PEI derivative and has 2 specific properties. 1) PEI is a cationic compound that can bind via ionic bonds with ASODN; it carries ASODN and absorbs H^+ in the lysosome, thus inactivating nucleic acid enzymes in the lysosome and protecting ASODN from degradation. 2) The PEI surface is occupied by many short RGD polypeptides, such as the integrin α_V subunit, that are natural ligands. These polypeptides specifically bind with α_V and enter target cells through endocytosis, thus realizing the targeted transfection [26]. Zhan et al. reported Cyclic RGD-poly(ethylene glycol)-polyethylenimine was more suitable for glioblastoma targeting gene transfer *in vivo* [27]. In the present study, the cell intake rate was maximized at 4 μl/2 μg PEI-RGD/^{125}I-(α_V) ASODN, and it decreased with increased dosage, likely because the receptor α_V was saturated on the HepG2 cell surface or the compound was toxic to the cells. Therefore, cytotoxicity tests were conducted under varying dosages. The results showed that, with the increased dosage, the compound inhibited HepG2 cells in a concentration-dependent manner. The latter results also correlated with the studied dosage range (r=0.879).

Because of the high cytotoxicity under high dosage and to reduce the effects during paired comparison, we selected the smallest dosage of 2 μl/1 μg for the control group. The following results were observed. 1) The inhibition rates were not significantly different between the PEI-RGD/^{125}I-(α_V)ASODN group and the PEI-RGD/(α_V) ASODN group ($P>0.05$), but both groups were differed significantly from the other groups ($P<0.001$). Additionally, at a low dosage (2 μl/1 μg), the toxic effects of the PEI-RGD/^{125}I-(α_V)ASODN group and the PEI-RGD/(α_V)ASODN group were not significantly different, but they were higher than in the other groups, indicating that the radiation bioeffect of ^{125}I was not obvious inside the cells. 2) The PEI-RGD group, ^{125}I-(α_V)ASODN group, (α_V)ASODN group, and ^{125}I group did not differ significantly from the control group ($P>0.05$), indicating that the 4 substances at low dosage were not cytotoxic. The likely causes for this result include the following: low-dosage PEI-RGD was not cytotoxic; without the operation of a vector, the ASODN transfection rate was low, leading to a low targeting capability; and the marked ^{125}I and free ^{125}I could not enter the nucleus and, thus, could not demonstrate radiation bioeffects against DNA.

To reduce the effects of compound cytotoxicity on HepG2 cell invasion, we selected the lowest dosage (2 μl/1 μg) for the cell invasion tests. The results showed that the inhibition rate of the PEI-RGD/^{125}I-(α_V)ASODN group was significantly different from those of the other control groups, indicating that the inhibitory effect on HepG2 cell invasion was greater than those in the other groups. These results may be attributed to the following factors. 1) The combination of PEI-RGD with α_V on the surface of HepG2 cells reduced the probability of α_V binding with other natural ligands (e.g., other cells, ECM, and basal membranes), indicating the competitive inhibitory effect [28,29]. 2) The combination of PEI-RGD with α_V induced endocytosis into the cytoplasm, thereby degrading α_V and reducing the density of α_V on the HepG2 cell surface. 3) Under the protection of PEI-RGD, (α_V)ASODN entering cells avoided the degradation by nucleases and bound with α_V mRNA, thereby interfering with the expression of α_V. 4) ^{125}I-(α_V)ASODN also entered the nucleus and bound to DNA. The marked ^{125}I produced Auger electrons

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and CK electrons via disintegration, forming local high-energy deposition within 10 nm (10-bp DNA chains) around the disintegration locus, which would induce non-repairable injuries, such as double-strand breaks (DSBs) [30].

Compared with the PEI-RGD/(α_v)ASODN group, the cytotoxicity of the PEI-RGD/^{125}I-(α_v) ASODN group was not significantly different, but the inhibitory effect on HepG2 cell invasion was significantly different, which indicated that although the ionizing radiation of ^{125}I did not significantly inhibit the growth of HepG2 cells, it weakened the invasion ability of the HepG2 cells. Moreover, the inhibition effects on cell invasion were significantly different between PEI-RGD/(α_v)ASODN and PEI-RGD, but the differences were not very large, indicating that the antisense technology did not satisfactorily inhibit the invasion of the HepG2 cells. Therefore, RNA interference technology should be used in future works, which is characterized by a higher inhibitory effect, longer working time, and greater stability [31,32].

Conclusions

This study showed that PEI-RGD/^{125}I-(α_v)ASODN can efficiently inhibit the growth and proliferation of HepG2 cells and can weaken their invasive ability. Although the radiation bioeffect of ^{125}I was not obvious inside the cells, it weakened the invasion ability of the HepG2 cells. We also found that the usefulness of antisense technology for inhibiting the invasion of HepG2 cells was limited.

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