维普资讯 http://www.cqvip.com

Effects of ^{125}I-Labeled Peptide Nuclear Acid Targeting Ki67 on the Growth of Implanted Human Renal Cell Carcinoma in Nude Mice

Jiacun Chen[1]
Junnian Zheng[1]
Song Wu[2]
Haibiao Lai[2]
Xiaoqing Sun[1]
Junjie Liu[1]

[1] Department of Urology, Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, China.
[2] Department of Urology, Zhongshan Hospital of Traditional Chinese Medicine, Zhongshan 528400, China.

OBJECTIVE To investigate the potential of ^{125}I-labeled anti-sense peptide nucleic acids (^{125}I-AS-PNAs) to inhibit the expression of the Ki-67 gene and growth of implanted human renal carcinoma cells in nude mice.

METHODS Anti-sense peptide nucleic acids (AS-PNAs) targeting the Ki67 gene were synthesized and labeled with ^{125}I by the Chloraseptine-T method. Drugs including PNAs and ^{125}I-AS-PNAs capsulated by cationic lipid were directly injected into tumors in nude mice. The Ki67 expression in tumors was detected by an immunohistochemical technique and Western blot. The apoptosis of tumor cells was detected by a TUNEL assay. Tumor volumes were measured every 3 days and tumor suppression rates were calculated at 12 days after treatment. Control groups were treated with AS-PNA, MM-PNAs (mismatch PNAs) and ^{125}I-Na.

RESULTS The Ki67 expression rate of tumors treated by ^{125}I-AS-PNAs [(15.3±1.8)%] was lower than that treated by AS-PNAs [(23.0±2.4)%] ($P<0.01$). The Ki67 protein production rate of tumors treated by ^{125}I-AS-PNAs [(43.6±3.5)%] was lower than that treated by AS-PNAs [(59.7±2.3)%] ($P<0.01$). The apoptosis rate of tumors treated by ^{125}I-AS-PNAs [(40.3±2.4)%] was higher than that treated by AS-PNAs [(31.1±2.0)%] ($P<0.01$). The volume of tumors treated by ^{125}I-AS-PNAs [(330.4±57.8) mm^3] was smaller than that treated by AS-PNAs[(513.2±64.2)mm^3] ($P<0.01$).

CONCLUSION ^{125}I-AS-PNAs targeted against the Ki67 gene have a greater inhibitory effect on the expression of the Ki67 gene and a larger apoptotic action on human renal carcinoma cells and can more efficiently inhibit tumor growth than AS-PNAs. ^{125}I-AS-PNAs targeting the Ki67 gene may be a promising anti-sense/anti-gene radiotherapy method for treating renal cell carcinoma.

KEYWORDS: peptide nucleic acids, Ki67 gene, gene radiotherapy, radionuclide, renal cell carcinoma, apoptosis.

Anti-sense/anti-gene radiotherapy is a new approach for tumor therapy, which combines the inhibitory effect of anti-sense drugs on gene expression with radionuclide damaging action on localized genes. Peptide nucleic acids (PNAs) are DNA analogues with the deoxyribose phosphate backbone replaced by aminoethyl glycine units.[1] The inhibition mediated by anti-sense peptide nucleic acids (AS-PNAs) is 10~100 fold more efficient than that of analogous antisense oligonucleotides (ASODNs).[2] Our in vitro studies have

Received July 19, 2004; accepted December 1, 2004.

维普资讯 http://www.cqvip.com

demonstrated that ^{125}I-labeled AS-PNAs (^{125}I-AS-PNAs) targeting of the Ki67 gene has a more powerful effect on the proliferation and apoptosis of human renal carcinoma cells than AS-PNAs.[3] In this study, we have investigate the effects of ^{125}I-AS-PNAs targeting the Ki67 gene as related to its expression in renal carcinoma cells and on the growth of implanted renal carcinoma in vivo.

MATERIALS AND METHODS

Materials and reagents

The human renal carcinoma cell line 786-0 was purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Ki67 primary antibody (mouse-anti-human-Ki67-monoclonal antibody) was obtained from Novocastra Laboratories Co. and a Ki67 immunohistochemistry detection kit was acquired from Lab Vision Co. A TUNEL apoptosis detection kit was purchased from R&D Co. and the cationic lipid Lipofection AMINE was a product of GIBCO Co. (USA).

PNAs synthesis

The sequences of AS-PNAs were directed against the start codon region at position 197-214 of Ki67 cDNA. The sequences used were as follows: AS-PNAs: Gly-GCGTCTCGTGGGCCAC-Lys, mismatch PNAs (MM-PNAs):Gly-GAGCATTACGCGATACGC-Lys. A C-terminal lysine residue and a N-terminal glycine residue were made with 2 oligomers to label ^{125}I and prevent PNAs from adjection respectively. AS-PNAs and MM-PNAs were synthesized and purified by the TAG Copenhagen Co. (USA).

Radiolabeling and encapsulation by lipid

AS-PNA and MM-PNA were labeled with ^{125}I using the Chloraseptine-T method. The specific activity of ^{125}I-PNA was 3.03 mBq/ μg. The molar ratio of iodine to PNA was more than 95%. The labeling ratio of ^{125}I-PNA was more than 95% . Nonradiolabeled AS-PNA was removed using a Sephadex G50 NICK column. The cationic lipids were mixed with ^{125}I-PNAs or PNAs for 45 min at room temperature in a lipid/^{125}I-PNAs (PNAs) ratio of 2 μl: 3 nmol.

Animal model establishment and treatment

Sixty BALB/C-nu nude mice (4-8 week old, male, 20-22 g), supplied by the experimental animal center of Chinese Medicine Academy of Sciences, were housed and cared for according to SPF standards. Tumor 786-0 cells (5×10^6) were injected subcutaneously directly into the lower left flank of each mouse. The mice bearing tumors of approximately 0.5 cm in diameter were divided into 5 groups as follows: ^{125}I-AS-PNAs (200 γ Ci, 10 nmol AS-PNAs/100 γ l RPMI-1640 medium), AS-PNAs (10 nmol AS-PNAs/100 γ l RPMI-1640 medium), MM-PNAs (10 nmol AS-PNAs/100γ l RPMI-1640 medium), ^{125}I-Na (200 γ Ci/100γ l RPMI-1640 medium) and control (100 γ l RPMI-1640 medium) group. The mice were injected with the drugs directly into the tumors every day for 4 days as described above. Four mice in each group were sacrificed at 3, 6 and 12 days after treatment. The mice were given drinking water containing 0.4% Lugol's solution to block the uptake of ^{125}I by the thyroid.

Tumor growth curves and suppression rate

Tumors were measured with a calipers in 2 perpendicular dimensions every 3 days, and the tumor volume was calculated using the formula volume=length × width^2 × π/6, after which the tumor growth curves were drawn. The suppression rate at 12 days was calculated using the formula: suppression rate=$|V_{treatment} - V_{control}| / V_{control} \times 100$.

Detection of Ki67 expression by immunohisochemistry

The sections were deparaffinized in xylene, rehydrated in graded ethanol, and incubated in 0.3% H_2O_2 solution in methanol for 10 min to block the endogenous peroxidase. The sections for Ki67 immunohischemistry were boiled in 10 mM sodium citrate (pH 6.0) for 10 min in a micro-wave oven and allowed to cool at room temperature. After incubation in an Ultra V Block solution for 5 min at room temperature, the sections were incubated overnight at

维普资讯 http://www.cqvip.com

4℃ with Ki67 primary antibody, then a secondary antibody (biotinylated goat anti-mouse) was applied for 10 min at room temperature. The sections were treated with streptavidin peroxidase for 10 min at room temperature, followed by DAB color development. Microscopic examination showed positive cell nuclei were brown. Five fields were randomly selected from every sample, and 200 cells were randomly selected from every field. The positive rate = (number of total positive cells /200) × 100.

Detection of Ki67 protein by Western blot analysis

Extracts of protein in tumors tissue were prepared as described. [4] Ki67 protein was separated by 10% SDS-PAGE electrophoresis. Once transferred, membranes were incubated with Ki67 primary antibody 1: 400 at a concentration of 1.5 μg/ml, followed by incubation of 1:4000 alkaline phosphatase-coupled rabbit-anti-mouse IgG antibody. Phosphatase activity was visualized using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Documentation of blots was performed with an imaging system (Gene Company, USA).

Detection of apoptosis by TUNEL assay

The sections were deparaffinized in xylene, rehydrated in graded ethanol, and incubated in a 0.3% H_2O_2 solution in methanol for 10 min to block the endogenous peroxidase, followed by procedures based on the instructions in the Apoptosis Detection Kit (R&D). Under microscopic analysis, positive cells were brown. The positive rate was calculated as described above.

Statistical analysis

All results were presented as x ± s. The statistical significance was determined using Student's *t* test ($P<0.05$) with SPSS software.

RESULTS

Effect of ^{125}I-AS-PNAs on tumor growth

Compared to the control groups tumor growth curves showed that ^{125}I-AS-PNAs and AS-PNAs treatments resulted in a marked suppression of tumor growth. The tumor suppression rate of ^{125}I-AS-PNAs and AS-PNAs were 70.9% and 54.8% respectively at 12 days after treatment. Tumor growth was not influenced significantly by treatment with MM-PNAs or ^{125}I-Na. ^{125}I-AS-PNAs had a significantly greater effect on inhibiting tumor growth than AS-PNAs (Table 1, Fig.1).

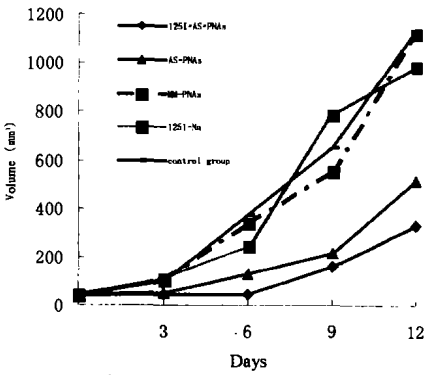

Fig.1. Tumor growth curves.

Table 1. Tumor volume and suppression rate (n=4)

group	Days after treatment					suppress rate★
	0	3	6	9	12	
^{125}I-AS-PNAs	40.2 ± 12.5	42.8 ± 14.2	44.8 ± 23.3	159.7 ± 36.9	330.4 ± 57.8▲	70.9
AS-PNAs	43.2 ± 11.9	49.7 ± 13.8	127.4 ± 23.6	214.5 ± 38.0	513.2 ± 64.2▼	54.8
MM-PNAs	41.3 ± 11.5	104.7 ± 18.2	339.1 ± 33.5	553.2 ± 50.9	1119.0 ± 138.1#	1.4
^{125}I-Na	42.8 ± 11.6	108.1 ± 19.1	243.5 ± 28.9	784.6 ± 84.3	984.8 ± 216.9#	13.2
control	40.5 ± 10.9	98.8 ± 14.9	375.4 ± 30.9	652.8 ± 70.2	1135.1 ± 190.7	-

★ suppression rate=$|V_t(12d)-V_c(12d)|/V_c(12d)\times 100$; *vs* MM-PNAs, control, $P<0.01$;

▲ *vs* AS-PNAs, MM-PNAs, ^{125}I-Na, control, $P<0.01$;

\# *vs* control, $P>0.05$.

维普资讯 http://www.cqvip.com

Effect of ^{125}I –AS –PNAs on Ki67 expression and protein production in tumors

Both ^{125}I-AS-PNAs and AS-PNAs significantly reduced the Ki67 positive expression rates and Ki67 protein of 786-0 tumor cells at various time points compared to the MM-PNAs, ^{125}I-Na, and control groups (P <0.01). The Ki67 positive expression rates and Ki67 protein of 786-0 tumor cells were not inhibited significantly by treatment with MM-PNAs or ^{125}I-Na (P >0.05).Treament with ^{125}I-AS-PNAs had greater inhibitory effect on Ki67 gene expression than AS-PNAs (P<0.01) (Table 2).

Effect of ^{125}I–AS–PNAs on inducing apoptosis

The TUNEL assay showed that the cells treated by ^{125}I-AS-PNAs and AS-PNAs exhibited typical apoptotic features. The apoptotic rates of the tumor cells treated with MM-PNAs were significantly lower than those treated with ^{125}I-AS-PNAs and AS-PNAs (P<0.01). There was no significant difference between the MM-PNAs group and control group (P>0.05). The apoptosis rate for tumor cells treated with ^{125}I-AS-PNAs was higher than that with AS-PNAs treatment (P <0.01). Compared to the control group, ^{125}I-Na treament showed some apoptotic activity due to nonspecific irradiation (P <0.05) (Table 3).

DISCSSION

Antisense oligodeoxynucleotides (AS-ODNs) have drawn a great deal of attention in recent years as specific tools for therapeutic applications for malignant cells. However, several obstacles including reduced affinity, non-specific binding, and nuclease degradation have plagued their use. PNAs are DNA analogues with the deoxyribose phosphate backbone replaced by aminoethyl glycine units. PNAs can bind single and double stranded DNA and RNA with high affinity and specificity. [5] PNAs are resistant to nuclease and proteases. [6] Norton and Piatyszek [7] reported that AS-PNAs targeting of human telomerase could inhibit the enzymtic activity 10~ 50 times more efficiently than inhibition by analogous AS-ODNs. In

Table 2. Effect of PNAs on Ki67 expression and protein production (%)

	3 day		6day		12 day	
group	Ki67 positive express rate	Ki67 protein rate	Ki67 positive express rate	Ki67 protein rate	Ki67 positive express rate	Ki67 protein rate
^{125}I-AS-PNAs	12.4± 2.1*	33.9± 3.6*	14.3± 1.6*	38.7± 4.1*	15.3± 1.8*	43.6± 3.5*
AS-PNAs	19.2± 1.5▲	58.3± 2.4▲	20.5± 2.3▲	57.9± 2.5▲	23.0± 2.4▲	59.7± 2.3▲
MM-PNAs	42.1± 3.2☆	97.9± 1.4☆	41.3± 2.5☆	98.8± 1.0☆	39.9± 2.1☆	98.1± 1.6☆
^{125}I-Na	38.5± 1.6☆	97.7± 2.1☆	37.4± 2.5☆	99.2± 0.5☆	37.4± 3.0☆	97.5± 2.1☆
control	42.5± 3.6	100	41.8± 2.1	100	39.1± 3.8	100

* *vs* AS-PNAs,MM-PNAs,^{125}I-Na,control, P<0.01; ▲ *vs* MM-PNAs, control, P<0.01; ☆ *vs* control, P>0.05.

Table 3. Effect of PNAs on cell proliferation (%)

group	3 day	6 day	12 day
^{125}I-AS-PNAs	33.7± 3.2*	37.6± 1.1*	40.3± 2.4*
AS-PNAs	19.5± 2.3▲	24.9± 1.9▲	31.1± 2.0▲
MM-PNAs	10.5± 1.8☆	11.8± 2.0☆	10.8± 1.9☆
^{125}I-Na	13.7± 1.5▼	14.3± 2.0▼	14.8± 1.7▼
control	11.2± 1.6	10.8± 2.0	11.7± 1.4

* *vs* AS-PNAs,MM-PNAs,^{125}I-Na,control, P<0.01; ▲ *vs* MM-PNAs, control, P<0.01; *vs* control, P<0.05, ☆ *vs* control, P>0.05.

维普资讯 http://www.cqvip.com

contrast to high selectivity of inhibition by AS-PNAs, AS-ODNs inhibit telomerase in a non-sequence selective fashion. The present study demonstrated that AS-PNAs against the Ki67 gene could diminish the tumor volume contributing to suppressing the Ki67 gene expression and inducing apoptosis of the tumor cells. However, AS-PNA did not completely inhibit expression of the target gene so that mRNA was continuously transcribed by the template DNA.

Anti-sense/anti-gene radiotherapy is based on the site-specific targeting of genetic sequences in the form of single- and double-stranded nucleic acids with radiolabeled anti-sense drugs.[8] In this way, anti-sense drugs are designed as carrier molecules to selectively deliver short-range, Auger electron-emitting radionuclides, such as iodine-125, to a designated DNA/RNA sequence from cellular oncogenes via site-specific duplex or triplex formation.[9] More of the lethal radiodamaging effects of the Auger electron emitters are precisely directed within the affected cells to particular mRNA or genomic DNA sites, while producing minimal damage to the rest of the genome and to other cellular components.[10] In our study, the effects of ^{125}I-AS-PNAs on inhibiting Ki67 gene expression and growth of tumors were significantly higher than that of AS-PNAs. ^{125}I-Na also showed some ability to induce apoptosis of tumor cells because of nonspecific irradiation, but the expression of Ki67 gene was not affected. This strongly suggested that ^{125}I-Auger electron emitters, were precisely delivered and bound to the Ki67 gene, which caused double strand breaks (DSB) and suppressed expression of Ki67 gene followed by apoptosis of tumor cells and inhibition of tumor growth.

AS-ODNs have been used as carriers for antisense/antigene radiotherapy in recent years. But because of its poor chemical and biological stability, the antisense/antigene radiotherapy approach needs to be improved. One of the improvements is to replace the ordinary AS-ODN by AS-PNA.[11] Indeed, such a replacement may provide the antisense/antigene radiotherapy approach with better sequence specificity intrinsic to AS-PNA along with an enhanced stability of hybrid duplexes and triplexes formed by AS-PNA with DNA/RNA targets.[12] Because AS-PNA have a non-natural polyamide backbone instead of the sugar-phosphate backbone of natural nucleic acids, AS-PNAs exhibit much higher chemical and biological stability compared with the biodegradable nuclease-sensitive natural oligonucleotides. In addition, PNA oligomers can selectively target double-stranded DNA via duplex- or triplex-inbinding modes.[11]

Tumor cell proliferation is a complex biological process controlled by many regulatory genes. Recently it was demonstrated that the Ki67 protein encoded by the Ki67 gene might be an absolute requirement for tumor cell proliferation.[13] The Ki67 protein is a DNA binding protein with a primary role of maintaining higher order structure for DNA during the process of mitosis. Detailed cell cycle analysis revealed the Ki67 protein is present in nuclei of proliferating (G1,S,G2, and mitotic phases)cells but not in nuclei of quiescent cells (G0 phrase).[14] The present study also found that Ki-67 downregulation was closely associated with or rather followed by apoptotic changes. It suggested that the tumor cells with a reduced Ki-67 product undergo apoptosis. This conclusion could strongly support the vital importance of this protein and its usefulness as an anticancer target. One plausible explanation for our findings is that cells that attempt mitosis in the absence of Ki-67 protein are unable to complete the process and are forced into apoptosis.

In conclusion, our results demonstrate that antisense/antigene radiotherapy strategies using ^{125}I-AS-PNAs against the Ki67 gene may be a promising approach in renal carcinoma therapy, and that the Ki67 gene plays a vital role in cell proliferation and apoptotic control of renal carcinoma cells in vivo.

REFERENCES

1 Nielsen PE, Egholm M,Berg RH, et al. Sequence-selective recognition of DNA by strand displacement with a Thymine-substituted polyamide. Science. 1991;254:1497-1500.

2 Norton JC, Piatyszek MA, Wright WE, et al. Inhibition of human telomerase acitivity by peptide nucleic acids. Nat Biotechnol. 1996;14:710-716.

3 Zheng JN, Sun XQ, Chen JC, et al. Effects of ^{125}I-labeled

维普资讯 http://www.cqvip.com

Ki67 gene targeting peptide nucleic acids on the proliferation and apoptosis of human renal carcinoma cell line. Chin J Nuclear Med. 2004;24:139–141.

4 Endl E, Gerdes J. Posttranslational modifications of the Ki67 protein coincide with two major checkpoints during mitosis. J Cell Physiol. 2000;182:371–380.

5 Nielsen PE,Egholm M. Srand displacement recognition of mixed adenine cytosine sequences in double stranded DNA by thymine–guanine PNA (peptide nucleic acid). Bioorg Med Chem. 2001;9:2429–2434.

6 Jesen KK, Orum H, Nielsen PE, et al. Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with BIAcore technique. Biochemistry. 1997; 36:5072–5077.

7 Norton JC, Piatyszek MA, Wright WE, et al. Inhibition of human telomerase acitivity by peptide nucleic acids. Nat Biotechnol. 1996;14:710–716.

8 Panyutin IG, Neumann RD. Gene radiotherapy; gene targeted versus targeted by gene product. J Nucl Med. 1998; 39:928–929.

9 Gaidamakova EK, Neumann RD, Panyutin IG. Site–specific strand breaks in RNA produced by ^{125}I radiodecay. Nucleic Acids Res. 2002;30:4960–4965.

10 Sedelnikova OA, Luu AN, Karamychev VN, et al. Development of DNA–based radiopharmaceuticals carrying Auger –electron emitters for antigene radiotherapy. Int J Radiat Oncol Biol Phys. 2001;49:391–396.

11 Uhlmann E, Peyman A, Breipohl G, et al. PNA: synthetic polyamide nucleic acids with unusual binding properties. Angew Chem Int Ed Engl. 1998;37:2796–2823.

12 Ray A, Norden B. Peptide nucleic acid (PNA): its medical and biotechnological applications and promise for the future. FASEB J. 2000;14:1041–1060.

13 Sauhney N, Hall PA. Ki67 –structure, function, and new antibodies. J Path.1992;168:161–162.

14 Gerdes J, Lemke H, Baisch H, et al. Cell cycle analysis of a cell proliferation–associated human nuclear antigen defined by the monoclonal antibody Ki67. J Immunol. 1984;133: 1710–1715.