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• GASTRIC CANCER •

Selection of optimal antisense accessible sites of survivin and its application in treatment of gastric cancer

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Abstract

AIM: To select the optimal antisense accessible sites of survivin, a highly expressed gene in tumor tissues, in order to explore a novel approach to improve biological therapy of gastric cancer.

METHODS: The 20 mer random oligonucleotide library was synthesized, hybridized with *in vitro* transcribed total survivin cRNA, then digested by RNase H. After primer extension and autoradiography, the antisense accessible sites (AAS) of survivin were selected. Then RNADraw software was used to analyze and choose the AAS with obvious stem-loop structures, according to which the complementary antisense oligonucleotides (AS-ODNs) were synthesized and transferred into survivin highly- expressing gastric cancer cell line MKN-45. Survivin expression was detected by RT-PCR and Western Blotting. Cellular growth activities were assayed by tetrazolium bromide (MTT) colorimetry. Cellular ultrastructure was observed by electronic microscopy, while apoptosis was detected by annexin V-FITC and propidium iodide staining flow cytometry.

RESULTS: Thirteen AAS of survivin were selected *in vitro*. Four AAS with stem-loop structures were chosen, locating at 207-226 bp, 187-206 bp, 126-145 bp and 44-63 bp of survivin cDNA respectively. When compared with non-tranfection controls, their corresponding AS-ODNs (AS-ODN$_1$, AS-ODN$_2$, AS-ODN$_3$ and AS-ODN$_4$) could reduce Survivin mRNA levels in MKN-45 cells by 54.3±1.1% (t = 6.12, P<0.01), 86.1±1.0% (t = 5.27, P<0.01), 32.2±1.3% (t = 7.34, P<0.01) and 56.2±0.9% (t = 6.45, P<0.01) respectively, while survivin protein levels were decreased by 42.2±2.5% (t = 6.26, P<0.01), 75.4±3.1% (t = 7.11, P<0.01), 28.3±2.0% (t = 6.04, P<0.01) and 45.8±1.2% (t = 6.38, P<0.01) respectively. After transfection with 600 nmol/L AS-ODN$_1$~AS-ODN$_4$ for 24 h, cell growth was inhibited by 28.12±1.54% (t = 7.62, P<0.01), 38.42±3.12% (t = 7.75, P<0.01), 21.46±2.63% (t = 5.94, P<0.01) and 32.12±1.77% (t = 6.17, P<0.01) respectively. Partial cancer cells presented the characteristic morphological changes of apoptosis, with apoptotic rates being 19.31±1.16% (t = 7.16, P<0.01), 29.24±1.94% (t = 8.15, P<0.01), 11.87±0.68% (t = 6.68, P<0.01) and 21.68±2.14% (t = 7.53, P<0.01) respectively.

CONCLUSION: The AAS of survivin could be effectively selected *in vitro* by random oligonucleotide library/RNase H cleavage method combined with computer software analysis, this has important reference values for further studying survivin-targeted therapy strategies for gastric cancer.

Key words: Gastric cancer; Survivin; Antisense accessible sites; Gene expression

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INTRODUCTION

Survivin is a novel member of inhibitors of apoptosis (IAPs) family, which expresses during human embryonal development and in most of tumor tissues, whereas lacks of expression in terminally differentiated normal tissues[1-4]. A series of studies indicate that survivin has a double function in blocking cell apoptosis and regulating cell proliferation, its overexpression correlates with occurrence and development of gastric cancer[5,6]. Antisense technique has become an efficient therapeutic method for gastric cancer through blocking gene expression and biological function of survivin[7]. However, since the target sequence is always folded into secondary and tertiary structures, resulting in the blocking effects on antisense accessible sites, which leads to the low inhibition activities of antisense oligonucleotides designed only by computer software, it is an attractive research focus on how to select optimal antisense accessible sites of targeted sequence[8-11]. In this research, the antisense accessible sites of survivin were selected *in vitro* through random oligonucleotide library/RNase H cleavage method combined with computer analysis software, in order to establish an experimental basis for further exploring the survivin-targeted therapy strategies for gastric cancer.

MATERIALS AND METHODS

Design and synthesis of random oligonucleotide library

According to literature[12], random oligonucleotide library was synthesized, with the following required standards: a length of 20 bp, a mixture of phosphoramidites at a ratio of 1.5:1.25:1.15:1.0 (A:C:G:U/T), and a sequence of 5'-mmmnnnnnnnn nnnnnnnnmm-

3'. G, A, T and C denote four kinds of deoxynucleotides, m denotes the methylation, n denotes random deoxynucleotides. Random oligonucleotide library was synthesized and purified by Shanghai Sangon Company.

Transcription of target gene in vitro

The plasmid pcDNA-SVV including full length of survivin cDNA was a kind gift from the Walter and Eliza Hall Institute of Medical Research (Australia). The plasmid was linearized by digestion with restriction enzyme *Nhe* I (Takara Biology Company) and transcribed into cRNA *in vitro* using a T_7 transcription kit (Promega Biology Company). The total reaction volume was 50 μL, and incubated at 37 ℃ for 2 h. Then the DNA template was removed by digestion with DNase I for 30 min. The products were extracted with phenol: chloroform: isoamyl alcohol (V/V 25:24: 1), precipitated with 100% ethanol, washed with 70% ethanol and dissolved in DEPC H_2O after drying at room temperature. The cRNA concentrations were measured with UV spectro- photometer, and determined with 1% agarose gel electrophoresis. The cRNA was preserved at -70 ℃.

RNase H cleavage reaction

The total volume for hybridization reaction between random oligonucleotide library and cRNA was 200 μL. The oligonucleotide library (60 μL, 300 nmol/L) was denatured at 95 ℃ for 5min, kept on ice for 5 min, added with cRNA 3 μL (30 pmol/L), 5×Tris buffer (200 mmol/L Tris, pH 8.0, 20 mmol/L $MgCl_2$, 5 mmol/L DTT) 40 μL, 10 U/μL RNase H (Takara Biology Company) 2 μL and 1% DEPC H_2O 95 μL. The reaction was conducted at 30 ℃ for 1h. Then the product was extracted with phenol: chloroform: isoamyl alcohol (V/V 25:24:1), added with 1/10 volume 3 mol/L NaAc and 2.5 volume 100% ethanol, precipitated at -20 ℃ for 30 min, washed with 70% ethanol and dissolved with DEPC H_2O.

Primer extension and selection of antisense accessible sites

According to human survivin cDNA sequence (429 bp) and protocol of the primer extension kit (Promega Corporation), two extension primers were designed with Primer 5.0 software: P_1 5'-CCAAGGGTTAATTCTTCAAACTGCTTC-3', P_2 5'-CCAAGTCTGGCTCGTTCTCAGTGGGGCAGT-3', which were synthesized by Shanghai Genebase Company and diluted with double distilled H_2O into 150 nmol/L. Primers and PhiX174 Hinf I DNA marker were labeled with ^{32}P-ATP (Beijing Furui Biology Corporation) with T_4 polynucleotide kinase. The 5' end primer extension reaction was conducted on the RNase H cleaved products with each labeled primer, and reaction volume was 20 μL. The reaction was continued at 42 ℃ for 30 min, terminated by adding 20 μL loading dye, then analyzed on 8% denaturing polyacrylamide gel. The gel was fixed with 8% acetic acid for 10 min at room temperature, dried up in vacuum at 80 ℃ for 1 h, exposed to X-ray film overnight at -70 ℃ for autoradiography. The secondary structures of full-length survivin cDNA were predicted with RNADraw software. The antisense accessible sites (AAS) with obvious stem-loop structure domains, were selected with results of random oligonucleotide libraries/RNase H cleavage *in vitro* combined with RNADraw analysis. Their complementary antisense oligonucleotides (AS-ODNs) were synthesized by Shanghai Gene-base Company.

Design of control AS-ODN

According to the secondary structure of survivin cDNA predicted by RNADraw, the antisense accessible site containing two stem-loop domains (4-23 bp) was selected (Figure 1). Its complementary antisense oligonucleotide, named as AS-ODN_0 (sequence: 5'-GGGGGCAACGTCGGGGCACC-3'), was synthesized as a control.

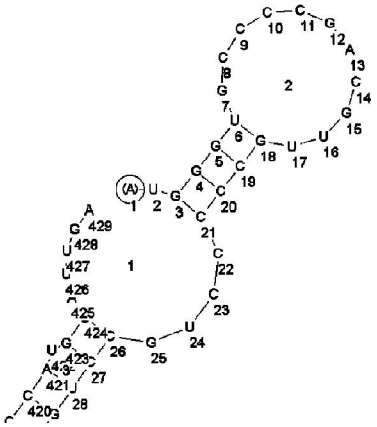

Figure 1 Secondary structure of antisense accessible sites (4-23 bp) of survivin designed only with RNADraw software.

Cell culture and gene transfection

The gastric cancer cell line MKN-45, a highly expressing survivin gene, was purchased from American Type Culture Collection (ATCC), and incubated in RPMI 1640 medium supplemented with penicillin/ streptomycin (100 U/mL and 100 μg/mL respectively) and 10% fetal bovine serum at 37 ℃ in a humidified atmosphere of 50 mL/L CO_2 and passaged every three days. MKN-45 cells at exponential phases of growth were inoculated into 24-well plates. The procedure for transfection of AS-ODN_0 and AS-ODNs was conducted according to the protocol of Lipofectamine 2000 kit (Gibco Company).

Survivin mRNA expression detection

Reverse transcription polymerase chain reaction (RT-PCR) was used. Eighteen hours after gene transfection, total RNA was extracted from cells of each group. Reverse transcription reaction was carried out using the following mixture containing 1μL 10 mmol/L 4×dNTP, 1μL 1 U/μL RNase inhibitor, 1μL 0.5 μg/μL Oligo dT primer, 0.5 μL AMV reverse transcriptase, 4 μL 5×AMV buffer, 4 μL RNA template, 8.5 μL ddH_2O. The reaction was conducted at 42 ℃ for 30 min, heated to 99 ℃ for 5 min to inactivate AMV reverse transcriptase and kept at 5 ℃ for 10 min. In reference to survivin cDNA sequence in GenBank (accession number: U75285), the primer pairs for survivin fragments were designed with Primer Premier 5.0 software: forward primer: 5'-CACCGCATCTCTACATTCAA-3', reverse primer: 5'-CACTTTCTTCGCAGTTTCCT-3'. The anticipated product was 345 bp in length. The primers for α-tubulin, including forward primer: 5'-CCGTCCTTTCCACTCA-3' and reverse primer: 5'-GTAATCT CGGCAACAC-3', served as an inner control with a product of 410 bp. PCR amplification was conducted in following condition: pre-denaturation at 95 ℃ for 5 min, denaturation at 95 ℃ for 1 min, annealing at 60 ℃ for 1 min, and extension at 72 ℃ for 90 s. After 30 amplification cycles the products were extended at 72 ℃ for 10 min. The PCR products were separated with electrophoresis on 1% agarose gel and photographed under ultraviolet radiation light.

Survivin protein expression assay

Western blotting was used. The total cellular protein was extracted and determined according to the Molecular Cloning Manual. Western blot was conducted. Blots were incubated sequentially with 1% fat free dry milk, rabbit polyclonal anti-survivin antibody (Santa Cruz Company) and peroxidase-conjugated second antibody, and evaluated using ECL Western blotting kit. Survivin

protein band intensities were determined densitometrically using the CMIASWIN computer imaging system.

Cell growth assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide (MTT) colorimetry method was used. MKN-45 cells were seeded at a density of 3×10^3/100 μL into 96-well chamber slides. The non-transfection controls, 600 nmol/L AS-ODN_0 transfection and 600 nmol/L AS-ODN transfection groups were designed, with each group having five wells. After transfected for 6, 12, 18 and 24 h, each well was added with 0.5% MTT 20 μL and incubated for another 4 h. The supernates were discarded, then DMSO 100 μL was added. When the crystals were dissolved, the optical density *A* values of the slides were read on enzyme-labeled minireader II at the wavelength of 490 nm. Cell growth inhibition rate (%) = (1- average *A* value of experimental group/ average *A* value of non-transfection control group)×100%.

Cellular ultrastructure observation

Cancer cells from three groups were collected, rinsed with PBS and fixed using 2.5% glutaraldehyde for 30 min, then washed with PBS. After routine embedding and sectioning, cells were observed under electronic microscope.

Cell apoptosis detection

Apoptosis was detected by annexin V-FITC and propidium iodide staining flow cytometry. Cells were collected, washed twice with cold PBS, resuspended with 100 μL binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl2, pH7.4) to 2-5×10^5/mL, and incubated with annexin V-FITC at room temperature for 10 min. After washed with binding buffer, cells were resuspended with 400 μL binding buffer containing 10 μL PI (20 μg/mL) and incubated on ice for 15 min. Apoptosis was analyzed by flow cytometry (BD Company, USA) at the wavelength of 488 nm.

Statistical analysis

Data were expressed as mean±SD and analyzed by SPSS10.0 statistical software.

RESULTS

In vitro selection of antisense accessible sites of survivin

As shown in Figure 2, after survivin cRNA was mixed with the random oligonucleotide library and digested by RNase H, there were obvious products on autoradiography through primer extension of P_1 and P_2, which were targeting coding regions of survivin mRNA. Thirteen antisense accessible sites were selected. Their product size of primer extension, 3' end cleaving sites by RNase H and corresponding antisense accessible sites are shown in Table 1. Secondary structures of these thirteen sites were analyzed with RNADraw software. As shown in Figure 3, four sites were found to have obvious stem-loop structures (at 207-226 bp, 187-206 bp, 126-145 bp, 44 -63 bp of survivin cDNA respectively). The antisense oligonucleotides AS-ODN_1-AS-ODN_4, complementary to these four sites, were synthesized (Table 2).

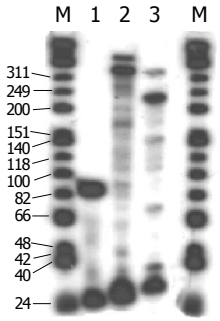

Figure 2 Primer extension analysis for the selection of antisense accessible sites of survivin by random oligonucleotide library/ RNase H cleavage method. M: PhiX174 Hinf I DNA marker. Lane 1: Primer extension positive control; lanes 2-3: Primer extension products of P_1 and P_2.

Table 1 Antisense accessible regions of survivin mRNA shown by primer extension analysis

Primer name	Product size of primer extension (bp)	3′ end site of primer extension (bp)	3′ end site of RNase H cleavage (bp)	Antisense accessible sites (bp)
P_1	42	255	254	235-254
	60	237	236	217-236
	70	227	226	207-226
	82	215	214	195-214
	90	207	206	187-206
	104	193	192	173-192
	118	179	178	159-178
	151	146	145	126-145
P_2	40	124	123	104-123
	70	94	93	74-93
	100	64	63	44-63
	118	46	45	26-45
	140	24	23	4-23

Table 2 Antisense accessible sites and their complementary oligonucleotide sequences selected by random oligonucleotide library/RNase H cleavage combined with RNADraw analysis

Serial number	Base sequence	Origination-end sites	Base pair
ODN_1	AGATGACGACCCCATAGAGG	207-226	20 bp
AS-ODN_1	CCTCTATGGGGTCGTCATCT		
ODN_2	GAGCTGGAAGGCTGGGAGCC	187-206	20 bp
AS-ODN_2	GGCTCCCAGCCTTCCAGCTC		
ODN_3	CTTCATCCACTGCCCCACTG	126-145	20 bp
AS-ODN_3	CAGTGGGGCAGTGGATGAAG		
ODN_4	AGGACCACCGCATCTCTACA	44-63	20 bp
AS-ODN_4	TGTAGAGATGCGGTGGTCCT		

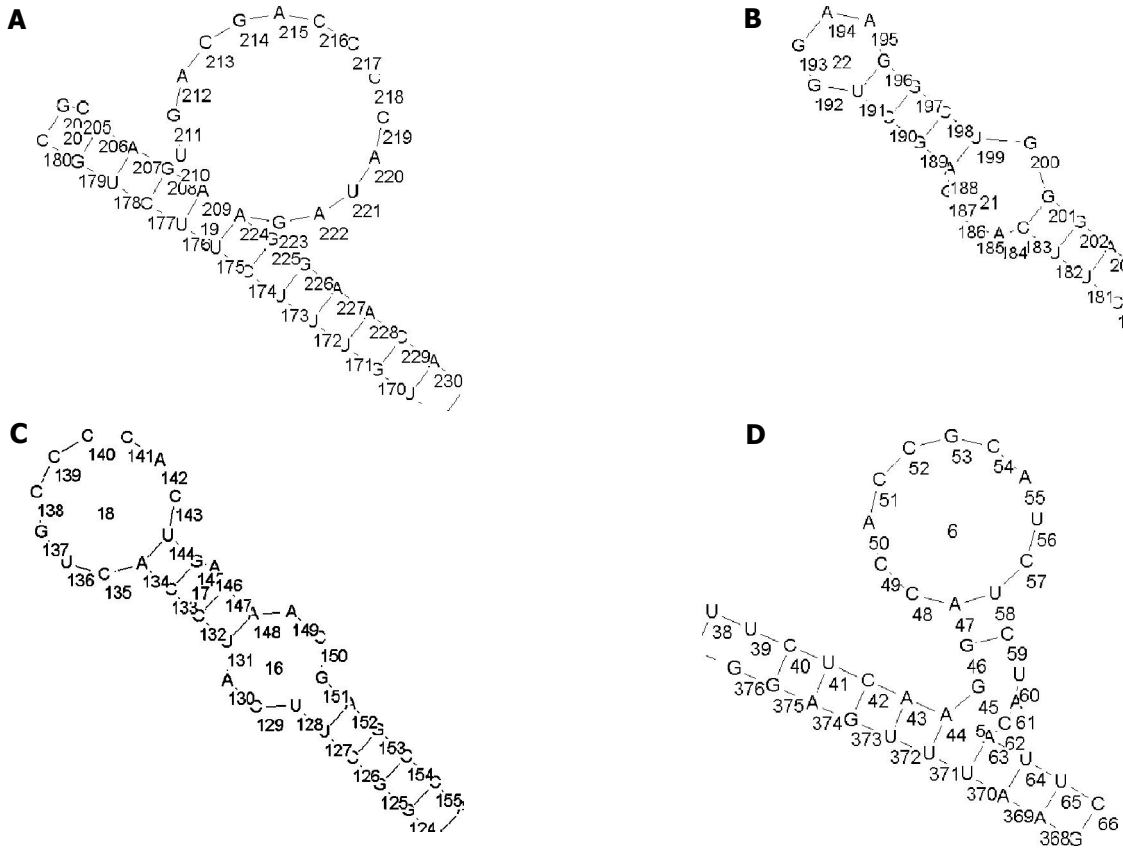

Figure 3 Four secondary structures of antisense accessible sites predicted by RNADraw software. A: ODN $_1$ (207-226 bp); B: ODN$_2$ (187-206 bp); C: ODN$_3$ (126-145 bp); D: ODN$_4$ (44-63 bp).

Blocking effects of antisense oligonucleotides on gene expression

The ratio of survivin/α-tubulin in non-transfection group was 0.918±0.006. Eighteen hours after transfection with AS-ODN$_0$ and AS-ODN$_1$-AS-ODN$_4$, the survivin/a-tubulin ratios were 0.749±0.006, 0.419±0.011, 0.128±0.009, 0.622±0.012 and 0.402±0.008 respectively (Figure 4A). Their inhibitory rates on survivin mRNA expression were 18.4±0.6% (t=5.02, P<0.05), 54.3±1.1% (t=6.12, P<0.01), 86.1±1.0% (t=5.27, P<0.01), 32.2±1.3% (t=7.34, P<0.01) and 56.2±0.9% (t=6.45, P<0.01) respectively. As shown in Figure 4B, Western blotting detection found obvious 16.5 KD protein bands in non-transfected MKN-45 cells. Computer imaging system demonstrated that the inhibitory efficiencies of AS-ODN$_0$ and AS-ODN$_1$-AS-ODN$_4$ on survivin protein expression were 12.6±1.1% (t=4.05, P<0.05), 42.2±2.5% (t=6.26, P<0.01), 75.4±3.1% (t=7.11, P<0.01), 28.3±2.0% (t=6.04, P<0.01) and 45.8±1.2% (t=6.38, P<0.01), respectively. The blocking effect of AS-ODN$_2$ was the highest among all these antisense oligonucleotides.

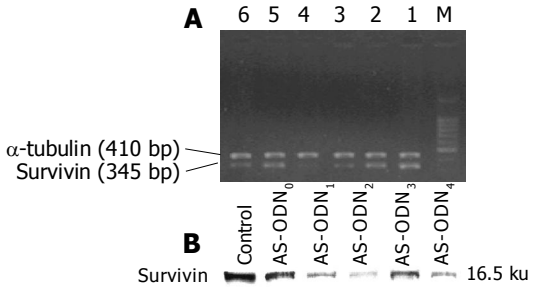

Figure 4 RT-PCR and Western blotting detection of the blocking effects of various antisense oligonucleotides on survivin mRNA and protein expression of MKN-45 cells. A: RT-PCR detection of the blocking effects of various antisense oligonucleotides on survivin mRNA expression of MKV-45 cells M: PCR marker (100-1 000 bp). Lane 1: Non-transfection Control; lane 2: AS-ODN$_0$; lane 3: AS-ODN$_1$; lane 4: AS-ODN$_2$; lane 5: AS-ODN$_3$; lane 6: AS-ODN$_4$; B: Western blotting detection of the blocking effects of various antisense oligonucleotides on survivin protein expression of MKN-45 cells.

Cell growth inhibition

After treatment with 600 nmol/L AS-ODN$_0$ and AS-ODN$_1$-AS-ODN$_4$, the growth activities of MKN-45 cells were reduced in a time dependent manner. Twenty-four hours after transfection, their growth inhibition rates on MKN-45 cells were 15.24±1.85% (t=5.44, P<0.01), 28.12±1.54% (t=7.62, P<0.01), 38.42±3.12% (t=7.75, P<0.01), 21.46±2.63% (t=5.94, P<0.01) and 32.12±1.77% (t=6.17, P<0.01) respectively. The growth inhibition effect of AS-ODN$_2$ was the highest in all antisense oligonucleotides (Figure 5).

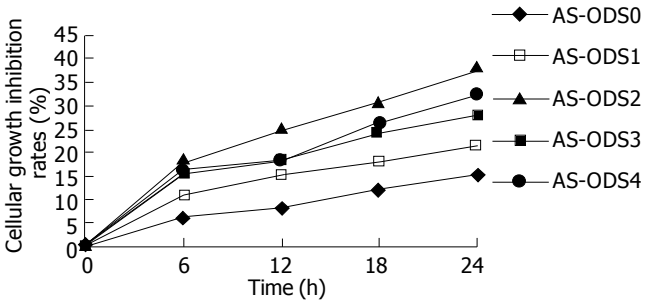

Figure 5 Growth inhibitory effects of various antisense oligonucleotides on MKN-45 cells.

Cell morphological features

Cancer cells in non-transfection control group grew rapidly, with a regular polygon shape. After transfection with 600 nmol/L AS-

$ODNS_{1-4}$, some cells presented reduced size, irregular shape, and mostly round profile. Under electronic microscope, some cells had characteristic morphological changes of apoptosis such as nuclear shrinkage, chromatin congregation around nuclear membranes, reduction of cell volume and integrity of nuclear membranes (Figure 6).

Induction of cell apoptosis

The apoptotic rate of MKN-45 cells in non-transfection group was 0.92±0.12%. After treatment with 600 nmol/L AS-ODN_0 and AS-$ODNS_{1-4}$ for 24 h, the apoptotic rates of MKN-45 cells were 5.02±0.26% (t = 4.17, P<0.05), 19.31±1.16% (t = 7.16, P<0.01), 29.24±1.94% (t = 8.15, P<0.01), 11.87±0.68% (t = 6.68, P<0.01) and 21.68±2.14% (t = 7.53, P<0.01) respectively. The apoptosis-inducing effect of AS-ODN_2 was the highest in all antisense oligonucleotides (Figure 7).

DISCUSSION

Gastric cancer is a common malignant neoplasm of the alimentary tract, its incidence is among the leading three kinds of cancers in different regions of China and has an increasing tendency[13-15]. It is one of the research focuses to explore effective methods for early prevention and treatment of gastric cancer. Survivin is a novel member of the apoptosis inhibitor gene family, which was identified by hybridization screening of human genome libraries with cDNA of effector cell protease receptor-1 (EPR-1) by Altieri *et al*[16] at Yale University in 1997. Interestingly, survivin expresses during embryonal development and in most human tumor tissues. It also expresses in many transformed cell lines, whereas lacks of expression in normal adult tissues[17], indicating that survivin participates in occurrence and development of neoplasms through inhibiting apoptosis, promoting cell proliferation and regulating mitosis and angiogenesis. These findings indicate that survivin is a potential neoplasm marker correlated with prognosis[18]. A series of researches demonstrate that both mRNA and protein levels of survivin in gastric cancer tissues are significantly higher than those in adjacent non-tumor gastric tissues, indicating that it can serve as a novel target for early diagnosis and treatment of gastric cancer[19,20]. Tu *et al*[7] stably transferred the antisense RNA vector for survivin into gastric cancer cells and found that cell growth was decreased with an increased rate of apoptosis, while these cells also exhibited decreased *de novo* gastric tumor formation and reduced angiogenesis.

In recent years, antisense oligonucleotide has been used to explore gene function, and exhibits a great potential in prevention and treatment of neoplasms[21-23]. Because the secondary or tertiary structure of target gene is found in its regions inaccessible to their complementary nucleic acids through base partnership, the selection of optimal antisense accessible sites of targeted sequence is one of the key factors influencing the blocking effects of antisense nucleic acids[24,25].

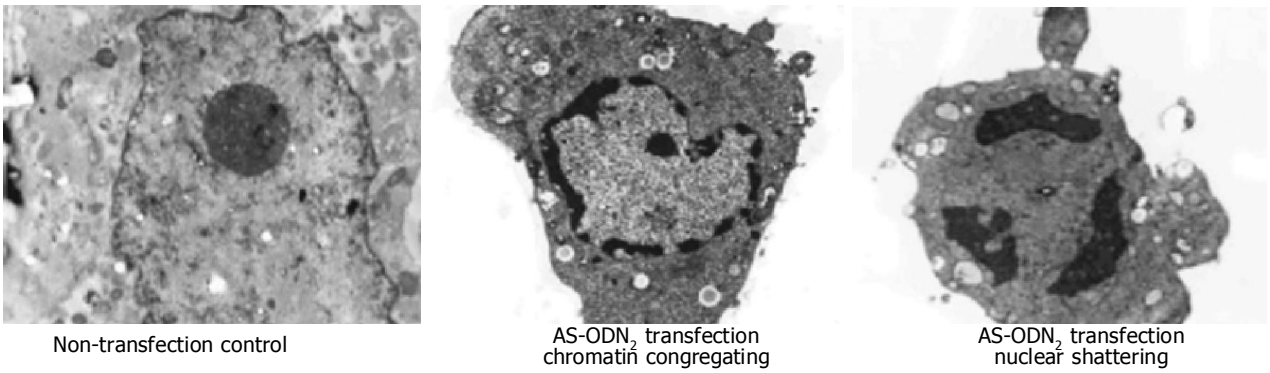

Figure 6 Morphological observation of gastric cancer cells after transfection with antisense oligonucleotides targeting survivin by electronic microscopy.

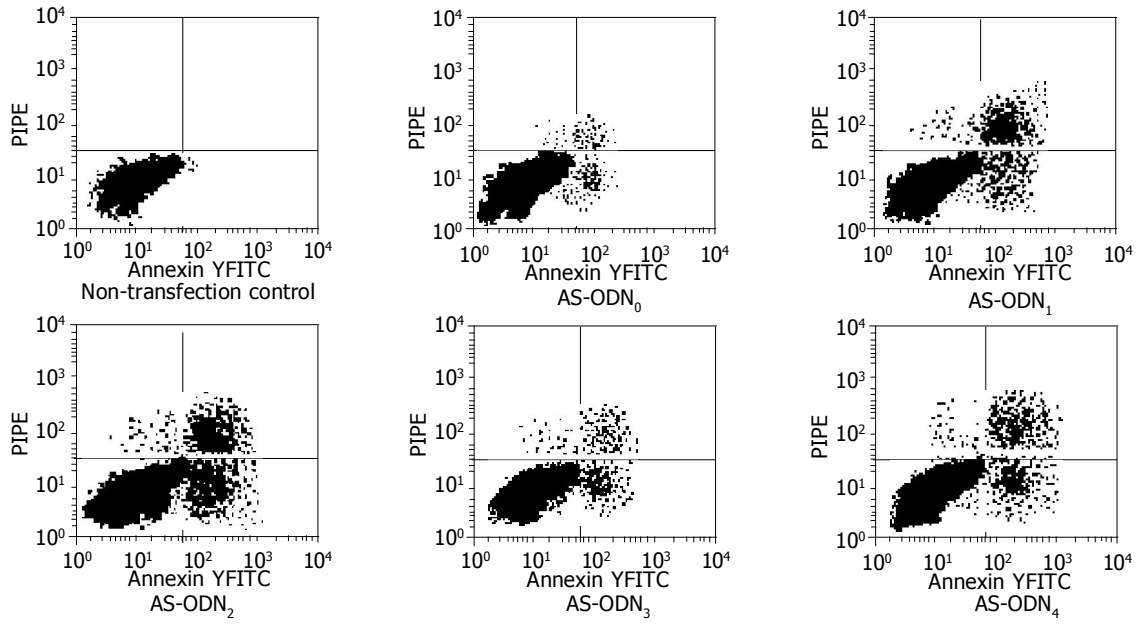

Figure 7 Flow cytometry detection of apoptosis-inducing effects of various antisense oligonucleotides on MKN-45 cells.

Random shooting method has been widely used to select antisense nucleic acids, in which a series of oligonucleotides (usually 50-100 pieces) targeting different regions of a special mRNA were synthesized and evaluated for their antisense activities or accessibilities to targeted sites. But generally only 2-5% antisense oligonucleotides are efficient. Moreover, this method can not determine all accessible sites of targeted mRNA[26]. By computer- predicting method, RNA secondary structures can be predicted through RNA fold softwares, such as MFOLD. Though it has been used for 25 years, it is not sufficient to design antisense oligonucleotides, except for few successful examples. The reason is that computer software can form different structures with similar free energies, which makes it difficult to determine folded structures[27-29]. Antisense hybridization screening was once considered as an efficient way to select antisense oligonucleotides, by which a large number of oligonucleotides could be synthesized and fixed in solid medium for preparation of screen-array, while corresponding analysis softwares for hybridization are also required. So, it costs a large amount of research expenditure[30].

In 1996, based on the fact that RNase H could specifically hydrolyze the phosphodiester backbone of the RNA strand in RNA-DNA duplex hybrid, Siew *et al* firstly brought forward a brand-new conception for the *in vitro* selection of antisense accessible sites by random oligonucleotide libraries/RNase H cleavage method. Firstly, a set of random oligonucleotide libraries, which were fixed in length and composed of possible sequences, were synthesized and incubated with targeted mRNA. Oligonucleotides, which were complementary to accessible sites in libraries, could form hybridization duplex with targeted mRNA. After RNase H cleavage, the sizes and sites of the cleaved products are determined by 5' end primer extension reaction, then the accessible sites of targeted sequence are selected[12]. This method does not require a complex resource of molecular biology and has full consideration of the blocking effects of thermodynamics on secondary or tertiary structure of mRNA. Using this method, Siew *et al* successfully selected 22 antisense accessible sites of human MDR-1 mRNA, and the maximal blocking rate of synthesized complementary antisense oligonucleotides to MDR-1 expression reached 95 percent. In order to explore the effects of random oligonucleotide libraries on selection of antisense accessible sites, Lloyd *et al*[31] introduced four kinds of random oligonucleotide libraries including 8, 12, 16 and 20 mer, and selected 34 antisense accessible sites of TNFα mRNA, in which the 20 mer library has the highest selection efficiency. Vickers *et al*[32] compared this method with RNAi (RNA interference) technique, and found the blocking effect of antisense oligonucleotides designed and synthesized by this method was close to that of siRNA.

In this study, thirteen antisense accessible sites of survivin were selected *in vitro* by the random oligonucleotide libraries/ RNase H cleavage method, then analyzed by RNADraw software. Four sites with obvious stem-loop structures were chosen to synthesize their complementary oligonucleotides. The results of cell transfection indicate that these four antisense oligonucleotides have significantly higher blocking effects on gene expression of survivin than that of simply designed by computer software. AS-ODN_2 against 187-206 bp region of survivin cDNA, had the best blocking effects. After transfected with 600 nmol/L AS-ODN_2, the growth activities of gastric cancer cells were significantly inhibited with obvious apoptotic cells. This demonstrates that the selected antisense oligonucleotides can block the biological function of survivin. This research establishes a basis for further exploring the roles of survivin in biological behaviors of gastric cancer and its regulation mechanisms. Meanwhile, it also provides a brand-new field of vision and an important method for the targeted therapy of gastric cancer, through antisense technology against survivin genes to carry biological therapeutic drugs[33,34].

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