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A novel integrated strategy (full length gene targeting) for mRNA accessible site tagging combined with microarray hybridization/ RNase H cleavage to screen effective antisense oligonucleotides

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Purpose: Down regulation of targeted gene by antisense oligonucleotides (ASOs) has been an effective approach for molecular therapy and the study of gene function. However, it is difficult to find optimal and effective ASOs. We describe a novel integrated strategy called full length gene targeting (FLGT), involving mRNA accessible site tagging combined with microarray hybridization/RNase H cleavage for screening effective ASOs in full length of target gene.
Methods: Initially, transcripts representing mRNA (cRNA) were hybridized with randomized oligonucleotides library, then oligonucleotides tags were sequenced, aligned to target mRNA, and found to be able to precisely define the accessible sites of the mRNA by TargetFinder softerware. Further, selected ASO probes were synthesized and used to construct microarrays. Target mRNA labeled α-^{32}P-UTP was hybridized to the microarrays, and the substrate heteroduplexes were followed by RNase H catalytic reaction on microarrays. Those ASOs with strong signal and shorter $T_{1/2}$ (time of 50% heteroduplex cleavage by RNase H) were selected in the combinatorial assays. Survivin, an inhibitor of apoptosis, was chosen as a target to screen ASOs by the FLGT process.
Results: Using the integrated strategy, five ASOs against survivin were selected and showed significant down regulation of survivin expression and inhibition of tumor cells growth in vitro. Furthermore, one ASO was used to further investigate its antitumor activity on Human hepatocellular carcinoma (HCC) orthotopic transplant model in mice.
Conclusions: This study demonstrated that FLGT is useful for screening effective ASOs. FLGT may become a useful tool for screening more effective ASOs in full length of target gene.

Inhibition of gene expression by antisense technology is widely used to determine the function of genes in cell culture and in animals. An antisense oligonucleotide (ASO) is a single-stranded, chemically modified DNA-like molecule that is 17-22 nucleotides in length and complementary to a target RNA. By pairing with their cognate mRNA, ASOs can knock-down gene expression specifically through mechanisms such as RNase H-mediated cleavage of the target mRNA [1-3], prevention of mRNA transport, modulation or inhibition of translation or splicing [4,5]. Moreover, ASOs can also be effective therapeutic agents. Several antisense compounds for disease treatment have been evaluated in clinical trials, including tumors, AIDS, hepatitis C, asthma, high cholesterol, diabetes, and ocular neovascular disease [6-13]. However, not all ASOs are sufficiently effective in inhibiting protein synthesis. Even with careful design, less than 20% ASOs were effective in screening ASOs [14]. The main reason is that interaction between targeted mRNA and ASOs is determined in part by their secondary and tertiary structures. The intramolecular folding of mRNA renders only 5-10% of most accessible sites to binding to complementary nucleic acids [15]. So an effective ASO is difficult to screen and select.

It is well recognized that selecting accessible sites of a target gene is an important step for obtaining effective ASOs. To address this problem, several experimental and theoretical approaches designed to test the accessibility of oligonucleotides to complementary regions in mRNA have been described, such as random “walking” method [16], oligonucleotides array [17,18], random oligonucleotides library/RNase H cleavage method [19-22], computer predicting method [23,24], and MAST (mRNA accessible site tagging) mapping [25]. Among these methods, oligonucleotides array and MAST mapping are considered efficient ways to select ASOs.

As described, ASOs knock down gene expression specifically through the most accepted mechanism: RNase- H-mediated cleavage of the target mRNA. We therefore supposed that investigating heteroduplex of oligonucleotides-mRNA with RNase H cleavage might be helpful for selecting optimal and effective ASOs. Based on the MAST method and oligonucleotides array, we developed a new integrated strategy called full length gene targeting (FLGT): mRNA accessible site tagging combined with microarray hybridization/RNase H cleavage for screening effective ASOs.

Survivin, an inhibitor of apoptosis, is overexpressed in most common human neoplasms [26,27]. Numerous clinical

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trials have demonstrated that survivin expression is closely associated with cancer progression, resistance to drugs, and poor prognosis [26,28,29]. Down regulation of survivin by antisense technique has become an efficient gene therapeutic method. In this report, survivin was chosen as a target to select effective ASOs by our FLGT process.

METHODS

Synthesis of oligonucleotides and randomized libraries: All DNA oligonucleotides with varied modification in this study were synthesized on an ABI8909 nucleic acid synthesis system (Applied Biosystems, Foster City, CA) and purified by OPC (oligonucleotides purification cartridge; Perkin-Elmer, Foster City, CA). Randomized DNA oligonucleotides library containing 17-mer randomized oligonucleotides were synthesized and the sequences were 5'-GAC GCA CTG A-N_{17}-ACG GTC GGA A-3'. The underlined sequences were fixed sequence for binding to PCR primer; N_{17} represented 17-mer randomized deoxynucleotides sequence. ASOs for transfection with cells were modified with phosphorothioate.

Preparation of targeted mRNA transcripts: cDNA fragments were generated by PCR and subcloned into the pSP64 Poly(A) vector (Promega, Madision, WI). They were used to produce the corresponding mRNA (cRNA) by in vitro transcription reactions driven by Sp6 RNA polymerase according to the procedures of the manufacturers (Promega). Briefly, standard 100 μl in vitro reactions were set up containing 2 mM ATP, CTP, GTP, UTP, 3.75 μg of DNA template and 10 μl RNA polymerase mix (SP6). After incubation 3 h at 37 °C, the DNA template was removed by digestion with DNase I for 30 min. The products (cRNA) were purified and dissolved in DEPC H_2O. The cRNA concentration was measured with an ultraviolet spectrophotometer.

For microarray hybridization, the radiolabeled cRNA was generated in the presence of 100 μCi [α^{32}P] UTP (3200 Ci/mmol, Amersham, Piscataway, NJ) in addition to 2 mM each ATP, CTP, GTP, UTP, and 1 mM UTP.

Hybridization of survivin transcripts with random oligonucleotides library: Hybridization was performed in 30 μl volume of selection buffer (10 mM Tris-HCl [pH 8.0], 1.5 mM $MgCl_2$ and 50 mM KCl). Briefly, the oligonucleotide library (120 μM) was denatured at 100 °C for 5 min, kept on ice for 10 min, and added with 0.8 μM cRNA. The reaction was conducted at 37 °C for 30 min. After reaction, the cRNA-oligonucleotides were gathered with RNeasy Mini Kit (Qiagen, Hilden, Germany), and eluted in nuclease-free water.

Amplification and sequencing of oligonucleotides (sequence tags): The cRNA-oligonucleotides, dissolved in nuclease-free water, was as a template for PCR amplification. Primers sequences for oligonucleotide amplification were as follows: forward, 5'-GCA AAA TGT TGA CGC ACT GA-3' that overlaps with the 5' fixed sequence; reverse, 5'-ACC ACT GGA ATT CCG ACC GT-3' that partially complements the 3' fixed sequence. The conditions for PCR were 30 cycles of 94 °C (30 s), 52 °C (1 min), and 72 °C (2 min). PCR products (amplified tags) were purified (Qiagen), subcloned into pDrive Cloning Vectors (Qiagen) and sequenced. Alignment of the oligonucleotides sequences to the target gene and the selec-

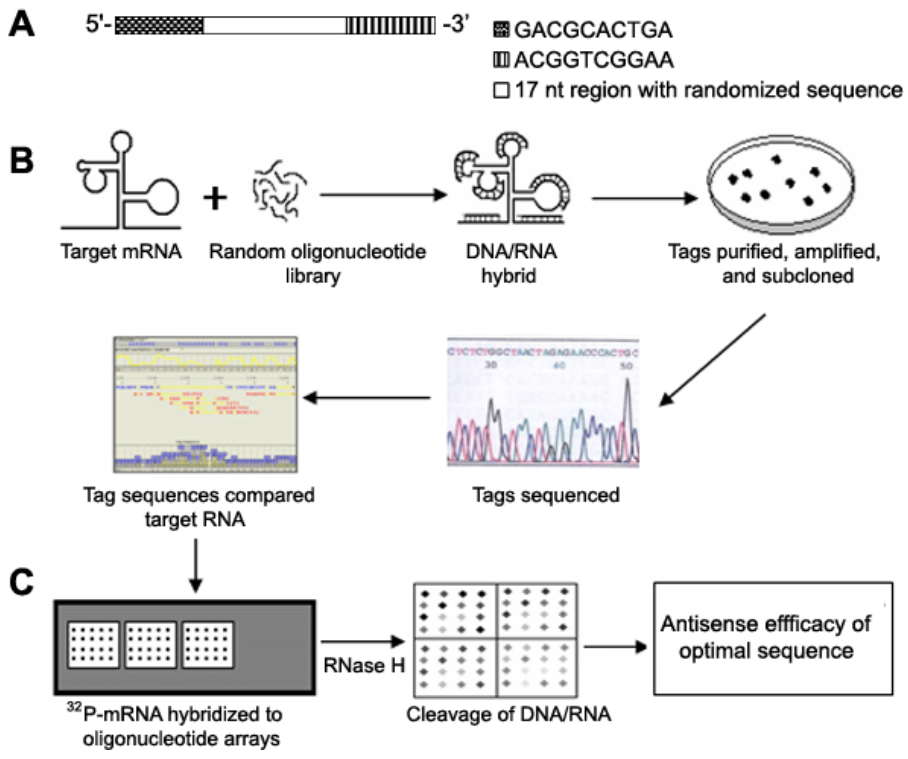

Figure 1. Schematic diagram of full length gene targeting (FLGT). **A**: An oligonucleotides library with 17 nt fully randomized sequence nested between two fixed sequences for binding to PCR primer. **B**: mRNA was synthesized by in vitro transcription and hybridized with fully oligonucleotides library. The oligonucleotide tags specifically bound to the mRNA were PCR amplified, cloned into vectors, sequenced and aligned to target gene. The selection of accessible sites and design of antisense oligonucleotides (ASOs) were accomplished by TargetFinder software. **C**: Oligonucleotide probes were designed and constructed microarrays. Radiolabeled transcripts (cRNA) hybridized to microarrays. The hybridization signal reflects the degree of binding affinity between cRNA and oligonucleotides. Subsequently, the heteroduplexes were followed by RNase H reaction for various times. According to the residuary signal intensity with time-course, RNase H cleavage ability for oligonucleotide-RNA duplexes was estimated by $T_{1/2}$ (the time of 50% heteroduplex cleavaged by RNase H).

tion of accessible sites were accomplished by TargetFinder software. The software designed by our laboratory is user friendly [30].

Microarray hybridization: Oligonucleotide probes were synthesized and purified as described. All probes were diluted in selection buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, and 1.5 mM $MgCl_2$) and spotted onto CSS-100 silylated glass slides (Xenopore, Hawthorne, NJ) using a noncontact piezoelectric microdispenser. Fifty picoliters of oligonucleotide solution were spotted at concentration of 15 μM. Microarray hybridization was performed in a closed chamber. Briefly, approximate 5-8 picomol of radiolabeled cRNA was in 10 μl hybridization buffer (1 mM NaCl, 10 mM Tris-HCl [pH 8.3], 1 mM EDTA, 0.01% SDS, and 0.2 U/μl yeast tRNA) and applied to the oligonucleotide microarray. Coverslips were placed over the microarrays, and the microarrays were placed in hybridization chambers and incubated for 3 h in a 37 °C water bath. Following hybridization, the microarrays were washed with hybridization buffer (not containing yeast tRNA) and exposed to a storage phosphor screen for 3 days. Subsequently, the storage phosphor screen was scanned with Typhoon 9410 scanner system (Amersham), and image analysis was conducted with ImageQuant 5.2. Data derived from hybridization signal represents affinity between oligonucleotides probes and mRNA.

RNase H cleavage in situ: ^{32}P-cRNA was hybridized to oligonucleotides arrays as described. After being washed with hybridization buffer, the oligonucleotides arrays were incubated in RNase H cleavage buffer and digested with RNase H at 37 °C for various times (2, 5 and 10 min, respectively). The RNase H buffer contained 100 mM KCl, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 0.1 mM DTT. Digestion reactions were carried out in a total volume of 20 μl with 0.125 U RNase H (2 U/μl; Promega). One control array was incubated in cleavage buffer without RNase H. Image analysis was performed as described. $T_{1/2}$, the time of 50% heteroduplex cleaved by RNase H, was calculated from the curve of signal intensity with time course, which represented ability of RNase H to cleave oligonucleotide-RNA duplexes. ASO-RNA heteroduplex with a high degradation rate (shorter $T_{1/2}$) indicated a good substrate with secondary structure for RNase H.

Functional analysis of selected antisense sequences against survivin in vitro: Human hepatocellular carcinoma (HCC) HepG2 cells, kindly provided by Beijing Medical University, were maintained in DMEM (Invitrogen, Carlsbad, CA) plus 10% newborn bovine serum (Gibco, Carlsbad, CA). For transient transfection experiments, we plated cells in 96-well or six-well plates and let them grow to 50-70% confluence. We then transfected with ASOs in the presence of lipofectin (8 μg/ml lipofectin for 0.8 μmol/l oligos; Invitrogen) according to the manufacturer's protocol. After incubation of ASOs with cells for 6 h, the media was replaced and cultures were incubated for various times before analysis of cell proliferation and the level of survivin mRNA.

Cell proliferation assay was performed with the CellTiter 96 Aqueous one solution cell proliferation assay kit (Promega). It was performed as per the instructions of the manufacturer.

The level of survivin mRNA in cells was analyzed by RT-PCR. Total RNA was extracted by using the Trizol reagent (Invitrogen). One μg total RNA was used to create cDNA by using the Super-Script II reverse transcriptase (Invitrogen). Subsequent PCR amplification was performed for 28 cycles of 94 °C (45 s), 55 °C (30 s) and 72 °C (40 s). A final extension was performed for 5 min at 72 °C. Under this condition, the amplification showed linearity (data not shown). PCR products were run on 2% agarose gels and the intensities were measured using an image analyzer Quantity one (Bio-Rad, Hercules, CA).

Analysis of in vivo antisense activity: Athymic (*nu*/*nu*), four- to six-week-old mice (BALB/c) were purchased from the Chinese National Cancer Institute (Beijing, China). The mice were kept under specific-pathogen-free conditions and used according to institutional guidelines. The HCM-Y89 tumor, derived from a surgical specimen of HCC that had been transplanted in liver of mice for several generations, was dissected into 1x1 mm pieces. Two pieces were implanted in the left liver of mice. After the orthotopic transplant model of HCC had been established for three days, the mice were randomly divided into four groups (eight mice per group). To closely mimic the clinical setting, we administered two oligonucleotides by intravenous injection. A10 was injected at 25 and 50 mg/kg/day, respectively, and sense oligonucleotides (sense) was injected at 50 mg/kg/day. A 30 μl volume of saline solution containing A10 or sense was given for four repeated cycles, and 5 consecutive days with one-day intervals between each cycle. Mice were monitored routinely for weight and general physical status. At the endpoint of treatment, the mice were

Table 1.

Name	Site*	Sequence	Hybridization intensity	$T_{1/2}$ (min)
A1	107-126	GGCCAGTTCTTGAATGTAGA	562.1	3.0
A2	414-433	CGCAGTTTCCTCAAATTCTT	1092.3	3.6
A3	467-486	CAGAGGCCTCAATCCATGGC	418.1	3.1
A4	564-583	TCCTAAGACATTGCTAAGGG	405.7	2.5
A5	596-615	GAAACATCTAATTTGAAAAT	451.8	2.5
A6	787-806	AGCTCTAGCAAAAGGGACAC	895.3	2.6
A7	995-1014	GTCATGCATCTACCAAAAAA	1142.8	2.9
A8	1072-1091	CAAACAAAATAAGAAAGCCA	615.8	2.8
A9	1211-1230	AGGAGTATCTGCCAGACGCT	797.7	3.1
A10	1238-1257	CCTGTCTAATCACACAGCAG	1367.4	2.5
A11	1486-1505	CCATCATCTTACGCCAGACT	1240.2	2.9
A12	1535-1554	GTAACAATCCACCCTGCAGC	519.7	3.1
B1	55-74	CAGGGGGCAACGTCGGGGCA	0	0
B2	524-543	CACCAGGGAATAAACCCTGG	0	0
B3	1511-1530	TGACAGGGAGGAGGGCGAAT	0	0
P1	36-55	ACCCATGCCGCCGCCGCCAC	868.6	2.7
P2	232-251	CCCAGCCTTCCAGCTCCTTG	947.9	2.7
P3	1099-1116	TGTGCTATTCTGTGAATT	1979.3	2.3
R	Random	ACGTCACGTACAACGAAGCT	0	0

Sequence of oligonucleotides probes used for microarrays hybridization and RNase H cleavage in situ. A1-A12 were selected ASOs probes targeting accessible survivin mRNA sites. B1, B2, and B3 were control oligonucleotides against survivin mRNA sites with poor or no accessibility, as negative controls. P1, P2, and P3 were also selected ASOs which have been found and reported by us and others, respectively, as positive controls. R was an unrelated oligonucleotides sequence. Sites were numbered according to the mRNA sequence presented in GenBank entry NM_001168.

sacrificed, tumors dissected and weighed. The percentage of tumor growth inhibition was calculated as follows:

Inhibitory rate (%)=($W_{control}$-W_{treat})/$W_{control}$x100, where W represents the tumor weight.

The protein levels of survivin in tumor tissue were analyzed by western blot. Protein lysates were prepared by using a procedure described by Tu et al. [31]. Equal amounts of protein were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto polyvinylidene fluoride membrane (Schleicher & Scheull Bioscience, Keene, NH). Membranes were immunoblotted with rabbit-antihuman (antisurvivin or anti-β-actin [Santa Cruz Biotech, Santa Cruz, CA]) antibodies. After washing, membranes were incubated with HRP-conjugated goat antirabbit antibody (Santa Cruz Biotech). Finally, the reactive band was visualized by an ECL-plus Detection Kit (Santa Cruz Biotech) and scanned by Gel Doc 1000 (Bio-Rad).

Statistical analysis: Results are expressed as means±SD. All statistical analyses were made with a two-sided Student's t-test. A $p<0.01$ was considered to be statistically significant.

RESULTS

Screening accessible sites of survivin mRNA in randomed oligonucleotides library: The experimental strategy of FLGT was described schematically in Figure 1. In this study, we chose survivin mRNA as a model system to validate the integrated strategy. Through hybridized with randomed oligonucleotides library, 15 accessible sites (A1-A12, P1, P2, and P3) were mapped and identified (Table 1). Interestingly, two accessible sites were reported by Olie [29] and Li et al. [32], and their antisense efficacies were also confirmed by both in vitro and in vivo [33-37]. Here, they were named P2 (targets nucleotides 232-251) and P3 (targets nucleotides 1099-1116), respectively. Moreover, we have previously found an effective ASO target-

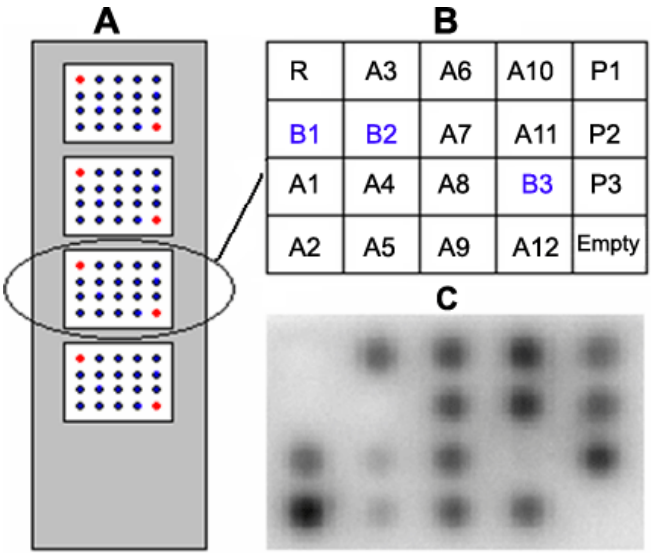

Figure 2. Analysis of microarray hybridization of selected ASO probes against survivin. **A**: Microarray sketch map. **B**: Schematic representation of a microarray. Table 1 shows the A, B, P, and R representing oligonucleotides. **C**: Hybridization image of ^{32}P-labeled transcripts to microarrays of oligonucleotides.

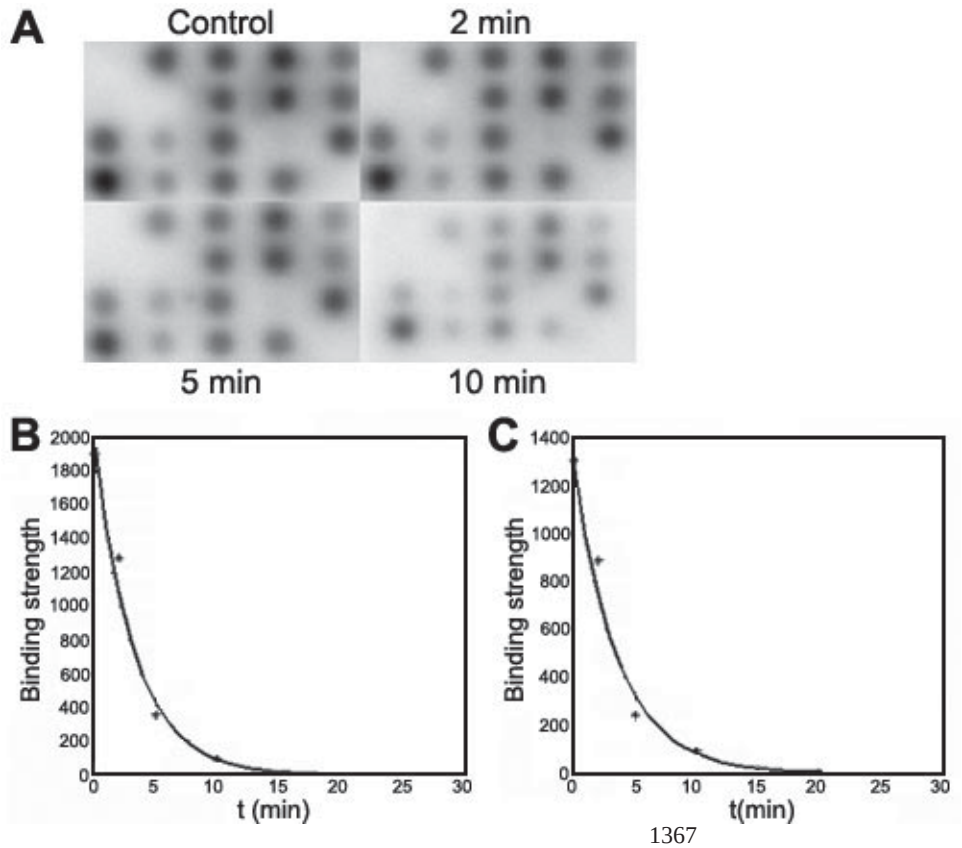

Figure 3. Cleavage of heteroduplex mediated by RNase H on microarrays in situ. **A**: A time course of 0.125 U RNase H reaction within 20 μl buffer. Heteroduplexes were incubated with RNase H for different times (2, 5, and 10 min) or without RNase H (control). **B** and **C**: Kinetic features of the cleavage reaction by RNase H. **B** and **C** were the best-fit curve for signal strength of oligonucleotides P3 and A10, respectively.

ing sites (36-55) by a computational neural network mode and Mfold server (data not shown), which was also found in this experiment and named P1.

Analysis of combinatorial microarray hybridization: In order to further screen and verify these selected accessible sites, we carried out microarray hybridization and RNase H cleavage reaction in situ. We designed 15 ASOs probes and constructed microarrays. As a comparison, three additional oligonucleotides (B1, B2, and B3) targeting survivin mRNA sites with poor and no accessibility and a random sequence (R) unrelated to survivin mRNA were included in the test. Probes sequences were also shown in Table 1. As shown in Figure 2, the image showed that all selected ASOs probes also formed heteroduplexes with survivin mRNA with different degree of hybridization signals. Remarkably, ASOs A2, A6, A7, A10, A11, P1, P2, and P3 showed strong signals. Whereas B1, B2, and B3, as with control R, showed poor or even no hybridized signals. Data of hybridized signals was also measured and is shown in Table 1. The hybridized value was from 405.7 to 1979.3 in 15 selected probes. P3 showed the best hybridization intensity (HI=1979.3), followed by A10 (HI=1367.4), A11 (HI=1240.2), A7 (HI=1142.8), and A2 (HI=1092.3). In this sense, these data reflected that the heteroduplexes on microarray correlated well with the results of screening oligonucleotides library.

Analysis of RNase H-mediated cleavage on microarray hybridization: Having tested these selected ASOs on microarray hybridization, we next observed the ability of RNase H cleavage for DNA-RNA heteroduplexes on microarray hybridization. As shown in Figure 3, the signal intensity of hybridization plot was weakened by RNase H, and the reduction rate of signal intensity was different in 15 hybridization plots. $T_{1/2}$ values of all hybridization plots were listed in Table 1. P3 not only displayed the best hybridization intensity, but also showed the minimum time of $T_{1/2}$, followed by A10, A11, A6, and P2. These data implied that DNA-RNA duplex could sensitize to RNase H activity and was cleaved by RNase H in situ. Another intriguing observation was that A2 with high hybridization intensity showed the maximum time of $T_{1/2}$ among all ASOs probes (Table 1). This will be further explored in the Discussion section.

Activity assay of selected antisense oligonucleotides in vitro: Next, we tested whether selected ASOs were related to an increased frequency of significant inhibition of survivin expression and inhibited tumor cells growth in vitro. As shown in Figure 4A, transfection of A7, A10, P1, P2, and P3 with high binding affinity inhibited significantly HepG2 cells growth with the inhibition rate by >50% at a concentration of 0.2 μM,

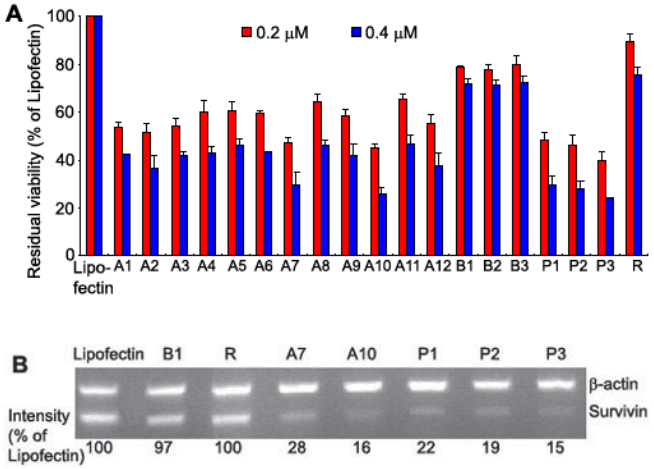

Figure 4. Function analysis of selected ASOs against survivin in vitro. **A**: Effect of ASOs on the growth of HepG2 cells, analyzed by MTS reagent. Cells were treated with oligonucleotides (A1-12, B1-3, and R, respectively) in the presence of lipofectin. Cell growth was determined in triplicate plates 72 h after the start of transfection using the MTS reagent. Each value represents the mean±SD of four independent experiments. **B**: Effects of ASOs on the level of survivin mRNA in HepG2 cells. Cells were transfected with 0.4 μM ASOs (P1, P2, P3, A7, and A10), or control oligonucleotides B1 and R for 24 h. Survivin mRNA was analyzed by RT-PCR and coamplified with β-actin mRNA. The number under each band is expressed as a percentage of lipofectin control, normalized by the corresponding β-actin level.

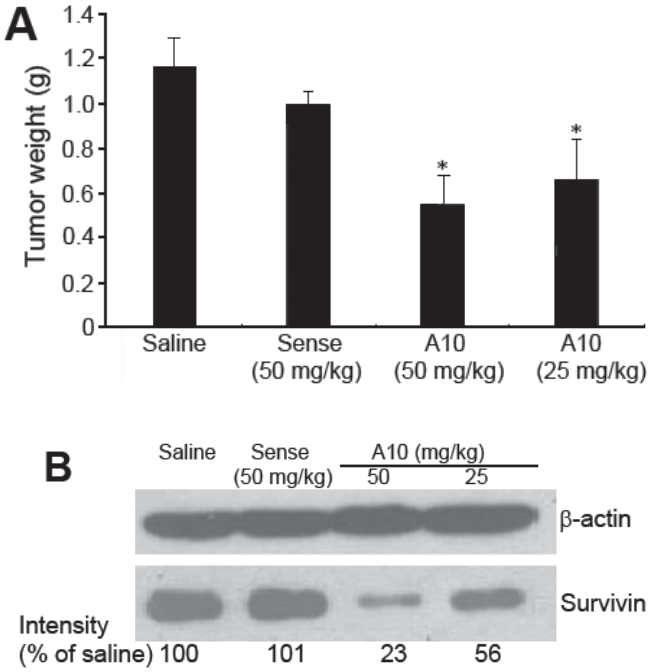

Figure 5. Function analysis of selected A10 against survivin in vivo. Nude mice bearing an orthotopic transplant of HCC for 3 days received intravenous oligonucleotides as described in Methods. **A**: Tumor weight as a function of treatment. Tumor weight was determined by electrobalance. All of the data are represented as the mean±SD of 8 mice. The asterisk indicates a $p<0.01$ compared with saline treatment. **B**: Analysis of survivin protein in tumor tissue. Equal amounts of protein from tumor lysates was immunoblotted as described in Methods. A representative sample of tumors in each group treated with saline, A10, or sense is shown, and the experiment was repeated three times with similar results. The number under each band is expressed as a percentage of saline treatment, normalized by the corresponding β-actin level.

whereas three controls, B1, B2, and B3, like control R, have poor activity for cell growth.

To examine whether the effects of these ASOs on tumor cells was correlated with downregulation of survivin mRNA, we selected five ASOs (A7, A10, P1, P2, and P3) with high inhibition of tumor growth to observe the level of survivin mRNA in cells by RT-PCR. As shown in Figure 4B, all five ASOs were found to knock down the expression of mRNA by >70% under the experimental conditions used here, whereas oligonucleotides B1 with poor accessibility, like random R, showed much lower levels of inhibition at a concentration of 0.4 μM. In this sense, we believe that mRNA accessible site tagging combined with microarray hybridization/RNase H cleavage in situ was reliable and an effective integrated strategy for screening effective ASOs.

Activity assay of selected antisense oligonucleotides in vivo: Having demonstrated the antisense activity of all ASOs in vitro, we further examined the selected ASO activity in nude mice bearing orthotopic transplant of HCC. A10 was chosen to be further tested in vivo. As shown in Figure 5A, A10 treatment (at a dose of 25 or 50 mg/kg/day) significantly inhibited tumor growth ($p<0.01$). Compared to the mock injections (saline), A10 reduced tumor weight by 43.1% and 52.6%, respectively. Compared to the treatment with control Sense, A10 (at dose of 50 mg/kg/day) also reduced tumor weight by 44.4%. To provide evidence that the antitumor activity of A10 was due to its ability to down-regulate survivin in tumor tissues, we analyzed the level of survivin protein by western blot. As shown in Figure 5B, A10 downregulated survivin protein in a dose-dependent manner, and we observed no changes of survivin protein in sense-treated mice.

DISCUSSION

It has been proposed that the efficacy of an ASO is dependent on the accessibility of the sequence [20,25]. A number of methods for finding accessibility of the sequence in RNAs have previously been described. In this study, we have developed a new integrated strategy (FLGT): mRNA accessible site tagging combined with microarray hybridization/RNase H cleavage in situ to screen accessible sites in full length of mRNA sequence and select more effective ASOs.

Through primary screening for randomized oligonucleotides library, we obtained 15 accessible sites of survivin mRNA. It is interesting that two of 15 ASOs have been reported by Olie [29] and Li et al. [32], and their antisense efficacy has been confirmed in vitro and in vivo [33-37]. These data suggests that FLGT might be an available and effective method for selecting accessible sites in full length of target mRNA. Oligonucleotides microarray is also an efficient way to select ASOs. In our integrated strategy, we used microarray hybridization as another screening approach.After performing hybridization on microarray, we found 15 ASOs probes could bind to the target gene with a different degree of binding affinity (Table 1). ASOs P3 and A10 have the maximal hybridization intensity among these probes. In contrast, B1, 2, 3 with poor or no accessibility in survivin mRNA showed no hybridization intensity as with control R (Figure 2). These data suggest that the results not only were consistent with that of primary screening from randomized oligonucleotides library, but also reflected and showed the binding affinity between ASOs and complementary sites of mRNA, which was important for the experiment.

As described in the introduction, the most accepted mechanism of how ASOs inhibit expression of target gene is that they form a mRNA-ASO duplex through Watson-Crick binding, leading to RNase H-mediated cleavage of the target mRNA [1-3] in living cells. A number of studies using RNase H mapping as a method developed for identification of mRNA accessible sites in solution have shown promising results [20-22]. In this study, we have, for the first time, used the RNase H cleavage method on microarray hybridization to select more effective ASOs. After microarray hybridization, the heteroduplexes of ASOs-RNA were digested with RNase H for various times. The high hybridization intensity with high degradation rate (shorter $T_{1/2}$) indicates this is a good substrate for RNase H, and the ASOs has antisense potency in living cells. Oligonucleotides P3 not only showed the best hybridization intensity, but also had the minimum time of $T_{1/2}$ in all candidate ASOs, followed by A10 and others (Table 1). In antisense activity experiments, those ASOs with high hybridization intensity and high degradation rate showed more inhibition of tumor growth and survivin expression in vitro (Figure 4). Based on these results, we conclude that there is a good correlation between $T_{1/2}$ of heteroduplexes degraded by RNase H on microarrays in situ and antisense activities in vitro. Surprisingly, an intriguing observation was that oligonucleotides A2 with high hybridization intensity, however, showed the maximum time of $T_{1/2}$ among all ASOs probes. One explanation for the results was that the mRNA binding to complementary A2 formed a stable DNA-RNA duplex and special local structure, which might hinder RNase H cleavage activity. In in vitro antisense activity, A2 also inhibited the HepG2 cells growth obviously, suggesting that in some cases RNase H-mediated cleavage in solution buffer or on microarrays did not completely reflect the practical environment in living cells, in which other pathways or associated factors might be involved. Although a $T_{1/2}$ indicator does not completely identify the effectiveness of ASO, it really helps us to select those "good" candidates.

Using the integrated strategy (FLGT), five effective accessible sites including published sites (P2 and P3) were identified in the full length of survivin mRNA, and the efficiency of ASOs on cells was validated in vitro. The results showed that the five selected ASOs have significantly inhibited tumor cell growth and decreased survivin expression. A10, a new selected antisense sequence against survivin, was used to further investigate its antitumor activity in vivo. We found A10 at two doses (25, 50 mg/kg/day, respectively) significantly inhibited tumor growth ($p<0.01$), and the inhibition was associated with knockdown of survivin protein in tumor tissues.

Since the first antisense drug, Vitravene (fomivirsen), was approved by the United States Food and Drug Administration (FDA) for the treatment of CMV retinitis in 1998 [7], a large number of ASOs are being studied using in vitro and in vivo

models, and are being explored as potential therapeutics against cancer, viral infections, cardiovascular disease, respiratory problems, inflammatory disorders, and hematological disease [6-13]. In December 2004, the FDA approved pegaptanib sodium (Macugen, a new oligonucleotides drug), an antivascular endothelial growth factor RNA aptamer, for the treatment of all types of neovascular age-related macular degeneration [12,13,38]. Pegaptanib sodium's approval represents a prospective future in antisense drug development as a therapeutic agent in clinical therapy. For this reason, we also believe that our FLGT strategy might provide a useful tool for screening effective ASOs in various fields, including eye research and therapy.

In summary, we described FLGT, an integrated strategy mRNA accessible site tagging, combined with microarray hybridization/RNase H cleavage to use in screening effective ASOs. Compared with current experimental methods for predicting effective antisense sequence, FLGT provides an integrative and useful tool with high throughput to select effective ASOs.

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