Anti-Cancer Drugs 1995, **6**, pp. 553–561

Inhibition of colon cancer cell proliferation by antisense oligonucleotides targeting the messenger RNA of the Ki-*ras* gene

Chouhei Sakakura, Akeo Hagiwara, Hiroyuki Tsujimoto, Kimihiko Ozaki, Tuguo Sakakibara, Takayuki Oyama, Masaharu Ogaki, Tsutomu Imanishi, Junya Yamazaki and Toshio Takahashi

First Department of Surgery, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kawaramachi-dori, Kyoto 602, Japan.

Point mutations that activate the Ki-*ras* proto-oncogene are present in approximately 50% of human colorectal tumors and the activated Ki-*ras* gene is considered to play an important role in colorectal cancer cell proliferation. Five different colon cancer cell lines and two kinds of control cell lines were treated with antisense oligonucleotides complementary to the messenger RNA of Ki-*ras*. Treatment with antisense oligonucleotides at concentrations between 10 and 40 μM significantly and dose-dependently inhibited cell growth, colony formation and Ki-*ras* protein production of the colon cancer cells with activated Ki-*ras*, but did not affect the normal cells and colon cancer cells without Ki-*ras* mutation. These results show that use of synthetic oligonucleotides is an effective way of producing antisense-mediated changes in the behavior of human colon cancer cells with an activated Ki-*ras* gene.

Key words: Antisense phosphorothioate oligonucleotides, antisense-mediated change, colon cancer, gene regulation Ki-*ras*.

Introduction

Colorectal cancers are one of the most common human malignancies and their carcinogenesis has been clarified in detail.[1] Colorectal cancers are associated with multiple genetic alterations, including activation of the Ki-*ras* proto-oncogene,[2–5] and inactivation of the tumor suppressor genes p53,[6,7] DCC,[8] MCC[9] and APC.[10]

Cellular proto-oncogenes may be activated in neoplastic processes by point mutations or gene amplifications.[11] The most notable class of oncogenes activated in human solid tumors is the *ras* oncogene family, all of which code for membrane-associated protein products of molecular weight 21 000 (p21). Activation of the *ras* gene is caused by single base mutations that result in an amino acid substitution yielding a protein with increasing transforming ability or by amplification of the gene.[12,13] *ras* p21 proteins have been demonstrated in many solid tumors and increased expression of p21 has been associated with neoplastic transformation.[14,15] A number of human carcinomas, including colon and pancreas cancer, have been shown to contain a Ki-*ras* gene activated by point mutations in codon 12, and to a lesser extent in codon 13 and 61.[5,16,17] Then it follows that inhibition of activated Ki-*ras* gene function will be one of the ways to inhibit colon cancer cell proliferation and change of cancer cell phenotype.

The present study was performed to determine whether antisense oligonucleotides complementary to the messenger RNA of the Ki-*ras* gene would inhibit Ki-*ras* expression and thereby inhibit the proliferation of colon cancer cells. At first we studied the genetic alteration of K-, H- and N-*ras* in all tested cells at codon 12, 13 and 61. SW1116 and WiDr cells contain one normal Ki-*ras* allele and one Ki-*ras* allele with a point mutation at codon 12 that converts Gly^{12} to Ala^{12}. DLD-1 and HCT 116 cells contain one normal Ki-*ras* allele and one Ki-*ras* allele with a point mutation at codon 13 that converts Gly^{13} to Asp^{13}. There are no mutations in H-*ras* or N-*ras* at codons 12, 13 or 61. COLO 201, WI-38 and WEHI-3 cells do not have mutations of the KI-*ras* gene.

If proliferation of cancer cells could be inhibited by these antisense oligonucleotides, their application might be a useful chemotherapeutic strategy for treating colon cancers.

This work was supported in part by a Grant-in Aid for Cancer Research from the Ministry and welfare, and from the Ministry of the Education, Science and Culture, Japan.

Correspondence to C Sakakura, The Biomembrane Institute, 201 Elliott Avenue, West, Suite 305, Seattle, WA 98119, USA. Tel: 206 285-1309; Fax: (206) 281-9893

Materials and methods

Cell culture

The human colon cancer cell lines DLD-1, HCT 116, SW1116, WiDr and COLO 201 in addition to the human myelomonocytic cell line WEHI-3 and human fibroblast WI-38 cells were cultured in RPMI 1640 (Nissui, Tokyo, Japan) with 10% fetal bovine serum (FBS) and 100 IU of penicillin at 37°C under standard conditions.

Oligonucleotide synthesis

Eighteen-base oligonucleotides were synthesized using the Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Foster City, CA) with a phosphorothioate substitution at each base. The oligomers were purified by two different HPLC methods,[18] and purity was assayed by polyacrylamide gel electrophoresis and HPLC. Purity range was above 95%. The antisense oligonucleotides were complementary to 18 bp sequences next to the start codon or overlapping the start codon of Ki-*ras* mRNA sequences, as shown in Figure 1. As controls, sense, scrambled and mismatched oligonucleotides were also synthesized. Sterile aliquots of 1 mM stock solutions were stored at −20°C and thawed on ice before use. Sequences of antisense, sense, random and mismatch oligonucleotides are as follows: antisense: CACAAGCTTATATTCAGT; sense: ACTGAATATAAGCTTGTG; scramble: ACTAGCTATACTAGCTAT; and mismatch: CACTTGCAAATATTCAGT (mismatched sequence underlined).

Oligonucleotide uptake

Random sequence phosphorothioate oligonucleotides were conjugated with fluorescein-5-isothiocyanate (Fluorescein-ON Phosphoramidite; Clontech, Palo Alto, CA) according to the procedure of Wachter *et al.*[19] Cells were seeded at a density of 5×10^3/ml in 60 mm tissue culture dishes. After

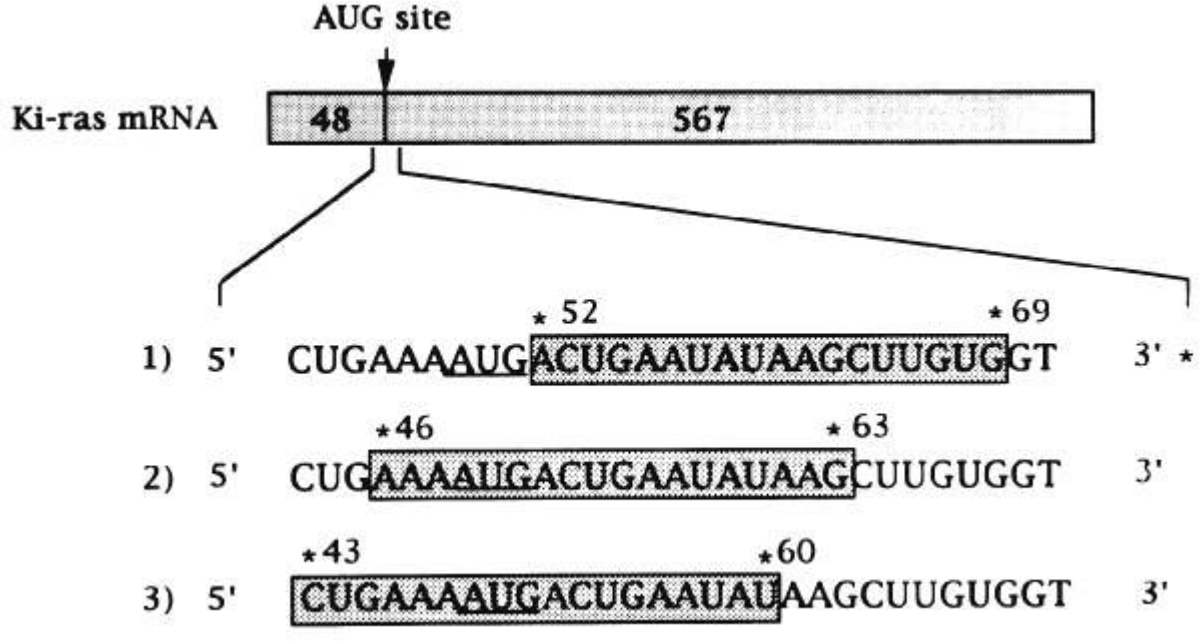

Figure 1. The sequences of Ki-*ras* mRNA targeted by the antisense oligonucleotides. Three targeted sequences are boxed with corresponding nucleotide number from sequences of Kusewitt (GenBank accession no. z12125). The start codon of the gene is underlined. The inhibitory potency of the antisense oligonucleotides was related to the small shift in the sequence targeted, but not significant statistically.

24 h, the media were changed. The labeled oligonucleotides were then added at a concentration of 5 μM to the medium. Plates were harvested at 0 min, 1 h, 2 h and then hourly. The cells were washed several times in cold phosphate buffer (PBS) and analyzed with flow cytometer (FACS 400; Becton Dickinson, Mountain View, CA).

Growth rate

Cells were plated at a density of 1×10^4 cells/ml into 24-well plates for 24 h. The media was then changed to one containing 10% FBS and various concentrations (10, 20 or 40 μM) of either antisense oligomer, sense, scrambled or mismatched oligomer or PBS were added. Daily, the cells were detached and separated with trypsin and counted. The dye exclusion test was done and only viable cells were counted at each data point. Only living cells were counted at each data point by the dye exclusion test in all experiments. In order to estimate the growth inhibitory rate they were counted after 96 h incubation with each kind of oligonucleotide. Each test was performed in triplicate and repeated at least three times for each concentration of oligonucleotide. The growth medium, with or without oligonucleotides, was changed daily.

Anchorage independence

Anchorage independence was assayed by seeding cells in 0.3% SeaKem low melting point agarose (FMC Bioproducts, Rockland, ME) dissolved in RPMI media with or without oligonucleotides. Suspensions, containing 50 or 500 cells/ml, were overlaid on a 0.6% agarose basal layer in 60 mm culture dishes and incubated at 37°C for 14–21 days. Foci containing more than 100 cells were counted.

Western blot analysis

Cells were cultured in complete medium containing 20 μM oligonucleotides for 48 h. Cells were trypsinized, flash-frozen in liquid nitrogen and incubated in lysis buffer [0.01 M Tris-HCl (pH 7.5) 0.144 M NaCl, 0.5% NP-40, 0.5% SDS and 0.1% aprotinin (1×10^6 cells/20 ml buffer)] for 30 min on ice and then vortexed. The lysates were centrifuged at 10 000 *g* for 10 min and subjected to 6–12% SDS–PAGE at 20 mA for 2.5 h. Each well was loaded with the equivalent of approximately 20 μl of cell extract (10 μg of protein). The proteins were transferred for 12 h at 100 V in a Polyblot (American Bionetics, Hayward, CA). Immunoblot was performed using anti-Ki-*ras* monoclonal antibody (Oncogene Science) at a dilution of 1:1000. The blots were incubated overnight with the anti-Ki-*ras* antibody and then with alkaline phosphatase-conjugated secondary antibody at a dilution of 1:5000.

Stastical analysis

Differences in inhibition of cell growth and colony formation were evaluated using Student's *t*-test.

Results

Oligonucleotide uptake

Flow cytometer (FACS 400) analysis revealed that most of the cells were capable of taking up the oligonucleotides. After 2 h of exposure, about 70–80% of the cells showed high intensity. Fluorescence microscopy revealed nuclear and cytoplasmic staining (data not shown, see ref. 27), and its intensity gradually became weaker and disappeared after 4–5 h in each cell line. All cell types showed a similar time course and pattern of staining (Figure 2).

Growth rate

When plated at an initial density of 1×10^4 cells/ml, all cell lines grew to maximum density within 5–6 days. At all times examined the growth of all colon cancer cell lines was inhibited by the presence of the antisense oligonucleotides, but to a lesser degree in WI-38 cells and WEHI-3 cells. Random sequence oligonucleotides showed no effect on the growth of any cell lines. Respective growth inhibition of DLD-1, HCT-116, WEHI-3 and WI-38 cells is shown in Figure 3. The other colon cancer cell line, WiDr cells, showed similar results. Treatment with 10–40 μM antisense oligonucleotides inhibited the growth of colon cancer cells in a dose-dependent manner, but each oligomer showed only a slight effect on the proliferation of WI-38 cells and WEHI-3 cells. Antisense oligonucleotides did not show the growth inhibitory effect on COLO 201 cells, which is a colon cancer cell line without any Ki-*ras* mutation. Other control oligonucleotides,

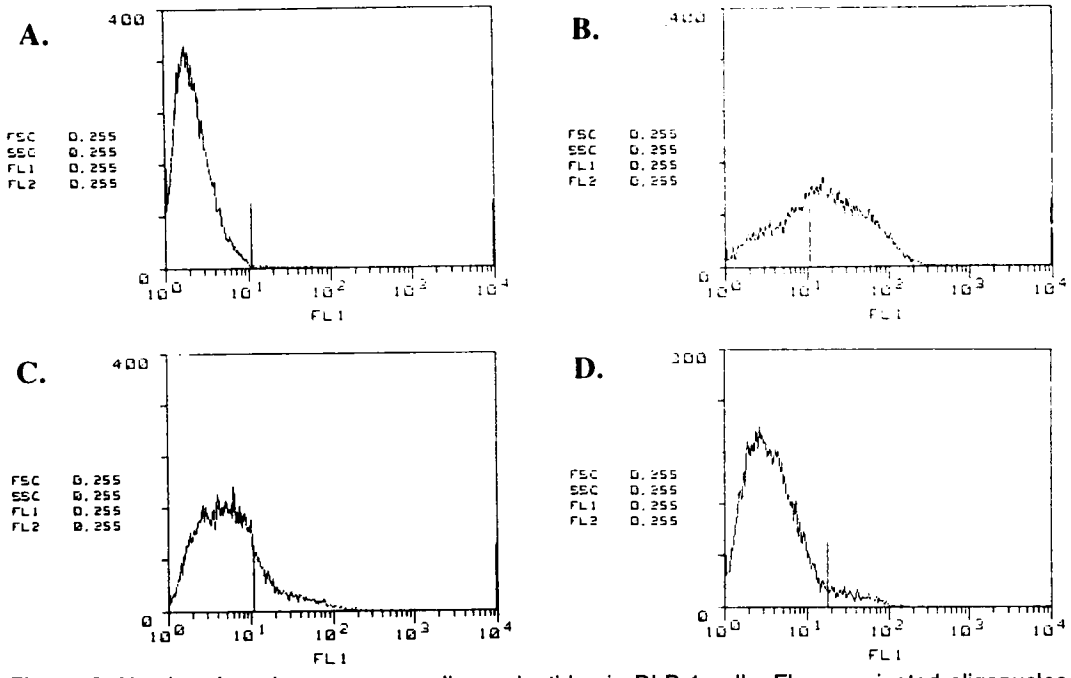

Figure 2. Uptake of random sequence oligonucleotides in DLD-1 cells. Fluoresceinated oligonucleotides were added to the culture, which was then analyzed by flow cytometry (FACS 400) after 0 min (A), 2 h (B), 3 h (C) and 4 h (D). All cell types showed a similar time course and pattern of staining.

scrambled, sense and mismatched oligomers, did not show any effect on cell growth (Figures 3 and 4).

When the composition of the antisense oligonucleotides was altered to make them complementary to a slightly 3′ region that spanned the start codon, differences were observed in the growth inhibitory effect. The inhibitory effect of three kinds of antisense oligonucleotides against DLD-1, HCT-116, SW1116, WiDr and WI-38 cells was compared. Antisense oligonucleotides starting at base 69 showed the strongest growth inhibition of each cell line in comparison with the other two kinds of antisense oligonucleotides starting at base 60 or starting at base 63. This antisense oligonucleotide (antisense 1) was used for all other experiments in this report. However, the difference in growth inhibition between antisense 1-treated and antisense 2- or antisense 3-treated cells was not statistically significant (Table 1).

Anchorage independence

The ability to form foci in soft agar was reduced by antisense oligonucleotide treatment in each colon cancer cell line but not COLO 201 cells ($p < 0.01$). Control oligonucleotides did not show any effect on colony formation (Table 2).

Effect of Ki-*ras*-specific antisense oligonucleotides on protein expression: Western blots

Immunoblots demonstrated a marked decrease in the level of Ki-*ras* protein after incubation with antisense oligonucleotides in DLD-1, HCT-116, SW1116 and WiDr cells (Figure 5, lanes 2, 5, 8 and 11), whereas no decrease was apparent in the cells exposed to scrambled oligonucleotides (Figure 5, lanes 1, 4, 7 and 10) or to PBS (Figure 5, lanes 3, 6, 9 and 12) (Figure 5, lanes 1–3: DLD-1; lanes 4–6: HCT-116; lanes 7–9: WiDr; lanes 10–12: SW1116). In WEHI-3, WI-38 and COLO 201 cells, endogenous Ki-*ras* protein was not detectable (Figure 5, lanes 13–15: COLO 201, lanes 16–18: WEHI-3; lanes 19–21: WI-38; lanes 13, 16 and 19: scrambled oligonucleotides; lanes 14, 17 and 20: antisense oligonucleotides; lanes 15, 18 and 21: PBS). Other control oligonucleotides, sense and mismatched oligomers, did not show any offset (data not shown).

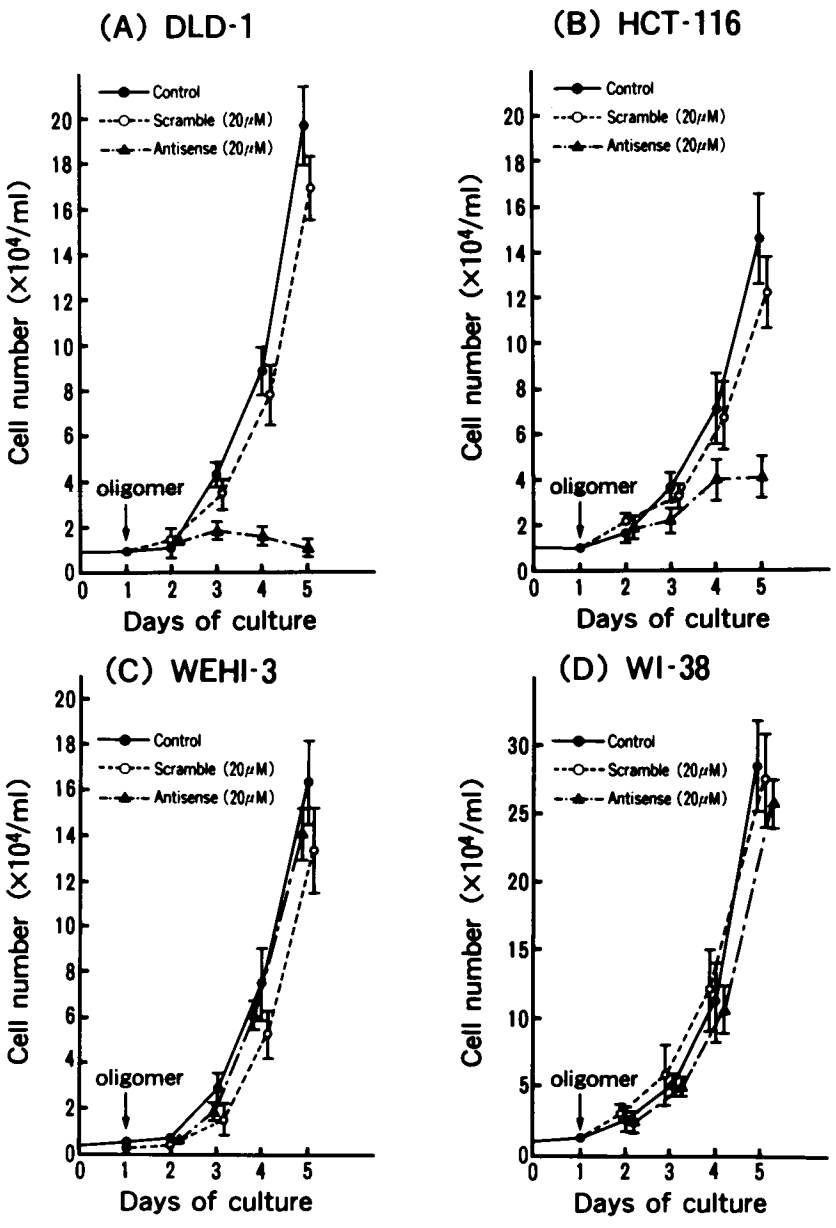

Figure 3. Growth curves of colon cancer cells and control cells. DLD-1, HCT-116, WEHI-3 and WI-38 cells were incubated with 20 μM antisense, scrambled oligonucleotides or PBS. Cells were seeded in growth medium on day 0 and incubated for 24 h. The cells were then incubated with 20 μM antisense or sense oligonucleotides or PBS in fresh medium. Medium with oligonucleotides was changed daily. The experiments were repeated three times. Results represent means ± SD from triplicate cultures. The difference between antisense-treated and control curves was statistically significant in DLD-1 and HCT-116 cells with Student's t-test ($p < 0.01$).

Discussion

One of the general approaches commonly explored for the treatment of colorectal cancer was a development of anticancer agents.[20,21] We have previously performed chemotherapy against colorectal cancers with many kinds of drug delivery systems and have obtained good results.[22,23] However, in some cases the anticancer agents were ineffective, probably because of multidrug resistance or low concentrations of drugs in the cancerous lesion. In this report we used antisense oligonucleotides and focused on the Ki-*ras* gene as a target for the treatment of colorectal cancer.

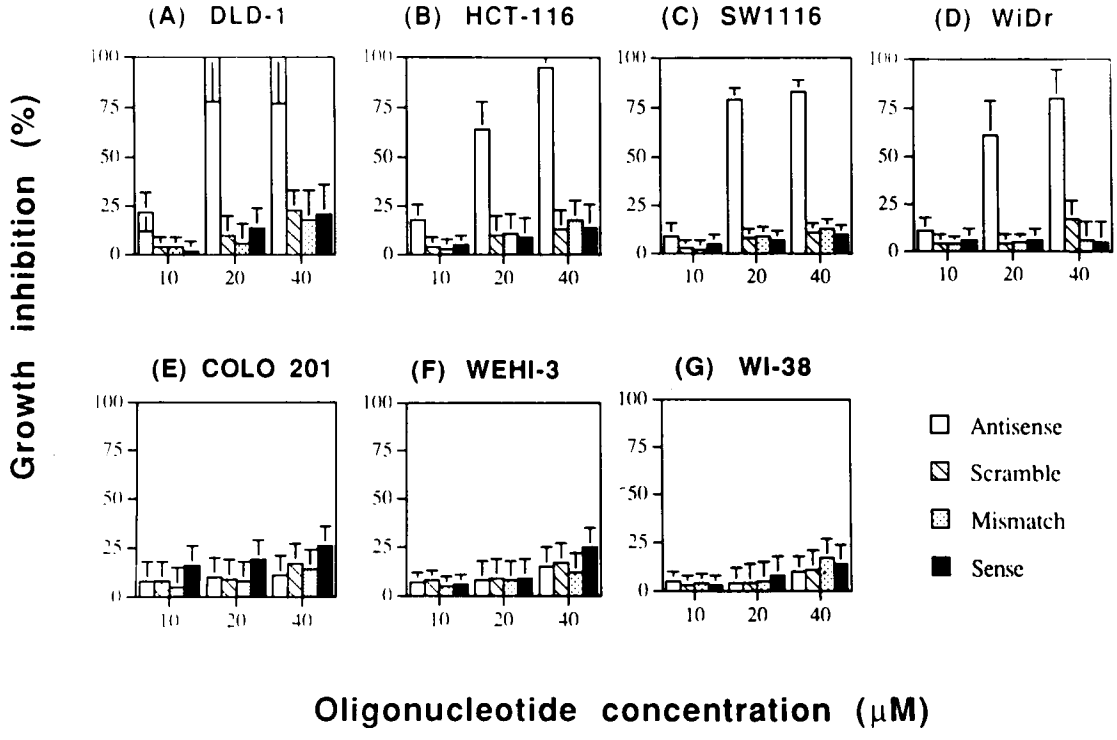

Figure 4. Concentration-dependence of cell growth by the antisense oligonucleotides. Percent inhibition was calculated with day 4 using the number of cells present in the control cultures incubated with PBS for comparison. Each point represents the mean ± SD of triplicate cultures. The experiments were repeated three times with similar results. The difference between antisense-treated (open bars) and scrambled oligonucleotide-treated (shaded bars, control) groups was statistically significant in the cell lines of DLD-1, HCT116, SW1116 and WiDr with Student's *t*-test ($p < 0.005$–0.01).

Antisense technology provides a valuable tool that can be used to interfere with the expression of specific genes. These modulators are complementary to specific mRNA sequences and frequently lead to modification of the phenotype of the cells.[24–26] We also have previously tested antisense oligonucleotides against several kinds of oncogenes and DNA replication factors, and obtained good results.[27,28] Previous reports show that H-*ras* and N-*ras* antisense oligonucleotides inhibited gene expression in a sequence-dependent manner,[29,30] and that Ki-*ras* antisense oligonucleotides inhibited the

Table 1. The inhibitory potency of the antisense oligonucleotides in colon cancer cell proliferation and its relation to a small shift in the targeted sequence of Ki-*ras* mRNA

First base of antisense oligonucleotides	Inhibition of cell growth (%)				
	DLD-1	HCT-116	SW1116	WiDr	WI-38
69 (antisense 1)	108.1 ± 8.9	63.3 ± 4.7	78.3 ± 4.2	59.3 ± 6.1	6.3 ± 2.1
63 (antisense 2)	75.6 ± 3.9	48.3 ± 1.9	43.9 ± 4.1	50.3 ± 5.5	5.3 ± 1.1
60 (antisense 3)	80.6 ± 4.3	58.3 ± 9.3	38.5 ± 4.5	48.2 ± 5.8	6.6 ± 1.4

Numbering is from sequenes of Kusewitt (GenBank accession no. z12125). Colon cancer cell line DLD-1, HCT-116, SW1116 and WiDr cells, and human fibroblast WI-38 cells were incubated with three kinds of 20 μM antisense oligonucleotides or PBS. Medium containing oligonucleotides was changed daily. After incubation for 72 h cell number was counted and percent inhibition of cell growth was calculated in comparison with non-treated cells. Results present means ± SD from triplicate cultures. Antisense 1 showed the strongest inhibition. The inhibitory potency of the antisense oligonucleotides was related to the small shift in the sequence targeted. However, the difference in growth inhibition between antisense 1-treated and antisense 2- or antisense 3-treated cell growth was not statistically significant.

Table 2. Effects of antisense oligonucleotides on focus formation by colon cancer cells in soft agar

Cell line	Antisense (20 μM)	Scrambled (20 μM)	Control (PBS)
DLD-1	12.3 ± 3.1	25.3 ± 3.6	28.6 ± 3.2
HCT 116	6.8 ± 2.2	24.2 ± 1.4	22.3 ± 2.8
SW 1116	14.2 ± 5.1	31.2 ± 4.8	35.3 ± 6.2
WiDr	5.8 ± 2.2	12.6 ± 3.3	14.4 ± 7.9
COLO 201	9.3 ± 1.2	9.0 ± 2.5	10.4 ± 3.5

Cells were seeded at 50 or 500 cells/ml into culture medium containing 0.3% agarose in the presence or absence of oligonucleotides. Suspensions were pipeted onto a basal layer of 0.6% agarose in 60 mm dishes and incubated for 14–21 days. Foci containing greater than 100 cells were counted. Results represent the means ± SD for triplicate cultures. The difference of colony number between antisense-treated and control groups in each cell line without COLO 201 was statistically significant with Student's *t*-test ($p < 0.01$).

growth of pancreas carcinoma,[31] but there is no report about Ki-*ras* antisense oligonucleotides against colorectal cancers.

The importance of *ras* protein in signal transduction pathways and carcinogenesis has been recently clarified.[5,15,32] Point mutations of the Ki-*ras* gene are found in 50% of human colorectal tumors as well as in other kinds of cancers.[33] The oncogenic form of *ras* proteins found in many cancer cells makes them work differently than normal *ras* proteins. GTPase activity inside the cell is greatly reduced and these proteins do not get switched off. Instead, they remain on for extended periods and flood the cell with continuous growth stimulatory signals.[34] It follows that inhibition of the growth signals for the Ki-*ras* gene with antisense oligonu-

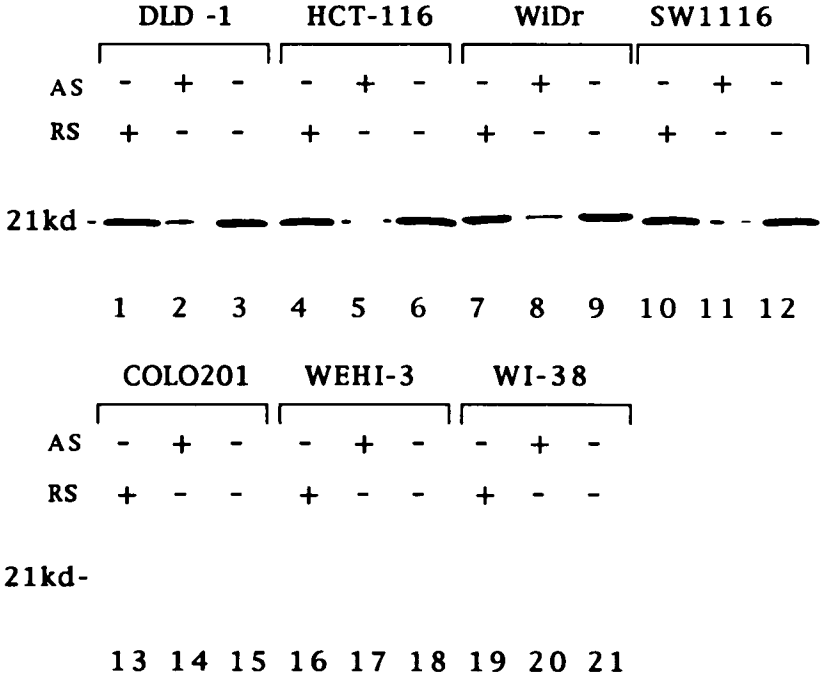

Figure 5. Western blot of oligonucleotide-treated colon cancer cells and control cells. Lanes 1–3: DLD-1; lanes 4–6: HCT-116; lanes 7–9: WiDr; lanes 10–12: SW1116; lanes 13–15: COLO201; lanes 16–18: WEHI-3; lanes 16–18: WI-38. Lanes 1, 4, 7, 10, 13, 16 and 19: scrambled oligonucleotides (20 μM); lanes 2, 5, 8, 11, 14, 17 and 20: antisense oligonucleotides (20 μM); lanes 3, 6, 9, 12, 15, 18 and 21: PBS. Cells (1×10^6) were treated with antisense or scrambled oligonucleotides or PBS for 48 h. Total protein (10 μg) was loaded to each lane. AS, antisense oligonucleotides; RS, random (equal to scrambled) oligonucleotides.

cleotides will be a potential way for the inhibition of colon cancer cell proliferation and the alteration of the cancer cell phenotype.

The growth rate of colon cancer cells with a Ki-*ras* mutation, DLD-1, HCT-116, SW1116 and WiDr cells, was inhibited by antisense oligonucleotides. However, these antisense oligonucleotides had no effect on the proliferation of normal hematopoietic cells WEHI-3, fibroblast WI-38 and colon cancer cell line COLO 201 without any Ki-*ras* mutation. As shown in Figure 5, expression of the Ki-*ras* gene in these cells was very low and could not be detected in Western blot analysis, so it follows that Ki-*ras*-specific antisense oligonucleotides will show a growth inhibitory effect on cancer cells with high Ki-*ras* gene expression or with a Ki-*ras* gene mutation.

The results of our investigations indicate that activated Ki-*ras* may be essential for the proliferation of colorectal cancer cells with a Ki-*ras* mutation. Although it is possible that an antisense strategy might ultimately be used *in vivo* to inhibit the proliferation of colon cancer cells as an anticancer agent, several problems needed to be solved. The first one is that although the oligonucleotides are avidly taken up into cells, some cells of each cell line do not take up oligonucleotides, and thus escape the antiproliferative effects of antisense oligonucleotides and continue to grow. According to FACS analysis as shown in Figure 2, the oligonucleotides were taken up by 70–80% of the cells within 2 h, and the intensity gradually became weaker and disappeared after 4–5 h in each cell line. So some cells fail to take up oligonucleotides and incorporated antisense oligonucleotides may be degraded in the cells. Thus it is important to deliver a large amount of antisense oligonucleotides to all cancer cells.

The seond problem is what part of the messenger RNA should be targeted by antisense oligonucleotides. Generally speaking, the 5′ portion of messenger RNA including the initiation site (AUG site) is often targeted in many cases.[26,30] However, in some cases the inhibitory effect of antisense oligonucleotides is related to the small shift in the targeted sequence.[27,35] As shown in Table 1, we used three different kinds of antisense oligonucleotides and all three oligomers showed growth inhibition. However, its effect was a little bit different and antisense 1 (starting at base 69) showed the strongest effect. According to the previous study by Chiang *et al.*, the antisense-mediated effect depends on the secondary structure of the targeted messenger RNA and a stable stem–loop structure is desirable as a targeted site.[36] So it is necessary to predict the secondary structure of the targeted messenger RNA and to use several different kinds of oligonucleotides for assays and to choose the most effective targeted sequence.

We conclude that gene targeted anticancer therapy could be effectively used *in vivo* specifically for colon cancer with Ki-*ras* expression. Others have previously proposed that the inhibition of cancer cells by antisense nucleic acids has important implications for the development of new cancer therapy,[37] and it is reported that oligonucleotides can be administered for treatment for cancer and vascular stenosis without any specfic toxicity in animal experiments.[38–41] Further studies will be required to determine the effectiveness *in vivo* as well as clarifying the mechanism of action.

References

1. Vogelstein B, Fearon ER, Hamilton SR, *et al.* Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988; **319**: 525–32.
2. Bos JL. *ras* Oncogenes in human cancer. *Cancer Res* 1989; **49**: 4682–9.
3. Burmer GC, Rabinovitch PS, Loeb LA. Analysis of c-ki-*ras* mutations in human colon carcinoma by cell sorting, polymerase chain reaction, and DNA sequencing. *Cancer Res* 1989; **49**: 2141–6.
4. Burmer GC, Loeb LA. Mutations in the KRAS2 oncogenes during progressive stages of human colon carcinoma. *Proc Natl Acad Sci USA* 1989; **86**: 2403–7.
5. Bos JL, Fearon ER, Hamilton SR, *et al.* Prevalence of *ras* gene mutations in human colorectal cancers. *Nature* 1987; **327**: 293–9.
6. Baker SJ, Fearon BE, Nigro JM, *et al.* Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* 1989; **244**: 217–21.
7. Shirasawa S, Urabe K, Yangagawa Y, *et al.* p53 mutations in colorectal tumors from patients with familial poliposis coli. *Cancer Res* 1991; **51**: 2874–8.
8. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759–67.
9. Kinzler KW, Nilbert MC, Vogelstein B, *et al.* Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. *Science* 1991; **251**: 1366–70.
10. Gorgen J, Thliveri A, Samowitz W, *et al.* Identification and characterization of the familial adenomatous polyposis coli. *Cell* 1991; **66**: 589–600.
11. Seemayer TA, Cavenee WK. Biology of disease. Molecular mechanism of oncogenesis. *Lab Invest* 1989; **60**: 85–99.
12. Land H, Parada LF, Weinburg RA. Cellular oncogenes and multi-step carcinogenesis. *Science* 1983; **222**: 771–8.
13. Tabin CJ, Bradley SM, Bargmann CI. Mechanism of activation of a human oncogene. *Nature* 1982; **300**: 143–9.
14. Sistonen IL, Alitalo K. Activation of c-*ras* oncogenes by mutations and amplification. *Ann Clin Res* 1986; **18**: 297–303.
15. Smith TR, Degudicibus ST, Stacey DW. Requirement for

c-*ras* proteins during viral oncogene transformation. *Nature* 1986; **320**: 540–3.
16. Almoguera C., Schbata D, Forfester K. Most human cancinomas of the exocrine pancreas contain mutant c-K-*ras* genes. *Cell* 1988; **53**: 549–54.
17. Smit V, Boot A, Smith A. K-*ras* codon 12 mutations occur very frequently in pancreatic adenocarcinomas. *Nuclei Acids Res* 1988; **16**: 7773–82.
18. Murakami, A., Tamura Y, Ide H, *et al. Chromatogr* 1993; **648**: 157–63.
19. Wachter L, Jabronski JA, Ramachandran KL, 1986. A simple and efficient procedure for the synthesis of 5′-aminoalkyl oligonucleotides. *Nucleic Acids Res* 1986; **14**: 7985–94.
20. Scheitauer W, Rosen H, Schiessel R, *et al.* Treatment of patients with advanced colorectal cancer with cisplatin, 5-fluorouracil, and leucovolin. *Cancer* 1991; **67**: 1294–8.
21. Lopez M. Adjuvant therapy of colorectal cancer. *Dis Colon Rectum* 1994; **37**: 86–91.
22. Takahashi T, Yamaguchi T, Kitamura K, *et al.* Clinical application of monoclonal antibody–drug conjugates for immunotargeting chemotherapy of colorectal carcinoma. *Cancer* 1988; **61**: 881–8.
23. Takahashi T, Yamaguchi T, Kitamura K, *et al.* Follow up study patients treated with monoclonal antibody drug conjugate: report of 77 cases with colorectal cancer. *Jpn Cancer Res* 1993; **84**: 976–81.
24. Helene C, Toulme JJ. Specific regulation of gene expression by antisense, sense and antigene nucleic acis. *Biochim Biophys Acta* 1990; **1049**: 99–125.
25. Szczylik C, Skorski T, Nicolaides NC, *et al.* Selective inhibition of leukemia cell proliferation by BCR-ABL antisense oligonucleotides. *Science* 1991; **23**: 562–5.
26. Steele C, Cowser LM, Shillitoe EJ. Effects of human papilloma type 18-specific antisense oligonucleotides on the transformed phenotype of human carcinoma cell lines. *Cancer Res* 1993; **53**: 2330–7.
27. Sakakura C, Hagiwara A, Tsujimoto H, *et al.* Inhibition of gastric cancer cell proliferation by antisense oligonucleotides targeting the messenger RNA encoding proliferating cell nuclear antigen. *Br J Cancer* 1994; **70**: 1060–6.
28. Sakakura C, Yamaguchi-Iwai Y, Satake M, *et al.* Growth inhibition and induction of differentiation of *t*(8;21) acute myeloid leukemia cells by the DNA binding domain of PEBP2 and the AML1/MTG8(ETO) specific antisense oligonucleotide. *Proc Natl Acad Sci USA* 1994; **91**: 11723–7.
29. Monia BP, Jonston JF, Ecker DJ, *et al.* Selective inhibition of mutant Ha-*ras* mRNA expression by antisense oligonuleotides. *J Biol Chem* 1992; **267**: 19954–62.
30. Skorski AM, Szczylik C, Ratajczac MZ, *et al.* Growth factor-dependent inhibition of normal hematopoiesis by N-*ras* antisense oligonucleotides. *J Biol Chem* 1992; **175**: 743–50.
31. Liu TH. The inhibitory effect of antisense oligonucleotides on the growth of human pancreatic adenocarcinoma cell lines. *Chung-Hua-Ping-Li-Hsueh-Tsa-Chih* 1993; **22**: 25–9.
32. Egan SE, Weinberg RA. The pathway to signal achievement. *Nature* 1993; **365**: 781–3.
33. Fearon ER, Cho KR, Nigro JM, *et al.* Identification of a chromosomal 18q gene that is altered in colorectal cancers. *Science* 1990; **247**: 49–55.
34. McCormick F. *ras* GTPase activating protein: signal transmitter and signal terminator. *Cell* 1989; **56**: 5–8.
35. Edith Speir BS, Stephen EE. Inhibition of smooth muscle cell proliferation by an antisense oligonucleotides targeting the messenger RNA encoding proliferating cell nuclear antigen. *Circulation* 1992; **86**: 538–47.
36. Chiang M-Y, Chan H, Zounes MA, *et al.* Antisense oligonucleotides inhibit intracellular adhesion molecule 1 expression by two distinct mechanisms. *J Biol Chem* 1991; **266**: 18162–71.
37. Calabretta B. Inhibition of protooencogene expression by antisense oligonucleotides: biological and therapeutic implications. *Cancer Res* 1991; **51**: 4505–10.
38. Agrawal S, Temsamani J, Tang JY. Pharmacokinetics, biodistribution, and stability of oligonucleotide phospholotioate in mice. *Proc Natl Acad Sci USA* 1991; **88**: 7595–9.
39. Ratajczak MZ, Kant JA, Lunger SM, *et al. In vivo* treatment of human leukemia in scid mouse model with c-*myb* antisense oligonucleotides. *Proc Natl Acad Sci USA* 1992; **89**: 11823–7.
40. Skorski T, Nieborowska-Skorska M, Nicolaides NC, *et al.* Suppression of Philadelphia leukemia cell growth in mice by BCR-ABL antisense oligonucleotide. *Proc Natl Acad Sci USA* 1994; **91**: 4504–8.
41. Shi Y, Hutchinson HG, Hall DJ, *et al.* Downregulation of c-*myc* expression by antisense oligonucleotides inhibits proliferation of human smooth muscle cells. *Circulation* 1993; **88**: 1190–5.

(Received 13 March 1995; received in revised form 25 April 1995; accepted 17 May 1995)