Antisense treatment directed against mutated Ki-ras in human colorectal adenocarcinoma

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Abstract

Background—Ki-ras (Ki-ras) mutations are common in gastrointestinal cancer and one codon 12 mutation, glycine to valine, is particularly aggressive in colorectal cancer.

Aims—To investigate if this valine point mutation could be targeted with antisense oligonucleotides and to determine the efficacy of any antisense/mRNA interaction.

Methods—Twenty nine antisense oligonucleotides were screened against target and control Ki-ras RNA in a cell free system and against target and control cell lines in culture.

Results—The activity and specificity of the oligonucleotides varied. Results for the individual oligonucleotides were consistent in a cell free model and in cell culture using two different uptake promoters. Only one oligonucleotide was specific in its cleavage of target Ki-ras mRNA in the cell free system and appeared specific in cell culture, although changes in Ki-ras mRNA and protein expression following a single treatment could not be detected. Experiments in the cell free system showed that the point mutation is relatively inaccessible to oligonucleotides. Other sites on the Ki-ras RNA molecule, away from the point mutation, can be targeted more effectively.

Conclusions—Successful targeting of the clinically relevant Ki-ras point mutation with antisense oligonucleotides is difficult because of RNA structure at the mutated site and is inefficient compared with other sites on the Ki-ras mRNA.

Keywords: Ki-ras mutation; antisense treatment; colorectal carcinoma

Colorectal cancer carries a poor prognosis and improvement in survival rates over the past 40 years has been disappointing. Progress in molecular genetics has demonstrated that colorectal cancer develops because of the accumulation of genetic abnormalities. These genetic changes offer potential as markers of disease for earlier diagnosis, as prognostic indicators, and as therapeutic targets. In a multicentre study of 2721 patients with colorectal cancer, we demonstrated that one of these characteristic genetic defects, the presence of a valine mutation on codon 12 of the Kirsten ras (Ki-ras) gene, increased the risk of death by 43% and was associated with poorer outcome than other Ki-ras mutations.

Antisense oligonucleotide therapy aims to target expression of a single protein that is critical for the progression of a specific cancer and relies on the introduction of synthetic oligonucleotides into cells where hybridisation—binding—to specific complementary target mRNA by Watson-Crick base pairing can occur. Such hybridisation can inhibit protein expression through a number of mechanisms, one of which is activation of RNase H, an ubiquitous intracellular enzyme. RNase H cleaves antisense/RNA duplexes destroying the RNA but leaving the antisense oligonucleotide free to bind to further complementary mRNA. If the antisense target is unique to the cancer and absent in normal cells, it is hoped that the antisense molecule will have no activity in normal cells. Preliminary clinical studies have demonstrated that treatment with antisense can be a safe relatively non-toxic strategy and there is already evidence of efficacy.

Antisense oligonucleotide design is critical for efficacy as the base pairing which maintains the secondary and tertiary structure of the target RNA may prevent hybridisation of some antisense molecules. The effect of RNA structure on oligonucleotide binding is difficult to predict and small differences in oligonucleotide structure can lead to large differences in hybridisation efficiency. Therefore, for each target RNA, large numbers of oligonucleotides need to be screened to identify which is most efficacious. Alternatively, libraries of random oligonucleotides can identify the most accessible regions of RNA.

There are no studies that have systematically investigated the efficacy of antisense treatment directed at a clinically relevant point mutation in gastrointestinal cancer. We investigated if one specific Ki-ras point mutation associated with a poor outcome could be targeted with antisense oligonucleotides. Our aim was to screen a series of antisense oligonucleotides to identify an active molecule with a bona fide antisense mechanism of action.

Methods

CELL LINES

SW480 human primary colorectal adenocarcinoma cells, with a homozygous mutation at codon 12 (glycine to valine: GGT→GTT) were used as the target cell culture model. Two other human colorectal carcinoma cell lines, HT29 (wild-type Ki-ras alleles at codon 12/13—mutated codon 61) and LoVo (heterozygous codon 13 GGC→GAC aspartate muta-
Antisense to Ki-ras

Codon 231

RNase H at 37°C transcripts incubated with RNase T1, T2, and mapped by comparison with 3' end labelled RNA molecules. RNase H cut sites were these conditions were obtained by titration of oligonucleotides and 0.13 units/reaction for accessible sites after incubation with 2.5 µg of DNA sequences (1–1000 nM) over a 30 minute time course. It was also used to screen for accessible sites after incubation with 2.5 µg of a heat quenched library of random sequence oligonucleotides and 0.13 units/reaction E. coli RNase H at 37°C for five and 15 minutes. These conditions were obtained by titration of oligonucleotide and RNase H concentration and time of incubation to ensure a single RNase H cut occurred approximately every 10 RNA molecules. RNase H cut sites were mapped by comparison with 3' end labelled transcripts incubated with RNase T1, T2, and B. cereus (data not shown). This eliminated the risk of secondary cuts following structural changes induced by primary cuts. (Full details will be published elsewhere, Ross et al in preparation.)

Antisense Treatment of Cell Lines

The bacterial toxin streptolysin O was used to permeabilise cells in monolayer culture by amending a protocol for cells in suspension. Conditions allowing temporary permeabilisation of cell membranes by streptolysin O without compromising long term cell viability were optimised using an MTT assay and by coinoculation with propidium iodide. Cells were seeded on day 0 to achieve 80% confluency at harvest. On day 1, media was removed and cells were washed twice with Dulbecco’s modified eagle medium. Each well was treated with a 15 µl solution containing 10 µM antisense oligonucleotide, and 7.5 units/ml streptolysin O reactivated at 4°C for one hour with 5 mM dithiothreitol and the volume made up with Dulbecco’s modified eagle medium. Cells assayed as controls were treated with an equivalent volume of media or control oligonucleotide. Cells were incubated for 30 minutes at 37°C, washed twice, and then serum containing media was replaced.

Cationic lipid transfection was used as an alternative to streptolysin permeabilisation. A screen of the Pfx lipid series (Invitrogen) identified Pfx-3 (1:1 mixture of a proprietary cationic lipid and DOPE at 2 mg/ml) as the optimal lipid for transfection of SW480 cells. Cells were seeded on day 0 as described above, on day 1 a solution of Pfx-3 24 µg/ml was made up in serum free OptiMEM medium (Gibco Life Technologies, Paisley, UK) with 1-glutamine 2 mM and CaCl2 100 mg/l. A 400 nM solution of oligonucleotide was also prepared in OptiMEM. Equal volumes of the two solutions were mixed and incubated at room temperature while the cells were washed twice in OptiMEM. Transfection solution was added to each well. Cells were incubated for four hours at 37°C, the transfection solution was removed, and serum containing medium replaced. Uptake of FITC labelled oligonucleotide was detected at four hours in approximately 80% of lipid treated cells. This effect persisted for at least 24 hours. There was almost no detectable fluorescence in cells treated without lipid (data not shown).

Cell Viability and Proliferation Assays

Viability was assayed by an MTT reaction and proliferation was measured by 3H thymidine incorporation. 3H thymidine was diluted in sterile phosphate buffered saline at 0.1 mCi/ml and 10 µl were added to each treated microwell 24 hours before the cells were harvested. The cells were washed with fresh media, trypsinised, and trapped on filter mats (Skantron) using an Inotech cell harvester. Filters were fixed in 100% ethanol, dried, and 3H thymidine incorporation was measured on an Inotech plate reader in a 90% argon/10% methane gas stream.

Analysis of Ki-ras Expression

mRNA

Single stranded high specific activity antisense RNA probes for human GAPDH (exons 5–8) and Ki-ras (exons 1–3) were prepared by in vitro transcription in the presence of [α-32P]UTP. RNase protection analysis of mRNA was performed directly in cell lysates, 5x105 cells assay, using a Direct Protect assay (Ambion, USA). Protected fragments were separated by electrophoresis on denaturing 8% polyacrylamide/7 M urea gels and detected by direct autoradiography.
Protein

Cells were recovered, washed twice, and resuspended at 10⁷/ml in ice cold lysis buffer (10 ml: 50 mM Tris HCl, 150 mM NaCl, pH 7.5, 1% (v/v) Nonindet P-40, 1% (w/v) sodium dodecyl sulphate, ×1 protease inhibitors; Complete mini tablets, Boehringer, Mannheim, Germany) passed through a 25 gauge needle and left on ice for 20 minutes. The suspension was centrifuged at 16 000 g at 4°C for 20 minutes. The supernatant was removed, a 25 µl aliquot was used for protein estimation, and the rest was stored at −70°C. Protein concentration was determined by Lowry assay (BioRad).

OLIGONUCLEOTIDES

Twenty nine different 13–17mer oligonucleotides (and their reverse sequence controls) complementary to a region encompassing the codon 12 glycine to valine Ki-ras point mutation were synthesised (Genosys, Cambridge, UK) and purified by high pressure liquid chromatography (fig 1). Oligonucleotides larger than a 17mer are not efficient at discriminating point mutations. To minimise the potential non-specific toxicity observed with phosphorothioate oligonucleotides, we initially used oligonucleotides with a phosphodiester backbone and two terminal phosphorothioate linkages at the 3' and 5' termini. Several oligonucleotides were also synthesised as full phosphorothioates. The 17mer randomer oligonucleotides for cell free studies were synthesised with a pure phosphodiester backbone. The antisense oligonucleotide PR4 was a phosphorothioated 17mer targeted against nucleotides 373–409.

Results

ANTISENSE TO K_i-ras mRNA IN A CELL FREE ASSAY

To determine if antisense oligonucleotides could show specificity for a point mutation, each oligonucleotide was incubated with RNase H and a 32P end labelled RNA corresponding to exon 1 of Ki-ras mRNA. This RNA either contained the target codon 12 valine mutation, a control codon 13 mutation, or a control wild-type sequence. Incubation of oligonucleotides, the RNA target sequence, and RNase H resulted in up to five cleavage products although some oligonucleotides were inactive (fig 2A, B). The intensity of individual cleavage products varied. However, the relative intensity, size, and number of products were reproducible. All the 13mers and 14mers were inactive, the 15mers showed poor activity while the 16mers induced increased cutting (fig 2A). However, the 17mers were the most active (fig 2B). It was apparent that the oligonucleotides skewed 5' to the mutation were the most active. Those skewed 3' were inactive. Comparison with RNase T1 sequencing lanes suggested that the products with oligonucleotide 17.2 in the SW480 lane represented RNase H cleavage around the mutation site (data not shown). Incubation with a control RNA carrying the aspartate 13 mutation resulted in the detection of cleavage products with oligonucleotides 17.1, and 17.3–17.5, and with the wild-type RNA also resulted in some cleavage except with 17.2. Thus oligonucleotide 17.2 appeared to be highly specific at inducing RNase H cleavage of the target RNA.

ANTISENSE TO K_i-ras mRNA IN CELL CULTURE

To compare the efficacy of the oligonucleotide series in the cell lines we used a direct permea-

Figure 1 The Ki-ras wild-type (HT29), mutated control (LoVo), and target (SW480) sequences, and the 29 different designs of oligonucleotides tested.
bilisation approach which was equally efficient in all three lines (data not shown). Viability, assessed using the MTT assay of mitochondrial activity, was not affected in any consistent way at one, three, five, or seven days in either SW480 or LoVo cells (data not shown) when cells were treated with a single dose of streptolysin O and each of the 29 antisense oligonucleotides or streptolysin O alone. Therefore, the effect of treatment was examined further using a proliferation assay.

For each antisense design, a greater reduction in thymidine incorporation at 24 hours was seen in SW480 cells than in the two control cell lines compared with untreated controls from the same line. Some oligonucleotides seemed to have greater specificity and their impact on 3H thymidine incorporation varied between cell lines. Figure 3 illustrates data from the 17mer series of oligonucleotides. The greatest specificity and efficacy was seen with oligonucleotide 17.2 which induced a 32.2% (SEM 0.06) reduction in thymidine incorporation in SW480 cells after a single dose but had minimal effects on the HT29 (wild-type) and LoVo (aspartate 13) control cell lines.

Data obtained with streptolysin O permeabilisation were followed up in SW480 cells using a cationic lipid to compare the 17mer antisense oligonucleotides with their control reverse sequence oligonucleotides. The three cell lines required different lipid formulations to achieve efficient transfection of DNA and therefore direct comparisons between cell lines using lipids could not be made.

When 3H thymidine incorporation was measured in cells treated with antisense or reverse sequence, results similar to those using streptolysin O were obtained (fig 4). Oligonucleotide 17.2 again seemed to be the most effective compared with its reverse sequence.

Figure 2 (A) The 13–16mer oligonucleotides incubated with the 3’ end labelled exon 1 RNA and E coli RNase H. A reverse sequence oligonucleotide to 17.2 (scr) was included as a control for background degradation of the target RNA. (B) The 17mer oligonucleotides incubated with the 3’ end labelled exon 1 RNA and E coli RNase H. A reverse sequence oligonucleotide to 17.2 (scr) was included as a control for background degradation of the target RNA.
and the other antisense designs. No activity was detected if 17.2 was added to media without enhanced uptake by permeabilisation or transfection (data not shown).

**Ki-ras EXPRESSION FOLLOWING ANTISENSE TREATMENT IN CELLS**

To confirm the specificity of the antisense effect seen with 17.2 in all of our systems, we examined Ki-ras mRNA and protein levels following antisense treatment. Changes in Ki-ras mRNA levels have been reported to occur 24 hours after antisense treatment. However, we were unable to detect changes in Ki-ras mRNA levels in SW480 cells 24 hours after treatment with the 17mer oligonucleotides transfected into cells using cationic lipids (fig 5A). One possible explanation for the absence of effect on Ki-ras mRNA expression was instability of the chimeric oligonucleotide. However, this was discounted as a fully phosphorothioated version of 17.2 was equally ineffective at reducing Ki-ras mRNA expression (fig 5B). In addition, we found that an active Ki-ras antisense that recognises wild-type Ki-ras could reduce Ki-ras expression effectively and specifically at 24 hours, demonstrating that our model system was capable of producing and detecting antisense effects (fig 5C).

An alternative explanation was that the effects we detected were due to steric blocking of the translation of Ki-ras mRNA, which would reduce protein levels without altering mRNA levels. However, no changes in Ki-ras protein expression were detected in any cells assayed at 8, 16, 24, 36, or 48 hours following oligonucleotide treatment (fig 6). It is also unlikely that changes in Ki-ras protein levels occurred earlier than eight hours and were missed. The half life of Ki-ras protein is not known. However, when cycloheximide, a general protein synthesis inhibitor, was added (50 µg/ml) to SW480 cells in culture and Ki-ras protein levels were assayed at various time points up to 54 hours, detectable Ki-ras protein did not disappear until 30 hours later (data not shown). It is likely that cycloheximide arrest of protein synthesis is more abrupt and possibly more complete than would be seen with the best antisense therapy. Therefore, it would seem reasonable that if an antisense effect is occurring, it will be seen at least 30 hours after treatment.

**SCREENING OF Ki-ras mRNA FOR SITES ACCESSIBLE TO OLIGONUCLEOTIDES AND RNase H**

These observations implied that the point mutated target site on Ki-ras mRNA was not easily accessible, thus perhaps explaining the modest effect on proliferation seen and lack of detectable effect on mRNA and protein expression. We noted that the best oligonucleotide (17.2) required an approximate 500-fold excess of oligonucleotide to achieve partial cleavage of the target exon 1 substrate RNA. Computer mapping suggests that codon 12 of Ki-ras lies in a structured base paired region of the mRNA (fig 7). The glycine to valine point mutation has a minimal effect on this structure, only slightly weakening the base pairing.
Antisense to Ki-ras

Discussion

In this study, we screened the efficacy of a large number of antisense molecules at inducing RNase H cleavage of a clinically important target, a point mutated Ki-ras mRNA. Different molecular structures were designed by changing the oligonucleotide length and skewing the sequence complementary to the point mutation in either direction away from the centre.

Our most effective oligonucleotide needed to penetrate the cell membrane to obtain efficacy and was completely inactive when added directly to the tissue culture media in the absence of cell permeabilisation or cationic lipid. In the presence of both uptake promoters, the oligonucleotides gave similar results. However, we saw no effect on cell viability, either in target or control cells, although we did see a consistent although modest effect on cell proliferation using 3H thymidine incorporation assays.

It has been suggested that oligonucleotides which have 3' or 5' terminal thymidines may interfere with 'H thymidine incorporation and hence give misleading results.31 We saw no evidence for this. Indeed, the most effective oligonucleotide, 17.2, had only one thymidine located six nucleotides from the 3' end. In addition, antisense and reverse sequence control oligonucleotides with the same base composition did not produce equivalent effects and the results with 3H thymidine incorporation in cell culture closely matched the results obtained in the cell free system.

Failure to demonstrate change in protein or RNA expression could be because the capped oligonucleotides were insufficiently stable to maintain an effect on RNA or protein. However, data from elsewhere suggest that the stability of end capped oligonucleotides in culture is not a significant issue32 and we still saw no effect on mRNA expression when a fully phosphorothioated 17.2 was used in cell culture. Our model system was capable of detecting antisense effects as an oligonucleotide designed to target a more accessible site completely inhibited Ki-ras expression. This suggested that the mutation site was not easily targeted and that the modest effects on prolif-

Figure 9 Comparing the activity of 17.2 and oligonucleotide PR4—which targeted one of the preferentially digested sites—in the cell free assay against the exons 1–3 Ki-ras mRNA containing the valine 12 point mutation. At a single time point, at 30 minutes, taken from the kinetic analysis, 17.2 required 100 times more oligonucleotide than PR4 to achieve a similar rate of cutting.

Table 1 Product cleaved (%)

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sw480 - gly12 to val12

HT29 - wild-type gly12 and gly13

Figure 7 Computer predicted secondary structure of oligonucleotides 190–330 of the Ki-ras mRNA (exon 1' nucleotides 182–203). The bold uppercase indicates sites mutated in the cell lines used in this study. The bold lowercase case indicated on the SW480 sequence indicates the sequence targeted by oligonucleotide 17.1 and the italic lowercase case that targeted by oligonucleotide 17.7.

Figure 8 A 3' end labelled RNA encompassing exons 1–3 was incubated with a random library of 17mer oligonucleotides and E. coli RNase H. A number of sites were preferentially digested but this did not include the point mutation at codon 12. 1. Marker lane, RNA+RNase T1 for 15 minutes at 68°C. 2. RNA+oligonucleotide library+RNase H for 15 minutes or five minutes (3). 4. The labelled RNA incubated in the absence of RNase H for 15 minutes or five minutes (5).

pairing and opening a small loop within this region.

However, computer predictions of RNA structure can be unreliable and therefore the accessibility of the structure around the point mutation to oligonucleotides and RNase H was investigated using a cell free screen. An end labelled RNA corresponding to the first three exons of the Ki-ras mRNA was incubated with a random library of 17mer oligonucleotides and RNase H. The random library of oligonucleotides contained a representation of all possible 17mer sequences. This assay therefore allows only 17mers complementary to the most accessible regions of the RNA to bind and activate RNase H. A parallel incubation with sequence specific RNases allowed precise positioning of cut sites induced by the randomer library. A number of sites were preferentially digested, although the codon 12 point mutation was not one of them (fig 8). This suggested that the target point mutation was not easily accessible either to oligonucleotides or RNase H.

We compared the activity of 17.2 and oligonucleotide PR4—which targeted one of the preferentially digested sites—in the cell free assay against the exons 1–3 Ki-ras mRNA containing the valine 12 point mutation. At a single time point taken from the kinetic analysis, it was clear that 17.2 was far less effective than PR4, requiring 100 times more oligonucleotide to achieve a similar rate of cutting (fig 9).
eration were caused by changes in mRNA and protein levels below the threshold of detection of western blotting or RNase protection. This seems the most likely explanation, particularly in view of the fact that in a comparison of this oligonucleotide to 17.2 in a cell free assay it was far more effective at inducing RNase H cleavage of Ki-ras mRNA. Other less likely explanations include transient early reduction in protein expression which was missed, effects resulting from RNase H mediated antisense actions against other mRNAs through partial complementarity with the Ki-ras antisense oligonucleotides, or antiproliferative effects generated through a non-antisense mechanism.

All of these data support the contention that the RNA structure of the target region can influence oligonucleotide efficacy, as in our models the oligonucleotides skewed 3' to the mutation were particularly ineffective whereas the most effective oligonucleotide was skewed 5' to the point mutation. When hybridising to the RNA, the more active oligonucleotides bound sites encompassing the stem loop carrying the point mutation, an adjacent stem loop, and the junction between these structures. In contrast, the most inactive molecules were restricted to the stem loop carrying the point mutation.

There is no doubt that targeting a point mutation is possible.24 25–29 No previous study has attempted to target a Ki-ras mutation systematically, although two studies have used two oligonucleotides chosen at random to target Ki-ras mutations and their results add weight to our findings. One used a 17mer antisense to a mutation in Ki-ras protein expression, which was missed, e 31 cleavage of Ki-ras mRNA. Other less likely mutations in view of the fact that in a comparison of this Ki-ras mRNA.

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