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Inhibition of Gastric Cancer Cell Cycle Progression by γ-Tubulin Antisense Oligonucleotides

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Abstract γ-Tubulin is an essential component involved in microtubule nucleation. The present work examined whether the fast proliferation of cancer cells can be retarded by the depletion of γ-tubulin expression. Two different gastric cancer cell lines and one control cell line were treated with antisense oligonucleotides complementary to the messenger RNA of γ-tubulin. The [^{3}H]-thymidine incorporation in the two gastric cancer cell lines, SNU-1 and SNU-216, was dramatically reduced by treatment with the γ-tubulin antisense oligonucleotides in a dosage-dependent manner. In contrast, the control cell line, NIH/3T3, showed no significant effect from the antisense oligonucleotides even at a high concentration. The ablation of γ-tubulin expression in the tumor cells resulted in an altered DNA synthesis during mitosis and it decreased the cell progression. Accordingly, the use of antisense oligonucleotides may be an effective way of inhibiting the proliferation of human gastric cancers.

Key words: γ-Tubulin, antisense oligonucleotide, cancer cell, proliferation

Microtubules are a critical organelle in eukaryotic cells and are involved in many cellular processes, including morphogenic events to maintain shape and polarity [20], intracellular organelle transport [15], and, especially, chromosome migration during mitosis [12]. Previous reports indicate that the dynamics of microtubules are essential for chromosome movement during mitosis, which involves dynamic interactions between the kinetochores of the chromosomes and the spindle microtubules [21]. Therefore, microtubules have been considered as the principal target of antimitotic compounds. For cancer therapy, a number of reagents have been applied to suppress the proliferation of cancer cells. However, only a few reagents are able to contribute nonspecifically to tumor cells. Hence, an anticancer drug should have a general cytotoxic effect against tumor cells [2]. For instance, taxol, which increases microtubule polymerization, inhibits mitosis in the prometaphase [25]. On the other hand, urushiol affected its profound cytotoxicity by triggering apoptosis in ovarian cancer cells [1]. The most potent mechanism of antimitotic drugs is the suppression of spindle microtubule dynamics rather than depolymerization or the excessive polymerizaion of spindle microtubules [13]. Many previous studies have reported that the assembly of mitotic spindle microtubules can be nucleated by the presence of γ-tubulin [11, 17, 23]. The microtubule-nucleating capacity of the animal cell centrosome requires a ring-shaped complex of proteins associated with γ-tubulin. Mutations in a microtubule-associated protein found at the poles of mitotic spindles result in abnormal spindle morphology, leading to mitotic arrest or to a loss of ability to restore microtubule-organizing center activity [3]. These studies suggest that the depletion of γ-tubulin can also result in the inhibition of cell mitosis through an appreciable change in microtubule dynamics.

Antisense oligonucleotides have became efficient agents, not only as molecular biological tools in understanding the role of proteins in a cell, but also as target-selective drugs that can modulate specific gene expression. There are several examples in previous literature where antisense oligonucleotides targeted at the open reading frame of many genes were used to inhibit tumor cell growth [4, 8, 18] or even as therapy in tumor patients [19].

The current study examined whether blocking γ-tubulin expression can arrest the growth of cancer cells. Furthermore,

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the possibility that antisense oligonucleotides can act as an antitumor agent against Korean stomach cancer cells was also investigated.

MATERIALS AND METHODS

Cell Culture and Antisense Treatment

The SNU-1, SNU-216, and NIH/3T3 cells were maintained in an RPMI 1640 medium (Life Technologies, Inc., U.S.A.) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, U.S.A.). A dose of 20mer, fully phosphorothioated oligodeoxynucleotides (antisense 5'-ATTTCCCTCGGCATCGCTCC-3' and missense 5'-AGAGTGTGCGCTGAGCCCGT-3' dissolved in RPMI culture media) was added to the cells in a 96-well culture plate. The cells were then synthesized using standard protocols and purified by an HPLC (IDT Inc., U.S.A.).

[^{3}H]-Thymidine Incorporation Assay

The cells were subjected to [^{3}H]-thymidine incorporation assay to determine the growth rate after oligonucleotide treatment for 48 h. The cells (4×10^6/ml) were seeded in 96-well culture plates and then pulsed with [^{3}H]-thymidine (1 μCi/m;) for 21 h after seeding. A glass fiber filter strip (type 240-1, Biomedical R&D Lab, U.S.A.) was used to harvest the cells, which were then soaked in a scintillation cocktail (Sigma, U.S.A.). The radioactivity of the ^{3}H was assayed using a liquid scintillation counter (Wallac, U.S.A.).

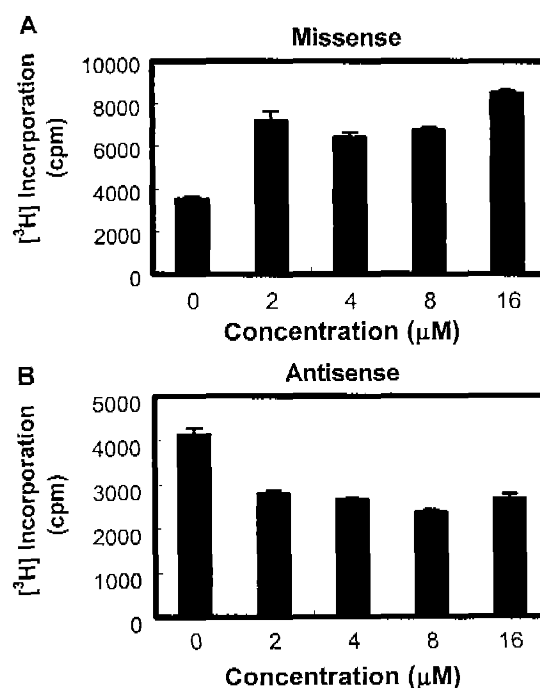

Fig. 1. Effect of antisense oligonucleotides on DNA synthesis of Korean stomach cancer cell, SNU-1.
A. Missense oligonucleotide treatment. B. γ-Tubulin antisense oligonucleotide treatment. SNU-1 cells were incubated for 48 h in the absence (0 μM) or presence of the oligonucleotides (2, 4, 8, or 16 μM) in RMPI media. The [^{3}H] incorporation rate was assayed after [^{3}H]-thymidine incubation for 20 h. Results represent a mean from triplicate cultures. Error bar indicates standard error (SE).

RESULTS AND DISCUSSION

Two Korean gastric cancer cell lines (SNU-1 and SNU-216) and a normal cell line (NIH/3T3) were used to test the inhibitory effect of γ-tubulin antisense oligonucleotides on cell cycle progression. As distinct from SNU-1, which has a point mutation on the hMLH1 gene encoding a mismatch repair system-related protein [16], SNU-216 has a point mutation in the *p53* gene, a tumor suppressor gene. SNU-1 is also known to be more sensitive to DNA damage by chemical than NIH/3T3 [7]. However, the characteristics of these tumor cell lines are not much known. The oligodeoxynucleotides used in this study were synthesized with the incorporation of phosphorothioated nucleotides to reduce the turnover rate, and purified by HPLC to exclude any possible non-antisense effect. The cells were plated at an initial density of 1×10^5 cells ml^{-1}. After 24 h incubation, the cells were treated with 0, 2, 4, 8, and 16 μM oligonucleotides. The cell cycle progression was examined by testing the incorporation of [^{3}H]-thymidine into DNA synthesis. As shown in Fig. 1B, the antisense oliogodeoxynucleotide of γ-tubulin resulted in a substantially reduced [^{3}H]-thymidine incorporation depending on the concentration of the oliogonucleotide. However, those cells treated with a missense oligonucleotide grew at the increasing proliferation rate as the control samples without oligonucleotide treatment (Fig. 1A). Martin *et al.* [10] originally suggested the hypothesis that the depletion of γ-tubulin may inhibit the nucleation of microtubule assembly in cell-cycle progression. However, little is known about the specific relationship between cell proliferation and the incorporation of γ-tubulin into microtubules. The present study provides several intriguing clues to this relationship. The importance of cytoskeleton for cell proliferation was already proved by the fact that S100A1 appears to regulate proliferation in PC12 cells by controlling tubulin levels [24]. The present study using antisense for inhibiting γ-tubulin synthesis also supports this hypothesis. However, several investigators pointed out the findings that, carried out with *S. pombe*, the γ-tubulin loss occurs relatively early before it is possible to observe the phenotype produced by the loss [5, 9]. This raises the possibility that some of the phenotypes observed are secondary rather than primary consequences of γ-tubulin loss.

Further evidence supporting a linkage between γ-tubulin expression and the gastric cancer cell-cycle progression emerged from the study of the other Korean gastric cancer cell line, SNU-216. The [^{3}H]-thymidine incorporation rates

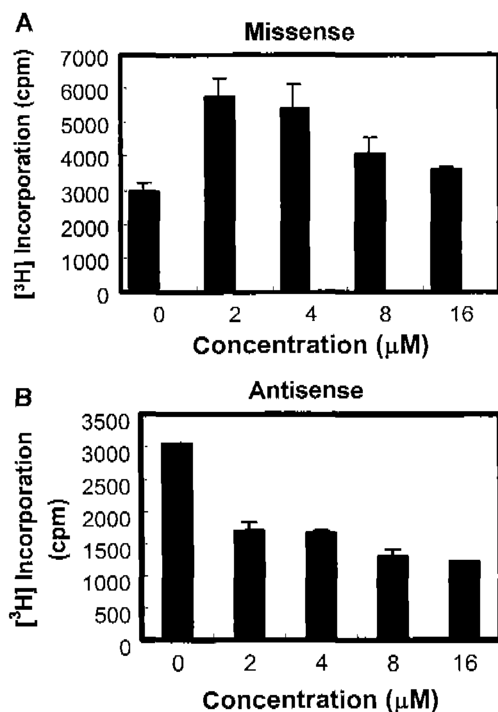

Fig. 2. Effect of antisense oligonucleotides on DNA synthesis of Korean stomach cancer cell, SNU-216.

A. Missense oligonucleotide treatment. B. γ-Tubulin antisense oligonucleotide treatment. SNU-216 cells were incubated for 48 h in the absence (0 μM) or presence of the oligonucleotides (2, 4, 8, or 16 μM) in RMPI media. The [³H] incorporation rate was assayed after [³H]-thymidine incubation for 20 h. Results represent a mean from triplicate cultures. Error bar indicates standard error (SE).

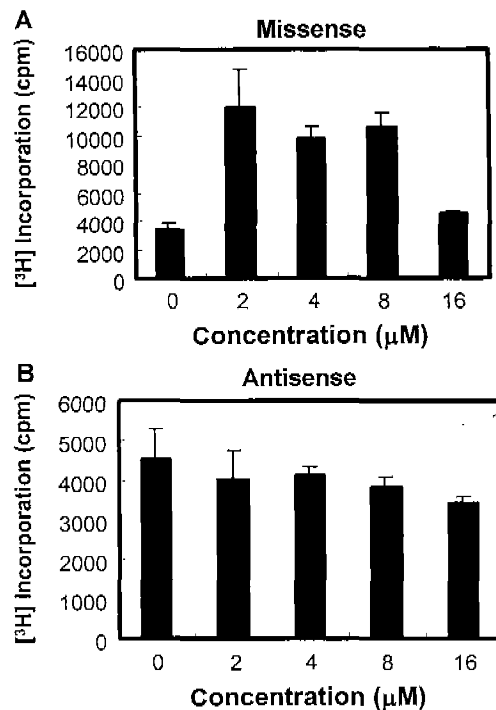

Fig. 3. Effect of antisense oligonucleotides on DNA synthesis of mouse fibroblast cell, NIH/3T3.

A. Missense oligonucleotide treatment. B. γ-Tubulin antisense oligonucleotide treatment. NIH/3T3 cells were incubated for 48 h in the absence (0 μM) or presence of the oligonucleotides (2, 4, 8, or 16 μM) in RMPI media. The [³H] incorporation rate was assayed after [³H]-thymidine incubation for 20 h. Results represent a mean from triplicate cultures. Error bar indicates standard error (SE).

of the SNU-216 cell line in a tissue culture are shown in Fig. 2. Although the SNU-216 and SNU-1 cells were both adenocarcinomas, the SNU-216 cells had a slower doubling time compared to SNU-1, which was 26 h. Regardless of the different doubling time, both cell lines showed the same retardation effect on the cell-cycle progression by inhibiting γ-tubulin expression (Fig. 1B and Fig. 2B). Another phenotypic feature of SNU-216 was that differentiation of SNU-216 was moderate, yet that of SNU-1 was very poor. When a high dose (16 μM) of antisense oligonucleotide was treated, the [³H]-thymidine incorporation was approximately 3-fold less than the nontreated sample.

A previous study demonstrating that normal cells, NIH/3T3, are much less sensitive to a DNA damage by chemicals than transformed cells, SUN-1, suggested that the retardation of spindle fiber formation resulted in different effects in tumor cells and normal cells [7]. To further examine this hypothesis, NIH/3T3 cells were treated with a γ-tubulin antisense oligonucleotide and the [³H]-thymidine incorporation rate was compared with that of samples treated with missense oligonucleotides. As shown in Fig. 3, there was no marked reduction in the NIH/3T3 cells compared with the SNU-1 and SNU-216 cells. This result suggested that γ-tubulin was essential for microtubule assembly, especially in transformed cells. Although γ-tubulin is absolutely required for the assembly of mitotic spindle microtubules [9], it has been established that, at the onset of mitosis, the centrosome suddenly recruits γ-tubulin and the nature of this dynamic exchange is microtubule-independent [6]. Therefore, it is still unknown whether the ablation of γ-tubulin inhibits cell-cycle progression through blocking microtubule assembly or some other way. Recent identification of a second expressed human γ-tubulin gene suggests that any pharmacological agents targeting one human γ-tubulin are likely to target both [21]. Treatment of antisense oligonucleotide against only one γ-tubulin may not be enough to completely inhibit the function of γ-tubulin in microtubule assembly.

The results presented in this study also provide a new target gene that can be caught by antisense oligonucleotides for arresting cancer cell proliferation. However, this gene may be a less effective target in slow-growing or fully differentiated cells because it is not absolutely necessary for microtubule assembly nucleation by γ-tubulin. There are also many problems with phosphorothioated oligonucleotides because of their side effects, due to the ability of phophorothioates

to bind to proteins, and specifically to heparin-binding proteins, and limit internalization by endocytosis. Accordingly, the usage of antisense oligonucleotides as anticancer drugs should be further investigated in detail. Several studies have reported that antisense oligonucleotide-targeted genes have already been used to inhibit tumor cell growth [4, 8, 14, 18, 19].

Interestingly, all the cell lines tested had common features yet distinct responses to the missense oligonucleotide treatment (Figs. 1A, 2A, and 3A). The mechanism by which the [^{3}H]-thymidine incorporation increased at a dose of 2 or 4 μM oligonucleotides in all three cell lines remains unknown. However, it would appear that a small amount of oligonucleotide was not enough to induce the breakdown of the γ-tubulin transcript by RNase H, and was used as material instead for the synthesis of nucleotide through the salvage pathway.

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