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Silencing of the p53R2 gene by RNA interference inhibits growth and enhances 5-fluorouracil sensitivity of oral cancer cells

Souichi Yanamoto[1], Tsutomu Iwamoto[2], Goro Kawasaki[3], Izumi Yoshitomi[4], Nobuyuki Baba[5], Akio Mizuno[6]

[1]Instructor, [2]Instructor, [3]Associate Professor, [4]Instructor, [5]Assistant Professor, [6]Professor and Chairman; Division of Oral and Maxillofacial Surgery, Department of Developmental and Reconstructive Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan

Corresponding author: Souichi Yanamoto, Division of Oral and Maxillofacial Surgery, Department of Developmental and Reconstructive Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan. Phone: 81-95-849-7698; Fax: 81-95-849-7700; E-mail: syana@net.nagasaki-u.ac.jp.

Abstract

The p53R2 gene encodes the ribonucleotide reductase (RR) small subunit 2 homologue, and is induced by several stress signals activating p53, such as DNA-damaging agents. The p53R2 gene product causes an increase in the deoxynucleotide triphosphate (dNTP) pool in the nucleus, which facilitates DNA repair and synthesis. We hypothesized that p53R2 would be a good molecular target for cancer gene therapy.

In this study, three human oral cancer cell lines (SAS, HSC-4 and Ca9-22), a human breast cancer cell line MCF-7, and a normal human fibroblast cell line NHDF were tested. We silenced the expression of p53R2 with the highly specific post-transcriptional suppression of RNA interference (RNAi). We investigated p53R2 expression with the reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. The sensitivity to anticancer agents was evaluated by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

The expression of p53R2 showed no association with the mutational status of p53. The cancer cell lines with higher p53R2 expression were more resistant to 5-FU. RNAi-mediated p53R2 reduction selectivity inhibited growth and enhanced chemosensitivity in cancer cell lines but not in normal fibroblasts.

These results suggest that basal transcription of p53R2 could be associated with the sensitivity to anticancer agents. Moreover, we assessed the possibility that p53R2 would be a good molecular target, and report that RNAi targeting of p53R2 could be useful for oral cancer gene therapy.

Key words: Head and neck/oral cancers; p53R2; RNA interference; 5-fluorouracil

1. Introduction

The sensitivity of cancer cells to anticancer agents is a useful factor for determining the most suitable treatment for individual cancer patients. Particularly, it is critical to reduce the area of tumor infiltration, because wide surgical resection of oral cancer causes various oral dysfunctions. The fluoropyrimidine drug 5-fluorouracil (5-FU) is widely used in the treatment of gastrointestinal, breast, head and neck cancers [1]. In fact, the combination of 5-FU with other anticancer agents such as cisplatin and methotrexate as a neoadjuvant chemotherapy has improved the response rate for advanced oral cancer [2]. However, some patients have a poor response to 5-FU-based chemotherapy. In these patients, a delay in commencing the most effective treatment may contribute to the poor overall prognosis. Therefore, new treatment strategies to improve the efficiency of anticancer agents are urgently needed.

The tumor suppressor gene p53 is the most frequently mutated gene in human cancers [3]. Although mutational inactivation of p53 has been reported to be the determinant of resistance to anticancer agents or radiation treatment in oral cancer cells [4-6], the significance of p53 in the sensitivity to chemotherapy and its underlying mechanisms still remain unclear. Recent studies have reported that several determinants of 5-FU sensitivity, including the 5-FU-targeting enzyme thymidylate synthase (TS), 5-FU-degrading enzyme dihydropyrimidine dehydrogenase (DPD) and 5-FU-anabolic enzyme orotate phosphoribosyl transferase (OPRT), play key roles in the 5-FU metabolism pathway [1, 7]. However, it is likely that these enzymes or the DNA damage response pathway evaluating downstream of p53 may also be associated with the response to 5-FU. Therefore, we believe that a downstream gene directly correlated with

the DNA damage response pathway may be a valuable therapeutic target to increase the sensitivity of 5-FU based chemotherapy.

Recently, the p53-inducible p53R2 gene has been isolated and shown to play a crucial role in DNA repair and synthesis after DNA damage [8]. Moreover, the expression and activity of p53R2 has been reported to be associated with the anticancer agent resistance of human cancer cells [1, 9]. We already reported that the presence of p53R2 expression was a predictive factor for the sensitivity to preoperative radiochemotherapy in oral squamous cell carcinoma [10]. The p53R2 gene encodes the ribonucleotide reductase (RR) small subunit 2 homologue, and is induced by several stress signals activating p53, such as DNA-damaging agents. The p53R2 gene product also causes an increase in the deoxynucleotide triphosphate (dNTP) pool in the nucleus, which facilitates DNA repair and synthesis [8, 9, 11].

We hypothesized that inhibition of p53R2 would increase the sensitivity to anticancer agents, and that p53R2 would be a good molecular target for cancer gene therapy. For this investigation, RNA interference (RNAi) directed against p53R2 was used. RNAi is a recently developed, small interfering RNA (siRNA) technique for the highly specific post-transcriptional suppression of gene expression, both *in vitro* and *in vivo* [12-14]. The cellular process resulting in enzymatic cleavage and breakdown of mRNA is initiated by the introduction into cells of double stranded 21-mer sense and antisense RNAs that are homologous to the sequence of the silenced gene. The siRNAs bind specifically to the cellular mRNA and activate a RNA degradation process [15].

In this study, we assessed the possibility that p53R2 would be a molecular target for cancer gene therapy, and reported for the first time that RNAi targeting of p53R2 inhibits growth and enhances the 5-FU sensitivity of oral cancer.

2. Materials and Methods

2. 1. Cell culture and drug treatment

The human oral squamous cell carcinoma cell lines, SAS (p53 wild-type), HSC-4 (p53 mutant) and Ca9-22 (p53 mutant) were obtained from the Human Science Research Resource Bank (Osaka, Japan). The human breast carcinoma cell line, MCF7 (p53 wild-type) was obtained from American Type Culture Collection (Manassas, VA). The normal human fibroblast cell line NHDF, derived from neonatal skin, was obtained from Clonetics (San Diego, CA). All of the cells were cultured under conditions recommended by their respective depositors. Cells were treated with 5-FU (Sigma Chemical Co., St. Louis, MO) at the various concentrations and time points.

2. 2. RNAi

For design of siRNA oligos targeting p53R2, a DNA sequence of the type AA(N_{19}) was selected corresponding to the nucleotides 349-369 (AAG AAA GAG TTC TCG CCG GTT) located 3' to the first nucleotide of the start codon of the human p53R2 mRNA. The sequence was submitted to a BLAST search against the human genome sequence to ensure that only the *p53R2* gene was targeted. The luciferase GL2 siRNA duplex was used as a nonspecific siRNA control. Human p53R2 siRNA and luciferase control siRNA were purchased from Greiner (Tokyo, Japan). Cells were transfected with double strand RNA using Oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2. 3. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol Reagent (Invitrogen) and first strand cDNA was synthesized from 1 μg total RNA using Oligo d (T) primer (Invitrogen) and ReveTra Ace (TOYOBO, Osaka, Japan). For PCR analysis, cDNA was amplified by Taq DNA polymerase (Takara, Otsu, Japan). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as the endogenous expression standard. Each PCR program involved a 3-min initial denaturation step at 94℃, followed by 26 cycles (for *p53R2*, *p53*, and *p21*), or 18 cycles (for *GAPDH*) at 94℃ for 30s, 53-55℃ for 30s and 72℃ for 1 min, on a PCR Thermal Cycler MP (Takara). Primer sequences were, for *p53R2*: F, TAT CTC GCC CTG CAT ATT CC and R, TTC CCT CAC CAT TGG CTA TC; for *p53*: F, TAC TCC CCT GCC CTC AAC AA and R, CAT CGC TAT CTG AGC GC; for *p21*: F, CCA AGA GGA AGC CCT AAT CC and R, CCC TTC AAA GTG CCA TCT GT; and for *GAPDH*: F, ATG TCG TGG AGT CTA CTG GC and R, TGA CCT TGC CCA CAG CCT TG. The amplified products were separated by electrophoresis on ethidium bromide-stained 2% agarose gels. Band intensity was measured by NIH Image 1.63.

2. 4. Western blot analysis

Cells were harvested by trypsinization, washed and pelleted by centrifugation. The cytoplasmic and nuclear proteins were purified using NE-PER^{TM} Nuclear and Cytoplasmic Extraction Reagents (Pierce Chemical Co., Rockford, IL). All subsequent manipulations were performed on ice. The cells were incubated in the cytoplasmic extraction reagent. The lysed cells were centrifuged at 15,000 rpm for 5 min to pellet the intact nuclei. The supernatant was removed (cytoplasmic fraction). The pelleted

nuclei were resuspended in the nuclear extraction reagent, vortexed vigorously for 15 sec and incubated on ice for 40 min with periodic vortexing every 10 min. At this point, the mixture was centrifuged at 15,000 rpm for 10 min. The supernatant was removed (nuclear fraction). The protein concentration of each sample was measured with micro-BCA protein assay reagent (Pierce Chemical Co.). The samples were denatured in SDS sample buffer and loaded onto an ExcelGel SDS Homogenous 12.5 (Amersham Biosciences, Piscataway, NJ). 10 μg of lysate protein was applied to each lane. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred onto a polyvinylidine difluoride membrane and immunoblotted with anti-p53R2 (Santa Cruz Biotechnology, Santa Cruz, CA) and then visualized using an ECL Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2. 5. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Cells were seeded in the 96-well plate at a concentration of 5×10^3 per well and incubated for 24 hours. The cells were treated with the various concentrations of 5-FU for 72 hours. To evaluate the effect of p53R2 siRNA, NHDF, SAS and HSC-4 cells were transfected with p53R2 siRNA or the luciferase control siRNA. After 24 hours, the cells were treated with 5-FU for 48 hours. For the MTT assay, cells were incubated with 0.5 mg/ml MTT (Sigma Chemical Co.). Four hours later, the medium was replaced with 100 μl dimethylsulfoxide (DMSO) (Sigma Chemical Co.) and vortexed for 10 minutes. Absorbance (A) was then recorded at 570 nm using Easy Reader 340 AT (SLT-Labinstruments, Salzburg, Austria). Cell viability (%) was calculated as a percentage of the absence of 5-FU.

2. 6. Statistical analysis

The continuous data are summarized as means ± SD. Significance was assessed by Student's *t* test. *P* values of less than 0.05 were considered to be significant.

3. Results

3. 1. Relationship between p53R2 expression and 5-FU sensitivity

p53R2 mRNA expression of oral cancer cell lines and the breast cancer cell line MCF7 were analyzed by semiquantitative RT-PCR. The basal expression of p53R2 mRNA was detectable in all cell lines, and the p53R2/GAPDH intensity ratio varied from 0.25 to 0.88 (Fig. 1A). The p53R2 mRNA expression level was significantly correlated with the sensitivity to 5-FU at the various concentrations ($p<0.05$), where low p53R2 mRNA expression levels were associated with high sensitivity to 5-FU (Fig. 1B).

3. 2. p53R2 mRNA expression in response to 5-FU

To explore the expression of p53R2 in response to 5-FU, we examined the expression of p53R2 mRNA by semiquantitative RT-PCR. All cell lines, whose mutational status of p53 had been characterized, were treated with 0.5 μM 5-FU (corresponding to approximately less than 10% of the growth inhibition dose of three oral cancer cell lines 48 h after 5-FU treatment, data not shown). Although three cell lines carrying wild-type p53 revealed a time-dependent induction of p53R2 mRNA expression, HSC-4 and Ca9-22 cells carrying the p53 mutant revealed a decrease of p53R2 mRNA expression at 6 h and 12 h after 5-FU treatment, respectively (Fig. 2). However, these two p53 mutant cell lines revealed an increase of p53R2 mRNA expression at 24 h after 5-FU treatment (Fig. 2, C and D). These data indicated that expression of p53R2 mRNA in response to 5-FU was significantly induced in a p53-dependent manner in p53 wild-type cell lines, but not in p53 mutant cell lines.

3. 3. Expression of p53R2 after treatment with p53R2 siRNA

SAS, HSC-4 and NHDF cells were transfected with p53R2 siRNA. At 72 h post-transfection, cells were harvested and p53R2 mRNA expression was analysed by semiquantitative RT-PCR. Oligofectamine reagent alone and luciferase control siRNA transfection had no effect on p53R2 mRNA expression. Moreover, p53 and p21 expression was unaffected by transfection with either p53R2 or luciferase control siRNA, and obvious reduction in p53R2 mRNA was observed with p53R2 siRNA at the final concentration of 200 nM (Fig. 3, A, B and C). Thus, we confirmed that transfection with p53R2 siRNA caused specific degradation of the p53R2 mRNA in human cells. Additionally, to explore whether p53R2 expression was reduced by p53R2 RNAi with the presence of 5-FU treatment, we examined p53R2 expression by semiquantitative RT-PCR and Western blot analyses. In all three cell lines, RNAi-mediated p53R2 reduction was confirmed both with and without 5-FU (Fig. 4, A, B and C). Moreover, to examine the nuclear accumulation of p53R2 product, the fractionated cytoplasmic and nuclear proteins were analyzed by immunoblotting. In our study, the expression of the p53R2 nuclear protein in p53 wild-type cell lines clearly increased in response to 5-FU treatment (Fig. 4A), but not p53 mutant cell lines (Fig. 4B). These results suggest that the nuclear accumulation of p53R2 in response to DNA damage could be deficient in p53 mutant cells.

3. 4. Effect of RNAi-mediated p53R2 reduction on growth and 5-FU sensitivity of oral cancer cells and normal fibroblasts

To determine the effect of p53R2 RNAi on cellular proliferation and 5-FU sensitivity, we transfected SAS, HSC-4 and NHDF cells with p53R2 siRNA, and tested

the cells with or without the addition of 0.5 μM 5-FU. This 5-FU concentration was determined corresponding to approximately less than 10% of the growth inhibition dose of SAS and HSC-4 cells 48 h after 5-FU treatment by MTT assay (data not shown). Luciferase control siRNA had no effect on cellular proliferation, compared with untreated cells (Fig. 5, A, B and C). In the absence of 5-FU, p53R2 siRNA had an inhibitory effect on cell growth of the SAS and HSC-4 cells (Fig. 5, A and B). However, in contrast to SAS and HSC-4 cells, p53R2 siRNA did not inhibit proliferation of the fibroblasts, which showed comparable growth rates to the control fibroblasts transfected with luciferase siRNA (Fig. 5C). In the presence of 5-FU, p53R2 siRNA significantly augmented the sensitivity of 5-FU in the SAS and HSC-4 cells but not in NHDF cell (Fig. 5). These results suggest that p53R2 siRNA selectively inhibits cell growth in the absence of DNA-damaging agents, and enhances 5-FU sensitivity in cancer cells but not in normal fibroblasts.

4. Discussion

Our previous study reported that expression of p53R2 could be associated with the sensitivity of preoperative radiochemotherapy in oral squamous cell carcinoma patients [10]. In addition, this study indicates that low p53R2 mRNA expression levels are associated with the high sensitivity to 5-FU in oral cancer cells *in vitro*. We have also shown that p53R2 RNAi can inhibit tumor growth and enhance 5-FU sensitivity in oral cancer.

RR is the pivotal enzyme that catalyzes conversion of ribonucleotide 5'-diphosphate to dNTP required for DNA repair and synthesis [16]. Human RR consists of three subunits, M1, M2 and p53R2. The RRM1 is a large peptide chain that contains the catalytic site, two allosteric effector-binding sites, and redox active disulfides that participate in the reduction of substrates [17, 18]. The RRM2 contains an oxygen-linked di-ferric center and one tyrosyl radical per monomer that are essential for enzymatic activity [17]. The p53R2 gene contains a p53-binding sequence in intron 1 and encodes a 351-amino-acid peptide that bears a striking similarity to RRM2 [8]. It has been shown that higher inherent levels of RRM2 expression is associated with resistance to a variety of anticancer agents, including gemcitabine, hydroxyurea, 2,2-difluorodeoxycytidine, and 5-FU [16, 19, 20]. Recently, several studies reported that the inhibition of endogenous RRM2 using phosphorothioate antisense oligonucleotides or siRNA decreases malignant cell proliferation, and enhances chemosensitivity [21, 22]. However, Yamaguchi *et al.* [11] suggested that RRM2 would be unsuitable as a molecular target because the inhibition of endogenous RRM2 interrupted the DNA synthesis of the normal cells, as well as cancer cells. In this study, the basal expression

level of p53R2 mRNA using RT-PCR was significantly associated with the sensitivity to 5-FU. This result suggests that p53R2 expression causes DNA repair through the activation of RR and prevents cell death in response to 5-FU.

Some authors reported that the transcription of p53R2 was induced by wild-type p53 [11, 23]. Xue *et al.* [9] suggested that in quiescent cells, p53R2 and RRM2 reside in the cytoplasm bound to p53. After cells are exposed to DNA damaging agents, p53R2 and RRM2 dissociate from p53 and translocate into the nucleus where both of RR subunits bind to RRM1 and form functional a tetramic RR holoenzyme [9]. This pathway depends on wild-type p53 [8, 9, 11, 23, 24]. In this study, the expression of the p53R2 nuclear protein clearly increased in response to 5-FU treatment in p53 wild-type cell lines, but not in p53 mutant cell lines. These results supported the possibility that mutant p53 interfered to release p53R2, preventing it from binding RRM1 [9]. Our results also indicated that the induction of p53R2 after 5-FU treatment revealed in p53 dependent manner in p53 wild-type cell lines. However, the basal expression level of p53R2 mRNA was not based on the mutational status of p53. Therefore, it was suggested that p53R2 was basally expressed at various levels regardless of the p53 status, but the transcription of p53R2 in response to DNA damage was up-regulated by the presence of wild-type p53.

In this study, we successfully demonstrated, by using siRNA to target the *p53R2* gene, that the expression of p53R2 was specifically inhibited. Tanaka *et al.* [8] reported that the suppression of p53R2 using antisense oligonucleotides reduced cell survival after genotoxic stress, such as γ -irradiation, UV irradiation, and exposure to adriamycin. Moreover, Yamaguchi *et al.* [11] suggested that inactivation of the p53R2-dependent pathway of DNA synthesis would cause failure of the normal repair

system; the p53-dependent apoptotic pathway might be activated instead, resulting in apoptosis to eliminate dangerously damaged cells. We examined the cell viability of p53R2 siRNA-transfected SAS cells (p53 wild-type), HSC-4 (p53 mutant) and NHDF (p53 wild-type) as a normal control in the presence and absence of 5-FU. Regardless of the p53 mutation status, treatment of cancer cells with siRNA-targeting p53R2 alone caused approximately 35% growth inhibition. Interestingly, in the fibroblast control cells, the suppression of p53R2 expression by RNAi did not result in growth inhibition. Therefore, these data suggest that p53R2 plays a critical role in cancer cell proliferation, and p53R2 RNAi-mediated growth inhibition is specific for cancer cells. In our study, p53R2 RNAi with 5-FU treatment significantly inhibited cell growth, compared with p53R2 siRNA alone both of p53 wild-typed SAS cells and p53 mutant HSC-4 cells. Furthermore, p53R2 RNAi with 5-FU treatment significantly inhibited cancer cell growth, compared with 5-FU treatment alone. Therefore, it was suggested that silencing of the p53R2 gene by RNAi enhanced the chemosensitivity to 5-FU.

Taken together, we consider that p53R2 is a potential target for cancer gene therapy. Moreover, RNAi technology is a remarkable therapeutic tool, and we speculate that the use of p53R2 RNAi together would reduce the necessary dose of anticancer agents, and it is expected that siRNA-targeting p53R2 may relieve the side effects of anticancer agents.

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Figure legends

Fig. 1. (A) Representative reverse transcription-PCR (RT-PCR) analysis for p53R2 in untreated MCF7, SAS, HSC-4 and Ca9-22 cells. The p53R2/GAPDH intensities are means±SD of triplicate experiments. (B) Growth inhibition by 5-fluorouracil (5-FU) in MCF7, SAS, HSC-4 and Ca9-22 cells. The cells were plated on 96-well plates and treated with 0, 0.5, 1, 5, and 10 μM 5-FU for 72 h. Cell viability was determined using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The data are presented as the means of three separate experiments, each performed in triplicate; *bars*, SD.

Fig. 2. Representative reverse transcription-PCR (RT-PCR) analysis for transcriptional induction of p53R2, p53 and p21 in NHDF (A), SAS (B), HSC-4 (C), Ca9-22 (D), and MCF7 (E) after 5-FU treatment. Cells were treated with 0.5 μM 5-FU and expression levels of p53R2, p53 and p21 were analyzed at 0, 2, 6, 12, 24, and 48 h after treatment. Band intensities were shown as the bar graphs.

Fig. 3. Representative reverse transcription-PCR (RT-PCR) analysis for p53R2 72 h following treatment with Oligofectamine reagent alone (Oligo.), luciferase control siRNA (Luc), or p53R2 siRNA (p53R2 RNAi) in SAS, HSC-4 and NHDF cells. Band intensities were shown as the bar graphs.

Fig. 4. Representative reverse transcription-PCR (RT-PCR) and Western blot analyses for the suppression of p53R2 in SAS (A), HSC-4 (B) and NHDF (C) cells. Cells were

transfected with luciferase control siRNA (Luc) or p53R2 siRNA (p53R2), and after 24 h treated with vehicle (−) or 5-fluorouracil (+) for 48 h. Western blot analysis shows p53R2 expression in the components (cytosolic and nuclear) of the cells.
Band intensities obtained from RT-PCR were shown as the bar graphs.

Fig. 5. Effect of p53R2 siRNA on cell growth. SAS (A), HSC-4 (B), and NHDF (C) cells were were transfected with luciferase control siRNAor p53R2 siRNA, and after 24 h treated with vehicle (5-FU −) or 5-fluorouracil (5-FU +) for 48 h. Cell viability (%) is shown as a percentage of the untreated control cells in the absence of 5-FU. *Columns* are presented as the means of three separate experiments, each performed in triplicate; *bars*, SD.