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Change of Telomerase Activity in Rectal Cancer with Chemoradiation Therapy

Telomerase, an enzyme associated with cellular immortality, is expressed by most malignant cells and is inactive in most normal somatic cells, with the exception of proliferative stem cells, germ cells and activated lymphocytes. Measuring telomerase activity clinically may provide useful diagnostic and prognostic information of cancer. The purpose of this study was to investigate the change in telomerase activity following chemoradiation in rectal cancer, which almost always produces positive enzymatic activity. A total of 24 tumor tissue samples were used in this study, consisting of 12 paired specimens before and 4 weeks after chemoradiation. Telomerase activity was determined by PCR-based telomeric repeat amplification protocol (TRAP) assay. The telomerase activity was positive in 10 out of 12 patients (83%) in pre-irradiated and post-irradiated states. The levels of telomerase activity was decreased in 8 out of 10 patients after chemoradiation (80%) and two cases showed no change in enzymatic activity. One case showed no activity in either sample. The other case showed no enzymatic activity in the pre-irradiated sample, but showed weak activity in the post-irradiated sample. These data indicate that telomerase activity in rectal cancer is reduced after neoadjuvant chemoradiation therapy, possibly suggesting a mechanism of downstaging following chemoradiation therapy in cancer.

Key Words: *Telomerase; Rectal Neoplasms; Drug Therapy; Radiotherapy*

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INTRODUCTION

Telomeres are protein-DNA structures at the ends of eukaryotic chromosomes and are composed of simple repetitive G-rich hexameric sequences (TTAGGG) (1-3.). They allow the cell to distinguish intact chromosomes from broken ones and to protect chromosomes from degradation. They are also substrates for novel replication mechanisms (4). Telomerase is a ribonucleoprotein polymerase that adds telomeric sequences to chromosome ends (5). It has been proposed that somatic cells are deficient in telomere maintenance and that the loss of terminal sequences with each round of DNA replication is the process that records their proliferative history, whereby short telomeres provide the signal for growth arrest at senescence (6). Avoiding telomeric shortening by expressing telomerase may contribute to the immortal phenotype (7, 8).

Recently, it has been reported that telomerase is expressed in most human cancers and immortalized cell lines, but is inactive in normal adult somatic cells except for testicular germ cells and stem cells present in bone marrow, lymphoid tissue, colonic crypts and the epidermis (9). Therefore, it has been postulated that reactivation of telomerase expression is necessary for the continuous proliferation of most cancerous cells (9). The cellular response to ionizing radiation involves the induction of cell cycle checkpoint arrests and programmed cell death, which produces double-srtand breaks in DNA and causes telomere-less chromosome ends. The radiation response appears to be the result of the inappropriate induction of cellular senescence mechanisms (10).

No data are currently published on telomerase activity in relation to radiation in rectal cancer. In this study, we examined the relationship between telomerase activity and chemoradiation in rectal cancer using a modification of the telomeric repeat amplification protocol (TRAP) assay.

MATERIALS AND METHODS

Patients and tumor tissue

Between January 1998 and December 1998, 12 patients with rectal cancer received preoperative chemoradiation therapy. They consisted of 9 men and 3 women in the fifth to eighth decades of life. Preoperatively, they were all T3N1M0 according to the American Joint Comittee on Cancer (11). After chemoradiation therapy, the pathology reports showed downgraded tumor stages: five were T2N0M0 and seven were T3N0M0. Among them, five underwent a low anterior resection and seven had an abdominoperineal resection. Those histologic types are shown in Table 1. A total of 24 tissue samples from 12 patients were examined in this study. The specimens were paired in relation to the time of chemoradiation. The tumor tissue obtained before chemoradiation was stored at -80℃ immediately after sigmoidoscopy-guided biopsy. After the completion of chemoradiation therapy, the patients were not given any therapy for a four-week, cooling time after radiation. Then, they underwent a radical operation. Tumor tissue was obtained immediately after separating the tumor from its systemic vascular supply, and the specimen was also stored at -80℃ until it was tested for telomerase activity.

Methods of chemoradiation

The chemotherapeutic regimen consisted of 5-fluorouracil (500 mg/m^2) on days 1 to 5 and days 28 to 32, and cisplatin (20 mg/m^2) on days 6, and 33. A total of 4,500 cGy was scheduled for external irradiation. This was spread over 25 doses (180 cGy/day), 5 days a week, for 5 weeks.

Telomerase assay

Extracts were prepared from frozen tissue stored at -80 ℃ by powdering the tissue in liquid nitrogen, followed by adding 200 μL of ice-cold lysis buffer (10 mM Tris-HCl [pH 7.9], 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 5 mM beta-mercaptoethanol, 0.1 mM phenylmethylsulfonyl-fluoride, 0.5% CHAPS[3-cholamido-propyl-dimethyl-amino-1-propanesulfonate]), and incubating for 30 min on ice. Then, it was centrifuged at 20,000 rpm for 20 min in 4℃. The supernatant was aliquoted, flash-frozen in liquid nitrogen and stored at -80℃. The protein concentration was determined by the Bradford assay (BioRad).

TRAP was used for the telomerase assays. The procedure was performed with a TRAPEZE Telomerase Detection Kit (Oncor, Gaithersburg, MD, U.S.A.). Aliquots of extracts, with or without 0.02-2.0 μg RNase were added to 50 μL reaction mixtures.

The extracts were incubated with 1 μL TS oligonucleotide sequence for 30 min to allow for elongation of the TS primer by telomerase. The elongated products were amplified by PCR through 35 cycles at 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 90 sec. Then, PAGE (polyacrylamide gel electrophoresis) was use to resolve the PCR products.

Positive telomerase activity in an extract was determined by the presence of a six-nucleotide ladder of TRAP assay products in PAGE that was sensitive to RNase pretreatment. For each run of the assay, HeLa extracts with three protein concentrations ranging from 0.02-0.2 μg/50 μL reaction mixture were assayed in parallel to serve as positive controls for comparison.

The level of telomerase activity was determined from the amount of extract. If the reaction was positive at a maximum dilution of 0.02, 0.2 and 2 μg, it was classified

Table 1. Age, sex and tumor stage of patients with rectal cancer

Patient No.	Age (year)	Sex	Preoperative stage (CT)*	Postoperative stage (pathology)*	Operation	Histology
I	75	M	T3N1M0	T3N0M0	LAR	W/D
II	78	M	T3N1M0	T3N0M0	LAR	MUC
III	70	F	T3N1M0	T3N0M0	LAR	W/D
IV	72	F	T3N1M0	T2N0M0	APR	W/D
V	67	M	T3N1M0	T2N0M0	APR	W/D
VI	75	M	T3N1M0	T2N0M0	LAR	W/D
VII	76	M	T3N1M0	T2N0M0	APR	W/D
VIII	59	M	T3N1M0	T3N0M0	APR	W/D
IX	55	M	T3N1M0	T3N0M0	APR	W/D
X	43	F	T3N1M0	T3N0M0	APR	W/D
XI	72	M	T3N1M0	T3N0M0	LAR	M/D
XII	42	M	T3N1M0	T2N0M0	APR	M/D

*TNM staging manual by American Joint Comittee on Cancer (1997)
LAR, low anterior resection; APR, abdominoperineal resection; W/D, well differentiated tubular adenocarcinoma; M/D, moderately differentiated tubular adenocarcinoma; MUC, mucinous adenocarcinoma

as strong, moderate and weak, respectively. No detectable activity was recorded as negative. The same procedures were used for the pre- and post-irradiated specimens.

RESULTS

Twelve patients with rectal cancer received preoperative chemoradiation therapy. Before irradiation, 10 out of the 12 rectal cancer patients showed positive telomerase activity (83%) that was sensitive to RNase pretreatment (Fig. 1).

After chemoradiation, 10 patients had positive telomerase activity that was sensitive to RNase pretreatment (Fig. 2).

With regard to the effect of chemoradiation therapy, 8 of 10 patients (80%) showed decreased telomerase activity after the therapy. Seven patients had high levels of telomerase activity before irradiation. They were converted to a moderate level of activity in five patients and a weak activity in two patients after irradiation. One patient showed moderate telomerase activity in pre-irradiated state, which was converted to no enzymatic activity (Fig. 3). One patient (Case VI) had moderate telomerase activity and one patient (Case IX) had weak activity at both states (Fig. 4). Another patient (Case VII) had no telomerase activity at either states (Fig. 5). The final patient (Case VIII) had no telomerase activity before irradiation, but weak activity afterwards (Fig. 6). The changes in telomerase activity in relation to chemoradiation are summarized in Table 2.

DISCUSSION

Telomerase is a ribonucleoprotein enzyme that can add TTAGGG repeat sequences to the end of chromosomes, thus stabilizing telomeres (5, 12). Telomerase contains RNA, which is used as a template to synthesize TTAGGG repeats directly onto telomere ends. Therefore, this

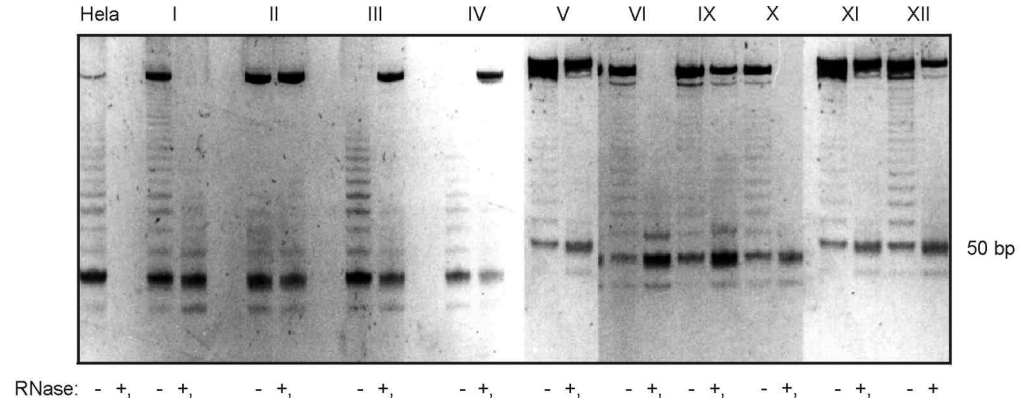

Fig. 1. Telomerase activity in tumor tissues from patients with rectal cancer, before chemoradiation therapy. −, without RNase pretreatment; +, with RNase pretreatment. Extracts of the HeLa cell (0.2 μg) are used as positive controls. This group of patients (Case No. I-VI, IX-XII) shows positive telomerase activity which was sensitive to RNase pretreatment.

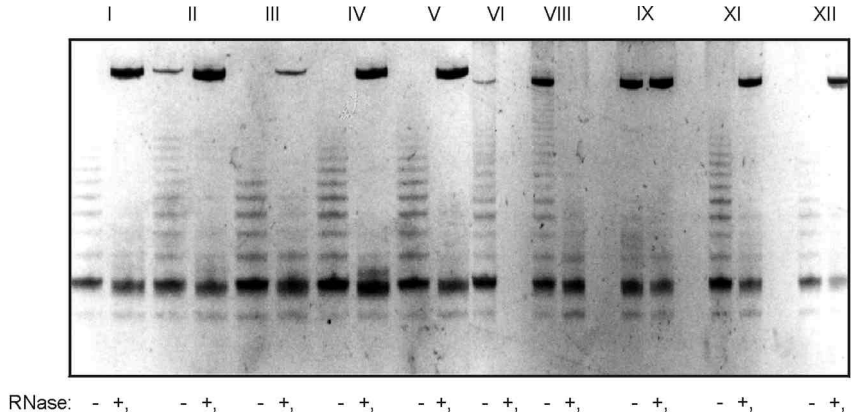

Fig. 2. Telomerase activity in tumor tissues from patients with rectal cancer, after chemoradiation therapy. −, without RNase pretreatment; +, with RNase pretreatment. This group of patients (Case No. I-VI, VIII, IX, XI, XII) shows positive telomerase activity which was sensitive to RNase pretreatment.

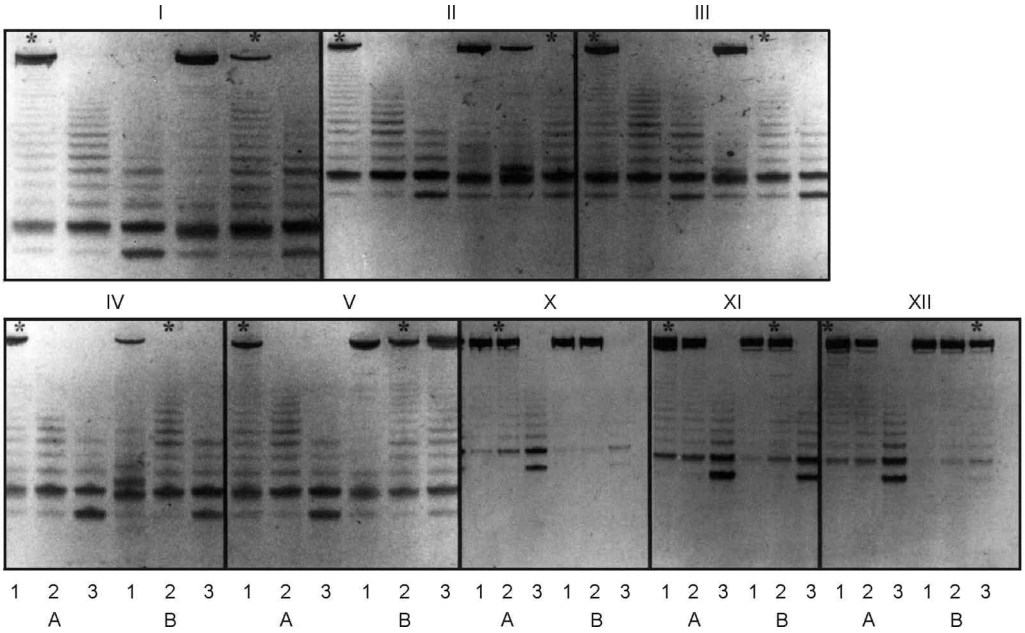

Fig. 3. Changes in telomerase activity with chemoradiation therapy (A, before chemoradiation; B, after chemoradiation; 1, 0.02 μg; 2, 0.2 μg; 3, 2 μg of tissue extract). Seven patients (Case No. I-V, XI, XII) show a strong telomerase activity before irradiation which was converted to a moderate activity in five patients (Case No. I, III-V, XI) and to a weak activity in two patients (Case No. II, XII). Case X showed moderate telomerase activity in pre-irradiated state which was converted to no enzymatic activity (*The lowest limit of telomerase activity).

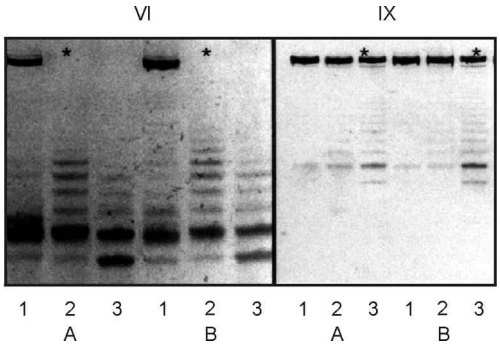

Fig. 4. Comparison of telomerase activity in two cases that show no change in telomerase activity after chemoradiation (positive in 0.2 μg of tissue extract). Other legends are same as in Fig. 3.

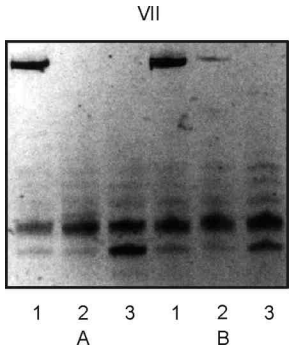

Fig. 5. Case VII shows no telomerase activity at either stage.

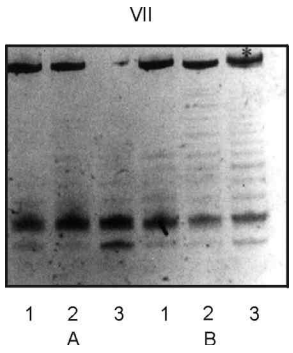

Fig. 6. Case VIII shows no telomerase activity before chemoradiation therapy and weak enzymatic activity after chemoradiation.

enzyme is sensitive to RNase pretreatment, as seen in this study. It was reported that progressive telomere shortening is halted in cancer cells by the presence of enzyme telomerase, which maintains and stabilizes the telomeres, allowing cells to divide indefinitely. In a recent study, telomerase activity was detected in solid tissue samples from 32 of 35 (95%) colorectal carcinomas examined, while it was not detected in 30 of 35 (86%) matched normal tissue samples from the same patients and was only weakly present in the remaining five (13). There was no correlation between telomerase activity and tumor stage. Telomerase activity was also found in inflammatory bowel disease lesions, although it was much weaker than in carcinomas (14). It was suggested that telomerase activity is a biomarker of cell proliferation, rather than a malignant transformation (15). It was proposed that detecting telomerase activity only indicates an increased potential for cellular immortality, and is not always synonymous with the acquisition of cancer.

Table 2. Changes in telomerase activity before and after chemoradiation therapy

Patient No.	Before chemoradiation	After chemoradiation
I	Strong	Moderate
II	Strong	Weak
III	Strong	Moderate
IV	Strong	Moderate
V	Strong	Moderate
VI	Moderate	Moderate
VII	None	None
VIII	None	Weak
IX	Weak	Weak
X	Moderate	None
XI	Strong	Moderate
XII	Strong	Weak

The methods used in determining telomerase activiy to date are all based on the TRAP assay originally described by Kim et al. (8). Briefly, the principle of TRAP assay depends on whether an extract of the proteins from a biopsy specimen, or of a pellet of cells from a clinical specimen, can extend a synthetic DNA oligonucleotide supplied in the reaction mix (8). The result is determined by PCR amplification of any extended sequences, followed by visualization of the presence or absence of a ladder of regularly spaced bands on an electrophoretic gel and analysis of their intensity. In this study, the TRAP assay for telomerase activity provided an extremely sensitive, rapid, and convenient assay procedure, with which the relationship of this enzyme to the existence of clinical disease could be investigated with sufficient samples (8). These investigators simultaneously described evidence that convincingly demonstrated telomerase activity in 90 of 101 tissue samples from 12 different varieties of human cancer. The activity was undetectable or only weakly so in 50 nonmalignant tissue samples. Several subsequent investigators confirmed these observations, and the issue of whether this assay could have a diagnostic use in clinical practice became an important priority for study (16-20). Nevertheless, this method has two problems: it does not quantify the level of enzymatic activity, and false-negative results can be caused by the presence of any tissue-derived *Taq* polymerase inhibitors. To quantify telomerase activity, we used the serial dilution technique, which was performed using three serial-diluted protein concentrations (21). Strong, moderate or weak enzymatic activity was determined by a positive reaction at a maximum dilution of 100×, 10× and 1×, respectively. In our study, there were four telomerase-negative tumors without regard to chemoradiation. When we added these tumor specimens to HeLa extracts as a positive control, the enzymatic activity of the HeLa extracts was not affected by the tumor tissue extract. This ruled out false-negative results in these tumor specimens (data not shown).

The cellular response to ionizing radiation involves the induction of cell cycle checkpoint arrests and programmed cell death. Since radiation produces double strand breaks in DNA, which results in telomere-less chromosome ends, the radiation response appears to be the result of inappropriate induction of cellular senescence mechanisms (10). It is speculated that chromosome healing after ionizing radiation is the result of conversion of double-strand breaks into chromosomes mediated by enzyme telomerase. Telomerase is a reverse transcriptase that has two distinct functions: to replicate pre-existing chromosome ends (telomeres) and to heal broken chromosomes by the de novo addition of telomeric sequences directly onto non-telomeric DNA. Slijepcevic et al. (22) suggested that telomere capture, a telomerase-independent mechanism, may be a more frequent mechanism for stabilizing chromosome healing. In this study, telomerase activity after radiation decreased rather than increased in all but one case. This increase was suspected to be due to sampling error in pre-irradiated state, i.e. delay in tissue storage into liquid nitrogen immediately after biopsy resulted in disappearance of telomerase activity. Therefore, telomerase may not be involved in chromosome healing. On the other hand, it was suggested that telomerase activity might be involved in chromosome healing after DNA damage (23, 24). It was reported that a pronounced increase in the proportion of G2 cells and a decrease in S-phase cells was found after irradiation, contributing a decrease in telomerase activity after radiation.

The primary mechanism of fluorodeoxyuridine as a radiosensitizer with early S-phase enrichment may be cell cycle redistribution (25). For this reason, chemoradiation is used in treating rectal cancer.

Horn et al. (26) reported significant down staging after preoperative radiation in rectal cancer. They also reported complete tumor regression in 4.4% of cases and decreased positive lymph nodes after preoperative radiation. In this study, down staging of the tumor occurred in all eight cases.

In conclusion, this study showed that telomerase activity in rectal cancer was reduced after chemoradiation therapy. The reduction of telomerase activity was related to down staging of the tumor and might be a mechanisms of less recurrence and better survival in rectal cancer patients after preoperative chemoradiation therapy.

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