Analysis of histological therapeutic effect, apoptosis rate and p53 status after combined treatment with radiation, hyperthermia and 5-fluorouracil suppositories for advanced rectal cancers

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Summary The tumour-suppressor gene p53 encodes a transcription factor that plays a critical role in the induction of G1 cell cycle arrest and apoptosis after DNA damage. To clarify the role of the p53 gene and apoptosis in combined hyperthermia, chemotherapy and radiation (hyperthermochemoradiotherapy, HCR therapy) for rectal cancer, we examined the histological response, rate of apoptosis, DNA fragmentation and p53 status in tumours from 28 patients undergoing HCR therapy before surgery and from 22 patients who did not have preoperative treatment. The therapeutic effect of HCR therapy was closely correlated with the rate of apoptosis; the correlation was statistically significant, suggesting that this effect occurs through apoptosis. The incidence of p53 mutations in the treated group was as follows: in tumours resistant to HCR therapy, four of seven (57.1%); intermediately sensitive, 7 of 13 (53.9%); or sensitive, three of eight (37.5%), suggesting that the therapeutic effect and apoptosis rate were related to the p53 status of the tumours to some extent, but the relation was not statistically significant. In the 22 control tumours (non-treated group), the apoptosis rate was 2.0 ± 1.1%, and there was no significant difference in p53 status compared with the HCR group. Our study indicates that the pathological response to HCR therapy correlates with the rate of apoptosis with statistical significance and that it induces the therapeutic effect more significantly in rectal cancer cells with wild-type p53, although HCR therapy-induced apoptosis also occurs in some rectal cancers with mutated p53. Therefore, this combination therapy can induce an additive or synergistic anti-tumour effect in rectal cancers with wild-type p53 as well as in those with mutated p53 through apoptosis, offering new therapeutic opportunities and a better prognosis.

Keywords: rectal cancer; p53; apoptosis; hyperthermochemoradiotherapy

Mutations in the p53 tumour-suppressor gene are among the most commonly identified genetic alterations in human cancers (Hollstein, et al, 1991; Levine et al, 1991). In addition to its clear role in tumour progression, recent evidence suggests that p53 is highly involved in the cellular response to ionizing radiation (Kastan et al, 1991; Kuert bitez et al, 1992). Levels of wild-type p53 increase after exposure to γ-radiation because of a post-translational stabilization of the protein (Kastan et al, 1991). Cells expressing wild-type p53 subsequently arrest the cell cycle at the G1/S boundary after radiation, whereas cells lacking wild-type p53, or containing a mutant form of the protein, continue to cycle or arrest in G1 (Kuerbitz et al, 1992).

Much of the evidence for the involvement of p53 in radiation-induced apoptosis is derived from work with cells from transgenic animals, isogenic apart from the status of the p53 gene. It has been shown that thymocytes from p53-deficient mice are completely resistant to the induction of apoptosis after γ-radiation, but are still sensitive to apoptosis induced by other cytotoxic agents (Lowe et al, 1993). More recently, Merritt et al (1994) reported that cells of the intestinal crypts of p53-null mice fail to undergo the rapid apoptotic cell death normally seen 3–4 h after the irradiation of mice with the wild-type gene.

Despite the apparent critical nature of the p53-dependent radiation-induced apoptotic response of haematopoietic cells and certain tissues derived from transgenic animals, the role of p53 in the DNA damage response of solid tumour cells and cell lines remains unclear. It has been found that extensive apoptosis occurs with the induction of wild-type p53 in a p53-negative cell line derived from human colon tumour (Shaw et al, 1992). In contrast, Brachman et al (1993) found that the radiosensitivity of 24 cell lines derived from head and neck cancers did not correlate with the mutational status of the p53 gene. Slichenmeyer et al (1993) found no direct influence of the p53 gene status or G1 checkpoint status on the sensitivity of colorectal carcinoma cell lines to radiation-induced cell death. Furthermore, they proposed that p53-dependent apoptosis is a cell type-specific phenomenon and that the G1 checkpoint may be more important in determining radiosensitivity (Slichenmeyer et al, 1993). Both hyperthermia and 5-fluorouracil (5-FU) can induce apoptosis in cancer cells (Barry et al, 1990; Takano et al, 1991; Shchepotin et al, 1994; Tomasovic et al, 1994), but the role of p53 in hyperthermia or 5-FU-induced apoptosis has not been ascertained.

Various protocols for the radiation therapy of rectal cancer have been developed, and their clinical outcomes have been examined carefully (Gerard et al, 1988; Neto et al, 1989; Pahlman et al, 1990; Stockholm Rectal Cancer Study Group, 1990). We have also

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developed a preoperative combination therapy using irradiation, intraluminal hyperthermia and 5-FU suppositories (HCR therapy) for rectal cancer to prevent local recurrences and improve survival. We have recently reported the results of a follow-up study, showing that the preoperative treatment improves the prognosis of these patients (Takahashi et al., 1982, 1993; Ichikawa et al., 1996) but increases post-operative complications, such as anastomotic leakage and pelvic infection (Takahashi et al., 1993). It is very important to predict the sensitivity of rectal cancers to this preoperative therapy to prevent patients from receiving ineffective treatments and to decrease the number of post-operative complications.

Considering these results, we therefore examined whether rectal cancer cells show apoptosis after HCR therapy and whether the effect of HCR therapy is related to the p53 status of the tumour.

MATERIALS AND METHODS

Patients
From 1990 to 1995, 110 patients with advanced cancer of the lower rectum admitted to the First Department of Surgery at Kyoto Prefectural University of Medicine were enrolled in this study.

Figure 1  (A) Extensive degenerative changes and fibrosis after HCR therapy in an ulcerated rectal cancer (HE staining, x 100). (B) Tunel staining nuclei (apoptotic cells) scattered in the same cancer (Tunel staining, x 200). (C) Fibrous changes in the centre of the main tumour. Pyknotic nuclei in fibrous tissue (HE staining, x 200). (D) These pyknotic cells are Tunel positive, apoptotic cells (Tunel staining, x 200). (E) Many apoptotic cells are seen in the lumens of cancerous tissue after HCR therapy (HE staining, x 200). The apoptotic cells have pyknotic or segmented nuclei, or apoptotic bodies. (F) These cells are positive on Tunel staining (x200)
Advanced rectal cancers were defined as those fixed to the surrounding structures. All cancers were located in the rectum less than 10 cm from the anal verge. The clinical evaluation of the tumours consisted of physical examination, barium enema, endoscopy, computerized tomography, magnetic resonance imaging and endoscopic ultrasonography. Our study was directed towards patients with American Joint Committee on Cancer Stage T3 and T4 tumours that were surgically resectable. Patients with distant metastases were excluded from this analysis. Patients were randomly selected, and the treatment group consisted of 28 patients who underwent combination therapy with radiation, intraluminal hyperthermia and 5-FU suppositories, and then had surgery 7 days later. The control group included 22 patients who underwent surgery without preoperative treatment.

Radiation

A 4-MeV linear accelerator or 60Co was used for the radiation therapy. A bilateral irradiation technique through anteroposterior portals was used to deliver the radiation to the area of the pelvis. The ports were approximately 12 cm in diameter, surrounding the carcinoma in the lower rectum. The target volume included the whole dorsal part of the pelvic cavity from the anus up to the promontorium. The inferior border was the bottom of the obturator foramen, and the lateral border was 1 cm lateral to the bony margin. Three times a week, 30 Gy was delivered in ten fractions.

Hyperthermia

To create hyperthermia in the rectal cancer, an intraluminal electrode was developed that consisted of a radiofrequency emitter and a cooling system. The radiofrequency system (Omron, HEH-500, Kyoto, Japan) was used as described previously (Takahashi et al., 1982, 1993; Ichikawa et al., 1996).

5-Fluorouracil suppository

Studies of colorectal cancer in vivo and in vitro have suggested an advantage when 5-FU is delivered in conjunction with radiation therapy (Takahashi et al., 1982, 1993; Ichikawa et al., 1996). Suppositories were made by dissolving 5-FU in a Wittepsol suppository base; each one contained 100 mg of 5-FU.

Protocol

The protocols for the preoperative treatment were as reported previously (Takahashi et al., 1982, 1993; Ichikawa et al., 1996). In order to get ethical approval, doctors always explained the protocol of HCR therapy and its possible complications, such as proctitis, diarrhoea, nausea and general fatigue, just before HCR therapy starts.

Pathological examination

In order to evaluate the histological changes induced by the preoperative treatment, the surgical specimens were subjected to
Table 1. p53 gene mutations and chromosome 17 allelic losses in rectal cancers treated with hyperthermochemoradiation therapy (HCR therapy)

| Case | Grade | Codon | Mutation | Amino acid | Number of 17p allele |
|------|-------|-------|----------|------------|---------------------|
| 1    | 1     | 159   | GCC→GTC | Ala→Val   | 2                   |
| 2    | 1     | 175   | CGG→CAC | Arg→His   | 1                   |
| 3    | 2     | 248   | CGG→TGG | Arg→Trp   | 1                   |
| 4    | 2     | 175   | CGG→CAC | Arg→His   | 1                   |
| 5    | 2     | 248   | CGG→TGG | Arg→Trp   | 1                   |
| 6    | 2     | 249   | AGG→AAG | Arg→Lys   | 1                   |
| 7    | 2     | 166   | TCA→TAA | Ser→Stop  | 1                   |
| 8    | 2     | 275   | 1-bp Insertion | Stop at 305 | 1 |
| 9    | 2     | 249   | AGG→AAG | Arg→Lys   | 1                   |
| 10   | 3     | 166   | TCA→TAA | Ser→Stop  | 1                   |
| 11   | 2     | 196   | 1-bp Deletion | Stop at 246 | 1 |
| 12   | 3     | 159   | GCC→GTC | Ala→Val   | 1                   |
| 13   | 3     | 275   | 1-bp Insertion | Stop at 305 | 1 |
| 14   | 3     | 159   | GCC→GTC | Ala→Val   | 1                   |

Figure 4. PCR-LOH analysis of a CA repeat at the p53 locus. Case numbers are given at the top of each panel. T, tumour DNA; N, normal DNA. Arrows indicate the allelic deletion in each case.

In situ end labelling (Tunel assay)

Tumour tissue from each patient was fixed and embedded in paraffin, and sections 5 μm thick were made according to routine histological procedures. The in situ detection of apoptotic cells was visualized according to the protocol of Wijsman et al (1993). After rehydration with phosphate-buffered saline (PBS), the slides were treated with terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP for 1 h at 37°C. After washing three times with PBS, peroxidase-conjugated avidin was applied, and the slides were incubated for another 1 h at 37°C, then incubated with DAB for another 15 min. Finally, the slides were washed three times with PBS and mounted with the mounting solution. The number of Tunel-positive cells was quantified in the light microscope and was expressed as cells per surface area. Five randomly chosen areas in each section were counted. Sections were also processed for routine staining with haematoxylin and eosin (HE).

DNA extraction

For the analysis of p53 mutation, genomic DNA was extracted from paraffin-embedded tissues. First, 5-μm-thick sections on glass slides were trimmed to remove as non-tumorous tissue as possible. Then they were stripped and deparaffinized with xylene; the DNA was extracted with PCR buffer containing Nonidet P-40 and Tween 20 in a total volume of 90 μl and was subjected to analysis of p53 mutations. For the analysis of DNA fragmentation on agarose gel, fresh endoscopic biopsy samples were taken through colon fibre examination during HCR therapy (day 5–10 after HCR therapy starts), and the DNA was extracted from biopsy specimens in six cases during HCR therapy, as described above.

PCR-SSCP (single-strand conformation polymorphism) analysis

A set of PCR primers flanking intron/exon junctions 4–8 of the p53 gene were used based on the genomic sequence data by Bukhman et al (1988). The nucleotide sequence of the primers were as follows: 5'-CTCTCTCCAGTACTCCCTCCTGC-3', 5'-GCCCGAGCTGCTACCATGCCT-3'. For exon 5, 5'-CGACAGGGTGGTGTTGCCTGCAAACT-3'. For exon 6, 5'-GGCTCTCATCTGGGCTGTG-3'. For exon 7, 5'-CTCTCTTGCTGCTCCTGCAAACT-3'. For exon 8 and 5'-GGGCTCTCATCTGGGCTGTG-3'. For exon 9. For each patient, the five primer pairs were used in individual PCRs to amplify the p53-coding sequences in genomic DNA from their tumour and from normal mucosa. PCR was performed in a thermal cycler (Astek, Fukuoka). The PCR reaction mixture (total 5 μl) contained genomic DNA (50 ng), 1 μM of each deoxynucleotidyl triphosphate (dNTP), 1 μM 32P-end-labelled primer, 6.7 mM magnesium chloride, 67 mM Tris-HCl (pH 8.8) and Taq polymerase (0.1 units, Perkin Elmer Cetus, Norwalk, CT, USA). Thirty cycles consisting of 60 s at 94°C, 45–60 s at 60°C and 2 min at 72°C were performed. The reaction mixture was denatured at 80°C for 5 min and was applied to a 6% polyacrylamide gel containing 45 mM

was performed in three or four different fields of microscopic view by clinical doctors and confirmed by the pathologists.

pathological investigation. The histological changes observed after the preoperative treatment were classified as follows: grade 0, no remarkable changes; grade 1, swelling of cells, enlarged vesicles, pyknosis of nuclei and vacuolated cytoplasm (< 25%); grade 2, cell nests consisting of markedly damaged cells, often exhibiting a moth-eaten appearance and simplified granular structures; and grade 3, extensive degenerative changes and fibrosis (< 75%), as previously described (Takahashi et al, 1982, 1993; Ichikawa et al, 1996). Estimation of histological therapeutic effect...
Tris-borate (pH 8.3) and 4 mM EDTA with or without 10% glycerol. The gel was dried on filter paper and exposed to autoradiographic film for 10 h at –80°C.

Direct sequencing
Samples with mobility shifts altered from the normal controls were reamplified without [γ-32P]ATP and were purified using the QIAquick-spin PCR purification kit (QiaGen, Chatsworth, CA, USA). The PCR products were directly sequenced by the dideoxy termination method using end-labelled sequencing primers and the T7 Sequencing kit (Pharmacia LKB). Products were electrophoresed on 8% polyarylamide gels containing 7 M urea. Sequencing was performed at least twice on both sense and antisense strands.

Allelic deletion analysis of 17p
To measure allelic deletions, PCR-LOH (loss of heterozygosity) analysis using a microsatellite marker (TP53) (Jones et al, 1992) within the p53 gene was performed (Baker et al, 1989). In a few cases in which the probe was not informative, additional markers (Gyapay et al, 1994) at chromosome 17p13 were analysed.

Statistical analysis
Associations between p53 mutations and the apoptosis rate were evaluated using the χ2 test. The criterion of significance was P < 0.05.

RESULTS
Histopathological analysis
Massive fibrosis was observed in the resected specimens after HCR therapy (Figure 1 A–D). Many apoptotic cells were found in the fibrous tissue (Figure 1 A–D) as well as in the lumens of the residual cancerous tissue (Figure 1 E and F). These apoptotic cells had pyknotic or segmented nuclei, or apoptotic bodies. The tumoricidal effects of the preoperative treatment were evaluated microscopically in the resected specimens. Of the 28 patients who had preoperative treatment, 21 cases (75%) demonstrated a histological anti-tumour effect (grade 1, seven cases; grade 2, 13 cases; grade 3, eight cases). Such histological findings could not be detected in cases without HCR therapy. Macroscopic tumour shrinkage did not always correlate with the histological effect (data not shown), so only the histological examination was used to estimate the anti-tumour effect in this study.

Tunel staining
Tunel-stained nuclei were scattered in the fibrous tissue and tubular lumens of the residual cancerous tissue (Figure 1B, D and F). Apoptosis was more easily observed in rectal cancers after treatment with HCR therapy than in non-treated rectal cancers. An intense Tunel signal was observed even in ordinary, non-pyknotic nuclei of tumour cells, and occasionally in nuclear fragments corresponding to apoptotic bodies. Necrotic foci of tumour cells and nuclear ghosts showed faint, diffuse staining, implying non-specific incorporation of nucleotides. Intense Tunel signal was observed in

Figure 5  PCR-SSCP analysis of the p53 gene. The case number is shown at the bottom of each lane. Lane N is control DNA. Cases 16 and 21 (exon 5) and 17 (exon 7) show different mobilities

Figure 6  Direct genomic sequence analysis of the p53 gene in the cases shown in Figure 5. Mutated sequences were compared with a control DNA (N) containing the normal p53 sequence. Case 16, codon 175 (G→A) in exon 5; case 21, codon 166 (C→A) in exon 5; case 17, codon 248 (C→T) in exon 7
some ordinary non-pyknotic nuclei of tumour cells that on conventional microscopic assessment of apoptosis would not be considered as undergoing apoptosis. No signals were detected in mitotic cells or inflammatory cells in the tumour stroma.

**DNA fragmentation on agarose gel**

Genomic DNA was extracted from fresh endoscopic biopsy specimens during HCR therapy in six cases. Typical DNA fragmentation was observed in case nos. 11 and 12 (wild-type p53) and case nos. 25 and 28 (mutant p53) (Figure 2). Typical DNA fragmentation was not detected in another two cases after HCR therapy, probably because of contamination of normal tissue or ineffectiveness of HCR therapy.

**Relation of therapeutic effect and apoptotic rate**

The number of apoptotic cells in the treated group increased with increasing grade of therapeutic effect (*P < 0.05, **P < 0.01 compared with control, Figure 3) significantly. Therapeutic effect correlated closely with the apoptotic rate, suggesting that this combination therapy acts through the induction of apoptosis.

**Allelic deletion analysis of 17p**

Chromosome 17p allelic loss was detected in 14 of 28 tumours (50%) by dinucleotide-repeat polymorphisms (Table 1). Thirteen of these 14 tumours also contained p53 gene mutations, but one case with allelic loss did not have a p53 mutation in the remaining allele (Table 1). Typical data is shown in Figure 4.

**SSCP analysis and direct sequencing**

Fourteen of 28 rectal cancers revealed mobility shifts in PCR-SSCP analysis (Figure 5). Typical data is shown in Figure 5. The position and type of the p53 gene mutation was identified by direct sequencing (Figure 6 and Table 1). The incidence of mutations in the treated group were distributed as follows: (four of seven; 57.1%) grade 1 tumours (resistant tumour), 7 of 13 (53.9%) grade 2 tumours (intermediate tumour) and three of the eight (37.5%) grade 3 tumours (sensitive tumour) (Table 1). The point mutations correlated well with allelic loss at chromosome 17 in this study, as other investigators have described (Baker et al., 1989).

**DISCUSSION**

Various adjuvant therapies for rectal cancers have aimed to prevent local recurrence, which is the most predominant prognostic factor for patients with advanced rectal cancer (Gerard et al., 1988; Neto et al., 1989; Pahlman et al., 1990; Stockholm Rectal Cancer Study Group, 1990). Among various adjuvant therapies, the most successful one is high-dose radiation or radiation combined with other therapies. We have developed a novel preoperative therapy combining radiation, intraluminal hyperthermia and 5-fluorouracil suppositories for advanced rectal cancer (Takahashi et al., 1982, 1993; Ichikawa et al., 1996). This therapy causes a striking tumoricidal effect and results in improved prognosis (Takahashi et al., 1982, 1993; Ichikawa et al., 1996).

Sensitivity or resistance of tumour cells to ionizing radiation and anti-cancer agents has substantial clinical consequences. There is evidence that ionizing radiation and several chemotherapeutic agents used to treat cancers act through the induction of apoptosis (Kastan et al., 1991; Kuerbitz et al., 1992; Lee et al., 1993; Lowe et al., 1993; Slichenmeyer et al., 1993; Kerr et al., 1994; Milas et al., 1994). 5-FU is one of the most commonly used chemotherapeutic agents for the treatment of cancers and has been shown to produce apoptosis in malignant cells (Barry et al., 1990; Shchpotin et al., 1994). Hyperthermia is also known to enhance the cytotoxic effect of radiation (Nevaldine et al., 1994; Bisht et al., 1995). Many experiments have shown the ability of mild hyperthermia to enhance apoptosis in human and murine neoplastic cells (Takano et al., 1991; Tomasovic et al., 1994). Although the mechanism of apoptosis induction by both hyperthermia and 5-FU has not yet been clarified, it is highly possible that these different treatments — irradiation, 5-FU and hyperthermia — result in additive or synergistic anti-tumour effects through apoptosis.

The p53 gene regulates apoptosis in some tissues (Lowe et al., 1993; Chiou et al., 1994; Dole et al., 1994) and has been shown to directly affect the sensitivity of cancer cells to these agents (Kerr et al., 1994; Milas et al., 1994). The p53-dependent induction of apoptosis by γ-radiation is of particular interest in the light of observations that suggest that p53 mutations increase resistance to ionizing radiation (Lee et al., 1993) and that radiation-induced apoptosis is abrogated in cells lacking wild-type p53 protein (Clarke et al., 1993; Lowe et al., 1993, 1994; Merritt et al., 1994). In contrast, the independence of radiosensitivity from p53 function has also been reported in colon adenocarcinoma RKO cells (Slichenmeyer et al., 1993) and head and neck cancer cell lines (Brachman et al., 1993). These differences appear to be related to the apoptotic potential of the cell lines used in these different studies. These studies highlight the importance of cellular context to the evaluation of the biological properties of the p53 tumour suppressor and its effector genes. In order to examine the mechanism of action of our therapy, and to predict tumour sensitivity to it, we examined the apoptosis rate and p53 status in rectal cancers and examined the relationship between the therapeutic efficacy, histological apoptosis rate and p53 status.

Many apoptotic cells (Tunel-positive cells) and massive fibrous changes were observed in the surgically resected specimens after HCR therapy (Figure 1). DNA electrophoresis revealed the typical pattern of DNA fragmentation, a marker of apoptosis (Figure 2). The therapeutic effect correlated with the apoptotic rate, suggesting that this combination therapy acts through the induction of apoptosis (Figure 3). The p53 status of the tumours correlated with therapeutic efficacy to some extent but the correlation was not statistically significant. This may indicate that it is difficult to predict the sensitivity of rectal cancers to HCR therapy using only p53 status. According to our preliminary experiments, endoscopic biopsy specimens removed and examined during HCR therapy show a high apoptosis rate in responsive tumours. This rate was higher than in surgically resected specimens after HCR therapy, probably because most apoptotic cells were destroyed and disappeared during preoperative HCR therapy (data not shown); this suggests that the apoptosis rate in endoscopic biopsy specimens taken during HCR therapy may be useful in determining the sensitivity of rectal cancers to HCR therapy. Indeed, the use of Tunel assay for assessment of DNA damage has been widely accepted as a reliable method of assessment of DNA fragmentation and hence apoptosis. However, as DNA damage may occur via mechanisms other than apoptosis, and as apoptosis has been shown to occur in colorectal carcinoma cell lines through both p53-dependent and p53-independent
mechanisms (Shaw et al, 1992), it cannot be assumed that all radiation-induced DNA fragmentation identifiable on TUNEL assays is due to apoptosis. Using TUNEL assay, we should concentrate on the relation of the therapeutic effect by HCR therapy and the apoptotic rate.

p53 gene regulates many kinds of genes, for example the apoptosis-related gene bax (Miyashita et al, 1995), the cell cycle regulator p21 (WAF1/CIP1) (Harper et al, 1993), the transcription regulator GADD45 (Kastan et al, 1992), MDM2 (Momand et al, 1992), etc. These genes may be defective in rectal cancers and thus may confuse the p53 status. Our study only looked at p53 status; further studies are necessary to analyze the state of these other genes and to determine whether the status of p53 and its regulated genes can be predictive for this treatment.

In summary, our study indicates that the pathological response to HCR therapy correlates with the rate of apoptosis with statistical significance and that it induces the therapeutic effect more significantly in rectal cancer cells with wild-type p53, although HCR therapy-induced apoptosis also occurs in some rectal cancers with mutated p53. Therefore, this combination therapy can induce additive or synergistic anti-tumour effect in rectal cancers with wild-type p53 as well as in those with mutated p53 through apoptosis, offering new therapeutic opportunities and a better prognosis.

**ABBREVIATIONS**

HCR therapy, hyperthermochemoradiotherapy; 5-FU, 5-fluorouracil; SSCP, single-strand conformation polymorphism

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