basis of sequence homology, eukaryotic pols can be divided into four main families, termed A, B, X and Y [9]. Family A includes mitochondrial pol γ as well as pols θ and ν; family B includes the three replicative pols α, δ and ε, and also pol ζ; family X comprises pols β, λ and μ as well as TdT; and last, family Y includes pols η, ι and κ in addition to REV1. The focus is on replicative pol inhibition as it supposes a concurrent antitumor effect because replicative pols, such as B-family pols, are essential for the cell division required for cancer cell growth. As a result of this laboratory's ongoing screening from natural materials and compounds, glycoglycerolipids from a fern and an alga have been identified that potently inhibit eukaryotic pol activities [10,11].

In higher plants, particularly in chloroplasts, the thylakoid membrane contains major glycoglycerolipids, such as monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol and sulfoquinovosyl diacylglycerol [12]. It is known that glycoglycerolipids are present in vegetables, fruits and grains [13,14], and it has been found here that spinach possesses the best glycoglycerolipid source, with the highest MGDG content, among the vegetables tested [15].

Cytidine analogs, such as gemcitabine (GEM, 2′,2′-difluoro-2′-deoxycytidine, dFdC) are widely used to treat a variety of cancers and remains in standard therapy for pancreatic cancer in adjuvant and palliative settings [16–18]. However, the GEM response rate is very low in pancreatic cancer, with only an 18% 1-year survival rate [19], which is attributed primarily to the lack of early detection and frequent metastases of primary tumors into lymph nodes and surrounding organs, such as liver and stomach [20–22]. In human cells, GEM must be metabolized by phosphorylation and catalyzed by deoxycytidine kinase to GEM-5′-monophosphate (GEM-MP), which can subsequently be phosphorylated sequentially to the di- and triphosphate forms, GEM-5′-diphosphate (GEM-DP) and GEM-5′-triphosphate (GEM-TP), respectively. Studies using LC/MS/MS have shown that GEM penetrates into cells and that clinically relevant levels of GEM was intracellularly phosphorylated for 24 h, with the converted GEMs, such as GEM-TP, pooling in the cells [23].

In this study, we focused our attention on evaluating human various cancer cell proliferation effects caused by a combination of GEM (Fig. 1A) or its phosphorylated compounds, such as GEM-TP (Fig. 1B), with spinach MGDG (Fig. 1C). Furthermore, the most effective timing of addition of these compounds was examined. In light of the results, their implications are discussed in terms of the observed properties of GEM and MGDG based on mammalian replicative pol inhibition as well as better treatment outcomes for human pancreatic cancer.

2. Materials and methods

2.1. Materials

GEM (Fig. 1A) was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dried spinach (*Spinacia oleracea* L.) was purchased from Kodama Foods Co., Ltd. (Hiroshima, Japan). Calf thymus DNA was purchased from Sigma-Aldrich, Inc., and four 2′-deoxynucleotide-5′-triphosphates (dNTPs), including 2′-deoxyadenine-5′-triphosphate, 2′-deoxycytidine-5′-triphosphates (dCTP), 2′-deoxyganine-5′-triphosphate and 2′-deoxythymidine-5′-triphosphate (dTTP), from GE Healthcare Life Sciences, Ltd. (Uppsala, SE). Radioactive [^{3}H]-dTTP (43 Ci/mmol) was obtained from MP Biomedicals, LLC (Solon, OH, USA). All other reagents were analytical grade from Nacalai Tesque Inc. (Kyoto, Japan).

2.2. Chemical synthesis of phosphorylated GEM

N^4-benzoylgemcitabine [24] was converted to N^4-benzoyl-gemcitabine 5′-diphosphate and 5′-triphosphate by selective phosphorylation with phosphoryl chloride in triethyl phosphate [25], followed by further phosphorylation using a phosphorimidazolidate method [26]. GEM-MP, GEM-DP and GEM-TP were obtained by

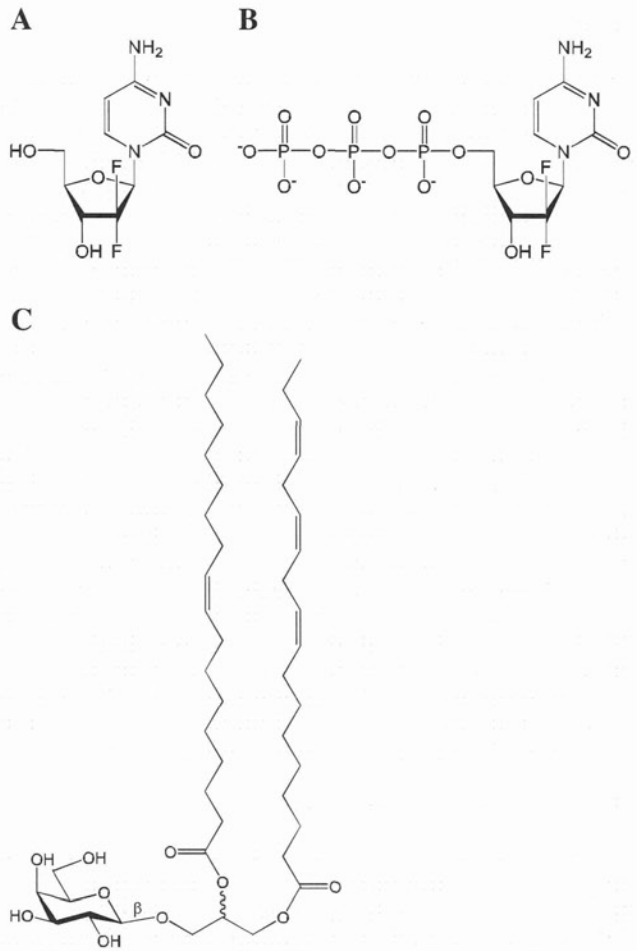

Fig. 1. Chemical structure of GEM (A), GEM-TP (B) and MGDG (C).

treatment of the corresponding N^4-benzoates with 1 M NH_4OH at room temperature for 1 day. When analyzed by high performance liquid chromatography (HPLC) equipped with a TSKgel DEAE-2SW column, (4.6 mm×25 cm, Tosoh Bioscience LLC, King of Prussia, PA, USA), elution using 0.15 M potassium phosphate buffer at pH 6.95 and containing 20% CH_3CN, and spectrophotometric detection at 270 nm, the purities of GEM-MP, GEM-DP and GEM-TP were confirmed to be greater than 99%, 96% and 98%, respectively.

2.3. Isolation of MGDG from spinach

Dried spinach was extracted with ethanol and the extract diluted to 70% aqueous ethanol and then subjected to Diaion HP-20 (Sigma-Aldrich, Inc.) column chromatography eluted with 95% aqueous ethanol. The eluted solution was evaporated to dryness, the residue redissolved in chloroform, and the resulting solution subjected to silica gel (PSQ60B, Fuji Silysia Chemical Ltd., Tokyo, Japan) column chromatography. After washing the column with chloroform/ethyl acetate (1/1, v/v), the column was eluted with ethyl acetate and the eluate purified using Sep-Pak C_{18} (Waters Corp., Milford, MA, USA) column chromatography eluted with methanol. The MGDG fraction was evaporated, yielding purified MGDG at ~98% of the chemical purity that can be obtained by normal-phase silica gel (Shiseido Co., Ltd., Tokyo, Japan) HPLC coupled with an evaporative light scattering detector (M&S Instruments Inc., Osaka, Japan) and eluted with chloroform/methanol (1/1, v/v).

2.4. Pol assays

Mammalian pols with high activities were purified according to our previous report [27], and standard pol reaction mixtures for pols α and β have been previously described [28]; those for pol γ and for pols δ and ε described by Umeda et al. [29] and Ogawa et al. [30], respectively; for pols η, ι and κ the same as for pol α; and for pol λ, μ and TdT the same as for pol β. For pol reactions, activated DNA, in the form of bovine deoxyribonuclease I-treated calf thymus DNA, and four dNTPs containing [^{3}H]-dTTP were used as the DNA template-primer substrate and nucleotide substrate, respectively.

The protocol for pol inhibitory assay was shown in Supplemental Fig. 1. MGDG was dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. Then, 4 μL aliquots of the solutions were mixed with 16 μL of each enzyme (0.05 units) in 50 mM Tris–HCl at pH 7.5, containing 1 mM dithiothreitol, 50% glycerol (by vol), and 0.1 mM EDTA, and held at 0 °C for 10 min. GEM or a phosphorylated GEM was dissolved in distilled phosphate buffered saline (PBS) at various concentrations, and 4 μL aliquots mixed with 16 μL of the standard pol reaction mixture containing the DNA template-primer/nucleotide substrates and held at 0 °C for 10 min. These MGDG–enzyme mixtures in 8 μL volumes were then added to 20 μL of GEM–pol reaction mixture, and incubated at 37 °C for 60 min. Activity without inhibitor was considered 100% and relative activity determined for each inhibitor concentration. One unit of pol activity was defined as the amount of each enzyme that catalyzed incorporation of 1 nmol dNTP into synthetic DNA template-primers in 60 min, at 37 °C and under normal reaction conditions [28].

2.5. Cell culture and cell viability assessment

Human pancreatic cancer cell lines, such as BxPC-3, MIAPaCa2 and PANC-1, and human other cancer cell lines, such as lung (A549), prostate (DU145 and PC3), cervix (HeLa), hepatocellular liver (HepG2) and breast (MCF-7), were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI1640 medium supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL) and 1.6 mg/mL $NaHCO_3$. A human normal cell line, primary human dermal fibroblast cells (PHDFCs) and its complete medium kit were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). GEM, phosphorylated GEMs and MGDG cytotoxicities were investigated by inoculating ~1×10^3 cells/well in 96-well microtiter plates and the addition of these compounds to various concentrations. After incubation for 72–96 h at 37 °C in a humidified atmosphere of 5% CO_2/95% air, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution was added to a final 0.6 mg/mL in Milli-Q purified water for 2 h [31], after which time the medium was discarded and the cells lysed in DMSO. The A_{540} was then measured in a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA).

2.6. Assessment of apoptotic cells

The induction of apoptosis by the test compounds was evaluated by a DNA fragmentation assay using the APO-Direct™ Assay Staining kit (BD Bioscience Pharmingen, San Diego, CA, USA). This assay is a single-step method for labeling DNA breaks with fluorescein isothiocyanate-2′-deoxyuridine-5′-triphosphate (FITC-dUTP) followed by analysis using flow cytometry (FACS Calibur, Becton, Dickinson & Co., Franklin Lakes, NJ, USA). MIAPaCa2 cells were treated with GEM (50 nM) alone, MGDG (70 μM) alone, or a GEM–MGDG combination for 24 h. Cultured cells were harvested by trypsinization, washed with PBS, fixed in 1% paraformaldehyde for 15 min, and then in 70% ethanol overnight at −20 °C. Fixed cells were next combined with a DNA labeling solution containing conjugated FITC for 30 min. The stained cells were then resuspended in PBS and subjected to flow cytometry analysis. The resulting data were analyzed with CellQuest software (Becton, Dickinson & Co.) and apoptotic cells quantitated as a percentage of the total number of cells.

3. Results

3.1. Effects of GEM and phosphorylated GEMs on mammalian pol activities

Initially, GEM (Fig. 1A) and its phosphorylated compounds, such as GEM-MP, GEM-DP and GEM-TP (Fig. 1B), were chemically synthesized and their *in vitro* biochemical action investigated. Next, the inhibition of four mammalian pols, calf pol α, rat pol β, human pol γ and human pol κ, by each compound at 10 μM was investigated. Pols α, β, γ and κ were used as representative of the B, X, A and Y families of pols, respectively [6–8]. Among these compounds, GEM-TP was the strongest inhibitor of these mammalian pols, while GEM had no effect (Fig. 2). The inhibitory effect of these four compounds on mammalian pols was ranked as follows: GEM-TP > GEM-DP > GEM-MP > GEM; therefore, the degree of GEM phosphorylation appeared important for pol inhibition. The inhibitory effect of GEM-TP on the activities of pols α and γ, which are DNA replicative pols in the nucleus and mitochondria, respectively, was stronger than that of pols β and κ, which are DNA repair-related pols. Table 1 shows the 50% inhibitory concentrations (IC_{50}s) of GEM-TP on the activities of 11 mammalian pols, showing a 4-fold stronger inhibition of B-family pols than X- and Y-families of pols. It is interesting that GEM-TP is an effective inhibitor of DNA replicative pols, such as pols α, δ and ε, causing a 50% inhibition of these pols at 3.4–3.8 μM.

3.2. Effects of the purified spinach MGDG on the activities of mammalian pols

MGDG, a yellow oily material, was purified from dried spinach (*Spinacia oleracea* L.), and the chemical structure analyzed by nuclear magnetic resonance, mass spectroscopy, and optical rotation. Its structure was characterized (Fig. 1C) [12], and the two acyl groups of MGDG found to be mainly palmitoleic acid (C16:1) and α-linolenic acid (C18:3).

The effects of the purified spinach MGDG on mammalian pols are shown in Table 1. This compound selectively inhibited the activities of calf pol α, and human pols γ, δ and ε, with inhibitory effects ranked as: pol ε > pol δ > pol α ≫ pol γ. The inhibitory effect on B-family pols α, δ and ε was stronger than on A-family pol γ, with IC_{50} observed at doses of 10.7–22.0 μM and 35.1 μM, respectively by family. On the other hand, MGDG did not influence the activities of X-family pols rat pol β, human pol λ, human pol μ and calf TdT, and Y-family pols human pol η, mouse pol ι and human pol κ, which

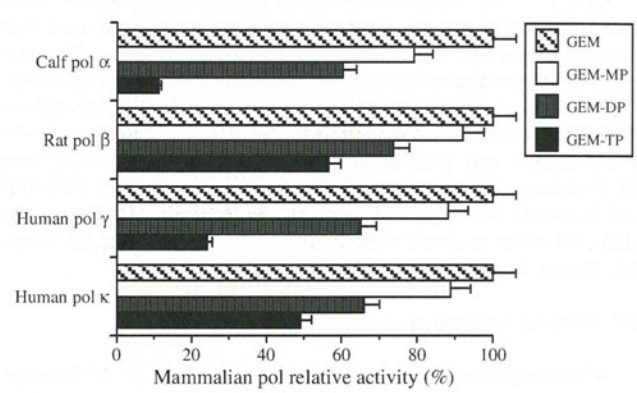

Fig. 2. Effect of GEM and phosphorylated derivatives (GEM-MP, GEM-DP and GEM-TP) on mammalian pol activities. Compounds at 10 μM incubated with each enzyme at 0.05 units; % of relative activity; activities in absence of compounds, 100%; data, means ± SD of three independent experiments.

Table 1
IC_{50} of GEM-TP and MGDG on mammalian pol activities.

Mammalian pols	IC_{50} (μM)	
	GEM-TP	MGDG
[A-Family]		
Human pol γ	7.9 ± 0.5	35.1 ± 2.1
[B-Family]		
Calf pol α	3.7 ± 0.3	22.0 ± 1.4
Human pol δ	3.8 ± 0.3	19.9 ± 1.2
Human pol ε	3.4 ± 0.2	10.7 ± 0.7
[X-Family]		
Rat pol β	18.5 ± 1.1	>200
Human pol λ	18.9 ± 1.2	>200
Human pol μ	17.2 ± 1.0	>200
Calf TdT	20.1 ± 1.2	>200
[Y-Family]		
Human pol η	15.7 ± 0.9	>200
Mouse pol ι	17.1 ± 1.0	>200
Human pol κ	16.0 ± 1.6	>200

Compounds incubated with each pol (0.05 units); 1 unit of pol activity, amount of enzyme catalyzing incorporation of 1 nmol of dNTP into synthetic DNA template-primers in 60 min at 37 °C under each enzyme's normal reaction conditions; enzyme activity in absence of inhibitor taken as 100%; data, mean ± SD (n = 3).

Table 2
Kinetic analysis of the inhibitory effects of GEM-TP and MGDG on calf pol α activity as a function of the DNA template-primer substrate dose and the nucleotide substrate concentration.

Compound	Conc. (μM)	Substrate	K_m[a] (μM)	V_{max}[a] (pmol/h)	K_i[b] (μM)	Inhibitory mode
GEM-TP	0	DNA[c]	7.80	55.6	1.35	Noncompetitive
	1	template-primer		27.8		
	2			18.5		
	3			13.9		
	0	Nucleotide[d]	1.65	29.2	0.67	Competitive
	1		3.23			
	2		6.25			
	3		11.1			
MGDG	0	DNA[c]	7.80	55.6	5.08	Noncompetitive
	5	template-primer		29.4		
	10			20.0		
	15			15.2		
	0	Nucleotide[d]	1.65	29.2	6.44	Noncompetitive
	5			12.5		
	10			8.00		
	15			5.88		

[a] Data obtained from Lineweaver–Burk plot.
[b] Data obtained from Dixon plot.
[c] That is, activated DNA.
[d] That is, four dNTPs.

suggested that MGDG was a selective inhibitor of DNA replicative pols, such as A and B-families of pols, among mammalian pol species.

3.3. Mode of inhibition of calf pol α by GEM-TP and MGDG

Next, to elucidate the mechanism of GEM-TP and MGDG's selective inhibition for mammalian pol species, the inhibitory mode of these compounds against calf pol α, a representative DNA replicative pol, was investigated. Activated DNA and four dNTPs were used as synthetic DNA template-primer substrate and nucleotide substrate, respectively, for kinetic analysis. The extent of inhibition as a function of the DNA template-primer substrate or nucleotide substrate concentration was measured (Table 2).

The collected data, expressed as Lineweaver–Burk plots (double reciprocal plots), showed that GEM-TP inhibited pol α activity noncompetitively with the DNA template-primer substrate because there was no change in the apparent Michaelis constant (K_m) of 7.80 μM, whereas decreases of 55.6, 27.8, 18.5 and 13.9 pmol/h in maximum velocity (V_{max}) were observed in the presence of GEM-TP at 0, 1, 2 and 3 μM, respectively. On the other hand, pol α inhibition by GEM-TP was competitive with the nucleotide substrate, with the V_{max} unchanged at 29.2 pmol/h and the K_m increased from 1.65 to 11.1 μM in the presence of 0–3 μM GEM-TP. The inhibition constant (K_i), obtained from Dixon plots, was found to be 1.35 μM for the DNA template-primer substrate and 0.67 μM for the nucleotide substrate. As the K_i value for the nucleotide substrate was ~2.0-fold less than that for the DNA template-primer substrate, it was concluded that GEM-TP had a greater affinity for the nucleotide substrate binding site than for the DNA template-primer binding site of the pol α protein.

Pol α inhibition by MGDG was noncompetitive with respect to both the DNA template-primer substrate and the nucleotide substrate. For the DNA template-primer substrate, there was no change in the apparent K_m (7.80 μM), while the V_{max} decreased from 55.6 to 15.2 pmol/h for the DNA template-primer substrate in the presence of 0–15 μM of MGDG. The induced inhibition of pol α activity by MGDG was noncompetitive with respect to the nucleotide substrate, with the K_m unchanged at 7.80 μM and the V_{max} for the nucleotide substrate 4.97-fold less in the presence of 15 μM of MGDG. From the Dixon plots, the K_i was 5.08 and 6.44 μM for the DNA template-primer substrate and the nucleotide substrate, respectively. Therefore, MGDG had a 1.3-fold greater affinity for the DNA template-primer substrate binding site than for the nucleotide substrate-binding site of the pol α protein. These results suggested that GEM-TP, a dCTP analog, competed with dCTP to interact with the nucleotide substrate binding site of pol α, whereas MGDG bound to another site on pol α.

To confirm the pol α noncompetitive inhibitory mode against DNA template-primer substrate by GEM-TP and MGDG, their binding should be precisely analyzed by gel mobility shift assay as described previously [32]. Mammalian pol α is made up of four subunits, i.e. p180, p68, p54 and p46 [6,33]. The largest subunit, p180, and the smallest subunit, p46, have the catalytic DNA polymerase and DNA primase activities, respectively. The other subunits, p68 and p54, have no known enzyme activities. We constructed the core domain (p110) in which we deleted the amino-terminal (1–329) and the carboxyl-terminal (1280–1465) regions of the largest (p180) subunit of mouse pol α, and then the recombinant proteins were expressed and purified [34]. The core domain of pol α protein (0.2 nmol) was bound to M13 single-stranded DNA (ssDNA, 2.2 nmol; nucleotide), such as DNA template-primer substrate, and were shifted in the gel. When the *I/E* ratio (the molecular ratios of inhibitor and enzyme) was 100, both GEM-TP and MGDG had no interfered with the complex formation between M13 ssDNA and pol α (Supplemental Fig. 2). These results suggested that GEM-TP and MGDG did not compete with M13 ssDNA and had no interfered with the binding of DNA template-primer substrate to the largest subunit of pol α.

3.4. Inhibition by GEM-TP of mammalian pol activities with or without MGDG

As the pol α inhibitory mode of GEM-TP against nucleotide substrate was different from that of MGDG in kinetic analyses, the question of whether MGDG could enhance pol inhibition by GEM-TP was investigated. As described in Materials and methods, GEM-TP and MGDG were preincubated with the standard pol reaction mixtures containing DNA template-primer/nucleotide substrates and pol enzymes. The concentrations of GEM-TP and MGDG added were one half of the IC_{50} for each pol and 20 μM, respectively. In this study, calf pol α, rat pol β, human pol γ and human pol κ were used as representative of pol families of B, X, A and Y, respectively.

The rate of pol relative activity by GEM-TP at one-half of the IC_{50} value for each mammalian pol was set at 1 (pol relative activity of ~75%), and the ratios of the activities by MGDG alone and in combination with GEM-TP are shown in Fig. 3. As 20 μM MGDG alone did not inhibit the activities of pols β and κ, the rate of pol relative activity was 1.33

(this pol relative activity at 100%), and pols α and γ were slightly inhibited. The combination of GEM-TP with MGDG significantly enhanced the inhibition of pols α and γ, with the inhibitory effect for pol α 2-fold stronger than that for pol γ. The effect of the GEM-TP/MGDG combinations on pols β and κ activity was the almost same as with GEM-TP alone. These results suggested that mixtures of GEM-TP and MGDG showed synergistic effects on the inhibitory activity of DNA replicative pols, such as with pols α and γ according to the differences in the mode of pol inhibition.

3.5. Effects of GEM, phosphorylated GEMs, and MGDG on cultured human cancer cells

As pols conduct cellular DNA synthesis [4–6] and are essential for DNA replication, repair, and subsequent cell division, inhibition of these enzymes will lead to cell death, particularly under proliferative conditions, such that pol inhibitors can be considered potential agents for cancer chemotherapy. GEM is clinically used in the treatment of various carcinomas and, in particular, possesses greater clinical benefit to pancreatic cancer patients compared with conventional medications [35]. Thus, the effect of GEM, phosphorylated GEMs and MGDG on the cultured cell growth was investigated in human various cancer cells, such as pancreatic carcinoma cell lines.

Here, GEM dose-dependently suppressed cell proliferation of three human pancreatic cancer cell lines, BxPC-3, MIAPaCa2 and PANC-1, with a 50% lethal dose (LD_{50}) of 3.0, 15.1 and 17.6 nM, respectively (Fig. 3A–C), suggesting that GEM's effect on BxPC-3 cell growth inhibition was the strongest among the three lines. GEM might have been able to penetrate these human cancer cells, undergo phosphorylation to GEM-TP, which then reached the nucleus, and inhibited pol activities, with the specific inhibition of DNA replicative pol activity leading to cell growth suppression. On the other hand, phosphorylated GEM, such as GEM-MP, GEM-DP and GEM-TP, had no effect on cancer cell growth, which suggested that these compounds did not penetrate the cells.

MGDG also suppressed the growth of these human pancreatic cancer cell lines (Fig. 4D), with the growth suppression involving concentrations similar to those for inhibition of mammalian pols α, γ, δ and ε by MGDG, suggesting that the cause of MGDG's intracellular influence in cancer cells may be its effect on pol activities, particularly

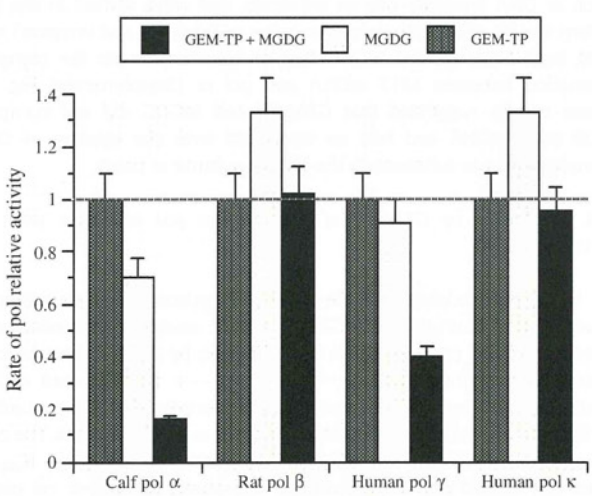

Fig. 3. Inhibitory effect of the combination of GEM-TP with MGDG on mammalian pol activities. GEM-TP and MGDG concentrations at one-half of IC_{50} of each pol (1.9, 9.3, 4.0 and 8.0 μM for pols α, β, γ and κ, respectively) and 20 μM, respectively; GEM-TP and MGDG preincubated with standard pol reaction mixtures, one containing DNA template-primer/nucleotide substrate and the other 0.05 units of pol enzyme, for 10 min at room temperature and these solutions were then mixed and incubated for 60 min at 37 °C; pol relative activity by GEM-TP at one-half of IC_{50} of each pol normalized to 1, and the ratios of the activity shown; data, means ± SD of three independent experiments.

on the replicative pols α, δ and ε. The cytotoxic dose was almost the same range of enzyme inhibitory concentrations (LD_{50} and IC_{50} for MGDG, 15.1–22.0 and 10.7–35.1 μM, respectively, Fig. 4D and Table 2), suggesting that MGDG penetrated human cancer cells and effectively inhibited nuclear and mitochondrial replicative pol activities. It was considered here that GEM penetrated into cells more effectively than MGDG, as GEM exhibited a ~1000-fold stronger toxicity than MGDG.

Next, additional experiments were carried out on GEM and MGDG in the latter part of this study. As shown in Table 3, the influence of GEM on the growth of human other cancer cell lines, such as lung (A549), prostate (DU145 and PC3), cervix (HeLa), hepatocellular liver (HepG2) and breast (MCF-7), and a human normal cell line, PHDFCs, was similar to that obtained for the pancreatic cancer cell lines. The LD_{50} values of MGDG for the three pancreatic cancer cell lines were >1.6-fold higher than those for other cancer cell lines. In contrast, MGDG exhibited no effect toward PHDFCs; therefore, MGDG from spinach, which is a food component, could selectively inhibit human cancer cell growth.

3.6. Cell growth inhibition by combinations of GEM with MGDG of cultured human cancer cells

The cell growth suppression effects of combined GEM and MGDG treatments were determined using various timed treatments in which three human pancreatic cancer cell lines were treated with mixtures of GEM & MGDG, GEM → MGDG, or MGDG → GEM [(a), (b), or (c) in Fig. 5A, respectively] for 48 h, the cells were cultured 48 h, and then the cytotoxicity assessed by MTT assay. The GEM and MGDG concentrations added were one half of the LD_{50} for each cell line and 20 μM, respectively.

With the rate of cell viability by GEM at one-half of the LD_{50} set at 1, as described above, the ratios of the viability by MGDG alone and the combination of GEM with MGDG were determined (Fig. 5B). The cell viability by 20 μM MGDG alone was 32.5–43.3% in the three human pancreatic cancer cell lines, and the ratio of cell viability of MGDG and GEM at one-half of the LD_{50} value was 0.43–0.58. In combining GEM and MGDG in treatments, the effects of schedules (a) (i.e., GEM & MGDG) and (b) (i.e., GEM → MGDG) were almost the same strength as MGDG alone, whereas schedule (c) (i.e., MGDG → GEM) showed synergistic effects on overall cytotoxicity, showing clearly that the latter combination was superior. The synergistic effect of schedule (c) on BxPC-3 cells, MIAPaCa2 cells and PANC-1 cells was 6.60, 28.7 and 13.0-fold stronger, respectively, than MGDG treatment alone; therefore, these effects showed the same tendency in all three cell lines. In contrast, the enhancement of cell growth suppression by combination of GEM and MGDG was not shown against the other human cancer cell lines tested, because the cytotoxic rations of MGDG/schedule (c) (i.e., MGDG → GEM) were 0.94–1.47 (Table 4). These results suggested that the timing of the application of GEM and MGDG might be very important for the suppression of human pancreatic cancer cell proliferation in the use of clinical GEM–MGDG combination treatments.

3.7. Apoptosis induction in cultured human pancreatic cancer cells by combinations of GEM with MGDG

As MIAPaCa2 cells showed the highest synergistic effect from schedule (c) on cell growth suppression among the human pancreatic cancer cell lines tested (Fig. 4A–C), the effects on this cell line were investigated further regarding the induction of apoptosis by a treatment combining 50 nM GEM with 70 μM MGDG as half time of schedule (c) (i.e., MGDG → GEM [GEM for 12 h after treated with MGDG for 12 h]) in Fig. 5A. In the DNA fragmentation assay using flow cytometry, the apoptotic cell rate of untreated MIAPaCa2 cells was normalized to 1, and treatment of both GEM alone and MGDG alone was found to slightly induce apoptosis (Fig. 6). On the other

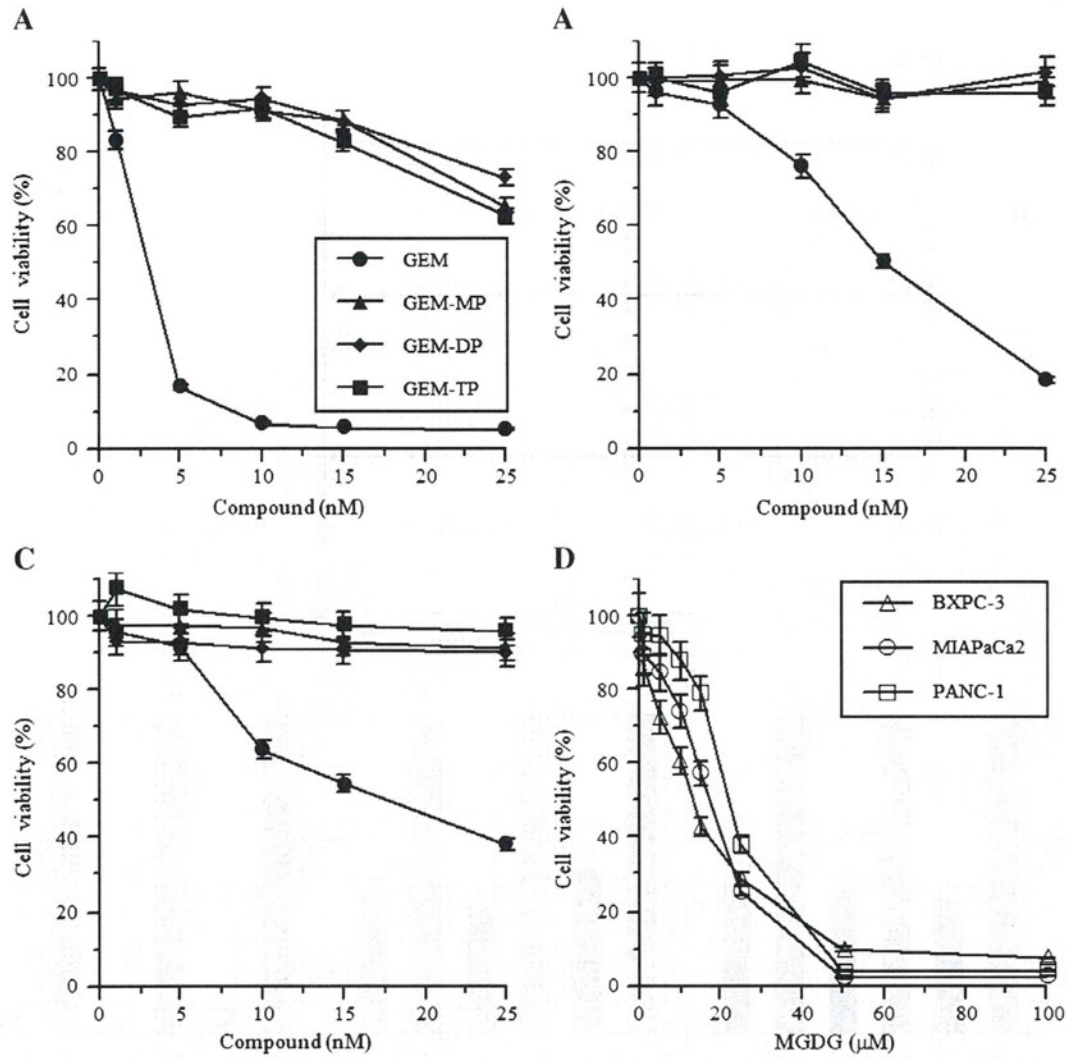

Fig. 4. Dose–response curves of growth inhibition on cultured human pancreatic cancer cell lines by GEM, phosphorylated GEM and MGDG. (A–C) GEM and phosphorylated derivatives, GEM-MP, GEM-DP and GEM-TP, added to cultures of BxPC-3 cells, MIAPaCa2 cells and PANC-1 cells (A–C, respectively); (D) MGDG added to cell cultures, each incubated with various concentration of each compound for 96 h; cell proliferation determined by MTT assay [31]; % of relative activity; activities in absence of compounds, 100%; data, means ± SD of three independent experiments.

hand, the present combination of GEM with MGDG significantly enhanced the appearance of apoptotic cells, suggesting that the synergistic effect on cell proliferation suppression may also induce apoptosis (Fig. 5B(c)). The direct DNA fragmentation of MIAPaCa2 cells was analyzed by electrophoresis, and these cells clearly underwent DNA fragmentation by GEM, MGDG and the combination of MGDG → GEM (Supplemental Fig. 3). It is thus considered that DNA damage in the cells was caused immediately with GEM treatment and that pol inhibition by phosphorylated GEM, such as GEM-TP and MGDG might have promoted apoptosis.

Table 3
LD_{50} of GEM and MGDG on the proliferation of human cancer and normal cells.

	Cell line	LD_{50}	
		GEN (nM)	MGDG (μM)
Cancer cells	BxPC-3 (pancreatic cancer)	3.0 ± 0.4	15.1 ± 1.7
	MIAPaCa2 (pancreatic cancer)	15.1 ± 1.6	18.8 ± 2.0
	PANC-1 (pancreatic cancer)	17.6 ± 1.8	22.0 ± 2.1
	A549 (lung cancer)	17.1 ± 1.7	8.0 ± 0.9
	DU145 (prostate cancer)	4.4 ± 0.5	9.4 ± 1.0
	HeLa (cervix cancer)	23.4 ± 2.2	8.8 ± 1.0
	HepG2 (liver cancer)	2.9 ± 0.3	0.33 ± 0.05
	MCF-7 (breast cancer)	3.6 ± 0.4	6.3 ± 0.7
	PC3 (prostate cancer)	7.7 ± 0.9	8.3 ± 1.0
Normal cells	PHDFCs (primary human dermal fibroblast cells)	5.0 ± 0.6	> 100

The 9 human cancer cell lines and a normal human cell line, PHDFCs, were incubated with each compound for 48 h. Cell viability was determined using the MTT assay; data, mean ± SD ($n = 5$).

4. Discussion

Pancreatic cancer remains one of the most deadly and chemo-resistant cancers. Multiple studies have evaluated various chemotherapeutic agents, but few have produced significant improvement in patient survival [36]. GEM remains the first-line drug for the treatment of advanced pancreatic cancer, either alone or in combination with other chemotherapeutic agents, but the inherent resistance of pancreatic cancer to currently available chemotherapeutic agents presents a major challenge [36–38]. Identification of robust new molecular targets and relevant pathways to produce greater sensitivity to chemotherapeutic agents is a top priority [36]. Several mechanisms, involving factors such as STAT3 and NF-κB, have been reported to induce GEM resistance in

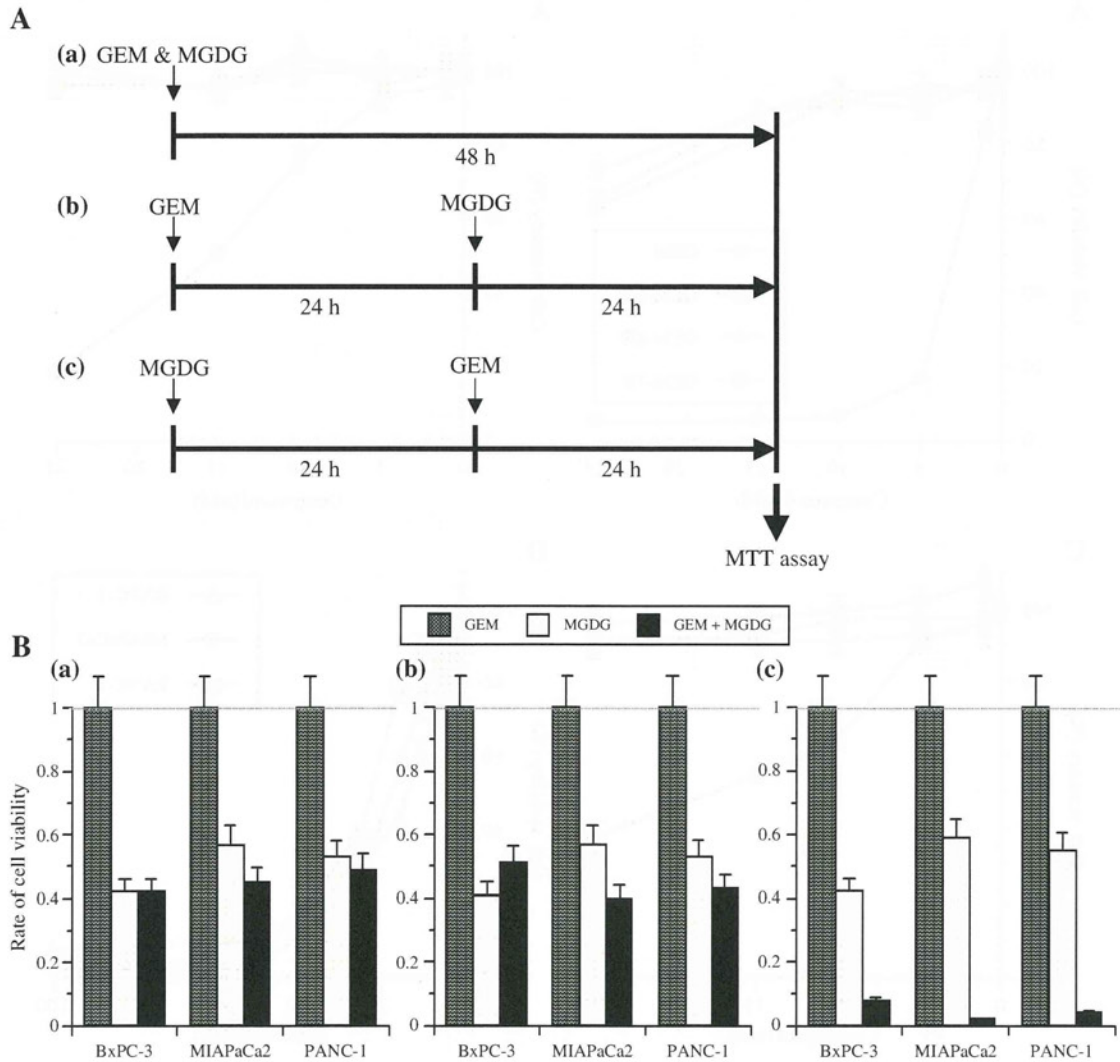

Fig. 5. Inhibitory effect of the combinations of GEM and MGDG on cultured human pancreatic cancer cell proliferation. (A) Treatments combining GEM and MGDG for three cell lines; GEM and MGDG concentrations added at one-half of LD_{50} of each cell line (1.5, 7.6 and 8.8 nM for BxPC-3 cells, MIAPaCa2 cells and PANC-1 cells, respectively) and 20 μM, respectively; (a), mixture of GEM and MGDG exposure for 48 h (i.e., GEM & MGDG); (b), GEM exposure for 24 h and then MGDG exposure for 24 h (i.e., GEM→MGDG); and (c), MGDG exposure for 24 h and then GEM exposure for 24 h (i.e., MGDG→GEM). (B) Cytotoxicity of cancer cells by MTT assay following treatments; cell viability by GEM at one-half of LD_{50} of each cell line normalized at 1, ratios of viability shown; data, means ± SD of three independent experiments.

preclinical models [39,40]. However, clinically, only one study has reported a survival improvement, of <2 weeks, with a combination of erlotinib and GEM [41], which highlights the urgent need to find novel agents against relevant molecular targets to produce new, more effective treatments. In this study, the focus was on DNA replicative pol species, especially the B-family pol α among 15 mammalian pols, as a new, metabolically critical molecular target to GEM treatment for human pancreatic cancer.

GEM is a nucleoside analog of dCTP, in which the hydrogen atoms on the 2′ carbon of deoxycytidine are replaced by fluorine atoms (Fig. 1A), and can replace dCTP, one of the building blocks of nucleic acids during DNA replication by pols. Thus, the inhibitory mode of GEM-TP for pol α, a DNA replicative pol, was predictably competitive with the nucleotide substrate (Table 2). In the cells tested, GEM was enzymatically phosphorylated, such as to GEM-TP, and then arrested cell growth (Fig. 4A–C), as only one additional nucleoside could be attached to the "faulty" nucleoside, resulting in cytotoxicity of both normal and cancer cells (Table 3) and induction of apoptosis (Fig. 6).

MGDG is a glycoglycerolipid non-nutrient compound found in vegetables, grains and fruits and, although its content varies among these plants [42], it is ingested daily in food. MGDG's chemical structure includes two acyl groups derived from two fatty acid molecules (Fig. 1). Wheat flour MGDG includes non-*n*-3 fatty acids, such as *n*-6 linoleic acid and saturated fatty acids [43], and the fatty acyl component appears to influence MGDG's antitumor effects. Therefore, the present findings suggested that researchers should observe and pay close attention to the lipid content and inherent fatty acid composition in MGDG studies.

In this study, MGDG, isolated from spinach (*Spinacia oleracea* L.), was found to a selective inhibitor of mammalian pols α, γ, δ and ε, while having no effect on other mammalian pols, such as repair-related pols β, η, ι, κ, λ, μ and TdT (Table 1). The mechanism of selective inhibition between A- and B-families pols, which are DNA replicative pols, and X- and Y-families of pols, which are DNA repair-related pols, by MGDG remains unclear and will require further study. This spinach MGDG prevented cell growth in three cancer cell lines (Fig. 4D) and, as the MGDG LD_{50}s on cell growth were almost the same of MGDG's IC_{50}s on pol activities, this inhibition was concluded to be mostly caused by direct effects on pol functions. MGDG appeared to be able to penetrate cancer cells and reach the nucleus and mitochondria, thus

Table 4
Effect of the combinations of GEM and MGDG on cell growth suppression against cultured human cancer cell lines.

Cell line	Rate of cell viability (%)				
	GEM[a]	MGDG[b]	GEM and MGDG[c]	GEM→MGDG[d]	MGDG→GEM[e]
BxPC-3	75±7	33±3	33±4	38±5	5.6±0.7
MIAPaCa2	75±7	43±5	34±4	30±3	1.5±0.2
PANC-1	75±7	39±4	36±4	32±4	3.0±0.4
A549	75±6	47±8	46±6	46±9	43±8
DU145	75±6	48±7	59±3	67±10	51±11
HeLa	75±9	48±9	40±8	47±9	33±6
HepG2	75±6	46±9	44±8	42±9	34±4
MCF-7	75±7	49±7	46±9	45±8	39±9
PC3	75±8	47±6	33±3	35±2	32±4

Cell line	Ratio of cytotoxicity		
	MGDG/GEM and MGDG	MGDG/GEM→MGDG	MGDG/MGDG→GEM
BxPC-3	1.00	0.88	6.60
MIAPaCa2	1.26	1.43	28.7
PANC-1	1.08	1.22	13.0
A549	1.09	1.09	1.16
DU145	0.81	0.72	0.94
HeLa	1.20	1.02	1.45
HepG2	1.05	1.10	1.35
MCF-7	1.07	1.09	1.26
PC3	1.42	1.34	1.47

Data, mean±SD ($n=5$).
[a] One-half of LD_{50} values of GEM were used.
[b] The used MGDG concentration for pancreatic cells (BxPC-3, MIAPaCa2 and PANC-1) was 20 μM. MGDG concentrations of A549, DU145, HeLa, HepG2, MCF-7 and PC3 were 10, 10, 10, 0.5, 8 and 10 μM, respectively.
[c] Mixture of GEM and MGDG exposure for 48 h (i.e., GEM & MGDG), the treatment schedule of Fig. 5A(a).
[d] GEM exposure for 24 h and then MGDG exposure for 24 h (i.e., GEM→MGDG), the treatment schedule of Fig. 5A(b).
[e] MGDG exposure for 24 h and then GEM exposure for 24 h (i.e., MGDG→GEM), the treatment schedule of Fig. 5A(c).

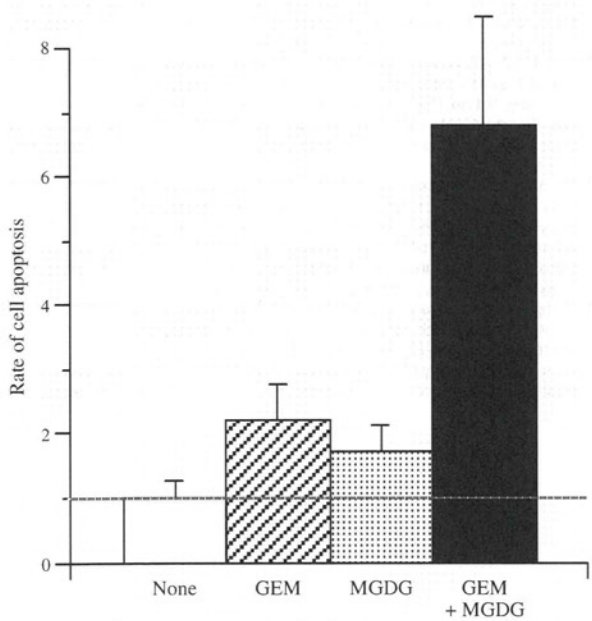

Fig. 6. Detection of apoptosis in cultured human pancreatic cancer cells by combination of GEM with MGDG. Half time of schedule (c) treatment (i.e., MGDG→GEM) using 50 nM GEM and 70 μM MGDG on MIAPaCa2 cells (Fig. 5A); treated cells cultured, harvested, stained with APO-Direct™ Assay Staining kit, analyzed using flow cytometry; nontreated apoptotic cell rate normalized to 1; ratios of apoptotic cell by GEM alone, MGDG alone, GEM–MGDG combination shown; data, means±SD of three independent experiments.

inhibiting mammalian pol α, γ, δ and ε activities and leading to cell growth suppression. It may be considered that the expression amounts and activities of pols α, δ and ε, all nuclear DNA replicative pols, as well as pol γ, a mitochondrial DNA replicative pol, in cancer cells are high and, thus, MGDG could inhibit various cancer cell proliferation, but did not influence normal cell growth (Table 3).

The present study is the initial investigation into the effects of combinations of GEM with MGDG, the latter a selective mammalian pol inhibitor. The results of this study indicated that GEM/MGDG enhanced the growth suppression of cells based on the inhibition of pol activities (Table 1, Figs. 2 and 3). Furthermore, treatment of cells by MGDG→GEM addition [schedule (c) of Fig. 5A] showed significantly greater anticancer effects, such as inductions of cell death and apoptosis, than other combined GEM and MGDG treatments in human pancreatic cancer cells (Fig. 5 and Table 4). It is not clear at this moment why MGDG-treated cells followed by GEM addition (i.e., MGDG→GEM) significantly enhanced cell proliferation suppression in human pancreatic cancer cell lines, but had no effect on the enhancement in the other human cancer cell lines; therefore, we will consider them more stringently in future studies. These results indicated that combining selective inhibitors of mammalian pols, such as MGDG and GEM, might have clinical potential in a pancreatic cancer treatment strategy.

In conclusion, the present findings suggest that an anticancer mechanism in which GEM easily enters and is quickly phosphorylated to GEM-TP within human pancreatic cancer cells and subsequently suppresses cell proliferation based on pol inhibition. Spinach MGDG was found to selectively inhibit the activities of mammalian DNA replicative pols. In particular, MGDG's inhibitory activity against pol α was different from that of GEM-TP and, therefore, might further enhance the chemotherapeutic potential of GEM against pancreatic cancer. Spinach MGDG could be a functional food for anticancer regimes without side effects.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2012.11.004.

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Radiotherapy for Stage I or II hypopharyngeal carcinoma

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Hypopharyngeal squamous cell carcinoma (HPSCC) is usually diagnosed at an advanced stage, and early-stage HPSCC is relatively rare. Because of the rarity of early-stage HPSCC, few reports have been published on the efficacy of radiotherapy (RT) in its treatment. We retrospectively reviewed the clinical records of 45 consecutive patients with Stage I and II HPSCC from May 1991 to June 2010. Patient characteristics were as follows: median age, 66 years (range, 44–90 years); male/female, 39/6; and T1/T2, 27/18. The irradiation dose ranged from 60 to 72 Gy (median: 70 Gy). Of the 45 patients, 21 underwent concurrent chemotherapy. With a median follow-up period of 62 months, the 5-year overall survival rate was 81%. Local failure occurred in 5 patients, and the 5-year local control rate was 83%. All local recurrences were successfully salvaged by surgery. The 5-year functional larynx preservation rate was 92%. Acute toxicity was manageable. Grade 3 laryngeal edema and Grade 3 hypothyroidism occurred in 1 patient each. No other late adverse events of Grade 3 or greater were observed. Based on these results, RT seemed to be an effective treatment modality for early HPSCC, with favorable organ preservation and acceptable adverse events. Early detection and accurate management of local recurrence and second malignancy was deemed to be critical.

Keywords: hypopharyngeal carcinoma; radiotherapy; chemotherapy; larynx preservation

INTRODUCTION

Hypopharyngeal squamous cell carcinoma (HPSCC) is usually diagnosed in the advanced stage, and early-stage HPSCC is relatively rare. In recent years, mainly owing to the development of laryngeal and gastrointestinal fiberscopes, HPSCC has tended to be found in an earlier stage. Although optimal treatment for early HPSCC has not been established, treatment options have included surgery and radiotherapy (RT) with or without chemotherapy. RT may be the treatment of choice in terms of functional preservation. However, because of the rarity of early-stage HPSCC, few reports have been published on the efficacy of RT. Nakamura *et al.* reported the results of chemoradiotherapy for early HPSCC [1]. In that article, chemoradiotherapy was started in the preoperative setting, and patients who achieved complete response after 30 to 40 Gy irradiation underwent further definitive chemoradiation. The authors reported equivalent disease-specific survival rates for early responders and for patients who underwent chemoradiotherapy and surgery. However, the effectiveness of curative chemoradiotherapy for all patient cohorts remains unclear. Nakamura *et al.* also reported the analysis of questionnaires from 10 institutions regarding early HPSCC treated with curative RT [2]. The authors collected the questionnaires from 115 patients treated between 1990 and 2001. The

results indicated the efficacy of RT for early HPSCC. However, deviation of treatment strategy might have existed in the multi-institutional questionnaire study. In our institution, definitive RT was performed as a first line treatment for Stage I and II HPSCC. Salvage surgery was performed for patients with local recurrence or non-responders. In this study, we retrospectively reviewed our single-institution results for definitive RT in Stage I and II HPSCC.

MATERIALS AND METHODS

Patients

From May 1991 to February 2010, 238 patients diagnosed with HPSCC were treated in our Division. Of these, 35 were treated with palliative intent, 2 preoperative, 73 postoperative, and 127 with definitive intent (82 Stage III/IV and 46 Stage I/II) (Table 1). Among the 46 patients with Stage I or II HPSCC, one patient was lost to follow-up after 4 months without any events. In this study, the remaining 45 patients with early (T1–2N0M0) HPSCC who underwent definitive RT were analysed. All patients were followed for at least 12 months or until any events. All patients had histologically proven squamous cell carcinoma. Patient characteristics are summarized in Table 2. There were 39 men and 6 women, with median age of 66 years (range, 44–90 years). Staging work-up included physical examination, laryngoscopy and computed tomography. Esophagogastroduodenoscopy was included as of May 2002, and PET scan was added in November 2006. According to the TNM classification of malignant tumors, 7th Edition [3], there were 27 patients with Stage I (tumor limited to one subsite of the hypopharynx and to ≤2 cm in the greatest dimension), 18 patients with Stage II (tumor that had invaded more than one subsite of the hypopharynx or an adjacent site or measured >2 cm but <4 cm in the greatest dimension, without fixation of the hemilarynx). The primary sites were the pyriform sinus in 35, the posterior pharyngeal wall in 6, and the postcricoid region in 4.

Table 1. Patient accrual according to treatment strategy and decade of accrual

	1991–2000	2001–10	Total
Preoperative	2	0	2
Postoperative	10	63	73
Palliative	17	18	35
Definitive (Stage III–IV)	15	67	82
Definitive (Stage I–II)	13	33	46[a]

[a]One patient was lost to follow-up after 4 months without any events and was excluded from this analysis.

Table 2. Patient characteristics

Characteristics	No. of patients
Total no. patients	45
Gender	
Male	39
Female	6
Age	
Median (range)	66 (44–90)
Tumor stage (from [3])	
Stage I	27
Stage II	18
Tumor differentiation	
Well	5
Moderately	15
Poorly	5
Unknown	20
Subsite	
Pyriform sinus	35
Posterior wall	6
Postcricoid region	4

Radiotherapy and chemotherapy

All patients underwent RT with radical intent, using 4-MV linear accelerator X-rays. No patient was treated with preoperative intent. A conventional fractionation schedule of 2 Gy/day was used. All patients received prophylactic lymph node irradiation. A prophylactic nodal area (including the retropharyngeal region and supraclavicular nodes) was irradiated up to 40–50 Gy with parallel-opposed lateral fields with a matched anterior lower neck field. The primary lesion was boosted with reduced fields after prophylactic nodal irradiation. The median total irradiated dose was 70 Gy (range: 60 to 72 Gy). Prescriptions for irradiation dose varied in accordance with the treating physician's preference. Concurrent chemotherapy was administered in 21 patients (Table 3). Inclusion criteria for chemotherapy were expanded to T2 disease beginning in the year 2000, and 16 out of 18 patients with T2 disease were treated after 2000; all 16 underwent concurrent chemotherapy. The two patients with T2 disease who were treated before 1999 did not receive chemotherapy. Five out of 27 patients with T1 disease underwent concurrent chemotherapy as per physician's preference. The regimen of chemotherapy is summarized in Table 4. Four patients received adjuvant chemotherapy with TS-1 (Oral fluoropyrimidine consisting of three components: tegafur, a prodrug of 5-FU; 5-chloro-2,4-dihydroxypyridine; and oxonic acid) (80 to 100 mg per body) as a part of feasibility study.

Follow-up

Patients were followed up monthly for the first year after completion of RT, every 3 months for the following 2 years, and then every 6 months until progression or death. Physical examination and laryngoscopy were performed at every visit. Computed tomography was performed 3 to 6 months after completion of RT, and thereafter performed annually. A PET scanner was installed in our Institute in 2005. PET scan was not performed routinely at follow-up examinations except in cases of suspected disease after computed tomography or physical examination. Esophagogastroduodenoscopy was performed at 1 to 2 year intervals, depending on the findings of routine follow-up examinations.

Statistical analysis

Survival was calculated from the date of initiation of RT to the date of any events or date last visited. Patients alive without relapse at the time of analysis were censored at their last follow-up. The progression-free survival (PFS) rate was calculated from the date of initiation of RT to the date of histologically-confirmed local recurrence, date of radiographic diagnosis of distant or nodal metastasis, or date of death from any causes. Local control rates and functional larynx preservation rates were calculated from the date of initiation of RT to the date of histologically-confirmed recurrence or date of surgical removal of larynx. Any death without local recurrence was censored for local recurrence. Any death with functional larynx was censored for functional larynx preservation rate. Survival rates were estimated using the Kaplan–Meier method. Univariate analyses with log-rank tests were performed to identify prognostic factors. Radiation dose, treatment interruption, use of chemotherapy, treatment period, tumor location, tumor stage, histological differentiation, age and gender were evaluated. All P values reported are 2-sided. For all statistical tests, differences were considered significant at the 5% level. Commercially available statistical software (StatView, 5.0; SAS Institute, Cary, NC) was used for analysis. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events v3.0 [4].

Table 3. Irradiated dose and chemotherapy

	1991–2000 ($n = 13$)	2001–10 ($n = 32$)	total
Irradiated dose			
60 Gy	5	4	9
66–70 Gy	7	28	35
72 Gy	1	0	1
Chemotherapy			
Induction	0	0	0
Concurrent	2	19	21
Adjuvant	0	4	4

RESULTS

Survivals and larynx preservation

Median follow-up periods for surviving patients and all patients were 62 and 53 months, respectively (range, 12–132 months and 8–132 months, respectively). The 5-year overall survival rate and PFS were 81% and 69%, respectively (Fig. 1). Causes of death were: primary disease (1 patient), other primary cancers (5 patients) and suffocation from aspiration (1 patient). Of the 5 patients who died of other primary cancers, 2 died of synchronous cancer (lung cancer and esophageal cancer), 1 died of recurrent metachronous cancer before treatment of HPSCC (lung cancer) and 2 died of metachronous cancer which arose after completion of the treatment for HPSCC (esophageal cancer and oropharyngeal cancer). Local recurrence occurred in 8 patients, and the 5-year local control rate was 83% (Fig. 2A). All patients with local recurrence were successfully salvaged with surgical resection. Of these 8 patients, 3 were salvaged with laryngeal preservation surgery and 5 were salvaged with laryngectomy. Another 2 patients underwent laryngectomy because of second primary head and neck cancers (cervical esophageal cancer and oropharyngeal cancer). The 5-year functional larynx preservation rate was 92% (Fig. 2B). Three of 7 laryngectomies were performed more than 60 months after initiation of RT (66, 68 and 125 months, respectively). The 6-year

Table 4. Chemotherapeutic agents for concurrent chemoradiotherapy

Chemotherapeutic agents	1991–2000	2001–06	2007–10	total
Cisplatin (70–80 mg/m^2)	0	4	6	10
Nedaplatin (70–80 mg/m^2)	0	6	1	7
Cisplatin + 5-FU (Cisplatin, 70 mg/m^2 on day 1; 5-FU, 700 mg/m^2 on days 1–4)	1	2	0	3
Low-dose cisplatin (5 mg/m^2, daily)	0	1	0	1

5-FU = 5-fluorouracil.

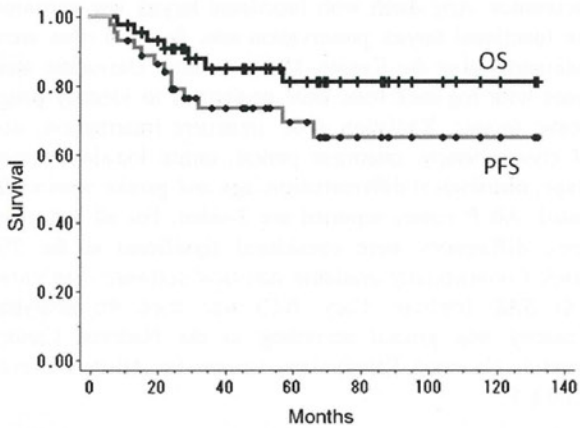

Fig. 1. Kaplan–Meier curves of overall survival rate (OS) and progression free survival rate (PFS). The 5-year overall survival rate and progression free survival rate for all patients were 81% and 69%, respectively.

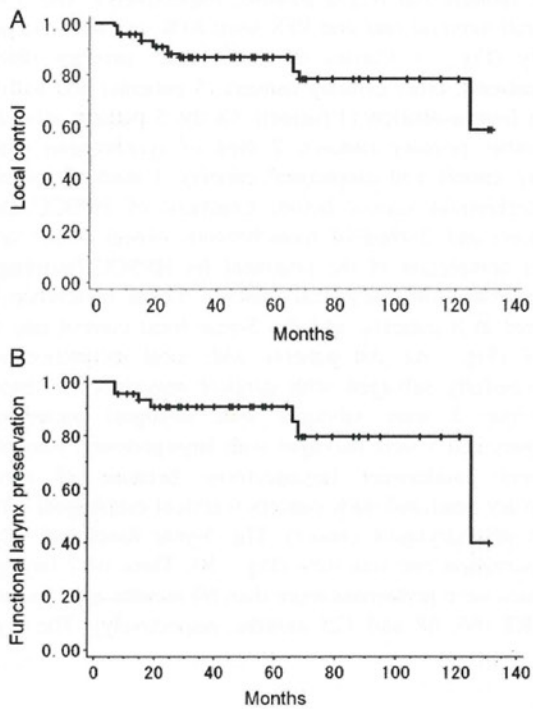

Fig. 2. Kaplan–Meier curves of (**A**) local control rate and (**B**) functional larynx preservation rate. The 5-year local control rate and functional larynx preservation rate for all patients were 83% and 92%, respectively.

functional larynx preservation rate was 79%. No cervical node metastasis was observed. Distant metastasis was observed in 1 patient.

Metachronous malignancies

Eight patients had previously treated metachronous malignancies before initiation of RT for HPSCC. All of these metachronous malignancies were judged to be cured at the time of initiation of RT.

Seven patients had synchronous malignancies. All of these malignancies were diagnosed at non-metastatic stages and were suitable for curative treatment. Six out of 7 synchronous malignancies were treated with RT concurrently with the HPSCC. One synchronous malignancy (esophageal cancer) was treated with endoscopic mucosal resection after completion of RT for HPSCC.

Fourteen patients developed metachronous malignancies in 17 sites during the follow-up period. Two patients received best supportive care (BSC). Patients in whom the other 15 malignancies were diagnosed without disseminated disease were treated with curative intent. Among 14 patients with metachronous malignancies after RT, 1 died of HPSCC, 2 died of metachronous malignancies, 1 died of suffocation from aspiration and 10 were alive and well at the time of analysis (Table 5).

Table 5. Synchronous and metachronous malignancies after radiation therapy

Synchronous malignancies	**No. of patients**
Esophagus	4
Larynx	2
Lung	1
Treatment	
RT	6
Endoscopic treatment	1
Metachronous malignancies after RT	No. of patients
Esophagus	6
Oropharynx	3
Lung	3
Prostate	2
Breast	1
Larynx	1
Hypopharynx[a]	1
Treatment	
Surgery	11
Endoscopic treatment	2
RT	1
Hormonal therapy	1
BSC	2

RT = radiation therapy, BSC = best supportive care.
[a]*De novo* carcinoma arising from contralateral pyriform sinus.

Table 6. Prognostic factors

Variable		5yr-PFS (%)	*P* value	5yr-LC (%)	*P* value
Dose	60 Gy	75	0.75	88	0.66
	66–72 Gy	68		81	
Interruption	< 5 days	74	0.18	84	0.55
	≥ 5 days	49		74	
Chemotherapy	yes	67	0.96	77	0.35
	No	73		87	
Period	1991–2000	62	0.41	68	0.16
	2001–10	72		89	
Subsite	PW	50	0.12	50	**0.01**
	Others	72		72	
Stage	I	67	0.84	80	0.93
	II	76		86	
Differentiation	w/d	0	**< 0.0001**	0	**< 0.0001**
	Others	77		91	
Age	< 70	65	0.43	80	0.61
	≥ 70	81		88	
Sex	Male	65	NA[a]	80	NA[a]
	Female	100		100	

PFS = progression free survival, LC = local control rate, PW = posterior wall, w/d = well-differentiated squamous cell carcinoma, NA = not assessed.

[a]*P* value was not assessed because no event occurred in the female arm.

Prognostic factors

We examined prognostic factors for PFS and local control, including radiation dose, treatment interruption, use of chemotherapy, treatment period, tumor location, tumor stage, histological differentiation, age and sex (Table 6). We found that well-differentiated squamous cell carcinomas ($n = 5$) were poor prognostic factors for PFS and local control ($P < 0.0001$). Tumors of the posterior wall ($n = 6$) were also associated with poor prognosis for local control ($P = 0.01$) (Fig. 3). The other factors did not show significant impact on PFS or local control.

Morbidity

No patient required a feeding tube or intravenous hyperalimentation during and after treatment. In the late period, laryngeal edema of Grade 3 (requiring temporal tracheostomy) was observed in 1 patient, and Grade 3 hypothyroidism (myxedema) was observed in 1 patient. The Grade 3 hypothyroidism was treated with levothyroxine sodium (Thyradin S). No other late toxicity of Grade 3 or greater was documented (Table 7).

Twenty-one patients underwent concurrent chemotherapy. Renal and hematologic toxicities related to chemotherapy were manageable (Table 7). Only 1 patient experienced febrile neutropenia.

DISCUSSION

Although several authors have reported the outcomes of RT for HPSCC, patients with Stage I and II hypopharyngeal cancer were relatively small cohorts in these studies [5–7]. In general, either RT or surgery with or without laryngeal preservation is selected as the initial treatment for T1–2 HPSCC. However, there have been only a few reports focusing on the efficacy of RT for early-stage hypopharyngeal cancer, and the optimal treatment approach remains controversial. RT has been recognized as an effective treatment modality for HPSCC. Mendenhall *et al.* achieved excellent local control in 80% of patients with T1–2 pyriform sinus carcinoma treated with RT alone [5]. Later, Amdur *et al.* also reported the results of RT for T1–2 pyriform sinus [6]. They included 101 patients with T1–2 carcinoma of the pyriform sinus and achieved local control rates for T1 and T2 tumors of 90% and 80%, respectively. However, of 101 patients, only 25 patients had Stage I or II disease. Their report showed relatively poor 5-year overall survival rates (57% for Stage I, 61% for Stage II, respectively). It was

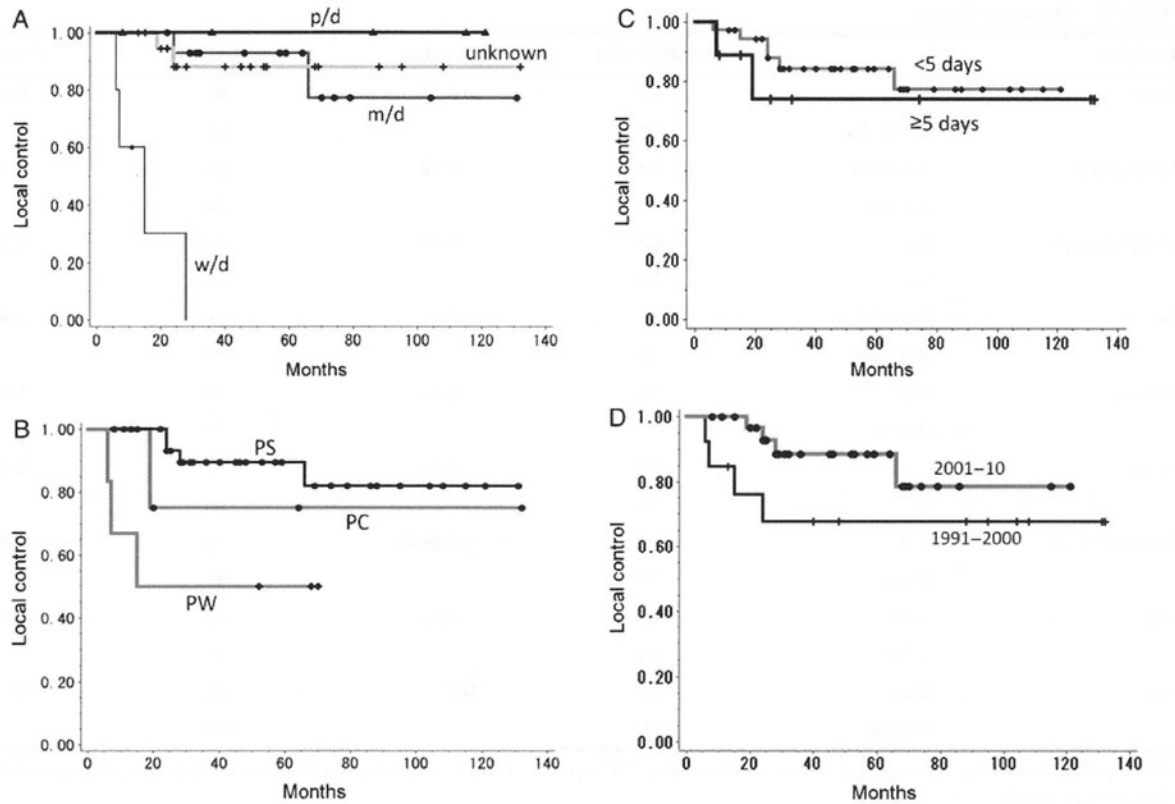

Fig. 3. Kaplan–Meier curves of local control rate according to (**A**) histological differentiation, ($P < 0.001$), (**B**) tumor location ($P = 0.01$), (**C**) treatment interruption ($P = 0.55$), (**D**) treatment period ($P = 0.16$). p/d = poorly differentiated squamous cell carcinoma, m/d = moderately differentiated squamous cell carcinoma, w/d = well-differentiated squamous cell carcinoma, PS = pyriform sinus, PC = postcricoid region, PW = posterior wall.

Table 7. Toxicity profiles (grade 3/4 toxicities)

Concurrent Chemotherapy	yes ($n = 21$)	no ($n = 24$)
	No. of patients (%)	No. of patients (%)
Early		
Renal dysfunction	0 (0)	0 (0)
Neutropenia	4 (18)	0 (0)
Anemia	0 (0)	1 (4)
Thrombocytopenia	2 (9)	0 (0)
Febrile neutropenia	1 (4.5)	0 (0)
Late		
Thyroid dysfunction	0 (0)	1 (4)
Laryngeal edema	0 (0)	1 (4)

difficult to determine the long-term efficacy of RT for early-stage HPSCC. Garden *et al.* reported that the 2-year actuarial local control rate for T1 and T2 tumors after RT alone was 89% and 77%, respectively [8]. These reports included patients with neck node metastasis, and neck nodes were managed with or without neck node dissection. Because these reports contained node-positive patients, the survival period was short. It may be difficult to elucidate the exact long-term benefit of RT for node-negative HPSCC, although these reports suggest the efficacy of curative RT for primary lesions. In our series, definitive RT resulted in a comparable local control rate (83%) in a neck node-negative patient cohort that achieved a relatively longer overall survival rate of 81% with a median follow-up period of 62 months.

In our series, all patients received prophylactic nodal irradiation and no patient experienced cervical node metastasis. Compared to other reported series for early stage HPSCC (Table 8), the nodal control rate in our study seemed to be favorable. There appears to be some potential benefit of prophylactic irradiation for early stage HPSCC. Local recurrence occurred in 8 patients. The incidence of local recurrence was comparable to other studies. All local recurrence was successfully salvaged with surgery. Early detection and adequate management for local recurrence seemed to be critical.

Table 8. Comparison of prophylactic irradiation, loco-regional control, and salvage surgery

Series	Treatment period	No. of Pts	median f/u (M)	5yr-OS (%)	Prophylactic RT yes/no	Prophylactic dose (Gy)	No. of nodal rec.	No. of local rec.	No. of salvage sx for local rec.
Nakamura[a] [1]	1976–2002	43	52	70	35/8 (81%)	30–50	3 (7%)	2 (5%)	2 (100%)
Nakamura[b] [2]	1990–2001	115	47	66	90/25 (78%)	36–50	14 (12%)	30 (26%)	26 (87%)
Yoshimura [10]	1988–2007	77	33	47	66/11 (86%)	20–50	11 (14%)	16 (21%)	12 (75%)
Current study	1991–2010	45	53	81	45/0 (100%)	40–50	0 (0%)	8 (18%)	8 (100%)

[a]Eleven of 43 patients received surgery after 30–40 Gy irradiation.
[b]Questionnaire collected from 10 institutions.
Pts = patients, f/u = follow-up period, M = months, OS = overall survival rate, RT = radiation therapy, rec. = recurrence, sx = surgery.

In our series, 5 patients died of second primary cancers. Yoshimura *et al.* also reported a high incidence of synchronous and metachronous malignancies [9]. In their report, patients with metachronous malignancies had poorer survival outcomes. In our series most metachronous malignancies were diagnosed at non-metastatic stages, and curative treatments were performed. Out of 14 patients with second malignancies, 9 were successfully treated and were alive and well at the time of analysis. We believe close follow-up and accurate management of local failure and metachronous malignancies can provide better outcomes. Careful follow-up and early detection of local recurrences and other malignancies are critical for survival and larynx preservation.

Several authors have reported the additional benefit of chemotherapy for advanced head and neck cancers [10–13]. However, the additional benefit of chemotherapy for early HPSCC remains controversial. Use of concurrent chemotherapy for these tumors differed among the reported articles. While Yoshimura reported that only 16 of 77 patients received concurrent chemotherapy [9], Nakamura reported that 39 of 43 patients received concurrent chemotherapy [1]. Inclusion criteria for concurrent chemotherapy were not documented in these articles. In our series, the treatment strategy included concurrent chemotherapy for T2 disease beginning in the year 2000. Chemotherapeutic agents were varied during the two decades of our study period. Since 2007, cisplatin alone has been the mainstay in our Institute. Out of 18 patients with T2 disease in our series, 16 were treated after 2000. All 16 underwent concurrent chemotherapy. In our results, T2 disease had a local control rate comparable with that of T1 disease. Thus, there may be some potential benefit of chemotherapy for T2 disease. T2 disease has a relatively wide range of tumor sizes (2 to 4 cm) and it may prove that concurrent chemotherapy is beneficial for larger tumors. However, it is still difficult to address the exact benefit of chemotherapy because of small sample sizes and lack of randomized data. Though adverse events related to chemotherapy were manageable, it is important to avoid unnecessary use of chemotherapy in patients who are likely to have tumor control with RT alone.

Well-differentiated squamous cell carcinoma and posterior wall tumors had poor outcomes for local control. Though these patients were a small cohort in our series, the poorer local control in posterior wall tumors was compatible with the report of Yoshimura *et al* [9]. Exact reasons for the poorer outcome in posterior wall tumors and well-differentiated tumors remain unclear. We might consider a more aggressive treatment strategy for these kinds of high-risk tumors, such as concurrent chemotherapy or volume-reduction surgery prior to RT.

Several authors have reported results of laryngeal preservation surgery for selected patients [14–17]. Though these reports also include node-positive disease, and it would be difficult to compare the long-term efficacy and local control rate with our result, they seemed to obtain comparable local control rates. However, postoperative mortality and morbidity is not negligible. Postoperative death rates of 2 to 10% were reported, and persistent swallowing difficulties and speech impairment were also reported. Radical RT for early-stage HPSCC may have some mortality and morbidity advantage in treatment without reducing local tumor control.

No patients in our series were treated with intensity-modulated RT (IMRT). IMRT is a conformal RT technique that can spare the major salivary glands and may reduce the incidence of long-term radiation-induced xerostomia. All patients with preserved larynxes in our series maintained ability in speech and swallowing. However, lack of saliva affects quality of life (QoL). Recently, the result of a randomized trial comparing conventional RT and parotid-sparing IMRT for head and neck cancers was reported [18]. Parotid-sparing IMRT was found to reduce the incidence of xerostomia and improve QoL. Because of the expected longer survival for early HPCSS, IMRT may be beneficial and should be considered for these patients.

CONCLUSION

In conclusion, RT for early HPSCC is deemed to be a feasible and effective treatment modality with minimal morbidity. In our cohort, 81% 5-year overall survival and 91%

functional larynx preservation rates were obtained during a follow-up period of 62 months. Salvage surgery with or without larynx preservation was reserved for recurrent disease. Early detection and adequate management of local recurrence and metachronous malignancies are critical in obtaining longer survival.

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Efficacy of intraoperative radiotherapy targeted to the abdominal lymph node area in patients with esophageal carcinoma

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We investigated whether intraoperative radiotherapy (IORT) during curative surgery for esophageal carcinoma is useful or not. The cases of 117 patients diagnosed with thoracoabdominal esophageal carcinoma who underwent curative surgery between 1986 and 2007 were reviewed: 72 patients received IORT (IORT group) and 45 did not (non-IORT group). Upper abdominal lymphadenectomy was performed in 115 patients (98.5%). Seventy patients (59.8%) received chemotherapy and 80 patients (68.4%) received external radiotherapy. IORT encompassed the upper abdominal lymph node area. A single-fraction dose of 20–30 Gy was delivered using high-energy electrons. Median follow-up duration for patients was 7.4 years. The 5-year overall survival rate did not significantly differ between the IORT and non-IORT groups. However, the 5-year abdominal control rate was significantly higher in the IORT group (89.2%) than in the non-IORT group (72.9%; $P = 0.022$). We next focused on a patient subgroup with a primary lesion in the lower thoracic or abdominal esophagus or measuring >6 cm in length since this subgroup is probably at high risk of upper abdominal lymph node metastasis. Of the 117 patients, 75 belonged to this subgroup, and among them 45 received IORT. Both univariate and multivariate analysis revealed the survival rate was significantly higher in patients who received IORT than in those who did not ($P = 0.033$ univariate; 0.026 multivariate). There were no obvious perioperative complications solely attributed to IORT. IORT for esophageal carcinoma will likely be effective for patients with a primary lesion in the lower thoracic or abdominal esophagus, or with a long lesion.

Keywords: esophageal carcinoma; intraoperative radiotherapy; abdominal controllability; overall survival; safety

INTRODUCTION

Esophageal carcinoma is one of the most difficult cancerous diseases to cure, despite the common use of multimodal therapy such as surgery, radiotherapy, and chemotherapy, and it has a poor prognosis [1, 2]. The reason for the poor prognosis is that most patients present with advanced stage disease, with the tumor metastasizing to the lymph nodes even in the early stages of the disease [3]. It is characterized by extensive local growth, lymph node metastasis, and

distant metastasis, and its spread is greatly affected by its vertical location because the esophagus is a long organ [4].

Management of lymph node metastasis is a critical issue in treatment of esophageal carcinoma; metastasis is considered to be one of the most important prognostic factors in patients who undergo curative surgery [5–8]. According to several studies, 58–74% of patients undergoing esophagectomy for thoracic esophageal carcinoma were diagnosed histologically as having lymph nodes metastasis[9–11]. Zhang *et al.* examined the 5-year survival rate by the number of metastatic lymph nodes and found a survival rate of 59.8% in patients with no metastatic lymph nodes, compared to just 33.4% and 9.4% in those with one metastatic node and two or more metastatic nodes, respectively [12]. Esophageal carcinoma in the upper thoracic esophagus frequently metastasizes to the cervical nodes, whereas that in the lower thoracic esophagus tends to metastasize to nodes in the upper abdominal area [4, 13, 14]. Tachimori *et al.* focused on the anatomical lymphatic drainage system in patients with a primary lesion in the lower thoracic esophageal area [15] and found metastasis in the perigastric lymph node area in a high proportion (65.6%) of patients with a pathological T2–4 tumor and in 39.5% of patients with a pathological T1 tumor. These findings suggest that the risk of metastasis to the perigastric lymph nodes, as well as to lower mediastinal lymph nodes, should be taken into consideration. Even after curative treatment for esophageal cancer, recurrence at the abdominal lymph node is still frequently observed and remains a critical problem [16–18]. Moreover, most patients with esophageal carcinoma have squamous cell carcinoma pathologically, considered to be more radiosensitive than adenocarcinoma in Japan [19].

Intraoperative radiotherapy (IORT) was developed by Abe *et al.* in 1964 and used mainly for the treatment of carcinoma of the stomach and pancreas [20]. This treatment modality was introduced into the US and other countries and applied to a variety of malignant tumors [21]. IORT delivers high-dose radiation directly to targeted tumors and potential tumor sites, while also sparing the surrounding important organs if they are manually displaced from the radiation field. IORT for advanced cervical node metastasis has afforded good local disease control with acceptable levels of toxicity [22].The efficacy of IORT on the tumor bed in the upper abdominal area, in addition to curative surgery, has also been demonstrated in patients with advanced gastric cancer [23]. However, the effectiveness of IORT for esophageal carcinoma has been poorly studied thus far: in 1993, Arimoto *et al.* reported successful prevention of mediastinal lymph node metastasis by IORT targeted at the upper mediastinum [24], while in 1999 Murakami *et al.* showed that IORT to the upper abdominal lymph node area was useful for esophageal carcinoma [25].

We started performing IORT at Tenri Hospital in 1992 to improve the control rate in the upper abdominal lymph node area in an effort to improve overall survival rates of patients with esophageal carcinoma. Our concern was that microscopic residual tumor in the upper abdominal area might cause abdominal recurrence, though it was performed with curative resection. Therefore, we inferred that IORT to the upper abdominal area in combination with local curative resection would have a good effect on treatment outcome. The purpose of this study was to evaluate the effectiveness of our treatment strategy for esophageal carcinoma, especially focusing on the contribution of IORT to overall survival and abdominal regional controllability.

MATERIALS AND METHODS

Patients

Cases were reviewed of patients diagnosed with thoracoabdominal esophageal carcinoma who underwent curative surgery at Tenri Hospital between July 1986 and July 2007. Cases of pathological Stage IVB disease, according to the International Union Against Cancer (UICC) 2002 staging system [26], were excluded. Similarly, cases of unsuccessful curative resection were excluded. The remaining 117 cases were examined in this study.

Outcomes were retrospectively compared between the group that received curative surgery with IORT targeted to the upper abdominal area (IORT group, $n = 72$) and the group that received curative surgery without IORT (non-IORT group, $n = 45$). Reasons why IORT was not performed in these 45 cases included timing of the treatment (IORT was introduced in April 1992), patient refusal, equipment problems, likelihood of a prolonged surgical procedure indicated in the preoperative assessment conference (e.g. involving extensive dissection of the pharynx, larynx, and stomach), and cancellation of IORT due to systemic deterioration during surgery attributed to increased bleeding, unstable vital signs, and unexpected delays during surgical procedures.

Table 1 shows the patient characteristics in each group. Pathological stage was determined according to the UICC 2002 staging system. None of the characteristics shown in Table 1, including patient medical conditions, differed significantly between the two patient groups.

Treatments

All patients received curative surgery. Subtotal esophagectomy was performed in most patients, while middle-lower thoracic and abdominal esophagectomy was performed when small primary lesions were present in the lower thoracic esophagus, when primary lesions were present in the abdominal esophagus or when gastrectomy was performed at the same time. Upper abdominal lymphadenectomy was performed in 115 patients (72 in the IORT group, 43 in the non-IORT group), mediastinal lymphadenectomy in

Table 1. Patient characteristics

	IORT group[a]	Non-IORT group	Total	*P*-value[b]
No. of cases	72	45	117	
Age, years	44–78	44–78	44–78	0.66
(median)	(60.5)	(63.0)	(62.0)	
Male	60	41	101	0.36
Female	12	4	16	
Performance status				0.44
0	49	23	72	
1	19	18	37	
2	3	4	7	
3	1	0	1	
4	0	0	0	
Primary site				0.73
upper thoracic esophagus	5	4	9	
middle thoracic esophagus	32	17	49	
lower thoracic esophagus	35	22	57	
abdominal esophagus	0	2	2	
Tumor length				0.89
>6 cm	29	19	48	
≤6 cm	42	26	68	
(no description)	(1)	(0)	(1)	
Histology				0.06
squamous cell carcinoma	71	40	111	
adenocarcinoma	1	5	6	
Pathological stage				0.44
0 and PCR[c]	4	1	5	
I	8	7	15	
IIA	22	10	32	
IIB	19	9	28	
III	12	16	28	
IVA	7	2	9	
Pathological T stage				0.99
Is and PCR	4	1	5	
1	18	10	28	
2	17	10	27	
3	31	22	53	
4	2	2	4	
Pathological N stage				0.33
0	35	26	61	
1	37	19	56	

Continued

Table 1. *Continued*

	IORT group[a]	Non-IORT group	Total	*P*-value[b]
Pathological upper abdominal lymph node metastasis				0.86
positive	23	15	38	
negative	49	30	79	
High risk subgroup; lesion localized in lower thoracic or abdominal esophagus or >6 cm in length				0.65
cases who belong to this subgroup[d]	45	30	75	
cases who do not belong to this subgroup	27	15	42	

[a]intraoperative radiotherapy, [b]*P*-value indicates statistical differences between IORT and non-IORT groups, [c]pathological complete response after preoperative chemothepray and radiotherapy, [d]lesion localized in the lower thoracic or abdominal esophagus or >6 cm in length is used for subgroup analysis in Table 2, as a high risk group.

113 patients (71 and 42, respectively), and cervical lymphadenectomy in 94 patients (65 and 29, respectively).

Of the 117 patients, 80 (68%) received external radiotherapy and 70 patients (60%) received chemotherapy. Details of chemotherapy and external radiotherapy in the IORT group and the non-IORT group are shown in Table 2. The radiation dose delivered during preoperative radiotherapy was 44 Gy (33–56 Gy), while that in postoperative radiotherapy was 50 Gy (30–60.4 Gy). However, less than 40 Gy was unavoidably delivered to 2 patients during preoperative radiotherapy and 3 patients during postoperative radiotherapy, while more than 55 Gy was delivered in 4 patients in preoperative and postoperative radiotherapy. In this study, external radiotherapy was provided as adjunctive therapy. Preoperative radiotherapy was performed to increase complete resectability in the subsequent surgery, while postoperative radiotherapy was performed to prevent recurrence. The target volume of the external radiotherapy was localized to the various sites of primary lesions. Therefore, only 5 patients with multiple or large abdominal lymph node metastases, or the primary lesion located in the abdominal esophagus, received external radiotherapy to the upper abdominal area. Regarding our concern about normal tissue tolerance, no patient in the IORT group received external radiotherapy to the upper abdominal area.

Table 2. Details of external radiotherapy and chemotherapy in the IORT group and the non-IORT group

	IORT group ($n = 72$)	non-IORT group ($n = 45$)
External radiotherapy	57 (79%)	23 (51%)
Upper abdominal area	0 (0%)	5 (11%)
Mediastinual area	53 (74%)	20 (44%)
Cervical area	22 (31%)	10 (22%)
Preoperative	32 (44%)	7 (16%)
Postoperative	10 (14%)	14 (31%)
Both	15 (21%)	2 (4%)
Chemotherapy	50 (69%)	20 (44%)
Preoperative	35 (48%)	9 (20%)
Postoperative	2 (3%)	9 (20%)
Both	13 (18%)	2 (4%)
Cisplatin and 5-fluorouracil	48 (66%)	17 (38%)
Nedaplatin and 5-fluorouracil	2 (3%)	0 (0%)
Cisplatin	0 (0%)	1 (2%)
S-1	0 (0%)	2 (4%)

Intraoperative radiotherapy

IORT was performed immediately after the surgical procedures in the chest area were completed and the esophagus was resected. In Tenri Hospital, the linear accelerator used for IORT was also used for radiotherapy for outpatients. With our patients, IORT was performed only on Mondays so that patients could be treated immediately after ultraviolet sterilization over the weekend. While patients were moved under general anesthesia between the operating room on the seventh floor and the radiation unit on the first basement floor, surgical incision sites were temporarily closed and an accompanying anesthesiologist continuously managed the artificial respirator. Before leaving the operating room, surgeons and radiation oncologists simulated