原著

The effect of radiosensitization by gemcitabine on mammalian cells

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Abstract:　Gemcitabine (4-amino-1- [3,3- difluoro -4- hydroxyl -5- (hydro-xymethyl) Tetrahydrofuran -2- yl] - 1H - pyrimidin -2- one; dFdC) is a deoxycytidine analogue which is well-known for its anti-tumor activity especially in pancreatic cancer. It is one of the more effective drugs sensitizing cells for radiation and its radio-sensitizing properties were demonstrated both in vivo and in vitro. Yet, the exact interaction of dFdC and radiation has not been elucidated. In this study, we examined the mechanisms by using 10 MV Lineac-X-irradiation on culture mammalian cells. A repair deficient clone, xrs5 cell line was used in addition to wild type rodent cell line (CHO). The sensitivity to irradiation or dFdC on each cell was evaluated by colony formation assays, and much higher cell killing effects were observed. To learn about the mechanism more, we examined p-53-binding protein 1 (53BP1) focus formation by the immunofluorescent method, which is known as a method for the detection of DNA double-strand breaks (DSBs). The results showed that the treatment of dFdC alone could induce 53BP1 foci, suggesting that DNA DSBs are induced by dFdC independently. When cells were irradiated after the dFdC treatment, enhanced cell-killing effects were observed in CHO cells but not in xrs5. These results strongly suggest that dFdC inhibits the repair for DNA DSBs.

Keyword：　gemcitabine, radiosensitization, CHO, xrs5, DNA DSBs

Introduction

The mortality rate of pancreatic cancer increases after 40 years of age and exceeds 120 deaths per 100,000 by the age of 80[1]. It is the fifth leading cause of cancer death in men and the sixth in women in Japan, and leads to an estimated 227,000 deaths per year worldwide[2]. The prognosis of this disease has not improved markedly, which remains dismal for patients with locally advanced or metastatic disease. Complete resection of the tumor is currently the only curative option, but only 10-20% of the patients have resectable tumors at diagnosis. Even with adjuvant therapy median overall survival of resected patients is still as low as 20% after 5 years in randomized phase III studies[3].

Chemotherapy is the mainstay of treatment for individuals with advanced disease, but is not helpful for those with poor performance status. Chemoradiotherapy downstages about 30% of patients with locally advanced disease to resectable pancreatic cancer, and these individuals go on to achieve median survival similar to that for those who are initially resectable without any preoperative treatment[4]. Gemcitabine (4-amino-1- [3,3- difluoro -4- hydroxyl -5- (hydro-xymethyl) Tetrahydrofuran -2- yl] - 1H - pyrimidin -2- one; dFdC) is standard for patients with advanced pancreatic cancer. However, most pancreatic cancers do not respond to dFdC alone: it induces a partial response in a few people and can alleviate symptoms in some with advanced tumors[5-7].

DFdC is one of the more effective drugs to sensitize cells for radiation, and its radio-sensitizing properties were demonstrated both in vivo and in vitro[8-11]. In preliminary experiments with human cell lung carcinoma cells, van Putten JWG et al. have reported that dFdC treatment retarded the rate and extent and reported no detectable effects on DNA double-strand break (DSB) repair[12]. On the contrary, Lawtence et al have reported no detectable effect on DNA DSB repair by dFdC[13]. Yet, the interaction of dFdC and radiation has not been elucidated. In this study, we aimed to evaluate the effect of sensitization by dFdC on cultured mammalian cells, and to get an insight into the mechanism of radio-sensitization by dFdC.

Materials and methods

Cell culturing procedures

The rodent cell lines were grown as monolayers at 5% CO_2 in a humidified 37°C incubator in plastic flasks (flasks Becton Dickinson, Billerica, MA). The Chinese hamster cell line, Chinese hamster ovary cells (CHO) and Ku80-deficient cells of CHO cells (xrs5) were grown on alpha-MEM medium (Gibco, Invitrogen). All of the media were supplemented with 10% bovine calf serum (FBS, Hyclone, South Logan, UT). All of the standard laboratory chemicals were purchased from Wako (Osaka, JP).

Treatment of cells

Exponentially growing cells were incubated with different concentrations of 0~5 μM dFdC (Tokyo Chemical Industry, JP) for 4 h or 24 h. After dFdC treatment, cells were trypsinized followed by neutralization of the trypsin with medium. Cell suspensions were diluted in fresh complete medium to a density of about 10^6 cells/ml. To study the effects of radiosensitization, cells were irradiated immediately using a 10 MV Lineac-X-irradiation (Primus, Siemens AG, DE) at a dose rate of 4.8 Gy/min.

Cell survival

Cell survival was assessed with clonogenic assay determined by plating 100 μL of and appropriately diluted samples to triplicate plastic Petri dishes (Becton Dickinson, NJ, USA), containing 5 ml of complete growth medium. After 6-8 days of incubation, colonies were fixed 70% ethanol and stained with Giemsa staining (Sigma-Aldrich, MO, USA). Colonies containing more than 50 cells were counted.

53BP1 foci formation

Cells grown on coverslips were incubated with or without 5 μM dFdC for 24 h at 37°C. After incubation, cells were immediately fixed using a 3.6% formaldehyde solution and washed with 0.5% Triton-X100 in glycine-PBS (50 mM glycine in PBS).

Subsequently, cells were incubated with a rabbit polyclonal antibody against Rad51 (Bethyl Laboratories, Montgomery, TX) at a concentration of 0.2 μl/100 μl TBS-DT for 2 h. Thereafter cells were washed with PBS to incubate with goat anti-rabbit Molecular Probes (Molecular Probes, Eugene, OR) which were labeled with Alexa Fluor®594 (Molecular Probes) of 2 μg/ml TBS-DT for 1 h. After washing with 0.1% Tween-20 and incubation with 2 μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 30 min, samples were analyzed with a laser scanning confocal microscope (IX81; Olympus, Tokyo, JP). Image analysis was performed on overlay projections of the Alexa- and DAPI-signal made by a confocal laser microscope. For analysis, 6 to 10 slices made through cell nuclei were compressed into one overlay projection. When two or more foci were observed, cells were scored as positive.

Statistical analysis

Significance of the difference was assessed by Student t test and Mann-Whitney test. A p value of < 0.05 was considered statistically significant. A standard deviation was demonstrated in a figure when each assay could be repeated at least three times. Statistical analyses were performed with SPSS (version 19.0; Tokyo, Japan).

Results

Sensitization by dFdC in CHO cells

When pre-treated with different concentrations of dFdC for 24 h, the extent of sensitization in CHO cells increased or slightly increased according to the concentration (Fig.1). We chose such long incubation time to give all cells influenced by dFdC more time. Next cells (10^6/ml) were exposed to 5 μM dFdC with different hours of pre-incubation. The extent of sensitization by dFdC slightly increased when pretreated with 5 μM dFdC for 4 h, while the cells pre-treated for 24 h showed significantly increased sensitization (**: $p < 0.01$; Fig.2). This data demonstrated that the status of pre-incubation with 5 μM dFdC for 4 h does not significantly affect the
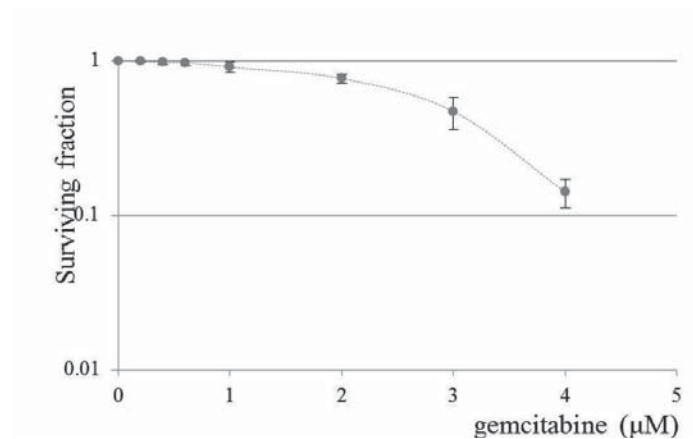
Fig.1 - Cell survival with different concentrations of gemcitabine in CHO cells. Cells (10^6/ml) were exposed to different concentrations of gemcitabine in 24 h. Cell survival was assessed with clonogenic assay. Each point represents mean and standard error from at least three experiments.
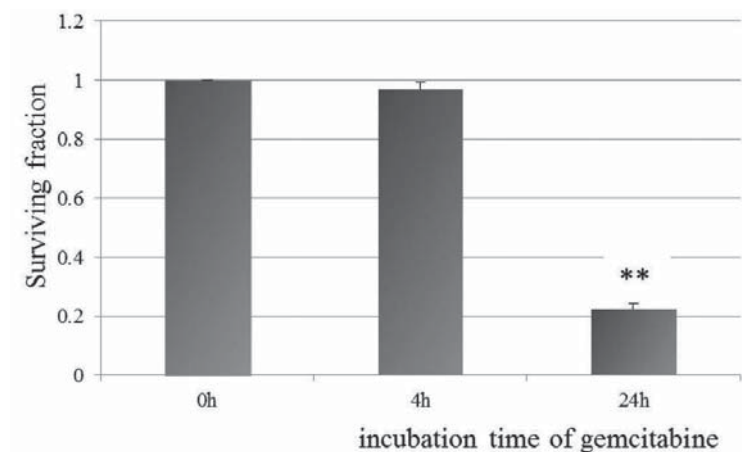

Fig.2 - Cell survival with or without gemcitabine (5 μM) in CHO cells. CHO cells (10^6/ml) were exposed to 5 μM gemcitabine with 4 or 24 hours of prei-ncubation. Cell survival was assessed with clonogenic assay. Data are the mean and standard error of three experiments. The difference in surviving fraction between 4 h and 24 h of gemcitabine pre-incubation was significant at $p < 0.01$ using a Student's t test.

cytotoxic effects of dFdC on CHO cells.

Sensitization by dFdC in cells proficient and deficient in NHEJ

Effect of dFdC of the CHO cells was compared with the xrs5 cell line which has deficient non-homologous end joining (NHEJ) repair (Fig.3). The magnitude of the enhancement of the cell killing effect by dFdC was more pronounced in xrs5 cells significantly (*: $p < 0.05$).

53BP1 foci formation

We tested the effect of dFdC on the formation of p53-binding protein 1 (53BP1) foci which are thought to represent sites of DNA DSBs. 53BP1 foci formation was observed in the CHO cells by pretreating with 5 μM dFdC for 24 h (Fig.4). We found that untreated CHO cells showed a normal pattern of small 53BP1 foci scattered in the nucleus (Fig.4A). While incubation with dFdC alone induced an increase in 53BP1 foci positive cells compared with untreated cells (Fig.4B). The number of foci was much more pronounced in xrs5 cells compared with the parental CHO cells significantly (**: $p < 0.01$) (Fig.5).
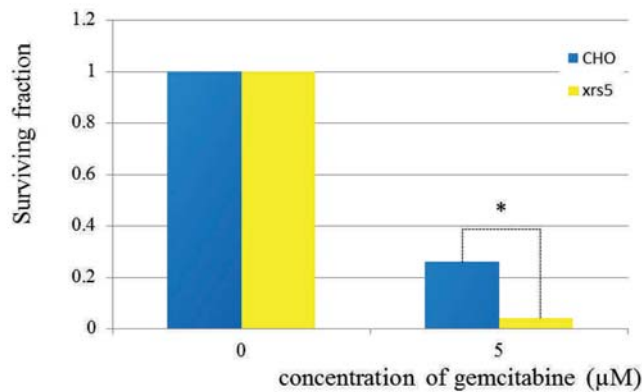
Fig.3 - Effect of gemcitabine on NHEJ-proficient CHO cells as compared with NHEJ-deficient xrs5 cells. Cells (10^6/ ml) were exposed with or without 5 μM gemcitabine for 24 h. Thereafter, their clonogenic ability was determined. Cell survival was assessed with clonogenic assay. The difference between CHO and xrs5 cell lines was significant at $p < 0.05$ using a Mann-Whitney test.
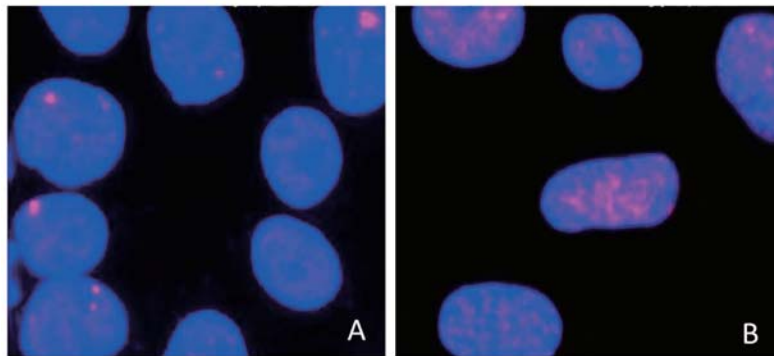

Fig.4 - Effect of gemcitabine on 53BP1 foci formation. CHO cells were incubated with or without 5 μM gemcitabine for 24 h before immediate fixation. Subsequently, cells were incubated with a rabbit polyclonal antibody against 53BP1, Alexa-conjugated goat anti-rabbit immunoglobulin, and DAPI. Shown are overlay projections of the Alexa- and DAPI-signal made by a confocal laser microscope. The pictures represent a typical pattern of 53BP1 foci in the nuclei of CHO cells. (A) Immunoflluorescent visualization of 53BP1 foci formation in nuclei of control cells. (B) 53BP1 foci formation in nuclei of cells fixated after a 24 h pre-incubation with gemcitabine.
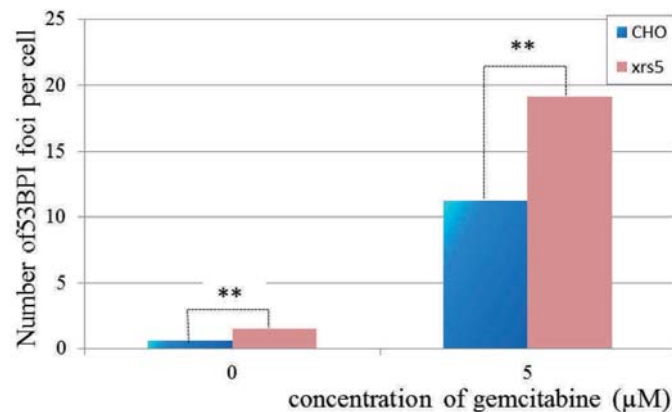

Fig.5 - Comparison of the effect of gemcitabine on 53BP1 foci formation of the parental CHO cell line and the NHEJ-deficient cell line xrs5. Cells were incubated with or without 5 μM gemcitabine for 24 h before immediate fixation. Subsequently, cells were incubated with a rabbit polyclonal antibody against 53BP1, Alexa-conjugated goat anti-rabbit immunoglobulin, and DAPI. The difference in the number of 53BP1 foci in nuclei between CHO and xrs5 cells was significant at $p < 0.01$ both with and without gemcitabine incubation using a Mann-Whitney test.

Radiosensitization

Effect of dFdC on cellular radio-sensitivity of CHO cells was seen in Fig.6. Cells (10^6/ml) were exposed to graded doses of X-rays without or with a 24 h pre-incubation of 5 μ M dFdC. The pre-incubation time of 24 h was adapted to avoid the cell cycle stage dependent. Consistent with other experiments, we found the CHO cell line to be hyper-radiosensitive. Unlike its parental CHO cell line, the xrs5 cells did not show radio-sensitization when pre-treated with dFdC.

Discussion

Gemcitabine (dFdC) is a deoxycytidine analogue which is well known for its antitumor activity. Intracellularly, dFdC is phosphorylated to its active metabolites by deoxycytidine kinase to dFdCMP, dFdCDP, and dFdCTP. DFdCTP is incorporated into DNA and as such can obstruct DNA replication and repair[14-16] (Fig.7).

Ionizing radiation induces an array of lesions in DNA, including base damage, single-strand breaks and DSBs, and damage to the phosphodiester backbone. DNA DSBs are generally thought to be the most relevant lesion in radiation-induced killing of cells, which are potentially dangerous to cells since they may lead to chromosome breakage and loss of
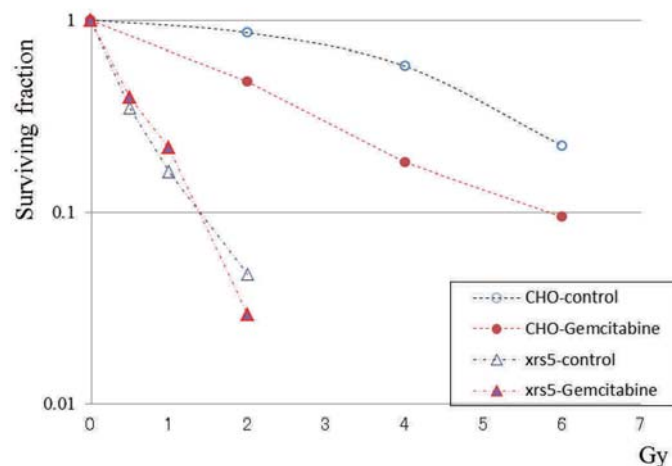

Fig.6 - Comparison of the effect of gemcitabine on cellular radio-sensitivity of the parental CHO cell line and the NHEJ-deficient cell line xrs5 represented by surviving fraction. Cells (10^6/ml) were exposed to graded doses of gamma rays without or with a 24 h pre-incubation with 5 μM gemcitabine. Cell survival was assessed with clonogenic assay. Plotted are the irradiation effect without pre-incubation of gemcitabine of the NHEJ-proficient CHO (◯) and the NHEJ-deficient xrs5 (△), compared with the effect with pre-incubation of gemcitabine of the CHO cells (●) and the xrs5 cells (▲).
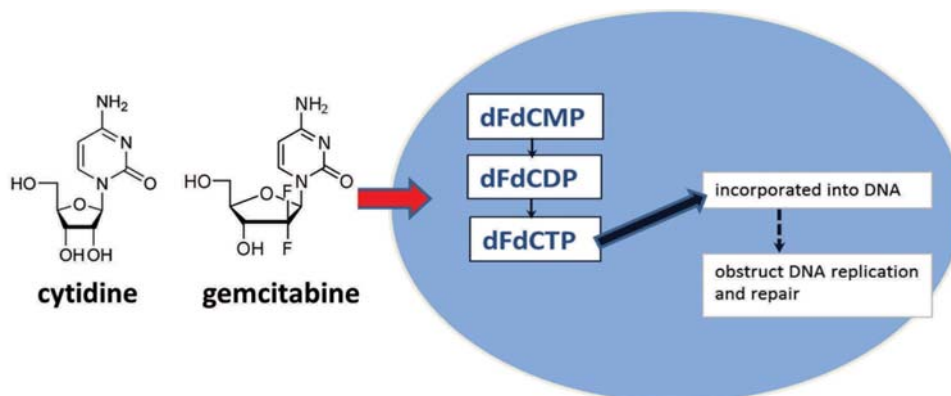

Fig.7 - The structural formula of gemcitabine hydrochloride. Chemically gemcitabine is a nucleoside analog in which the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms. DFdCTP is incorporated into DNA and as such can obstruct DNA replication and repair.

genetic information. In lower eukaryotes, such as yeast, DSBs are repaired by Rad52-dependent homologous recombination (HR). Rad52 binds to DNA ends, thereby protecting them from exonuclease activity and activating end-to-end interaction and HR[17]. While in vertebrates, DSBs are primarily repaired by Ku-dependent NHEJ. In mammals, NHEJ is the most prominent cellular DNA repair pathway of radiation-induced DNA DSBs, so that DSBs are primarily repaired by Ku-dependent NHEJ[18-19].

In preliminary experiments with human cell lung carcinoma cells, it has been reported that the dFdC treatment retarded the rate and extent of the repair of DNA DSBs[20-21]. However, other reports have reported no detectable effects on DNA DSB repair by dFdC[14]. In this study, the CHO cells showed the sensitization of dFdC according to the concentration. The extent of sensitization was more exaggerated in xrs5 cells. This is explained by the fact that the xrs5 cells, rodent cell line mutated in the gene XRCC5 encoding Ku80 have been found to be deficient in DNA repair[19-20], as Ku80 is a protein which binds to DNA DSB ends and is required for the NHEJ pathway of DNA repair.

To test whether or not dFdC targets on the DNA DSBs, we detected dFdC-induced foci formation of 53BP1 in cells. 53BP1, a member of the BRCT protein family, it is hyperphosphorylated and relocalizes to a number of nuclear foci in response to DNA damage. 53BP1 binds to the central domain of p53 which is required for site-specific DNA binding[21]. It is known that the assembly of 53BP1 foci is not cell cycle stage dependent, and ionizing irradiation-induced 53BP1 foci occur in almost all cells except those in mitosis[22]. The 53BP1 localizes rapidly to discreeting foci within the nucleus of cells exposed to DNA DSB-inducing agents and propose that these foci represent sites of DSBs[23]. In this study, the 53BP1 protein was detected by immunofluorescent antibodies in a small number of discrete foci in the nucleoplasm of a small fraction of cells in non-treated cultured CHO cells. After dFdC damage, the number of foci with focally concentrated 53BP1 protein increased in CHO cells, and gross effects of dFdC were seen in xrs5 cells. It did

demonstrate that actually dFdC induced DNA DSBs independently.

Next, we tested the effect of dFdC on cellular radio-sensitivity of CHO and xrs5 cells. In this study, cells with Ku80 expression (CHO) showed a different extent of radio-sensitization at given concentrations of dFdC. On the contrary, cells without Ku80 (xrs5) showed lower dFdC-mediated radio-sensitization. This could not be explained by the fact that the xrs5, Ku80-deficient cells appeared sensitive to the direct toxic action of dFdC. Hence, this would seem that a functional NHEJ pathway, which is required for the repair of the majority of radiation-induced DNA DSBs in mammals, could be a prerequisite for radiosensitization by dFdC. However, John WG *et al*. have reported that NHEJ and its individual components are not essential for radio-sensitization by dFdC[24]. They showed that the Ku-80 deficient cells even showed the highest levels of radio-sensitization using rodent cell lines. This may be influenced by the differentiation of incubation time; they used 4 h for pre-incubation and we used 24 h to avoid the cell cycle stage dependent. Actually it has been reported that dFdC effects on cell cycle redistribution and dNTP pools may contribute to dFdC-mediated radio-sensitization[13]. To see the most likely pathway as a target for dFdC-mediated radio-sensitization, we need a more detailed examination on the effects of long-path repair pathway such as HR.

In conclusion, we examined the sensitivity to each irradiation or dFdC on wild type rodent cell line (CHO) and a repair deficient clone, xrs5 cell line which is deficient in DNA DSBs repair. The results showed that the treatment of dFdC alone can induce 53BP1 foci, suggesting that DNA DSBs are induced by dFdC independently. When cells were irradiated after dFdC treatment, enhanced cell killing effects were observed in CHO cells but not in xrs5. These results strongly suggest that dFdC inhibit the repair for DNA DSBs, and this could be one of the mechanisms of radiosensitization by dFdC.

References

1) Matsuno S, Egawa S, Fukuyama S, Motoi F, Sunamura M, Isaji S, Imaizumi T, Okada S, Kato H, Suda K, Nakao A, Hiraoka T, Hosotani R, Takeda K. Pancreatic Cancer Registry in Japan: 20 years of experience, *Pancreas*., **28(3),** 219-230, 2004.

2) Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer, *Lancet*., **378(9791)**, 607-620, 2011.

3) Brunner TB, Scott-Brown M. The role of radiotherapy in multimodal treatment of pancreatic carcinoma, *Radiat Oncol*., **5(64)**, 1-12, 2010.

4) Gillen S, Schuster T, Meyer Zum Büschenfelde C, Friess H, Kleeff J. Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages, *PLoS Med*., **7(4)**, e1000267, 2010.

5) Oh SY, Kim HJ, Kim TH, Lee GW, Kim HG, Jeong CY, Kwon HC, Kang JH. Pilot study of irinotecan/oxaliplitin (IROX) combination chemotherapy for patients with gemcitabine- and 5-fluorouracil- refractory pancreatic cancer, *Invest New Drugs*, **28(3)**, 343-349, 2010.

6) Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD.   Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial, *J Clin Oncol*., **15(6)**, 2403-2413, 1997.

7) Storniolo AM, Enas NH, Brown CA, Voi M, Rothenberg ML, Schilsky R. An investigational new drug treatment program for patients with gemcitabine: results for over 3000 patients with pancreatic carcinoma, *Cancer*., **85(6)**, 1261-1268, 1999.

8) Shewach DS, Hahn TM, Chang E, Hertel LW, Lawrence TS. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells, *Cancer Res*., **54(12)**, 3218-3223, 1994.

9) Lawrence TS, Chang EY, Hahn TM, Hertel LW, Shewach DS. Radiosensitization of pancreatic cancer cells by 2',2'-difluoro-2'-deoxycytidine, *Int J Radiat Oncol Biol Phys*., **34(4)**, 867-872, 1996.

10) Joschko MA, Webster LK, Groves J, Yuen K, Palatsides M, Ball DL, Millward MJ. Enhancement of radiation-induced regrowth delay by gemcitabine in a human tumor xenograft model, *Radiat Oncol Investig*., **5(2)**, 62-71, 1997.

11) Milas L, Fujii T, Hunter N, Elshaikh M, Mason K, Plunkett W, Ang KK, Hittelman W. Enhancement of tumor radioresponse in vivo by gemcitabine, *Cancer Res*., **59(1)**, 107-114, 1999.

12) van Putten JWG, Groen HJM, Smid K, Peters GJ, Kampinga HH. End-joining deficiency and radiosensitization induced by gemcitabine, *Cancer Res*., **61(4)**, 1585-1591, 2001.

13) Lawrence T. S, Eisbruch A, Shewach D. S. Gemcitabine-mediated radiosensitization, *Semin. Oncol*., **24(7)**, 24-28, 1997.

14) Tashiro S, Walter J, Shinohara A, Kamada N, Cremer T. Rad51 accumulation at sites of DNA damage and in postreplicative chromatin, *J Cell Biol*., **150(2)**, 283-291, 2000.

15) Wang ZM, Chen ZP, Xu ZY, Christodoulopoulos G, Bello V, Mohr G, Aloyz R, Panasci LC. In vitro evidence for homologous recombinational repair in resistance to melphalan, *J Natl Cancer Inst*., **93(19)**, 1473-1478, 2001.

16) Ross GM, Eady JJ, Mithal NP, Bush C, Steel GG, Jeggo PA, McMillan TJ. DNA strand break rejoining defect in xrs-6 is complemented by transfection with the human Ku80 gene, *Cancer Res*., **55(6)**, 1235-1238, 1995.

17) Van Dyck E, Stasiak AZ, Stasiak A, West SC. Binding of double-strand breaks in DNA by human Rad52 protein, *Nature*, **398(6729)**, 728-731, 1999.

18) Kanaar R, Hoeijmakers JH, van Gent DC. Molecular mechanisms of DNA double strand break repair, *Trends Cell Biol*., **8(12)**, 483-489, 1998.

19) Schy WE, Hertel LW, Kroin JS, Bloom LB, Goodman MF, Richardson FC. Effect of a template-located 2',2'-difluorodeoxycytidine on the kinetics and fidelity of base insertion by Klenow (3'-->5'exonuclease-) fragment, *Cancer Res*., **53(19)**, 4582-4587, 1993.

20) Singleton BK, Priestley A, Steingrimsdottir H, Gell D, Blunt T, Jackson SP, Lehmann AR, Jeggo PA. Molecular and biochemical characterization of xrs mutants defective in Ku80, *Mol Cell Biol*., **17(3)**, 1264-1273, 1997.

21) Iwabuchi K, Li B, Massa HF, Trask BJ, Date T, Fields S. Stimulation of p53-mediated transcriptional activation by the p53-binding proteins, 53BP1 and 53BP2, *J Biol Chem*., **273(40)**, 26061-26068, 1998.

22) Maser RS, Monsen KJ, Nelms BE, Petrini JH. hMre11 and hRad50 nuclear foci are induced during the normal cellular response to DNA double-strand breaks, *Mol Cell Biol*., **17(10)**, 6087-6096, 1997.

23) Anderson L, Henderson C, Adachi Y. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage, *Mol Cell Biol*., **21(5)**, 1719-1729, 2001.

24) van Putten JWG, Groen HJM, Smid K, Peters GJ, Kampinga HH. End-joining deficiency and radiosensitization induced by gemcitabine, *Cancer Res*., **61(4)**, 1585-1591, 2001.