ZD1839 (IRESSA®) Stabilizes $p27^{Kip1}$ and Enhances Radiosensitivity in Cholangiocarcinoma Cell Lines

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Abstract. *The prognosis of cholangiocarcinoma patients is extremely poor despite the aggressive multidisciplinary cancer therapies that have been used clinically (1). Recently, molecular target therapy has attracted attention. Epidermal growth factor receptor (EGFR) tyrosine kinase (TK) is a promising target for anticancer therapy. ZD1839 (IRESSA®) is an orally active, selective inhibitor of EGFR-TK. This study examined the effects of ZD1839 in TFK-1 and HuCCT1, the human cholangiocarcinoma cell lines that express EGFR. Somatic mutations in the TK domain of the EGFR gene are associated with the sensitivity of lung cancers to ZD1839 (2). In the analysis of the EGFR sequence, no mutations were found in TFK-1 and HuCCT1. The TFK-1 and HuCCT1 cells showed almost the same sensitivity to ZD1839. It is shown that ZD1839 induced apoptotic cell death of TFK-1 cells as indicated by apoptotic morphological changes and an enhancement of TUNEL-positive cells. ZD1839 produced a dose-dependent inhibition of cellular proliferation in TFK-1. Cell cycle analysis demonstrated that ZD1839 induces G1 arrest. Moreover, concurrent evaluation of the expression of $p27^{Kip1}$ protein and Jun activating domain-binding protein 1 (Jab1) with ZD1839 by Western blotting analysis was performed. It was found that ZD1839 activity causes an increase of $p27^{Kip1}$ stability that correlates with Jab1 down-regulation. Thus, ZD1839 affects key cellular pathways, controlling cell proliferation and apoptosis. Furthermore, the treatment of TFK-1 with ZD1839 reduced the cell survival after radiation exposure. ZD1839 in combination with radiation produced a dose-dependent and synergic inhibitory effect on cellular proliferation. In conclusion, these results suggest that ZD1839 may have clinical activity against cholangiocarcinoma.*

The prognosis of cholangiocarcinoma patients is extremely poor despite the aggressive multidisciplinary cancer therapies that have been used clinically. In spite of newly developed treatments, the overall five-year survival rate has remained only several percent. One reason for the poor prognosis is the persistence and advancement of micro-metastases or perineural spaces that occur prior to surgery (3, 4). Other treatments such as adjuvant chemotherapy, intraluminal brachytherapy (ILBT) or the use of expandable metallic biliary stents (EMBS) are offered to patients in order to improve the quality of life, but the outcome of this disease is invariably fatal.

Effective treatments involving immunotherapy or gene therapy are needed to improve the prognosis of this disease. Radiation therapy is a key management strategy for many epithelial tumor types. It has been reported that chemotherapy with ILBT was useful in bile duct cancer patients for whom excision is not possible (5). In the cholangiocarcinoma cell line TFK-1, growth inhibition was demonstrated using a recombinant bispecific diabody by employing a refolding system in E x 3 diabody mediated T-LAK cells (6). Recently, the most common target in cancer gene therapy is tumor suppressor gene p53, but p53 mutations occur in only 30 to 35% of cholangiocarcinomas (7, 8). Thus, p53-targeted gene therapy cannot be applied for cholangiocarcinoma patients and a more suitable gene target is needed.

Epidermal growth factor receptor (EGFR) plays an important role in the regulation of cell proliferation, differentiation, development and oncogenesis through signal transduction (9). This signal transduction pathway can also lead to cell proliferation in tumor growth as well as the

Abbreviations: EGFR, Epidermal growth factor receptor; TK, tyrosine kinase; Jab1, jun activating domain-binding protein; HRP, horseradish peroxidase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; S.D., standard deviation; PCR, polymerase-chain-reaction; IC_{50}, concentration of the drug that inhibits cell growth by 50% .

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progression of invasion and metastasis. There is accumulating evidence that the over-expression of EGFR is associated with metastasis and a poor prognosis in patients with cancer. EGFR is considered to be a tumor-specific therapeutic target. ZD1839 (Iressa®) is an EGFR tyrosine kinase (TK) inhibitor that was developed for various kinds of carcinoma. Its antitumor effect for carcinoma has been reported to be as strong as monad target drugs, but most of its mechanisms of action have not been clarified. Somatic mutations in the TK domain of the EGFR gene are associated with the sensitivity of lung cancers to ZD1839 (10, 11). The mutations were either small, in-frame deletions (exon 19) or amino acid substitutions clustered around the ATP-binding pocket of the tyrosine kinase domain (exon 21). EGFR mutations may be a determinant of cholangiocarcinoma cell's sensitivity to ZD1839.

ZD1839 was shown to increase the protein level of p27Kip1 in a previous report, but the mechanism was not clarified. Cyclin dependent kinase (CDK) inhibitor $p27^{Kip1}$, a member of the $p21^{Waf1}$ family, is a negative regulator of cell cycle progression (12, 13). Recently, a pattern of low p27Kip1 expression has been shown to be predictive of increased mortality in breast cancer, gastric cancer, lung cancer, and other malignant tumors (14-17) Previous reports have demonstrated the role of $p27^{Kip1}$ in the induction of apoptosis (18). A previous study also showed that the over-expression of $p27^{Kip1}$ triggers apoptosis in several different human cancer cells including the cholangiocarcinoma cell line TFK-1 (19). In the present study, the cellular and molecular mechanisms of action of ZD1839, especially those of the $p27^{Kip1}$ protein, were examined in human cholangiocarcinoma cell lines.

A series of studies have evaluated the antiproliferative effects of combined treatment with ZD1839 and a variety of cytotoxic drugs with different mechanisms of action (20). In a series of recently published abstracts, it was described that ZD1839 had a capacity to modulate the radiation response in human tumor cell lines and xenografts (21-23). These data provide a rationale to support clinical trials combining ZD1839 with radiotherapy in human cholangiocarcinoma.

Materials and Methods

Materials. ZD1839 [4-(3-chloro-4-fluorophenylamino-)-7-methoxy-6-(3-(4-morphlinyl) propoxy)-quinazoline] was provided by AstraZeneca (Macclesfield, United Kingdom). ZD1839 was dissolved in DMSO for the *in vitro* study. Anti-$p27^{Kip1}$ (F-8), anti-actin (I-19), and horseradish peroxidase-linked (HRP) antibodies (goat anti-mouse for $p27^{Kip1}$ and Jab1; donkey anti-goat for actin) were purchased from Santa Cruz Biotechnology, Inc (California, USA). Anti-Jab1 (611618) antibodies were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Cycloheximide (C7698) and Protease inhibitor cocktail (P2714) were obtained from Sigma-Aldrich (St. Louis, USA).

Cell cultures. Two human cholangiocarcinoma cell lines, TFK-1 (24) and HuCCT1 (obtained from Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan), were used in this study. The cells were cultured in RPMI 1640 medium (Gibco BRL, Gaithersberg, USA) supplemented with 10% CELLect™ Gold fetal bovine serum (FBS), 10000 unit/mL penicillin, 10000 μg/mL streptomycin sulfate and 25 μg /mL amphotericin B (Gibco BRL) at 37˚C in an atmosphere of 5% CO_2 and 95% air. Experiments were performed when cells reached 80% confluence.

Cell growth studies. To determine the exponential doubling time at different concentrations of ZD1839, the TFK-1 and HuCCT1 cells were seeded at a density of 1×10^5 cells per 10 cm diameter well. After 24 hours (time 0), the cells were treated with various concentrations of ZD1839 for the indicated periods. After the treatment, the number of viable cells was determined by the Trypan blue dye exclusion method using a hemocytometer.

Cell proliferation assay. For the growth inhibition assay, cells were seeded into 96-well microtitration plates (200 μL per well) to obtain exponential growth for the whole duration of the experiment: the initial cell densities were 5,000 cells per well. After 24 hours, the cells were treated with various concentrations of ZD1839 from 0 to 100 μM for the indicated periods. After 48 hours treatment, MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay kit; PROMEGA, Madison, WI, USA) was performed to examine the antiproliferative effect of ZD1839. TFK-1 cells were incubated with 1 mg/mL MTS for another 1 hour. The absorbance of each well was measured in a microplate reader at 492 nm. The percentage cell growth was calculated by comparison of the A492 reading from the treated *versus* the control cells.

Mutational analysis of EGFR in cholangiocarcinoma cell lines. Genomic DNA was derived from TFK-1 and HuCCT1. All sequencing reactions were performed in both forward and reverse directions, and all mutations were confirmed by PCR amplification of an independent DNA isolate. For drug sensitive tumors with mutations, sequences from exon 18 to exon 21 were also determined from at least two independently derived PCR products.

Cell cycle analysis. Control or ZD1839-treated cells were removed with a cell scraper, washed with phosphate-buffered saline (PBS), fixed in 95% ethanol, and stored at 4˚C before the DNA analysis. Analysis of the cell cycle distribution and the Sub-G1 (sub-diploid) population was performed by measuring the fluorescence activity of propidium iodide stained DNA of the permeabilized and fixed cells using a FACS Calibur instrument (Becton Dickinson, Mansfield, MA, USA). The resulting DNA distributions were analyzed by FACScan for the proportion of cells in G_0, G_1, S and G_2-M phases of the cell cycle.

Measurement of apoptotic cells; TUNEL method. For the detection of apoptosis by the TUNEL (TdT-mediated dUTP-biotin nick end labeling) method, TFK-1 cells were cultured without or with 10 μM and 20 μM ZD1839 for the indicated time periods. After 48 hours the cells were removed with a cell scraper for further analysis and washed two times with PBS. TUNEL assay was performed using MEBSTAIN Apoptosis Kit (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan) according to the manufacturer's directions. FACS analysis was performed using a FACS Calibur instrument (Becton Dickinson).

Immunostaining of $p27^{Kip1}$ protein. TFK-1 cells were seeded at a density of 5×10^5 cells. After 24 hours, the cells were treated without or with ZD1839 (at 10 μM). After another 48 hours, the cells were fixed with 4% paraformaldehyde. Immunostaining of the ZD1839 treated cells was performed by the EnVision-polymer technique (EnVision+ kit; Dako, Kyoto, Japan). Primary anti-$p27^{Kip1}$ antibodies (Santa Cruz) were applied at 1:200 dilution with 0.1% Triton-X, and the cells were incubated overnight at 4˚C. To block the endogenous peroxidase activity, the cells were incubated with 0.3% hydrogen peroxide (H2O2) and methanol for 10 minutes. After blocking, the cells were reacted with EnVision-horseradish peroxidase for 1 hour at room temperature, and visualized with 3,3'diaminobenzidine (DAB) with 0.01% H_2O_2.

Western blotting analysis. TFK-1 cells were seeded at a density of 5×10^5 cells per well. Twenty-four hours after seeding, the cells were treated with 0, 10 and 20 μM of ZD1839. After another 48 hours, the cells were washed twice with ice-cold PBS and scraped in ice-cold PBS. Cells were lysed into EBC lysis buffer [NP40 1g/L, 40 mM Tris (pH 8.0), 100mM NaCl] supplemented with Protease inhibitor Cocktail (P2714, Sigma). The lysates were clarified by centrifugation, and the protein concentration was determined by the Bradford method (Bio-Rad, USA). The samples were then mixed with 4x SDS-PAGE sample loading buffer, boiled, separated on Tris-Glysine gels (Novex; Invitrogen life technologies, Hercules, CA, USA), and transferred onto PVDF membrane (Sequi-BlotTM; Bio-Rad Laboratories). Blots were probed with anti-p27Kip1 (F-8; Santa Cruz), anti-Jab1 (BD Biosciences), and anti-actin (I-19; Santa Cruz). Signals were visualized by using horseradish peroxidase-conjugated antibodies (goat anti-mouse for p27Kip1 and Jab1, donkey anti-goat for actin; Santa Cruz) and an enhanced chemiluminescence kit (Super Signal West Pico Chemiluminescent Substrate; PIERCE, Rockford, IL, USA).

Cycloheximide treatment of Cells; p27Kip1 stability assay. Cells were seeded at 5×10^5 cells per well. After 24 hours, they were treated with ZD1839 (at 20 μM) and/or cycloheximide (10 μg/mL). The cells were harvested at different time points after treatment and used for Western blotting analysis.

Combination effect of ZD1839 with radiation. TFK-1 cells were seeded in 96-well microtitration plates (200 μL per well) at a density of 500 cells per well to obtain exponential growth for the whole duration of the experiment. Twenty-four hours after seeding, the cells were treated with various concentrations of ZD1839. After another 24 hours, they were subjected to X-ray irradiation generated by a Hitachi MBR-1520R apparatus (Hitachi, Tokyo, Japan) operated at 150 kV and 20 mA with 0.1 mm Cu and 0.5 mm Al filters. The absorbed dose rate was 0.79 Gy/min. The cells were irradiated at graded doses (0, 2Gy, 4Gy, 8Gy).

Growth inhibition was assessed 7 days after the irradiation using the MTS assay. Results were expressed as the relative percentage of absorbance compared with controls without ZD1839. Dose effect curves were established for TFK-1 using a total of 4 doses (0, 2, 4, 8Gy). The cell sensitivity to the tested drugs was expressed by the IC_{50} (concentration leading to 50% cell survival). The combined effect was analysed by isobolographic analysis(25).

For the detection of apoptosis resulting from the combined therapy of ZD1839 with irradiation, the TUNEL method was employed. TFK-1 cells were seeded in 10 cm dishes at a density of 5×10^5. After 24 hours, the cells were cultured without or with 20 μM ZD1839. After another 24h, the cells were irradiated at graded doses (0 or 4Gy). Three days after the irradiation, the cells were removed with a cell scraper for further analysis and washed two times with PBS. The TUNEL assay was performed according to the manufacturer's directions. FACS analysis was performed using a FACS Calibur instrument (Becton Dickinson).

Analysis of data. In this study, all data are presented as mean±S.D. of separate experiments. Statistical differences were calculated using the Student's *t*-test and considered significant at the **: $p<0.01$ or *: $p<0.05$ level. All figures shown in this article were obtained from at least three independent experiments. Immunoblots were scanned using a scanner, and densitometry was performed using the NIH image program.

Results

Antiproliferative effect of ZD1839. The EGFR expression of TFK-1 and HuCCT1 was evaluated using a FACS Calibur instrument (Figure 1A). The cells were treated with various concentrations of ZD1839 (0, 1, 10, 20 and 30 μM), and viable cells were counted daily by the Trypan blue dye exclusion method. As shown in Figure 1, ZD1839 inhibited the growth of TFK-1 and HuCCT1 cells in a dose-dependent manner. Morphological examination of TFK-1 cells using a phase-contrast microscope showed that the anti-proliferative effect induced by a low dose of ZD1839 was mainly cytostatic. However, long-term persistent exposure of the cells to high dose ZD1839 (30 μM) showed a cytotoxic effect, because floating dead cells were observed after 4 days of incubation (data not shown).

The antiproliferative effects of ZD1839 in the cholangiocarcinoma cell lines were examined using the MTS assay. TFK-1 and HuCCT1 cells were treated with various concentrations of ZD1839 (0, 0.0032, 0.016, 0.8, 4, 20, 100 μM). After two days incubation, the cells were almost completely suppressed by ZD1839 administration. The concentrations of the drug inhibiting cell growth by 50% (IC_{50}) were determined in all cell lines. ZD1839 inhibited the growth of cells in a dose-dependent manner in the TFK-1 (IC_{50} 32.2±2.1 μM) and HuCCT1 cell lines (IC_{50} 30.2±1.6 μM), (data not shown).

Nucleotide-sequence analysis of cell lines. For mutational analysis of the EGFR coding sequence, DNA was extracted from the cell lines and uncloned polymerase-chain-reaction (PCR) fragments were sequenced and analyzed in both sense and antisense directions for the presence of heterozygous mutations. All sequence variants were confirmed by multiple independent PCR amplifications. No mutations were found in the TFK-1 and HuCCT1 cells (Figure 2). The antiproliferative effect of ZD1839 was observed with an IC_{50} of 30-35 μM in the cholangiocarcinoma cell lines. The sensitivity to ZD1839 was almost the same.

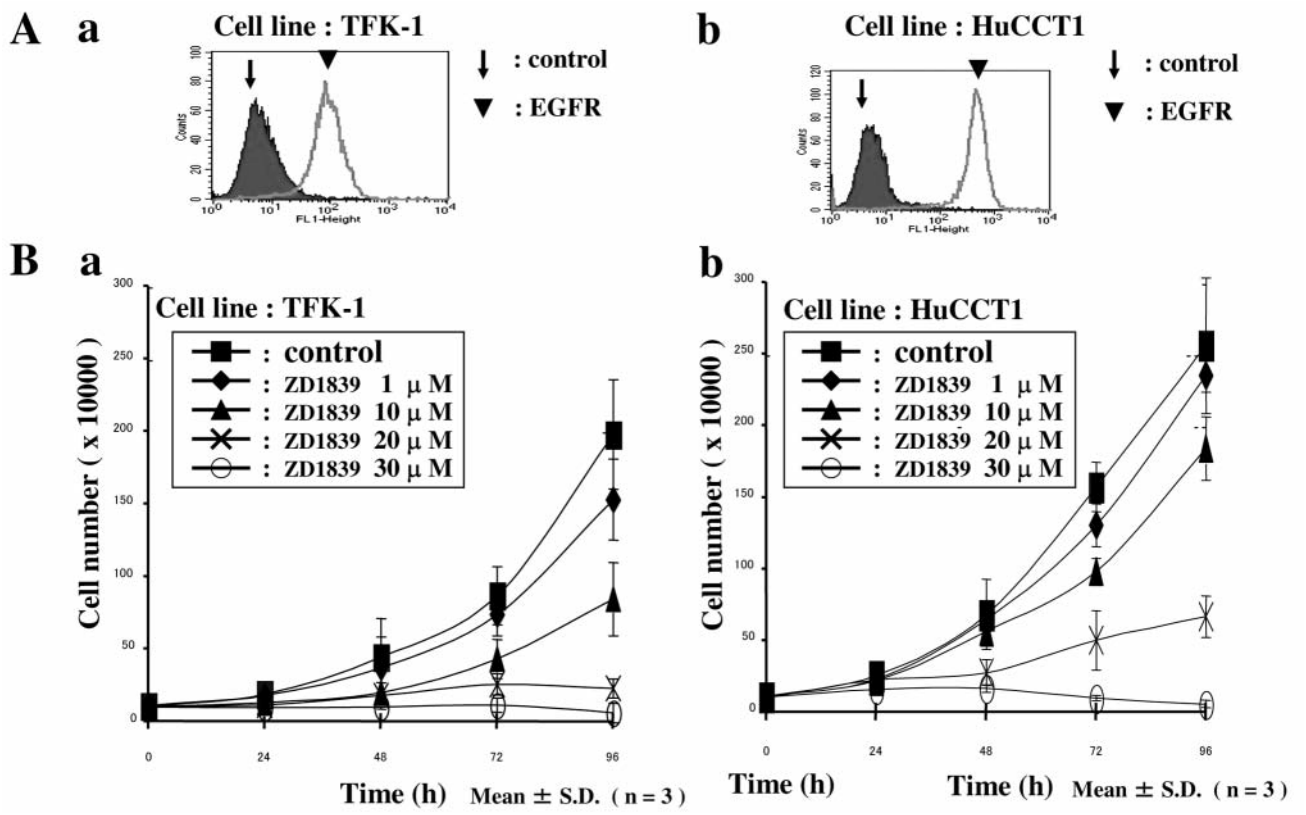


Figure 1. *Antiproliferative effects of ZD1839. A: EGFR expression was evaluated by a FACS Calibur instrument. (a): TFK-1 cells. (b): HuCCT1 cells. B: Dose- and time-dependent response. Human cholangiocarcinoma TFK-1 and HuCCT1 cells were treated with various concentrations of ZD1839 (0, 1, 10, 20, 30 μM) for the indicated periods (24, 48, 72, 96 hours). After treatment, the cell number was measured by the Trypan blue dye exclusion method. (a): TFK-1 cells. (b): HuCCT1 cells.*

ZD1839 induces G1 cell cycle arrest. To determine the effect of ZD1839 on the cell cycle progression of TFK-1 cells, flow cytometry analysis was performed on cells treated with 10, 20 and 30 μM or without ZD1839 for 48 hours. As shown in Figure 3, treatment with ZD1839 caused a dose-dependent increase in the proportion of cells in the G1 phase, with a corresponding decrease in the proportion of cells in the S phase in comparison with the control cultures. These results indicated that ZD1839 mediated a prolongation of the cell cycle progression in the G1 phase in TFK-1 cells.

ZD1839 induced apoptotic cell death in TFK-1 cells. After continuous incubation of TFK-1 cells with ZD1839 for 48 hours, a significant cytotoxic effect was observed. To investigate whether the cytotoxic effect of ZD1839 was due to the induction of apoptosis in TFK-1, the TUNEL assay was performed on cells treated with 0, 10 and 20 μM ZD1839 for 2 days. The TUNEL assay revealed a dose-dependent induction of apoptosis in ZD1839 treated TFK-1 cells (Figure 4).

Regulation of cell cycle G1-related proteins by ZD1839; $p27^{Kip1}$ stabilization. The cell cycle progression is regulated through positive and negative cell cycle regulatory molecules, such as CDKs, cyclins, CDK inhibitors (CDKI), p53 and pRb. The G1 arrest induced by ZD1839 was further confirmed by examining its effect on the expression of several key regulators of the G1-S phase transition. To elucidate the specific cell cycle regulatory proteins responsible for the G1 block mediated by ZD1839 in TFK-1 cells, focused was upon the $p27^{Kip1}$ involved in the G1 phase of the cell cycle. No influence on the p53 and CDKs was recognized in this experiment (data not shown).

The expression of $p27^{Kip1}$ in TFK-1 cells was examined by immunostaining (Figure 5A). $p27^{Kip1}$ protein was immunostained in the nucleus. In the TFK-1 cells, the control samples were almost immunostain-negative, but the over expression of p27Kip1 was detected after treatment with ZD1839.

Cells were treated with or without ZD1839 under the protein synthesis inhibitor cycloheximide. Western blotting

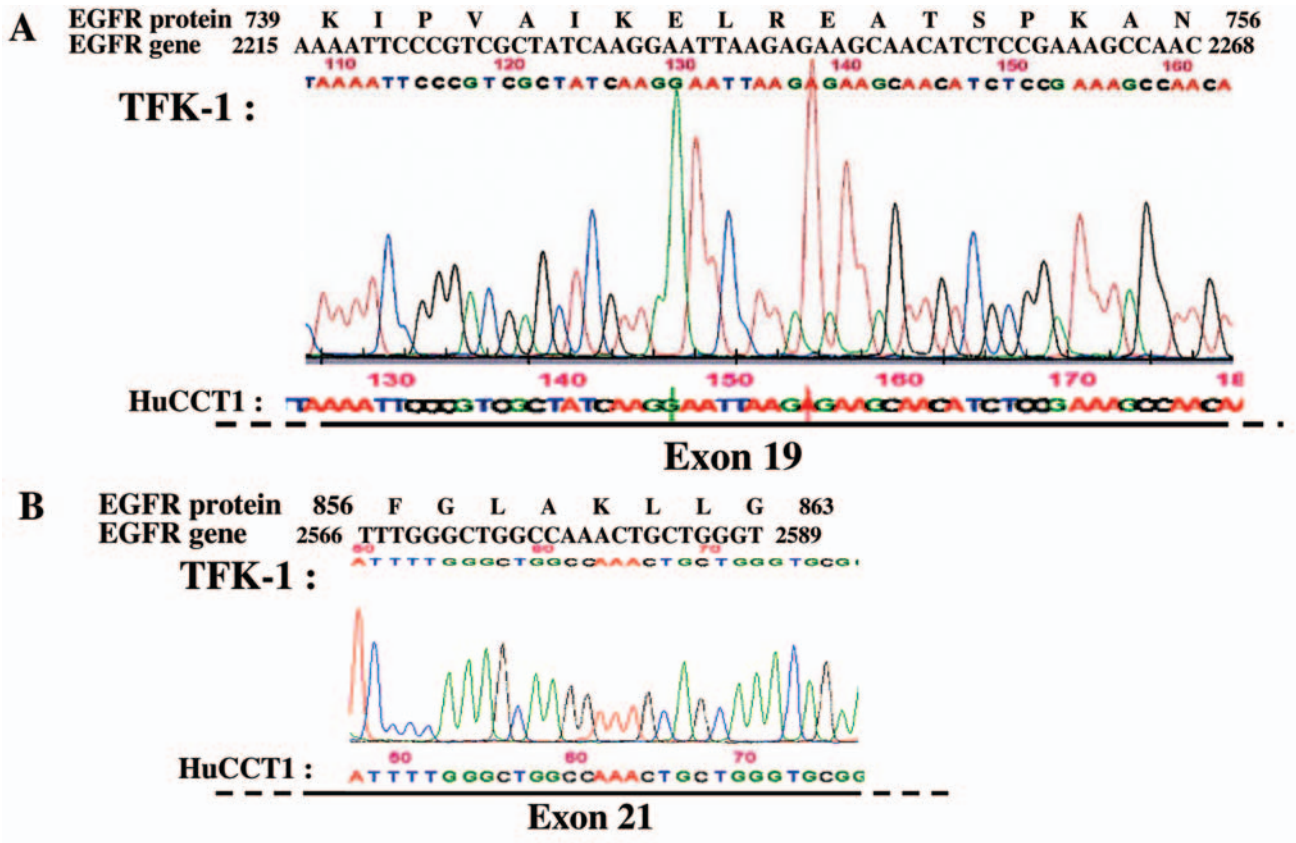


Figure 2. *Nucleotide-sequence analysis of cell lines. No mutations were founded in exon 19 (A) and exon 21(B) of EGFR from TFK-1 and HuCCT1 cells.*

analysis was performed using antibodies specific for $p27^{Kip1}$ and actin. The protein level of $p27^{Kip1}$ was measured at various time points after treatment with cycloheximide (Figure 5B). In ZD1839-treated cells, the levels of $p27^{Kip1}$ showed a time dependent increase. Interestingly, the level of $p27^{Kip1}$ declined much more rapidly in ZD1839-untreated cells than in ZD1839-treated cells (Figure 5C). This result suggested that ZD1839 enhanced the $p27^{Kip1}$ stability.

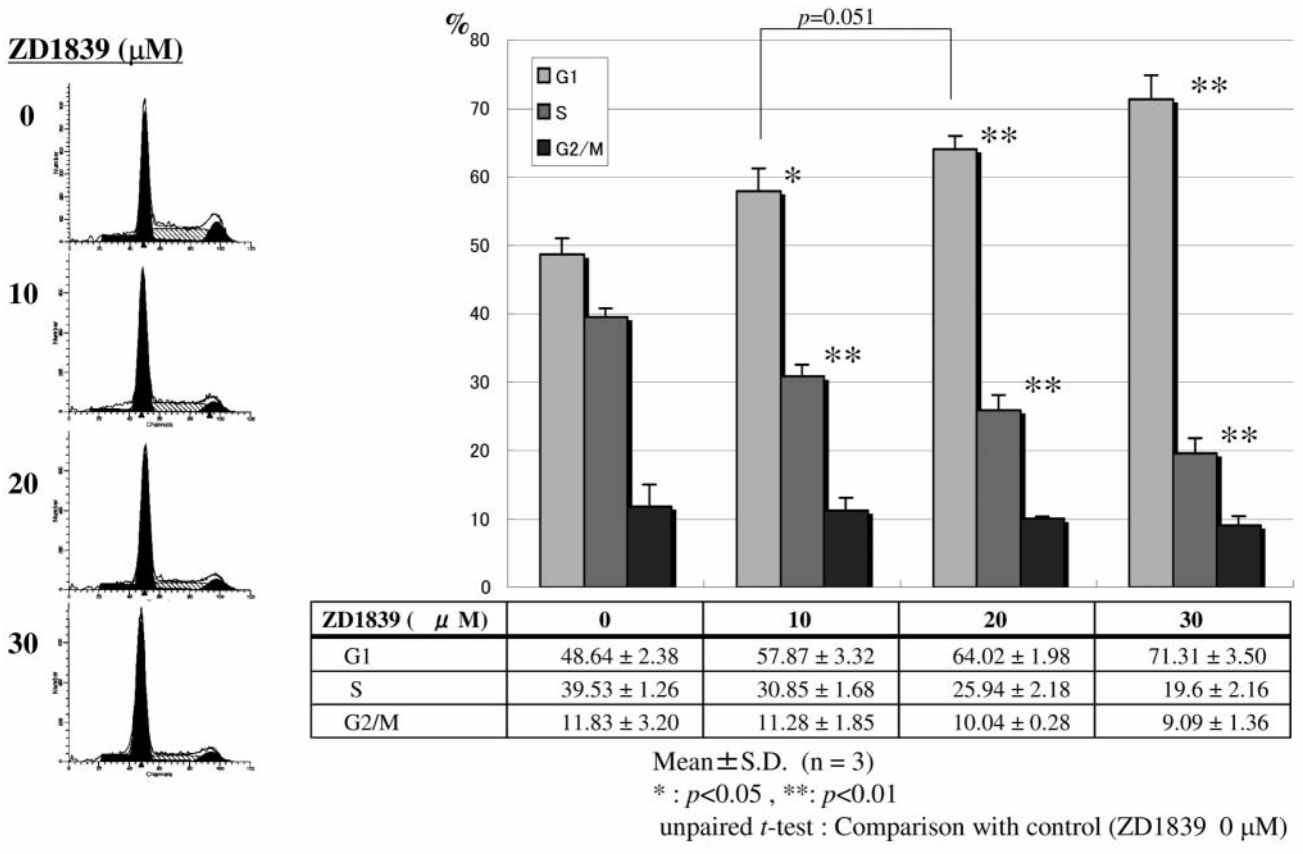
ZD1839 regulates the protein level of Jab1, a key molecule in the degradation of $p27^{Kip1}$. Protein extracts were prepared from cells treated with 10, 20 μM or without ZD1839 for two days. Blotting was performed using antibodies specific for CDKIs ($p27^{Kip1}$), Jab1 and actin. It was found that treatment with ZD1839 resulted in a significant reduction of Jab1 proteins. The $p27^{Kip1}$ time- and dose- dependent increase was related to the Jab1 down-regulation (Figure 6). In TFK-1 cells, ZD1839 stabilizes the $p27^{Kip1}$ protein by reducing Jab1. Stabilization of $p27^{Kip1}$ protein by the down-regulation of Jab1 might be important in the ZD1839-triggered G1 cell cycle block and apoptosis.

ZD1839 enhances radiosensitivity. To investigate whether ZD1839 and X-ray irradiation have synergic effects on the growth inhibition of TFK-1 cells, growth-inhibitory assays were performed (Figure 7A). The results demonstrate that the combined treatment significantly reduced the cell survival compared with control in TFK-1 cells. The isobolographic analysis indicated a synergistic effect (Figure 7B).

The differences in the effects between ZD1839 and radiation were further evaluated. The enhancement of radiation-induced apoptosis by ZD1839 was examined using TUNEL assay. As shown in Figure 7C, radiation alone (4Gy) induced apoptosis to a similar degree compared with control. However, combined treatment with radiation (4Gy) and ZD1839 (at 20 μM) resulted in a substantially more potent induction of apoptosis. This approach also confirmed the potentiation of radiation-induced apoptosis in TFK-1 cells by ZD1839.

Discussion

As a target for cancer cells, epidermal growth factor receptor (EGFR) has attracted attention (26). ZD1839 (Iressa®) is an orally active, selective EGFR tyrosine kinase (TK) inhibitor



ZD1839 (μ M)	0	10	20	30
G1	48.64 ± 2.38	57.87 ± 3.32	64.02 ± 1.98	71.31 ± 3.50
S	39.53 ± 1.26	30.85 ± 1.68	25.94 ± 2.18	19.6 ± 2.16
G2/M	11.83 ± 3.20	11.28 ± 1.85	10.04 ± 0.28	9.09 ± 1.36

Mean±S.D. (n = 3)
* : $p<0.05$, **: $p<0.01$
unpaired *t*-test : Comparison with control (ZD1839 0 μM)

Figure 3. *Cell cycle analysis. Cell cycle analysis by flow cytometry of TFK-1 cells treated with ZD1839. Effect of ZD1839 on cell cycle distribution of human cholangiocarcinoma cells. A clear G1 arrest was demonstrated in TFK-1 at 48h after ZD1839 treatment.*

that blocks the signal transduction pathways implicated in cell proliferation. In this report, it is demonstrated that ZD1839 is capable of inhibiting cellular proliferation and augmenting the response to radiation in cholangiocarcinoma cell lines. Somatic mutations in the TK domain of the EGFR gene are associated with the sensitivity of lung cancers to ZD1839 (10-11). In this analysis of the EGFR sequence, no mutations were found in TFK-1 and HuCCT1 cells. The sensitivity to ZD1839 was almost the same in TFK-1 and HuCCT1, and was relatively low. The lack of a mutation was one of the reasons for the reduced sensitivity.

In this study, the apoptosis induced by ZD1839 in TFK-1 cells was examined. The effect of ZD1839 was cytostatic, but higher doses increased apoptotic cell death. Previous studies have shown that ZD1839 has numerous effects on tumor cells including cell cycle arrest, an increase in apoptosis and a reduction in cell proliferation. It was found that treatment with ZD1839 (at concentrations ≥10 μM) for more than 48 hours resulted in apoptotic cell death in TFK-1 cells. The induction of apoptosis by ZD1839 has been reported in several cell lines derived from carcinomas of different hystotypes. The present cell cycle analyses revealed

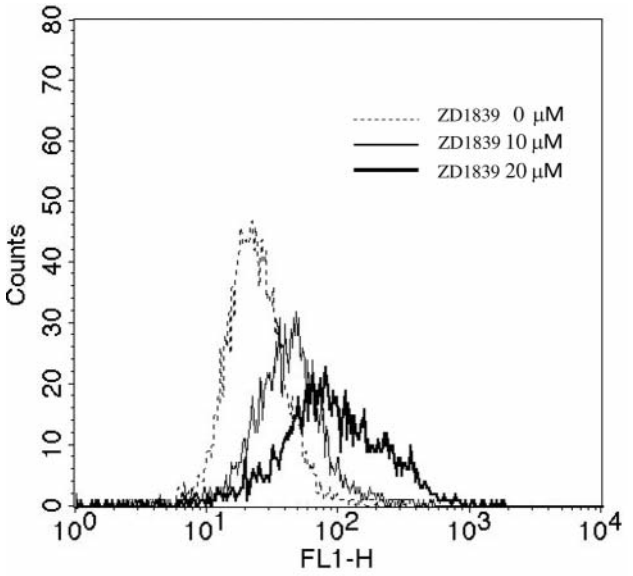


Figure 4. *Effect of ZD1839 on apoptosis in TFK-1 cells. TFK-1 cells were treated with or without ZD1839. At 48 hours after treatment, the apoptotic cells were determined by the TUNEL method as described in "Materials and Methods."*

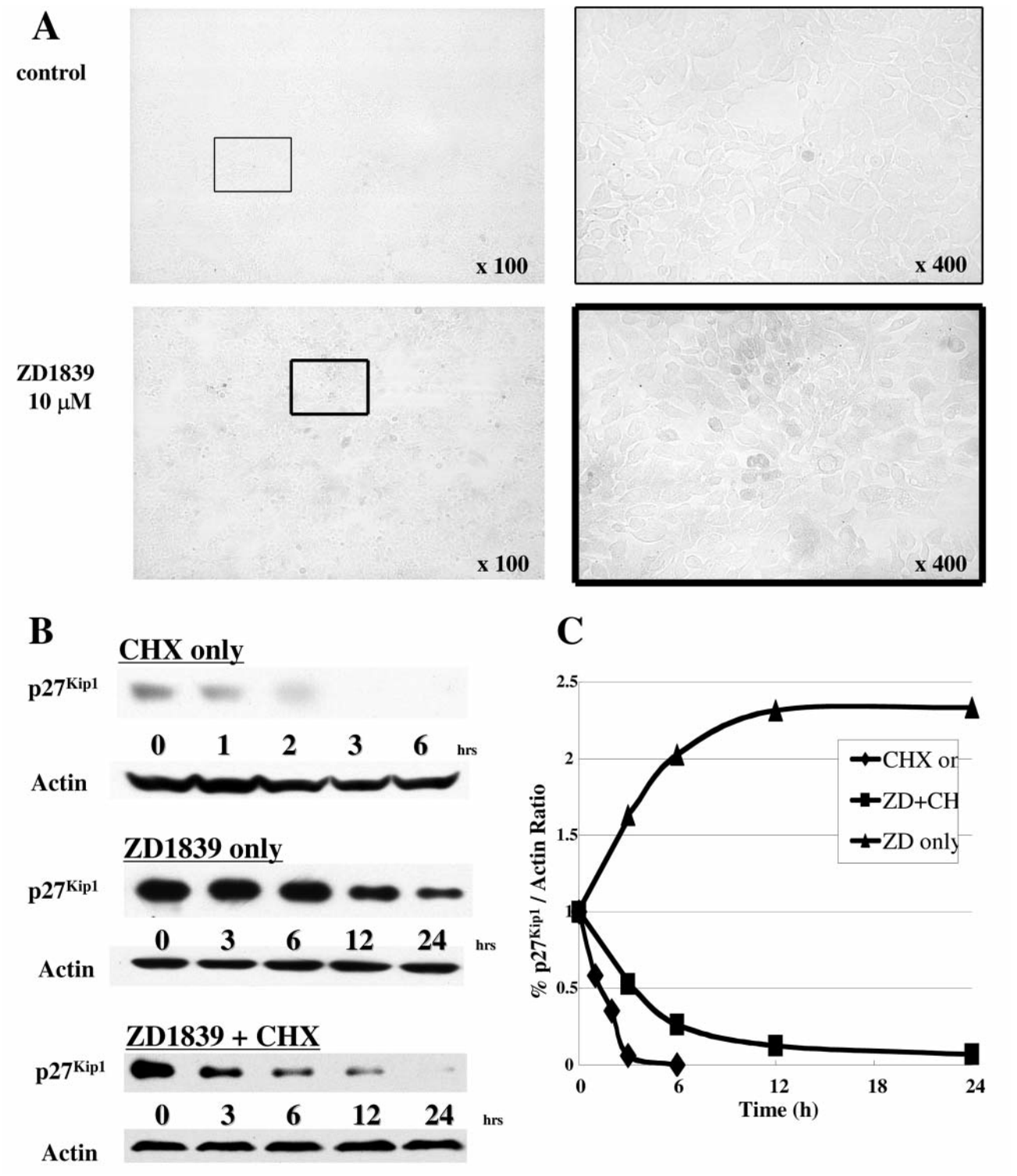


Figure 5. *A: Immunostaining of p27Kip1 protein. TFK-1 cells were treated without or with ZD1839 (at 10 μM). At 48 hours after treatment, the cells were fixed with 4% paraformaldehyde and stained with monoclonal antibody against p27^{Kip1} protein. The overexpression of p27^{Kip1} was detected at the nuclear level after treatment with ZD1839. B: Analysis of p27^{Kip1} protein expression in TFK-1 cells treated with ZD1839. Cells were treated with the protein synthesis inhibitor cycloheximide (at 10 μg/mL) for the indicated period of time. The cells were harvested and extracts were prepared, and the level of p27^{Kip1} was measured by Western blotting analysis as described in "Materials and Methods." C: Blots were also probed with polyclonal antibodies against actin to show the equivalent loading of protein.*

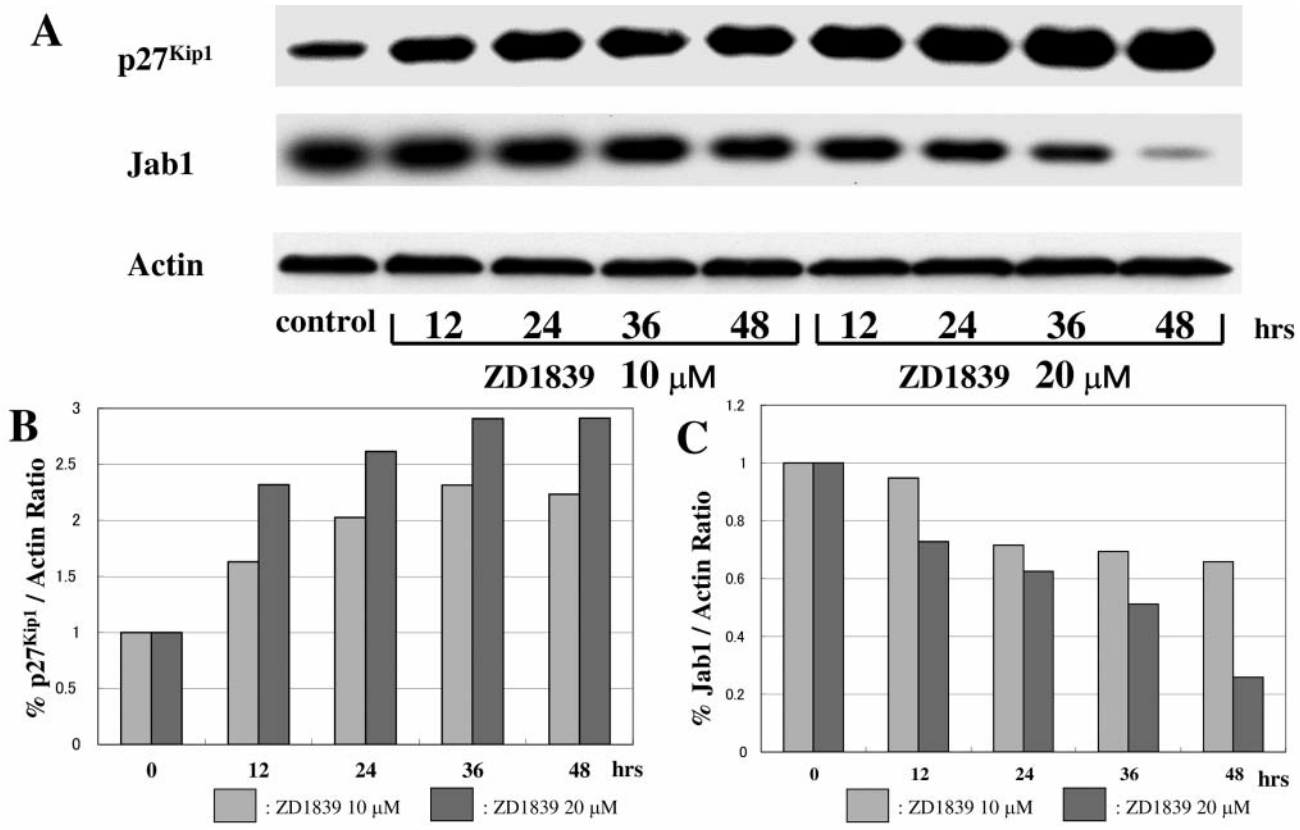


Figure 6. *The levels of p27Kip1 and Jab1 were measured by Western blotting analysis as described in "Materials and Methods." Blots were also probed with polyclonal antibodies against actin to show the equivalent loading of protein.*

prominent G1 arrest of TFK-1 cells after exposure to various concentrations of ZD1839, and this was accompanied by a significant decrease in the S phase. This result is consistent with those of other investigators, who showed that ZD1839 induced G1 arrest in several types of human cancer cell lines. Cell cycle control is a highly regulated process that involves a complex cascade of events.

The proliferation of mammalian cells is under strict control, and the cyclin-dependent kinase inhibitory protein p27Kip1 is an essential participant in this regulation both *in vitro* and *in vivo*. In various human cancers, reduced $p27^{Kip1}$ expression correlated well with a poor prognosis (16, 27, 29). The physiological role of $p27^{Kip1}$ is linked to the inhibition of G1-related CDKs kinase activities. $p27^{Kip1}$ is able to arrest the growth of cells in the G1 phase of the cell cycle (30). Previous studies demonstrated that the ZD1839-induced G1 arrest was associated with an elevation of $p27^{Kip1}$ in human head and neck cancer cell lines(31). The induction of $p27^{Kip1}$ protein after ZD1839 administration is well known, but the mechanism had not been clarified. The molecular mechanism of EGFR signals in up-regulating $p27^{Kip1}$ was investigated. The results suggested that $p27^{Kip1}$ plays a key role in the ZD1839-induced cell cycle perturbation by decreasing Jun activating domain-binding protein 1 (Jab1) (32) which leads to G1 growth arrest. Jab1 is a key molecule in the degradation of $p27^{Kip1}$ (33, 34). Jab1 has been described as a co-activator of AP1 transcription factor, and is a subunit of a large protein complex (called the COP9 signalosome). Jab1 specifically transports p27Kip1 from the nucleus to the cytoplasm, and reduces the protein level of $p27^{Kip1}$ by accelerating its degradation. Recently, the potential role of Jab1 was described in epithelial ovarian tumors (35), pancreatic cancer (36) and pituitary tumors (37). The control of Jab1 could be a novel target for experimental therapies. The present study showed that the ZD1839-induced apoptotic cell death was accompanied by an increase in $p27^{Kip1}$ stability.

Additionally, this study shows that ZD1839 potentiates the antitumor effect of radiotherapy in human cholangiocarcinoma cell lines. Cellular apoptosis represents another process that may influence the ultimate response of a particular tumor to ionizing radiation (38). ZD1839 was previously shown to increase the radiation-induced tumor growth delay in human squamous cell carcinomas (SCCs) (23), non-small-cell lung cancer (NSCLC) (21, 39), and breast cancer (40). These

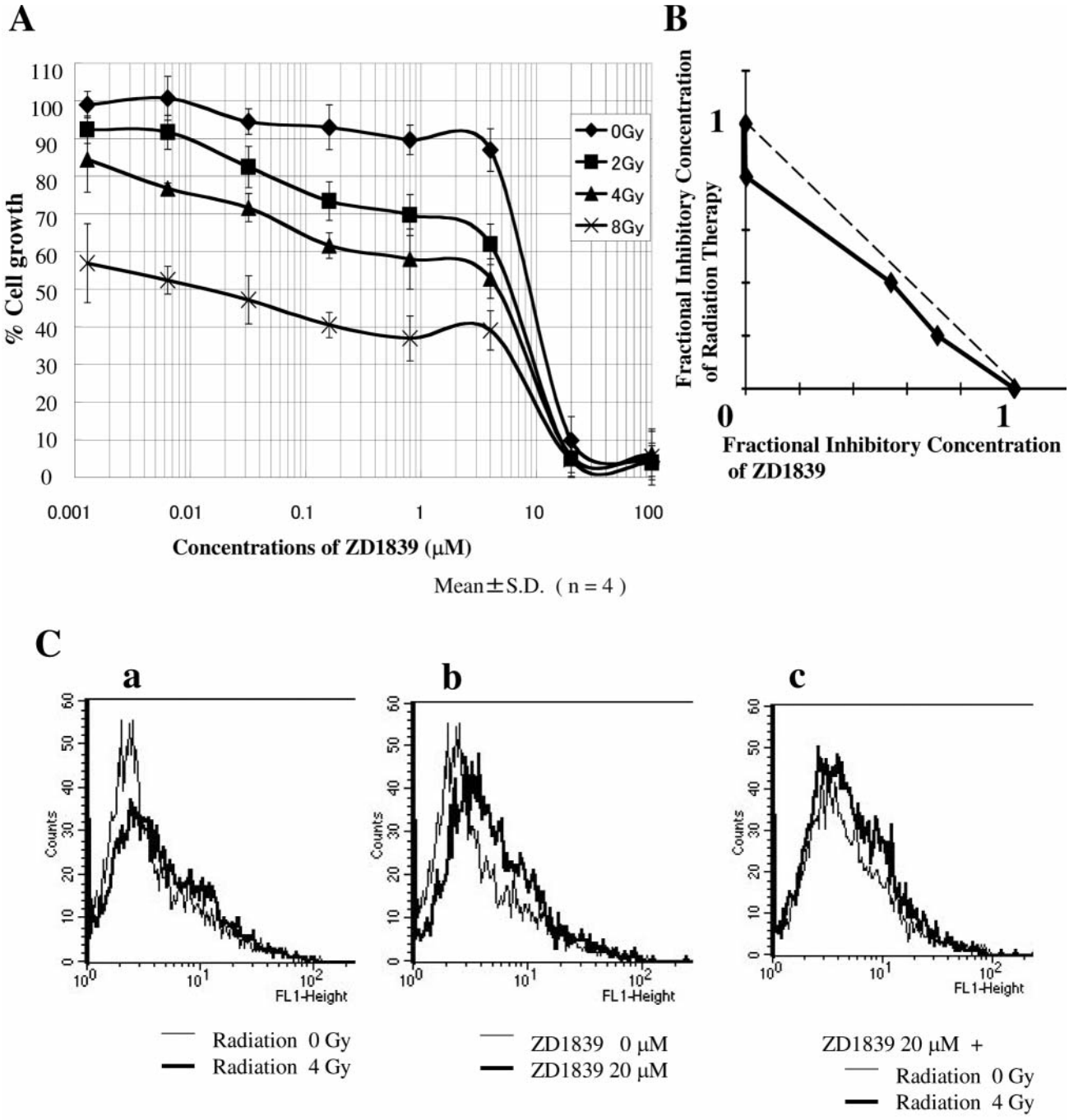


Figure 7. *A: Effect of ZD1839 on radiosensitivity. The influence of ZD1839 on radiosensitivity was examined by MTS assay in TFK-1 cells after exposure of various doses of radiation as described in "Materials and Methods." Cells were exposed to ZD1839 for 24 hours before irradiation. Control cells were exposed to radiation without ZD1839 treatment. Lines represent fitted curves as estimated by linear quadratic regression. B: The isobolographic analysis indicated that these cooperative effects in TFK-1 cells were synergistic. C: Enhancement of radiation-induced apoptosis by ZD1839 was examined using a TUNEL assay. Cells were exposed to ZD1839 (at 20 μM), X-ray irradiation (4Gy).*

findings showed that treatment with ZD1839 not only enhanced cell death after radiation but also increased the fraction of tumor cells that succumbed to radiation-induced apoptosis.

In conclusion, ZD1839 (Iressa®) has numerous effects on human cholangiocarcinoma cells including cell cycle arrest, an increase in apoptosis and a reduction in cell proliferation. In human cholangiocarcinoma cell lines (no mutation in the

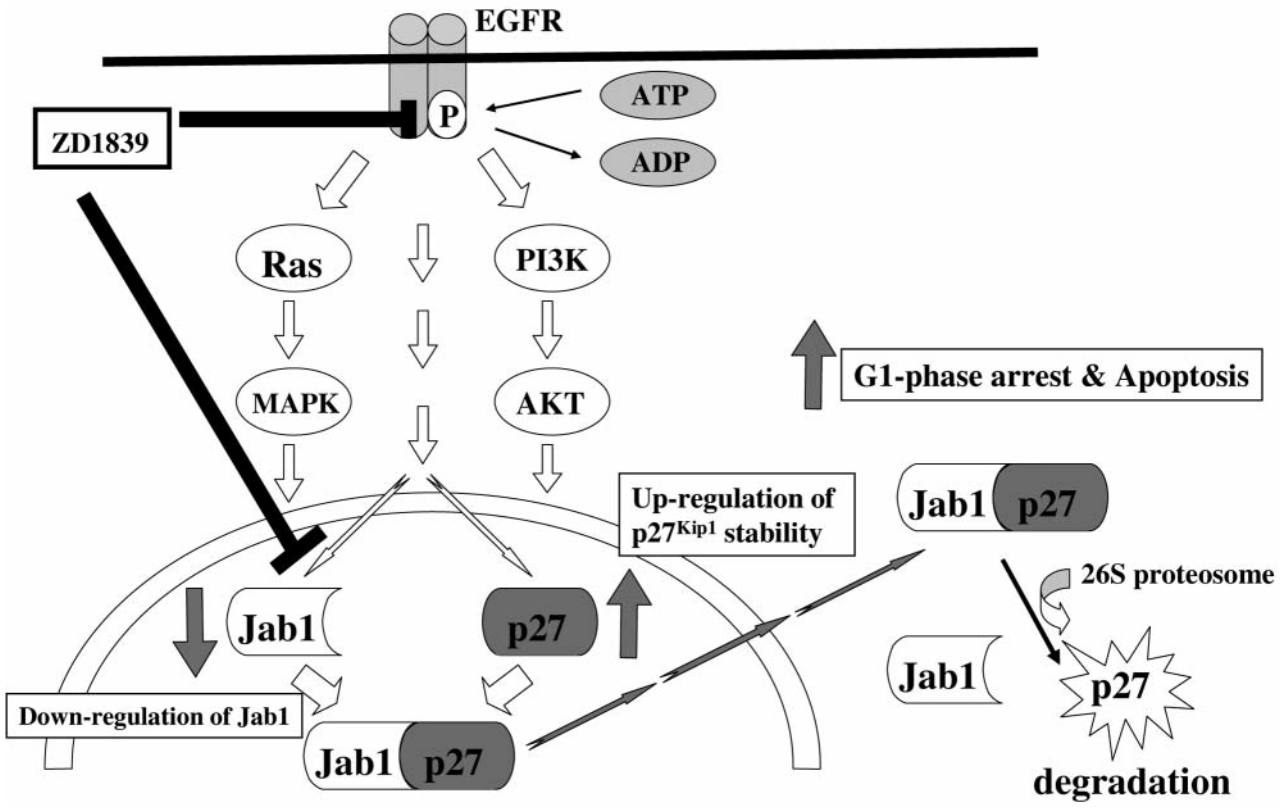


Figure 8. *ZD1839 influences $p27^{Kip1}$ protein up-regulation by down-regulating Jab1 protein*

EGFR sequence), it was found that the proliferative rate of tumor cells was reduced due to alterations in the pathways regulating the $p27^{Kip1}$ stability. ZD1839 influences $p27^{Kip1}$ protein up-regulation by down-regulating Jab1 protein (Figure 8). More work will obviously be needed to identify the various factors that play a role in the regulation of $p27^{Kip1}$ stability in human malignancies. ZD1839 in combination with radiation produced a dose-dependent inhibition, showing a synergic effect against cellular proliferation in TFK-1 cells.

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