Cell Cycle Perturbation and Acquired 5-Fluorouracil Chemoresistance

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Abstract. *Acquired chemoresistance is one of the obstacles for success of 5-fluorouracil (5-FU)-based cancer chemotherapy. Some molecular mechanisms of acquired 5-FU resistance are still unknown. We have recently demonstrated down-regulation of a group of cell cycle related genes in acquired 5-FU resistant human cancer cell lines. In this study, the bivariate distribution of propidium iodide versus BrdU in acquired 5-FU resistant colon ($H630_{R10}$) and breast ($T47D_{FU2.5}$) cancer cell lines was compared with their parental cell lines using flow cytometric analysis. The resistant cell lines showed significantly lower labelling index ($T47D_{FU2.5}$) and cell cycle delay in G1 and G1/S boundary and prolonged DNA synthesis time ($H630_{R10}$). Both resistant cell lines demonstrated significantly prolonged potential doubling time (T_{pot}). The protein expression levels of some G1 and S phase transition-related genes were also analysed by Western blot. CDK2 protein and Thr-160 phosphorylated CDK2 were remarkably reduced in the resistant cell lines. Cyclin D3 and cyclin A were also decreased in the resistant cells. Total pRB expression was unaltered but hypophosphorylation of pRB (Ser780, Ser795 and Ser807/811) was detected in the resistant cancer cells. Our data suggest that there may be a slow down in cell cycle traverse preventing incorporation of 5-FU metabolites into DNA and also providing cancer cells with sufficient time to correct the mis-incorporated nucleotides. The cell cycle perturbation may be involved in acquired 5-FU resistance.*

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5-Fluorouracil (5-FU) has been effectively used in the treatment of colorectal, pancreatic, breast, head and neck, gastric and ovarian cancers (17). 5-FU blocks DNA synthesis by inhibition of thymidylate synthase (TS) activity and also induces DNA and RNA strand breaks and apoptosis by direct incorporation into DNA and RNA. Chemoresistance is still one of the major obstacles for the success of 5-FU based chemotherapy. Mechanisms of 5-FU resistance have been intensively investigated but many details are still largely unknown (reviewed in 10). Although it has been widely thought that TS is a main molecular mechanism governing 5-FU sensitivity and targeting TS is a key strategy to reverse 5-FU resistance (7, 9, 24), the causal relationship between TS levels and 5-FU sensitivity is still quite controversial (1, 20). 5-FU resistance may also be induced by p53 gene mutation (3, 4), mismatch repair gene deficiency (12), deregulation of pyrimidine metabolism related enzymes and over-expression of anti-apoptotic factors (23).

5-FU only targets cycling cells and modulates the cancer cell cycle status *via* one of the following three modes: i) loss or accumulation of S-phase cells; ii) G2/M block; and iii) G1/S arrest (22). 5-FU directed DNA and RNA damage occurs in S and G1 phases, respectively (11, 13, 14). It has been reported that *de novo* 5-FU resistant cancer cell lines have a lower growth rate and disturbed cell cycle status. The cytotoxicity (IC_{50}) of 5-FU to asynchronous cancer cells is conversely correlated with the cell doubling time and BrdU labelling index (8, 13). The relationship between 5-FU chemosensitivity and cell cycle status in acquired 5-FU resistant cells is still not fully elucidated.

We have recently demonstrated a much slower proliferation rate in acquired 5-FU resistant cancer cell lines. In comparison with sensitive cancer cells, monovariate propidium iodide (PI) FACS analysis demonstrates a higher proportion of 5-FU resistant cancer cells were arrested in G0/G1 phase. In addition, DNA microarray profiling identified significant

down-regulation of some cell cycle related genes in the 5-FU resistant cancer cell lines (23).

In order to gain insight into the relationship between cell cycle aberration and acquired 5-FU chemoresistance, bivariate distribution of DNA synthesis (BrdU incorporation) *versus* DNA content (PI staining) was analysed in 2 pairs of acquired 5-FU resistant and sensitive cancer cells using flow cytometry. In parallel, the expression and/or functional status of key cell cycle regulators were analysed using western blotting. The results indicate that attenuation of cell cycle traverse may play a prominent role in acquired 5-FU resistance in human cancer cells. The data derived from this study provides further insight into the relationship between cell cycle perturbation and acquired 5-FU resistance in cancer cell lines.

Materials and Methods

Cell culture. The 5-FU resistant ($H630_{R10}$) and relevant parental (H630) colon cancer cell lines were generously provided from Prof. P.G. Johnston (The Queen's University of Belfast, Department of Oncology). The 5-FU resistant breast cancer cell line $T47D_{FU2.5}$ was generated by continuously culturing the drug sensitive parental T47D cells in medium containing increasing concentrations of 5-FU in a stepwise procedure. In comparison with relevant parental cell lines, the $H630_{R10}$ and $T47D_{FU2.5}$ are 37- and 35-fold resistant to 5-FU, respectively (23). The cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin. The resistant cell lines were cultured in the above medium supplemented with appropriate concentrations of 5-FU ($H630_{R10}$: 10 μM and $T47D_{FU2.5}$: 2.5 μM). The drug resistant cell lines were cultured in drug-free medium for 2 weeks before cell cycle analysis or harvesting for Western blotting analysis.

Western blotting analysis. Cells (80% confluence) grown in 75 cm flasks were washed in ice-cold PBS and lysed in 500 μl RIPA buffer. The lysate was centrifuged for 5 min in a microfuge and the supernatants retained. The protein (200 μg/cell line) was electrophoresed through a 10% SDS-PAGE and transferred to a PVDF membrane (Millipore) using an electrophoretic transfer chamber (Millipore). The blots were blocked for non-specific binding by incubating the membranes for 1 hour in TBS-T with 5% non-fat milk, which was also used to dilute primary [total pRb, p53, p21: Pharmingen, CA, 1:500; cdk2, cyclin A, cyclin D1, cyclin D3, cyclin E and p27: Santa Cruz, CA, 1:250; phosphor-cdk2 (Thr-160), phosphor-Rb (Ser-780, Ser-795, Ser-807/811): Cell Signaling, MA, 1:500] and secondary (Amersham, UK, 1:5,000) antibodies. The signal was detected using ECL Western blotting detection kit (Amersham, UK).

BrdU cell cycle analysis. Samples were processed according to the method described by Begg *et al.* (2). Briefly, the cells were labelled with 25 μM BrdU for 15 min. After culture in BrdU-free medium for appropriate time (0, 2, 4, 8, 12 and 24h), the cells were harvested by trypsinization and labelled with anti-BrdU antibody (DAKO; 1:40 diluted in PBT) and FITC conjugated anti-mouse IgG antibody (Sigma; 1:40 diluted in PBT) for 30 min at RT. After propidium iodide (PI, 10 μg/ml) staining, the cells were subjected to flow cytometric analysis (FACSCAN, Becton Dickinson). The green (FITC; BrdU) and red (PI; DNA) fluorescence signals were collected using the following filters: 515-545 nm (green) and 650 nm (red). At least 10,000 events were collected and analysed using CellQuest software (BD Biosciences).

Table I. *Cell cycle parameters of 5-FU resistant and sensitive cell lines.*

	$H630_{WT}$	$H630_{R10}$	$T47D_{WT}$	$T47D_{FU2.5}$
LI (%)	50.06 (3.78)	53.46 (2.41)	33.84 (1.13)	18.12 (0.62)
T_s (h)	7.77 (1.18)	21.65 (4.96)	5.05 (0.64)	4.89 (1.20)
T_{pot} (h)	12.40 (2.11)	32.70 (3.98)	11.93 (1.53)	21.57 (5.31)

[1]Data is mean from 3 experiments with SD in parentheses; [2]LI: $T47D_{WT}$ *vs.* $T47D_{FU2.5}$, $p<0.005$; T_S: $H630_{WT}$ *vs.* $H630_{R10}$, $p<0.01$; T_{pot}: $H630_{WT}$ *vs.* $H630_{R10}$, $p<0.001$; $T47D_{WT}$ *vs.* $T47D_{FU2.5}$, $p<0.05$.

Samples were analysed using the method reported by Begg *et al.* (2). The typical flow cytometric output is shown in Figure 1. Figure 1A is the DNA histogram of PI stained cells. The BrdU background was determined by staining the BrdU labelled cells with isotype-matched primary and then the FITC conjugated secondary antibody. Three gates were set in the BrdU/PI cytogram (Figure 1B). Total BrdU-unlabelled (BrdU–/PI+, Box 1), and BrdU-labelled G1 (Box 2) and S + G2/M phase cells (Box 3) could be identified.

The cycling cells were determined using a calculated labelling index (CLI):

$$CLI = \frac{LuD + [LD \times 0.5]}{uL + LuD\ [LD \times 0.5]}$$, LuD = labelled undivided cells (Box 3),

LD = labelled divided cells (Box 2) and uL = all unlabelled cells (Box 1). S-phase duration (T_S) was calculated from the following formula:

$$T_s = \frac{0.5}{RM - 0.5} \times t$$, where, $$RM = \frac{F_L - F_{G1}}{F_{GM} - F_{G1}}$$,

F_L is the mean PI fluorescence of the BrdU-labelled cells and F_{G1} and F_{GM} are the mean PI fluorescence values of G1 and G2/M cells, respectively.

At time zero (immediately after labelling), FL will be approximately halfway between the G1 and G2/M values, *i.e.* RM will be approximately 0.5. With time, F_L will approach the fluorescence of G2/M cells and RM will approach unity. RM will reach 1.0 only when the cells initially at the beginning of S have progressed to G2/M, *i.e.* in a time equal to TS.

The potential doubling time T_{pot} was calculated using the following formula: $T_{pot} = \frac{T_s}{LI}$,

where λ is a correction factor for the nonlinear distribution of cells through the cell cycle and usually taken to be 0.8 for tumour samples (21). Two factors influence the doubling time of tumour volume: proportion of non-cycling cells and cell loss (death). T_{pot} takes account of the non-cycling cell population but ignores the cell loss.

Results

Cell cycle status of 5-FU resistant and sensitive cell lines. The potential doubling time (T_{pot}) of resistant cancer cell lines was 2 ($T47D_{FU2.5}$) to 2.6 ($H630_{R10}$) times longer than the relevant sensitive cell lines (Table I). Cell cycle analysis indicated that

the underlying mechanisms for the longer T_{pot} in the 2 resistant cell lines were distinct. The LI of the 5-FU resistant and sensitive H630 cell lines were similar (Table I), indicating that the proportion of cycling cells did not differ significantly between the resistant and sensitive CRC cells. In comparison with $H630_{WT}$, the traverse of 5-FU resistant $H630_{R10}$ cells through the cell cycle was delayed in G1 and early S-phase for 4-6 hours (Figure 2) leading to a 2.89-fold longer S-phase time (T_S). In contrast, $T47D_{FU2.5}$ cells have a lower proportion of cycling cells than $T47D_{WT}$ but both cell lines travelled throughout the cell cycle at comparable speed (Figure 2). $T47D_{FU2.5}$ manifested 1.87-fold lower LI than their sensitive counterparts. Thus, the long T_{pot} of 5-FU resistant $T47D_{FU2.5}$ breast cancer and $H630_{R10}$ CRC cells appear to be caused by distinct mechanisms, involving either blockage of cell cycle entry or attenuation of S-phase transition, respectively.

Expression of key cell cycle regulatory proteins in resistant and sensitive cell lines reveals distinct molecular differences between 5-FU resistant cell lines. Cyclin D-CDK4 complexes influence the cell cycle during early G1 phase, primarily through phosphorylation of the retinoblastoma protein, pRB, resulting in inactivation of this protein as a transcriptional repressor of genes critical for S-phase entry and completion (18). There was no significant difference in CDK4 and cyclin D1 levels between sensitive and resistant cell lines (Figure 3A). In contrast, cyclin D3 and cyclin A were significantly down-regulated in the resistant cell lines. The expression of cyclin E was unchanged (Figure 3A).

A striking down-regulation of CDK2 expression was detected in both resistant cancer cell lines (Figure 3A). Since threonine-160 phosphorylation is essential for CDK2 kinase activity, we tested the Thr-160 phosphorylation status of CDK2. Figure 3A shows that Thr-160 phosphorylated CDK2 was also significantly decreased in the resistant cell lines.

Retinoblastoma protein plays a central role in cell cycle regulation. Phosphorylation of pRB releases E2F thus driving cells in the cell cycle. Our data demonstrated that although total pRB levels were not altered in resistant $T47D_{FU2.5}$ cells, pRB was hypophosphorylated in the resistant T47D cells at serine 780 and 795, preferential sites for cyclin D- and cyclin E-CDK complexes, respectively (Figure 3B). There was no significant pRB phosphorylation change detected in $H630_{R10}$ cells (Figure 3B).

CDK inhibitors are key negative regulators of the cell cycle and antagonise the catalytic activity of cyclin-CDK complexes. We therefore examined the expression of some of these proteins in our matched cell lines. The expression of $p21^{WAF1}$ was only modestly increased in both resistant cell lines. The increased expression of $p27^{Kip1}$ was observed in $H630_{R10}$ but not in $T47D_{FU2.5}$ cells (Figure 3C). The p53 gene (*TP53*) is mutant in both the T47D and H630 cell lines [(5, 16) and Dr. Chu E, personal communication]. The expression levels of p53 protein were not significantly different between the resistant and sensitive cell lines.

Discussion

In this study, cell cycle status in 2 pairs of acquired 5-FU resistant and parental cell lines was investigated by PI and BrdU bivariate flow cytometric analysis. Although both resistant cell lines manifested significantly longer T_{pot} (Table I), the underlying causes of cell cycle attenuation were different. In comparison with their sensitive counterparts, there were less $T47D_{FU2.5}$ cells entering the cell cycle. Flow cytometric analysis showed significantly lower LI in the $T47D_{FU2.5}$ cells. In contrast, prolonged S-phase transition time was the major cause for the extended T_{pot} in $H630_{R10}$ cells (Table I and Figure 2).

5-FU is an anticancer drug mainly targeting S-phase cells. The metabolites of 5-FU can only incorporate into the DNA and RNA of cycling cells. In addition, TS inhibition induced deoxynucleotide imbalance can only damage the cells undergoing DNA synthesis. Thus, attenuation of cell cycling may protect the resistant cancer cells from 5-FU cytotoxicity. Furthermore, prolonged G1 and S phases may also provide cancer cells with more time to repair damage induced by 5-FU. Hence, forcing resistant cancer cells into cell cycle might be another potential strategy to reverse 5-FU chemoresistance.

The eukaryotic cell cycle is highly regulated to ensure the correct sequence of events during genome replication. The cell cycle phase transitions are sequentially activated by cyclin-CDK complexes which in turn are regulated at multiple levels (18). To gain insight into the molecular mechanisms underlying the distinct cell cycle aberrations observed in the 5-FU resistant cell lines, the expression status of G1 and S phase regulatory proteins was investigated. As previously reported (23), CDK2 expression was strikingly down-regulated in the 5-FU resistant cell lines. We have now shown that active threonine-160 phosphorylated CDK2 also dramatically decreased in these cells.

Cyclin E, cyclin D1 and CDK4 expression was comparable in both resistant and sensitive cell lines. CDK2 is a key component for G1 and S phase transition. Without cyclin E-CDK2 activity, most types of cells will be arrested in G1 and S phase (18). Cyclin D1-CDK4/6 cannot replace cyclin E-CDK2 function. In contrast, cyclin E can correct cyclin D1-null phenotype indicating that cyclin D1 is not strictly necessary for G1 transition (15). Therefore, the low CDK2 activity induced cyclin E-CDK2 deficiency may confer the G1 and S phase delay in the 5-FU resistant cell lines. Cyclin A expression was reduced in the resistant cell lines. Cyclin A-CDK2 complex is involved in S-phase

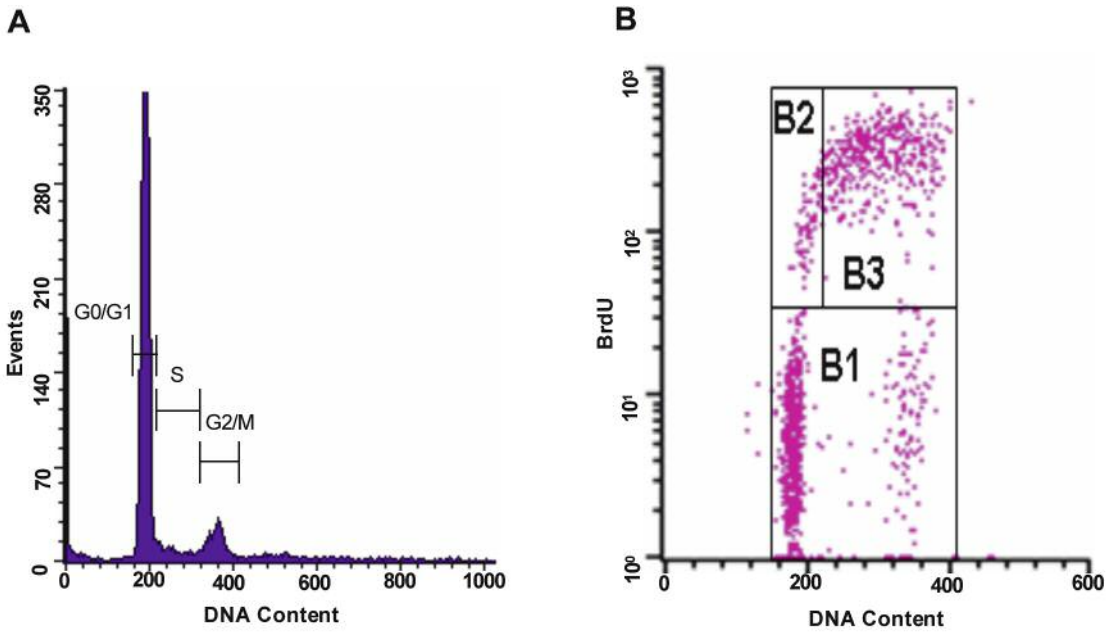

Figure 1. *A typical output of the flow cytometric analysis of cell kinetics. A. DNA histogram. B. Bivariate cytogram of BrdU versus DNA content. B1 = Box 1; B2 = Box 2; and B3 = Box 3.*

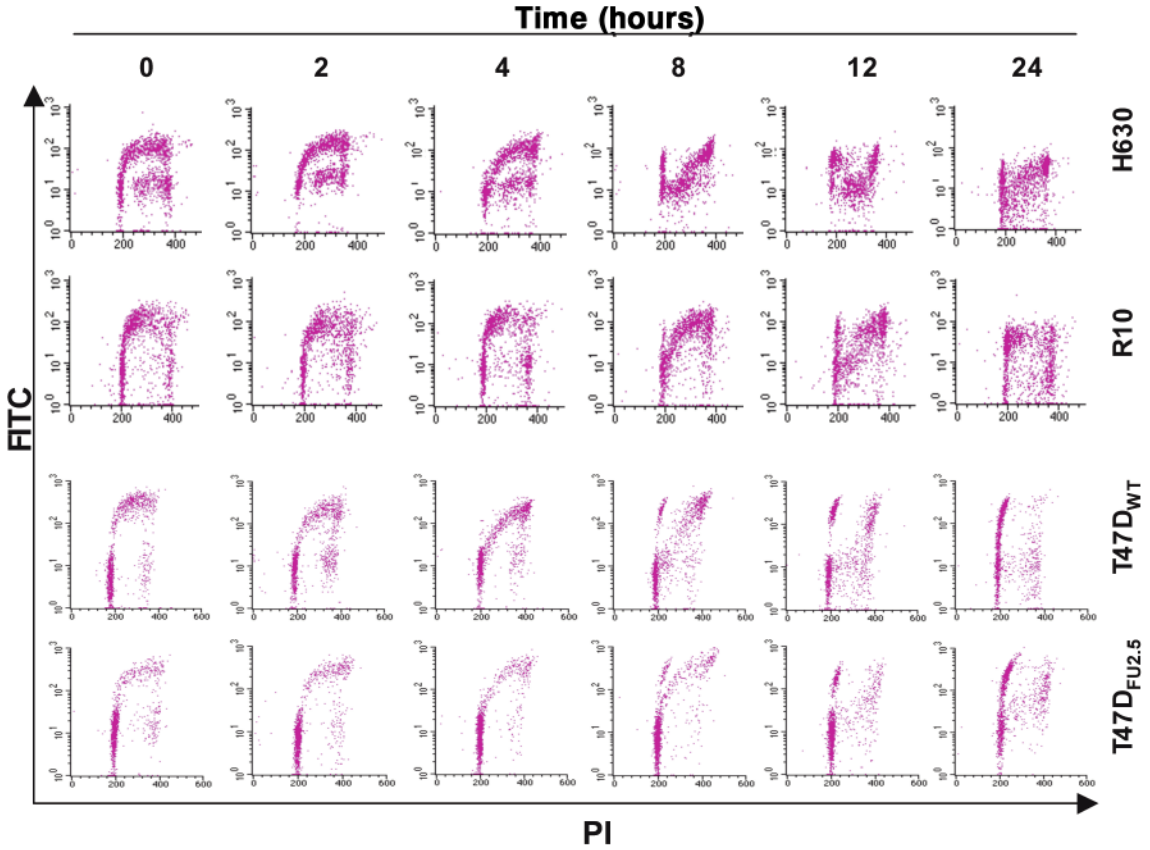

Figure 2. *The bivariate distribution of BrdU (y-axis) versus DNA content (PI, x-axis) measured by flow cytometry in 5-FU resistant and sensitive cell lines.*

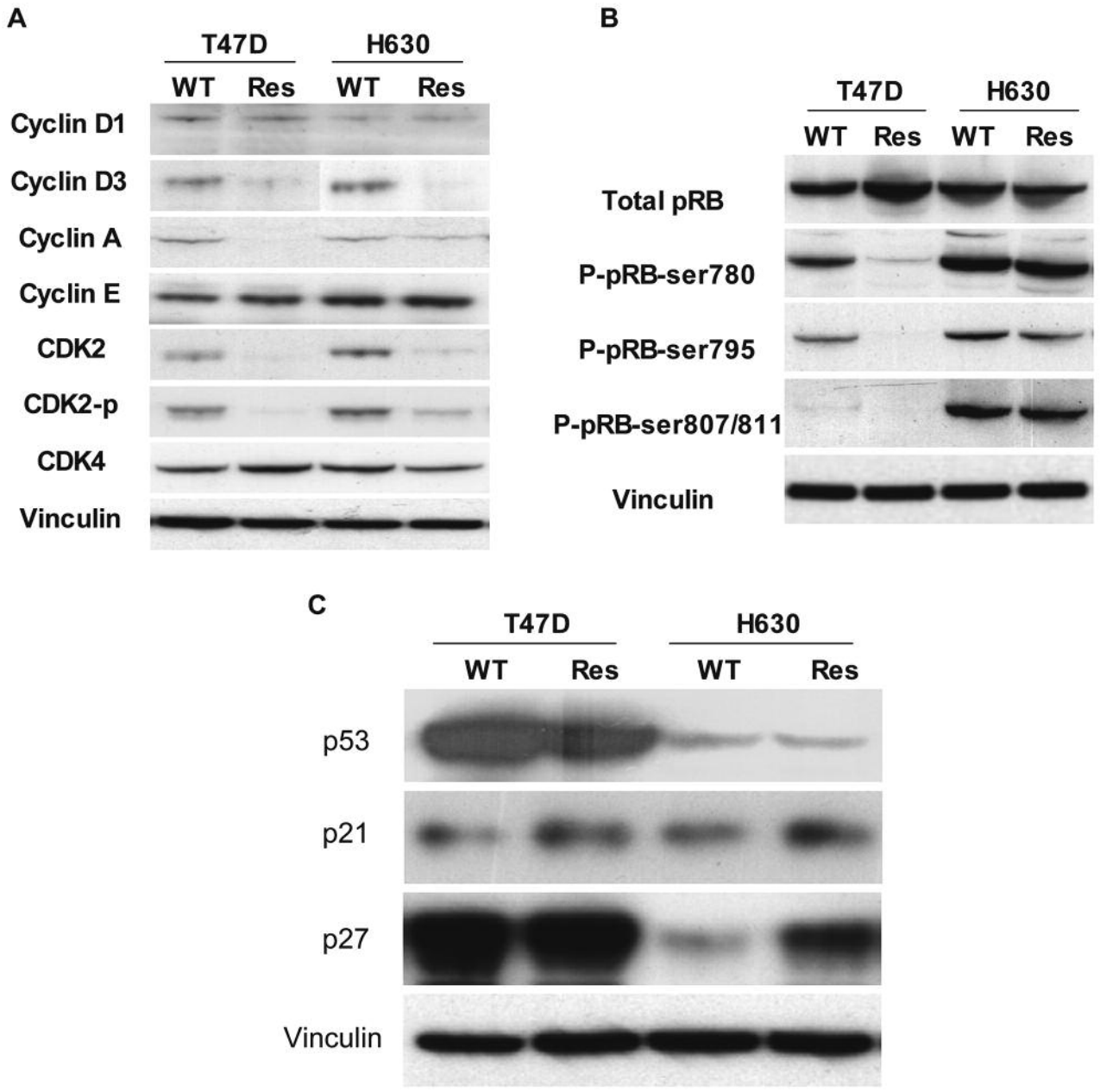

Figure 3. *Western blot analysis of the expression of cell cycle-related genes in 5-FU resistant and sensitive cell lines. A. CDK and cyclins. B. RB isoforms. C. p53, p21^{WAF1} and p27^{Kip1}.*

traverse. The reduced cyclin A expression may also play a role in S-phase delay in the 5-FU resistant cell lines. Cyclin D3 was also decreased in the resistant cell lines. However, unlike that of cyclin D1, the roles of cyclin D3 in the cell cycle remain largely unknown.

Retinoblastoma protein is the primary substrate of CDK2. pRB functions as a 'docking' site for a series of proteins that must be tightly controlled throughout the cell cycle. One group of proteins controlled by pRB is E2F transcription factor family. The hypophosphorylated pRB binds to and inhibits E2F transcriptional activity. Cyclin E-CDK2 catalyses the hyperphosphorylation of pRB and frees E2F transcription factors which will trigger the downstream gene expression and induce cell cycling (6). Although the total pRB expression was comparable in 5-FU resistant and sensitive cell lines, three major phosphorylation sites (Ser-780, Ser-795 and Ser-807/811; Figure 3) of the pRB in T47D$_{FU2.5}$ cells was remarkably hypophosphorylated. The reduced

phosphorylation of pRB at principal sites for cyclin-CDKs correlates with the decrease in CDK2 in these cells and may contribute to the cell cycle delay in $T47D_{FU2.5}$ cells. CDK2 inhibition also arrests RB-null cells, indicating that in addition to pRB, CDK2 has other cell-cycle targets (15). Further work needs to be done to elucidate the molecular events downstream of CDK2 in the 5-FU resistant H630 cells.

There was only a modest increase in $p21^{WAF1}$ expression in both resistant cell lines and $p27^{Kip1}$ protein expression was higher in $H630_{R10}$ cells. These inhibitors play distinct roles within the cell, with $p21^{WAF1}$ having a major role in p53-dependent and independent cell cycle arrest, while $p27^{Kip1}$ appears to be a negative regulator of cell proliferation in response to extracellular cues (19). It is widely accepted that cyclin-CDK2 complexes represent the principal target through which these inhibitors exerted their effects on cell proliferation. Further work is required to establish the significance of these alterations in determining the phenotype of the 5-FU resistant cells.

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References

1 AbdAlla EE, Blair GE, Jones RA, Sue-Ling HM and Johnston D: Mechanism of synergy of levamisole and fluorouracil: induction of human leukocyte antigen class I in a colorectal cancer cell line. J Natl Cancer Inst *87*: 489-496, 1995.

2 Begg AC, McNally NJ, Shrieve DC and Karcher H: A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. Cytometry *6*: 620-626, 1985.

3 Boyer J, McLean EG, Aroori S, Wilson P, McCulla A, Carey PD, Longley DB and Johnston PG: Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan. Clin Cancer Res *10*: 2158-2167, 2004.

4 Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Kinzler KW and Vogelstein B: Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest *104*: 263-269, 1999.

5 Butt AJ, Firth SM, King MA and Baxter RC: Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. J Biol Chem *275*: 39174-39181, 2000.

6 Chau BN and Wang JY: Coordinated regulation of life and death by RB. Nat Rev Cancer *3*: 130-138, 2003.

7 Copur S, Aiba K, Drake JC, Allegra CJ and Chu E: Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. Biochem Pharmacol *49*: 1419-1426, 1995.

8 Didelot C, Mirjolet JF, Barberi-Heyob M, Ramacci C and Merlin JL: Radiation could induce p53-independent and cell cycle – unrelated apoptosis in 5-fluorouracil radiosensitized head and neck carcinoma cells. Can J Physiol Pharmacol *80*: 638-643, 2002.

9 Johnston PG, Behan KA, Allegra CJ and Drake JC: Fluorouracil: active in ZD1694 (tomudex)-resistant cell lines with markedly elevated thymidylate synthase levels. J Natl Cancer Inst *87*: 1558-1559, 1995.

10 Longley DB, Harkin DP and Johnston PG: 5-Fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer *3*: 330-338, 2003.

11 Maybaum J, Ullman B, Mandel HG, Day JL and Sadee W: Regulation of RNA- and DNA-directed actions of 5-fluoropyrimidines in mouse T-lymphoma (S-49) cells. Cancer Res *40*: 4209-4215, 1980.

12 Meyers M, Wagner MW, Hwang HS, Kinsella TJ and Boothman DA: Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle responses. Cancer Res *61*: 5193-5201, 2001.

13 Mirjolet JF, Barberi-Heyob M, Merlin JL, Marchal S, Etienne MC, Milano G and Bey P: Thymidylate synthase expression and activity: relation to S-phase parameters and 5-fluorouracil sensitivity. Br J Cancer *78*: 62-68, 1998.

14 Myers CE: The pharmacology of the fluoropyrimidines. Pharmacol Rev *33*: 1-15, 1981.

15 Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM and Pagano M: Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol Cell Biol *15*: 2612-2624, 1995.

16 Park JG, Oie HK, Sugarbaker PH, Henslee JG, Chen TR, Johnson BE and Gazdar A: Characteristics of cell lines established from human colorectal carcinoma. Cancer Res *47*: 6710-6718, 1987.

17 Pinedo HM and Peters GF: Fluorouracil: biochemistry and pharmacology. J Clin Oncol *6*: 1653-1664, 1988.

18 Sherr CJ: Cell cycle control and cancer. Harvey Lect *96*: 73-92, 2000.

19 Sherr CJ and Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev *13*: 1501-1512, 1999.

20 Sobrero A, Kerr D, Glimelius B, Van Cutsem E, Milano G, Pritchard DM, Rougier P and Aapro M: New directions in the treatment of colorectal cancer: a look to the future. Eur J Cancer *36*: 559-566, 2000.

21 Steel A: Growth kinetics of tumours: cell population kinetics in relation to the growth. Oxford, Clarendon Press, 1977.

22 Tokunaga E, Oda S, Fukushima M, Maehara Y and Sugimachi K: Differential growth inhibition by 5-fluorouracil in human colorectal carcinoma cell lines. Eur J Cancer *36*: 1998-2006, 2000.

23 Wang W, Cassidy J, O'Brien V, Ryan KM and Collie-Duguid E: Mechanistic and predictive profiling of 5-Fluorouracil resistance in human cancer cells. Cancer Res *64*: 8167-8176, 2004.

24 Wang W, Marsh S, Cassidy J and McLeod HL: Pharmacogenomic dissection of resistance to thymidylate synthase inhibitors. Cancer Res *61*: 5505-5510, 2001.

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