

# Discrimination Effectiveness of CK18 on Cell Death Modes in Colon Cancer Cells

## [Kolon Kanseri Hücrelerinde CK18'in Hücre Ölüm Modlarını Ayırma Etkinliği]

<sup>1</sup>\*Zekiye Sultan Altun,

<sup>1</sup>\*Serpil Tanrıverdi Akhisaroglu,

<sup>2</sup>Janserey Batu,

<sup>3</sup>Halil Ateş,

<sup>4</sup>Hatice Giray,

<sup>2</sup>Semra Koçturk

<sup>1</sup>Dokuz Eylül University, Institute of Oncology, Izmir, Turkey

<sup>2</sup>Dokuz Eylül University, School of Medicine, Department of Biochemistry, Izmir, Turkey

<sup>3</sup>Dokuz Eylül University, Medical Faculty, Department of Hematology, Izmir, Turkey

<sup>4</sup>Dokuz Eylül University, Medical Faculty, Department of Public Health, Izmir, Turkey

\* These authors contributed equally to the study

### Yazışma Adresi

[Correspondence Address]

### Semra Koçturk

Dokuz Eylül University, School of Medicine, Department of Biochemistry, İnciraltı, 35340 Izmir, Turkey

Telephone: 90 232 4124407

Fax: +90 232 4124439

E-mail: semra.koçturk@deu.edu.tr

Registered: 15 April 2009; Accepted: 12 October 2009

[Kayıt tarihi : 15 Nisan 2009 ; Kabul tarihi : 12 Ekim 2009]

### ABSTRACT

**Objectives:** Cytokeratin-18 is an intermediate filament that could be a candidate cell death marker in monitoring chemotherapy efficiency. The aim of this study was to evaluate cell death determination effectiveness of CK18 ELISA assay in HCT-116 colon cancer cells treated with clinical compatible dosages of FOLFIRI chemotherapy combination.

**Methods:** Cytotoxicity of different FOLFIRI combinations was evaluated with MTT assay in HCT-116 cells. Apoptotic and non apoptotic cell deaths were evaluated with M30 and M65 CK18 ELISA assays. Flow cytometric Annexin V assay was used to verify apoptotic /non-apoptotic cell death ratios found with CK18 ELISA assays.

**Results:** FOLFIRI (15/1) treatments significantly increased apoptotic M30 levels at 24 and 48 hr ( $p < 0.05$ ) but did not cause any change at 72 hr ( $p > 0.05$ ). While low levels of M65 were found at 24 and 48 hr ( $p > 0.05$ ), high levels of M65 were detected at 72 hr ( $p > 0.05$ ). CK-18 and Annexin V results were correlated with each other.

**Conclusion:** It was demonstrated for the first time that CK 18 ELISA assay has the capacity to determine apoptotic and non apoptotic cell death in colon cancer cells caused by FOLFIRI treatment in a time dependent manner. We concluded that CK18 measurement may be useful for assessment of chemotherapy efficiency in an earlier time as a therapy monitoring marker in colon cancer cells.

**Key words:** CK18, cell death, apoptosis, colon cancer, FOLFIRI

### ÖZET

**Amaç:** Sitokeratin-18 bir arafilemant olup kemoterapi etkinliğinin izleminde kullanıma aday görülen bir hücre ölüm belirteçidir. Bu çalışmanın amacı; CK 18 ELISA yönteminin hücre ölümünü belirleme etkinliğini klinikle uyumlu dozlarda FOLFIRI kemoterapi kombinasyonu ile muamele edilmiş insan kolon kanser hücre hattında değerlendirmektir.

**Yöntem:** Farklı FOLFIRI kombinasyonlarının sitotoksitesiteleri MTT yöntemi ile HCT-116 hücrelerinde değerlendirildi. Apoptotik ve nonapoptotik hücre ölümleri M30 ve M65 CK18 ELISA yöntemi ile değerlendirildi. Akış sitometrik Anneksin V yöntemi CK18 ile saptanan apoptotik/non apoptotik hücre ölüm oranlarını doğrulamak için kullanıldı.

**Bulgular:** FOLFIRI (15/1) uygulaması apoptotik M30 düzeylerini anlamlı olarak 24 ve 48 saatte arttırdı ( $p < 0.05$ ) ancak 72. saatte anlamlı bir değişiklik oluşturmadı ( $p > 0.05$ ). Düşük M65 düzeyleri 24. ve 48. saatlerde saptanırken ( $p > 0.05$ ) 72. saatte yüksek M65 düzeyleri saptandı ( $p > 0.05$ ). CK18 ve Anneksin V yöntem sonuçları birbirleriyle uyumlu oldu.

**Sonuç:** CK18 ELISA yönteminin FOLFIRI uygulaması ile zamana bağlı olarak apoptotik ve nonapoptotik hücre ölümünü belirleme kapasitesi olduğu ilk defa gösterildi. CK18 ölçümünün kolon kanser hücrelerinde bir izlem belirteci olarak kemoterapi etkinliğini daha erken zamanda değerlendirmek için kullanışlı olabileceğini önermekteyiz.

**Anahtar kelimeler:** sitokeratin 18, hücre ölümü, apoptoz, kolon kanseri, FOLFIRI

## Introduction

Colorectal cancer (CRC) is the third most common cancer type in the world (1). Approximately 84-92 % of patients with CRC are treated by surgical resection, but more than half of these patients subsequently developed disease recurrence (2). Although chemotherapy is a choice for patients with recurrent tumors, intrinsic or acquired chemo-resistance is frequently encountered during chemotherapy. Due to this reason, to predict which patients will respond to a particular treatment, modality is becoming increasingly important (3). Currently, FOLFIRI (5-FU/Irinotecan) is one of the chemotherapeutic combinations used for metastatic colorectal cancer patient's treatment. At the present time many cancer therapy combinations are available but the clinician ought to receive guidance as to which patients should be treated with which drug. Ideally, biological markers should be available for predicting whether a specific tumor will be sensitive to treatment or not during chemotherapy cure. Unfortunately, many years of research has failed to identify reliable markers for prediction of sensitivity to chemotherapy during treatment. A good marker that predicts the sensitivity of chemotherapeutic agent may give a chance to clinicians to change the chemotherapy combination in a short time. An alternative approach to treatment monitoring is to develop methods for assessment of cell death in real time (3). Recently an attractive alternative is to measure the levels of cytokeratins, released macromolecules from death cells during chemotherapy.

Apoptosis is a commonly described cellular outcome of treatment with many anticancer drugs and defects in the apoptotic machinery are believed to contribute to therapy resistance (4-7). However, whether apoptosis is the primary anti proliferative mechanism of the anticancer drugs in solid tumors is controversial and non-apoptotic cell death modes are also possible (8). Evaluation of apoptotic and non-apoptotic responses is important in determining the sensitivity of anticancer drugs, chemotherapy usefulness and resistance. For these reasons an ideal marker should have a potential to determine different cell death modes together.

Cytokeratins are members of the family of intermediate filament proteins and are found primarily in epithelial cells (9). Cytokeratin 8, 18 and 19 are expressed by most types of carcinomas, including those of the breast, prostate, lung, colon and ovary (10). Cytokeratins are released from tumor cells and provide useful serum markers to evaluate the clinical progression of patients with epithelial malignancies. Cytokeratins are also released into the circulation where they may comprise partly degraded intermediate filament complexes. Soluble cytokeratin fragments can also be produced by caspase-cleavage during apoptosis, and these fragments are relatively stable (11-14).

The cytokeratin tumor marker field has recently been

developed by the introduction of a monoclonal antibody which recognizes a neo-epitope of CK18 exposed after caspase cleavage during apoptosis (15). Cytokeratin (CK) 18 is cleaved by caspases at two distinct sites (Asp238 and Asp396) during apoptosis (10). Cleavage after Asp396 leads to exposure of the M30 neoepitope (CK18-Asp396-NE). Soluble CK18 cleaved at this site is detected by the M30-ELISA assay that uses antibodies M30 and 5. Total soluble full-length CK18 and soluble COOH-terminal fragments are measured with the M65-ELISA assay that uses antibodies 6 and 5. M30 antibody detects only caspase-cleaved fragments of CK18, but not the native protein. An ELISA assay based on the M30 and M65 antibody has been developed and made commercially available (16). Recently it has been postulated that M30 as a selective biomarker of apoptotic cell death (15) and M65 as a marker for intact CK18 that is released from cells undergoing non-apoptotic cell death (17). While augmentation in the M30 activity correlates with high levels of caspases during apoptotic cell death, releasing of uncleaved CK18 (M65) correlates with necrotic cell death (15). Therefore CK18 is potentially both a quantitative and qualitative biomarker for cell death. Previous studies have provided evidence that serum CK18 is derived from tumor cells (10, 18) and it was encouraged as a clinically useful biomarker in breast, prostate, endometrial and esophageal cancer except for colon cancer (10, 18-21). Up to date, there were restricted studies related to the determination of apoptotic/ non-apoptotic cell death in colon cancer cells in the literature (22-24). In addition, there was no research related to assess cell death modes in the colon cancer cells (HCT-116) with CK18 assay and confirmed the assay by flow cytometric Annexin V assay.

The aim of our study is to evaluate effectiveness of CK18 ELISA assay in HCT-116 colon cancer cells treated with clinical compatible dosages of FOLFIRI (5FU/Irinotecan) combination and verify the mode of cell death, found with CK18 (M30/M65) ELISA, with flow cytometric Annexin V assay.

## Material and Methods

### *Chemotherapeutic drugs*

FOLFIRI combinations were prepared with 5 fluorouracil (Ebewe, Pharma) and irinotecan (Camptosar, Pfizer) drugs. Doxorubicin (1 µg/mL) (Adriplastina, Carlo Erba) was used as a positive control for apoptotic cell death in all experiments. All drugs were kindly provided from our oncology clinics which were normally used for patients. All drug combinations were prepared freshly for all experiments.

### *Cell line and culture conditions*

Human colon cancer cell line (HCT-116) was obtained from Prof. Dr. Henning Walczak (German Cancer Research Centre) as a gift. Cells were grown in McCoy's

(Gibco) medium supplemented with 10 % FBS (Gibco), 2 mM L-glutamine (Gibco) and 1 % penicillin/streptomycin (Gibco) under a humidified atmosphere, 95 % air and 5 % CO<sub>2</sub> at 37 °C. Cells were seeded at 1.8 x 10<sup>5</sup> cells/well in 6-well plates (Greiner) 24 hr before the addition of chemotherapeutic drugs. In the next day, fresh medium containing FOLFIRI combinations (5FU/irinotecan) was added to the wells. At times indicated in the treatment schedule, culture medium and cells were collected separately from subconfluent monolayer with trypsin/EDTA (Gibco). The studies were carried out using cells from passages 3-7. In all experiments untreated cells and Doxorubicin treated cells were included as negative and positive controls respectively. All experiments were carried out with six separately seeded wells (n=6) with three replicates.

### **Treatment schedule**

According to previous reports the drug treatment schedule was formed (25). FOLFIRI combinations were prepared with two cytotoxic drugs (5FU/Irinotecan). Twenty-four hours after seeding (0 hr), medium of cells were removed and replaced with combination of drugs. Cells were incubated with FOLFIRI combinations at 24, 48 and 72 hours. FOLFIRI (5FU/Irinotecan) combinations were used in 1/1, 5/1, 10/1 and 15/1 ratios for cell growth inhibition experiments.

### **Cell growth inhibition assay**

Inhibition of cell growth in response to FOLFIRI combination (5FU/Irinotecan) was assessed by 3-(4,5-dimethylthiazol-2-yl)-tetrazolium 2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Kit I (Roche Diagnostics, GmbH Penzberg, Germany) assay according to manufacturer's instructions.

### **MTT**

Briefly, cells were dispersed by trypsin-EDTA treatment and 5x10<sup>4</sup> cells/mL resuspended in McCoy's medium containing 10 % FBS and seeded into 96-well culture plates with six replicates. After 24 hours of plating, incubation was continued for another 24, 48 and 72 hours with presence of different ratios of FOLFIRI combinations, presence of doxorubicin (1 µg/ml) as positive control or in absence of drugs as negative control. At the end of the incubation periods, the reaction was terminated by adding 10 µL MTT reagent to each well. The reaction was allowed to proceed for 4 hour at 37 °C. The formazan crystals were dissolved by adding solubilization solution. After overnight incubation, the intensity of the color, reflects number of living cells, was measured at a wavelength of 595 nm by microplate reader (Thermo, Instruments Inc, USA). All assays were performed with 6 replicates and values were compared to the corresponding controls.

### **Cytokeratin 18 ELISA (M30/M65) Assays**

To quantify the apoptosis-associated M30 neopeptide

in tissue culture media, M30-Apoptosense an enzyme-linked immunosorbent assay (ELISA Kit, PEVIVA) was used. HCT-116 colon cancer cells were seeded in 96 well plates with a density of 10.000 cells per well, in 200µl medium. Treatment with FOLFIRI (5FU/Irinotecan) was initiated after 24 hr of seeding. At the end of the incubation periods 25µl medium was used for M30-Apoptosense assay and performed to the instructions of manufacturer. Briefly, 25µl standards, controls and samples were added to their assigned wells precoated with a mouse monoclonal antibody, followed by the horse-radish peroxidase-conjugated monoclonal antibody (M30) as tracer. For the formation of the solid phase/antigen/labeled antibody sandwich the microtiter plates were agitated on shaker for 4 hr at a speed of 600 rpm. After washing, substrate was added and the reaction was stopped with H<sub>2</sub>SO<sub>4</sub> after 20 min incubation. The absorbance was measured in a microplate reader at 450 nm. Assay performance was confirmed with the low and high controls that supplied with kits. M65-ELISA assay (ELISA Kit, PEVIVA) was used for detecting total soluble CK18.

The amount of M30/M65 antigens in the samples were calculated from a standard curve and expressed as Unit per Liter (U/L). Apoptotic and non-apoptotic cell death ratios (%) were calculated by CK18 measurements using the formulas (Apoptotic cell death=(M30/M65 levels)\*100, Non-apoptotic cell death=(M65-M30/M65 levels)\*100).

### **Annexin V/Propidium Iodide (PI) Flow Cytometry**

Apoptotic/non-apoptotic cell death ratios were also measured by flow cytometry. Annexin V assay gives the ratio of cell death according to staining of cells with Annexin V or PI dyes. Annexin V dye detects phosphatidylserine (PS) exposure on the outer leaflet of cell membrane (26). Assay was achieved briefly with 1.8x10<sup>5</sup> cells/well plated in 6-well plates and after 24 hours of plating, the medium was changed with FOLFIRI combination for 24, 48 and 72 hr incubations. At the end of the incubation periods, the cells were collected and resuspended in 500 µL of binding buffer (10 mM HEPES, NaOH (pH 7.4), 140 mM NaCl, 5 mM CaCl<sub>2</sub>) and 5 µL of Annexin V-fluorescein isothiocyanate (FITC, BD Biosciences), 5 µL of propidium iodide (PI, BD Biosciences). The samples were incubated at room temperature for 15 min in the dark, and analyzed by flow cytometer (FACSort) (Becton Dickinson, Sunnyvale, U.S.A.) using Cell Quest Software. The ratio of the cells in the Annexin V (+)/PI (-) fractions were regarded as early apoptosis, Annexin V (+)/PI (+) as late apoptosis or necrosis, and Annexin V (-)/PI (-) as viable. Each experiment was repeated three times.

### **Statistics**

All statistical analyses were performed using the SPSS

11.0 software program. Results were expressed as mean±S.E.M. Continuous variables were compared with the Mann-Whitney U test. Relationship analysis was evaluated with Spearman correlation coefficients (27). All treatment experiments were repeated at least three times to generate statistically relevant data.  $p < 0.05$  was considered statistically significant.

## Results

### Cell growth inhibition effect of FOLFIRI (5FU/Irinotecan)

MTT experiments showed that combinations used in the experiments (1/1, 5/1, 10/1, 15/1) have growth inhibition effect in a time dependent manner. No significant growth inhibition effect was observed at 24 hr for any combination. However significant inhibitions were observed at 48 and 72 hours in a time dependent manner and maximal inhibition (60 %) was obtained at 72 hr for the 15/1 FOLFIRI (5FU/Irinotecan) (30µg/mL-2 µg/mL) combination. This ratio was also compatible with the *in vivo* dosage being used in colon cancer patient's current therapy. Therefore the 15/1 ratio of FOLFIRI combination was chosen as an appropriate dosage for all of our experiments (Figure 1).

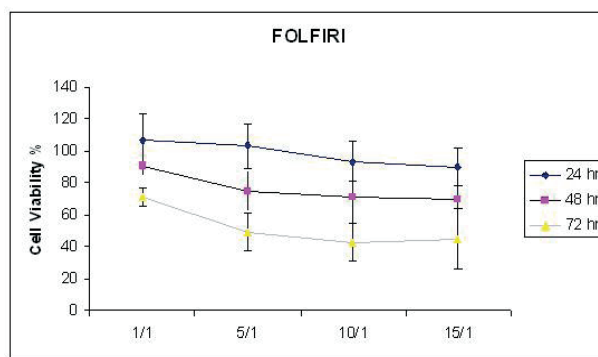
### Assessment of FOLFIRI induced apoptosis by CK18 and Annexin V assays

FOLFIRI (15/1-combination) treatments significantly increased M30 levels at 24 and 48 hours ( $p < 0.05$ ) but did not cause any change at 72 hr ( $p > 0.05$ ) compared with control (Figure 2A and Table 1). In addition, low levels of M65 were found in FOLFIRI treated group at 24 and 48 hr incubations ( $p > 0.05$ ) but high level of M65 was detected at 72 hr ( $p > 0.05$ ) (Figure 2B and Table 1). Measuring the ratio of caspase-cleaved CK18 (apoptotic) to total CK18 (apoptotic + non-apoptotic) released from cells is a convenient method to assess cell death modes (3, 19). To compare CK18 results with the Annexin V assay the results were calculated as a ratio of cell death with an equation ( $(M30/M65) * 100 = \text{apoptotic}$ ,  $(M65 - M30/M65) * 100 = \text{non-apoptotic}$ ). After calculation of cell death ratios; non-apoptotic cell death ratios were found 51.7 %, 48.7 %, 89.3 % and apoptotic cell death ratios were found 48.3 %, 51.3 %, 10.7 % at 24, 48 and 72 hr respectively (Figure 3A, B and Table 2). M30 and M65-CK18 assay results showed that FOLFIRI treatment caused nearly equal levels of apoptotic and non-apoptotic cell death at 24 hr and 48 hr and it caused mostly non-apoptotic cell death at 72 hr.

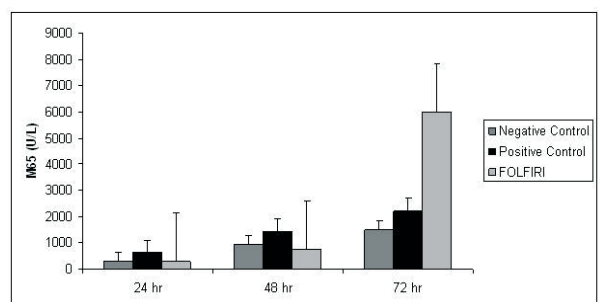
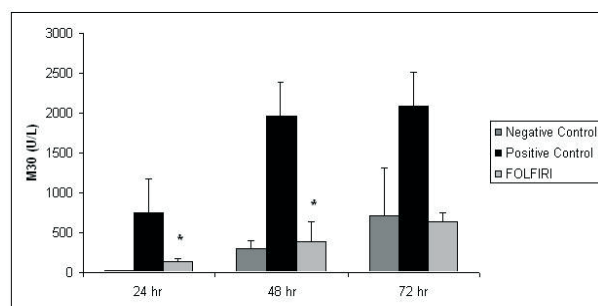
According to staining of Annexin V or PI dyes to the cells, cell death ratios were obtained by flow cytometry. Results showed that early apoptosis ratios were 68 %, 27 %, 0.75 % and sum of the late apoptosis and necrosis ratios (non-apoptotic cell death) were 32 %, 73 %, 99 % at 24 hr, 48 hr and 72 hr respectively (Figure 3A, B, E, F, G). Annexin V assay indicated that maximum apopto-

tic cell death occurred at 24 hr and cell death mode converts to non-apoptotic cell death with time.

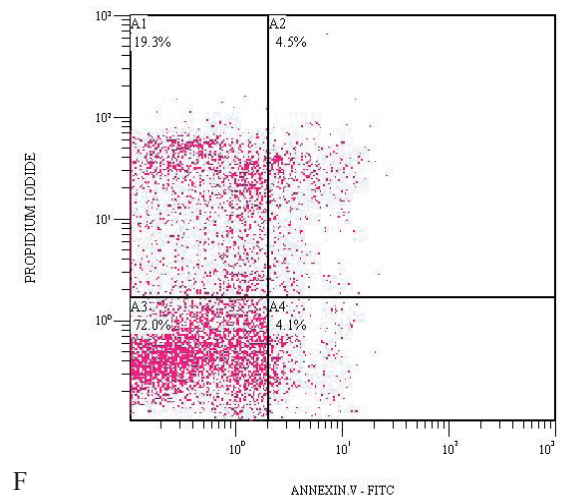
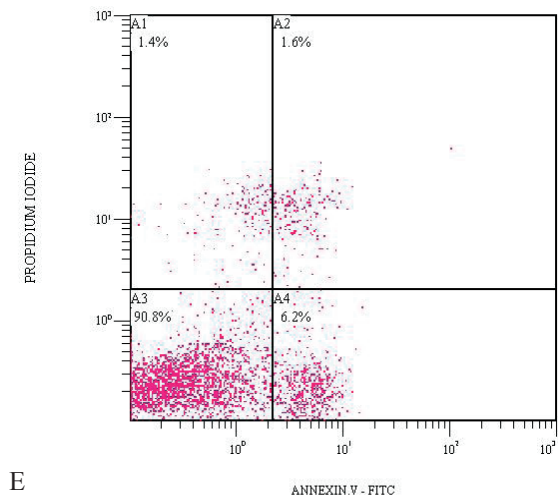
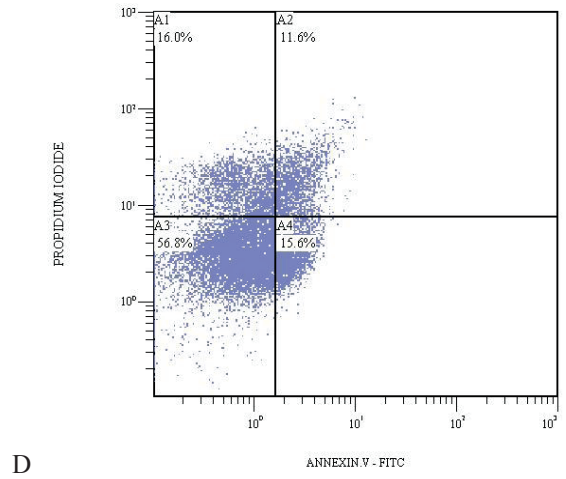
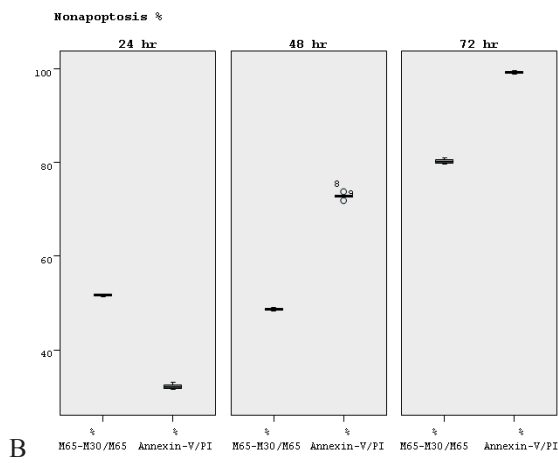
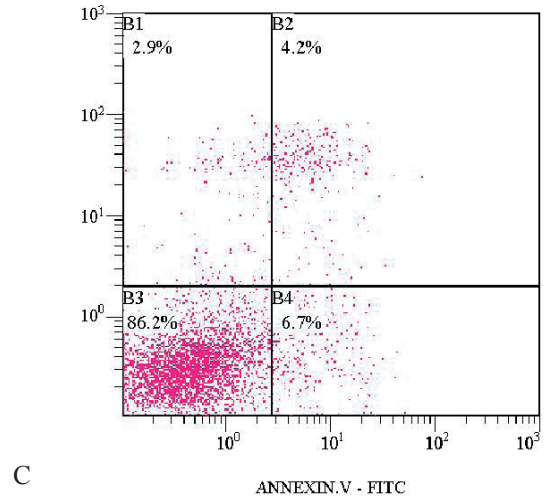
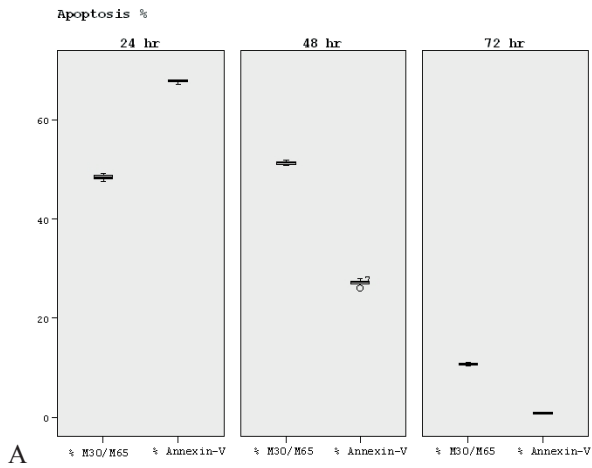
The apoptotic cell death ratios obtained by CK18 and Annexin V assays were correlated in a time dependent manner ( $\rho = 0.429, 0.435$  and  $0.086$  at 24, 48 and 72 hr respectively). Evaluation of compatibility of the apoptotic cell death ratios of CK18 and Annexin V assays indicated moderate correlation at 24 and 48 hr and poor correlation at 72 hr. Non-apoptotic cell death results of CK18 and Annexin V (PI) assays showed moderate correlation only at 48 hr ( $\rho = -0.080, 0.445$  and  $-0.113$  for 24, 48 and 72 hr respectively). Obtained Annexin V results were compatible with CK18 in terms of apoptotic and non-apoptotic cell death ratios (Fig 3A, 3B and Table 2).

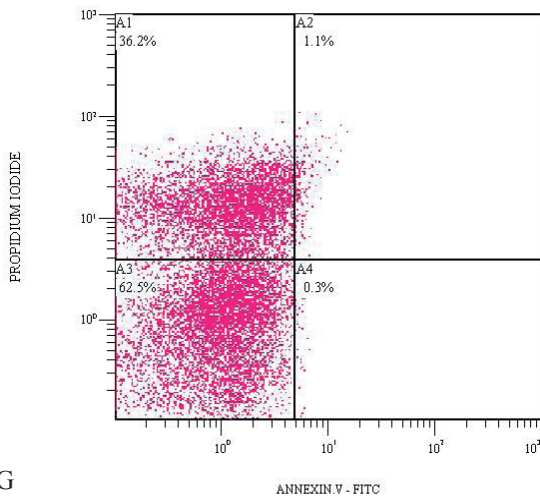


**Figure 1.** Cell growth inhibition effects of FOLFIRI combination (5FU/Irinotecan: 1/1, 5/1, 10/1 and 15/1). Each point represents the mean ± SEM of at least three independent experiments (n=6).



**Figure 2.** The levels of caspase-cleaved CK-18 (M30) (A) and total CK-18 (M65) (B) in HCT-116 human colon cancer cells at 24, 48 and 72 hours treated with FOLFIRI combination (5FU/Irinotecan:15/1). Results are the mean of three independent experiments; bars represent mean ± S.E.M. \*  $p < 0.05$ , indicates statistically significant difference between FOLFIRI treated and negative control groups.





G

**Figure 3.** Box plot graphics of apoptotic (A) and non-apoptotic (B) cell death ratios obtained from CK-18 and Annexin-V assays at 24 hr, 48 hr and 72 hr.

Representative images from Annexin V assays: C and D; negative and positive controls, E, 24 hr, F, 48 hr, G, 72 hr of FOLFIRI treated groups.

## Discussion

Until to date there were no ideal guides to determine of which patients have to use which chemotherapeutic drugs. Biological markers should ideally detect the response of specific tumor therapy. Some markers carrying that specialty are possible both in some of tumors and therapies following the treatment. An alternative approach in following of treatment is the evaluation of cell death modes in real time (3). It is generally believed that anticancer therapy induces tumor apoptosis (28, 29).

Apoptosis is an attractive clinical end point for the assessment of treatment efficiency and cytokeratin molecules are suggested for the assessment recently (3, 10, 30). Cytokeratins have been detected in circulation of cancer patients and widely used as serum tumor markers in some cancer types (10).

We previously showed that treatment of colon cancer patients could monitories with CK-18 assay (M30 and M65) using sera samples (31) but we didn't assess the conformity of CK 18 assay with a reference method, for instance a flow cytometric Annexin V assay. In this *in-vitro* study we assessed the accuracy of CK18 assay with Annexin V assay in aspect to apoptotic or non-apoptotic cell death discrimination in colon cancer cell line. We also determined cell death type caused by treatment of the cells with different FOLFIRI combinations. When apoptotic ratios of CK18 (M30) and Annexin V assays were evaluated, results show that FOLFIRI significantly induced apoptosis both at 24 and 48 hr. However, obtained apoptotic ratio by Annexin V assay was higher than obtained with M30 assay at 24 hr. This difference may be explained with the power of Annexin V assay and discrimination ability of Annexin V dye on early apoptotic cells (32). This suggestion was also supported with the results for 48 hr. At this time point, higher apoptotic ratio was obtained with M30 assay than with Annexin V assay. Although no differences were detected by M30 assay at 24 hr and 48 hr, different ratios were obtained from Annexin V assay at the same time points. These differences might also be explained with the discrimination power of Annexin V assay for apoptotic and non-apoptotic cell death. For higher apoptotic ratios of M30 assay at 48 hr, we suggest that M30 assay has no

**Table 1.** M30 and M65 levels in negative and positive controls and FOLFIRI (5Fu/Irinotecan) (15/1) treated cells.

	M30 (U/L)		
	24 hr Median (min-max)	48 hr Median (min-max)	72 hr Median (min-max)
Negative Control (n=6)	23.3 (8.3- 49.3)	297.8 (88.3- 507.3)	102.5 (32.5-2430)
Positive Control (Doxorubicin treated) (n=6)	643.1 (631.3-976.8)	1973.8 (1758.8-2137.5)	2176.3 (1906.3-2179.4)
FOLFIRI treated (n=6)	128* (115.3-178.8)	391.3* (255.3-518.3)	632.8 (541.3-748.8)

	M65 (U/L)		
	24 hr Median (min-max)	48 hr Median (min-max)	72 hr Median (min-max)
Negative Control (n=6)	318.4 (305.1-331.8)	922.2 (916.9-926.9)	1486.2 (1456-1558.2)
Positive Control (Doxorubicin treated) (n=6)	627 (591.8-727.3)	1612.9 (964-1747.3)	2227 (2218.4-2235.1)
FOLFIRI treated (n=6)	290.6 (264-320.7)	691.8 (624-952.9)	6105 (5206.2-6721.8)

In all experiments untreated cells and Doxorubicin treated cells were included as negative and positive controls respectively. Values are shown as median and range. \*p<0.05 versus negative control.

**Table 2.** Apoptotic and non-apoptotic cell death ratios from CK-18 and Annexin V assays.

Annexin-V (Apoptosis %)			
	24 hr Median (min-max)	48 hr Median (min-max)	72 hr Median (min-max)
Negative Control (n=6)	6.9 (6.7-7.3)	8.18 (7.8-8.7)	9.25 (8.8-9.7)
FOLFIRI treated (n=6)	67.85 (67.2-68.1)	27.2 (26.0-27.9)	0.74 (0.7-0.9)
Annexin-V (Non-apoptosis %)			
	24 hr Median (min-max)	48 hr Median (min-max)	72 hr Median (min-max)
Negative Control (n=6)	7.1 (6,9-7,4)	7.8 (7.4-8.1)	9.1 (8.8-9.4)
FOLFIRI treated (n=6)	32.1 (31.6-33.1)	72.9 (71.9-73.8)	99.3 (98.9-99.7)
CK-18 (Apoptosis %)			
	24 hr Median (min-max)	48 hr Median (min-max)	72 hr Median (min-max)
Negative Control (n=6)	8 (7.8-8.3)	32 (32.1-32.6)	48 (48.0-49.0)
FOLFIRI treated (n=6)	48.25 (47.6-49.1)	51.35 (50.7-51.8)	10.65 (10.3-11.0)
CK-18 (Non-apoptosis %)			
	24 hr Median (min-max)	48 hr Median (min-max)	72 hr Median (min-max)
Negative Control (n=6)	92 (92.0-92.6)	67.7 (67.4-68.1)	52.1 (51.8-52.4)
FOLFIRI treated (n=6)	51.65 (51.4-52.0)	48.65 (48.2-49.0)	80.2 (79.7-81.0)

Apoptotic and non-apoptotic cell death ratios from CK-18 assays were calculated from the formulas:  $((M30/M65)*100=apoptotic, ((M65-M30/M65)*100) = non-apoptotic$ .

Apoptotic and non-apoptotic cell death ratios from Annexin-V assays were expressed as follows: Early apoptosis ratios as apoptotic cell death and sum of the late apoptosis and necrosis ratios as non-apoptotic cell death. Results are expressed as median and range.

ability to discriminate early and late apoptotic cell death. Moderate compatibility between M30 and Annexin V results at 24 hr ( $p=0.429$ ) expressed that apoptotic cell death is predominant at the early time point and then non-apoptotic death takes place. Kramer *et al.* (10) concluded that apoptotic cell death of tumor cells was not a dominant cell death mode for *in vivo* of many tumors and monitoring of total cell death modes during therapy might be important than only monitoring apoptosis. When we evaluate M65 results as non-apoptotic and M30 results as apoptotic cell death ratio our results agree with this suggestion.

We also assessed non-apoptotic cell death ratio with Annexin V and CK18 (M65) assays. Almost the same ratios were obtained at 24 and 48 hr by CK18 (M65) assay and no significant difference was found between the results ( $p<0.05$ ). However correlation analysis between M65 and Annexin V assays showed moderate correlation only at 48 hr ( $p=0.445$ ). Obtained close ratios of apoptotic and non-apoptotic deaths at 24 and 48 hr expressed that both of the cell death modes are effective at these time points and CK18 ELISA assays have compatible capacity of cell death determination with Annexin

V assay. Although the results point out that Annexin V assay was more sensitive in the discrimination of early and late cell death modes than CK18 ELISA assays, the results also suggested that CK18 ELISA method has a capacity to determine apoptotic/non-apoptotic cell death modes in FOLFIRI treated colon cancer cells *in vitro*. If the undesirable side effects of chemotherapeutic agents and the importance of early evaluation of treatment response during chemotherapy are taken into consideration, CK18 assay can be suggested for therapy monitoring due to easier usage and more cost effective than cytometric Annexin V assay. To monitorize therapeutic response and apoptotic, non-apoptotic cell deaths, we suggest that synchronous analysis of M30 and M65 levels is necessary. Colorectal cancer is the most common malignancy affecting both women and men worldwide. To evaluate the clinical utility of CK18 larger clinical studies are required. Although this *in-vitro* study and our *in-vivo* results (31) confirmed each other, we need more studies including larger groups with colon cancer patients.

In conclusion the clinical efficiencies of a chemotherapeutic drug generally require long observation periods

and always are assessed in patients. Additionally it is uncertain whether measurements of tumor cell death *in vivo* (by imaging, serum biomarkers or by other methods) will be strictly correlated with patient survival. A therapy may induce cell death of sensitive population of cells but other cell populations can continue to proliferate. A biomarker should be useful for demonstration of a drug effect in early phase of treatment and also gives information about the modes of cell death. Although our previous data have suggested that serum CK18 measurements may be useful for assessing colorectal cancer patient's treatment effects (31), we also suggest that CK18 ELISA method has a capacity to determine apoptotic/non-apoptotic cell death modes in FOLFIRI treated colon cancer cells *in vitro* by this study.

As a result, it was demonstrated for the first time that CK 18 ELISA assay has capacity to determine apoptotic and non-apoptotic cell death in colon cancer cells caused by FOLFIRI treatment in a time dependent manner. Based on moderate correlations between Annexin V and CK18 assays, we concluded that CK18 measurement may be useful for assessment of chemotherapy efficiency in an earlier time as therapeutic response marker in colon cancer cells.

## Acknowledgements

This study was supported by a grant from Dokuz Eylül University Research Foundation (Project 2006.KB.SAG.21).

## References

- [1] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. (2008) Cancer statistics, 2008. *CA Cancer J Clin.* 58: 71-96.
- [2] Nelson H, Petrelli N, Carlin A, Couture J, Fleshman J, Guillem J, Miedema B, Ota D, Sargent D. (2001) National Cancer Institute Expert Panel. Guidelines 2000 for colon and rectal cancer surgery. *J Natl Cancer Inst.* 93: 583-96.
- [3] Linder S, Havelka AM, Ueno T, Shoshan MC. (2004) Determining tumor apoptosis and necrosis in patient serum using cytokeratin 18 as a biomarker. *Cancer Lett.* 214: 1-9.
- [4] Viktorsson K, Lewensohn R, Zhivotovsky B. (2005) Apoptotic pathways and therapy resistance in human malignancies. *Adv Cancer Res.* 94: 143-96.
- [5] Dive C, Gregory CD, Phipps DJ, Evans DL, Milner AE, Wyllie AH. (1992) Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim Biophys Acta.* 1133: 275-85.
- [6] Eliopoulos AG, Kerr DJ, Herod J, Hodgkins L, Krajewski S, Reed JC, Young LS. (1995) The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. *Oncogene.* 11: 1217-28.
- [7] Reed JC. (1995) Bcl-2 family proteins: regulators of chemoresistance in cancer. *Toxicol Lett.* 82-83: 155-8.
- [8] Brown JM, Attardi LD. (2005) The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer.* 5: 231-7.
- [9] Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell.* 31: 11-24.
- [10] Kramer G, Erdal H, Mertens HJ, Nap M, Mauermann J, Steiner G, Marberger M, Bivén K, Shoshan MC, Linder S. (2004) Differentiation between cell death modes using measurements of different soluble forms of extracellular cytokeratin 18. *Cancer Res.* 64: 1751-6.
- [11] Eguchi, Y., Shimizu, S., and Tsujimoto, Y. (1997) Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res.* 57: 1835-1840.
- [12] Teicher, B. A. (1994) Hypoxia and drug resistance. *Cancer Metastasis Rev.* 13: 139-168.
- [13] Caulin C, Salvesen GS, Oshima RG. (1997) Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *J Cell Biol.* 138: 1379-1394.
- [14] Ku NO, Liao J, Omary MB. (1997) Apoptosis generates stable fragments of human type I keratins. *J Biol Chem.* 272: 33197-33203.
- [15] Leers MP, Kölgen W, Björklund V, Bergman T, Tribbick G, Persson B, Björklund P, Ramaekers FC, Björklund B, Nap M, Jörnvall H, Schutte B. (1999) Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol.* 187: 567-72.
- [16] Hägg M, Biven K, Ueno T, Rydlander L, Björklund P, Wiman KG, Shoshan M, Linder S. (2002) A novel high-throughput assay for screening of pro-apoptotic drugs. *Invest New Drugs.* 20: 253-259.
- [17] Schutte B, Henfling M, Kölgen W, Bouman M, Meex S, Leers MP, Nap M, Björklund V, Björklund P, Björklund B, Lane EB, Omary MB, Jörnvall H, Ramaekers FC. (2004) Keratin 8/18 breakdown and reorganization during apoptosis. *Exp Cell Res.* 297: 11-26.
- [18] Kramer G, Schwarz S, Hägg M, Havelka AM, Linder S. (2006) Docetaxel induces apoptosis in hormone refractory prostate carcinomas during multiple treatment cycles. *Br J Cancer.* 94: 1592-8.
- [19] Ueno T, Toi M, Bivén K, Bando H, Ogawa T, Linder S. (2003) Measurement of an apoptotic product in the sera of breast cancer patients. *Eur J Cancer.* 39: 769-74.
- [20] Cummings J, Ranson M, Lacasse E, Ganganagari JR, St-Jean M, Jayson G, Durkin J, Dive C. (2006) Method validation and preliminary qualification of pharmacodynamic biomarkers employed to evaluate the clinical efficacy of an antisense compound (AEG35156) targeted to the X-linked inhibitor of apoptosis protein XIAP. *Br J Cancer.* 95: 42-8.
- [21] Brattström D, Wagenius G, Sandström P, Dreilich M, Bergström S, Goike H, Hesselius P, Bergqvist M. (2005) Newly developed assay measuring cytokeratins 8, 18 and 19 in serum is correlated to survival and tumor volume in patients with esophageal carcinoma. *Dis Esophagus.* 18: 298-303.
- [22] Sheard MA, Vojtesek B, Simickova M, Valik D. (2002) Release of cytokeratin-18 and -19 fragments (TPS and CYFRA 21-1) into the extracellular space during apoptosis. *J Cell Biochem.* 85: 670-7.
- [23] Hostanska K, Nisslein T, Freudenstein J, Reichling J, Saller R. (2004) Cimicifuga racemosa extract inhibits proliferation of estrogen receptor-positive and negative human breast carcinoma cell lines by induction of apoptosis. *Breast Cancer Res Treat.* 84: 151-60.
- [24] Hostanska K, Nisslein T, Freudenstein J, Reichling J, Saller R. (2005) Apoptosis of human prostate androgen-dependent and -independent carcinoma cells induced by an isopropanolic extract of black cohosh involves degradation of cytokeratin (CK) 18. *Anticancer Res.* 25: 139-47.
- [25] Rougier P, Van Cutsem E, Bajetta E, Niederle N, Possinger K, Labianca R, Navarro M, Morant R, Bleiberg H, Wils J, Awad L, Herait P, Jacques C. (1998) Randomised trial of irinotecan versus fluorouracil by continuous infusion after fluorouracil failure in patients with metastatic colorectal cancer. *Lancet.* 352: 1407-12.
- [26] Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using



- fluorescein labelled Annexin V. *J Immunol Methods*. 184: 39-51.
- [27] Aksakoglu G. (2006) *Sağlıkta Araştırma ve Çözümleme*, s. 286, DEU Rektörlük Basımevi, İzmir.
- [28] Kaufmann SH and Earnshaw, WC (2000) Induction of apoptosis by cancer chemotherapy. *Exp Cell Res*. 256: 42–49.
- [29] Meyn RE, Stephens LC, Hunter NR, Milas L. (1995) Kinetics of cisplatin induced apoptosis in murine mammary and ovarian adenocarcinomas. *Int J Cancer*. 60: 725–729.
- [30] Van de Wiele C, Lahorte C, Oyen W, Boerman O, Goethals I, Slegers G, Dierckx RA. (2003) Nuclear medicine imaging to predict response to radiotherapy: a review. *Int J Radiat Oncol Biol Phys*. 55: 5–15.
- [31] Batu J, Koçtürk S. (2008) Thesis: Efficiency of serum Cytokeratin18 levels for monitoring of colorectal cancer patients under chemotherapy. Dokuz Eylül University School of Medicine Department of Biochemistry-İzmir, Turkey.
- [32] Chen WS, Liu JH, Liu JM, Lin JK. (2004) Sequence-dependent effect of a cyclooxygenase-2 inhibitor on topoisomerase I inhibitor and 5-fluorouracil-induced cytotoxicity of colon cancer cells. *Anticancer Drugs*. 15: 287-94.