Cyclin D1 level, ploidy status, and S-phase fraction in colorectal cancer patients

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Background **Colorectal carcinoma (CRC) is a common neoplasm representing the third most common cancer worldwide. In Egypt, it constitutes 5% of the total estimated cancers. Cyclin D1 is overexpressed in CRC. The prognostic value of cyclin D1 together with ploidy status and S-phase fraction (SPF) is still conflicting.**

Aim **The aim of this study was to assess the prognostic value of cyclin D1, ploidy status, and SPF in CRC patients and evaluate its impact by correlating with histopathological characters.**

Materials and methods **A total of 50 specimens of CRC were collected from National Cancer Institute, Cairo University. The upregulation of cyclin D1 was assessed by means of immunohistochemistry using tissue microarray assay. The ploidy status and SPF were also determined using flow cytometry and compared with cyclin D1 expression as prognostic factors for CRC.**

Results **Of the 50 cases examined, cyclin D1 was overexpressed in 78% of cases. On the other hand 24.4% of the cases were aneuploid with high SPF. A significant relation was detected between high tumor stages and glandular adenocarcinoma type with cyclin D1 overexpression as well as between SPF and ploidy status. *Egypt J Pathol* 35:144–149 © 2015 Egyptian Journal of Pathology.**

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Keywords: colorectal cancer, cyclin D1, ploidy status, S-phase fraction

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Introduction

According to the American Cancer Society, colorectal carcinoma (CRC) is the third most common cancer (9%) in both male and female populations. It is the third most common cause of cancer-related deaths in both sexes (Siegel *et al.*, 2013).

In Egypt, CRC strikes at early age with large tumor size, advanced stage, and high-grade tumors that carry more mutations (Mokhtar *et al.*, 2007).

Currently, tumor stage at diagnosis is still the most important prognostic factor in CRC. However, several studies on molecular markers tried to identify high-risk patients who may benefit from adjuvant treatment. Aberrations in the cell-cycle checkpoints have been shown to be of prognostic significance in CRC (Wangefjord *et al.*, 2011).

G1/S-specific cyclin D1 is a protein that in humans is encoded by the *CCND1* gene. They are called cyclins as they are synthesized and destroyed during each cell cycle. There are eight types of cyclins (A–H), which directly control cell-cycle progression. Each type of cyclin binds specifically to different cyclin-dependent kinase to form distinct complexes at definite phases of the cell cycle to drive the cell from one phase to another. Cyclin D1 is a cell-cycle checkpoint, which can function as a transcriptional coregulator. It regulates the progression from G1 phase of the cell cycle to S phase and thus it organizes cellular decision between proliferation and growth arrest (Li *et al.*, 2014).

The degradation of this protein is crucial for S-phase progression because acute overexpression or failure of elimination, causing G1 arrest, prevents S-phase entry. This degradation is not only associated with its sequestration to the cytoplasm but also inhibition of apoptosis in cancerous cells (Alao, 2007). Cyclin D1 is known to be upregulated in a variety of tumor types including one-third or more of CRC. This upregulation may be due to gene amplification and/or overexpression. Despite a well-established role of cyclin D1 in cell-cycle progression, previous data on cyclin D1 and clinical outcome in CRC have been conflicting (Li *et al.*, 2014).

Ploidy status is the number of homologous sets of chromosomes in the nucleus of a living cell.

Recent works have highlighted ways in which aneuploidy could have either tumor-promoting or tumor-suppressing effects. Aneuploidy could be just another type of mutation with potential beneficial or harmful effects that depend on the chromosomes involved. The prognostic significance of high DNA content (DNA ploidy or index) in CRC has been investigated with controversial results. However, it was associated with significantly higher tumor recurrence rate (Xynos *et al.*, 2013).

The synthesis phase (S-phase) is the part of the cell cycle in which DNA is replicated, occurring between the G1 phase and the G2 phase. The G1/S transition is a major checkpoint in the regulation of the cell cycle. The prognostic value of S-phase fraction (SPF) in evaluating CRC was investigated by many authors and it was a significant independent prognostic factor for disease-free, overall survival, and higher probability of metastasis (Bell and Dutta, 2002).

Aim

The aim of this study was to assess the correlation between cyclin D1, ploidy status, and SPF and different

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clinicopathological criteria in a number of CRC patients with contribution to the prognosis of this disease.

Patients and methods

This study was approved by the Ethical Committee of the National Cancer Institute.

Patients

The present study was conducted on 50 Egyptian CRC patients who underwent surgical colectomy at National Cancer Institute, Cairo University, during the period between 2006 and 2012. None of the patients had received chemotherapy or radiotherapy before surgical intervention. All related clinicopathological data of the patients were collected, including age, sex, histological type, and stage.

Methods

Hematoxylin and eosin stain

Hematoxylin and eosin (H&E)-stained slides were obtained to confirm the diagnosis and revise the stage and grade of selected CRC cases. Histopathological examination was performed on routinely fixed paraffin-embedded tissue sections. Samples were fixed in neutral formalin, embedded in paraffin, sectioned at 5 µm, stained with H&E, and finally examined microscopically. Histological typing and grading were carried out according to the WHO classification criteria, and the pathologic stage was determined according to the American Joint Committee on Cancer (TNM's staging system).

Immunohistochemical studies

Tissue microarray assay (TMA) construction: A representative tumor tissue was localized on the donors' paraffin blocks. Extraction of tissue cores with a diameter of 1.5 mm from the original block was done. The cores were re-embedded in duplicate on a recipient paraffin block. (A recipient block containing 93 tissue cores, each arranged in 11×9 sectors, was constructed.) Five-µm sections of the tissue array block were prepared and stained with H&E to identify tumor tissue on the array slide.

Immunohistochemical staining procedures of TMA slides: Slides were deparaffinized in xylene twice for 10 min, and rehydrated through descending grades of ethanol (100, 95, 85, 70%) to distilled water. The slides were then washed twice in PBS, followed by incubation for 15 min with 3% hydrogen peroxide block to reduce nonspecific background staining. The slides were washed four times in PBS and ultraviolet block was applied and incubated for 5 min at room temperature. Primary antibody enhancer was added and incubated for 10 min at room temperature. The following antibody was used: monoclonal mouse anti-human cyclin D1 (clone DSC-6; Dako, Denmark; 1 : 20–1 : 40 dilution). The slides were then washed four times in PBS. Biotinylated horseradish peroxidase and streptavidin polymer was added in the dark and incubated for 15 min at room temperature. The slides were then washed four times in PBS and incubated with peroxidase-compatible chromogen, followed by washing with distilled water four times. Finally, the slides were counterstained with Mayer's hematoxylin and the coverslip was mounted using an aqueous mounting media.

Evaluation of TMA immunostaining scores: Immunostaining intensity of cyclin D1 was scored as negative if less than 10% of tumor cells reacted positively. Positivity was further classified into low and high on the basis of the intensity of the stain. A positive reaction was evident as brown stain located in either the nucleus and the cytoplasm or in the cytoplasm alone.

DNA ploidy and S-phase fraction analysis

DNA flow cytometric analysis was carried out using the Modfit Computer Software in FAC scan flow cytometer (Becton and Dickinson Inc., New Jersy, USA). Standardization and initial alignment were performed according to the manufacturer's instructions using the quality control particles kit (Becton and Dickinson Inc.).

Samples were prepared for DNA analysis from formalin-fixed paraffin-embedded tissues. Briefly, 3–7 sections of 5-µm thickness each were placed in 15 ml glass centrifuge tubes and deparaffinized using three incubations for 30 min each with 10 ml of descending concentration of xylene. Samples were rehydrated using a sequence of 10 ml of 100, 95, 70, and 50% ethanol incubations for 10 min each at room temperature. Tissues were washed three times with distilled H_2O and then left in distilled H_2O at 4°C overnight before enzymatic digestion. A volume of 1 ml of 0.5% pepsin solution (P7012; Sigma, Missouri, USA) was added in saline and adjusted to pH 1.5 with HCl. This mixture was then incubated in water bath for 2 h at 37°C with brief vortexing at 10 min intervals. The mixture was filtered through a 37-mm nylon mesh and then washed with 5 ml of PBS. Nuclei were counted by taking 0.1 ml of suspension stained with trypan blue and then counted on a hemocytometer. Normal colon paraffin-embedded block and fresh lymphocytes were used as external controls to identify reference peak for human diploid G0/G1 population. Staining was performed using the cycle test plus DNA reagent kit (Becton and Dickinson Inc.) according to manufacturer's protocol. At least 10 000 cells were acquired using FAC scan flow cytometer for each case. The standard suspension was run first to adjust the diploid G0/G1 reference peak (2N). Results were stored as DNA histograms and were stored in list mode for subsequent analysis.

Interpretation of DNA histogram

Cellular debris was excluded before analysis by gating on DNA content. The quality of DNA histogram was controlled by coefficient of variation of less than 7%. A sample was considered diploid if the flow cytometry histogram showed a single symmetric G0/G1 peak at 2N position similar to reference peak and aneuploid if the histogram showed two distinct G0/G1 populations (one diploid peak and one or more peaks with abnormal DNA content).

The SPF (estimated as percentage of cells occupying the region between the mean channel number for G0/G1 and G2/M) was measured automatically. The cutoff value for the SPF was considered in relation to the mean SPF of the CRC cases.

Statistical analyses

Data management and analysis were performed using Statistical Analysis Systems (SAS Institute, North Carolina, USA). Numerical data were summarized using medians and ranges. Categorical data were summarized as percentages. Comparisons between groups with respect to numeric variables were tested using the Mann–Whitney nonparametric test. Comparisons between categorical variables were tested using the χ^2-test or Fisher's exact test for small sample size. Correlations between numeric variables were tested using Spearman's correlation coefficients. All P values were two-sided. P values less than 0.05 were considered significant.

Results

Clinicopathological findings

The mean age of the studied cases was 52 years. Nineteen (38%) cases were less than 50 years of age and 31 (62%) cases were 50 years or older. A total of 31 (62%) cases were male and 19 (38%) cases were female (the male-to-female ratio = 1.6). Forty (80%) cases were colonic, whereas only 10 (20%) cases were rectal. Twenty-two (44%) cases were classified as grade I–II and 28 (56%) cases were of grade III. Forty-one (82%) cases were classified as stage III–IV and nine (18%) cases were of stage II. Thirty-three (66%) cases were lymph node-negative, whereas 17 (34%) cases were of stages N1–N2. Eight (16%) cases were less than 5 cm and 42 (84%) cases were 5 cm or greater. Finally, 42 (84%) cases were glandular adenocarcinoma type and eight (16%) cases were mucinous adenocarcinoma type (Table 1).

Table 1 The clinicopathological findings of 50 colorectal carcinoma cases studied

Clinicopathological parameters	*N* (%)
Age	
<50	19 (38)
≥ 50	31 (62)
Sex	
Male	31 (62)
Female	19 (38)
Tumor site	
Colon	40 (80)
Rectum	10 (20)
Grade	
I–II	22 (44)
III	28 (56)
Stage	
pT classification	
T2	9 (18)
T3–T4	41 (82)
pN classification	
N0	33 (66)
N1–N2	17 (34)
Tumor size	
<5	8 (16)
≥ 5	42 (84)
Histopathological types	
Glandular adenocarcinoma	42 (84)
Mucinous adenocarcinoma	8 (16)

Cyclin D1 expression

Thirty-nine (78%) cases showed cyclin D1 overexpression and 11 (22%) cases showed reduced expression. There was a significant correlation between cyclin D1 expression and both the T-stage and the histologic type of the tumor ($P = 0.027$ and 0.005, respectively) (Table 2).

Flow cytometry findings

DNA content analysis (ploidy status)

Normal lymphocytes and normal colon tissue samples showed a single diploid peak (reference peak) representing G0/G1 cells (2N). The coefficient of variation of G0/G1 peak as calculated using the flow cytometric statistics software ranged from 5.05 to 6.56%, with a mean value of 5.805%.

All 50 cases were selected for ploidy status evaluation; however, only 45 cases could be analyzed because of debris and fixation artifacts in five samples. Thirty four cases (75.6%) were diploid and the remaining 11 cases (24.4%) were aneuploid (compared to normal diploid peak) with an additional single peak in its mean channel. No significant difference was found between ploidy status and any of the clinicopathological parameters examined (Table 3).

All 50 cases were analyzed for 2-SPF; calculation of SPF was possible for 44 cases only. A high SPF of 70% was reported in aneuploid tumors (a median of 18.7), whereas 75.6% of the diploid tumors showed low SPF with a median of 8.7. A significant correlation was reported between SPF and ploidy status ($P = 0.041$), and a borderline significance was reported between SPF and the expression levels of cyclin D1. There was no correlation between SPF and other clinicopathological parameters (Table 4).

Of the 11 aneuploid cases, only nine cases were available for DNA-index calculation, four (44%) cases were hypotetraploid, three (33%) were triploid, and two (22%) were tetraploid (Figs 1–4).

Discussion

CRC is a complex disease with a high mortality rate. It is influenced by lifestyle, environmental factors, and family history (Finne and Aguilar 2000). Former studies have clearly demonstrated that cyclin D1 overexpression, DNA ploidy, and SPF correlate with the occurrence and prognosis of CRC (El-Kinaai *et al.*, 2004). In the current work, cyclin D1 expression was measured using immunohistochemistry. DNA analysis and determination of SPF were also carried out using flow cytometry for a more extensive understanding of CRC.

In the present study, the average age of CRC patients was 52 years. Male sex represented 62% of patients. These results are parallel to those reported by El-Kinaai *et al.* (2004). As regards the site of tumors, the majority of cases (80%) were colonic and the remaining were rectal. These results are in agreement with those of El-Kinaai *et al.* (2004). However, El-Hennawy *et al.* (2003) detected more frequent affection of rectum.

Cyclin D1 regulates the transition from the G1 to the S phase. The mechanisms likely to activate the oncogenic properties of the cyclins include chromosomal translocations, gene amplification, and aberrant protein overexpression (Bahnassy *et al.*, 2004). In this study, cyclin D1

Table 2 Correlation of cyclin D1 expression level and clinicopathological parameters

Clinicopathological parameters	Number of cases	Expression of cyclin D1		*P*-value*
		High	Low	
Age (years)				
<50	19	15 (78.9)	4 (21.1)	1.000
≥ 50	31	24 (77.4)	7 (22.6)	
Sex				
Male	31	26 (84)	5 (16)	0.413
Female	19	13 (68.4)	6 (31.6)	
Site				
Colon	40	32 (80)	8 (20)	0.373
Rectum	10	7 (70)	3 (30)	
Grade				
I–II	22	18 (82)	4 (18)	0.693
III	28	21 (75)	7 (25)	
Stage				
T2	9	3 (33.3)	6 (66.7)	0.027*
T3–T4	41	36 (88)	5 (12)	
Lymph nodes				
N0	33	26 (78.8)	7 (21.2)	1.000
N1 + N2	17	13 (76.5)	4 (23.5)	
Type				
Glandular adenocarcinoma	42	37 (88)	5 (12)	0.005*
Mucinous adenocarcinoma	8	2 (25)	6 (75)	

*$P \leq 0.05$ is considered significant.

Table 3 Correlation between ploidy status and clinicopathological parameters

Clinicopathological parameters	Number of cases	Ploidy status		*P*-value*
		Aneuploid	Diploid	
Age (years)				
<50	18	4 (22)	14 (78)	1.000
≥ 50	27	7 (26)	20 (74)	
Sex				
Male	27	8 (30)	19 (70)	0.711
Female	18	3 (17)	15 (83)	
Site				
Colon	37	9 (24)	28 (76)	1.000
Rectum	8	2 (25)	6 (75)	
Stage				
T2	8	2 (25)	6 (75)	1.000
T3–T4	37	9 (24)	28 (76)	
Lymph nodes				
N0	33	8 (24)	25 (76)	1.000
N1 + N2	12	3 (25)	9 (75)	
Type				
Glandular adenocarcinoma	38	11 (29)	27 (71)	0.31
Mucinous adenocarcinoma	7	0 (0)	7 (100)	

*$P \leq 0.05$ is considered significant.

Table 4 Correlation between S-phase fraction, clinicopathological parameters, and immunohistochemical findings

Measurements	Number of cases	Median	Range	*P*-value*
Age (years)				
50	18	14.0	3.4–43.7	0.560
≥ 50	26	10.5	2.0–39.7	
Sex				
Male	26	10.3	3.4–28.8	0.280
Female	18	15.3	2.0–43.7	
Site				
Colon	11	17.7	5.7–43.7	0.264
Rectum	33	12.3	2.0–39.7	
Grade				
I–II	21	10.8	2.0–43.7	0.750
III	23	13.7	3.0–39.7	
Stage				
T2	7	0.06	0.0–0.12	0.827
T3–T4	37	0.00	0.0–4.25	
Lymph nodes				
N0	33	11.8	2.0–39.7	0.154
N1 + N2	11	17.7	5.7–43.7	
Type				
Glandular adenocarcinoma	37	12.8	2.0–43.7	1.000
Mucinous adenocarcinoma	7	12.6	6.8–20.4	
Expression cyclin D1				
High	35	14.6	3.6–43.7	0.066*
Low	9	5.6	3.0–39.6	
Expression site of cyclin D1				
Cytoplasmic and nuclear	26	10.3	3.4–43.7	0.325
Cytoplasmic	18	15.1	7.3–28.8	
Ploidy status				
Aneuploid	11	18.7	8.7–39.7	0.041*
Diploid	33	8.7	2.0–43.7	

*$P \leq 0.05$ is considered significant.

was overexpressed in 78% of cases, and this was more than 58.7% detected by Holland *et al.* (2001).

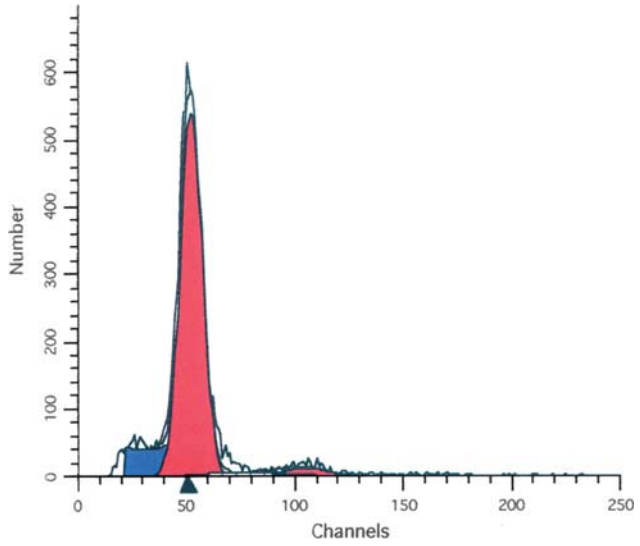
In the current study, we detected cyclin D1 overexpression in 88% of the late stages T3 and T4 tumors. There was a significant correlation between cyclin D1 overexpression and tumor stage ($P = 0.027$). In addition, cyclin D1 was overexpressed in 88% of adenocarcinoma type with a significant correlation ($P = 0.005$). These results are in agreement with those of Palmqvist *et al.* (1998).

In the present study, we failed to record a significant correlation between cyclin D1 overexpression and positive lymph node metastasis, which was successfully recorded by Bahnassy *et al.* (2004).

In contrast, Holland *et al.* (2001) and Bondi *et al.* (2004) failed to detect significant correlation between cyclin D1 overexpression and any of the clinicopathological criteria. This controversy in results may be due to the multiple and complex role of cyclin D1 or/and the difference in the sampling of studied cases (Bondi *et al.*, 2004).

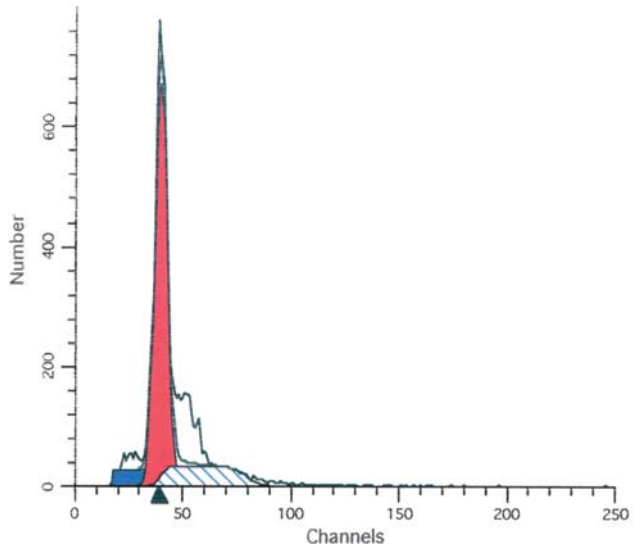
Colorectal cancer can be divided into those with a normal or diploid DNA content and those with an abnormal, aneuploid, DNA content. The negative effect of aneuploidy has been dilemmatic (Araujo *et al.*, 2007). In the present study, 24.4% of cases were aneuploid. Nevertheless, we did not find any correlation between ploidy status and clinicopathological parameters. These results are close to those of Giaretti (1994), who reported DNA

Fig. 1



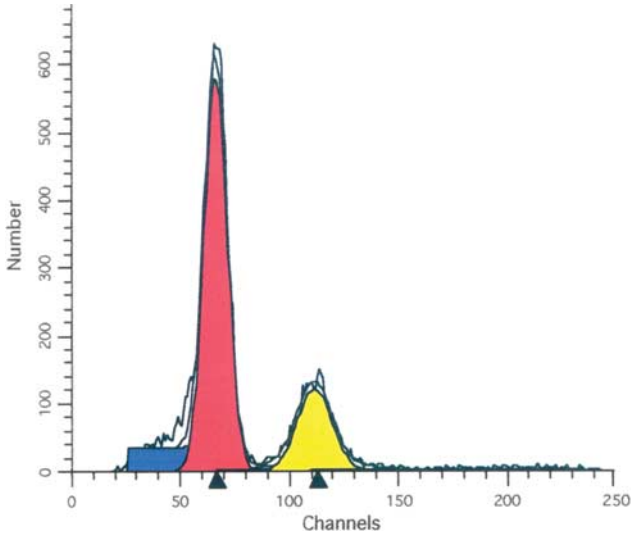
DNA frequency histogram showing a single diploid peak with a low S-phase fraction of colorectal carcinoma case.

Fig. 2



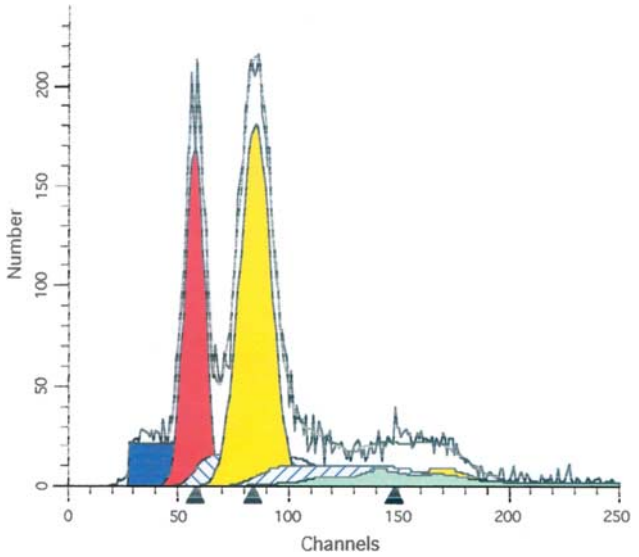
DNA frequency histogram of a grade 3 colorectal carcinoma case, showing a single diploid peak with high S-phase fraction (22.5%).

Fig. 3



DNA frequency histogram of grade 2 colorectal carcinoma case, showing diploid and aneuploid peaks (DI = 1.48) and low S-phase fraction (5.6%). DI, DNA index.

Fig. 4



DNA frequency histogram of grade 2 colorectal carcinoma CRC case, with diploid and aneuploid peaks (DI = 1.4) and high S-phase fraction (24.96%).

aneuploidy in 35% of cases. In Egyptian cases, Zalata *et al.* (2002) reported aneuploidy in 46.25% of cases, which are significantly correlated with rectal tumors. In contrast, a higher percentage (56.7%) of aneuploidy was obtained by El-Kinaai *et al.* (2004). Moreover, Purdie and Piris (2000) reported a 73% of aneuploid tumors with a significant correlation with tumor site, histological type, and grade of differentiation.

Many factors could explain the diversity in the results of DNA ploidy in CRC. Possible differences could result from heterogeneity of the abnormal DNA patterns gathered under the common term of aneuploidy. It has been shown that aneuploid tumors are usually associated with worse prognosis and poor response to therapy compared with diploid ones (Araujo *et al.*, 2007).

SPF represents the cells prepared for mitosis by their active doubling of DNA (number of cells in the S phase), which gives relevant information about the proliferation potential of the tested cell population. A high SPF has been associated with a poor prognosis (Schutte *et al.*, 1987).

The present results achieved a positive correlation between high SPF and overexpression of cyclin D1 with a borderline significance ($P = 0.066$). This result is

parallel to that of Bahnassy *et al.* (2004), who detected a positive correlation between cyclin D1 overexpression and histone H3 (used to identify the SPF when histone H3 reaches its peak). In addition, high SPF was associated with aneuploid tumors (70% of aneuploid tumors showed high SPF with a median of 18.7%) with a statistically significant correlation ($P = 0.041$). These results are in line with those of El-Kinaai *et al.* (2004), who recorded a high SPF in 76% of aneuploid tumors with a median of 21%.

The study could not achieve any correlation between SPF and histopathological findings. In contrast, Wang *et al.* (2001) found a nearly significant correlation between SPF and distal tumors. Moreover, Ando *et al.* (1993) reported a significant correlation between SPF and poorly differentiated type carcinomas with tendency to poorer survival.

Among the aneuploid cases available for DNA-index calculation, in the present work, 44% of cases were hypotetraploid, 33% were triploid, and 22% were tetraploid. These findings are lower than those of El-Kinaai *et al.* (2004), who reported 52.9, 33, and 22% in corresponding regions.

Conclusion

In conclusion, cyclin D1 is overexpressed in advanced stage CRCs and in highly proliferative tumor cell in SPF. The majority of tumor cells with high SPF are of aneuploid type. Overexpression of cyclin D1 and aneuploid tumors of high SPF carry poorer prognosis.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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