Background/Aims: The purpose of our analysis was to determine the prognostic value of molecular markers for identifying high-risk TNM stage II colon cancer patients, the association with various clinical and pathological features, and possible relation to survival.

Methods: In 191 colon cancer patients who underwent a potentially curative resection, clinical and pathological factors (age, tumour site, histological grade of malignancy, pT stage, presence of venous, lymphatic, and perineural invasion) and tumour molecular markers were analysed. Molecular markers were assessed immunohistochemically in sections of paraffin-embedded tissues. Patients were followed for a median of 8.7 years. The 5-year survival rate was estimated using the Kaplan-Meier statistical method.

Results: From 1 Jan. 1994 to 31 Dec. 2000, 191 patients underwent radical resection for T3-4 N0M0 colorectal cancer without adjuvant chemotherapy. A significant decrease in survival was identified in older patients, patients with tumours pT4 and with perineural invasion. We found no significant differences in survival of patients with expression of MLH1, Cyclin D1 and reduced overexpression of E-cadherin.

Conclusions: The results of our study indicate that the presence of perineural invasion, pT4 stage and the patient’s age are significantly correlated with the expected survival in radically resected TNM stage II colon cancer patients, while immunohistochemical markers are not related to survival.

Key words: Colon cancer, UICC stage II, tumour markers, prognosis.

INTRODUCTION

The prognosis of colorectal carcinoma depends on the anatomic extent of the disease (TNM classification and revised UICC TNM classification), and on its amenability to radical (R0) resection (residual tumour (R) classification) 1.

Patients who die from the disease most commonly succumb to the effects of secondary metastases in the liver. The prognosis of this cancer is influenced by a variety of factors present at the time of initial diagnosis. These include: age, gender duration of symptoms, presence of bowel obstruction, tumour location, need for blood transfusion and the quality of surgical intervention 2,3,7.

Treatment of colorectal cancer is unsuccessful if the aggressive tumour phenotype is identified at surgery. The most important variable influencing long-term outcome is the AJCC/UICC TNM stage, with depth of invasion and infiltration of lymph nodes or distant organs (Table 1) 7.

Stage D was not included in the original Dukes’ staging system, but has become commonly used to represent distant metastasis.

Table 1: Comparison of the two most commonly used pathological staging systems for colorectal cancer 4.

Postoperative adjuvant chemotherapy is indicated in patients with node-positive, Dukes’ stage C disease. Although up to 40% of patients with Dukes’ B colorectal cancer will develop recurrent disease during their lifetime, the role of adjuvant chemotherapy in this setting is still unclear 5.

In patients with metastatic recurrence further curative resection is rarely possible. Palliation by radiotherapy or surgery is feasible in a small proportion of remaining cases. Partial response rates to cytotoxic chemotherapy, ranging from 18% to 48%, have been reported for regimens based on 5-fluorouracil and folic acid 6; some of these studies established a survival benefit of chemotherapy 7.

Identifying the categories of patients with high-risk colorectal cancer would greatly help us improve treatment strategies and outcome in patients with the node-negative disease 8.
Evidence is accumulating that several prognostic molecular markers may be useful in defining individual patients’ risk after radical surgery and in selecting patients who might benefit most from adjuvant chemotherapy.

**PATIENTS AND METHODS**

**Patients**

Blocks of paraffin-embedded tissue were obtained from 191 patients with colon carcinoma who underwent resection at the Department of Abdominal Surgery, University Medical Centre Ljubljana, during the period 1 Jan. 1999 – 31 Dec. 2000. The patient group consisted of 106 (55.5%) men and 85 (44.6%) women, with a mean age of 67 years; range 38 - 92 years. Among 191 colon tumours, 69 (36.1%) were located in the sigmoid colon, 39 (20.4%) in the ascending colon, 32 (16.7%) in the coecum, 15 (7.8%) in the hepatic flexure, 15 (7.8%) in the transverse colon, 13 (6.8%) in the descending colon and eight (4.2%) in the splenic flexure.

The tumours were staged using the UICC TNM classification. There were 181 (94.8%) patients with T3N0M0 tumours and ten (5.2%) patients with T4N0M0 lesions.

All patients were treated by surgery (Table 2).

By the UICC R classification, all tumour resections are classified as radical and curative procedures (R0). None of the patients received preoperative radiotherapy or postoperative radiotherapy and chemotherapy. All of them were followed for a median of 8.7 years (3 – 13 years) according to our protocol for colorectal cancer patients. The follow-up was scheduled every three months during the first two years after surgery, and every six months thereafter.

The follow-up schedule consisted of physical examination, tumour markers determination (CEA, Ca 19-9), chest X-ray, abdominal ultrasonography, CT scanning and endoscopy. In patients with elevated tumour markers or clinically suspected metastatic disease, imaging examinations, including chest X-ray, ultrasonography, CT scan and colonoscopy, were performed when necessary.

**IMMUNOHISTOCHEMICAL METHODS**

**Tumour samples**

Colon cancer cases were retrieved retrospectively from the files of the Department of Pathology, Institute of Oncology. The specimens were formalin-fixed and paraffin-embedded. For the validation study we selected 191 colon cancers received between 1994 and 2000.

**Tissue microarray technique**

A map of receiver block was prepared with coordinates for each sample to correctly identify the tumour. Under a microscope, areas of interest that were non-necrotic and rich in tumoral glands were marked out accurately with an indelible pen on the whole section of each donor block. The tissue microarrayer was used. Cores 2.0 mm in diameter were punched from the donor blocks and positioned in the recipient paraffin array blocks. The array blocks were incubated at 37°C for 30 min. to improve adhesion between cores and paraffin of the recipient blocks. They were cut at room temperature with a standard microtome.

**Description of the array blocks of the study**

For the validation study two blocks of 124 cores were prepared: each colon cancer was punched six times.

---

**TABLE 1**

<table>
<thead>
<tr>
<th>COMPARISON OF THE TWO MOST COMMONLY USED PATHOLOGICAL STAGING SYSTEMS FOR COLORECTAL CANCER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dukes’ stage</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>Tumour invading the submucosa or muscularis propria</td>
</tr>
<tr>
<td>Tumour penetrating muscular coat of the bowel</td>
</tr>
<tr>
<td>Evidence of tumour in regional lymph nodes regardless of local tumour invasion</td>
</tr>
<tr>
<td>Distant metastases, regardless of local tumour invasion or lymph node status</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>TYPES OF RESECTION IN COLON CANCER PATIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of resection</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Right hemicolectomy</td>
</tr>
<tr>
<td>Left hemicolectomy</td>
</tr>
<tr>
<td>Segmental resection</td>
</tr>
<tr>
<td>Sigmoid resection</td>
</tr>
<tr>
<td>Total and subtotal colectomy</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
### IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was performed using the EnVision ChemMate (DAKO Corporation, Carpinteria, CA, USA) method on sections of formalin-fixed paraffin-embedded tissue samples. After antigen retrieval performed by 10 min microwave treatment in TRIS-EDTA at 110°C, and blockade of endogenous peroxidase activity with H2O2, the sections were incubated with antibody for 25 min. The reaction product was visualized using the ChemMate EnVision detection kit. (Table 3)

### STATISTICAL METHODS

Survival rates are based on the data provided by the Cancer Registry of Slovenia. The 5-year survival rate was determined by the Kaplan-Meier method and defined by death of any cause (with or without tumour). Differences between survival curves were tested using the log rank test. A P value of was considered statistically significant. The multivariate Cox proportional hazards model was used to check for possible confounding statistically significant variables from univariate analysis.

### RESULTS

The crude 5-year survival and the 10-year survival for all resected patients was 77.2% and 58.0%, respectively. The tumour-specific 5-year survival rate was 88.2%, and the tumour-specific 10-year survival was 80.0%.

The results of univariate survival analysis for 191 patients with stage II colon cancer are listed on Table 4. Among the original nine clinicopathological variables three had statistically significant univariate associations with survival. There was no significant correlation between histological grade of malignancy and lower survival. There was a strong association, however, between age and decreased survival. The number of lymph nodes examined ranged from 2 to 66, with a median of 17.3.

Lymphatic invasion was found in 12 (6.3%) specimens of this series. Vascular invasion was present in six (3.1%) and perineural invasion in four (2.1%) specimens. Patients showing perineural invasion had a significantly poorer prognosis (0.0227)

Cut-off indicates the percentage of cells with positive membranous staining. E-cadherin shows reduced overexpression. Positivity of Cyclin D1, Mhl1 and reduced overexpression of E-cadherin, were not correlated with reduced survival (Table 5). Table 4 shows lower survival rates for patients with neural invasion, T4 stage and strong association between decreased survival and age.

### DISCUSSION

The results of the study indicate independent influence of some prognostic variables on survival of patients with stage II colon cancer. Clinical and pathological markers were found to be the major prognostic determinants. Close collaboration between surgeon and pathologist is essential to accurate clinicopathological staging. Clinico-pathological stage defined in this way proved to be the dominant, independent variable affecting survival of patients with stage II colon cancer.

The results of investigations into the association of tumour site and survival of colon cancer patients have varied. Some authors maintained that the site of the lesion does not influence survival, and others that cancer of the left colon is associated with poorer survival. The results of our study suggest that there is no significant influence of tumor site on long-term outcome.

Histological grade has not been found to have a significant effect on survival. Despite this variability, histological grade has repeatedly been shown by multivariate analysis to be a stage-independent prognostic factor. Specifically, it has been demonstrated that high tumour grade is an adverse prognostic factor.

The variables that we have used to define the direct spread of the tumour – T3: tumour invades through the muscularis propria into the subserosa; T4: tumour directly invades other organs or structures – were shown to carry prognostic information. Previous univariate and multivariate analyses have confirmed the prognostic importance of direct spread of node positive tumours. In our series intramural spread (pT stage) was a significant indicator of survival in univariate analysis (p= 0.0054). It has been shown that 12 to 15 lymph nodes predict for regional node negativity. The number of uninvolved lymph nodes in the resected specimen is another factor predicting tumour recurrence. According to Chaplin et al. examination of six or fewer nodes is related to poor prognosis. There seem to be two explanations for this observation:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clon</th>
<th>Produced by</th>
<th>Dilution</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMLH1</td>
<td>G168-15</td>
<td>BD</td>
<td>1:10</td>
<td>MV, TRIS-EDTA, pH9, 98°C, 45'</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>SP4</td>
<td>TFS</td>
<td>1:50</td>
<td>MV, TRIS/EDTA, pH9, 98°C, 45'</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>HCH-38</td>
<td>Dako</td>
<td>1:10</td>
<td>MV, TRIS-EDTA, pH9, 98°C, 45'</td>
</tr>
</tbody>
</table>

TFS: Thermo Fisher Scientific, MV™ microwave
a) the lack of sufficient nodes may signify inadequate resection and may be associated with a higher recurrence and lower survival rate, because of down-staging during the pathologic examination, and

b) variability of the host-specific response to the tumour. The lymphocytic infiltrate has been demonstrated to be related significantly to the paracortical hyperplasia in regional lymph nodes in Dukes’ stage B colorectal carcinoma, which is associated with a better prognosis\textsuperscript{26}. Therefore, if fewer than 12 nodes are found, regional negativity is not reliable. It may be argued that a large lymph node harvest (median 17.3) would influence the significance of regional negativity. The presence of fewer than 12 isolated nodes has not been shown in univariate analysis to be associated with a significant decrease in survival (p=0.5096).

We have identified vascular invasion in 3.1% of patients in our series. The results of univariate analysis did not confirm the reported significant independent effect of the presence of venous invasion on prognosis in colon cancer patients treated by resection. Talbot et al. have reported poorer survival in patients with the presence of venous invasion in node-positive tumours\textsuperscript{17}.

In several studies, both lymphatic and perineural invasion have been shown by multivariate analysis to be indicators of poor prognosis\textsuperscript{11,18}. In our present study, univariate analysis showed that perineural invasion was associated with a significantly decreased survival (p=0.0227). Multivariate analysis suggests that gender is a powerful independent prognostic variable.

Significant differences in survival rates between men and women have been reported by US Centers for Disease Control\textsuperscript{19}. The observed gender differences are not in keeping with the findings of a prospective study of 11,000 patients with colorectal cancer that demonstrated higher survival rates in women than in men\textsuperscript{20}.

In the present study, which included only UICC TNM stage II (T3-4 N0M0) colon cancer patients, immunohistochemical markers Cyclin D1, Mhl1 and E-cadherin revealed no significance in multivariate survival analysis. E-cadherin is a member of cadherin family of calcium-dependent adhesive molecules that function as mediators of cell adhesion. It is a transmembrane protein with a large extracellular domain and a small cytoplasmic component, which interacts with cytoplasmic proteins, particularly β-catenin. Loss of E-cadherin is associated with increased cell invasiveness\textsuperscript{9}. Normal membranous E-cadherin expression is essential for establishing cell polarity and maintaining epithelial integrity and cellular differentiation\textsuperscript{21}.

Reduced E-cadherin expression has been found to correlate with an increased rate of local recurrence and metastatic spread in epithelial-type cancers at various sites, such as prostate, bladder, breast, lung, stomach, colon and rectum\textsuperscript{22}.

Immunohistochemistry for hMLH1 expression may represent a practical test for identifying DNA mismatch repair-deficient tumours, and has been used in retrospective prognostic analysis in colorectal cancer\textsuperscript{23}. This phenomenon is known as microsatellite instability (MSI) and is due to the silencing of the mismatch repair genes, MLH1 and MLH2\textsuperscript{24}.

Cyclin D1 (CCND1) is a protein that plays a key role in cell cycle control, particularly in the transition from G1 to S phase, which is regulated by cyclin-dependent kinases\textsuperscript{27}. The overexpression of the CCND1 gene occurs in more than one-third of all colorectal cancers\textsuperscript{28}. The strongest evidence to date has linked the CCND1 A870G poly-
morphism with an increased risk of colorectal cancer and adenomas.29,30,31

CONCLUSIONS

The results of our study indicate that the presence of perineural invasion, pT4 stage and the patient’s age are significantly correlated with the expected survival in radically resected TNM stage II colon cancer patients, while immunohistochemical markers are not related to survival.

SUMMARY

PROGNOSTičKI ZNAČAJ KLINIČKIH, PATOLOŠKIH I IMUNOHISTOHEMIJSKIH MARKERA KOD II STADIJUMA KOLOREKTALNOG KARCINOMA.

Cilj: Cilj ove analize je da se odredi prognostički značaj molekulskih markera u identifikaciji visokorizičnih pacijenata II stadijuma karcinoma kolona TNM, ocjenjivanjem određenih kliničkih i patoloških parametara i u odnosu na preživljavanje.

Metode: Kod 191 pacijenta obolelog od karcinoma kolona koji su podvrgnuti potencijalno kurativnoj resekciji, analizirani su klinički i patološki parametri (godine, lokalizacija tumora, histološki gradus maligniteta, pT stadijum, venska, limfatična i perineuralna invazija) i tumorski molekulsni markeri. Molekulsni markeri su mereni imunohistohemijski u presecima parafinskih preparata tkiva. Srednje vreme praženja pacijenata iznosilo je 8.7 godina. Petogodišnje preživljavanje određivano je Kaplan-Meier statističkom metodom.


Zaključak: Rezultati naše studije pokazuju da prisustvo perineuralne invazije, pT4 stadijum i starost pacijenta imaju značajnu korelaciju sa očekivanim preživljavanjem kod radikalno operisanih pacijenata II stadijuma kolorektalnog karcinoma po TNM-u, dok immunohistohemski markeri nisu pokazali povezanost sa preživljavanjem.

TABLE 5

THE RESULTS OF MOLECULAR MARKER ASSAYS (CYCLIN D1, E-c cadherin AND hMhll) FOR ALL PATIENTS

<table>
<thead>
<tr>
<th>Marker</th>
<th>% positive cases</th>
<th>cut-off</th>
<th>Univariate analysis (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>18.3</td>
<td>.90</td>
<td>0.2505</td>
</tr>
<tr>
<td>E-cadherin*</td>
<td>43.5</td>
<td>&gt;95</td>
<td>0.3029</td>
</tr>
<tr>
<td>Mhll</td>
<td>35.6</td>
<td>&gt;90</td>
<td>0.7505</td>
</tr>
</tbody>
</table>

Positivity of Cyclin D1, Mhll and reduced overexpression of E-cadherin, were not correlated with reduced survival

REFERENCES