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## **p16 expression in colorectal adenocarcinoma: marker of aggressiveness and morphological types**

Running head: p16 expression in colorectal carcinoma

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## **STRUCTURED ABSTRACT**

*Aim:* The aim of the present study is to investigate the clinicopathological roles of p16 expression in a large cohort of patients with colorectal adenocarcinoma with tight methodology and close follow-up.

*Methods:* p16 protein expression was investigated in 194 patients (102 men, 92 women) with colorectal adenocarcinomas by immunohistochemistry. The findings were correlated with their clinicopathological features.

*Results:* p16 protein was detected in 80% (155 of 194) of patients with colorectal carcinoma. The p16 protein was more often detected in male patients with colorectal cancers (86% versus 73%,  $p=0.03$ ). p16 protein expression was more often seen in carcinomas in the rectum, sigmoid and descending colon compared to more proximal colon (90% versus 61%,  $p=0.001$ ). The p16 protein was more often detected in well or moderately-differentiated colorectal adenocarcinoma than poorly-differentiated colorectal adenocarcinoma (84% versus 63%,  $p=0.009$ ). The level of expression of p16 protein is related to the lymph nodal status ( $p=0.004$ ) and the TNM staging of the colorectal carcinoma ( $p=0.008$ ).

*Conclusion:* p16 protein expression was common in colorectal adenocarcinomas. The expression correlated with gender of the patient, distal location, differentiation and staging of the tumour. The findings suggest that p16 plays an important role in cancer pathogenesis and has implications for improving the clinical management.

**KEYWORDS:** p16, expression, colorectal adenocarcinoma

## INTRODUCTION

Colorectal cancer is amongst the most common malignancy found in the Western world and usually ranks high in incidence and mortality among malignancies in those countries [1]. Globally, colorectal cancers accounted for about 1 million new cases in 2002 (9.4% of the world total). In terms of incidence, colorectal cancers rank fourth in frequency in men and third in women. There is at least a 25-fold variation in occurrence of colorectal cancer worldwide. The highest incidence rates are in North America, Australia/New Zealand and Western Europe. The incidence of colorectal cancer is also increasing in many Asian countries [2]. The main pathology type of the colorectal cancer is adenocarcinoma, though some other variants can occur [3, 4]. Surgery is the mainstay of treatment, with adjuvant chemotherapy and radiotherapy for specific subgroups of patients. The disease imposes a significant health threat despite the fact that survival from the disease has been improved. There is evidence that research contributes directly to improve the care of patients with colorectal cancer by more accurately refining prognosis and selecting the most appropriate adjuvant therapy for individual patients with colorectal carcinoma [5]. In order to do this, novel tissue-based prognostic indicators should be sought at the molecular level.

The p16 suppressor gene is one of the most commonly studied candidates in the pathogenesis of human neoplasia [6]. p16 gene encodes p16 protein that competes with cyclin D for binding to CDK4. This inhibits the ability of the cyclin D-CDK4 complex to phosphorylate Rb (retinoblastoma) protein, thus causing cell cycle arrest at late G<sub>1</sub> phase. In recent years, the status of p16 alternations in cancer can be studied by immunohistochemistry. Strong p16 expression has been reported in many neoplasia

[7,8]. Nevertheless, to date, the major clinical application of p16 expression has been in the field of cervical pathology [9].

In colorectal cancers, the clinicopathological roles of p16 protein expression have been studied but with no agreement between their findings [10-18]. This is likely related to small patient numbers. Therefore, we investigated p16 expression in a large consecutive cohort of patients with colorectal cancer with good quality follow-up in order to determine more definitely its relationship with the different clinicopathological parameters. The current series is the one of the largest studies to critically determine the roles of this biomarker in colorectal cancer.

## **MATERIALS AND METHODS**

### *Data collection*

One hundred and ninety-four consecutive patients (102 men, 92 women) with primary colorectal adenocarcinoma were recruited from the Townsville Hospital in North Queensland. The age, gender, clinical presentation, management and survival data of these patients were prospectively collected in a computerized database. The actuarial survival rate of the patients was calculated from the date of surgical resection of the colorectal carcinomas to the date of death or last follow-up. Management was by a preagreed standardized multidisciplinary protocol supervised by a senior specialist colorectal surgeon. None of the rectal cancer patients had undergone neoadjuvant radiotherapy or chemotherapy prior to surgery. Pathological diagnosis was also standardized and reviewed by committee criteria, chaired by a senior academic pathologist with a special interest in the field. Follow-up was routinely at 3 monthly intervals for the first 2 years, 6 monthly for the subsequent 3 years, and yearly thereafter. Serum carcino-embryonic antigen (CEA) levels were taken before each visit and imaging investigations performed as clinically appropriate. Colonoscopy was performed at 1 year after surgery, and repeated as appropriate to the findings and other clinical features at follow-up. Informed consent was obtained for the Townsville Hospital ethics committee.

The tissue samples were collected prospectively. The site and size (maximum length) of the colorectal cancers were recorded. Standard blocks were then taken, fixed in 10% formalin and embedded in paraffin wax. Histological sections were cut and stained for haematoxylin and eosin for light microscopic examination. The histological subtypes were classified using the World Health Organization (WHO) criteria [19].

Mucinous carcinoma was defined as an adenocarcinoma with more than 50% of the tumour composing of extracellular mucin. The carcinomas were staged according to TNM (Tumour, Lymph node and Metastases) classification adopted in American Joint Committee on Cancer [20].

### *P16 staining*

The antibody p16<sup>INK4a</sup> used was a mouse monoclonal antibody supplied by Biocare Medical (Cat # CM020, Walnut Creek, CA, USA). From the selected paraffin blocks, 4µm paraffin sections were de-waxed in xylene and re-hydrated through graded alcohols and water. Tissue endogenous peroxidase activity was blocked by incubating sections with 3% H<sub>2</sub>O<sub>2</sub>. Antigen retrieval was performed by incubation in 10mM citrate buffer (pH = 6.0) in a pressure cooker for 20 minutes. After the slides were cooled, they were rinsed with distilled water and then washed with Tris Buffer Saline (TBS, pH = 7.6). From this stage onwards, TBS was used as the washing solution throughout the immunohistochemistry staining procedure. Sections were then treated with 10% normal goat serum to block the non-specific binding. Endogenous biotin activity of tissue was blocked by the Biotin blocking system (Dako). Sections were incubated in p16 primary antibody at a dilution 1:25, for 60 minutes at 37<sup>0</sup>C. The secondary antibody, biotinylated goat anti mouse (at a dilution of 1:400, Dako) was applied for 30 minutes at 37<sup>0</sup>C, followed by the incubation of 1:100 StreptABC (Avidin biotin complex, Dako) for 30 minutes at 37<sup>0</sup>C.

### *Assessment of immunostaining*

The slides were then immersed into the liquid diaminobenzidine and substrate-chromogen system (Dako). Finally, the sections were counter stained with Mayer's haematoxylin and dehydrated through graded alcohol, cleared in xylene. Negative controls were sections treated using the same techniques as above, but with omission of the primary antibody. In each experiment, paraffin blocks of colorectal carcinoma known to be strongly positive for p16 protein were used as positive controls. Examination of p16

staining was done using a standard light microscope. Brown nuclear stain was regarded as positive. The positive cases of p16 were graded semi-quantitatively into 3 categories; 1+, 2+, 3+ (1+: < 30% of the tumour cells were positive; 2+:  $\geq 30$  to <70% of the tumour cells were positive; 3+:  $\geq 70\%$  or more of the tumour cells were positive).

### *Statistical analysis*

Comparisons between groups were performed using the *chi*-square test, Fisher's exact test and student t-test. Fisher's exact test or likelihood ratio was used for categorical variables. Student t-test with Yates correction was used for continuous variables. The significance of various parameters on survival was analysed by the Kaplan-Meier method with log-rank test and multivariate Cox's regression. Significance level was taken at  $p < 0.05$ . All statistical tests were performed with the program, Statistical Package for Social Sciences (SPSS version 14.0, Chicago, IL).

## **Results**

### **Clinico-pathological data**

The median age of the patients was 64 (range, 24 to 92) years. The mean diameter of the tumours was 4.5 cm (range, 0.7 to 16.0cm). The tumours were located in caecum in 11% (n=22), ascending colon in 8% (n=16), transverse colon in 15% (n=30), descending colon in 5% (n=9), sigmoid colon in 25% (n=48) and rectum in 36% (n=69). In the proximal portion of the large intestine (caecum, ascending colon and transverse colon), 58% of the tumors occurred in women. Conversely, in the distal portion of the large intestine (descending colon, sigmoid colon and rectum), 58% of the tumours were noted in men. The gender difference in tumour location was statistically significant (p=0.04).

In this series, 37 mucinous carcinomas in the colorectum were noted. The clinicopathological features of 36 cases were documented in our previous study [3]. The other colorectal cancers were 157 typical adenocarcinomas. Overall, the adenocarcinomas were well-differentiated in 9% (n=17), moderately-differentiated in 73% (n=142) and poorly-differentiated in 18% (n=35). The adenocarcinomas as classified according to TNM were: stage I in 26% (n=50), II in 32% (n=62), III in 30% (n=58) and IV in 12% (n=24).

### **p16 expression and clinicopathological parameters**

p16 protein was detected in 80% (155 of 194) of patients with colorectal adenocarcinoma (Table 1). The non-neoplastic mucosa showed no evidence of p16 protein expression (Figure 1A). Staining was found to be grade 1 in 32% (n = 62), grade 2 in 27% (n = 52) and grade 3 in 21% (n = 41) of the patients. In grades 2 and 3 staining,

the p16 protein expression was located at the nuclei as well as the cytoplasm of the tumour cells. These high levels of p16 protein expression were regarded as p16 overexpression (Figure 1B).

There was no significant difference between the frequency of p16 protein expression in mucinous carcinoma and typical adenocarcinoma. On the other hand, p16 expression showed significant correlation with the differentiation of the carcinomas (Figure 1C). The p16 protein was more often detected in well or moderately-differentiated colorectal adenocarcinoma than poorly-differentiated colorectal adenocarcinoma (84% versus 63%,  $p=0.009$ ). Also, the level of p16 protein expression was higher (with grade 2 or 3 expression) in well or moderately-differentiated colorectal adenocarcinoma ( $p=0.034$ ). In addition, within a tumour, the p16 expression was often seen in well-differentiated glandular structures rather than isolated poorly-differentiated tumour cells.

The p16 protein was more often detected in male patients with colorectal cancers (86% versus 73%,  $p=0.03$ ). The expression did not differ from those without with respect to age at presentation ( $p=0.44$ ). Also, p16 protein expression was often seen in carcinomas in the distal colorectum (the rectum, sigmoid colon or descending colon) than in proximal portions of the colon (90% versus 61%,  $p=0.001$ ). This difference in p16 protein expression between the proximal and distal colorectum was significant in both males and females ( $p=0.0001$  and  $0.005$  respectively). Also, the level of p16 protein expression in the distal colorectum was often higher than the proximal colon ( $p=0.003$ ).

The level of expression of p16 protein is related to the lymph nodal status and the TNM staging of the colorectal carcinoma. Sixty-one per cent (25 of 41) of patients with high grade (grade 2 or grade 3) p16 expression had lymph node metastases whereas only

35% (53 of 153) patients with grade 1 or negative p16 expression had lymph node metastases ( $p=0.004$ ). In addition, 61% (25 of 41) patients with grade 2 or grade 3 p16 protein expression had advanced stages (stage 3 or 4) carcinoma whereas only 37% (57 of 153) patients with grade 1 or negative p16 protein expression had carcinoma in advanced stages ( $p=0.008$ ).

### **Survival analysis**

Follow-up data were available for all the patients. Twenty-nine percent ( $n=57$ ) of the patients died of causes related to the colorectal cancer in the study period. The overall median survival rate for patients with the cancer was 61 months. By Kaplan-Meier log-rank survival and Cox-multivariate analysis, survival of the patients with colorectal cancer was only associated with the TNM stages of the tumours (Figure 2A). The mean survival of patient with early stages (stages 1 or 2) cancers was 70 months whereas that of advanced stages (stages 3 or 4) was 41 months ( $p=0.0001$ ).

Survival of patients was not significantly related to patients' age, gender and tumours' differentiation, location, p16 protein expression ( $p>0.05$ ). Patients with p16 protein overexpression had slightly poorer survival rates than other patients. The difference in survival was more prominent in female patients (Figure 2B). The mean survival was 74 months in female patients with low level or negative p16 protein expression whereas the mean survival was 53 months in patients with p16 protein overexpression. However, Kaplan-Meier analysis did not show that the level of p16 expression was significantly predicative of survival ( $p=0.1$ ). Patients with loss of p16 expression did not show any survival difference from other patients ( $p > 0.05$ ).

## DISCUSSION

The relationships between p16 protein expression and colorectal adenocarcinoma have been investigated in a few studies [10-18] (Table 2). The frequency of expression of p16 protein in colorectal adenocarcinoma reported varied from 17% to 99%, with the majority of studies showed p16 expression in more than two third of colorectal cancers. In this study, we noted that p16 protein was expressed in 80% of the colorectal adenocarcinoma and approximately half (48%) of the tumors showed p16 overexpression. The frequency of p16 protein expression noted in the study was similar to the frequency of p16 protein expression in colorectal mucinous adenocarcinoma [3]. Also, we noted no significant differences between typical adenocarcinomas and mucinous adenocarcinomas. We are the first to describe significantly higher p16 protein expression in males with colorectal adenocarcinoma. The overall high prevalence of p16 expression in colorectal adenocarcinoma implies that p16 alternations played an important role in the pathogenesis of this cancer.

p16 is a nucleoprotein, the presence of staining in both the nuclei and the cytoplasm supports the finding that p16 gene is overexpressed. The change in the subcellular location of the over-expressed nucleoprotein may account for the pathogenesis of colorectal adenocarcinoma. It is apparent that the overexpression of p16 rather than the loss of its protein contributes to the pathogenetic mechanism of colorectal adenocarcinoma. Because of the frequent overexpression of p16 protein in colorectal adenocarcinoma, p16 overexpression may be use as a marker for colorectal adenocarcinoma for selected patients with histological diagnostic difficulty.

In the recent years, researchers have demonstrated that cancers located in the right (proximal) and left (distal) colorectum can be distinguished by clinical and molecular

criteria [21-23]. Proximal colorectal tumours, when compared to distal tumours, were more often found in older age, females and benefit from 5-fluorouracil-based-chemotherapy. Right sided tumours also showed significantly less nuclear  $\beta$ -catenin and p53 overexpression than left-sided tumours [21-23]. With regard to p16 protein expression, Schineier-Stock et al noted that there was a loss of p16 expression in proximal tumors in a study of 43 patients with colorectal cancers [13]. Our group also noted that p16 protein expression was more often noted in mucinous carcinoma of the distal colorectum [3]. In this study, we confirmed the findings in 194 colorectal adenocarcinomas. The difference in p16 protein expression between the two sites of the large intestine was independent of the difference in gender distribution of the right and left sided tumours. Thus, the differences in gene expression in different location in colorectum exist and this may have important implications with specifically targeted therapeutic regimens in the future.

There is often a phenotypic-genotypic correlation with regard to cell cycle proteins. For instance, our group demonstrated that p53 expression was related to the differentiation in many different carcinomas [24-26]. For p16 protein expression, Palmqvist et al. have noted that the expression was more in well-differentiated adenocarcinoma and Tada et al noted that the expression was lost in mucinous or poorly-differentiated adenocarcinoma [10, 14]. In this study, p16 expression was demonstrated to be more often found in well and moderately-differentiated colorectal carcinomas. P16 overexpression was also commonly seen in these grades of colorectal adenocarcinomas. In addition, within a tumour, the p16 expression was often seen in well-differentiated glandular structure rather than isolated poorly-differentiated tumour cells. Thus, p16 expression was shown to be a marker for differentiation in colorectal adenocarcinoma.

The prognostic role of p16 protein have been investigated in four studies [12,14,15,18]. Norrie et. al. and Tada et. al. reported no relationship of patients' survival with p16 protein expression. Zhao et. al. noted that the survival of patients was poorer with weak p16 protein expression survival [18]. Also, Cui et al reported that both loss of p16 protein expression and p16 protein overexpression were associated with poorer survival rates [15]. In the current study with a larger cohort of patients, we also noted that patients with negative or low level expression of p16 protein enjoyed good survival whereas those with high level of p16 protein expression had poorer survival.

Unfortunately, the difference did not reach statistical significance. Thus, this large study with prospective collection of data, tight methodology and close follow-up suggest the p16 protein overexpression or loss of p16 protein expression did not alone correlate with prognosis of patients with colorectal cancer. On the other hand, it was confirmed that high levels of p16 protein expression were associated with advanced tumour stages and lymph node metastases. Thus, p16 protein overexpression was demonstrated to be a marker of aggressive biological behaviour and could be used as a potential prognostic marker in colorectal adenocarcinoma.

In conclusion, the clinicopathological roles of p16 protein expression were studied in a depth in a large cohort of patients with colorectal adenocarcinoma. The expression correlated with location, differentiation and staging of the tumour. These findings imply that p16 alternation is important in the pathogenesis of colorectal adenocarcinoma and p16 protein expression can be a potential biomarker for selected patients with colorectal adenocarcinoma.

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## **Figure legend**

Figure 1. p16 protein detected by immunohistochemistry: (A) showing the non-neoplastic colonic epithelium was negative for p16 protein and adenocarcinoma was positive (B) showing the high percentage of positive immunostaining in the nuclei and cytoplasm of the moderately-differentiated adenocarcinoma. (C) showing the focal immunostaining in poorly-differentiated adenocarcinoma.

Figure 2. (A)The relationship between patients's survival and stages of the colorectal carcinoma (B) The relationship between survival of female patients and level of expression of p16 (p16 +: overexpression; p16 -: negative or low expression).

**Table 1.****Clinicopathological parameters of p16 positive and negative colorectal adenocarcinoma**

Parameters	positive (n=155)	negative (n=39)	p value
Male: female	88:67	14:25	<b>0.03</b>
Mean age (years)	65	64	0.67
Mean tumour size (cm)	4.4	4.7	0.44
Location			
Proximal colon	42	27	<b>0.0001</b>
Distal colorectum	113	12	
Tumour histology			
Conventional	127	30	0.50
Mucinous	28	9	
Grade			
Well/Mod	133	26	<b>0.009</b>
Poor	22	13	

**Table 2 Relationship between immunohistochemical expression of p16 and clinicopathological data and colorectal adenocarcinoma**

Author/year/country	%positive	demographic data	pathological features	prognosis
Palmqvist/2000/ Sweden	75/92(82%)	-	more in grade 1 tumour, not correlate with stage, tumour type,	-
McKay/2002/UK	184/249(74%)	not correlate with age, gender	not correlate with grade, differentiation	-
Norrie/2003/Australia	148/161 (92%)	not correlate with gender	loss expression associated with grade 3 tumour not correlate with type, site	no relationship
Schneider-Stock/ 2003/Germany	29/ 43 (67%)	not correlate with age, gender	loss expression in proximal tumour not correlate with grade and stage	-
Tada/2003/Japan	82/84 (98%)	-	loss expression in mucinous/grade 3 tumour, advanced T stage & lymphatic invasion not correlate with stage, lymph node metastases	no relationship
Cui/2004/Japan	45/117 (38%)	not correlate with age, gender	strong expression associate with size not correlate with grade, stage, site tumour type, lymph node metastases	worst in overexpression or loss expression
Carneiro/2006/Brazil	5/30 (17%)	-	not correlate with grade	-
Zhao/2003 & 2006/ UK & China	73/74 (99%)	-	not correlate with grade and stage	poorer in low expression
Present study	155/194 (80%)	correlate with gender, not with age	strong expression in distal colorectum well/moderate differentiation, advanced stages, lymph node metastases, not correlate with tumour type, size	no relationship

Figure 1A

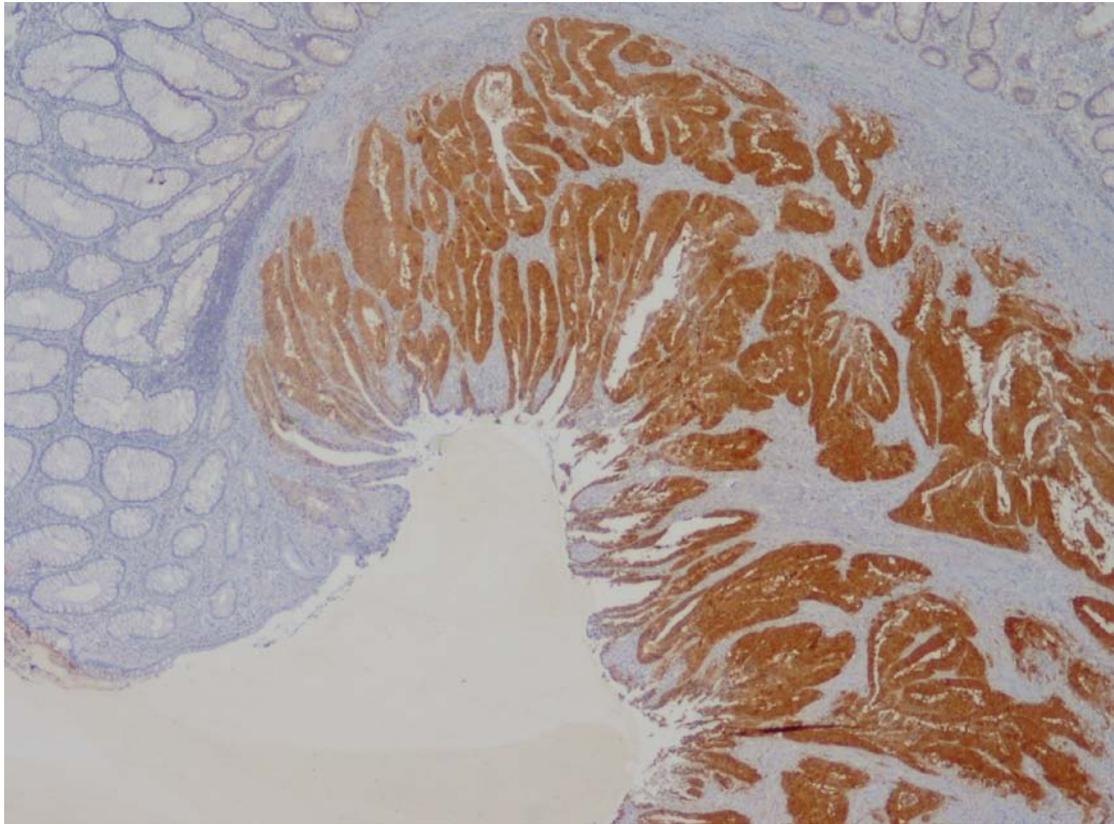


Figure 1B

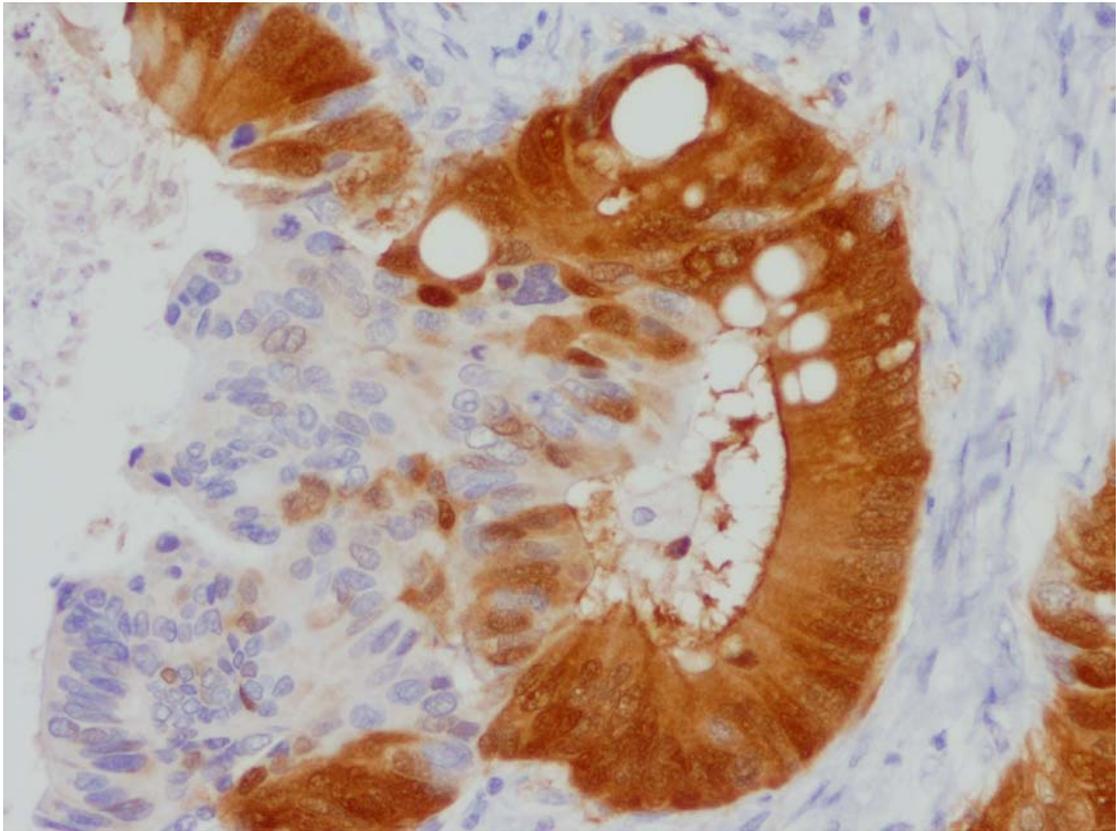


Figure 1C

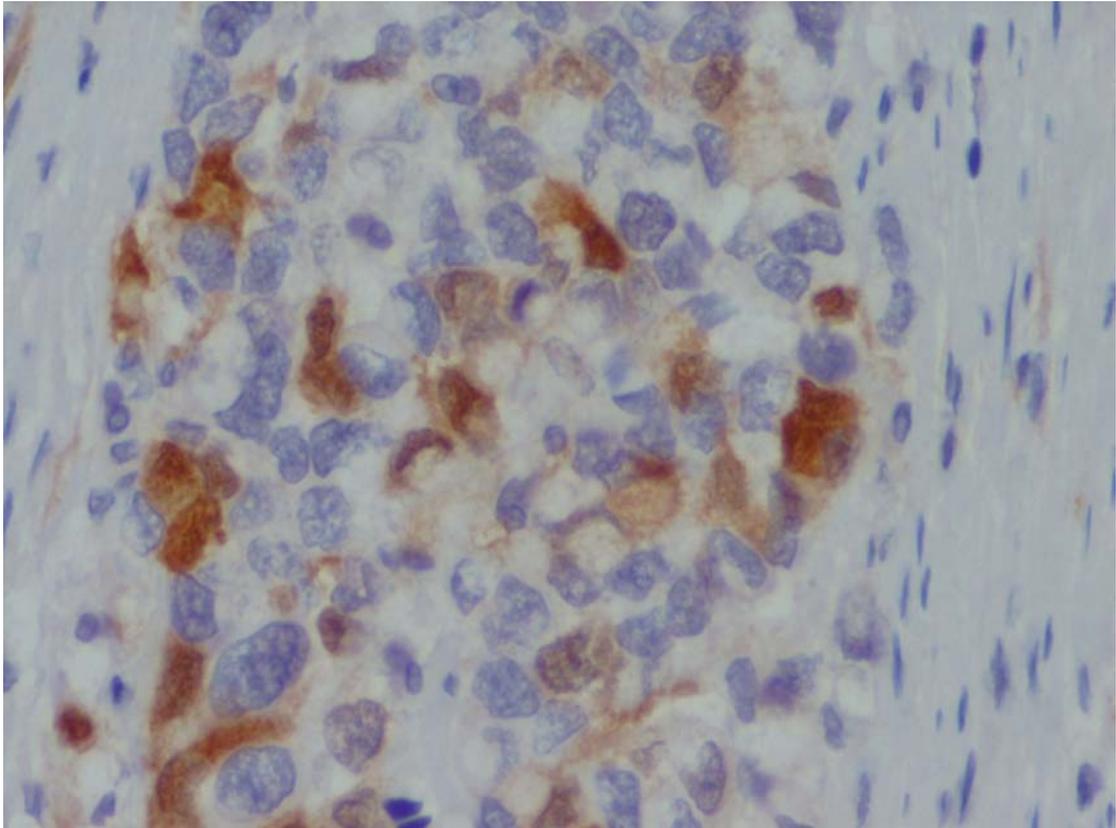


Figure 2A

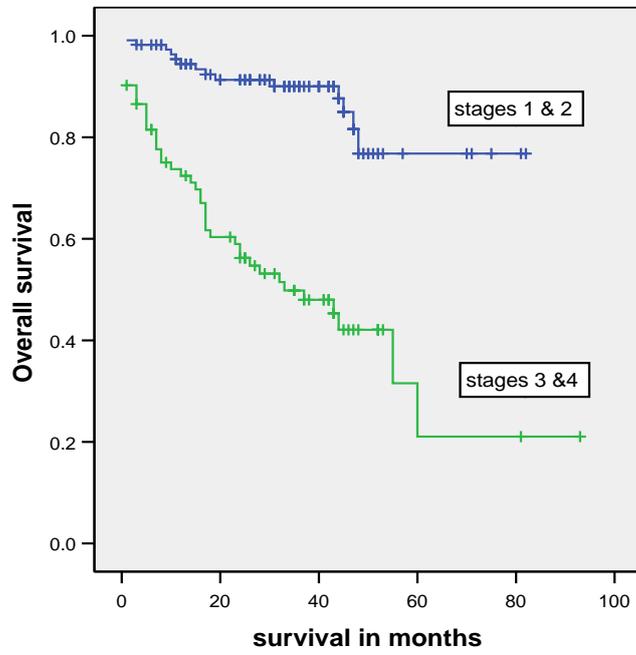


Figure 2B

