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Analysis of proteasomal subunit expression reveals Rpt4 as a prognostic marker in Stage II colorectal cancer

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Abstract

Colorectal cancer is a leading cause of cancer related deaths worldwide. Early diagnosis and treatment is the key to improving survival rates and as such a need exists to identify patients who may benefit from adjuvant chemotherapy. The dysregulation of the ubiquitin-proteasome system (UPS) has been implicated in oncogenesis and cancer cell survival, and proteasome inhibitors are in clinical use for a number of malignancies including multiple myeloma. In this study we examined the protein expression of several key components of the ubiquitin-proteasome system in colorectal cancer using immunohistochemistry to determine expression levels of ubiquitinylated proteins and the proteasomal subunits, 20S core and Rpt4 in a cohort of 228 colon cancer patients. Multivariate Cox analysis revealed that neither the intensity of either ubiquitinylated proteins or the 20S core were predictive in either Stage II or III colon cancer for disease free survival or overall survival. In contrast, in Stage II patients increased Rpt4 staining was significantly associated with better disease free survival (Cox proportional hazard ratio 0.605; $p=0.0217$). Our data suggest that Rpt4 is an independent prognostic variable for Stage II colorectal cancer and may aid in the decision of which patients undergo adjuvant chemotherapy.

Introduction

Colorectal cancer is the third leading cause of cancer – related death in the US with half a million deaths worldwide each year [1]. This is in part due to up to 75% of patients having locally advanced disease at presentation and despite advances in surgery and therapeutics, up to 40% of patients will experience disease relapse or distant progression [2]. To date the identification of patients most likely to develop disease recurrence relies mainly on clinical and pathological staging strategies; however there is considerable variability in survival among similarly staged patients [3]. For clinicians, the ultimate challenge lies in selecting those patients most likely to derive benefit from chemotherapeutics and experience minimal side effects. Undoubtedly a need exists to identify novel molecular markers which predict patient outcome and identify patients who may benefit from adjuvant chemotherapy. Advances in research and technology may now have the potential to assist in the integration of cancer biology and patient clinicopathological parameters, facilitating an improved clinical decision pathway. Currently surgical resection is the mainstay treatment for colorectal cancer patients in the presence and absence of 5-fluorouracil based adjuvant chemotherapy, although not all patients treated with chemotherapy will derive a benefit from it [4, 5]. Indeed, particularly in patients with Stage II disease the advantage of adjuvant chemotherapy remains contentious [6-8].

The ubiquitin-proteasome system is the major pathway for the degradation of the intracellular proteins and as such can modulate a variety of signaling pathways. Proteins destined for degradation are tagged with a chain of ubiquitin moieties to a lysine residue in the target protein in a series of enzyme catalyzed reactions which ultimately results in

the targeting of proteins to the proteasome complex [9]. The proteasome complex itself is a large multi-subunit complex composed of a 20S catalytic unit and 19S regulatory subunits [10, 11]. Several lines of evidence have pointed to the dysregulation of the ubiquitin-proteasome pathway in cancer where it is thought to be involved in oncogenesis and cancer cell survival [12]. Indeed, much research has centered on targeting the ubiquitin-proteasome system in a number of different malignancies [13], with the proteasome inhibitor, bortezomib, currently in clinical use as a therapy for multiple myeloma [14].

Previous studies have demonstrated elevated levels of 19S proteasome subunits in ovarian carcinoma tissues [15] and higher levels of the 19S proteasomal subunit, Rpt1, were observed in breast cancer tissue specimens compared to patient matched normal adjacent tissue as controls correlating with increased proteasomal activity [16]. However, to our knowledge no study to date has examined the potential of proteasomal subunits as prognostic markers in colorectal cancer. In this study we examined the immunohistochemical expression of several components of the UPS namely the levels of ubiquitinylated proteins, the 20S proteasomal catalytic core and the 19S regulatory subunit, Rpt4 and their correlation with clinicopathological features and prognosis in a large cohort of Stage II and III colorectal tumors.

Materials and Methods

Patients and Tissue Specimens

Tissue microarrays (TMAs) were constructed from 228 colorectal cancer cases taken from a phase III trial of adjuvant 5-flurouracil – based chemotherapy compared to postoperative observation alone [17]. The study group consisted of 228 non-consecutive patients with demographic variables including gender and age at surgery recorded. Histological stage II and III, tumor grade, TMN stage and presence or absence of vascular invasion were included in the analysis and whether the patients were randomised to adjuvant chemotherapy with 5-FU or no treatment was noted (Table 1). Time to disease progression, including age at recurrence, site of recurrence and time to recurrence were also verified. At the time of analysis, a median follow of 6.5 years was available for analysis. Full ethical approval was granted for this study. TMAs consisted of paraffin embedded colorectal cancer specimens from patients with a positive diagnosis of colorectal adenocarcinoma, stage II and III [18]. Morphologically representative tissue areas from tumor specimens and normal adjacent tissue were arrayed on tissue arrays and subsequently utilized to determine expression levels of ubiquitinylated proteins, 20S Core and Rpt4. Expression was then correlated to patient demographics, adjuvant treatment regimens and histological parameters.

Immunohistochemistry

Sections 4 μm in thickness were cut from array blocks and floated onto adhesive slides. Sections were then baked at 55° C overnight. All staining was carried out on a BondMax

automated immunostainer from Vision BioSystems. Sections were loaded onto the system and the relevant program was started. The BondMax system dewaxed the slides and then carried out and antigen retrieval was performed. Sections were stained with antibodies against ubiquitinylated proteins (Cell Signaling Technology, MA, USA), the 20S core (Enzo Life Sciences, Lausen, Switzerland) or Rpt4 (Enzo Life Sciences). Optimisation steps were carried out to determine appropriate antibody dilution, which were 1:1500 for ubiquitinylated proteins; 1:200 for the 20S core and 1:1000 for Rpt4. Following detection using diaminobenzidine the sections were then counterstained lightly with haematoxylin, and mounted with coverslips.

Immunohistochemical Evaluation

Immunostained slides were scored for ubiquitinylated proteins, 20S and Rpt4 using a standardised scoring system. Each slide was evaluated using light microscopy with respect to intensity. An intensity score was assigned that represents the average intensity of the positive cells (0 = no staining; 1 = weak; 2 = moderate; 3 = strong). Slides were scored by two independent pathologists (SC and EK) blinded to the clinic-pathological data and entered into a database. In all cases with scoring discrepancies between individual pathologists, slides were reviewed and scoring assigned following consensus agreement. As multiple cores for each patient were arrayed, subsequent statistical analysis was carried out using median intensity scores.

Statistical Analysis

Univariate and multivariate analysis was carried out using logistic regression and Cox's proportional hazard model. Univariate analysis of overall and progression-free survival was performed by the Kaplan-Meier method and log-rank test and carried out in SPSS for Windows 15.0 (SPSS Inc, Chicago, IL). Staining intensity was stratified for Kaplan-Meier survival plot and log rank analysis according to the following criteria; median intensity values of less than 2 were considered weak staining and greater or equal to 2 were considered strong staining. Variables included in the multivariate analysis included gender, age at surgery, stage of colorectal cancer, tumor stage, intensity of staining for the individual proteins of interest and whether the patient was randomized to receive 5-flurouracil chemotherapy. A *p*-value of less than 0.05 was considered significant.

Results

Clinicopathological features and patient outcome

The study was performed on a tissue microarray constructed from surgical resection samples of Stage II and Stage III colorectal cancer patients which were collected as part of a trial of adjuvant 5-fluorouracil based chemotherapy compared to postoperative observation alone [17]. The demographics of the patients are shown in Table 1 along with the clinicopathological features of tumor site, grade of differentiation and vascular invasion (Table 1). As expected, increasing stage was significantly associated with risk of distant recurrence and death (Log rank test; $p<0.05$). The results compare favorably with internationally acceptable 5 and 10 year survival rates for Stage II and III colonic carcinoma [19]. There was no statistically significant improvement in overall and disease free survival in stage II patients who received chemotherapy compared to those who underwent observation (Chi-squared test; $\chi 2(1)=0.415$, $p> 0.05$). Evidence based practice advocates the use of adjuvant chemotherapy in stage III CRC, and while no statistically significant benefits in either overall or disease free survival were observed in this study, a trend towards significance was achieved (Chi-squared test; $\chi 2(1)=3.326$, $p = 0.068$), which is likely attributable to the smaller number of Stage III patients involved in this study.

Expression of ubiquitin-proteasome system proteins in tumor tissue and correlation with clinical outcome

The tissue microarrays (TMAs) were prepared and representative sections of tumor tissue stained with each of the antibodies are shown in Figure 1. The TMAs were scored for the

expression of ubiquitinylated proteins as well as the proteasomal subunits, 20S core and Rpt4 as described in the materials and methods section. Survival analyzes were performed with 6.5 year median follow up clinical data available. Kaplan Meier survival curves were generated to determine if a significant relationship existed between the expression levels of ubiquitinylated proteins, the 20S core subunit or Rpt4 expression scores and both disease free and overall survival in our cohort of Stage II and III patients.

***Ubiquitinylated Proteins*:** The expression of ubiquitinylated proteins was detected in 96% of tumor specimens and localized to both the cytoplasm and nucleus of cells. The intensity of ubiquitinylated proteins staining was not found to be a predictive of time to disease free survival in stage II colon cancer, however, a trend towards significance exists (Hazard Ratio (HR) =0.658; p=0.0605). Analysis of stage III disease patients revealed that levels of ubiquitinylated proteins were not predictive of predict disease recurrence (Table 2; Figure 2A). Overall survival analysis of ubiquitinylated protein expression showed that it was not a predictor of death in either stage II or III patients (Table 3; Figure 3A).

***20S proteasome core*:** Next we examined the expression of the 20S proteasome core. Expression was detected in 96% of tumor specimens and was consistent with the known cytoplasmic and nuclear localization of proteasomes [20]. Univariate and multivariate analysis of our cohort of stage II and III patients indicated that the expression of the 20S proteasome core was not predictive of those patients more likely to experience disease recurrence (Table 2; Figure 2B). Likewise, in the same cohort of stage II and III patients,

20S core staining did not predict those patients who were likely to die from their disease (Table 3; Figure 3B).

***Rpt4 subunit*:** Finally we examined the relationship between the expression levels of the 19S proteasome subunit, of Rpt4, and disease free and overall survival. In Stage II patients, increased expression of Rpt4 significantly predicted patients who were less likely to experience disease recurrence. This effect was also evident when analyzed by Kaplan Meier analysis (Figure 2C). There was no similar correlation between Rpt4 expression and disease free survival in Stage III patients (Table 2; Figure 2C). Interestingly, although Log Rank analysis revealed that increased Rpt4 intensity in Stage II patients also predicted those who were more likely to have survived (OR = 0.648, *p=0.0467*) Rpt4 expression was not predictive of time to death in univariate analysis or multivariate analysis (Table 3; Figure 3C). There was no significant association between Rpt4 intensity and overall survival in Stage III patients (Table 3).

Discussion

Discrepancies in survival of patients with node negative colorectal carcinoma are most often attributable to the presence of micro-metastases that are too small and thus undetectable at the time of staging by conventional pathological staging mechanisms [21], leading to heterogeneity in survival among patients who have comparable disease stages. Identification of prognostic biomarkers would select out high risk patients who are likely to experience disease recurrence and would theoretically benefit from further adjuvant therapies, while avoiding unnecessary toxicity to those who will not benefit, remains the rationale for targeted patient treatment modalities.

With this in mind we employed tissue microarray technology to assay the immunohistochemical expression of a number of components of the ubiquitin-proteasome pathway in a large cohort of node negative stage II and node positive stage III patients. Immunohistochemical staining with an antibody which recognized mono- and poly-ubiquitinylated proteins and subsequent correlation with clinico-pathological data revealed that the intensity of staining of ubiquitinylated proteins was not a prognostic marker for disease free survival and time to death in either Stage II or III colorectal cancer patients. Similar results were evident when the tissue microarray was stained with an antibody recognizing the 20S core. In contrast, increased Rpt4 expression was found to be a significant positive prognostic marker for disease free survival and time to death in Stage II patients but not in Stage III patients.

The ubiquitin-proteasome pathway plays a major role in the facilitating protein degradation and in such a manner modulates a number of cellular signaling pathways including those involved in cell cycle regulation and apoptosis [22], both of which are affected in colon cancer. The 19S regulatory subunit of the proteasome, of which Rpt4 is an integral component, plays a role in substrate unfolding prior to entering the catalytic core chamber [23, 24] and represents a potential target for development of novel chemotherapeutic treatment modalities. Currently the benefit of adjuvant chemotherapy to patients with Stage II disease remains controversial [6-8]. We have determined that decreased Rpt4 expression is significantly associated with decreased time to disease progression in Stage II patients. This finding may support the potential of Rpt4 as a prognostic biomarker in identifying patients who are likely to experience disease recurrence and thus would benefit from further adjuvant therapies and those who are less likely to develop recurrence and therefore would not gain any survival advantage from additional treatments. Furthermore, a recent study has linked a member of the ubiquitin-like modifier family, Ubiquitin D (UBD), as a potential prognostic marker in stage II-III colorectal cancer [25]. In future studies it may be interesting to combine Rpt4 expression alongside UBD to enhance the identification of patients who may benefit from adjuvant chemotherapy. In conclusion, we have identified a novel finding that increased expression of the 19S proteasomal subunit Rpt4 correlated with increased disease free and overall survival in a cohort of Stage II colon cancer patients. Such observations may be of future use for clinicians in identifying patients who will benefit from adjuvant chemotherapy.

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Table 1: Clinicopathological features of colon cancer patient cohort.

Table 2: Effect of individual marker scores on disease free survival as assessed using Cox regression proportional analysis.

Table 3: Effect of individual marker scores on overall survival as assessed using Cox regression proportional analysis.

Figure 1: Representative images of immunohistochemical expression of ubiquitinylated proteins, the 20 S core and Rpt4 expression in tumor colonic tissue specimens. (A) Ubiquitinylated proteins (B) 20S core (C) Rpt4. Sections were scored for intensity staining.

Figure 2: Kaplan-Meier survival curve of disease free survival for ubiquitinylated proteins (A), 20S core (B) and Rpt4 (C). Intensity staining scores of 0-1 were classified as weak staining and scores of 2-3 classified as strong staining.

Figure 3: Kaplan-Meier survival curve of overall survival for ubiquitinylated proteins (A), 20S core (B) and Rpt4 (C). Intensity staining scores of 0-1 were classified as weak staining and scores of 2-3 classified as strong staining.

TABLE 1: CLINICOPATHOLOGIC DETAILS OF COLORECTAL CANCER PATIENT COHORT (N=228)		
	Chemotherapy	No Treatment
Age (years)		
Mean	64.58	63.69
Median	66	65
Range	45.75 - 80.96	35.01 - 79.63
Gender		
Male	64 (56.6%)	71 (61.7%)
Female	49 (43.4%)	44 (38.3%)
Stage		
II	69 (61.1%)	76 (66.1%)
III	44 (38.9%)	39 (33.9%)
Tumour Site		
Right Colon	46 (40.7%)	41 (35.7%)
Left Colon	42 (37.2%)	48 (41.7%)
Synchronous	3 (2.7%)	0 (0%)
Rectum	22 (19.5%)	26 (22.6%)
Grade of differentiation		
Grade 1	9 (8%)	10 (8.7%)
Grade 2	86 (76.1%)	86 (74.8%)
Grade 3	14 (12.4%)	13 (11.3%)
Not specified	4 (3.5%)	6 (5.2%)
Vascular invasion		
No	58 (51.3%)	62 (53.9%)
Yes	27 (23.9%)	23 (20%)
Not specified	28 (24.8%)	30 (26.1%)

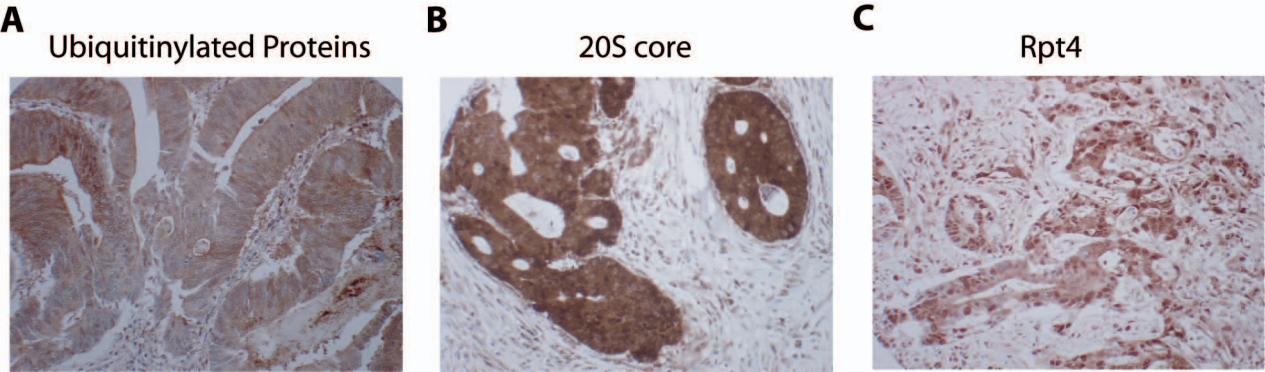
Marker	Disease Free Survival							
	Stage II				Stage III			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Ubiquitinylated proteins	0.658 (0.425 - 1.019)	0.0605	0.676 (0.435 - 1.050)	0.0817	1.192 (0.885 – 1.587)	0.2302	1.1117 (0.824 – 1.514)	0.4758
20S proteasomal core	0.806 (0.566 - 1.147)	0.2307	0.791 (0.554 - 1.131)	0.1991	1.150 (0.882 - 1.500)	0.3023	1.136 (0.855 - 1.509)	0.3795
RPT4 proteasomal subunit	0.605 (0.394 – 0.929)	0.0217	0.613 (0.398 – 0.945)	0.0265	1.097 (0.825 - 1.459)	0.5233	1.026 (0.758 - 1.390)	0.8665

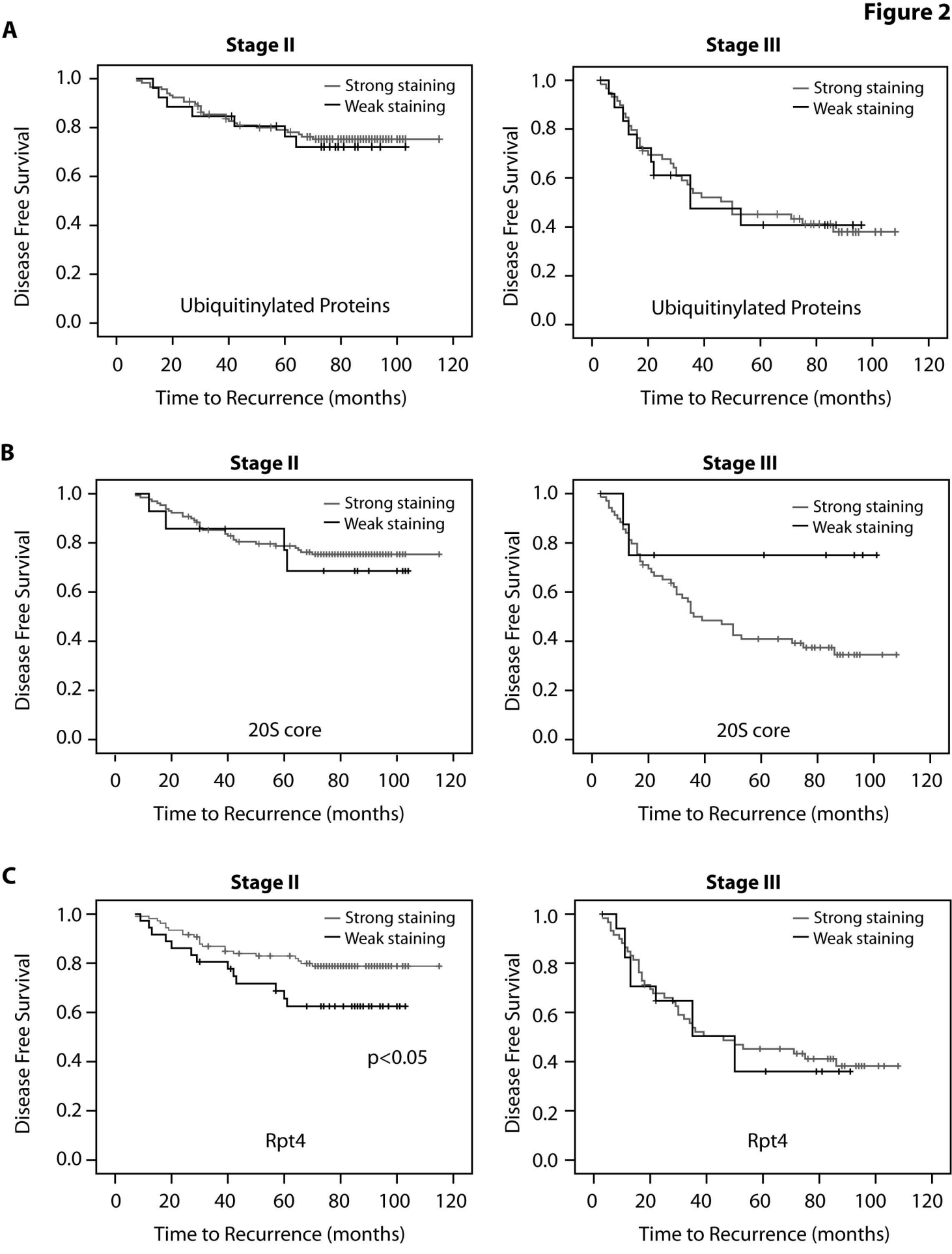
Table 2

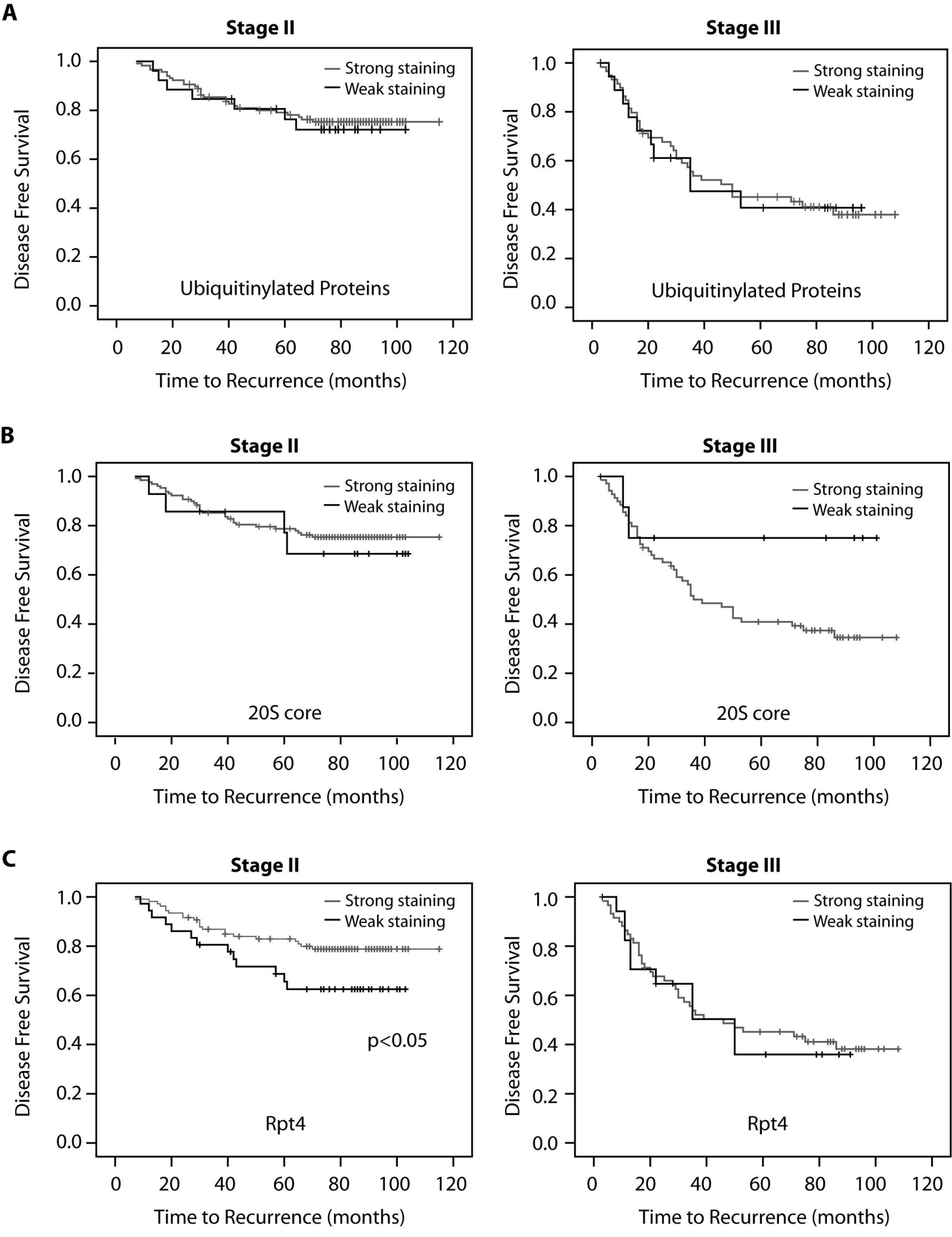
Marker	Overall Survival							
	Stage II				Stage III			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Ubiquitinylated proteins	0.831 (0.596 - 1.158)	0.2746	0.798 (0.565 - 1.127)	0.2005	1.356 (0.863 - 2.130)	0.1869	1.264 (0.572 - 2.796)	0.5624
20S proteasomal core	0.841 (0.583 - 1.213)	0.3529	0.913 (0.639 - 1.306)	0.619	1.072 (0.832 - 1.380)	0.593	1.033 (0.785 - 1.359)	0.8154
RPT4 proteasomal subunit	0.841 (0.616 - 1.148)	0.2757	0.842 (0.606 - 1.170)	0.3059	1.018 (0.775 - 1.337)	0.8996	0.958 (0.719 - 1.278)	0.7724

Table 3

Figure 1




A
Stage II
Stage III
Strong staining
Weak staining
Disease Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
Ubiquitinylated Proteins
0
20
40
60
80
100
120
Time to Recurrence (months)
B
Stage II
Stage III
Strong staining
Weak staining
Disease Free Survival
20S core
Time to Recurrence (months)
C
Stage II
Stage III
Strong staining
Weak staining
Disease Free Survival
p<0.05
Rpt4
Time to Recurrence (months)


A
Stage II
Strong staining
Weak staining
Disease Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
Ubiquitinylated Proteins
0
20
40
60
80
100
120
Time to Recurrence (months)
Stage III
Strong staining
Weak staining
Disease Free Survival
Ubiquitinylated Proteins
Time to Recurrence (months)
B
Stage II
Strong staining
Weak staining
Disease Free Survival
20S core
Time to Recurrence (months)
Stage III
Strong staining
Weak staining
Disease Free Survival
20S core
Time to Recurrence (months)
C
Stage II
Strong staining
Weak staining
Disease Free Survival
p<0.05
Rpt4
Time to Recurrence (months)
Stage III
Strong staining
Weak staining
Disease Free Survival
Rpt4
Time to Recurrence (months)