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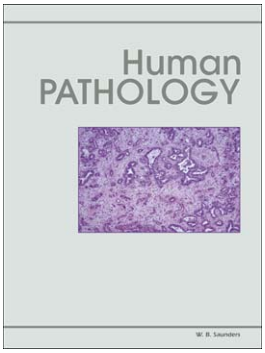

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Should the grading of colorectal adenocarcinoma include microsatellite instability status?

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Abstract

Adenocarcinomas of the colon and rectum are graded using a two-tiered system into histologic low-grade and high-grade tumors based on the proportion of gland formation. The current grading system does not apply to subtypes of carcinomas associated with a high frequency of microsatellite instability (MSI), such as mucinous and medullary carcinomas. We investigated the combined effect of histologic grade and MSI status on survival for 738 patients with colorectal carcinoma (48% female; mean age at diagnosis 68.2 years). The proportion of high-grade adenocarcinoma was 18%. MSI was observed in 59 adenocarcinomas (9%), with higher frequency in high-grade tumors compared with low-grade tumors (20% vs. 6%; P<0.001). Using Cox regression models, adjusting for sex, age at diagnosis and stratifying by the American Joint Committee on Cancer (AJCC) stage, microsatellite stable (MSS) high-grade tumors were associated with increased hazard of all-cause and colorectal cancer-specific mortality: hazard ratio (HR) 2.09 (95% confidence interval (CI), 1.58-2.77) and 2.54 (95% CI, 1.86-3.47), respectively, both P<0.001. A new grading system separating adenocarcinoma into low-grade (all histologic low-grade and MSI high-grade) and high-grade (MSS histologic high-grade) gave a lower Akaike information criterion value when compared with the current grading system and, thus, represented a better model fit to stratify patients according to survival. We found that patients with a high-grade adenocarcinoma had significantly shorter survival than patients with low-grade adenocarcinoma only if the tumor was MSS, suggesting that the grading of colorectal adenocarcinoma with high-grade histologic features should be made according to the MSI status of the tumor.

Introduction

Most colorectal carcinomas are adenocarcinomas of usual type (adenocarcinoma NOS, not otherwise specified). Since the first grading systems were established [1, 2], pathologists have routinely included histologic grade in their reports of resected colorectal adenocarcinomas. Adenocarcinomas are graded into well-, moderately or poorly differentiated tumors (grades 1, 2, 3, respectively) depending on the proportion of gland formation in the least differentiated component of the tumor away from the invasive edge, according to the World Health Organization (WHO) criteria [3]. Despite low levels of agreement among pathologists on this subjective assessment [4, 5], histologic grading has been shown to be an independent prognostic factor for colorectal carcinoma [6-9]. This is particularly true for the poorly differentiated subgroup that has been most consistently found to be associated with adverse clinical outcome. The WHO and the American Joint Committee on Cancer (AJCC) recommend a two-tiered histologic grading system: low-grade for well- and moderately differentiated adenocarcinomas (50-100% gland formation) and high-grade for poorly differentiated adenocarcinomas (0-49% gland formation) [3, 10].

Testing tumors for microsatellite instability (MSI) by immunohistochemistry for mismatch repair (MMR) proteins MLH1, MSH2, PMS2 and MSH6 and/or by molecular-based methods is routinely performed for patients diagnosed with colorectal carcinoma, primarily to screen for Lynch syndrome. Up to 15% of all colorectal carcinomas demonstrate MSI, more frequently secondary to acquired methylation of *MLH1* (sporadic cases) than caused by a germline mutation in an MMR gene (Lynch syndrome). MSI has been reported to be a strong positive prognostic factor by multiple independent studies [11-13]. Some histologic subtypes of colorectal carcinomas are more commonly observed in MSI tumors, including medullary carcinomas, mucinous adenocarcinomas and signet ring cell carcinomas [14]. The adverse

prognosis associated with the poor differentiation of most of these tumor subtypes contrasts with the positive prognosis associated with MSI. Consequently, the current WHO histologic grading does not apply to these subtypes of colorectal carcinoma. Additionally, the WHO recommends that mucinous carcinomas should be graded according to their MSI status, regardless of their morphologic appearance [3]. Such an MSI-based grading principle could potentially be applied to colorectal adenocarcinomas of usual type to more effectively stratify patients by prognosis. To test this hypothesis, we investigated the survival of a large series of patients diagnosed with colorectal adenocarcinoma with respect to histologic grade and MSI status.

Patients and Methods

Study Sample

Incident colorectal carcinomas were identified from participants enrolled of the Melbourne Collaborative Cohort Study (MCCS), a prospective cohort study of 41,514 people (17,045 males and 24,469 females) recruited between 1990 and 1994 [15]. Participants were aged 27 to 75 years with almost all aged between the ages 40-69 years at baseline. The study protocol was approved by the Cancer Council Victoria's Human Research Ethics Committee and the Human Research Ethics Committee of the Queensland Institute of Medical Research under protocol P799. Written informed consent was obtained from all study subjects for the investigators to review their medical records.

Data Collection

Clinical data were collected from medical charts, colonoscopy reports and pathology reports. Paraffin-embedded tissue blocks were collected from hospital pathology departments where the patient underwent colectomy. Tissue sections were cut for pathology reviews,

immunohistochemistry and DNA extraction. All surgically resected carcinomas underwent standardized review by two pathologists (Jeremy Jass and Christophe Rosty) to assess for a set of histologic features including, histologic type (adenocarcinoma, mucinous carcinoma, others) and tumor grade. Adenocarcinoma of usual type is defined by a carcinoma of intestinal type forming glandular structures with variability in size and configuration, and with frequent mucus and cellular debris in the lumen. In poorly differentiated adenocarcinomas, gland formations had to be present even if only focally. Following the 2010 WHO histologic grading system, adenocarcinomas were classified as high-grade if <50% gland formation was present in the least differentiated area of the lesion (**Figure 1**) [3]. This area had to be present in at least one microscopic field at magnification x40, away from the tumor invading edge. Tumors showing complete absence of differentiation were classified as 'other subtype', which included undifferentiated carcinoma and medullary carcinoma. The diagnosis of medullary carcinoma required sheets of neoplastic cells with typical vesicular nuclei and conspicuous tumor infiltrating lymphocytes without any gland formation. If more than 50% of the tumor exhibited mucinous differentiation, defined by the presence of pools of extracellular mucin containing clusters of carcinomatous cells or individual tumor cells including signet ring cells, it was classified as a mucinous carcinoma. Signet ring cell carcinoma was defined by the presence of signet ring cells within mucin pools or in a diffuse infiltrative pattern occupying >50% of the tumor. Tumors were staged using the AJCC criteria [10].

Immunohistochemical and Molecular Analysis

Immunohistochemistry was performed on available tissue sections to assess the tumor expression of MMR proteins MLH1, PMS2, MSH2 and MSH6 [16]. MSI status was determined using a 10-loci panel in tumor DNA as previously described [17]. Tumors were

deemed MSI if loss of immunohistochemical expression of at least one MMR protein was demonstrated and/or ≥30% of MSI markers were unstable. All other tumors were deemed microsatellite stable (MSS).

Statistical Analysis

Statistical analyses were performed with Stata version 11.1 (College Station, TX: StataCorp LP). Time of observation was from the date of diagnosis of the colorectal carcinoma until death or March 2013, whichever came first. Kaplan-Meier methods were used to estimate separately for overall and disease-specific survival by MSI status (MSI vs. MSS) for all histologic types and by histologic grade (low-grade vs. high grade) and MSI status (MSI vs. MSS) for the group of adenocarcinoma only. Survival was compared between groups using the log-rank test. Cox regression models were used to estimate hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) for all-cause mortality and colorectal cancer-specific mortality associated with histologic grade and MSI status, after adjusting for: sex, age at diagnosis (<60, 60-70, >70 years), and stratifying by AJCC stage since hazards were non-proportional across stages. The proportional hazards assumption was assessed using graphic methods and tests based on Schoenfeld residuals. A two-tailed P value was used for all analyses and values less than 0.05 were considered to be statistically significant. We calculated the Akaike information criterion (AIC) for each Cox model to assess goodness-of-fit [18]. The AIC is a method used to measure and compare the relative quality of statistical models. AIC takes into account the goodness of fit and the complexity of the model. In this context, AIC provides information on the best prognostic model using a set of variables. The lower the AIC value is, the more informative the statistical model is.

Results

We identified 1046 incident carcinomas of the colon or rectum among MCCS participants between the study baseline (1990-1994) and 31 December 2009. Histopathologic review was undertaken for 795 of the tumors. Complete data for MSI status and histologic grade were available for 738 tumors, which were classified as adenocarcinoma of usual type (668 cases, 91%), mucinous carcinoma (58 cases, 8%), signet ring cell carcinoma (8 cases, 1%), and 'other subtype' including 3 undifferentiated carcinomas and 1 medullary carcinoma (**Table 1**). There were 356 females (48%) and the mean age at diagnosis was 68.2 years (standard deviation, 8.2 years). The proportion of high-grade tumors was 18% of adenocarcinomas. MSI was observed in 59 adenocarcinomas (9%), with higher frequency in high-grade tumors compared with low-grade tumors (20% vs. 6%; P<0.001), in 23 (40%) of mucinous carcinomas, in 3 (38%) of signet ring cell carcinomas, in the medullary carcinoma and in none of the 3 undifferentiated carcinomas (**Table 1**).

There were 377 deaths during a median follow-up of 7.5 years (range 2 days to 20.4 years), of which 237 (63%) were attributed to colorectal cancer. There was evidence of significantly better overall survival and disease-specific survival for MSI tumors, compared with MSS tumors of all histologic types (P=0.038 and P=0.0003, respectively) (**Figure 2**). In the subgroup of adenocarcinoma only, there was evidence of significantly lower overall survival and disease-specific survival for high-grade MSS tumors, compared with the other three tumor groups (MSI low-grade, MSS low-grade, and MSI high-grade) (**Figure 3**). When modeled together, high-grade was associated with an increased hazard for all-cause mortality and colorectal cancer-specific mortality: Hazard ratio (HR) 3.02 (95% CI, 2.32-3.92) and 4.20 (95% CI, 3.13-5.64), respectively, both P<0.001 (**Table 2**). After adjustment for sex, age and AJCC stage, MSS high-grade tumors were associated with an increased hazard for all-

cause and colorectal cancer-specific mortality: HR 2.09 (95% CI, 1.58-2.77) and 2.54 (95% CI, 1.86-3.47), respectively, both P<0.001 (**Table 3**).

Based on these results, a new grading system that includes MSI status is proposed that categorizes adenocarcinomas as low-grade if histologically low-grade (MSS and MSI) or high-grade with MSI, and as high-grade if histologically high-grade and MSS (**Table 4**). The AIC values for the adjusted Cox model for all-cause and colorectal cancer-specific mortality according to MSI status and histologic grade were 2806.7 and 1859.9. Omitting MSI status made little difference, with AIC values of 2806.9 and 1863.0. Replacing histologic grade with the new proposed grading system separating tumors into 2 groups based on the combined effect of MSI and histologic grade gave an AIC value of 2803.0 for all-cause mortality and 1856.0 for colorectal cancer-specific mortality, representing a better model fit. Similarly, for colorectal cancer-specific mortality the Cox model according to the new grading system gave the lowest most favorable AIC value.

Discussion

Our aim was to investigate the combined effect of histologic grade and MSI status on the survival of patients diagnosed with colorectal adenocarcinoma. Our finding of improved survival associated with MSI in colorectal carcinomas of any histologic subtype is consistent with previous reports [11-13]. This supports the recent WHO recommendation to grade mucinous carcinoma according to their MSI status [3]. We found that patients with high-grade adenocarcinoma had significantly lower survival than patients with low-grade adenocarcinoma only when the tumor was MSS. Patients with an MSI high-grade tumor had similar survival compared with patients with low-grade tumors of MSI or MSS subgroups. For better stratification of patients by survival, these results suggest that the grading of

histologically high-grade colorectal adenocarcinomas should take account of MSI status. The high-grade category should be restricted to adenocarcinomas displaying high grade histologic features and that are MSS (**Table 4**). The significant difference in survival between the MSI high-grade and the MSS high-grade subgroups suggests that the positive effect of MSI on survival is stronger than the negative effect of high histologic grade. However, our results should be interpreted with caution due to small numbers of death in the MSI subgroup. Conversely, MSI does not seem to have any effect on low-grade tumors, with no significant difference in survival between patients with MSI low-grade tumors and patients with MSS low-grade tumors, but the proportion of MSI in low-grade tumors is low (6% in this series) and studies with larger numbers would be required to identify such an effect.

A grading system incorporating MSI with the traditional histologic grades would potentially affect 10-20% of all colorectal carcinomas classified as histologically high-grade adenocarcinomas. From this subgroup, 20-50% of tumors are expected to be MSI and would then be reclassified as low-grade adenocarcinomas. In this study, the proportion of MSI in high-grade adenocarcinomas was 20%, which is low compared with other studies that report up to 50% of MSI in high-grade tumors [19]. This difference may be due to the lack of standardization to define an adenocarcinoma as histologically high-grade [3, 4, 8, 20]. While the WHO clearly sets the cutoff for gland formation to 50% to separate low-grade from high-grade adenocarcinomas, it is further specified that grading is based upon the least differentiated component, with the invading edge of tumor regarded as suboptimal to evaluate tumor grade [3]. Unfortunately, the definition of the least differentiated area remains unclear and this makes comparisons between series difficult. In our study, adenocarcinomas were graded according to the area showing the least differentiated component which had to be present in at least one field at magnification x40. Using these criteria may have resulted in a

greater proportion of adenocarcinomas with high grade histologic features (18%) compared with studies that assessed the whole analyzed tumor area for histologic grading as recommended by the AJCC and the College of American Pathologists (CAP) guidelines. When histologically high-grade, MSI adenocarcinomas are often more homogeneous with >50% of the tumor showing poor differentiation. It is, therefore, expected that both grading systems would include comparable numbers of MSI high-grade adenocarcinomas. Heterogeneity is frequently observed in MSI carcinomas but usually by the frequent occurrence of minor mucinous carcinoma components in an otherwise low-grade adenocarcinoma [14, 21]. The difference in proportions of high-grade adenocarcinomas is likely to be explained by the inclusion of tumors with overall <50% gland formation (i.e. called low-grade according to the AJCC/CAP system) but with minor areas of poorly differentiated adenocarcinoma that were sufficient to call the tumor high-grade histologically, as in this study and recommended by the WHO. Focal areas of poor differentiation have been referred to as poorly differentiated clusters by some authors and it has been reported to be associated with other histologic variables of poor prognosis, such as tumor budding, infiltrating tumor front and nodal metastasis [22, 23]. Ueno et al. proposed a histologic grading system based on the number of poorly differentiated clusters, which better stratified patients by clinical outcome than the traditional grading system based on the proportion of gland formation in the tumor [24, 25]. Reporting tumors with these aggressive morphologic features as low grade is counterintuitive as is the reporting of poorly differentiated MSI tumors as high grade. Most histologically high-grade adenocarcinomas characterized by poorly differentiated clusters are likely to be MSS and, therefore, not affected by the incorporation of MSI in the final grade. Our results on incorporating MSI to the grading of colorectal adenocarcinoma with high grade histologic features need to be verified when tumors are graded according to the AJCC/CAP system. However, these findings would

suggest that the WHO grading system of adenocarcinoma may be more appropriate for patient prognostication than the AJCC/CAP system, which may undergrade tumors with a focal poorly differentiated component present in <50% of examined tumor, possibly secondary to sampling error. We propose that one microscopic field at magnification x40 away from the invading edge could be used as the minimum area containing poor differentiation for a colorectal adenocarcinoma to be classified histologically as a high-grade tumor.

In 1986, Jass et al. [9] established a grading system using a Cox regression model based on data from 447 resected rectal adenocarcinomas. The variables that best predicted survival included tubule configuration (regular, irregular, none), lymphocytic infiltration (marked, moderate, little or none) and tumor growth pattern (expanding, infiltrating). The best score (a score of 0) was attributed to tumors showing marked lymphocytic infiltration and an expanding growth pattern. The Jass grading system was, therefore, heavily influenced by these two variables which have been subsequently found to be typical characteristics of MSI colorectal adenocarcinoma [26]. That is, our proposed criteria for integrating MSI status with the histologic grading of colorectal adenocarcinoma can be seen as a simplified version of the Jass grading system. Poor reproducibility of the Jass grading system has been reported by some authors [27] and this might have hampered its uptake by pathologists. A growing number of pathology laboratories are now implementing upfront MSI testing for all newly diagnosed colorectal carcinomas, usually by immunohistochemistry. The reliability of MMR immunohistochemistry and its good concordance with molecular methods make MSI status an easy and reproducible parameter to be widely used in pathology laboratories [28]. Moreover, MMR immunohistochemistry has been found to be equally reliable in biopsy

samples and resection specimens [29], allowing appropriate grading at time of the initial diagnosis from the endoscopic biopsy.

In conclusion, we found that patients with colorectal adenocarcinoma are better stratified by survival when the MSI status of the tumor is incorporated in the grading system. Patients with high-grade MSI adenocarcinomas had similar survival to patients with low-grade adenocarcinomas. We propose that the grading of colorectal adenocarcinoma with high-grade histologic features, present in at least one microscopic field at magnification x40, should be made according to the MSI status of the tumor. As molecular pathology increasingly supplements morphology, pathologists will be able to integrate clinically useful biologic markers into their routine pathology practice.

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Table 1. Number of colorectal cancer patients (N=738) and deaths by histologic type, histologic grade and microsatellite instability status.

		Patients, N (%)	Females, N (%)	Mean age, years	Deaths	
					All cause, N (%)	Colorectal cancer, N (%)
Adenocarcinoma						
Low-grade						
	MSS	514 (94)	229 (45)	67.8	244 (47)	142 (28)
	MSI	35 (7)	25 (71)	70.8	15 (43)	4 (11)
High-grade						
	MSS	95 (80)	50 (53)	68.9	75 (79)	67 (71)
	MSI	24 (20)	17 (71)	70.4	13 (54)	5 (21)
Mucinous carcinoma						
	MSS	35 (60)	16 (46)	68.4	16 (46)	11 (31)
	MSI	23 (40)	11 (48)	66.8	5 (22)	2 (9)
Signet ring cell carcinoma						
	MSS	5 (62)	5 (100)	61.8	5 (100)	4 (80)
	MSI	3 (38)	0 (0)	73.8	2 (67)	1 (33)
Undifferentiated carcinoma (all MSS)		3	3 (100)	62.6	2 (67)	1 (33)
Medullary carcinoma (MSI)		1	0	75	0	0

Abbreviations: MSS: Microsatellite stable; MSI: Microsatellite instable

Table 2. Hazard ratios for all-cause and colorectal cancer-specific mortality with no adjustment in the subgroup of adenocarcinoma patients.

Variable		All-cause		Colorectal cancer	
		HR (95% CI)	P	HR (95% CI)	P
MSI (main effect)		0.88 (0.52-1.49)	0.637	0.39 (0.15-1.06)	0.066
High-grade (main effect)		3.02 (2.32-3.92)	<0.001	4.20 (3.13-5.64)	<0.001
Interaction term		0.43 (0.20-0.95)	0.037	0.45 (0.12-1.74)	0.248
Four-group comparisons:					
Low-grade					
	MSS	1 (Referent)		1 (Referent)	
	MSI	0.88 (0.52-1.49)	0.637	0.39 (0.15-1.06)	0.066
High-grade					
	MSS	3.02 (2.32-3.92)	<0.001	4.20 (3.13-5.64)	<0.001
	MSI	1.15 (0.66-2.01)	0.624	0.75 (0.31-1.82)	0.521

Abbreviations: HR: Hazard ratio; CI: Confidence interval; MSS: Microsatellite stable; MSI: Microsatellite instable

Table 3. Hazard ratios for all-cause and colorectal cancer-specific mortality with adjustment for sex, age-group and stratified by AJCC stage in the subgroup of adenocarcinoma patients.

Variable		All-cause		Colorectal cancer	
		HR (95% CI)	P	HR (95% CI)	P
Female		0.84 (0.66-1.05)	0.128	0.91 (0.68-1.20)	0.497
Age-group (years):					
<60		1 (Referent)		1 (Referent)	
60-70		1.24 (0.85-1.80)	0.267	1.11 (0.73-1.70)	0.614
70+		1.92 (1.35-2.74)	0.000	1.27 (0.85-1.89)	0.245
MSI (main effect)		1.10 (0.63-1.92)	0.731	0.62 (0.23-1.71)	0.360
High-grade (main effect)		2.09 (1.58-2.77)	<0.001	2.54 (1.86-3.47)	<0.001
Interaction (MSI and grade)		0.50 (0.22-1.13)	0.097	0.53 (0.14-2.07)	0.364
Four-group comparisons:					
Low-grade					
	MSS	1 (Referent)		1 (Referent)	
	MSI	1.10 (0.63-1.92)	0.731	0.62 (0.23-1.71)	0.360
High-grade					
	MSS	2.09 (1.58-2.77)	<0.001	2.54 (1.86-3.47)	<0.001
	MSI	1.15 (0.63-2.07)	0.653	0.85 (0.34-2.08)	0.715

Abbreviations: HR: Hazard ratio; CI: Confidence interval; MSS: Microsatellite stable; MSI: Microsatellite instable

Table 4. Proposed new grading criteria for colorectal adenocarcinoma combining histologic grade (tumor differentiation) and microsatellite instability (MSI) status (MSI versus microsatellite stable (MSS))

Proportion of gland formation*	Tumor differentiation	Histologic grade	MSI status	New grade
0-49%	Poor	High-grade	MSS	High-grade
0-49%	Poor	High-grade	MSI	Low-grade
50-100%	Moderate or well-differentiated	Low-grade	MSS	Low-grade
50-100%	Moderate or well-differentiated	Low-grade	MSI	Low-grade

* in at least one microscopic field at magnification x40

Abbreviations: MSS: Microsatellite stable; MSI: Microsatellite instable

Figure legends

Figure 1. Examples of colorectal adenocarcinoma with high histologic grade and different microsatellite instability status. A. Histologically high grade adenocarcinoma associated with a microsatellite stable (MSS) phenotype showing only focal gland formations with 'dirty necrosis' and overall poor differentiation. B. Histologically high grade adenocarcinoma with a microsatellite instability (MSI) phenotype showing tumor infiltrating lymphocytes and very focal gland formations.

Figure 2. Kaplan-Meier survival curves showing overall survival (A) and colorectal-cancer specific survival (B) according to microsatellite status (MSI and MSS) for all histologic types.

Figure 3. Kaplan-Meier survival curves showing overall survival (A-C) and colorectal-cancer specific survival (D-F) according to histologic grade (low-grade versus high-grade) and microsatellite status (MSI and MSS) (A and D), histologic grade only (B and E), and the new grading system with high-grade MSS vs other (C and F), in colorectal adenocarcinoma patients.

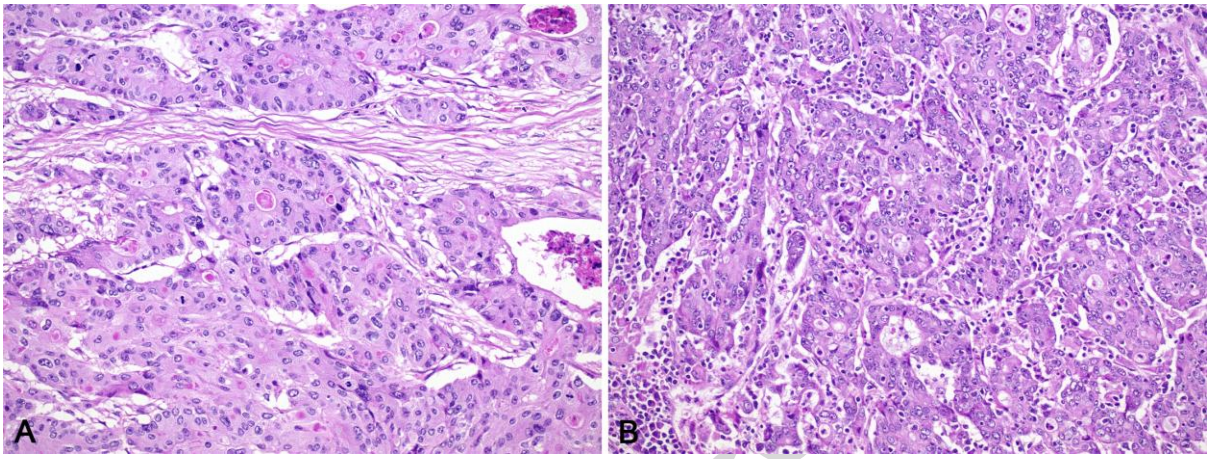

Figure 1

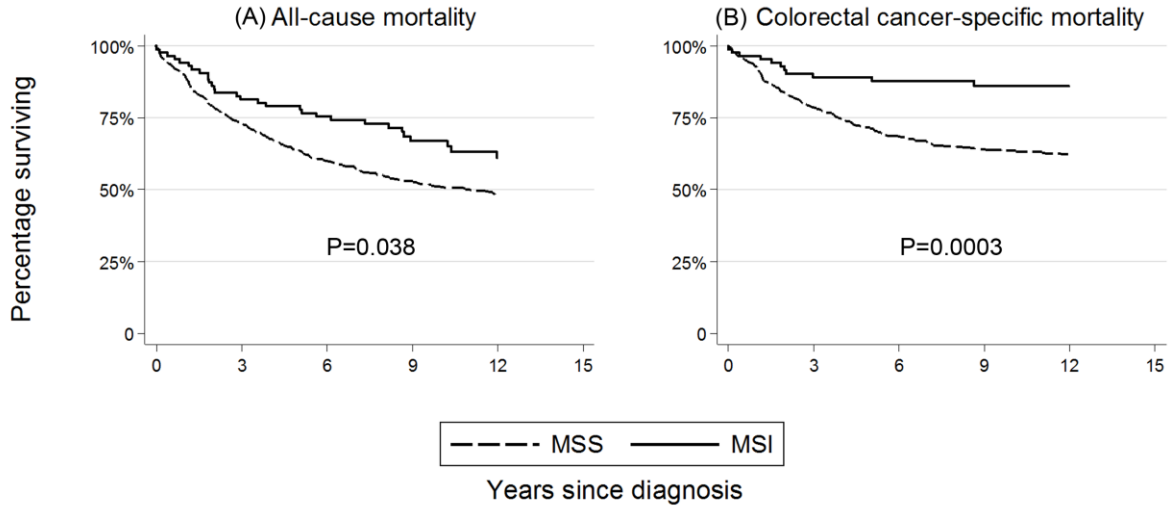

Figure 2

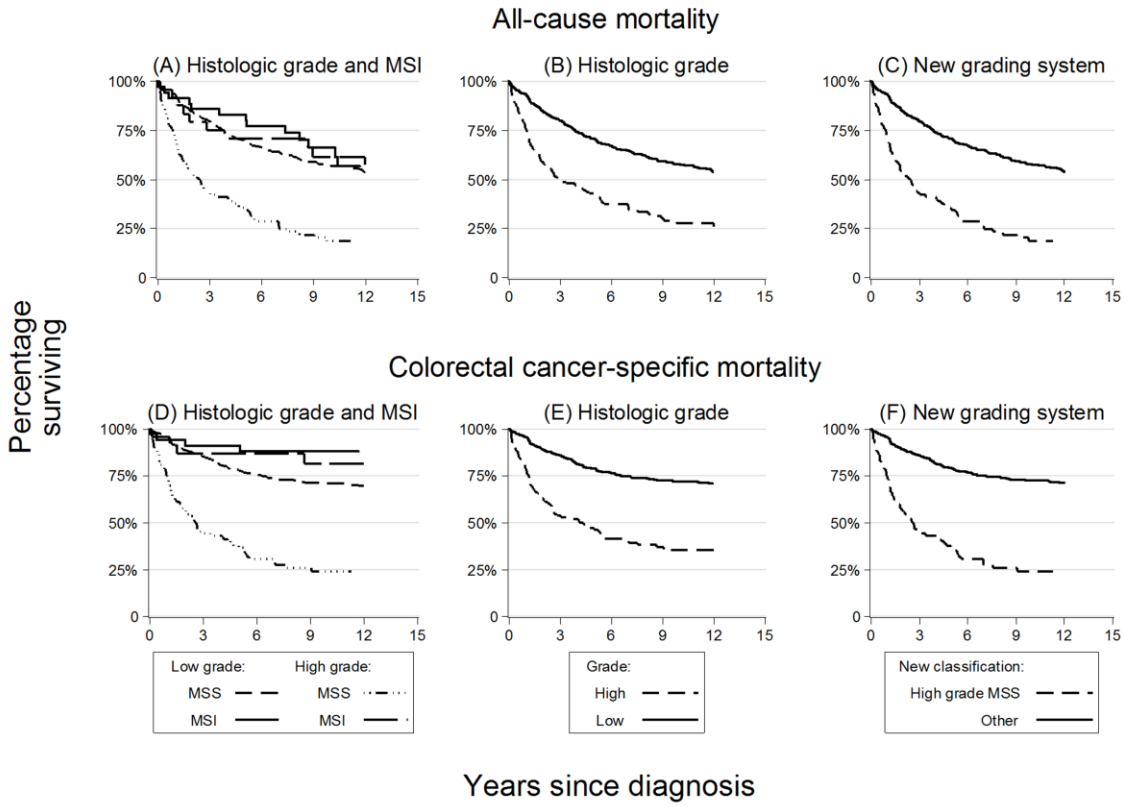

Figure 3