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Conflicts of interests

The authors declare no conflicts of interest.

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Abstract

The aim of the present study was to investigate the prognostic value of BMI1 protein expression in primary tumors of stage II colon cancer patients.

BMI1 protein expression was assessed by immunohistochemistry in a retrospective patient cohort consisting of 144 stage II colon cancer patients. BMI1 expression at the invasive front of the primary tumors correlated with mismatch repair status of the tumors. Furthermore, BMI1 expression at the luminal surface correlated with T-stage, tumor location, and the histological subtypes of the tumors. In a univariate Cox proportional hazard analysis no statistical significant association between risk of relapse and BMI1 protein expression at the invasive front (HR: 1.12; 95% CI 0.78-1.60; *p*=0.53) or at the luminal surface of the tumor (HR: 1.06; 95% CI 0.75-1.48; *p*=0.70) was found. Likewise, there was no association between 5-year overall survival and BMI1 expression at the invasive front (HR: 1.12; 95% CI 0.80-1.56; *p*=0.46) or at the luminal surface of the tumor (HR: 1.16; 95% CI 0.86-1.60; *p*=0.33).

In conclusion, BMI1 expression in primary tumors of stage II colon cancer patients could not predict relapse or overall survival of the patients, thus having a limited prognostic value in stage II colon cancer patients.

Keywords

BMI1, Biomarkers, Colon Cancer, Recurrence

Abbreviations

BMI1, B-cell-specific Moloney murine leukemia virus insertion site 1; dMMR, Mismatch repair deficiency; IHC, immunohistochemistry; MMR**,** Mismatch repair; *MLH1, MutL homolog 1*; *MSH2*, *MutS protein homolog 2*; *MSH6*, *MutS protein homolog 6*; *PMS2, Postmeiotic Segregation Increased 2*; SOX9, sex-determining region y-box 9 (SOX9)

Introduction

Colorectal cancer is one of the most common cancers and accounts for the second highest mortality rate amongst cancers(1). Approximately one third of the patients are diagnosed with stage II colon cancer(2). The main treatment of stage II colon cancer is surgical resection of the tumor. The patients are offered adjuvant therapy if they are considered in high risk of relapse. The stratification of high-risk patients is based on histopathological features composed of depth of invasion (T4 tumor), low differentiation, presence of veinor perineural invasion, margin involvement, tumor perforation, and number of sampled lymph nodes (<12 lymph nodes). Despite of proper surgical intervention and stratification of the patients, approximately 20% of the stage II colon cancer patients have relapse of their cancer. Thus, prognostic and predictive markers for stage II colon cancer relapse are highly desired.

One of the hallmarks of cancer is genomic instability and the mismatch repair (MMR) system has gained attention in relation to colon cancer. Germline mutations in central MMR genes, including *MutL homolog 1* (*MLH1*), *Postmeiotic Segregation Increased 2* (*PMS2*), *MutS protein homolog 2* (*MSH2*), and *MutS protein homolog 6* (*MSH6*) are associated with Lynch Syndrome. However, loss of MMR genes is not limited to Lynch Syndrome but is also found in 15% sporadic colorectal cancers, mainly due to *MLH1* promoter hyper-methylations(3). MMR status of sporadic colorectal tumors has been reported to have a prognostic significance(4).

B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) is a component of the polycomb repressive complex 1 which plays a central role in chromatin modification. The protein has been reported to target the *Ink4a*/*Arf* locus which encodes cell cycle regulators exerting key functions in the retinoblastoma protein and p53 signaling pathways(5,6). It has been proposed as a marker of quiescent stem cells in the small intestinal crypt which is a population of stem cells becoming activated upon injury(7,8). Within the last decade the theory of cancer stem cells and their potential role in tumor initiation, progression, recurrence, and therapy resistance has emerged. Several intestinal stem cell markers have been described as having a potential prognostic significance, including BMI1(9). In line with these studies, the polycomb protein has been found to play role in cancer initiation and tumor growth(10). In terms of the prognostic significance of BMI1 expression in colorectal tumors the data is conflicting(11–13). We hypothesized that there may be an association between the BMI1 protein expression in primary tumors of stage II colon cancer patients and their risk of relapse. We addressed the hypothesis by investigating BMI1 expression by immunohistochemistry in tumors from stage II colon cancer patients following the REMARK guidelines (14).

Materials and Methods

Patient cohort

The enrollment, exclusion, and characteristics of patients in this retrospective study cohort have been described in a previously published paper(15). Briefly, the patient cohort included primary tumors from 144 patients diagnosed and treated for primary stage II colon cancer at Glostrup University Hospital, Gentofte University Hospital, and Herlev University Hospital, Denmark. The patients were enrolled consecutively from January 2005 to August 2008 and follow-up ended the 28th of April 2014. The inclusion criteria of the study was stage II colon cancer. Patients who had been diagnosed with other primary cancers prior to or after their primary stage II colon cancer diagnosis was excluded from the study. Likewise, patients under the age of 50 and patients with a history of inflammatory bowel diseases were excluded in an effort to exclude inheritance and chronic inflammation as confounders. Patients presenting multiple or synchronous tumor at diagnosis was excluded. Moreover, patients who relapsed within 3 months or died less than a month after primary surgery was excluded from the study.The MMR status of the primary tumor as well as histopathological risk factors, tumor location, age, and gender was registered as previously described(15). The study was approved by the Scientific Ethics Committee of the Capital Region of Denmark (H-1-2013-028) and by the Data Protection Agency of the Capital Region of Denmark (2007-58-0015).

Tumor tissue and immunohistochemistry

The tumor tissue was processed as part of the diagnostic routine as formerly described(15). 3µm full slides were incubated for 45 min. at 60°C. The staining was performed by the EnVision™ FLEX, High pH detection system (Dako, Glostrup, Denmark) using the automated Autostainer Link 48 (Dako, Glostrup, Denmark) according to manufacturer's instructions. Both a monoclonal BMI1 antibody (Mouse, clone F6, cat. no. 05-637, Merck Millipore, Darmstadt, Germany) and polyclonal BMI1 antibody (Rabbit, HT-99, cat no. sc-10745, Santa Cruz Biotechnology, Heidelberg, Germany) were tested for detection of BMI1 protein expression. The BMI1 antibody from Millipore (diluted 1+200 with EnVision Flex+ Linker (Dako, Glostrup, Denmark)) was selected as the most optimal antibody and therefore used further in the study. Mayers hematoxylin was used for counterstaining by the automated slide stainer Tissue-Tek®Prisma®/Film® (Sakura, Alphen aan den Rijn, Netherlands). For each run a control tissue slide consisting of normal tissue from colon, small intestine, testis, ventricle, and breast was included. The stability of the epitope was tested by staining normal colon tissue which had been subjected to 10% neutral buffered formalin for 3, 27, 51, and 123 hours, respectively.

The BMI1 protein expression was evaluated both at the invasive front and at the luminal surface independently by a specialized pathologist and a trained molecular biologist. While the invasive front was defined as the area where the tumor periphery invades deepest into the tissue, the luminal surface was considered the luminal surface of the neoplastic glands.

Five random areas at the invasive front and the luminal surface of the tumors were selected using the image analysis software Visiopharm Integrator System (version 4.5.6.516, Visiopharm, Hoersholm, Denmark). The immunohistochemical staining reaction was scored as previously described(15) evaluating both percent positive tumor cells and intensity for a final overall score by multiplying the intensity score with the percent score. Tumors with overall score 0 was rerun for confirmation. The positive stromal cells and lymphocytes were used as an internal control for the staining for each tissue slide. In cases of inter-observer disagreement a consensus score was generated by evaluating the slides once more and the pathologist made the final decision. All analysis was conducted blinded to patient outcome.

Statistics

Due to a low number of some of the histopathological risk factors, the patients were grouped as having a risk factor if either of the following histological risk factors were present: T4 tumor grade, low differentiated histology (unless the tumor was dMMR), tumor perforation, vein infiltration, nerve infiltration, or less than 12 lymph nodes sampled at primary resection.

In all statistical analysis, BMI1 was analyzed as a continuous variable. Correlations between clinicopathological variables and BMI1 expression were investigated at the invasive front and at the luminal surface. Spearman rank correlation was used to investigate the association between age and the BMI1 expression level. Associations between BMI1 expression levels and categorical variables were explored by rank test for location (Mann-Whitney U and Kruskal-Wallis). The median, range, and interquartile range (Tukey's Hinge) was presented to improve the overview of potential differences in the clinicopathological subgroups and the BMI1 expression.

Time to relapse was the primary endpoint and was analyzed by univariate Cox proportional hazards models containing the BMI1 expression at the invasive front or at the luminal surface as continuous variable. Time to relapse was defined as time from surgical resection of the primary colon tumor to local relapse or distant metastasis. Patients who died during follow-up were censored. The secondary endpoint was 5-year overall survival which was investigated by univariate analysis as well. 5-year overall survival was defined as time from surgery to death of any cause. The hazard ratio is presented with a difference of three in BMI1 units. The clinicopathological variables were not tested in the models since this has been published in a previous study(15). The assumptions for the Cox proportional hazards model were assessed using cumulative sums of martingale residuals.

The statistical analysis was performed using SPSS Statistics 22 (IBM, Armonk, N.Y., USA) and SAS (version 9.3, SAS Institute, Cary, N.C., USA). p-values of ≤ 0.05 were considered significant.

Results

Patient characteristics

The basic patient characteristics of the cohort and the MMR status has been previously described(15). Table 1 provides an overview of patient characteristics of the study. The invasive front of the primary tumors was evaluated in all of the 144 stage II colon cancers. However, the luminal surface of the tumors was only accessible for evaluation from 141 of the stage II colon cancers.

BMI1 expression

We initially tested two antibodies targeting the BMI1 protein. The monoclonal BMI1 antibody (Mouse, clone F6, cat. no. 05-637, Millipore) was superior to the polyclonal BMI1 antibody (Rabbit, HT-99, cat no. sc-10745, Santa Cruz Biotechnology) in terms of specificity. Thus, the former was used for all subsequent analysis. Additionally, differences in fixation time did not affect the BMI1 protein staining using the selected antibody.

High expression of BMI1 was observed in the nuclei of epithelial cells at the bottom of the colon crypts with a decreasing expression towards the lumen and with no expression at the luminal surface (Figure 1). The endothelial cells, smooth muscle cells, and perineural cells also expressed nuclear BMI1. Additionally, a number of lymphocytes and stromal cells such as fibroblasts and/or myofibroblasts were positive for BMI1. An example of the expression of BMI1 in normal colon tissue is presented in Figure 1.

The expression of BMI1 in stage II colon cancer tissues was heterogeneous at both intratumoral and intertumoral levels. The number of BMI1 positive cells and the intensity of the staining varied widely in the tumors. Within the individual tumor the BMI1 expression could vary from highly positive at the lumen and very low expression at the invasive front or vice versa. Examples of high and low expression of BMI1 are presented in Figure 1.

The prognostic value of BMI1

BMI1 expression at the invasive front correlated significantly with MMR status and age (Table 1). However, the correlation between dMMR and BMI1 was weak. Moreover, the r-value of the Spearman rank correlation was quite low indicating a very weak correlation between BMI1 expression and age of the patients. At the luminal surface BMI1 correlated significantly with tumor location, T-stage, and the histological subtype of the tumors (Table 1). This correlation was not significant at the invasive front. There were no significant correlations between gender, the histological risk factor variable, or the remaining histological risk factors and BMI1 expression at neither the invasive front nor the luminal surface (Table 1).

Univariate Cox proportional hazards analysis showed no significant association between risk of relapse and BMI1 expression at the invasive front or at the luminal surface of the tumors (Table 2). Likewise, there was no significant association between 5-year overall survival and the BMI1 expression at two sites in the tumors (Table 2).

Discussion

Within the last decade stem cells and their role in cancer has been focus for much attention. Meanwhile several potential intestinal stem cell markers has been reported and investigated in clinical prognostic settings(9). One of the potential stem cell markers of the intestine is BMI1. We set to investigate the prognostic value of the expression of BMI1 in primary tumors from a comprehensive cohort of patients diagnosed with stage II colon cancer.

Since no current national or international guidelines are present for BMI1 protein expression analysis we sought to score BMI1 in what was the most informative manner in our opinion, by evaluating both the invasive front and the luminal surface. We have previously reported that dMMR was associated with an low risk of relapse(15). In the present study we found that the BMI1 expression at the invasive front correlated with MMR status, however the correlation was weak, suggesting that the correlation is of less importance. We also found that the BMI1 expression at the luminal surface correlated with T-stage, tumor location, and histological subtype of the tumor. None of the other studies investigating BMI1 as a prognostic marker has found correlations between BMI1 and the histological subtype or tumor location(11–13,16). However, none of the other studies have investigated both the luminal surface and the invasive front of the individual tumors. A study found a correlation between BMI1 expression and T-stage investigating the BMI1 expression by tissue microarray(12). The study differs from ours by the use of tissue microarray. This could be an explanation to the discrepancies in results, as the tissue microarray provides a minor reflection of the tumor. Moreover, none of the other published studies have included MMR status as a variable. Conclusively, the correlation between BMI1 and the specific clinicopathological features is contradicting.

The primary objective of our study was to investigate if the protein could predict relapse of the stage II colon cancer and as a secondary endpoint investigate if it was associated with overall survival of the patients. We found that the BMI1 was not associated with neither of the prognostic endpoints, suggesting that BMI1 is not feasible as a prognostic marker for stage II colon cancer patients. To our knowledge this is the first study investigating the prognostic value of BMI1 expression in only stage II colon cancer patients. Other studies have included all colon cancer stages(11–13), therefore it cannot be excluded that BMI1 might only be relevant in less or more advanced stages than stage II. Therefore, our study should optimally be verified in another cohort before a final consensus of the prognostic value of BMI1 can be presented.

The BMI1 expression was analyzed as a continuous variable in the study since we had no valid cut point for high, moderate, and low BMI1 expression. Thus, we did not find a rational argument for grouping the expression of BMI1 in certain subgroups based on the retrieved data. Other studies have dichotomized the expression of BMI1 into high and low expression or positive and negative staining which might be a contributing cause to the discrepancies seen in the studies in between and compared to our study. Another contributing cause could be the different antibodies used in the studies and the staining protocols applied(12,13). We tested two antibodies to ensure the most optimal staining of BMI1 and found that one of the antibodies was superior with respect to specificity compared to the other antibody.

Optimally, a biomarker identifying high risk patients should also provide information on the benefit of adjuvant therapy. Unfortunately, we did not have data on adjuvant therapy. It would have been interesting to further explore whether the patients included in the study had benefitted from adjuvant therapy. A limitation to our study is that patients were excluded from the cohort if they had had another primary cancer diagnosis prior to or after the primary stage II colon cancer diagnosis. This constitutes a selection bias of the patient cohort, posing another explanation of why our results might differ from previous studies. Insufficient reporting of patient materials and methods including patient inclusion and exclusion criteria, antibody specifications, and statistical considerations of the different cohorts further complicates the comparison across studies.

The understanding of BMI1 as a biomarker appear to be complicated with our study showing no association to overall survival or relapse; another study showing an association between positive BMI1 expression of primary colon tumors and lower overall survival of the patients(12); and a third study reporting that high BMI1 expression in colon tumors is associated with a longer survival than patients with low BMI1 expression(13). Since BMI1 acts in a complex with other polycomb proteins the latter authors constructed a variable consisting of several polycomb proteins, including BMI1 and observed that the best survival and longest recurrence free period was found when all of these polycomb proteins combined were highly expressed in the tumor samples compared to using them as singular biomarkers(13). This indicates that BMI1 might not be optimal as a singular biomarker but may have a prognostic significance in combination with other markers. Unfortunately, in the present study it was not possible to also investigate the remaining polycomb proteins. We have previously shown that the transcription factor sex-determining region y-box 9 (SOX9) can predict relapse of stage II colon cancer patients. Additional studies are necessary to confirm the prognostic value of SOX9 and to explore whether other biomarkers together with SOX9 could improve stratification of high-risk stage II colon cancer patients(15).

In conclusion, we could not demonstrate that BMI1 expression in primary tumors of stage II colon cancer patients predicts relapse of cancer nor have a significant effect on overall survival of the patients. Further studies are needed to find optimal biomarkers for prediction of relapse to improve the personalized treatment of stage II colon cancer patients.

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Table 1. Patient baseline characteristics

	No. of Patients	BMI1 Invasive Front (n=144)			BMI1 Luminal Surface (n=141)		
		Median (range)	Inter-quartile range	*p*-value	Median (range)	Inter-quartile range	*p*-value
Total No. of Patients	144						
Age, years				0.01 (R=-0.21)[a]			0.08 (R=-0.15)[a]
Median (Range)	73 (50-90)						
Gender				0.25[b]			0.44[b]
Female	74 (51.4%)	8 (0-12)	4-8		8 (0-12)	8-8	
Male	70 (48.6%)	8 (0-12)	8-8		8 (0-12)	8-12	
Tumor Location				0.13[b]			<0.01[b]
Right	73 (50.7%)	8 (0-12)	4-8		8 (0-12)	7-8	
Left	71 (49.3%)	8 (2-12)	8-8		8 (0-12)	8-12	
Histological Risk Factors				0.53[b]			0.16[b]
Yes	70 (48.6%)	8 (0-12)	8-8		8 (0-12)	8-8	
No	74 (51.4%)	8 (2-12)	4-8		8 (1-12)	8-12	
T-stage				0.45[b]			<0.01[b]
T3	123 (85.4%)	8 (0-12)	6-8		8 (0-12)	8-12	
T4	21 (14.6%)	8 (2-8)	8-8		8 (2-12)	4-8	
Histological subtype				0.51[c]			0.02[c]
High differentiation	112 (77.8%)	8 (0-12)	8-8		8 (0-12)	8-12	
Low differentiation	15 (10.4%)	8 (3-12)	5.5-8		8 (3-12)	8-10	
Mucinous	17 (11.8%)	8 (0-12)	4-8		8 (0-12)	5-8	
Vein infiltration				0.41[b]			0.76[b]
Yes	29 (20.1%)	8 (0-12)	4-8		8 (0-12)	8-10	
No	115 (79.9%)	8 (0-12)	8-8		8 (0-12)	8-12	
Nerve infiltration				0.41[b]			0.68[b]
Yes	13 (9.0%)	8 (3-12)	8-8		8 (0-12)	8-8	
No	131 (91.0%)	8 (0-12)	4-8		8 (0-12)	8-12	
Lymph nodes sampled				0.30[b]			0.27[b]
<12	27 (18.8)	8 (0-12)	6-10		8 (0-12)	8-12	
≥12	117 (81.3)	8 (0-12)	8-8		8 (0-12)	8-8	
Tumor perforation				0.33[b]			0.85[b]
Yes	2 (1.4%)	5.5 (3-8)	3-8		8 (8-8)		
No	142 (98.6%)	8 (0-12)	8-8		8 (0-12)	8-12	
MMR status				0.01[b]			0.30[b]
pMMR	111 (77.1%)	8 (0-12)	8-8		8 (0-12)	8-12	
dMMR	33 (22.9%)	8 (0-12)	3-8		8 (2-12)	8-8	

[a]Spearman rank correlation; [b]Mann-Whitney U test; [c]Kruskal-Wallis test. The "Histopathological risk factor" variable is based on the presence of one or more of the risk factors in italics. Left sided tumors include tumors of the left flexur, descendens, or sigmoideum. Right sided tumors include tumors of the cecum, ascendens, right flexur, or transversum. Abbreviations: dMMR, Mismatch repair deficient; MMR, Mismatch Repair; n, number of patients analyzed; pMMR, Mismatch repair proficient; BMI1 Invasive Front, BMI1 expression at the invasive front of the tumor; BMI1 Luminal Surface, BMI1expression at the luminal surface of the neoplastic glands.

Table 2. Univariate Cox proportional hazards models containing relapse and 5-year overall survival as endpoints.

		Univariate Analysis Endpoint: Relapse		Univariate Analysis Endpoint: 5-Year Overall Survival	
Variable	**n**	**Hazard Ratio (95% CI)**	***p*-value**	**Hazard Ratio (95% CI)**	***p*-value**
BMI1 Expression					
Invasive front	144	1.12 (0.78-1.60)	0.53[a]	1.12 (0.80-1.56)[a]	0.46[a]
Luminal surface	141	1.06 (0.75-1.48)	0.70[a]	1.16 (0.86-1.60)[a]	0.33[a]

[a]BMI1 as a continuous score. The hazard ratio is presented with a difference of 3 in BMI1 units. Abbreviations: CI, Confidence interval; Invasive Front, BMI1 expression at the invasive front of the tumor; Luminal Surface, BMI1expression at the luminal surface of the neoplastic glands; n, number of patients analyzed.

Figure 1. Immunohistochemical staining of BMI1 at the invasive front (x10 magnification). (A) BMI1 expressed in normal colon. (B) Low expression of BMI1 in stage II colon cancer tissue. (C) High expression of BMI1 in stage II colon cancer tissue.