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1   **Title: The Prognostic Value of Polycomb Group Protein BMI1 in Stage II Colon Cancer Patients**

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25   **Conflicts of interests**

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29

30   **Abstract**

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

33   BMI1 protein expression was assessed by immunohistochemistry in a retrospective patient cohort consisting
34   of 144 stage II colon cancer patients. BMI1 expression at the invasive front of the primary tumors correlated
35   with mismatch repair status of the tumors. Furthermore, BMI1 expression at the luminal surface correlated
36   with T-stage, tumor location, and the histological subtypes of the tumors. In a univariate Cox proportional
37   hazard analysis no statistical significant association between risk of relapse and BMI1 protein expression at
38   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;
39   95% CI 0.75-1.48; $p=0.70$) was found. Likewise, there was no association between 5-year overall survival
40   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
41   the tumor (HR: 1.16; 95% CI 0.86-1.60; $p=0.33$).

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

44

45   **Keywords**

46   BMI1, Biomarkers, Colon Cancer, Recurrence

47   **Abbreviations**

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

52   **Introduction**

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

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

69   B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) is a component of the polycomb
70   repressive complex 1 which plays a central role in chromatin modification. The protein has been reported to
71   target the *Ink4a/Arf* locus which encodes cell cycle regulators exerting key functions in the retinoblastoma
72   protein and p53 signaling pathways(5,6). It has been proposed as a marker of quiescent stem cells in the
73   small intestinal crypt which is a population of stem cells becoming activated upon injury(7,8). Within the last
74   decade the theory of cancer stem cells and their potential role in tumor initiation, progression, recurrence,
75   and therapy resistance has emerged. Several intestinal stem cell markers have been described as having a
76   potential prognostic significance, including BMI1(9). In line with these studies, the polycomb protein has
77   been found to play role in cancer initiation and tumor growth(10). In terms of the prognostic significance of
78   BMI1 expression in colorectal tumors the data is conflicting(11–13). We hypothesized that there may be an
79   association between the BMI1 protein expression in primary tumors of stage II colon cancer patients and
80   their risk of relapse. We addressed the hypothesis by investigating BMI1 expression by
81   immunohistochemistry in tumors from stage II colon cancer patients following the REMARK guidelines
82   (14).

83

84

85   **Materials and Methods**

86   *Patient cohort*

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

101   *Tumor tissue and immunohistochemistry*

102   The tumor tissue was processed as part of the diagnostic routine as formerly described(15). 3µm full slides
103   were incubated for 45 min. at 60°C. The staining was performed by the EnVision™ FLEX, High pH
104   detection system (Dako, Glostrup, Denmark) using the automated Autostainer Link 48 (Dako, Glostrup,
105   Denmark) according to manufacturer's instructions. Both a monoclonal BMI1 antibody (Mouse, clone F6,
106   cat. no. 05-637, Merck Millipore, Darmstadt, Germany) and polyclonal BMI1 antibody (Rabbit, HT-99, cat
107   no. sc-10745, Santa Cruz Biotechnology, Heidelberg, Germany) were tested for detection of BMI1 protein
108   expression. The BMI1 antibody from Millipore (diluted 1+200 with EnVision Flex+ Linker (Dako, Glostrup,
109   Denmark)) was selected as the most optimal antibody and therefore used further in the study. Mayers
110   hematoxylin was used for counterstaining by the automated slide stainer Tissue-Tek®Prisma®/Film® (Sakura,
111   Alphen aan den Rijn, Netherlands). For each run a control tissue slide consisting of normal tissue from
112   colon, small intestine, testis, ventricle, and breast was included. The stability of the epitope was tested by
113   staining normal colon tissue which had been subjected to 10% neutral buffered formalin for 3, 27, 51, and
114   123 hours, respectively.

115   The BMI1 protein expression was evaluated both at the invasive front and at the luminal surface
116   independently by a specialized pathologist and a trained molecular biologist. While the invasive front was
117   defined as the area where the tumor periphery invades deepest into the tissue, the luminal surface was
118   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

152 The basic patient characteristics of the cohort and the MMR status has been previously described(15). Table
153 1 provides an overview of patient characteristics of the study. The invasive front of the primary tumors was
154 evaluated in all of the 144 stage II colon cancers. However, the luminal surface of the tumors was only
155 accessible for evaluation from 141 of the stage II colon cancers.

156 *BMI1 expression*

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

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

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

172 *The prognostic value of BMI1*

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

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

184   **Discussion**

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

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

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

212   The BMI1 expression was analyzed as a continuous variable in the study since we had no valid cut point for
213   high, moderate, and low BMI1 expression. Thus, we did not find a rational argument for grouping the
214   expression of BMI1 in certain subgroups based on the retrieved data. Other studies have dichotomized the
215   expression of BMI1 into high and low expression or positive and negative staining which might be a
216   contributing cause to the discrepancies seen in the studies in between and compared to our study. Another

217 contributing cause could be the different antibodies used in the studies and the staining protocols
218 applied(12,13). We tested two antibodies to ensure the most optimal staining of BMI1 and found that one of
219 the antibodies was superior with respect to specificity compared to the other antibody.

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

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

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

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251

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288

289

290 **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	
<i>T-stage</i>				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	
<i>Histological subtype</i>				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	
<i>Vein infiltration</i>				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	
<i>Nerve infiltration</i>				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	
<i>Lymph nodes sampled</i>				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	
<i>Tumor perforation</i>				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	

291 ^aSpearman rank correlation; ^bMann-Whitney U test; ^cKruskal-Wallis test. The “Histopathological risk factor” variable is based on the presence of one
292 or more of the risk factors in italics. Left sided tumors include tumors of the left flexur, descendens, or sigmoideum. Right sided tumors include
293 tumors of the cecum, ascendens, right flexur, or transversum. Abbreviations: dMMR, Mismatch repair deficient; MMR, Mismatch Repair; n, number
294 of patients analyzed; pMMR, Mismatch repair proficient; BMI1 Invasive Front, BMI1 expression at the invasive front of the tumor; BMI1 Luminal
295 Surface, BMI1 expression at the luminal surface of the neoplastic glands.

297 **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)	<i>p</i> -value	Hazard Ratio (95% CI)	<i>p</i> -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

298 ^aBMI1 as a continuous score. The hazard ratio is presented with a difference of 3 in BMI1 units. Abbreviations: CI, Confidence interval; Invasive
299 Front, BMI1 expression at the invasive front of the tumor; Luminal Surface, BMI1 expression at the luminal surface of the neoplastic glands; n,
300 number of patients analyzed.

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