

## Expression of DNA Double-Strand Break Repair Proteins ATM and BRCA1 Predicts Survival in Colorectal Cancer

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**Abstract** **Purpose:** The double-strand break (DSB) is the major DNA lesion leading to chromosomal aberrations and faithful repair is crucial for maintaining genomic instability. Very little is known about the expression of DNA DSB repair proteins in colorectal cancer. To address this issue, we examined the expression pattern of DSB repair key proteins ATM, BRCA1, BRCA2, Ku70, and Ku80 and their putative role in patients survival in a large series of colorectal cancer. **Experimental Design:** 342 sporadic colorectal cancer were subjected to immunohistochemistry by using specific antibodies for the various proteins investigated. Staining results were compared with clinicopathologic data, patient survival, as well as expression of mismatch repair proteins MLH1 and MSH2. **Results:** The expression pattern of both ATM and BRCA1 predicted survival in all colorectal cancer patients as well as in the small subgroup of patients that received adjuvant therapy. Low expression of ATM and BRCA1 was associated with loss of MLH1 or MSH2 expression. **Conclusions:** This is the first study to show a relationship between the expression of DNA DSB repair proteins ATM and BRCA1 and survival in colorectal cancer patients. Studies in tumors from large randomized trials are now necessary to validate our pilot data and establish the clinical usefulness of the immunohistochemical assay in predicting response to a particular adjuvant therapy regimen. Furthermore, our results indicate a possible link between expression of DNA mismatch repair and DNA DSB repair proteins in sporadic colorectal cancer, which warrants further investigation.

Genetic instability has been proposed to be a driving force in colorectal carcinogenesis (1). Two major types of genetic instability have been described in colorectal cancer: chromosomal instability and microsatellite instability (2). Up to 15% of colorectal cancer show a high frequency of microsatellite instability which is caused by a defect of the DNA mismatch repair pathway (3). The vast majority of colorectal cancer, however, shows chromosomal instability, which is characterized by gains and losses of whole chromosomes or large chromosomal regions.

Whereas a large number of genes that trigger chromosomal instability have been identified in yeast in the past (reviewed in

ref. 4), the underlying mechanisms leading to chromosomal instability in colorectal cancer remain to be characterized. The DNA double-strand break (DSB) is regarded as the most critical of all DNA lesions (5, 6) and it has been shown that defects in the cellular response to DSBs can lead to genetic alteration, chromosomal instability, and ultimately malignant transformation (7).

In mammalian cells, DSBs can be repaired by two distinct pathways: homologous recombination and nonhomologous end joining (8). DSB repair by nonhomologous end joining involves the formation of a Ku70/Ku80 protein heterodimer and recruitment of DNA-dependent protein kinase C to the site of DNA damage (9). DSB repair by homologous recombination is mediated by a large number of different proteins including BRCA1 and BRCA2 (10). The ATM kinase seems to be the primary activator and master controller of the cellular response to DNA DSBs and phosphorylates key players of the DNA damage response network initiating cell cycle arrest, apoptosis, and DNA repair (11). The high frequency of chromosomal aberrations in cell mutants underlines the importance of DNA DSB repair for the maintenance of genomic integrity (for review, see refs. 12–15). Furthermore, it has been shown that mice deficient for either BRCA1 or BRCA2 develop a wide range of carcinomas, most commonly breast and lung but also endometrial, gastric, and colon cancer (12).

Several studies in the past reported an increased risk of colorectal cancer in patients with BRCA1 or BRCA2 germ-line mutations (16–19) whereas two more recent investigations could not confirm such an elevated risk (20, 21). Loss of heterozygosity at the BRCA1 gene locus was shown to be

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associated with shorter survival in colorectal cancer (22). Frequent allelic imbalances at the *ATM* locus have been reported in colorectal cancer (23–25). Methylation of the *ATM* promoter and concurrent reduced *ATM* protein expression were found in a small series of colorectal adenomas and carcinomas, suggesting that *ATM* may have a role in the early stage of colorectal cancer development (26). Another study suggested a constitutive activation of the *ATM*-*Chk2* pathway in colorectal adenomas and speculated that activation of this pathway might limit progression of adenomas towards carcinomas (27).

It is still controversial whether the overall expression of *Ku70* and *Ku80* is reduced or increased in colorectal cancer (28, 29). However, the expression pattern of *Ku70* and *Ku80* was associated with tumor radiosensitivity, tumor stage, and survival in rectal cancer, indicating that *Ku70*/*Ku80* immunohistochemistry may be a useful variable to select patients for chemotherapy (30).

Overall, the currently available literature on DNA DSB repair and colorectal cancer is still very sparse and controversial; however, these findings seem to argue for a role of the DNA DSB repair network in colorectal carcinogenesis and led us to hypothesize that loss of expression of key proteins of one or both DNA DSB repair pathways may be related to advanced tumor stage and poorer patient survival in colorectal cancer.

Thus, we analyzed the expression of DNA repair proteins *ATM*, *BRCA1*, *BRCA2*, *Ku70*, and *Ku80* by immunohistochemistry in a series of 342 colorectal cancer and explored their association with clinicopathologic data, patient survival, expression of mismatch repair proteins *MLH1* and *MSH2*, and tumor cell proliferation.

## Materials and Methods

**Patients.** The present study was done in accordance with local ethics regulations. This series included 342 tumors from 330 consecutive patients with colorectal adenocarcinomas, all of whom underwent curative surgery at the Department of Surgery of the Marien-Hospital in Duesseldorf, Germany, between January 1990 and December 1995.

Eight patients had two synchronous tumors and two patients had three synchronous tumors at different sites of the large bowel. After surgery, 14 patients received chemotherapy, 17 patients received radiotherapy, and 7 patients both. The median age of the patients was 69.72 years (range, 28.52–88.30 years); 149 patients were male (45%) and 181 patients were female. Seventy five (21.9%) tumors were located in the cecum or ascending colon, 35 (10.2%) at the right flexure, transverse colon, or left flexure, 109 (31.9%) in the descending or sigmoid colon, and 123 tumors (36%) in the rectosigmoid or rectum.

Histopathologic data such as depth of invasion (pT), lymph node involvement (pN) according to the tumor-node-metastasis classification (31), tumor differentiation (grading) according to the WHO classification (32), and blood and lymphatic vessel invasion were available for all tumors. As tissue for histopathologic diagnosis of distant metastasis (pM category) was received in a few cases only, this variable was excluded from further analysis.

Data about overall survival and disease-free survival were available from 330 patients. Patients who died within 30 days after surgery were excluded from this study. The median follow up time was 4 years (range, 5 months–11.4 years). Two hundred twenty-two (69%) patients were alive at the end of the study period whereas 107 (34%) patients had survived for >5 years (median survival, 7 years; range, 5.05–11.38 years). Local recurrent disease was observed in 25 patients, distant metastasis in 49 patients, and both in 8 patients. Patients with recurrent disease had a median survival of 2.3 years (range, 0.46–11.38 years).

**Immunohistochemistry.** Full tissue sections of 342 paraffin-embedded colorectal cancer were processed for immunohistochemical staining of *Ku70*, *Ku80*, and *Ki67*. *BRCA1*, *BRCA2*, *ATM*, *MLH1*, and *MSH2* immunohistochemistry was done on tissue microarrays constructed from the same series as described by Simon et al. (33), sampling at random a minimum of three cores of 0.6-mm diameter from each tumor. Antigen retrieval, blocking procedures, and the modified ImmunoMax method used have previously been described (34). For further details of the immunohistochemical procedures, see Table 1. Negative controls were done by using antibody diluent instead of primary antibody.

**Assessment of immunohistochemical staining pattern.** Colorectal cancer cases that could not be assessed due to complete loss of tissue, absence of tumor cells in the tissue microarray core, or other technical difficulties were recorded as "noninformative." For *Ku70*, *Ku80*, *Ki67*, *MLH1*, *MSH2*, *ATM*, and *BRCA2*, a positive tumor cell was defined as immunoreactivity present in the cell regardless of its staining intensity. As a very striking difference between the staining intensity of tumor cells

**Table 1.** Primary antibodies and detection methods

Antibody (clone)	Dilution	Incubation temperature and duration	Antigen retrieval buffer*	Immunohistochemical detection method
<i>ATM</i> (ATX08)	1:25	4°C/20 h	CB	ImmunoMax <sup>†</sup>
<i>BRCA1</i> (SG11)	1:60	37°C/1 h	CB	ChemMate <sup>‡</sup>
<i>BRCA2</i> <sup>§</sup>	1:500	4°C/20 h	CB	ImmunoMax <sup>†</sup>
<i>Ku70</i> <sup>§</sup>	1:1,000	37°C/0.5 h	CB	NBA-kit <sup>  </sup>
<i>Ku80</i> <sup>§</sup>	1:3,000	37°C/0.5 h	CB	NBA-kit <sup>  </sup>
<i>MSH2</i> (FE11)	1:50	37°C/1 h	AUS	EnVision <sup>†</sup>
<i>MLH1</i> (G168-728)	1:70	4°C/20 h	AUS	EnVision <sup>†</sup>
<i>Ki67</i> (MIB 1)	1:500	37°C/1 h	TEC	ChemMate <sup>‡</sup>

NOTE: Antibodies were supplied from Lab Vision Corp, Fremont, CA (*ATM* and *BRCA2*); Zymed Laboratories, San Francisco, CA (*BRCA1*); Oncogene Research Products, San Diego, CA (*p53* and *MSH2*); BD Biosciences, San Jose, CA (*MLH1*); Dianova GmbH, Hamburg, Germany (*Ki67*); and DPC Biemann, Bad Nauheim, Germany (*Ku70* and *Ku80*).

\*CB: 10 mmol/L citrate buffer, pH 6. AUS: ready-made antigen unmasking solution from Vector. TEC: 0.25 g Tris base, 0.5 g EDTA, 0.32 g citrate, pH 7.8.

†For details of the ImmunoMax method, see ref. 34.

‡DakoCytomation Ltd., Ely, Cambridgeshire, United Kingdom.

§Rabbit polyclonal antibody.

||Zymed Laboratories.

**Table 2.** DNA DSB repair protein expression pattern in CRC percentage of positive tumor cells\*

	<5%	5-25%	26-50%	51-75%	>75%	Total
BRCA1	42 (13)	64 (21)	40 (12)	73 (23)	99 (31)	322
BRCA2	27 (10)	66 (23)	91 (32)	72 (26)	26 (9)	282
ATM	71 (22)	181 (57)	35 (11)	23 (7)	10 (3)	320
Ku70	0 (0)	0 (0)	0 (0)	0 (0)	342 (100)	342
Ku80	0 (0)	0 (0)	0 (0)	0 (0)	342 (100)	342

NOTE: Values in table expressed as n (%).

and that of nonneoplastic cells was noted for BRCA1, we defined BRCA1 positivity as tumor cells having immunoreactivity of a similar or stronger intensity compared with nonneoplastic cells in the same tissue core.

For MLH1 and MSH2, colorectal cancer cases were categorized as being either positive or negative. For all other antibodies, the percentage of positively stained tumor cells was determined and primarily recorded as a continuous variable by two independent observers both blinded to any histopathologic or clinical variables. A case was categorized as "negative" for a certain antibody when immunoreactivity was present in <5% of tumor cells. Tumor cell proliferation was analyzed using the Ki67 immunoreactivity as previously described (35).

**Statistical analysis.** Statistical analysis was done using the statistical software package SPSS 11.0 (SPSS, Inc., Chicago, IL). All analyses were done for the whole data set as well as stratified by clinical stage. Comparisons of the percentage of positive tumor cells for different groups were done using the Mann-Whitney test (for two groups) or the Kruskal-Wallis test (for more than two groups). Spearman's rank correlation and Wilcoxon signed-rank test were used to determine the associations between expression levels of the different proteins. Analyses of overall and disease-free survival were done using the Kaplan-Meier method (36) and differences between groups were tested by log-rank test. Multivariate analysis was done using the Cox proportional hazards model (37). *P* < 0.05 was considered statistically significant.

**Results**

**Expression of DNA DSB repair proteins in normal colon mucosa.** Immunoreactivity of BRCA1, ATM, Ku70, and Ku80 was localized in the nucleus whereas BRCA2 immunoreactivity showed a predominant apically located granular cytoplasmic staining pattern. Immunoreactivity of all antigens was observed in all colorectal cancer. Whereas Ku70 and Ku80 immunoreactivity was present in all epithelial cells throughout the crypt, BRCA1, BRCA2, and ATM immunoreactivity was predominantly present in cells located within the lower two thirds of the colonic crypts, a staining pattern identical to that seen for MSH2 and MLH1.

**Expression of DNA DSB repair proteins in colorectal cancer.** Immunohistochemical staining for BRCA1, BRCA2, and ATM was informative for 322, 282, and 320 colorectal cancer, respectively. Ku70 and Ku80 immunoreactivity could be assessed in all 342 colorectal cancer. The subcellular localization of the immunoreactivity was similar to that observed in normal colon epithelium for all antigens.

Two hundred eighty colorectal cancer (87%) were classified as BRCA1 positive (median percent of positive tumor cells, 62.9; range, 5-95%); 255 colorectal cancer (90.4%) as BRCA2 positive (median percent of positive tumor cells, 41.7; range, 5-95%); 249 colorectal cancer (77.8%) as ATM positive (median percent

of positive tumor cells, 15.0; range, 5-92.5%); and 342 colorectal cancer (100%) as Ku70 and Ku80 positive with >90% of positively stained nuclei in all colorectal cancer (Table 2).

**Expression of mismatch repair proteins MLH1 and MSH2 in colorectal cancer.** Staining for MLH1 and MSH2 was nuclear and informative in 330 colorectal cancer. Forty colorectal cancer (12.1%) were classified as MLH1 negative and 11 colorectal cancer (3.3%) as MSH2 negative.

**Expression of proliferation marker Ki67 in colorectal cancer.** Immunohistochemical staining for Ki67 was nuclear and positive in all colorectal cancer (median percent of positive tumor cells, 48.2; range, 7.1-96.2%).

**Association of DNA DSB protein expression with each other, with expression of Ki67, and with mismatch repair proteins MLH1 and MSH2.** ATM expression correlated positively with expression of BRCA1 (*P* < 0.001) and was lower than expression of BRCA1 (*P* < 0.001), BRCA2 (*P* < 0.001), and Ki67 (*P* < 0.001). BRCA1 expression correlated positively with expression of BRCA2 (*P* = 0.003), was higher than expression of BRCA2 (*P* < 0.001), but not different from Ki67 expression (*P* = 0.976). BRCA2 expression was lower than Ki67 expression (*P* < 0.001). No association was found between expression of Ku70 or Ku80 and any of the other proteins.

**Table 3.** BRCA1 expression and relationship with clinicopathologic variables in CRC

	n	BRCA1 expression (%)		P
		Negative (<5%)	Positive (>5%)	
<b>pT category</b>				
pT <sub>1</sub>	24	0 (0)	24 (100)	0.13
pT <sub>2</sub>	61	6 (10)	55 (90)	
pT <sub>3</sub>	212	31 (15)	181 (85)	
pT <sub>4</sub>	25	5 (20)	20 (80)	
<b>pN category</b>				
pN <sub>0</sub>	211	26 (12)	185 (88)	0.75
pN <sub>1</sub>	69	10 (15)	59 (85)	
pN <sub>2</sub>	42	7 (17)	35 (83)	
<b>Stage</b>				
Stage I	72	6 (8)	66 (92)	0.6
Stage II	137	20 (15)	117 (85)	
Stage III/IV*	113	16 (14)	97 (86)	
<b>Grading</b>				
G1 <sup>†</sup> /G2	245	30 (12)	215 (88)	0.49
G3/G4 <sup>‡</sup>	77	12 (16)	65 (84)	
<b>Blood vessel invasion</b>				
Negative	296	39 (13)	257 (87)	0.92
Intramural	11	1 (9)	10 (91)	
Extramural	15	2 (13)	13 (87)	
<b>Lymphatic vessel invasion</b>				
Negative	233	33 (14)	200 (86)	0.33
Positive	89	9 (10)	80 (90)	
<b>Proliferative activity (Ki67)</b>				
Low	160	19 (12)	141 (88)	0.54
High	162	23 (14)	139 (86)	

\*Stage IV only six tumors.

†G1 only four tumors.

‡G4 only two tumors.

**Table 4.** BRCA2 expression and relationship with clinicopathologic variables in CRC

	BRCA2 expression (%)			P
	n	Negative (<5%)	Positive (≥5%)	
<b>pT category</b>				
pT <sub>1</sub>	20	1 (5)	19 (95)	0.62
pT <sub>2</sub>	53	7 (13)	46 (87)	
pT <sub>3</sub>	189	18 (10)	171 (90)	
pT <sub>4</sub>	20	1 (5)	19 (95)	
<b>pN category</b>				
pN <sub>0</sub>	188	15 (8)	173 (92)	0.25
pN <sub>1</sub>	59	9 (15)	50 (85)	
pN <sub>2</sub>	35	3 (9)	32 (91)	
<b>Stage</b>				
Stage I	62	6 (10)	56 (90)	0.29
Stage II	124	8 (6)	116 (94)	
Stage III/IV*	96	13 (14)	83 (86)	
<b>Grading</b>				
G1 <sup>†</sup> /G2	210	15 (7)	195 (93)	0.018
G3/G4 <sup>‡</sup>	72	12 (17)	60 (83)	
<b>Blood vessel invasion</b>				
Negative	259	24 (9)	235 (91)	0.15
Intramural	10	0 (0)	10 (100)	
Extramural	13	3 (23)	10 (77)	
<b>Lymphatic vessel invasion</b>				
Negative	201	17 (8)	184 (92)	0.32
Positive	81	10 (12)	71 (88)	
<b>Proliferative activity (Ki67)</b>				
Low	141	13 (9)	128 (91)	0.84
High	141	14 (10)	127 (90)	

\*Stage IV only six tumors.

†G1 only four tumors.

‡G4 only two tumors.

In comparison with MLH1-negative cases, MLH1-positive colorectal cancer showed higher expression of ATM ( $P = 0.024$ ) and BRCA1 ( $P = 0.016$ ) but lower expression of Ki67 ( $P = 0.014$ ). ATM and BRCA1 expression was also higher in MSH2-positive colorectal cancer ( $P = 0.006$  and  $P < 0.001$ , respectively) compared with MSH2-negative cases. No association was observed between BRCA2, Ku70, or Ku80 expression and mismatch repair protein expression.

**Association of DNA DSB protein expression with clinicopathologic variables.** High BRCA2 expression was associated with low grade of tumor differentiation ( $P = 0.018$ ). No statistical associations were found between Ku70, Ku80, ATM, BRCA1, and BRCA2 and pT, pN, blood and lymphatic vessel invasion, or tumor stage (Tables 3–5).

**Association of DNA DSB protein expression with patient survival.** All patients with BRCA1- or ATM-positive colorectal cancer were found to have a significantly longer overall survival [ $P = 0.0103$  (Fig. 1A) and  $P = 0.0097$  (Fig. 1B), respectively]. Patients with BRCA1-positive colorectal cancer receiving adjuvant therapy had a significantly longer overall and disease-free survival ( $P = 0.0049$  and  $P = 0.0262$ , respectively; Fig. 1C and D). Patients with ATM-positive colorectal cancer

receiving adjuvant therapy had a significantly longer disease-free survival ( $P = 0.0046$ ; Fig. 2).

Adjusting the multivariate survival analysis model for the known prognostic marker pT, pN, and extramural BVI, BRCA1, and ATM expression proved to be an independent prognostic marker of overall survival (Table 6) but not of disease-free survival (data not shown).

No associations were found between patient survival and BRCA2, Ku70, or Ku80 expression.

## Discussion

Our study shows that the expression of Ku70 and Ku80, key proteins of the nonhomologous end joining pathway of DNA DSB repair, is very similar in colorectal cancer and normal colon mucosa and no relationship of Ku70 and Ku80 expression to any of the clinicopathologic variables including patient survival could be established. Our findings are in contrast to results from a Japanese series of rectal cancer (30) which showed a survival benefit of high Ku70 expression. High expression of Ku70 and Ku80 in colorectal cancer, as shown in our study, could be compensatory due to altered expression of proteins involved in the homologous recombination pathway

**Table 5.** ATM expression and relationship with histopathologic variables in CRC

	ATM expression (%)			P
	n	Negative (<5%)	Positive (≥5%)	
<b>pT category</b>				
pT <sub>1</sub>	24	5 (21)	19 (79)	0.95
pT <sub>2</sub>	61	13 (21)	48 (79)	
pT <sub>3</sub>	208	48 (23)	160 (77)	
pT <sub>4</sub>	27	5 (30)	22 (70)	
<b>pN category</b>				
pN <sub>0</sub>	209	48 (23)	161 (77)	0.75
pN <sub>1</sub>	69	13 (19)	56 (81)	
pN <sub>2</sub>	42	10 (24)	32 (76)	
<b>Stage</b>				
Stage I	73	17 (23)	56 (77)	0.94
Stage II	134	31 (23)	103 (77)	
Stage III/IV*	113	23 (20)	90 (80)	
<b>Grading</b>				
G1 <sup>†</sup> /G2	242	57 (24)	185 (76)	0.3
G3/G4 <sup>‡</sup>	78	14 (18)	64 (82)	
<b>Blood vessel invasion</b>				
Negative	294	66 (22)	228 (78)	0.53
Intramural	11	1 (9)	10 (91)	
Extramural	15	4 (27)	11 (73)	
<b>Lymphatic vessel invasion</b>				
Negative	231	53 (23)	178 (77)	0.6
Positive	89	18 (20)	71 (80)	
<b>Proliferative activity (Ki67)</b>				
Low	159	36 (23)	123 (77)	0.85
High	161	35 (22)	126 (78)	

\*Stage IV only six tumors.

†G1 only four tumors.

‡G4 only two tumors.

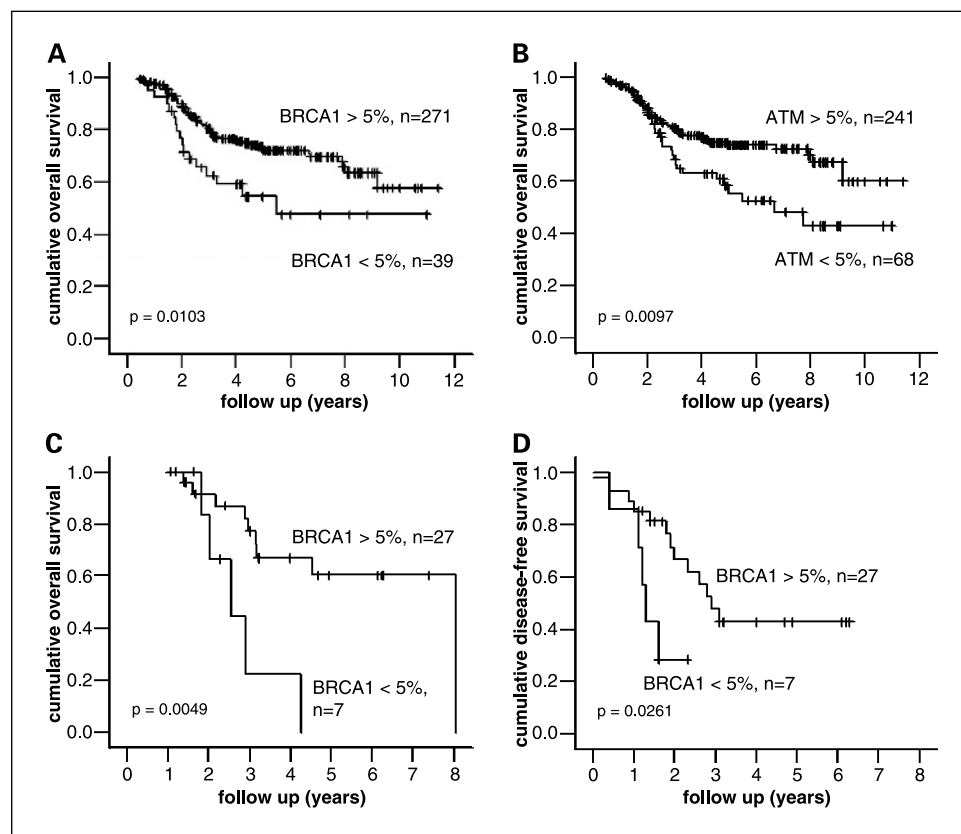


Fig. 1. Overall survival of colorectal cancer patients with BRCA1-positive tumors versus those with BRCA1-negative tumors (A) and of colorectal cancer patients with ATM-positive tumors versus those with ATM-negative tumors (B). C and D, overall survival and disease-free survival of colorectal cancer patients with BRCA1-positive tumors who received adjuvant therapy.

of DNA DSB repair as an interplay between these two repair pathways has been shown (38).

Our investigation provides evidence that the expression of ATM, the key regulator of the biological response to DNA damage, as well as the expression of BRCA1 and BRCA2, proteins involved in the homologous recombination pathway of DNA DSB repair, is markedly reduced in sporadic colorectal cancer. Similar findings have been reported in two much smaller series of colorectal cancer (ref. 39,  $n = 38$ ; ref. 26,  $n = 47$ ) whereas Garcia et al. (ref. 40,  $n = 8$ ) and Romagnolo et al. (ref. 41,  $n = 5$ ) reported a higher expression of BRCA1 in colorectal cancer.

However, no statistical association could be established between reduced expression of ATM, BRCA1, or BRCA2 and tumor stage [depth of invasion (pT) and lymph node involvement (pN)], suggesting that reduced expression of these proteins may be an early event in colorectal carcinogenesis. This concept is supported by a study which reported reduced expression of ATM in colorectal adenomas (26).

Our data show a relationship between loss of MLH1 and MSH2 expression and reduced expression of ATM and BRCA1, suggesting that microsatellite instability at the respective gene locus may be one of the underlying mechanisms of reduced expression. Inactivating mutations in mononucleotide repeats of the coding region of ATM and BRCA1 (42) as well as ATM deletions (43) have been described in colorectal cancer with microsatellite instability. The relationship between loss of MLH1 and MSH2 expression and DNA DSB repair proteins could also indicate that the expression of DNA DSB repair proteins may not only be impaired in colorectal cancer with chromosomal instability, as originally hypothesized, but may also be a characteristic feature of colorectal cancer with microsatellite

instability. However, apart from using MLH1 and MSH2 expression as surrogate markers of microsatellite instability, we did not investigate microsatellite instability in our series. Nonetheless, identification of colorectal cancer with concomitant microsatellite instability and impaired expression of DNA DSB repair proteins may be of clinical importance as it has been shown that microsatellite instability-positive colorectal cancer cell lines with impaired DNA DSB repair activity are more sensitive to DNA DSB-producing chemotherapeutic agents than microsatellite instability-negative colorectal cancer (44).

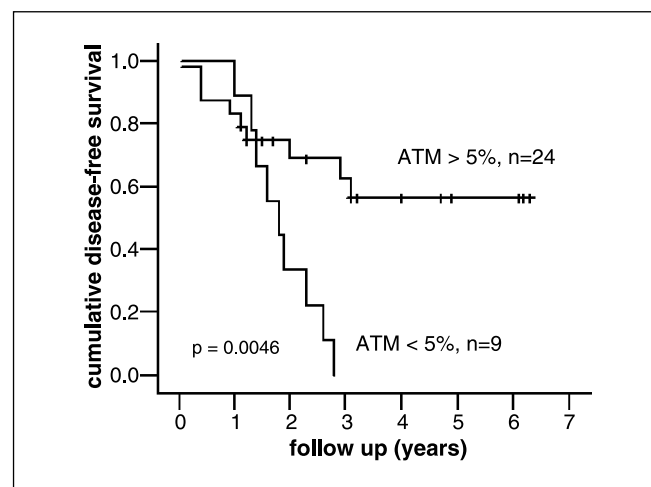


Fig. 2. Disease-free survival of colorectal cancer patients with ATM-positive tumors who received adjuvant therapy.

**Table 6.** Multivariate analysis of BRCA1 and ATM expression adjusting the Cox proportional hazards model for the known prognostic factors depth of invasion (pT category), lymph node involvement (pN category), and blood vessel invasion

Covariables	Hazard ratio (95% confidence interval)	P
pT category	1.822 (1.200-2.769)	0.005
pN category	1.828 (1.397-2.391)	<0.001
Blood vessel invasion	2.155 (1.036-4.484)	0.040
BRCA1 expression	0.565 (0.330-0.965)	0.037

Covariables	Hazard ratio (95% confidence interval)	P
pT category	1.836 (1.227-2.749)	0.003
pN category	1.808 (1.376-2.375)	<0.001
Blood vessel invasion	2.100 (1.011-4.360)	0.047
ATM expression	0.596 (0.379-0.937)	0.025

Further studies in colorectal cancer are necessary (a) to establish whether there is a functional link between the expression level of DNA DSB repair proteins and activity of the DSB repair pathway; (b) to show that DNA mismatch repair and DNA DSB repair are indeed interconnected pathways as hypothesized by Wang et al. (45); and (c) to investigate the possible regulatory mechanism of ATM and BRCA1 expression in colorectal cancer.

Our study showed that the Ki67 index, which was used to determine the percentage of proliferating cells per case, was significantly higher than the percentage of ATM-, BRCA1-, or BRCA2-positive cells per case. Thus, the possibility that the tumor cells simply have a reduced expression of these proteins

because the cells are not dividing seems to be unlikely. Furthermore, there was no correlation between Ki67 index and expression level of ATM, BRCA1, and BRCA2, suggesting that the protein expression level is not related to high or low proliferative activity.

Arguably, the most significant among our results is the relationship between loss of expression of ATM or BRCA1 and poorer patient overall survival. Our study is the first to report that the expression of DNA DSB repair proteins ATM and BRCA1 is a prognostic marker in colorectal cancer, which are independent from established prognostic markers such as pT, pN, and extramural blood vessel invasion. These findings would be consistent with the proposition put forward by Bartkova et al. (27) that tumor progression may rely on the selection of cells defective in their DNA damage components such as ATM.

As DNA DSB repair proteins have been suggested to play an important role in the cellular response to chemotherapy (46) as well as to radiotherapy (47), we further analyzed the subgroup of patients with colorectal cancer who received adjuvant chemotherapy, radiotherapy, or both. Interestingly, patients with BRCA1-negative, stage II, or stage III colorectal cancer showed significantly shorter overall and disease-free survival. The same was true for patients with stage III disease and ATM-negative tumors. Although conclusions from our observations are limited due to the small number of patients who received adjuvant therapy ( $n = 38$ ), the differences in both overall and disease-free survival are striking and suggest that ATM and BRCA1 protein expression levels may influence response to adjuvant therapy. It is now necessary to validate our findings in colorectal cancer from large randomized clinical trials to assess whether a simple immunohistochemical assay of ATM and BRCA1 expression done in routinely paraffin embedded tissue is able to predict patient's response to a particular adjuvant therapy and may help to select optimal therapy for individual patients in the near future.

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