Quantitative Analysis of BRCA1, BRCA2 and Hmsh2 mRNA Expression in Colorectal Lieberkühnien Adenocarcinomas and Matched Normal Mucosa: Relationship with Cellular Proliferation

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Abstract. The human DNA mismatch repair gene hMSH2 is involved in the development of sporadic and hereditary nonpolyposis colorectal cancer. An increased risk of colorectal cancer has also been suggested in BRCA1 and BRCA2 mutation carriers. To address the relationship between the expression level of these genes and colorectal tumorigenesis, we studied BRCA1, BRCA2 and hMSH2 mRNA expression by real-time quantitative RT-PCR in 72 colorectal Lieberkühnien adenocarcinomas and matched normal mucosa. We investigated the relationship between mRNA levels and various clinicopathological parameters. The mean expression of BRCA1 3’ and BRCA2 3’ (mRNA pool), BRCA1 ex11 (with exon 11), BRCA2 ex12 (with exon 12) and hMSH2 mRNAs were increased in tumor samples. BRCA1 and BRCA2 mRNAs expressions were altered according to colon tumor site: BRCA1 3’ and BRCA2 3’ mRNAs levels were highest, respectively, in the right colon and left colon. No difference in hMSH2 mRNA levels was detected in relation to clinicopathological parameters. The mean SPF value was significantly higher in tumor than in non-tumor colonic tissue, and a high SPF value was correlated with high BRCA2 mRNA levels. BRCA2 3’ mRNA levels tended to decrease as the Dukes’ stage increased. In conclusion, the mechanisms of colorectal carcinogenesis seem to differ according to the right or left position of the tumor.

Colorectal cancer is an important public health issue because about 5% of persons will develop this disease. Sporadic colorectal cancer accounts for approximately 70% of all cancer. There are several molecular pathways to colorectal cancer, particularly the pathway involving abnormalities of DNA mismatch repair (MMR). Germ-line mutations in MMR genes have been reported in hereditary nonpolyposis colorectal cancer (HNPCC) (1, 2). Susceptibility to HNPCC is caused by mutations in one of the MMR genes (hMSH2, hMLH1, hPMS1, hPMS2, hMSH6). The majority of alterations have been found in hMSH2 and hMLH1, which account for 45-65% of HNPCCs (3, 4). Mutations in other MMR genes appear to be rare, absent and/or associated with atypical families (1-5%). Mutations of hMSH2 and hMLH1 genes are found in about 15% of sporadic colorectal carcinomas and are associated with microsatellite instability (5, 6). However, most sporadic colorectal carcinomas seem to be related to epigenetic rather than genetic changes (7). On the other hand, an increased risk of colorectal cancer has been found in BRCA1 and BRCA2 mutation carriers (8, 9). Germ-line mutations in the breast susceptibility genes BRCA1 and BRCA2 confer a high risk for developing breast and ovarian cancer (10, 11). Women who develop two of these cancers may be expected to have a constitutionally increased risk of developing subsequent cancers to which mutations in these genes predispose. Cancers for which an increased relative risk has been found in BRCA1 and BRCA2 mutation carriers include colon, pancreatic, gall-bladder cancers and melanoma (12, 13). BRCA1 and BRCA2 regulate multiple nuclear processes including DNA repair, cell cycle checkpoints and transcription (14). These proteins have been shown to interact with DNA repair proteins. Wang et al. demonstrated an interaction between BRCA1 and hMSH2 in vitro and in vivo (15). Moreover, BRCA1 and
BRCA2 are part of a large multisubunit protein complex of tumor suppressors, DNA damage sensors and signal transducers. This complex, named BASC for BRCA1-Associated Genome Surveillance Complex, is composed of DNA repair proteins: hMSH2, ATM, BLM, hMSH6, hMLH1, RAD51, the RAD50-MRE11-NBS1 complex and the RFC1-RFC2-RFC4 complex. BASC may serve as a sensor of abnormal DNA structures and/or as a regulator of post-replication repair (16). The BRCA1 and BRCA2 splicing variants may have possible cellular roles (35), which could explain why, despite its vital cellular functions, BRCA2 3' (mRNA pool), BRCA1 3' (transcripts containing exon 11) and BRCA2 3' (mRNA pool), BRCA1 ex11 (transcripts containing exon 11) and BRCA2 ex12 (transcripts containing exon 12) mRNAs. These mRNA levels were compared to various clinicopathological parameters in order to clarify their roles in colorectal carcinogenesis.

**Materials and Methods**

**Patients and samples.** Seventy-two pairs of histologically verified sporadic colorectal cancer (with no familial or inherited predisposition) and adjacent normal colorectal mucosa were obtained at the time of surgery and collected as frozen samples in Centre Paul Papin, Angers (France). None of the patients received radiotherapy or chemotherapy before surgery. There were 38 men and 34 women, with a mean age of 69.8 years (range 32 to 90 years). All samples were staged according to Dukes' classification modified by Gunderson and Sosin (17). All tumors were Lieberkühnien adenocarcinomas and ranged from poorly- to well-differentiated. The clinical and histological characteristics of the patients are reported in Table I.

**Flow cytometric (FCM) DNA-ploidy analysis and S-phase (SPF) measurement.** For each patient, one tumor fragment was compared with a matched non-tumor colonic tissue. DNA analyses were performed in the same experiment by preparing a mechanical dissociation of the thawed samples. Vindelov’s protocol was used to stain nuclear DNA (18). FCM analysis was performed with a FACSscan flow cytometer (Becton Dickinson, CA, USA) equipped with Modfit 5.2 software (Verity Software House, Topsham, ME, USA). Every cell suspension was analyzed in the same conditions in order to locate the DNA-diploid peak on DNA histograms according to an external standardization procedure using normal colonic cells positioned at the fifth part of the red fluorescence scale.

A tumor sample was DNA-diploid (D) when it provided a unimodal histogram with a G0G1 peak in the same channel as normal cells (+/-1%) (Figure 1-A). A sample was classified as aneuploid when a second peak exceeded 5% of total cells, and tetraploid when this proportion exceeded 10% with a corresponding DNA index between 1.9 and 2.1 (Figure 1-B). For some near-diploid cases, the tumor cell suspension was mixed with non-tumor cells before staining in order to ensure the position of the diploid peak. Finally, tumors were characterized as multiploid with different aneuploid populations (Figure 1-C).

The S-phase fraction was calculated when the background was lower than 20% of total acquired events and the coefficient of variation of the G0G1 peak was lower than 5%. When a unimodal histogram was obtained, the diploid option of the software was used. In case of abnormal DNA content, only the aneuploid SPF measurement was taken into account, provided that the aneuploid fraction exceeded 30% of cells. In every case, the rectangular option was always used.

**Real-time RT-PCR.** Colorectal tumors and normal mucosa were ground thoroughly with a french press and the powder was dispersed in a crucible with the appropriate amount of RT-™ buffer (β-mercaptoethanol and GITC (guanidinium isothiocyanate buffer), from the RNAsample kit (Amersham Pharmacia Biotech, Uppsala, Sweden). For BRCA1,
Figure 1. DNA histograms obtained in three different colonic carcinomas.

Left column: DNA histograms obtained in normal colonic tissues
Right column: DNA histograms obtained in tumor samples
DNA index are shown above each G0G1 peak.
**BRCA2** and **hMSH2** mRNA analysis, probes and primers (Table II) were designed using the Primer Express software (Applied Biosystems, ZA Courtabœuf, Villebon/Yvette, France) so that they overlapped splice junctions (exons 23-24 for **BRCA1** ex 3’, probes, exons 11-12 for **BRCA1** ex 11 probe, exons 26-27 for **BRCA2** 3’ probe, exons 12-13 for **BRCA2** ex 12 probe, exons 9-10 for **hMSH2** probe), to distinguish PCR products generated from genomic versus cDNA templates. The sequence of forward primers, TaqMan® probes and reverse primers were, respectively, for **BRCA1** ex 3’: 5’-5566CAGAGGACAACTTCACATGAGGCACCTG5586-3’, 5’-5588AATTGGGCGAGTGGTGGAGGCACCTG5561-3’, 5’-5566CAGACCCACAGATGCTGCTTCA5586-3’; for **BRCA2** ex 3’: 5’-1479AATAATGAATGATGAATGAAATGCAGAAGC1498-3’, 5’-1498CAGCCAGAGATCTT5423-3’; for **hMSH2** amplification: 5’-9794CCAAGTGGTCCACCCCAAC9812-3’, 5’-9818ACTGTACTTCAGGGCCGTACACTGCTCAAA9847-3’, 5’-9897CAATTAGGAGAAGACATCAGAAGC9870-3’; for **BRCA2** ex 12 amplification: 5’-7120GAATACAAAGAAAATCTTAAACGGCT7143-3’, 5’-7145GAACCTCCAGATTGGCCACAATAAAAGATC7168-3’, 5’-7220GTATTCCGCTTCTAAAGGAAACATGATG7184-3’, 5’-7201CTAATCCTGCCATCTCTCTAACAGTG7195-3’; for **hMSH2** amplification: 5’-1451AATAATGAATGATGAATGAAATGCAGAAGC1470-3’, 5’-1457AGCACTCCAGATGGCACAATAAAAGATC1475-3’, 5’-1477AAAATAATCGAAAGGAACGGGCTTGGAA1498-3’, 5’-1498CAGCCAGAGATCTT5423-3’; for **BRCA2** ex 3’ amplification: 5’-1451AATAATGAATGATGAATGAAATGCAGAAGC1470-3’, 5’-1457AGCACTCCAGATGGCACAATAAAAGATC1475-3’, 5’-1477AAAATAATCGAAAGGAACGGGCTTGGAA1498-3’, 5’-1498CAGCCAGAGATCTT5423-3’; for **BRCA2** ex 12 amplification: 5’-1451AATAATGAATGATGAATGAAATGCAGAAGC1470-3’, 5’-1457AGCACTCCAGATGGCACAATAAAAGATC1475-3’, 5’-1477AAAATAATCGAAAGGAACGGGCTTGGAA1498-3’, 5’-1498CAGCCAGAGATCTT5423-3’; for **BRCA1** ex 11 amplification: 5’-4157AAAGGGGAGGCTGGTCTGAA1475-3’, 5’-4177AAAAATATCTAAAGAGGCAAGGCAAAGCATGGATTCAAACTTA4214-3’, 5’-5646CTACACTGTCCAA9847-3’, 5’-9897CAATTAGGAGAAGACATCAGAAGC9870-3’. All doubly-labelled probes, 18S rRNA probe, were designed using the Primer Express software (Applied Biosystems). Each sample was tested in triplicate.

Flow cytometric DNA analysis

**DNA ploidy.** The DNA content was always normal in healthy colonic mucosa, while 47 tumors (65.3%) were classified as aneuploid (Figure 1-B and 1-C). Among these, the DNA index showed very scattered values (range 0.9-3.1) and 6 tumors were multiploid. The frequency of aneuploidy did not correlate significantly with anatomic site. DNA ploidy did not correlate with Dukes’ stage, the frequency of aneuploidy being 65.9% in Dukes’ B stage and 70.8% in Dukes’ C.

**S-phase fraction (SPF).** Owing to good quality DNA histograms (debris mean 7.7% and CV mean 2.9%), SPF could be calculated in 71 out of 72 tumors. The mean SPF value was significantly higher in tumor than in non-tumor colonic tissue (15.1% and 3.1%, respectively, p<0.005). The diploid SPF mean value was not significantly lower than the aneuploid SPF mean value (13.5% and 16.7%, respectively, p=0.078). SPF did not correlate with tumor site or Dukes’ stage (SPF mean values were 15.9%, 14.9% and 13.9% in Dukes’ B, C and D, respectively).

### Table II. Mean values and 75th percentiles of mRNA gene expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of cases</th>
<th>Mean value</th>
<th>SD</th>
<th>75th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRCA1</strong> ex 3’</td>
<td>72</td>
<td>4.9</td>
<td>4.8</td>
<td>6.3</td>
</tr>
<tr>
<td><strong>BRCA1</strong> ex 11</td>
<td>72</td>
<td>3.5</td>
<td>3.3</td>
<td>5.4</td>
</tr>
<tr>
<td><strong>BRCA2</strong> ex 3’</td>
<td>71</td>
<td>12.0</td>
<td>13.6</td>
<td>14.0</td>
</tr>
<tr>
<td><strong>BRCA2</strong> ex 12</td>
<td>71</td>
<td>9.7</td>
<td>11.1</td>
<td>11.4</td>
</tr>
<tr>
<td><strong>hMSH2</strong></td>
<td>72</td>
<td>3.5</td>
<td>11.4</td>
<td>4.9</td>
</tr>
</tbody>
</table>

The mean value (± SD) corresponds to mRNA expression levels in tumor samples normalized to the normal colonic tissue. Each measurement was performed on two extractions and three RT.

**Statistical analysis.** Mean values of two series were compared using the Student’s t-test and Anova test when more than two series were concerned. Two-sided p values of <0.05 were considered significant.
Quantitative analysis of BRCA1, BRCA2 and hMSH2 mRNA expression. The mean values of mRNA levels for each gene are reported in Table II. The distribution of these values indicated a few tumors with high mRNA expression. For each gene, tumors were dichotomized, using the 75th percentile as a cut-off point for high and low expression levels (Table II). Thus, a quartile of tumors with high mRNA gene expression was discriminated.

Table III. Mean expression (± SD) of mRNA according to tumor site.

<table>
<thead>
<tr>
<th></th>
<th>Right colon</th>
<th>Left colon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20*</td>
<td>52</td>
</tr>
<tr>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
</tr>
<tr>
<td>BRCA1 3'</td>
<td>6.89a</td>
<td>5.38</td>
</tr>
<tr>
<td>BRCA1 ex11</td>
<td>3.61</td>
<td>3.25</td>
</tr>
<tr>
<td>BRCA2 3'</td>
<td>7.01</td>
<td>5.14</td>
</tr>
<tr>
<td>BRCA2 ex12</td>
<td>6.52</td>
<td>5.61</td>
</tr>
<tr>
<td>HMSN2</td>
<td>3.53</td>
<td>2.33</td>
</tr>
</tbody>
</table>

The level of mRNA expression in colorectal tumor was normalized to the non-tumor colonic tissue. Each measurement was performed on two extractions and three RT. Statistical analysis was performed using Student’s t-test.

Correlations with histological characteristics. BRCA1 ex11, BRCA2 ex12 and HMSN2 mRNA expression were not altered according to tumor site. BRCA1 3’ mRNA levels were highest in the right colon (p=0.038) and BRCA2 3’ mRNA levels were highest in the left colon (p=0.005) (Table III). BRCA2 expression showed a discrepancy with Dukes’ stage, with BRCA2 3’ mRNA levels decreasing as the histological stages progressed by Dukes’ classification (Table IV).

Table IV. Mean expression of mRNA BRCA2 3’ according to Dukes’ stage.

<table>
<thead>
<tr>
<th>Dukes’ stage</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA2 3’</td>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td></td>
<td>6.4</td>
<td>15</td>
<td>8.7</td>
<td>3.6</td>
</tr>
<tr>
<td>SDa</td>
<td>1.8</td>
<td>0.028</td>
<td>16.6</td>
<td>6.1</td>
</tr>
<tr>
<td>P (Anova test)</td>
<td></td>
<td></td>
<td>0.0013</td>
<td></td>
</tr>
</tbody>
</table>

The level of mRNA expression in colorectal tumor was normalized to the non-tumor colonic tissue. Each measurement was performed on two extractions and three RT. Statistical analysis was performed using Anova test.

a number of tumors
SD: standard deviation

Gene mRNA expressions are expressed according to 75th percentile (52 cases are classified in the “low” group and 20 cases are classified in the “high” group). Statistical analysis was performed using Student’s t-test.

Correlation with FCM results. BRCA1, BRCA2 and HMSN2 mRNA levels were statistically independent of ploidy. In contrast to the other mRNA, a significant correlation was observed between SPF and BRCA2 3’ mRNA expression (p=0.0013). Moreover, the mean SPF value appeared to be higher in a quartile of tumors exhibiting BRCA2 3’ mRNA levels higher than 14.0 (Table V). This result is more clearly observed in tumors of the left colon, which exhibit the highest mRNA expression values (Table III).

Table V. Mean SPF values according to gene mRNA expression.

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>Mean SPF (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1 3’</td>
<td>&lt; 6.3</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>&gt; 6.3</td>
<td>14.0</td>
</tr>
<tr>
<td>BRCA1 ex11</td>
<td>&lt; 5.4</td>
<td>15.6</td>
</tr>
<tr>
<td></td>
<td>&gt; 5.4</td>
<td>15.4</td>
</tr>
<tr>
<td>BRCA2 3’</td>
<td>&lt; 14.0</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>&gt; 14.0</td>
<td>19.9</td>
</tr>
<tr>
<td>BRCA2 ex12</td>
<td>&lt; 11.4</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>&gt; 11.4</td>
<td>17.1</td>
</tr>
<tr>
<td>HMSN2</td>
<td>&lt; 4.9</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>&gt; 4.9</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Gene mRNA expressions are expressed according to 75th percentile (52 cases are classified in the "low" group and 20 cases are classified in the "high" group). Statistical analysis was performed using Student’s t-test.

a NS: no significant value.
considerable increase in expression is observed when cells enter the cell cycle (22, 23). Marra et al. suggested that increased hMSH2 protein expression is required when DNA replication in S-phase is activated and followed by mitosis. These observations are consistent with our results, where the mean SPF value was significantly higher in tumors than in normal colonic tissues. Thus, the up-regulation of hMSH2 mRNA expression in colorectal adenocarcinomas seems to be induced by the high proliferation and, consequently, the high SPF values in tumors.

Epidemiological studies hint at a BRCA1 and BRCA2 role in hereditary colorectal cancer (12, 13). The BRCA1 and BRCA2 genes account for about 5-10% of breast cancer. BRCA1 and BRCA2 mutations appear to be responsible for up to 80% of hereditary breast cancer (10, 11), but not for sporadic breast cancer where BRCA1 and BRCA2 expression is down-regulated mainly by epigenetic changes (24-26). We found an up-regulation of BRCA1 and BRCA2 mRNA levels in sporadic colorectal tumor compared with normal colonic tissue. BRCA1 and BRCA2 mRNA expression is reported to be up-regulated in vitro in certain malignant human ovarian, prostate and breast cancer cell lines (27) and in vivo in no BRCA1-associated sporadic breast cancer (28, 29). Moreover, BRCA1 and BRCA2 expression increases during late G1- and S-phase of the cell cycle (30, 31). Our present observations, that the mean BRCA1 and BRCA2 mRNA levels and SPF values were significantly higher in colorectal adenocarcinomas than matched normal mucosas, seem to be consistent with this report. One possible explanation for these results is that BRCA1 and BRCA2 mRNA expression is induced by proliferation, as demonstrated in previous studies (32, 33). The BRCA1 and BRCA2 proteins are believed to exert growth-inhibitory action. Thus, an increase of their mRNA level in sporadic colorectal cancer may represent a negative feedback mechanism for curbing proliferation in fast-growing cells.

Among the clinicopathological parameters, BRCA1 and BRCA2 mRNA expressions differed with tumor site. Thus, BRCA1 3’ mRNA level was higher in the right colon and BRCA2 3’ mRNA level was higher in the left colon. Previously, the same relationship was established with p53 expression, which showed more p53 expression in distal than proximal neoplasms (34). These data support the hypothesis that mechanisms of colorectal carcinogenesis may differ in the right and left colon. On the other hand, only BRCA2 expression varied with SPF and Dukes’ stage, as BRCA2 3’ mRNA level tended to decrease as the histological stage increased in Dukes’ classification (Table V). Our results indicate that the cell cycle of tumor cells is disturbed through the reduction in BRCA2 3’ mRNA expression, leading to uncontrolled cell proliferation. The tumor cells may acquire more rapid growth activity and more malignant potential as the BRCA2 3’ mRNA expression level decreases.

The number of known BRCA1 and BRCA2 mRNA variants is relatively high, with remarkably different expression patterns (10, 35-37). Although their possible functions are still unclear, the splicing variants may have possible cellular roles (38). Splicing variants of these genes could explain the current paradox that, despite its vital cellular functions, mutations of this gene are associated with tissue-specific tumor formation in the breast and the ovary. The variants that would be expected to differ greatest at the functional level from the full-length species are those lacking exon 11 (A11BRCA1) for BRCA1 and exon 12 (A12BRCA2) for BRCA2, as a result of their remarkable size difference and the absence of many functional domains involved in protein-protein interactions (36, 37, 39). In order to define a possible implication of splice variants in colorectal cancer, we quantified BRCA1 3’ and BRCA2 3’ (mRNA pool), BRCA1 ex11 (transcripts with exon 11) and BRCA2 ex12 (transcripts with exon 12). All the mean values of BRCA1 and BRCA2 mRNA expression were increased in colorectal tumor in comparison with normal tissue. Moreover, the contribution of BRCA1 and BRCA2 splice variants, in particular A11BRCA1 and A12BRCA2 mRNA, appears weak in the up-regulation of BRCA1 and BRCA2 mRNA levels. These results suggest than BRCA1 and BRCA2 mRNA variants seem not to be implicated in the carcinogenesis of colorectal Lieberkühnien adenocarcinomas.

In conclusion, pathologically decreased hMSH2, BRCA1 and BRCA2 mRNA levels do not seem to account for all tumors in this population-based series of colorectal Lieberkühnien adenocarcinomas. On the contrary, our data suggest that these mRNA are up-regulated in response to proliferation. Moreover, mechanisms of colorectal carcinogenesis seem to differ according to tumor site and only BRCA2 mRNA seems to be up-regulated with proliferation and genomic instability in high Dukes’ stage of tumors. In order to better understand colorectal tumorogenesis, the genetic mechanisms inducing alterations of hMSH2, BRCA1 and BRCA2 mRNA expression need to be studied.

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