Abstract. Background: Microsatellite instability (MSI) and expression of cell cycle-related markers may predict a favorable outcome in colorectal cancer patients. The aim of this study was to elucidate the molecular profiles of patients with rectal cancers treated with neoadjuvant chemotherapy (5-Fluorouracil and CPT-11), radiotherapy and surgery that correlate with response to therapy. Patients and Methods: Fifty-seven patients with rectal cancer were treated with the same preoperative chemotherapy regimen, radiotherapy (45 to 54 Gy) followed by surgery. The microsatellite status, the expression of the mismatch repair proteins MLH1 and MSH2 and p21WAF1/C1PI, p27, bcl-2, topoisomerase II (topo II) and Ki-67 were assessed in the pretreatment biopsies. The response to adjuvant therapy was categorized in the resected specimens as complete response (CR, no microscopic residual tumor present) and partial response (PR, tumor present). Results: p21WAF1/C1PI expression characterized the CR with 12 out of 30 tumors (40%) positive for this marker. None of the patients whose tumors did not express p21WAF1/C1PI (10 patients) was a CR (p=0.011). Overall, the tumors with CR also showed higher expression of bcl-2, topo II and p27. However, p53 was more frequently expressed in the PR tumors. Tumors with high microsatellite instability showed CR (3/5, 60%) more often than PR, whereas tumors with stable microsatellites showed PR (26/36, 80%) more often than CR (p=0.099). Conclusion: We conclude that a molecular profile characterized by high microsatellite instability with loss of mismatch repair protein expression and p21WAF1/C1PI is predictive of an improved response to neoadjuvant treatment with 5-FU, CPT-11 and radiation therapy.

Colorectal cancer is the third most common malignancy in both men and women, and the third most common cause of cancer-related death in the US. However, the incidence and the mortality of these cancers are now decreasing in the US (1). High microsatellite instability (MSI-H) as a marker for the DNA mismatch repair deficient "Mutator Pathway" of carcinogenesis and immunohistochemical expression of cell cycle-related proteins have been found to predict a favorable outcome in colorectal cancer patients (2,3). The mutator pathway of carcinogenesis is a well-recognized pathway of carcinogenesis, particularly in a subtype of colorectal cancers. The underlying problem is a DNA mismatch repair (MMR) deficiency. MLH1 and MSH2 are the MMR proteins that are implicated most frequently. MMR genes are either mutated in the germline, resulting in hereditary non-polyposis colorectal cancer (HNPCC), or MLH1 is inactivated by hypermethylation of the promoter, as a somatic, epigenetic phenomenon. A characteristic feature of tumors with MMR deficiencies is microsatellite instability, as spontaneous errors in DNA replication can no longer be repaired. Both microsatellite analysis and immunohistochemical assessment of MSH2, MLH1, MSH6 and PMS2, the MMR proteins most frequently involved, serve as screening tools for MMR proficiency (4,5). Interestingly, colorectal cancers with MSI-H – whether sporadic or hereditary – differ from microsatellite stable tumors (MSS) in many aspects, which include age, site, prognosis and response to chemotherapy (6,7).

Cell differentiation and proliferation are regulated through cell cycle proteins that allow for proliferation, arrest and repair, as well as apoptosis. The cell cycle
proteins regulate each other and are influenced by external factors, thus ensuring normal proliferation of intact cells and repair or elimination of damaged cells. However, in cancer, these regulatory mechanisms are disrupted or altered. P53 and p21WAF1/CIP1 play an important role in surveillance of external insults (8). Although the role of p53 during normal development is not fully understood, loss of normal p53 function is an important event in the development of colorectal carcinoma.

P21WAF1/CIP1 is induced by wild-type p53 protein. It inhibits the kinase activity of cyclin/CDK complexes, leading to G1 arrest. P27 protein acts as a negative regulator of the cell cycle. In some studies, reduced expression of p27 in colorectal cancer was described as a predictor of poor survival and increased likelihood of lymph node metastases (9,10). Bel-2 is an anti-apoptotic protein that promotes cell survival. Overexpression of bel-2 has an antiproliferative effect and thus prolongs the cell cycle (11). This might explain why, by univariate and multivariate analysis, bel-2-positive colon cancers showed a better survival rate compared to bel-2-negative tumors (12,13). However, other studies have failed to reproduce these results (14).

CPT-11 is a potent topoisomerase I (topo I) inhibitor. Initially, CPT-11 received approval by the Food and Drug Administration (FDA) for use in fluorouracil-refractory stage IV colorectal cancer (15). However, more recent studies indicate an advantage of using CPT-11 alone or in combination as the first line treatment of colon cancer (16).

Topo I is a nuclear enzyme that plays a key role in DNA replication, transcription, and relaxing torsional strains that occur during different cell processes (17). The mechanism through which topo I achieves this critical function is by making breaks at the site of supercoiling of DNA and then resealing single-stranded DNA.

Topo I inhibitors prevent resealing of DNA and eventually lead to cell death through apoptosis. Thus a functional p53-dependent apoptotic pathway (p53 and p21WAF1/CIP1) appears to be required to achieve a good response to CPT-11 (18).

Preliminary in vivo and clinical studies demonstrate a synergistic effect of CPT-11 and radiation and suggest a radiosensitizing activity of CPT-11. It has been suggested that CPT-11 may potentiate the lethal effects of ionizing radiation by binding to the DNA-topo I adducts at DNA single-strand breaks. Subsequently, the stabilized CPT-11-topo I-DNA complexes interact with advancing replication forks during S-phase, converting single-strand breaks into irreversible DNA double-strand breaks, resulting in cell death. Fractionated irradiation synchronizes the tumor cell population, leading the majority of cells into S-phase, thereby sensitizing them to CPT-11 treatment (19).

So far, only a small number of studies have focused on a correlation between cell cycle parameters, the MMR proficiency and response to chemotherapy and radiotherapy. The goal of this study was to characterize a molecular profile of rectal tumors that predicts better response to an adjuvant treatment regimen consisting of CPT-11, 5-Fluorouracil (5-FU) and radiation.

**Patients and Methods**

**Patient population.** Fifty-seven patients, with newly diagnosed rectal cancer, were enrolled in an IRB approved phase I/II clinical trial of 5-FU, CPT-11 and radiation prior to surgical resection, at Thomas Jefferson University Hospital, Philadelphia, PA, USA. Informed consent was obtained at the time of enrolment in the clinical trial. The mean age of the patients was 50 years (30-76 years) and the male/female ratio was 44/13. Based on family history the patients were categorized as follows: 2 Familial Adenomatous Polyposis (FAP), 16 HNPCC according to the Bethesda Criteria (7), 6 familial, not fulfilling the criteria for FAP or HNPCC, 14 sporadic, and 19 without clinical information. The patients were treated preoperatively with 5-FU (300 or 225 mg/m2/day by continuous infusion 5 days per week) and escalating doses of CPT-11 (30 to 60 mg/m2 weekly x 4) and radiation (45-54 Gray). Surgery was performed 6-10 weeks after the preoperative therapy was completed. The response to therapy was classified as complete response (CR, no microscopic residual tumor present in the resection specimen) or as partial response (PR, residual but reduced tumor present in the resection specimen). All patients experienced a reduction of tumor size, Hematoxylin and eosin-stained slides of the original biopsy and the resection specimen were reviewed and microsatellite analysis and a panel of immunostains were performed on the original biopsy in each case.

**Immunohistochemical staining.** Five-μm sections, containing tumor tissue and normal colonic mucosa as internal positive control, were cut onto neoprene-coated slides. After routine deparaffinization
and rehydration, including an endogenous peroxide block with methanol-peroxide for 30 min, the sections were microwaved for nonenzymatic epitope retrieval in 200 ml ChemMate H.I.E.R. Buffer pH5.5-5.7 (Ventana Medical Systems, Tuscon, AZ, USA) at 800W for 5 min. Fifty ml H2O were replenished and an additional microwaving step followed at 800W for 5 min (US patent number 5,244,787). The slides were cooled by keeping them in the buffer for 20 min at room temperature. The immunostains were performed using the avidin-biotin-peroxidase amplification system in an automatic immunostainer (Techmate 1000, Ventana Medical Systems). After blocking in normal serum for 20 min, the slides were incubated with the primary antibodies overnight at room temperature at the concentrations listed in Table I. The biotinylated secondary antibody was applied for 30 min and the 30-min incubation with ABC complex was followed by 3 applications of DAB/0.02% hydrogen peroxide. The slides were washed with PBS between the incubations. A Harris hematoxylin counterstain blued with 3% lithium carbonate was used.

After assessing the internal positive controls, the immunohistochemical stains were evaluated as follows:

- **MSH2 and MLH1**: Any nuclear staining was counted as positive for both MSH2 and MLH1.
- **p53**: Tumors with distinct nuclear staining in > 50% of cells were considered positive.
- **p21WAF1/CIP1**: Tumors were considered positive when ≥ 5% of the nuclei were stained.
- **p27**: Tumors with nuclear staining in ≥50% of the cells were considered positive.
- **Topoisomerase II** (topo II) and **Ki-67**: Tumors with distinct nuclear staining in >70% of cells were considered to have an elevated proliferation rate.
- **Bel-2**: Tumors with cytoplasmic staining in >50% of cells were considered positive.

These cut-off values were selected based on our previous experience and the available literature. The positive control for p53 consisted of colorectal carcinoma sections with known p53 positivity. For MSH2, MLH1, topo II, Ki-67 and bel-2, sections of normal appendix were used as a positive control. The positive controls for p21WAF1/CIP1 and p27 were appendix and placenta. The negative controls consisted of the same tissues as the positive controls incubated with class-matched, non-specific mouse monoclonal antibodies as the primary antibody under the same conditions as the respective primary antibody.

**Microsatellite analysis.** Microdissection was performed on paraffin sections stained briefly with methylene blue. Areas of interest were selected by two surgical pathologists (TBE and JP), scraped off the slide with a scalpel blade and subjected to proteinase K digestion at a final concentration of 2 µg/ml overnight at 55°C (Qiagen, Valencia, CA, USA). DNA was extracted with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s recommendations.

A panel of 6 microsatellite markers, including the five markers recommended by the National Cancer Institute and the International Collaborative Group for HNPCC, was analyzed (20). The panel consists of 3 mononucleotide markers (BAT25, BAT26, BAT40), and 3 dinucleotide markers (D2S123, DSS346, D17S250). The primer sequences have been published elsewhere (5) and the PCR conditions were as follows for any of the markers: 50mM KCl, 10mM Tris-HCl pH 8.3, 2.5mM MgCl2, 0.2mM dNTPs (Roche, Indianapolis, IN, USA), 0.33µM each primer (Perkin Elmer, Norwalk, CT, USA), 0.4uA AmpliTaq Gold Polymerase (Perkin Elmer), 1µl DNA solution. The following cycling steps were carried out on a Perkin Elmer 9600 or 9700 thermal cycler: 95°C 12 min (94°C 15 sec, 55°C 15 sec, 72°C 30 sec) x10, (89°C 15 sec, 55°C 15 sec, 72°C 30 sec) x30, 72°C 30 min. Each forward primer had a fluorescent tag at the 5’ end that could be detected by an ABI automated fluorescence sequencer (FAM, TET, HEX). The primers were obtained from Perkin Elmer (Norwalk, CT, USA). After successful amplification, the PCR products were diluted 10- to 25-fold in H2O, mixed with loading buffer (final concentration: 50% formamide, 0.1x Size Standard TAMRA (Perkin Elmer), 25µg Dextran Blue, 5mM EDTA), denatured, loaded, run and analyzed on ABI 377 sequencers equipped with GeneScan Software (Applied Biosystems, Incorporated, Foster City, CA, USA) according to the manufacturer’s recommendations.

Additional peaks at a microsatellite locus in the tumor DNA compared to the normal DNA from the same patient were interpreted as MSI. Loss of heterozygosity (LOH) was diagnosed if one of the alleles was reduced by ≥40%. Cases with MSI in more than 40% of the loci were interpreted as exhibiting high microsatellite instability (MSI-H); cases that did not show MSI or LOH in any of the 6 markers in the microsatellite panel were called microsatellite stable (MSS). In cases with MSI in less than 40% of the markers or with LOH, 4 additional microsatellite markers (D10S197, D18S58, D18S69 and MYCL1) were analyzed. Instability at less than 40% of microsatellite loci was called low microsatellite instability (MSI-L) and instability at more than 40% of the loci was classified as MSI-H. However, the MSI status did not change from MSI-L to MSI-H in any case after amplifying the additional 4 loci. The presence of LOH was not included as MSI but recorded separately. The criteria for the evaluation of the microsatellite analysis were identical with the one suggested by the National Cancer Institute and the International Collaborative Group-HNPCC (20).

**Statistical methods.** Statistical analysis was performed using the c2 and Fisher’s exact test (Instat 1.14, GraphPad Software).

**Results**

**Cell cycle and proliferation markers.** There was a highly significant correlation between response to radiochemotherapy and the level of p21WAF1/CIP1 expression. More p21WAF1/CIP1-positive tumors (12/30, 40%) were complete responders than p21WAF1/CIP1-negative tumors (0/10, 0%) (p=0.011) (Figure 1-A). The group of bel-2-positive tumors was more likely to be complete responders (3/5, 60%) than bel-2-negative (7/30, 23%) (p=0.12) (Figure 1-C). With regards to p53, more p53-negative cases were complete responders (9/26, 35%) than cases with positive p53 (4/21, 19%) (p=0.19) (Figure 1-B). However, there was no correlation between Ki-67, topo-II, or p27 expression and therapy response (Table II).

**Correlation between response to therapy and MMR status.** Among the 5 patients with MSI-H, 3 (60%) showed complete response, compared to only 7/36 (20%) of cases...
with MSS \((p=0.099)\). However, one of the two MSI-H cases with partial response had a history of ulcerative colitis and the tumor expressed both MMR proteins MLH1 and MSH2 by immunohistochemistry, indicating an intact but insufficiently functioning MMR system. When this patient is removed from the MSI-H group, there is a statistically significant correlation between response to therapy and MMR status \((p=0.04)\) (Figure 2-A). These results are corroborated by the immunohistochemical analysis of MMR proteins where CR is seen in 3/4 (75\%) of all cases with absence of either MSH2 or MLH1, indicating a MMR deficiency. One patient with MSI-H and a loss of MLH1 only had a partial response. On the other hand, CR was seen in only 7/36 (20\%) of cases that expressed both MMR proteins \((p=0.67)\) (Figure 2-B).

In contrast to the complete pathological response, complete clinical response did not correlate with the cell cycle markers nor the MMR status. Among the complete clinical responders, 14/30 tumors were \(p21^{\text{WAF1/CIP1}}\)-positive and 2/11 were \(p21^{\text{WAF1/CIP1}}\)-negative \((p=0.09)\). In the group of patients with complete clinical response 6/20 tumors expressed p53 and 11/26 were p53-negative \((p=0.53)\). Among the 5 patients with MSI-H, 3 showed complete clinical response, compared to 11/35 with MSS and 5/10 with MSI-L \((p=0.319)\).

**Discussion**

The combined regimen CPT-11, 5-FU and radiotherapy is known to have a synergistic effect when treating patients with rectal cancer (16). CPT-11 acts by inhibiting topo I, which relaxes supercoiled DNA during replication by making transient breaks of one strand (21). The CPT-11-topo I complex prevents reannealing of the double-strand, thus leading to fragmentation of the DNA. In an attempt to repair the damage, cells arrest the cell cycle at the G2 checkpoint to allow for DNA repair. This requires a functional p53 and a functional MMR system. If these two conditions are met, the CPT-11-induced damage should be able to be repaired, resulting in a suboptimal treatment response. However, in the background of MMR deficiency, there should be an excellent response to CPT-11 because of the inability to repair the damage induced by CPT-11. Similarly, if p53 is mutated, tumor cells will not be able to maintain the G2 arrest so that, even with a functional MMR system, the cells cannot completely repair the damage before moving to the M-phase. Thus, some response to CPT-11 would be expected, but not necessarily complete remission. Finally, if p53 is mutated and the MMR system is

**Table II. Immunohistochemical markers and response to therapy.**

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<th>Marker</th>
<th>Number of CR/total n (%)</th>
<th>(p)-value</th>
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<tr>
<td>bcl-2 [pos ≥ 50%]</td>
<td>3/5 (60%) 7/30 (23%)</td>
<td>0.12</td>
</tr>
<tr>
<td>p53 [pos ≥ 50%]</td>
<td>4/21 (19%) 9/26 (53%)</td>
<td>0.19</td>
</tr>
<tr>
<td>p21 [pos ≥ 5%]</td>
<td>12/30 (40%) 0/11 (0%)</td>
<td>0.011</td>
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<tr>
<td>TopoIIA [pos ≥ 70%]</td>
<td>7/21 (33%) 5/22 (23%)</td>
<td>0.66</td>
</tr>
<tr>
<td>p27 [pos ≥ 50%]</td>
<td>6/18 (33%) 6/26 (23%)</td>
<td>0.68</td>
</tr>
<tr>
<td>Ki-67 [pos ≥ 70%]</td>
<td>10/29 (35%) 3/16 (19%)</td>
<td>0.44</td>
</tr>
<tr>
<td>MLH1 and MLH2</td>
<td>7/36 (20%) 3/5 (100%)</td>
<td>0.67</td>
</tr>
</tbody>
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With one of the two MSI-H cases with partial response having a history of ulcerative colitis and the tumor expressing both MMR proteins MLH1 and MSH2 by immunohistochemistry, indicating an intact but insufficiently functioning MMR system. When this patient is removed from the MSI-H group, there is a statistically significant correlation between response to therapy and MMR status \((p=0.04)\) (Figure 2-A). These results are corroborated by the immunohistochemical analysis of MMR proteins where CR is seen in 3/4 (75\%) of all cases with absence of either MSH2 or MLH1, indicating a MMR deficiency. One patient with MSI-H and a loss of MLH1 only had a partial response. On the other hand, CR was seen in only 7/36 (20\%) of cases that expressed both MMR proteins \((p=0.67)\) (Figure 2-B).

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deficient, all cells damaged by CPT-11 during the S-phase would carry the damage through the M-phase and end up dying through p53-independent apoptosis that might involve BAX, BcL-XL, and bcl-2 (19). The influence of the p53 and hMLH1 status in the cellular response to CPT-11 on carcinoma cells has recently been demonstrated (18). These authors showed that the percentage of apoptotic cells after treatment with CPT-11 was higher in p53-positive, hMLH1-negative cells than in p53-positive, hMLH1-positive cells, which supports the evidence that an intact hMLH1 molecule may prevent CPT-11-induced apoptosis (18). In advanced colorectal cancers, a better response to CPT-11 is noted in patients that display a MSI-H phenotype and loss of BAX expression (22). Consequently, the status of the cell cycle proteins and the status of the MMR system play a critical function in determining the response to CPT-11.

A recent study showed that patients with colon cancers displaying microsatellite instability had a better five-year survival rate compared to tumor with microsatellite stability or low instability (3). Interestingly, patients with microsatellite

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<td><img src="image1.png" alt="Graph A" /></td>
<td><img src="image2.png" alt="Graph B" /></td>
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**Figure 2. Correlation between mismatch repair status and response to therapy.**

A: MSI-H, MSI-L, and MSS: high microsatellite instability, low microsatellite instability and microsatellite stable.
B: MMR: mismatch repair deficiency.
CR: complete pathological response, PR: partial response.
stable tumors responded better to fluorouracil-based adjuvant chemotherapy and showed overall improved survival (3).

Our study revealed a molecular profile that is predictive of CR for rectal cancers treated adjuvantly with CPT-11, 5-FU and radiation. This profile consists of positive staining for p21\textsuperscript{WAF1/C1PI} and MMR deficiency evidenced by MSI-H and absence of immunostaining for one of the MMR proteins MSH2 or MLH1.

This is the first study observing a correlation of p21\textsuperscript{WAF1/C1PI}-positivity and complete response to this combined therapeutic regimen of chemotherapy and radiation. The majority of tumors with MSI-H that are negative for one of the MMR proteins showed complete response, indicating that the underlying mechanism of molecular carcinogenesis follows the mutator pathway. Our findings confirm previous studies, which indicate that the status of p53 and MMR proteins can have an important effect on the sensitivity and/or response to cytotoxic agents (23). There was a trend towards bcl-2 being more often positive and p53 more often negative in CR. These findings go along with previous publications describing bcl-2-positivity in MMR-deficient tumors, whereas p53 is typically wild-type (negative stain) (11). It has also been described that p21\textsuperscript{WAF1/C1PI} is positive in all colorectal cancers with MSI-H, but only in 45 to 55\% of MSS and MSI-L tumors (11). Even though the small number of cases limits our data so that the IHC for MMR protein expression do not reach statistical significance, several important observations can be made.

One patient with a history of ulcerative colitis developed a rectal cancer that was characterized by MSI-H and the presence of intact MSH2 and MLH1 protein by IHC. This might indicate that, although the MMR system was intact, its function might not have sufficed to keep up with the high cell turnover in the inflammatory bowel disease. Previous studies have found MSI in inflammatory bowel disease without an underlying abnormality of the MMR system (24,25). However, additional replication errors, including microsatellite instability could not be repaired sufficiently.

The other patient with MSI-H who only achieved PR had lost MLH1 expression and the rest of the immunohistochemical panel was typical for MMR-deficient tumors with lack of p53 and p21\textsuperscript{WAF1/C1PI} expression.

Clinical complete response did not correlate with any of the factors analyzed in this study. Following neoadjuvant therapy the vast majority of tumors are no longer detected grossly, although small collections of tumor cells are still present in the wall of the rectum. The fact that pathological response correlates with a subgroup of rectal cancers with distinct molecular features emphasizes the importance of using microscopically assessed pathological response in these patients. Since 5-FU only had a favorable influence on the outcome of MMR-proficient tumors (3), the question arises whether it could be omitted from our neoadjuvant protocol comprising CPT-11, 5-FU and radiation when treating MMR-deficient patients.

This study gives evidence that a subgroup of rectal cancers characterized by MSI-H, the absence of one of the MMR proteins, expression of p21\textsuperscript{WAF1/C1PI} and possibly wild-type p53 protein and presence of bcl-2, responds particularly well to an adjuvant treatment protocol consisting of CPT-11, 5-FU and radiation. On the other hand, MMR, proficient, p21\textsuperscript{WAF1/C1PI}-negative tumors respond only partially, probably because the double-strand breaks caused by the treatment are still repaired to some extent by the DNA repair machinery.

We suggest that testing for these proteins may be performed in rectal cancer biopsies to help tailor the treatment approach that should be given to the different molecular subtypes of rectal cancer in order to optimize the response.

Acknowledgements

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References


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