Abstract. While sporadic cases of colorectal cancer (CRC) most commonly arise via the well-characterized chromosomal instability pathway (CIN), most other cases develop via a serrated neoplasia pathway (CIMP), in which methylation of CpG islands results in silencing of DNA nucleotide mismatch repair (MMR)-related genes, and a high level of microsatellite instability (MSI). MSI-high tumors typically show proximal location, mucinous histology, poor differentiation, and lymphocytic infiltration. Cell-free circulating DNA (CFD) may become elevated in CRC patients compared to healthy individuals. Because of these biological differences, we hypothesized that compared to MMR-proficient tumors MMR-deficient CRCs may produce higher CFD blood levels. Patients and Methods: Forty-one patients with newly-diagnosed CRC from all stages were studied for MMR-proficiency status, and CFD and carcinoembryonic antigen (CEA) blood levels. MMR proficiency was evaluated in formalin-fixed, paraffin-embedded tissues by immunohistochemistry (IHC) for MLH1/MSH2. CFD plasma levels were measured with SYBR gold nucleic acid gel staining on fluorometry. MMR-proficiency status was studied by clinicopathological parameters, CFD and CEA blood levels. Results: Tumors were MMR-proficient, and -deficient in 16 patients (39%), and 25 patients (61%), respectively. The mean age of MMR-deficient patients was approximately 10 years higher than that of MMR-proficient patients (61.2±8.4 years versus 71.9±9.7 years, p=0.07). MMR-deficient tumors were more often proximally-located, (p=0.018). The mean CFD plasma levels in MMR-proficient, and MMR-deficient patients were 795±431 ng/ml, and 906±494 ng/ml, respectively (p=0.68). The mean CEA serum levels in MMR-proficient and MMR-deficient patients were 10.4±17.6 μg/l, and 15±48 μg/l, respectively (p=0.68). Conclusion: Compared to MMR-proficient CRCs, MMR-deficient tumors occurred in older patients, and were more commonly proximally-located. Despite the presence of distinct biological and histopathological characteristics, both tumor types produced similar CFD blood levels.

Colorectal cancer (CRC) is one of the most common malignancies in the western world, also constituting a major cause of cancer-related mortality (1). Molecular profiling has revealed at least two distinct molecular pathways underlying most CRCs. Sporadic cases (approximately 70% of all cases) mainly arise via the well-characterized chromosomal instability pathway (CIN), an allelic imbalance at several chromosomal loci (including 5q, 8p, 17p, and 18q), and chromosome amplification and translocation, which together contribute to tumor aneuploidy (2, 3). Most other remaining cases develop via a serrated neoplasia pathway (CIMP), in which molecular abnormalities consistently involve methylation of CpG islands, activating mutations of the mitogen-activated protein kinase pathways components BRAF or KRAS, and a high level of microsatellite instability (MSI) (4, 5). MSI is a measure of the inability of the DNA nucleotide mismatch repair (MMR) system to correct errors that often occur during DNA replication. It is controlled by several genes, most importantly MLH1 and MSH2 (6). MSI-high CRCs are characterized by proximal location, mucinous histology, poor differentiation, and lymphocytic infiltration (7).

Despite differences in molecular pathways, blood levels of cell-free circulating DNA (CFD) may commonly become elevated in all CRC types (8). Elevated CFD levels have been associated with tumor burden and malignant progression, and were prognostic for CRC patients (9-11). Most CFD in cancer patients originates from dying malignant cells (12, 13). However, increased levels have also been quantified in other patients with benign lesions, inflammatory diseases and...
tissue trauma (8). Since the biology of MMR-deficient CRC differs in many aspects from that of MMR-proficient tumors, we anticipated that an impaired DNA-correcting system in MMR-deficient CRCs may result in higher CFD blood levels compared to MMR-proficient tumors.

**Patients and Methods**

Following approval of the Soroka Medical Center (SMC) Institutional Review Board, we studied 41, newly-diagnosed patients with CRC of all stages who were treated at the SMC from 2010-2012. Thirty-eight of these patients had been included in a previous study, reported by Czeiger et al. (10). CFD and carcinoembryonic antigen (CEA) blood levels were determined one week before surgery. CFD plasma levels were measured according to a method previously described by Goldshtein et al. (14). In short, SYBR gold nucleic acid gel stain (Invitrogen™, Carlsbad, USA) was diluted first at 1:1,000 in dimethyl sulfoxide (Sigma-Aldrich, Rehovot, Israel) and then at 1:8 in PBS. Ten microliters of serum samples or DNA standard solutions were applied in duplicate to black 96-well plates (Greiner Bio-One, Frickenhausen, Germany); 40 μL of diluted SYBR gold was added to each well (final dilution, 1:10,000), and fluorescence was measured with a 96-well fluorometer (Spectrafluor Plus, Tecan, Durham, NC) at an emission wavelength of 535 nm and an excitation wavelength of 485 nm. CEA serum levels were measured using the Architect CEA assay (Abbott Laboratories, Abbott Park, IL). Mismatch-repair proficiency in formalin-fixed, paraffin-embedded tissues was studied by immunohistochemistry (IHC) for MLH1/MSH2, according to a method described by Lanza et al. (15). One block of tumor tissue, including adjacent normal mucosa, was selected per case. For staining MLH1, an anti-MLH1 antibody (clone G1680728; PharMingen, San Diego, CA, USA) was used according to a method described by Machin et al. (16). For Staining MSH2, an anti-MSH2 antibody (clone G168-728; Oncogene Research Products, Cambridge, MA, USA) was used according to a method described by Thibodeau et al. (17). Staining was performed on a Ventana automated IHC slide staining system (Ventana XT-machine, Ventana/Roche®, Strasbourg, France) following the manufacturer’s instructions. 3, 3’-DAB was used as a chromogen, and hematoxylin was used as counterstain. Table I summarizes antibodies, suppliers, dilutions and techniques used for IHC of MSH1 and MLH2. Evaluation of IHC staining results was performed on light microscopy by a pathologist (NTV) blinded to previously obtained clinicopathological features. Carcinomas with normal expression of MLH1, and MSH2 gene products (presence of nuclear immunostaining in a large proportion of neoplastic cells) were classified as MLH1-positive and MSH2-positive, respectively. Only nuclear staining was scored. The staining with each antibody was evaluated without knowing the results obtained by the other antibody. Nuclear immunostaining of normal epithelial cells, lymphocytes, and stromal cells served as an internal positive control in each case. Tumors that positively-stained for both antibodies were classified as MMR-proficient (MMR+ve). Tumors that negatively-stained for at least one antibody were classified as MMR-deficient (MMR-ve).

**Table I. Antigodies and technical details employed for immunohistochemical evaluation of mismatch repair proteins, MSH1 and MLH2.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone/Ab</th>
<th>Source</th>
<th>Dilution</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-MLH1 Ab</td>
<td>G168-15 PharMingen, San Diego, CA, USA</td>
<td>Machin P et al. (16)</td>
<td>1:10</td>
<td>Ventana BenchMark XT automated slide preparation system</td>
</tr>
<tr>
<td>Anti-MSH2 Ab</td>
<td>FE11, Oncogene Research Products, Cambridge, MA, USA;</td>
<td>Thibodeau SN et al. (17)</td>
<td>1:25</td>
<td>Ventana BenchMark XT automated slide preparation system</td>
</tr>
</tbody>
</table>

**Results**

The demographic and clinicopathological characteristics of all CRC patients according to MMR-proficiency status are presented in Table II. The mean age was 67.7±10.5 (range=42-86) years. Gender distribution for males and females was similar. In the majority of patients, a family history of cancer could not be determined. Adenocarcinoma and mucinous-type CRC were diagnosed in 32 (79%) and 9 patients (20%), respectively. The tumor was proximally-located (ascending and transverse colon), and distally-located (descending and sigmoid colon, and rectum) in 17 (41%), and 24 patients (59%), respectively. With regard to stage, stage 2 tumors were most commonly diagnosed (18 patients, 44%). The demographic and clinicopathological characteristics of patients according to MMR-proficiency status are presented in Table III. IHC for MLH1 and MSH2 was positive in 16/41 (39%), and in 36/41 (87%) of patients, respectively. All five patients that negatively-stained for anti-MSH2 antibody also showed a negative stain for anti-MLH1 antibody. Figure 1 shows immunohistochemical staining for MSH1 and MLH2.
MLH1 and MSH2. Table III shows staining results for both antibodies. MMR-proficiency, and MMR-deficiency were classified in 16 (39%), and 25 (61%) patients, respectively. The mean age of MMR-deficient patients was approximately +10 years compared to MMR-proficient patients (71.9±9.7 years versus 61.2±8.4 years, p=0.07). MMR-deficient tumors were more often proximally-located, while MMR-proficient tumors were more commonly distally-located (p=0.018). Place of birth, family history of cancer, histological type, and grade of differentiation, surgical or clinical stage were similar in both groups (Table IV). CFD and CEA blood levels according to MMR-proficiency status are presented in Table V. The mean CFD plasma levels in MMR-proficient, and MMR-deficient patients were 795ng/ml ±431, and 906ng/ml ±494, respectively (p=0.68). The mean CEA serum levels in MMR-proficient and MMR-deficient patients were 10.4±17.6 μg/L, and 15±48 μg/L, respectively (p=0.46).
Discussion

CRC is a heterogeneous disease with different molecular pathways leading to different phenotypes: genetic and epigenetic alterations act to dysregulate conserved signaling pathways involved in cellular metabolism, proliferation, differentiation, survival, and apoptosis. Apart from the exclusive group of hereditary cancer syndromes, at least two distinct molecular pathways have been recognized in the transformation process of sporadic CRC: the CIN and CIMP pathways (2-5). Silencing of two MMR-related genes, MLH1 and MSH2 is responsible for most CRC-transformed tumors that arise via the CIMP pathway (19, 20) and silencing of these genes may practically be measured on IHC for their corresponding proteins (21). Sixty-one percent of the patients included in our study were classified as MMR-deficient, showing silencing of at least one MMR-related gene. All MMR-deficient tumors negatively-stained for the anti-MLH1 antibody, although 5 patients (11%) negatively-stained for both anti-MLH1 and anti-MSH2 antibodies. This finding suggests a widespread hypermethylation of promoter CpG island loci in these five patients, which resulted in the inactivation of at least two tumor-related genes. The relatively high proportion of MMR-deficient tumors in our study (61%) according to

Table V. CEA and CFD blood levels in patients with colorectal cancer according to mismatch repair status.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mismatch repair proficiency</th>
<th>p-Value</th>
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<tbody>
<tr>
<td></td>
<td>Patients n=41 MMR+ve n (%)</td>
<td>MMR-ve n (%)</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>CFD (ng/ml)</td>
<td>Median 832 758 840 0.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean 864±469 795±431 906±494</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range 120-2591 120-1551 168-2591</td>
<td></td>
</tr>
<tr>
<td>CEA (µg/L)</td>
<td>Median 2.1 1.9 2.2 0.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean 13.2±39 10.4±17.6 15±48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range 0.5-235 0.9-66 0.5-235</td>
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</table>

Figure 1. Immunohistochemical stains for mismatch repair proteins in biopsies of patients with colorectal cancer (with diaminobenzidine, ×100). (A-B) Anti-MLH1 antibody: (A) positive, (B) negative; (C-D). Anti-MSH2 antibody: (C) positive, (D) negative.
current literature suggests that cases included in our study were highly-selective for unclear reasons, and did not represent the expected distribution of MMR-proficiency status in our Institute. The mean age of MMR-deficient patients in our study was approximately 10 years younger than MMR-deficient patients ($p=0.07$). Two patterns of methylation have been described in CRC (22): the first pattern is age-related with low methylation level that increases incrementally with age. The second pattern, occurring in CIMP-tumors encompasses all cases of sporadic MSI-high cancers arising from sessile-serrated adenomas, usually having a proximal location. MMR-deficient tumors in our study were associated with proximal location ($p=0.018$). This finding is consistent with the biology of MMR-deficient tumors which usually develop via the CIMP pathway. These tumors are typically characterized in addition to their proximal location, by an expanding growth pattern, mucinous features, poor differentiation, lack of “dirty necrosis,” and lymphocytic reactions (7, 23, 24).

Stage 2 CRCs, as opposed to stage 3 CRCs are considered to be enriched with MMR-deficient tumors and a high-frequency MSI phenotype (25). We have previously shown that CFD blood levels in stage 2 CRC patients were paradoxically higher than in stage 3 patients (10). We anticipated that an enriched population of MMR-deficient tumors in stage 2 CRC patients could have explained this paradox. CFD has been proposed to be released into the blood circulation by apoptotic and necrotic cells originating in the tumor. Secretion has also been suggested as a potential source of CFD (26). Necrotic and apoptotic cells are usually phagocytized by macrophages or other scavenger cells that engulf necrotic cells and release digested DNA into the tissue environment. We, therefore, postulated that the high mutation rate and poor differentiation, combined with intense lymphocytic reaction typical of MMR-deficient tumors could result in increased CFD blood levels in these patients. However, we showed that CFD blood levels in MMR-deficient patients were similar to levels in MMR-proficient patients, suggesting a comparable magnitude of CFD release to the circulation by tumors developing via the two different mechanisms. As with CFD, blood levels of CEA, another prognostic marker, were similar in MMR-proficient, and MMR–deficient cases. It is important to state however, that the method we used for measuring CFD total blood level was anticipated that an enriched population of MMR-deficient cases. It is important to state however, that the method we used for measuring CFD total blood level was unable to detect for differences in CFD composition $e.g.$ it could not detect the presence of specific marker mutations in each group.

In summary, through the present study we showed that compared to MMR-proficient CRCs, MMR-deficient tumors occur more frequently in older patients, and are more commonly proximally-located. Despite their distinct biological and histopathological characteristics, both tumor groups produce similar CFD blood levels.

References


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