Differing DNA Methylation Patterns and Gene Mutation Frequencies in Colorectal Carcinomas from Middle Eastern Countries

Annie O. Chan,1,2,12 Amr S. Soliman,2,5 Qing Zhang,2,3 Asif Rashid,1 Ahmed Bedeir,1 P. Scott Houlihan,1 Nadia Mokhtar,6 Nidal Al-Masri,8 Ugur Ozbek,10 Rami Yaghani,9 Ayten Kandilci,10,11 Sherif Omar,7 Yersu Kapran,10 Ferhunde Dizdaroglu,11 Melissa L. Bondy,2,3 Christopher I. Amos,2,3 Jean-Pierre Issa,4 Bernard Levin,3 and Stanley R. Hamilton1

Abstract  Purpose: The epidemiology of colorectal carcinoma is well known to differ among countries but the molecular characteristics are usually assumed to be similar. International differences in molecular pathology have not been studied extensively but have implications for the management of patients in different countries and of immigrant patients. Experimental Design: We evaluated the CpG island methylator phenotype pathway characterized by concordant methylation of gene promoters that often silences transcription of the genes, the microsatellite instability pathway, and K-ras and p53 gene status in 247 colorectal carcinomas from the three selected Middle Eastern countries of Egypt, Jordan, and Turkey. Results: Colorectal carcinoma from Egypt had the lowest frequencies of methylation. In multinomial logistic regression analysis, Jordanian colorectal carcinoma more frequently had methylation involving the p16 tumor suppressor gene (odds ratio, 3.5; 95% confidence interval, 1.2-10.6; P = 0.023) and MINT31 locus (odds ratio, 2.3; 95% confidence interval, 1.0-5.1; P = 0.041). The K-ras proto-oncogene was more frequently mutated in colorectal carcinoma from Turkey (odds ratio, 2.9; 95% confidence interval, 1.2-6.7; P = 0.016), but p53 overexpression was more common in both Jordanian and Turkish colorectal carcinoma than in Egyptian cases (odds ratio, 2.5; 95% confidence interval, 1.2-5.5; P = 0.019; and odds ratio, 3.6; 95% confidence interval, 1.8-7.1; P = 0.0003, respectively). The findings in Turkish colorectal carcinoma were most similar to those reported for Western cases. Conclusions: Colorectal carcinoma from Middle Eastern countries have differing DNA methylation patterns and mutation frequencies that indicate dissimilar molecular pathogenesis, probably reflecting different environmental exposures. These molecular differences could affect prevention strategies, therapeutic efficacy, and transferability of clinical trial results.

The epidemiology of colorectal carcinoma in developing countries differs from that of developed countries. Colorectal carcinoma in developing countries, including those in the Middle East, is usually characterized by low incidence, young age of onset, left-sided location, poor differentiation, and paucity of precursor adenomas (1–9). International studies as well as studies in immigrants suggest that environmental factors, especially lifestyle and dietary differences, play a major part in the observed epidemiologic differences. Morphologic and genetic progression to colorectal carcinoma in an adenoma-adenocarcinoma sequence and in hereditary colorectal carcinoma syndromes are well described (reviewed in ref. 10). Chromosomal instability and mutation of the K-ras proto-oncogene and the p53 suppressor gene are common.

Authors’ Affiliations: 1Department of Pathology, Division of Pathology and Laboratory Medicine; 2Department of Epidemiology; 3Division of Cancer Prevention; 4Department of Leukemia, Division of Cancer Medicine, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; 5Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, Michigan; Departments of 6Pathology and 7Surgical Oncology, The National Cancer Institute at Cairo University, Cairo, Egypt; Departments of 8Pathology and 9Surgery, Jordan University for Science and Technology, Irbid, Jordan; 10Department of Genetics, Institute for Experimental Medicine; 11Department of Pathology, Faculty of Medicine, Istanbul University, Istanbul, Turkey; and 12Department of Medicine, The University of Hong Kong, Hong Kong, People’s Republic of China

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Requests for reprints: Stanley R. Hamilton, Division of Pathology and Laboratory Medicine, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 85, Houston, TX 77030. Phone: 713-792-2040; Fax: 713-792-4094; E-mail: shamito@mdanderson.org.

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Microsatellite instability (MSI) due to abnormal nucleotide mismatch repair that results in numerous mutations, especially in repeated nucleotide sequences (microsatellites), is a second important molecular pathway to colorectal carcinoma. Recent studies have shown that aberrant methylation of CpG islands is also common in colorectal carcinoma (11, 12). CpG islands are 0.5 to 2 kb regions rich in cytosine-guanosine dinucleotides that are present in the 5’ region of about half of all human genes. The recently discovered CpG island methylator phenotype (CIMP) has widespread concordant hypermethylation of the promoters of numerous genes that often results in transcriptional silencing of those genes. Most studies of the molecular characteristics of colorectal carcinoma have been reported from Western countries but the three major molecular pathways of chromosomal instability, MSI, and CIMP are usually assumed to be similar in tumors in developing countries.

Environmental influences on molecular pathways in colorectal neoplasia are indicated by several lines of evidence (13). Low dietary folate is associated with K-ras proto-oncogene mutation (14, 15). Western diet characterized by high calories, fat, refined carbohydrates, and animal proteins is associated with p53 mutation and overexpression of the gene product (13, 16, 17). Cigarette smoking and alcohol consumption are related to MSI (18) and chronic inflammatory bowel disease is related to aberrant methylation (19).

Evaluation for microsatellite instability. MSI was determined by fluorescently labeled PCR amplification with five markers from the panel described by the National Cancer Institute conference on MSI: BAT25, BAT26, D2S123, D5S346, and D17S250 (23). High levels of MSI were defined by shifts of bands compared with control DNA in at least 30% of the evaluated sites. Tumors were classified as CIMP-negative if all of the evaluated genes and loci were unmethylated, CIMP-low if one of the evaluated sites was methylated, and CIMP-high if two or more of the evaluated sites were methylated.

Fig. 1. Methylation-specific PCR amplification of the p16 gene. Tumors T2 and T4 have methylation of p16 as indicated by the presence of a band in the methylated lane (M) as well as the unmethylated lane (U).

Materials and Methods

Patients and specimens. The cases were surgical resection specimens of consecutive patients with colorectal carcinoma at hospitals in Egypt, Jordan, and Turkey where the research collaborators were attending physicians. Inclusion criteria were resection of colorectal carcinoma and availability of a pathology tissue block of the primary tumor. Exclusion criteria were history of, or pathologic evidence for, familial adenomatous polyposis or idiopathic inflammatory bowel disease, or family history of hereditary nonpolyposis colorectal cancer syndrome. The use of the specimens was approved by the Institutional Review Board of the M.D. Anderson Cancer Center.

The study groups were as follows: 93 patients from the National Cancer Institute of Cairo University in Egypt in 1999 and 2000, including 44 patients from our previously published study (20); 59 patients from the Medical School of Jordan University for Science and Technology in Irbid in 1995 through 1999; and 95 patients from Istanbul Medical School in Turkey in 1996 through 1999. The demographics and pathologic characteristics of our study groups were similar to those reported in previous studies from the three countries (4–7). Tumor tissue and nonneoplastic control tissue from each formalin-fixed, paraffin-embedded resection specimen were microdissected and DNA was extracted as reported previously (20).

Methylation-specific PCR amplification of the hMLH1 mismatch repair gene; and methylated in tumor (MINT) loci MINT1, MINT2, and MINT3. Bisulfite treatment of DNA was followed by methylation-specific PCR, as described previously (11, 12). Detailed protocols can be found at the website www.mdanderson.org/methylation/. Data were acquired as percentage of methylation by determining the density of the methylated band relative to the sum of the methylated and unmethylated bands with a Bio-Rad imager (Bio-Rad, Richmond, CA) and densitometer, as in our previous studies (22). A gene or locus was classified as methylated when the methylated band was ≥10% based on dichotomous discrimination of methylation status from nonmethylatable control colorectal mucosa. CIMP classification was based on the number of methylated sites to evaluate possible associations with methylation of multiple genes and loci in a tumor. Tumors were classified as CIMP-negative if all of the evaluated genes and loci were unmethylated, CIMP-low if one of the evaluated sites was methylated, and CIMP-high if two or more of the evaluated sites were methylated.
**Table 1. Demographic and clinicopathologic characteristics of colorectal carcinomas from Egypt, Jordan, and Turkey**

| Characteristics               | Percentage of cases (n) | P  
|------------------------------|-------------------------|--------
| Demographics                 |                         |        |
| Age, y (mean ± SD)           | Egypt (n = 93)           | Jordan (n = 59) | Turkey (n = 95) |        |
| Age range                    | 42 ± 17                 | 50 ± 17    | 60 ± 12          | <0.0001 |
| Male sex                     | 58.0 (54)               | 50.9 (29)  | 49.5 (47)        | 0.46    |
| Tumor site                   |                         |           |                  |        |
| Right colon                  | 25.8 (24)               | 38.6 (22)  | 32.6 (31)        |        |
| Left colorectum              | 74.2 (69)               | 61.4 (35)  | 67.4 (64)        |        |
| Rectum                       | 45.2 (42)               | 33.4 (19)  | 33.7 (32)        | 0.19    |
| Extent of tumor (stage)      |                         |           |                  |        |
| Localized (I and II)         | 48.3 (42)               | 59.7 (34)  | 57.6 (53)        |        |
| Metastatic (III and IV)      | 51.7 (45)               | 40.4 (23)  | 42.4 (39)        | 0.31    |
| Tumor histopathology         |                         |           |                  |        |
| Poor differentiation         | 38.1 (32)               | 17.9 (10)  | 23.2 (22)        | 0.02    |
| Nonmucinous                  | 53.3 (49)               | 66.1 (37)  | 77.9 (74)        | 0.01    |
| Mucinous-partial             | 30.4 (28)               | 23.2 (13)  | 12.6 (12)        |        |
| Mucinous-diffuse             | 16.3 (15)               | 10.7 (6)   | 9.5 (9)          |        |
| Signet-ring cells            | 25.8 (24)               | 14.6 (8)   | 6.4 (6)          | 0.001   |
| Medullary                    | 6.1 (3)                 | 6.9 (4)    | 3.1 (3)          | 0.53    |

*Variation in the denominators used for calculating percentage results from missing data.

1P values for differences in frequencies were calculated with the two-sided χ² test except as indicated.

1P value was calculated with the two-sided F test.

**Immunostaining for hMLH1 gene product.** All colorectal carcinoma were evaluated for presence or absence of nuclear expression of hMLH1 mismatch repair gene product by immunohistochemistry, as described previously (20), for comparison to hMLH1 methylation and MSI status due to the known occurrence of discordance that can occur between promoter methylation and gene product expression (20, 24). Tumor cell nuclei were assessed as positive or negative relative to internal positive-control staining of nonneoplastic cell nuclei.

**K-ras mutation analysis.** K-ras mutation in exon 1 was analyzed by PCR and automated sequencing as previously described (20). Mutations were confirmed by use of the reverse primer.

**Immunostaining for p53 gene product overexpression.** Overexpression of p53 gene product by immunohistochemistry was evaluated as described previously (20). A labeling index of >40% of nuclei was used to categorize overexpression, based on our previous studies that showed that this level of overexpression is ~80% accurate as an individual immunohistochemical surrogate indicator of p53 gene mutation status in colorectal carcinoma (25, 26).

**Statistical analysis.** Data were entered into the Statistical Analysis System package (version 8.01, SAS Institute, Cary, NC). ANOVA was used to evaluate differences in mean ages of onset among countries. To compare frequencies, we used goodness of fit χ² tests, or Fisher exact tests when the expected number in a cell was <5.

Logistic regression analysis was used to evaluate tumor-related characteristics while adjusting for potential concomitant variables, such as age, that may reflect different ascertainment patterns for the study populations. To calculate odds ratios as estimates of relative risks and to adjust for other factors, we conducted binary logistic regression using the LOGISTIC procedure. We used backward selection methods with α = 0.1 to select models but then dropped any variables that did not reach a significance of 0.05 in the final models. For correlated predictors, we chose one variable from each group to include in the model and evaluated each other variable in a separate model. To compare demographic, clinical, and molecular findings among countries, we used multinomial logistic regression analysis as implemented in the CATMOD procedure of the Statistical Analysis System package. Cases with incomplete data were excluded from the multinomial analyses. All statistical tests were two sided.

**Results**

**Demographic and clinicopathologic characteristics.** The ages of the patients varied among the countries (P < 0.0001; Table 1). Egyptian patients were on average the youngest (42 ± 17 years old) and Turkish patients the oldest (60 ± 12 years old) but the gender distribution was similar among the three countries. Left-sided colorectal carcinoma predominated in all three groups (61.4-74.2%). The frequencies of localized tumors (stages I and II) and metastatic tumors (stages III and IV) did not vary significantly among countries (P = 0.31).

Tumor histopathology differed among the three Middle Eastern countries (P = 0.001-0.02). Carcinomas from Egyptian patients had the highest prevalence of poor differentiation (38.1%). The frequency of mucinous carcinoma and signet-ring cell component also differed among countries (P = 0.01 and P = 0.001, respectively). These histologic types were more frequent among Egyptian colorectal carcinoma (16.3% and 25.8%, respectively), whereas Turkish cases had the lowest frequencies (9.5% and 6.4%) and Jordanian carcinomas had intermediate frequencies (10.7% and 14.6%).

**CpG island methylation pattern.** The frequency of methylation at MINT31, MINT2, and hMLH1 differed among the three countries, with Egyptian cases having the lowest frequency of aberrant methylation of individual markers and Jordanian cases the highest frequencies (P = 0.01-0.04; Table 2). When the
colorectal carcinomas were classified for CIMP status based on the panel of five markers, some evidence of variability among countries was noted (P = 0.06 for CIMP-negative and P = 0.07 for CIMP-high). Colorectal carcinoma from Egypt had the highest prevalence of CIMP-negative tumors (46.2%) and the lowest prevalence of CIMP-high tumors (21.5%).

Microsatellite instability status. Egyptian colorectal carcinoma had a low frequency of high-level MSI (16.7%) and the lowest frequencies of hMLH1 methylation and loss of hMLH1 protein expression (8.7% and 4.7%, respectively; Table 2), but only hMLH1 methylation varied significantly among countries (P = 0.02). High-level MSI and loss of hMLH1 were also infrequent in the Jordanian cases (15.1% and 5.6%, respectively). Adjustment for the histopathologic features of differentiation, signet ring cell component, and mucinous component yielded odds ratios similar to those in Table 4 although the association between p16 methylation and Jordanian origin of the colorectal carcinoma had more frequent K-ras mutation than Egyptian cases (P = 0.016). Colorectal carcinoma from both Jordan and Turkey was more frequently associated with p53 overexpression (P = 0.019 and P = 0.0003, respectively). Adjustment for age and site of the tumors and using Egyptian colorectal carcinoma as the reference group based on our previous studies (4, 20, 21). Colorectal carcinoma from Jordan was more frequently methylated at p16 (P = 0.023) and MINT31 (P = 0.041) and showed a trend toward CIMP-high (P = 0.052). Turkish colorectal carcinoma had more frequent K-ras mutation than Egyptian cases (P = 0.016).

Discussion

The epidemiology of colorectal carcinoma in the three Middle Eastern countries we studied was reported previously to differ: Colorectal carcinoma from Egypt and Jordan are characterized by frequent young onset, rectal location, and mucinous histology, in contrast to Turkey (4–7). We confirmed these previously reported demographic and pathologic characteristics, indicating that the tumors we studied were representative of the countries of origin. Most of the existing literature on the molecular characteristics of colorectal carcinoma

### Table 2. Molecular characteristics of colorectal carcinomas from Egypt, Jordan, and Turkey

<table>
<thead>
<tr>
<th></th>
<th>Percentage of cases (n)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Egypt (n = 93)</td>
<td>Jordan (n = 59)</td>
<td>Turkey (n = 95)</td>
</tr>
<tr>
<td><strong>Methylation markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16 gene</td>
<td>10.3 (7)</td>
<td>27.1 (13)</td>
<td>19.7 (14)</td>
</tr>
<tr>
<td>MINT31</td>
<td>33.3 (30)</td>
<td>56.9 (33)</td>
<td>50.0 (47)</td>
</tr>
<tr>
<td>MINT2</td>
<td>22.7 (20)</td>
<td>43.4 (23)</td>
<td>31.9 (30)</td>
</tr>
<tr>
<td>MINT1</td>
<td>19.6 (18)</td>
<td>27.6 (16)</td>
<td>21.5 (20)</td>
</tr>
<tr>
<td>hMLH1 gene</td>
<td>8.7 (8)</td>
<td>20.4 (11)</td>
<td>23.2 (22)</td>
</tr>
<tr>
<td><strong>Methylation pathway status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIMP-high</td>
<td>21.5 (20)</td>
<td>34.5 (20)</td>
<td>36.8 (34)</td>
</tr>
<tr>
<td>CIMP-low</td>
<td>32.3 (30)</td>
<td>39.7 (23)</td>
<td>29.5 (28)</td>
</tr>
<tr>
<td>CIMP-negative</td>
<td>46.2 (43)</td>
<td>25.9 (15)</td>
<td>34.7 (33)</td>
</tr>
<tr>
<td><strong>MSI status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI-H</td>
<td>16.7 (8)</td>
<td>15.1 (8)</td>
<td>22.7 (20)</td>
</tr>
<tr>
<td>MSI-L</td>
<td>14.6 (7)</td>
<td>0</td>
<td>6.8 (6)</td>
</tr>
<tr>
<td>Microsatellite stable</td>
<td>68.8 (33)</td>
<td>85.9 (46)</td>
<td>70.5 (62)</td>
</tr>
<tr>
<td>Loss of hMLH1 protein</td>
<td>4.7 (2)</td>
<td>5.6 (3)</td>
<td>14.3 (12)</td>
</tr>
<tr>
<td>K-ras mutation</td>
<td>18.4 (16)</td>
<td>33.3 (16)</td>
<td>34.2 (28)</td>
</tr>
<tr>
<td>p53 overexpression</td>
<td>42.1 (37)</td>
<td>62.8 (27)</td>
<td>69.5 (68)</td>
</tr>
</tbody>
</table>

Abbreviations: MSI-H, high levels of MSI; MSI-L, low levels of MSI.

*Variation in the denominators used for calculating percentage results from missing data.

*P values for differences in frequencies were calculated with the two-sided \( \chi^2 \) test.
originated from Western countries but our previous molecular pathology study of colorectal carcinoma in patients from Egypt identified distinctive molecular characteristics that included a low frequency of \( K\text{-ras} \) mutation in poorly differentiated mucinous and signet-ring cell carcinoma in young patients (20). Our current study confirmed these previous molecular findings in an expanded set of Egyptian cases and also identified for the first time a low frequency of CpG island methylation. In addition, we now report the results of molecular characterization of colorectal carcinoma from two other Middle Eastern countries (i.e., Jordan and Turkey) and found differences between those countries and Egypt.

The international differences in molecular pathology of colorectal carcinoma have implications for pathogenesis. We found in multivariate analysis that colorectal carcinoma from Jordan had more frequent methylation than the other two Middle Eastern countries. Of note, the Jordanian cases contrasted sharply with the neighboring country of Egypt despite the geographic and cultural similarities between the two countries. Among individual markers, methylation of the \( p16 \)
tumor suppressor gene and the MINT31 locus were more frequent in Jordanian colorectal carcinoma (Tables 2 and 3). Such geographic variation in methylation of specific genes (p16 and estrogen receptor) has been reported in hepatocellular carcinoma and related to hepatitis and cirrhosis (27). In addition, methylation is a common feature of chronic inflammatory disease involving the colorectal mucosa in patients with ulcerative colitis (19). Environmental factors have also been shown to influence methylation in lung cancer: methylation of p16 and other genes is associated with cigarette smoking (28, 29). Our study indicates that geographic variation in methylation also exists in colorectal carcinoma, possibly as a result of different environmental exposures. We have thus extended the observations that methylation of specific genes is variable in different tumor types, geographic locations, and environmental exposures (30).

Current understanding of clinicopathologic correlates with molecular characteristics of colorectal carcinomas is based mainly on Western data. Some of our findings in Middle Eastern cases (Table 3) corroborate previous reports in Western cases (12, 20). The molecular characteristics of Turkish colorectal carcinoma were most similar to those reported from Western countries for the frequencies of high-level MSI, K-ras mutation, and p53 protein overexpression (12, 15, 16, 20). On the other hand, mucinous tumors in Egypt and nearby Jordan were predominantly rectal in location (Table 1), in contrast to the mainly right-sided location in Western populations (31). Our findings suggest that studies to explore the associations among left- and right-sided mucinous tumors, CIMP status, MSI status, and gene mutations are necessary to clarify the molecular pathogenesis in different areas of the world, which we have now established to have differences.

Strategies for prevention of colorectal carcinoma can evolve from understanding of pathogenesis. Numerous epidemiologic studies have addressed environmental factors in the etiology of colorectal carcinoma but little is known about environmental-genetic interactions in the multistep process of colorectal tumorigenesis. Ras proto-oncogene mutation in colorectal adenomas has been associated with low dietary folate (14), although the relationship was less clear in patients with colorectal carcinoma (15). MSI in colonic cancers has been linked to cigarette smoking and alcohol consumption (13, 18) and p53 mutation in colorectal carcinoma was reported to be associated with a Western diet (13, 16, 17) but not cigarette smoking (13). Because the prevalences of the molecular pathways for colorectal carcinoma are different among the three Middle Eastern countries we studied, we speculate that unique environmental exposures or lifestyle factors in the Middle East may interact with genetic factors and be reflected in the differing molecular pathways. For example, numerous wild edible plants are available and consumed exclusively in Jordan (32). The extracts of several of these plants that are consumed for treatment of gastrointestinal disorders possess anti-inflammatory activity, possibly due to inhibition of cyclooxygenase-2 that is important in colorectal neoplasia (33). Analogous to international epidemiologic studies, population differences in molecular pathology offer opportunities for better understanding of gene-environment interactions. International differences in molecular pathology may enhance insights into the molecular pathogenesis of colorectal carcinoma, as well as the development of novel preventive strategies directed at environmental factors.

Finally, our findings suggest that the lessons learned from molecular studies in Western countries, where most of this research has been conducted, are unlikely to be directly applicable to other areas of the world where tumors have a different molecular pathogenesis. Molecular characteristics of colorectal carcinoma are known to influence tumor biology, including prognosis and response or resistance to chemotherapy (reviewed in refs. 34–37). As a consequence, differences in methylation and in genetic alterations may influence treatment response rates in different patient populations. Colorectal carcinoma in recent immigrants is likely to have the molecular characteristics of patients in their native country rather than those in their adopted country, analogous to the characteristics of other types of cancer (2). International differences in molecular pathology may also affect the results of clinical trials in colorectal carcinoma so that the response rates in one patient population may not transfer well to other countries with different tumor molecular pathology.

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References


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