

Distinct Genetic and Epigenetic Signatures of Colorectal Cancers According to Ethnic Origin

Taina T. Nieminen¹, Soheir Shoman², Saad Eissa², Päivi Peltomäki¹, and Wael M. Abdel-Rahman^{1,3}

Abstract

Background: The outcome of colorectal cancer varies depending on ethnic origin. Egyptian colorectal carcinoma is surprisingly young-age disease with high proportion of rectal and advanced stage cancers.

Methods: We characterized 69 sporadic Egyptian colorectal cancers for promoter methylation at 24 tumor suppressor genes, microsatellite instability, and expression of mismatch repair, p53, and β -catenin proteins. Data were compared with 80 Western colorectal carcinoma of sporadic and familial origin from Finland.

Results: Egyptian colorectal carcinomas showed significantly higher methylation of the microsatellite stable (MSS) tumors as reflected by the average number of methylated genes per case ($P = 0.00002$) and tumor suppressor gene methylator phenotype (TSGMP), defined here as methylation of ≥ 5 genes, ($P = 0.0001$) compared with the sporadic Western cancers. The TSGMP was associated with advanced stage in the Egyptian cancers ($P = 0.0016$). Four genes were differentially methylated between Egyptian and Western cases, of which the association of *CDKN2B/p15* methylation with Egyptian origin was outstanding ($P = 4.83E-10$). Egyptian carcinoma also showed significantly lower frequency of nuclear β -catenin localization than the sporadic Western cancers ($P = 0.00006$) but similar to that of the familial Western subset designated as familial colorectal cancer type X.

Conclusions: We show novel pathway in colon carcinogenesis marked by high methylation of MSS cancers, remarkable *CDKN2B/p15* methylation, and low frequency of Wnt signaling activation.

Impact: Our findings highlight the possible effect of environmental exposures in carcinogenesis through DNA methylation and should have applications in prevention, molecular diagnosis, prognosis, and treatment. *Cancer Epidemiol Biomarkers Prev*; 21(1); 202–11. ©2011 AACR.

Introduction

Colorectal carcinoma is a notorious disease which annually affects one million individuals worldwide and more than half of these patients succumb to the disease. The available knowledge of factors that determine the development and clinical course of colorectal cancers is mainly derived from Western countries. Cancers in the Eastern and Middle Eastern countries appear to include novel clinical and molecular categories as implied by

different incidence rates, familial clustering, and/or younger age at onset compared with the West (1, 2). In particular, colon cancer is a surprisingly young-age disease and has a more unfavorable outcome in Egypt (3). More than one third of Egyptian colorectal carcinomas affect young people before 40 years and 60% affect people younger than 50 years, whereas only 15% of patients were older than 60 years in a multicenter study in Egypt (4). A comparison of the age-specific colorectal cancer incidence rates of a representative sample from Egypt with the data from the Surveillance, Epidemiology and End Results (SEER) of the National Cancer Institute, Bethesda, MD, showed higher rates of colorectal cancer among Egyptians than in North Americans until age 30 to 34, followed by constant rates in Egyptians (<20 of 100,000) and a marked increase in North Americans (>100 of 100,000) in 60+ age groups (3). This high prevalence in the young could not be explained by Lynch syndrome (hereditary non-polyposis colorectal cancer, HNPCC) as few, if any, of the young patients fulfilled the Amsterdam criteria nor could it be explained by other known hereditary syndromes including familial adenomatous polyposis. Adenomas and synchronous or metachronous tumors are not common features of colorectal carcinoma in Egypt. The high prevalence in young people could not be attributed to

Authors' Affiliations: ¹Department of Medical Genetics, University of Helsinki, Helsinki, Finland; ²Department of Pathology, National Cancer Institute, Cairo University, Cairo, Egypt; and ³College of Health Sciences, University of Sharjah, and Sharjah Institute for Medical Research, Sharjah, United Arab Emirates

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Corresponding Author: Wael M. Abdel-Rahman, College of Health Sciences, University of Sharjah, Sharjah, United Arab Emirates. Phone: 791-6-5057556; Fax: 791-6-5057502; E-mail: whassan@sharjah.ac.ae and Department of Medical Genetics, University of Helsinki, Biomedicum Helsinki, P.O. Box 63, Helsinki, 00014, Finland. E-mail: Wael.Abel-Rahman@helsinki.fi

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Bilharziasis, a prevalent parasitic disease in Egypt, either (4, 5). Egyptian colorectal carcinomas usually present in an advanced stage (4).

Classical genetic pathways that contribute to colorectal cancer development involves among others: deregulated Wnt signaling pathway, resulting from either inactivating mutation of *APC* or activating mutation of β -catenin, loss of wild-type p53, chromosomal instability, and/or microsatellite instability (MSI) but exceptions were noted mainly in cell lines (6, 7). CpG island methylator phenotype was shown to play a significant role in colon carcinogenesis through widespread silencing of tumor suppressor genes (TSG; ref. 8). Genetic instability and/or methylator phenotype might provide a favorable route in colorectal carcinogenesis to facilitate the accumulation of a large number of mutations required for the full blown invasive cancer (9). Interestingly, the methylator phenotype is usually associated with MSI as a result of the mismatch repair gene *MLH1* promoter silencing (10). It was suggested that the methylator phenotype is somehow dictated or modified by different environmental exposures to carcinogens (8).

The available reports on molecular profiles of colon cancer in the east highlight some interesting areas, including epigenetics, for future explorations (11). However, the extensive epidemiologic reports on the ethnic variations in colorectal cancers are generally not accompanied by corresponding data on the genetic basis of this phenomenon. We, therefore, took the opportunity to conduct a comprehensive investigation into the molecular basis of Egyptian colorectal carcinoma with accurate clinical data available and compared the molecular and clinical profiles with those of the Western counterparts, with the purpose to dissect factors that are universal versus unique in the etiopathogenesis of this cancer.

Materials and Methods

Patients and samples

This study was carried out on a total of 149 colorectal adenocarcinomas, general features of which are given in Table 1. The Egyptian tumors represented a consecutive series collected by the collaborating pathologists, after getting the required approval, from formalin-fixed,

paraffin-embedded tissue blocks of surgical resection specimens submitted to pathology laboratories at the National Cancer Institute, Cairo, Egypt, which serve patients from large area including mid and north Egypt in addition to the great Cairo area. The Western patients were from population-based cohorts corresponding to certain hospital districts in Finland (Finn sporadic series) or a selected group from a nationwide registry of hereditary colorectal cancer families (Finn familial, FCCX series) representing familial non-polypotic colorectal cancers not associated with germ line mutation in the mismatch repair genes *MLH1*, *MSH2*, *MSH6*, or *PMS2* (12, 13). DNA was extracted from paraffin-embedded specimens by standard techniques. Mutation screening, MSI, methylation, and immunohistochemical analyses were carried out in previous studies for the FCCX group (10, 12, 13). For Finn sporadic samples, MSI and methylation analyses were conducted previously (10). β -Catenin, p53, and other data reported here on the Finn sporadic samples were generated in this study. All data from the Egyptian series were generated in this study. The work was conducted at Helsinki under the approval of the institutional review boards of the Helsinki University Central Hospital.

MSI analysis

MSI status was determined using the mononucleotide repeat markers *BAT25* and *BAT26* from the Bethesda panel (14) as previous reports (15, 16) have shown that mononucleotide repeats are sensitive and specific for the MSI-high phenotype. Tumors with at least one unstable marker were considered to have MSI. The forward primers were fluorescently labeled with FAM, and PCR products were run on ABI3730 sequencer/genotyper and results analyzed using GeneMapper v3 software (Applied Biosystems).

Immunohistochemistry

Four-micrometer sections from formalin-fixed, paraffin-embedded tissues were dewaxed and rehydrated to distilled water then sections were subject to heat-induced target retrieval in 1 mmol/L EDTA buffer, pH 8.0, for 5 minutes at 750 W followed by 5 minutes at 450 W in a microwave oven. After cooling, the slides were washed in TBS, pH 7.2, and subsequent staining steps were carried

Table 1. General characteristics of the Egyptian versus Westerns (Finnish) materials

	Egypt	Finn sporadic	Finn familial (FCCX)
No. of tumors	69	61	19
No. included in MS-MLPA	43	51	19
Age range (average)	18–78 (54.8)	52–95 (73.6)	35–77 (56.2)
Gender (M:F)	1.23:1	0.5:1	1.23:1
Tumor site (right:left:rectal)	15:21:33	35:17:4 ^a	7:5:7
MSI frequency	19/61 ^a (31%)	16/61 (26%)	1/19 (5%)

^aVariation in the numbers or denominator used for calculating percentages results from missing data.

out manually with the Dako EnVision+ System, Peroxidase (DAB), according to manufacturer's instructions (Dako). In addition, after blocking endogenous peroxidase activity, and prior to incubation with the primary antibody, the sections were incubated with 10% normal (non-immune) goat serum (Dako) for 30 minutes. The primary antibodies were purified mouse monoclonal anti- β -catenin antibody (clone 14, BD Transduction Laboratories), anti-MLH1 (clone G168-15, Pharmingen), anti-MSH2 (clone FE-11, Calbiochem/Oncogene Research), anti-MSH6 (clone 44, Transduction Laboratories), and anti-p53 (clone DO7, DakoCytomation). Nonimmune mouse IgG1 (Dako) was used as a negative control. Paired tumor and normal mucosa were in the same section and the normal tissues were used as internal reference for evaluation of staining results. The cutoff level for nuclear β -catenin expression was 10% and for p53 stabilization was 30% as described previously (13).

Methylation-specific multiplex ligation-dependent probe amplification

We used the SALSA MS-MLPA Tumor suppressor Kit ME001-C1 (MRC-Holland) to study promoter methylation of 24 TSGs in tumor and normal samples according to manufacturer's instructions (17). This kit contains 26 probes for 24 TSGs, which were described in detail in our previous publication (10). For each reaction, 200 to 800 ng paraffin-extracted DNA was used. Each methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) reaction generates 2 aliquots, one undigested and one digested and these can be used for copy number and methylation detection, respectively. MS-MLPA uses methylation-sensitive enzyme *HhaI* and probes have a recognition sequence against this enzyme. Only templates with methylated *HhaI* sites, resistant to *HhaI* cleavage, generate a signal after probe ligation. Amplification products were separated by capillary electrophoresis and analyzed with ABI Prism GeneMapper 4.0 (Applied Biosystems). In every assay, normal DNA derived from healthy individuals' lymphocytes was included as a control for the lack of methylation and tumor cell line (RKO) was included as a reference for frequent methylation. Methylation dosage ratios were calculated as described in our previous study (10), in which a value of 0.15 (corresponding to 15% of methylated DNA) was used as cutoff level for methylation. However, in the present investigation, a dosage ratio of 0.25 gave the best discrimination between paired normal and tumor DNAs from the Egyptian series and was regarded to indicate promoter methylation. The 0.25 cutoff value was subsequently applied to all series reported here for the sake of consistency.

Statistical analysis

The Fisher exact probability test was used to evaluate differences between groups in frequencies of advanced stage, poor differentiation, MSI, abnormal immunohistochemistry, or methylation of individual genes. When

analyzing differences in the average number of methylated genes per sample, the Student *t* test for independent samples was applied. Analyses were carried out using MS Excel and/or VassarStats Web-based statistical program (18). All reported *P* values were 2-tailed and *P* values < 0.05 were considered significant.

Results

Young- versus old-age Egyptian carcinomas

The overall clinicopathologic and molecular profiles of the Egyptian series are shown in Table 2 together with a comparison between the young age group of ≤ 50 years ($n = 45$) and old age group of >50 years ($n = 24$). The cutoff level of 50 years was chosen to match the majority of published literature in the field as well as the age threshold recommended by the Amsterdam Criteria (19) and Bethesda guidelines (20) for Lynch syndrome and familial colorectal cancer diagnosis and molecular screening. Mismatch repair protein expression was carried out for all 61 cases and protein loss of MLH1, MSH2, or MSH6 could explain 13 of the 19 MSI cases. A significant though expected difference was the tendency for MLH1 protein loss in old age versus MSH2 loss in young age (Table 2). Methylation frequencies were not significantly different. Of note, the percentages of patients in each age group and the general clinicopathologic features matched the previously published literature from Egypt (4) so that high overall frequency of rectal cancers (48%), advanced stage (68%), poor differentiation (45%), and extracellular mucin production (50%) were evident.

Clinicopathologic and molecular characteristics of Egyptian cancers stratified by MSI status

Egyptian series is divided into 2 groups according to the MSI status in Table 3. The MSI frequency was 19 of 61 (31%) and 13 of these MSI cases could be explained by MLH1, MSH2, or MSH6 protein loss. There were no major differences between Egyptian MSI and microsatellite stable (MSS) cancers regarding the 2 prognostic factors stage and grade. Right-sided location in the colon was associated with MSI ($P = 0.04$), whereas location in the left colon was associated with MSS phenotype ($P = 0.034$). Rectal cancers which form around half of the Egyptian cancers in our series as well as previous reports (4) were divided almost equally between MSS and MSI categories. Stabilization of p53, nuclear β -catenin, and TSG methylation were not different in the MSI versus MSS carcinoma.

Comparison of Egyptian and Finnish cancers relative to molecular parameters

β -Catenin and p53 expression. The frequencies of nuclear β -catenin and p53 stabilization are shown in Table 4. p53 stabilization frequencies were not significantly different in the Egyptian series compared with either the Finn sporadic or the FCCX group. In regard to β -catenin localization, Egyptian carcinomas showed significantly lower frequency of nuclear β -catenin, indicating

Table 2. Characteristics of young- versus old-age Egyptian colorectal carcinoma

	All (n = 69) ^a	≤50 y (n = 45) ^a	>50 y (n = 24) ^a	P
Age, y, range (mean)	18–78 (54.8)	18–50 (42.4)	55–78 (65.2)	
Gender (M:F)	38:31	24:21	14:10	0.8
Tumor site				
Right colon (%)	15/69 (22%)	10/45 (22%)	5/24 (21%)	1
Left colon (%)	21/69 (30%)	14/45 (31%)	7/24 (29%)	1
Rectosigmoid and rectum (%)	33/69 (48%)	21/45 (47%)	12/24 (5%)	0.8
Extent of tumor (stage)				
Early (Dukes A&B)	21/65 (32%)	14/45 (31%)	7/20 (35%)	0.78
Advanced (Dukes C&D)	44/65 (68%)	31/45 (69%)	13/20 (65%)	0.78
Histology				
Poor differentiation	31/69 (45%)	17/45 (38%)	14/24 (58%)	0.13
Extracellular mucin	35/69 (50%)	19/45 (42%)	16/24 (67%)	0.07
Mismatch repair status				
MSI	19/61 (31%)	11/39 (28%)	8/22 (36%)	0.57
MLH1 protein loss	6	1	5	0.029
MSH2 protein loss	6	5	1	0.029
MSH6 protein loss ^b	1	1	0	ND
p53 Stabilization	43/68 (63%)	26/44 (59%)	8/24 (33%)	0.26
Nuclear β-catenin	28/68 (41%)	19/42 (45%)	12/22 (55%)	0.4
Methylation				
Average no. of methylated genes/case	5	4.8	5.6	0.18
TSGMP phenotype ^c	22/43 (51%)	14/28 (50%)	8/15 (53%)	0.39

Abbreviation: ND, not determined.

^aVariation in the denominator used for calculating percentages results from missing data.

^bOnly MSH6 loss not accompanied by MSH2 loss is reported here. The remaining MSI cases expressed MLH1, MSH2, and MSH6 by immunohistochemistry which was carried out for all cases in this study.

^cPromoter methylation present in ≥5 of 24 TSGs.

low frequency of active Wnt signaling, than the Finn sporadic. In contrast, the Egyptian series did not differ relative to FCCX tumors, which were previously reported by us to show remarkably low levels of active Wnt signaling (13).

Overall frequencies for promoter methylation. Egyptian colorectal carcinomas showed significantly higher overall methylation than the Western cancers, as shown in Table 4. For the various methylation parameters given in Table 4, the overall frequency in each series is shown first followed by breakdown comparison between MSI and MSS subsets in the Egyptians and sporadic Finn, whereas division into MSI and MSS was not applicable to FCCX which was MSS as a rule. The average number of methylated genes/case was significantly higher in the Egyptian series than the Finn sporadic ($P = 0.0025$) and FCCX ($P = 0.00039$). Subset analysis showed that this difference was a reflection of the situation in the MSS subset ($P = 0.00002$), not the MSI subset (Table 4). Using a cutoff value of ≥5 methylated genes of 24 to define a "tumor suppressor gene methylator phenotype" (TSGMP), the TSGMP phenotype was clearly associated with MSI in the Finn sporadic series (69% in MSI vs. 9% in MSS; $P = 0.000056$) but not in the Egyptian series (50% vs.

52%; $P = 1$; Table 4). Tumors with TSGMP showed a statistically nonsignificant tendency to right-sided location in the Finn sporadic series ($P = 0.067$) but not in the Egyptian series. Interestingly, TSGMP was associated with advanced stage (defined as stage C and D) in the Egyptian cancers (86% TSGMP cases in advanced stage cancers, $P = 0.0016$) but not in the Finn sporadic or FCCX (0% TSGMP cases in advanced stage cancers, $P = 0.19$). TSGMP was not associated with patient age in the Egyptian series ($P = 0.39$), and no significant association was observed between the TSGMP phenotype and tumor grade or other clinicopathologic or molecular feature in the Egyptian or other series.

CDKN2B and other differentially methylated genes. Methylation frequency of individual genes in all series is given in the Supplementary Table S1. Different comparisons were made between related groups and subsets and displayed in Fig. 1. Only those genes with methylation frequency of ≥20% in tumors from any series are included in these comparisons. Representative examples of the MS-MLPA tracing from three subsets are shown in Fig. 2. *CDKN2B*, *DAPK1*, *RASSF1*, *MLH1*, and *HIC1* showed significantly differential methylation among groups (Fig. 1; Table 4). A remarkable finding in this study was

Table 3. Characteristics of MSS versus MSI Egyptian colorectal carcinoma

	MSS (n = 42) ^a	MSI (n = 19) ^a	P
Age, y, range (mean)	18–72 (64.3)	35–78 (61.5)	
Proportion of young ≤50 y	28/42 (67%)	11/19 (58%)	0.57
Gender (M:F)	25:17	11:8	1
Tumor site			
Right colon (%)	6/42 (14.3)	7/19 (36.9)	0.04
Left colon (%)	17/42 (40.5)	2/19 (10.5)	0.034
Rectosigmoid and rectum (%)	19/42 (45.2)	10/19 (52.6)	0.78
Extent of tumor (stage)			
Early (Dukes A&B)	27/41 (66%)	12/18 (67%)	1
Advanced (Dukes C&D)	14/41 (34%)	6/18 (33%)	1
Histology			
Poor differentiation	16/42 (38%)	9/19 (47%)	0.57
Extracellular mucin	19/42 (45%)	10/19 (53%)	0.78
p53 Stabilization	24/42 (57%)	6/18 (33%)	0.16
Nuclear β-catenin	19/41 (46%)	8/19 (42%)	0.79
Methylation			
Average no. of methylated genes/case	5	5.6	0.24
TSGMP phenotype ^b	17/33 (52%)	5/10 (50%)	1

^aVariation in the denominator used for calculating percentage results from missing data.

^bPromoter methylation present in ≥5 of 24 TSGs.

the selectively high frequency of *CDKN2B* methylation in the Egyptian series ($P < 4.83E-10$) regardless of MSI status (Fig. 1; Table 4). Within a given case, the MS-MLPA technique has the ability to quantify the methylation dosage from 0, indicating no methylation, to 1, indicating complete methylation. Taking advantage of this feature, we observed that the *CDKN2B* average methylation dosage ratio/case ranked first (together with *ESR1*) with an average dosage of 0.46 in the Egyptian series. This level also represented the highest average methylation dosages observed in our previous study of a diverse group of colorectal cancers (10).

Methylation in paired normal tissue

Forty paired samples of normal colon from the Egyptian ($n = 14$) and Finnish ($n = 26$) series were investigated to determine the extent and locus-specific spectrum of methylation in the normal tissue, if present. The majority of normal DNA (31 of 40, 78%) did not show any instance of methylation at any gene of 24. Cases showing any instance of methylated genes in the normal DNA accounted for 3 of 14 (21%) in the Egyptians and 6 of 26 (23%) in the Finns. The presence of promoter methylation was thus significantly associated with tumors in all series when compared with matching normal tissue ($P = 3.9E-08$ Egyptians and $3.8E-09$ Finns). No gene was methylated more than twice in normal DNAs from Egypt or Finland and TSGMP phenotype was observed in only one paired normal DNA from the Egyptian series (with only 5 genes methylated with low dosage ratios ranging from 0.25 to 0.30). Only *ESR1* and *CDKN2A* were methylated in 2 normal DNAs

from Egypt and 2 normal DNAs from Finland, *GSTP1* was methylated in 2 normal Finnish samples, whereas other genes (*CDH13*, *TP75*, *PTEN*, *CDKN2B*, *APC*, *CASP8*) showed only single instances of methylation. The remaining 15 genes did not show any methylation in any normal DNA.

Discussion

The Egyptian colorectal carcinoma series that we studied here showed intriguing epidemiologic and molecular differences compared with its Western counterparts. We have observed frequent young age onset, poor and mucinous differentiation and advanced stage cancers, and rectal carcinoma accounted for almost half of our series. Therefore, this series is well representative of the country of origin. Analyses carried out in our study revealed that Egyptian colorectal cancers are most likely to represent a homogenous group of tumors with only subtle differences between subsets defined by MSI or other parameters. This finding is consistent with the results from another young age Eastern series (21). However, we cannot exclude the possible existence of subtypes classified according to other parameters (22). The data presented here show that methylation of promoter region of distinct TSGs are associated with the pathogenesis of the Egyptian colorectal carcinoma. We have used a powerful technique, MS-MLPA, to simultaneously detect and quantify methylation in a large set of TSGs and promoters. This fruitful approach uncovered some unique pathogenic features in the Egyptian colorectal cancers including: (i) frequent

Table 4. Molecular and immunohistochemical profiles of colorectal carcinoma from Egypt compared with Western cancers

	Egypt	Sporadic (Finn)	P (Eg vs. Finn sporadic)	FCCX (Finn)	P (Eg vs. FCCX)
No. of tumors ^a	69	61		19	
MSI	19/61 (31%)	16/61 (26%)	0.69	1/19 (5%)	NA ^b
<i>p53</i> Stabilization	43/68 (63%)	29/57 (51%)	0.2	8/16 (50%)	0.4
Nuclear β -catenin	28/68 (41%)	45/58 (78%)	0.00006	7/16 (43%)	1
Average no. of methylated genes/case	5	3	0.0025	2.6	
MSS	5	2	0.00002	NA	
MSI	5.6	6	0.4	NA	0.00039
TSGMP phenotype (≥ 5 genes methylated)	22/43 (51%)	14/51 (27%)	0.021	3/19 (16%)	0.011
MSS	17/33 (52%)	3/35 (9%)	0.0001	NA ^p	
MSI	5/10 (50%)	11/16 (69%)	0.42	NA	
Distinct methylated genes					
<i>CDKN2B</i> (all cases)	29/43 (67%)	1/51 (2%)	4.83E-10	0/19 (0%)	4.83E-10
MSS	22/33 (67%)	0/35 (0%)	4.83E-10	NA	
MSI	7/10 (70%)	1/16 (6%)	0.0013	NA	
<i>DAPK1</i> ^c					
MSI	5/10 (50%)	0/16 (0%)	0.0038	NA	
<i>MLH1</i> ^c					
MSI	1/10 (10%)	10/16 (63%)	0.014	NA	
<i>HIC1</i> ^c					
MSI	0/10 (0%)	7/16 (44%)	0.022	NA	

^aVariation in the denominator used for calculating percentage results from missing data.

^bNA: not applicable as FCCX is a special, mainly, MSS series.

^cThese genes showed different methylation at MSI but not MSS subset.

methylation of MSS cancers reflecting dissociation of TSGMP and *MLH1* methylation; (ii) targeting of *CDKN2B/p15* irrespective of MSI status; (iii) targeting of different sets of genes in different MSI subsets (*DAPK1*, *MLH1*, *RASSF1*, *HIC1*); and (iv) the association of TSGMP with advanced stage.

CDKN2B/p15 (p15^{INK4b}) gene resides, together with 2 other related genes *ARF* and *CDKN2A/p16* (p16^{INK4a}) within a 35 kb stretch of the human genome. The locus is known as *INK4a/ARF/INK4b* locus and is deleted in a wide spectrum of tumors including melanoma, pancreatic adenocarcinoma, glioblastoma, certain leukemias, non-small cell lung cancer, and bladder carcinoma. The binding of the INK4 proteins to CDK4 and CDK6 abrogates the binding of these kinases to D-type cyclins, inhibiting CDK4/6-mediated phosphorylation of retinoblastoma (pRb) family members. Hypophosphorylated Rb family proteins potently binds E2F to effect a G1 cell-cycle arrest (23, 24). Deregulation of pRb pathway is very common in human cancers (24), but alterations of the major molecules of this pathway are rarely observed in colorectal cancer. Loss of *CDKN2A/p16* expression in colorectal carcinoma associated with promoter methylation was observed in small fractions that did not exceed a quarter of the cohorts studied (10, 25). Hypermethylation of *CDKN2B/p15* was reported mainly in glial tumors, leukemias, and myelo-

dysplasia (26). We searched the literature for data on *CDKN2B/p15* methylation in colorectal cancers and found that *CDKN2B/p15* was actually analyzed in large cohorts of Western colorectal carcinoma from the United States but no methylation was found (27). Together with the results from the Finnish cohorts studied here, these data suggest that the lack of methylation in *CDKN2B/p15* in Western material is a nonrandom event. Interestingly, we found an article from China showing *CDKN2B/p15* methylation in 68% of colorectal cancer specimens of Chinese origin (28).

The frequent methylation of MSS cancers and the selective methylation of different sets of genes in carcinoma of Eastern origin might be related to common environmental or dietary factors. In support of this hypothesis are the epidemiologic studies which associated smoking with methylation of specific genes including *CDKN2A/p16* in lung cancer (29, 30). Experiments on mice have shown that early nutritional exposure to folate modified the epigenetic state of transposable elements (31). In human, folic acid supplementation was associated with DNA hypermethylation in patients with colorectal adenoma (32). The available data on folate and cancers suggest that while folate may prevent early tumors and preneoplastic lesions, it could promote the transformation of these lesions to cancer once developed (33). In common with

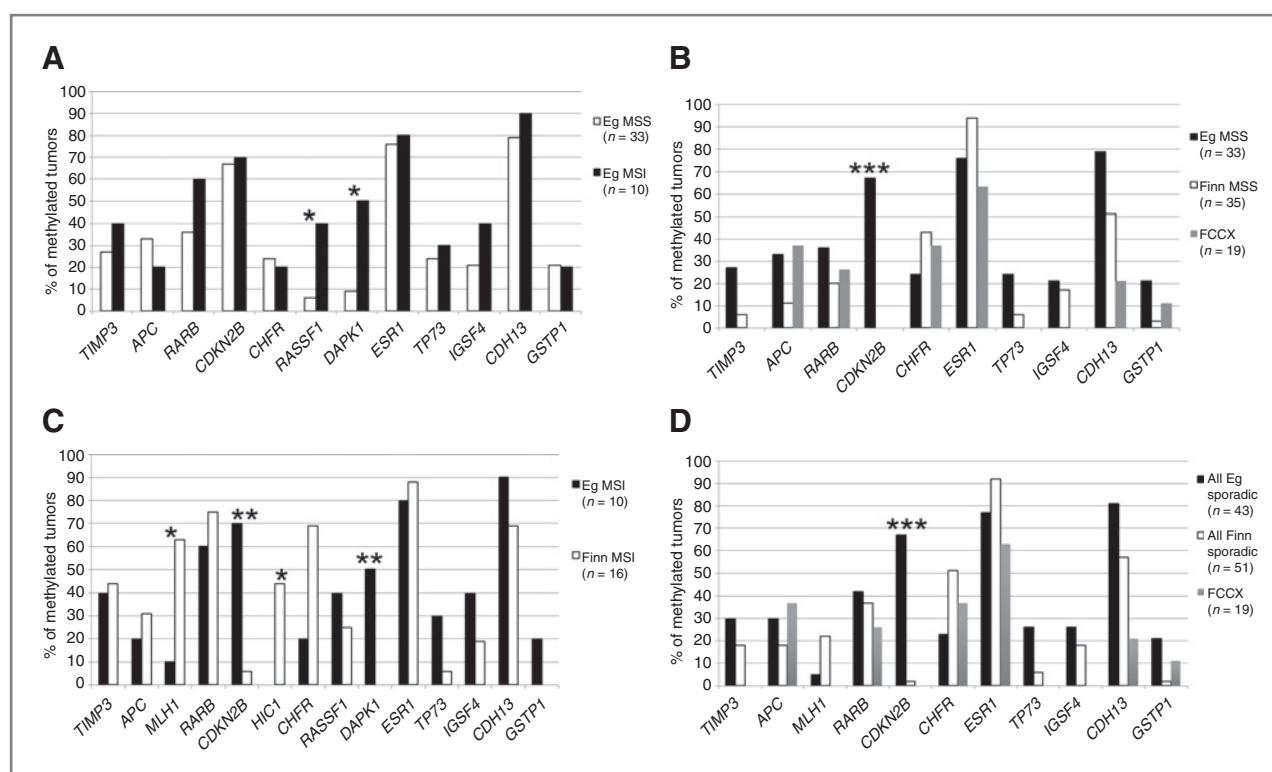


Figure 1. Comparison of the percentages of tumors with promoter methylation at indicated genes in different subsets. Only TSGs which showed promoter methylation in at least 20% of the total number of cases in any subset are included in this comparison. Significant differences at level $P < 0.05$, $P < 0.01$, and $P < 0.001$ are indicated by *, **, and ***, respectively. A, Egyptian MSS versus MSI; B, Egyptian MSS versus Finn sporadic MSS versus Finn FCCX; C, Egyptian MSI versus Finn sporadic MSI; D, all Egyptian tumors versus all Finn sporadic, versus Finn FCCX.

other developing countries, many adverse environmental factors are likely to exist in Egypt. These include the indiscriminate use of pesticides, herbicides, and fertilizers in agricultures, the mixing of more than one type of these chemicals, and the uncontrolled use of antibiotics and hormones in farming. Inappropriate farming practices, such as crop residue burning, releasing potential carcinogens into the air and soil as well as the likely contamination of irrigation water with industrial waste add to the problem. Indeed, a recent study has shown that history of pesticide exposure and more frequently eating food directly from farms were significantly associated with a higher risk of colorectal carcinoma in Egypt (34). However, as the literature on this issue is still sparse (35–37), the hypothesis that environmental exposures could induce cancers through epigenetic changes remains to be confirmed in experimental models and large series of clinical samples.

The Egyptian carcinomas showed MSI rates higher than the Western but MSI was not associated with TSGMP as seen in the Western material. The high frequency of methylation of MSS cancers in the Egyptian series implies that the combination of TSGMP and *MLH1* methylation is not selected for in the development of these subsets contrary to a well established association between methylation phenotypes and *MLH1* silencing in many other

series from the published literature. The reason behind this remains obscure. The ‘too much instability’ argument might be an explanation assuming that these tumors have other as yet unknown forms of genetic instability (9). MSI tumors usually have a good prognosis, and the loss of the tight association between MSI and methylation might explain our finding of more advanced stage Egyptian cancers with TSGMP.

The low frequency of nuclear β -catenin in the Egyptian series that was also noted in the FCCX could be partially explained by the TSGMP phenotype. Our previous findings of mutations in specific genes such as *FGF9* could also explain some of these tumors that lack nuclear β -catenin (38). The question remains why β -catenin protein expression should be membranous in a high proportion of colorectal carcinomas of Egyptian and FCCX origin and whether or not a distinct pathogenic mechanism is responsible for the development of this particular phenotype.

The high prevalence of the Egyptian carcinoma in the young could not be explained by Lynch or other hereditary syndromes (4, 5). This was also true in our series. We also did not find any evidence to suggest that this high prevalence in the young could represent a heritable methylation trait because methylation was virtually absent in blood or adjacent normal tissue. This finding is consistent

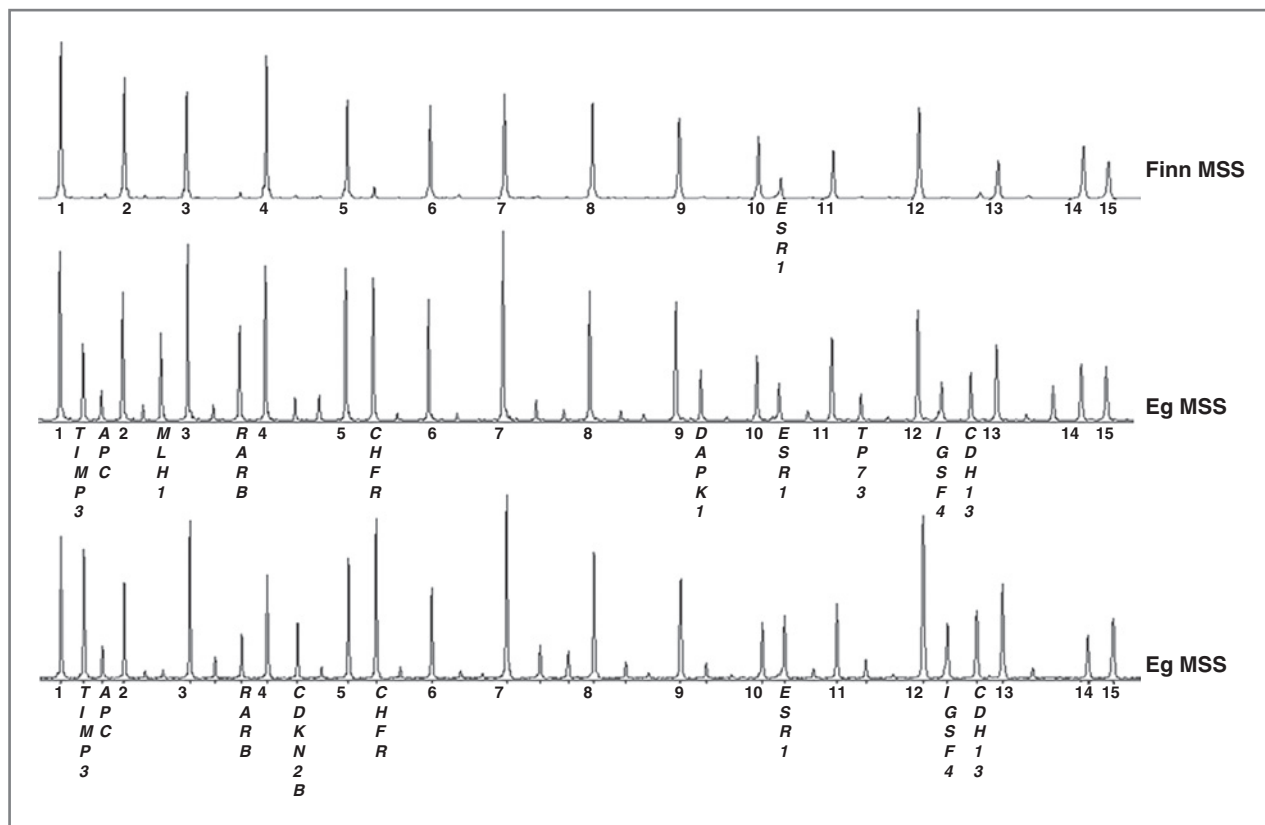


Figure 2. Examples of MS-MLPA plots representing 3 different subsets: Finn sporadic MSS (top), Egypt MSI (middle), Egypt MSS (bottom). Twenty-four different tumor suppressor loci were interrogated with specific probe pairs (2 pairs of probes were included for RASSF1), combined with a set of control probes (labeled 1–15). The fragment size on the x-axis identifies the exact gene or promoter, and area (height) for a given peak is proportional to the degree of methylation. The control loci 1–15 yield peaks invariably, whereas additional visible peaks represent amplification products obtained only if a given tumor suppressor locus (names shown) is methylated.

with our previous data from well-characterized familial colorectal and endometrial tumors of as yet unknown predisposition (10). A few articles exist in the literature that claim that DNA methylation within normal mucosa may be associated with pathway-specific predisposition to cancer through the "field effects" (39). However, our series provide no clear evidence for field effects because methylation in normal tissue was generally rare and when present, showed no apparent association with TSGMP in tumors.

In summary, by comparing colorectal carcinomas from Egypt and Finland, we have shown that some features of the "classical" colorectal cancer pathway (40) are universal, such as frequent stabilization of p53, whereas some features related to Wnt signaling, TSG methylation, or genomic instability status appear unique to certain subsets defined by clinical and family characteristics (e.g., FCCX) or ethnic origin. Our findings are clinically important because, for example, small-molecule inhibitors of the Wnt pathway are becoming available as novel therapeutic agents in colorectal cancer (41), and information of the Wnt signaling status may guide the treatment of the respective subsets of cancers. The presence versus absence

of a methylator phenotype, combined with the mode of genomic instability, may predict treatment response and prognosis of colorectal cancers (42). The involvement of the retinoblastoma pathway through *CDKN2B/P15* methylation in colorectal carcinogenesis is a novel finding. Therefore, apart from opening the door for identifying critical pathogenic mechanisms in cancers of different ethnic origins, the novel molecular profiles revealed in this study are likely to have more general implications in the diagnosis, prognosis, and therapy of colorectal cancers. The possible influence of environmental factors is an important issue which has to be kept in mind when judging molecular data as accurate knowledge of these effects and applying appropriate strategies to limit these exposures could help in prevention of many cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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