Colorectal carcinoma from Saudi Arabia

Analysis of MLH-1, MSH-2 and p53 genes by immunohistochemistry and tissue microarray analysis

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ABSTRACT

Objective: To document the incidence and role of p53 and DNA mismatch repair proteins in colorectal carcinomas, and to evaluate the relative frequency of major molecular pathways in colorectal cancers from Saudi Arabia.

Methods: We collected the formalin fixed, paraffin embedded tissues from 154 colorectal tumors (83 patients from King Faisal Specialist Hospital and Research Centre and 71 from Saudi Aramco Dhahran Health Centre) between January 1989 and December 2003. We analyzed the p53 and mismatch repair gene expression (hMSH-2, hMLH-1) by immunohistochemistry in tissue microarray format.

Results: Expression loss of at least one mismatch repair gene was found in 33.8% of cases and significantly associated with the right-sided tumor location (p=0.0047). The p53 positivity was observed in 57.5% of tumors, and was inversely linked to expression loss of mismatch repair genes (p=0.0102).

Conclusion: The strong confirmation of the previously established associations between tumor phenotype, and mismatch repair gene alteration provided strong evidence for the validity of our experimental approach. Together with the higher incidence of right sided location in Saudi (46.6%) than in Western colon cancers (34.9%), the observed high prevalence of mismatch gene expression loss in Saudi tumors argues for a higher importance of microsatellite instability in this population. If confirmed, it will be interesting to see whether an increased level of familial or sporadic microsatellite instability cases is causing this variation.

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Colorectal cancer is the fourth most common cancer across all age groups in Saudi Arabia.[1] According to established models, colorectal cancers can be subdivided into 2 distinct forms: those belonging to the chromosomal instability pathway/ microsatellite stable (MSS), and those belonging to the microsatellite instability (MSI) pathway.[2] The first and more common pathway is characterized

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by the sequential inactivation of a series of tumor suppressor genes such as adenomatous polyposis coli (APC) (localized on chromosome 5q), p53 (chromosome 17p), and genes on chromosome 18q (deleted in colorectal carcinoma (DCC), Smad2, and Smad4). Immunohistochemically these tumors often show a nuclear accumulation of inactivated p53 protein.[2] The second genetic pathway involves defective mismatch repair genes such as human homologues of MutS (hMSH-2), human homologues of MutL (hMLH-1), human post meitotic segregation (hPMS-1), hPMS-2, hMSH-6, and hMSH-3.[3-5] This leads to a high level of MSI, which can be found in a significant proportion of hereditary non-polyposis colorectal cancers (HNPCC), and approximately 10-20% of sporadic tumors. Approximately 95% of the germline mutations occur in hMLH-1 or hMSH-2. The MSI in these sporadic cases is mostly due to methylation and subsequent inactivation of the hMLH-1 promoter. The gold standard for diagnosis of MSI is molecular by polymerase chain reaction technology examining DNA sequences of normal and tumor tissue at 5 or more different chromosomal loci. Immunohistochemistry is a less expensive alternative for identifying MSI tumors through demonstration of loss of either MLH-1 or MSH-2 protein expression.

Almost all information on molecular pathways in colorectal cancer is currently derived from the study of Western patients. However, others and we have recently shown that massive differences in molecular features can be found between tumors from patients of different ethnic background.[6-8] To determine, whether this may also apply to colorectal cancer, we investigated the hallmarks of the 2 most relevant colon cancer pathways (MSH-2/MLH-1 and p53 expression) in a series of 154 colorectal cancers in a tissue microarray (TMA) format. The TMA allows a rapid parallel and highly standardized analysis up to 1,000 different tumor tissues on one microscope slide.

Methods. ***Tissues.*** Formalin fixed, paraffin embedded tissues from 154 colorectal tumors were collected including 83 patients treated at King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia between January 1989 and December 2003 as well as 71 patients treated at the Saudi Aramco Dhahran Health Centre between January 1989 and December 2003. Of the 154 patients 149 were from Saudi Arabia, 2 from Palestine, 1 from Lebanon, 1 from Sudan, and 1 patient from USA. The Duke's system was used for staging. The greatest dimension of the tumor and the tumor sites were recorded from the patient files wherever possible. The tumor location was classified as right-sided (proximal to, and including, the splenic flexure), and left-sided (distal of the splenic flexure). The hematoxylin and eosin (H & E) slides were reviewed and classified according to the World Health Organization classification.[9] Histologic grading was based on the amount of gland formation as follows: well-differentiated tumors >95% glands, moderately differentiated tumors 50-95% glands, poorly differentiated tumors 5-50% glands, undifferentiated cancers <5% glands. All mucinous and signet-ring carcinomas were classified as poorly differentiated. For analysis, poorly differentiated and undifferentiated tumors were grouped together. Additional recorded features included presence or absence of invasion of small lymphovascular emboli or perineural invasion, the advancing front of the tumor according to Jass et al[10] (expansive versus infiltrative), and the extension of peritumoral lymphocytic response. Moreover, the number of tumor infiltrating intraepithelial lymphocytes (IELs) in 10 high power field (HPF) of tumor were counted, using the H & E slides only. Based on this count of tumor infiltrating lymphocytes (TIL), a tumor was regarded as TIL positive (TIL+) if there were at least 4 IELs per HPF.[11] Tissue microarray construction was as described before.[12] Briefly, tissue cylinders with a diameter of 0.6 mm were then punched from representative tumor regions of each donor tissue block and brought into recipient paraffin block using a homemade semiautomatic robotic precision instrument. An overview of a H & E stained colon cancer TMA section is shown in **Figure 1**.

Immunohistochemistry (IHC). Standard indirect immunoperoxidase procedures were used for detection of MLH-1, MSH-2 and p53. The antibodies, their dilutions and sources, pretreatment conditions, and scoring criteria are described in **Table 1**. The antigen retrieval was carried out for 5 minutes at 750 W, and then for 15 minutes at 250 W. After antigen retrieval, the slides were cooled for 90 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxidase in methanol for 10 minutes. Staining visualization was in 3,3'-diamino benzidine with H_2O_2 as a substrate (Dako). The sections were then lightly counterstained with Gills hematoxylin. Staining examples for each antibody are shown in **Figure 2**. Loss of expression was recorded when none of the tumor nuclei stained for either hMLH-1 or hMSH-2. Positive staining of nuclei in intact adjacent crypt bases, and lymphocytes served as an internal control. Those tumors showing loss of expression for hMLH-1 or hMSH-2 in all tumor nuclei were regarded as MSI, and those tumors with intact expression- weak, moderate or intense staining in any tumor nuclei were called "intact".

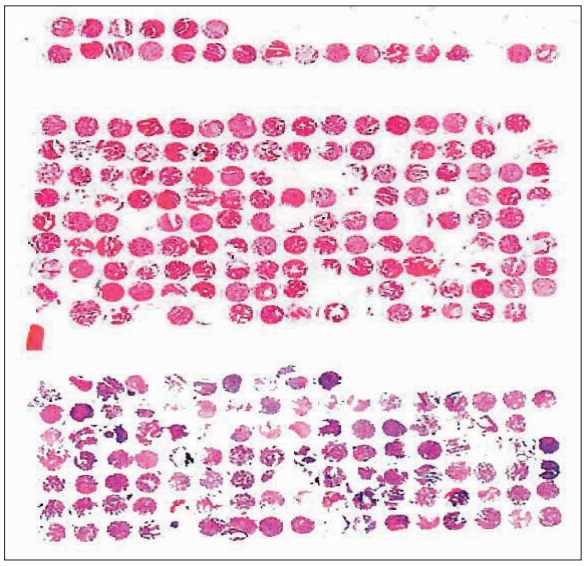

Figure 1 - Overview of the hematoxylin and eosin stained sections of the colon cancer tissue microarray. The tissues were distributed into 2 array blocks according to the source hospitals.

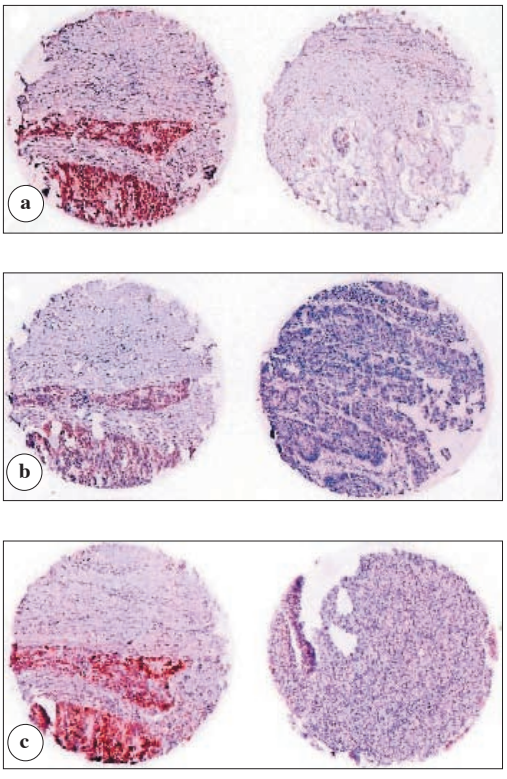

Figure 2 - Examples of mismatch repair enzyme immunostaining; **a)** p53, **b)** MLH1, and **c)** MSH2. Left hand images represent positive findings and the right hand images are negative staining results.

The immunostaining results showed some variability in staining intensities between runs. Tumors were only used for statistical analyses if both MLH-1 and MSH-2 were interpretable.

Statistical Analysis. Chi-square tests were used to examine relationship between nominal variables. Overall survival time was calculated from the date of diagnosis until death or date of last follow-up. Univariate survival analysis was carried out using Cox proportional hazards regression model. A probability value of less than 0.05 was considered significant.

Results. ***Clinical and pathological features.*** All clinical and morphological features are summarized in **Table 2** with the immunohistochemical results. A total of 154 patients were included in the study, but the number of tumors with data for the individual parameters varied between 61 tumor size, and 154 as some of the data could not be retrieved from the patient files or histologic sections.

Mismatch repair gene analysis. Loss of expression of at least one of the 2 examined mismatch repair genes was observed in 52 of 154 interpretable tumors (33.8%). Among tumors with mismatch repair gene expression loss, loss of MLH-1 expression was seen in 93.9%, loss of MSH-2 expression in 27.5%, and loss of both MLH-1, and MSH-2 was seen in 8.9% of the cases. The MLH-1 was negative, but MSH-2 was not interpretable in 5 samples due to loss of tissue in the array slide or lack of internal positive control. Similarly, MSH-2 was negative but MLH-1 was not interpretable in 4 samples. All these cases without interpretable MSH-2 and MLH-1 results were excluded from statistical analyses. Mismatch repair gene expression loss was significantly associated with the right-sided tumor location (p=0.0047). No significant association was observed with several other clinical and pathological tumor features, for example; grade or Duke's stage.

The p53 genes. The p53 positivity was observed in 57.5% of 153 interpretable tumors. Positive staining was inversely related to loss of mismatch repair gene expression (p=0.0102).

Discussion. Immunohistochemistry was used for detection of mismatch repair gene alterations in this study. Although, molecular MSI testing is the gold standard for diagnosing mismatch repair gene defects, several previous studies had suggested a >95% specificity of IHC analysis for MSI analysis.[13-16] In our study, a higher frequency of mismatch repair gene expression loss (35%) was found than in most previous studies. Most reports on Western patients

Table 1 - Antibodies used for immunohistochemistry study.

Antigen	Antibody clone	Dilution	Antigen retrieval	Source	Interpretation
MLH-1	G168-15	1:20	EDTA buffer pH 8 with cooling time of 90 minutes	BD pharmingen	Complete absence with an internal positive control
MSH-2	FE11	1:100	EDTA buffer pH 8 with cooling time of 90 minutes	Zymed	Complete absence with an internal positive control
p53	DO-7	1:50	Citrate buffer pH 6.0	Dako	Positive if >10% of tumor nuclei showed unequivocal staining

EDTA - ethylenediaminetetraacetic acid

Table 2 - The p53, MSH-2/MLH-1 immunostaining and clinico-pathological features.

Parameters	N (on TMA)	MSH-2 and MLH-1 alteration, or both		p53 alteration*	
		(%)	*P*-value**	(%)	*P*-value**
Gender					
Male	68	(27.9)	0.0652	(53)	0.3530
Female	75	(42.7)		(60.8)	
Age					
≤40 years	23	(47.8)	0.1905	(50)	0.4627
>40 years	120	(33.3)		(58.5)	
Tumor site					
Right (proximal) colon	73	(46.6)	0.0047	(52.8)	0.3006
Left (distal) colon	67	(23.9)		(61.5)	
Tumor size (cm)					
0 - 3	16	(37.5)	0.5485	(60)	0.7348
4 - 6	30	(26.7)		(56.7)	
> 6	17	(41.2)		(47.1)	
Tumor grade					
Well differentiated	8	(37.5)	0.3632	(50)	0.5427
Moderately differentiated	118	(32.2)		(61.2)	
Poorly differentiated	23	(47.8)		(50)	
Tumor stage (Dukes staging)					
A	53	(41.5)	0.4290	(53.9)	0.1033
B	57	(36.8)		(46.4)	
C	23	(26.1)		(72.7)	
Histological type					
Adenoca. with mucin	15	(40)		(40)	
Mucinous carcinoma	22	(40.9)		(59.1)	
Medullary carcinoma	1	(0.0)	NS	(0.0)	NS
Mixed carcinoma	1	(0.0)		(100)	
Adenocarcinoma	117	(33.3)		(59.7)	
Tumor margin					
Expansive	28	(39.3)	0.3707†	(50)	0.6930†
Infiltrative-dissecting	14	(24.3)		(50)	
Infiltrative-diffuse	21	(38.1)		(52.4)	
Tumor infiltrating lymphocytes					
Positive	71	(40.1)	0.1352	(52.9)	0.2843
Negative	85	(29.4)		(61.5)	
Peri tumor lymphocytic response					
Absent	74	(31.1)	0.5831	(51.4)	0.3433
Present	59	(35.6)		(59.7)	
Lymphovascular emboli/peri neural infiltration					
Present	46	(34.8)	0.9774	(62.2)	
Absent	110	(34.6)		(55.6)	0.4456

* p53 immunohistochemistry positive, ** Chi-Square test, † expansive versus infiltrative, NS - not significant, TMA - tissue microarray

showed 10-20% of colorectal cancers having MSI.[3,13,17-22,23] The high number in our data set may be partly attributable to reduced antigenicity of some tissue samples. It is well known that tissue fixation is rarely homogeneous, and in large section analysis, regional staining deficiencies are often observed. In a TMA study where samples measuring only 0.6 mm in diameter are analyzed, staining heterogeneity can lead to an unspecified number of false negative cases.

However, a large number of studies have previously shown that representative research information can be obtained in TMA studies.[23-31] Studies investigating the validity of the TMA approach have previously shown that virtually all established associations between molecular features and clinico-pathological parameters can also be identified in TMA settings. This was also true in many studies analyzing only one tissue spot per tumor although.[23,29,32-36] Also, in this study the previously known associations of mismatch repair gene expression loss with clinico-pathological features including right-sided tumor location,[17,37-38] and lack of p53 alterations[39] could be reproduced. This strong confirmation of previously established associations strongly suggests that both our mismatch repair gene expression loss data and clinico-pathological parameters are valid. That the frequency of colon cancers with a mismatch repair defect could indeed be higher in Saudi Arabia than in Europe is also supported by the high frequency of right-sided colon carcinomas (46.6%). Using the same criteria for distinguishing right and left side colon cancer, we found only 34.9% right-sided colon cancers in a Swiss series of 1401 consecutive colorectal cancers. Differences in the lifestyle or in the epidemiology of HNPCC between Saudi and Swiss populations could potentially serve as explanations for our observations.

The p53 positivity, a hallmark of the second colon cancer pathway was found in 57.5% of our cancers. This is comparable to data obtained from the Western literature describing p53 positivity in 51.5% of colon cancers. This also applies for the inverse relationship between p53 positivity, and mismatch repair gene expression loss observed in this study.[39]

In summary, our data show that valid information can be obtained from a TMA composed of 154 colon carcinomas from Saudi Arabia. As in Western tumors, 2 main pathways are involved, which are characterized by either p53 alterations or mismatch repair gene inactivation. The data suggest that the relative fraction of tumors with mismatch repair gene defects may be higher in Saudi Arabia than in Europe. Further studies validating this hypothesis on the level of MSI are needed. If confirmed, it will be interesting to determine whether this high frequency of mismatch repair alterations is caused by an increased level of familial or sporadic cases with MSI.

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