

# Frequency of Mismatch Repair Protein Deficiency in a Puerto Rican Population with Colonic Adenoma and Adenocarcinoma

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**Abstract.** *Background/Aim: Microsatellite instability (MSI) results from genetic alterations involving the mismatch repair (MMR) genes MLH1, PSM2, MSH2, and MSH6. MSI has been implicated in both sporadic CRC and Lynch syndrome. The aim of the study was to assess the frequency of alterations in MMR protein expression in both primary colorectal cancer and precursor lesions among Puerto Rican patients. Patients and Methods: A retrospective study of 84 Puerto Rican patients was performed to assess the frequency of MMR protein expression alterations in both primary CRC and precursor lesions using tissue microarray and immunohistochemistry. Results: The loss of expression of both MLH1 and PMS2 proteins was present in 6.3% of adenomas, 9.1% of adenomas with high-grade dysplasia and 9.4% of colon adenocarcinomas. Negative nuclear staining for both MSH2 and MSH6 proteins was found in 2.4% of colon adenocarcinomas. Conclusion: When compared to prior reports, this study suggests a lower frequency of MSI among the Puerto Rican population. The higher prevalence of MLH1 mutations correlates with previous studies of protein expression among the Hispanic community including Colombian, Uruguay and Brazilian populations.*

Colorectal cancer (CRC) is the third most common cancer in

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the United States and the fourth most common cause of death worldwide. Studies suggest that approximately 20% of colorectal cancers arise due to microsatellite instability, a mechanism characterized by deletions or insertions within repetitive sequences of a distinct cluster of genes known as the mismatch repair genes (MMR). The MMR system is responsible for correcting mismatches generated during DNA replication. Novel studies have unveiled new roles of these genes defects in carcinogenesis involving DNA damage signaling caused by exogenous carcinogens through synergistic interaction with p53-homologous proteins, meiotic crossover promotion, reparative recombination prevention, somatic hypermutation process of immunoglobulin diversification, trinucleotides expansion associated with multiple neurodegenerative conditions and modulation of microRNA (1).

Microsatellite instability is the hallmark of hereditary nonpolyposis colorectal cancer (HNPCC) which accounts for 12-15% of sporadic colon cancer. This diagnosis harbors distinct clinical features such as an autosomal-dominant inheritance pattern with up to 90% of colorectal cancer penetrance with an onset during 4th or 5th decades of life, tumor location at the ascending colon, lymphocytes infiltration, mucinous histology and higher risk of developing extracolonic malignancies especially gastric and endometrial cancers (1, 2). These microsatellite genetic alterations also account for 85% of hereditary colorectal cancer cases as well (3). These tumors are distinguished by multiple molecular mechanisms leading to the common denominator of MMR impairment including truncation of protein and subsequent loss of expression or hypermethylation of MLH1 promoter region and additional mutation of the proto-oncogene *BRAF* which plays a major role in growth factor signaling process (4).

The most frequently observed genetic alterations among MMR proteins involve the MLH1, MSH2, PMS2 and MSH6

proteins. The MSH2 and MSH6 bind together to form a complex known as MutSalpha that predominantly identifies base pairs mismatch. MLH1 and PMS2 also form a complex known as MutLalpha whose main role consists in interacting with the MutSalpha and proliferating cellular nuclear antigen (PCNA) in order to conduct excision and DNA re-synthesis. Therefore, mechanisms leading to loss of expression of any of these proteins cause an inability to repair DNA mismatches and consequently accumulation of mutations that favor oncogenesis (5-7). Researchers have stated that the clinical phenotype depends on the specific gene mutations. It has been described that colorectal cancer incidence is higher among individuals with MLH1 mutation (8, 9). The most frequently observed genetic alteration among sporadic CRC with microsatellite instability was hypermethylation of the 5-end CpG region of the MLH1 gene, therefore affecting the promoter function and consequently protein transcription and expression (1, 10). However, individuals with MSH2 genetic alterations have higher risk of developing extracolonic malignancies (11). Genetic alterations of secondary proteins such as PMS2 and MSH6 are associated with early tumor development expressing high microsatellite instability and late tumor development during the 6th decade of life expressing low microsatellite instability respectively (12-14).

The mutation spectrum has been shown to vary among the Hispanic populations. It has been described that MLH1 loss-of-expression mutations are more prevalent among non-Hispanic populations with colorectal cancer than other MMR genes mutations (15, 16). Similarly, higher prevalence of MLH1 mutations has been described among several Hispanic populations from Colombia, Uruguay, Brazil, Portugal and Spain. (17). However, Caribbean Puerto Ricans MMR studies suggest a higher prevalence of MSH2 mutations among Puerto Rican patients compared to MLH1 mutations among Portuguese and Spanish patients despite of shared Spanish ancestry (18). The aim of the study is to assess the frequency of alterations in MMR protein expression in both primary colorectal cancer and precursor lesions of Puerto Rican patients.

## Patients and Methods

*Study design.* We conducted a retrospective study including 84 CRC cancer patients among the Puerto Rican population from St. Luke's Episcopal Hospital at Ponce, Puerto Rico between 2010 and 2013. The pathology reports and H&E slides of each case were evaluated by pathologists (SG and DC) with interest in Gastro-intestinal pathology, at both Institutions, to confirm the diagnosis and mark the regions of interest (normal colon, adenoma, HGD, and carcinoma). All tissue samples were collected, to construct tissue microarrays (TMA). When present, the tissue types included normal colonic mucosa (NM), adenoma (AD), high grade dysplasia adenoma (HGD) and colon adenocarcinoma (CA). Study criteria included individuals older than 18 years old with colorectal cancer. Those patients with inflammatory bowel disease or family history

of it, were excluded from the study. To construct the TMAs, three core tissue cylindrical punches (diameter 1 mm) were taken from each "donor" paraffin-embedded tissue block and precisely arrayed into a new "recipient" paraffin block using Beecher Instruments (Manual tissue arrayer Model MTA-1). The constructed TMAs contained 84 cases of colonic adenocarcinoma, 11 cases of adenoma with high grade dysplasia, 16 cases of adenoma, and 30 cases of non-neoplastic colonic mucosa.

*Mismatch repair protein expression.* All samples included in the TMAs consisted of formalin fixed paraffin embedded tissues. Four micrometer sections were taken from each TMA and stained with hematoxylin and eosin. The mismatch repair protein expression of the tissues was assessed by immunohistochemistry using the following antibodies: MLH 1 (Clone ES05, Dako, Carpinteria, CA, USA), PMS2 (Clone EP51, Dako), MSH2 (Clone FE11, Dako) and MSH6 (Clone EP49, Dako). The immunostains were performed using the automated Ventana Ultra instrument. The immunostains were interpreted as positive if >10% of the available tissue for each sample was staining.

*Ancestry analysis of Colon TMA tumors.* A set of 106 single nucleotide polymorphisms (SNPs) that can discriminate indigenous American, African, and European ancestry was used to estimate the proportion of genetic ancestry in all cases tested. This panel has been described previously (30). SNPs included were selected for their large difference in allelic frequencies between ancestral populations and have a well-balanced distribution across the 22 autosomal chromosomes. Genotyping of the ancestry informative markers was done using a multiplex PCR coupled with single base extension methodology with allele calls using a Sequenom analyzer. All of the 84 CRCs, the 11 AD with HGD and the 16 AD present in the TMAs were subjected to ancestry analysis. Out of the 106 AIMS genotyped, 5 were excluded due to a genotyping rate <80%. Genotype frequencies at all remaining markers did not significantly deviate from those expected under the Hardy-Weinberg equilibrium law. Individuals with a call rate of <80% were excluded from the analysis. A total of 6 duplicates were also genotyped and concordance was >90%. For each sample, global genetic ancestry proportions were estimated using ADMIXTURE (31) under a supervised model with k of 3.

*Statistical analysis.* In order to evaluate the distinct expression of mismatch proteins expression of MLH1, PMS2, MSH2, and MSH6 in Puerto Rican population, a paired T-test was performed. The determined *p*-value measured as statistically significant was less than 0.05.

## Results

*Clinicopathological features.* A total of 95 samples were collected from 64 male and 31 female CRC/HGD patients ranging from 26 to 93 years old. Eighty-four tissues were colon adenocarcinoma from the right colon (40.4%), transverse colon (3.57%), splenic flexure (1.19%), left colon (10.7%), sigmoid colon (14.3%), rectosigmoid (27.4%) and rectum (2.38%). Eleven tissues were high-grade dysplasia (HGD) adenoma from the right colon (45.4%), transverse colon (9.1%), left colon (18.2%), sigmoid colon (9.1%),

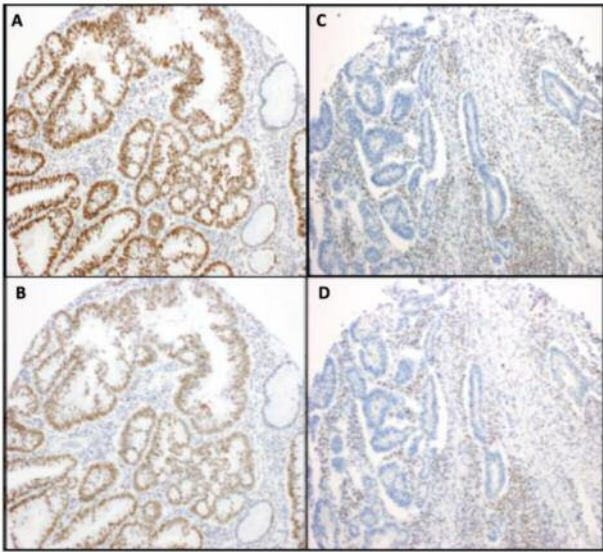


Figure 1. *MLH1* and *PMS2* proteins are expressed normally in microsatellite stable tumors (Image A and Image B respectively). Loss of *MLH1* and *PMS2* from tumor tissue in microsatellite instable colonic adenocarcinoma tumor (Image C and Image D respectively).

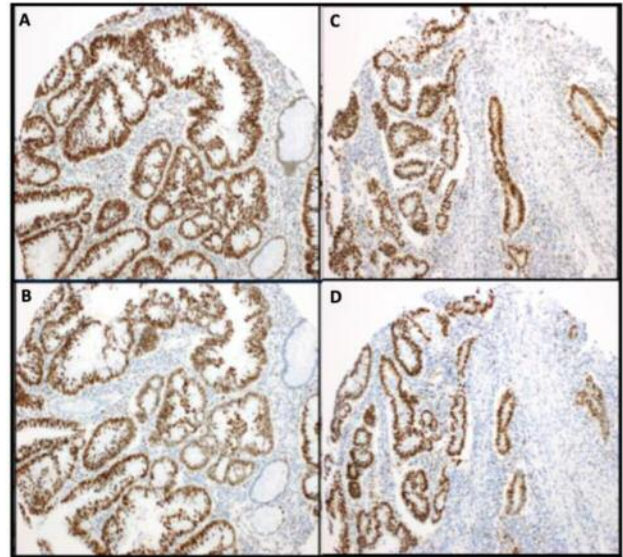


Figure 2. *MSH2* and *MSH6* proteins are expressed normally in microsatellite stable tumors (Image A and Image B respectively). Loss of *MSH2* and *MSH6* from tumor tissue in microsatellite instable colonic adenocarcinoma tumor (Image C and Image D respectively).

rectosigmoid (9.1%) and rectum (9.1%). Most tissues of colon adenocarcinoma were moderately differentiated (67.9%) and stage IIA or extending into serosa (23.8%). The average adenocarcinoma size was 4.27 centimeters, with the median being 4 centimeters and ranging from 0.6 to 12.5 centimeters. Clinicopathological characteristics of colon adenocarcinoma and HGD-adenoma respectively are detailed in Table I.

**Frequency of mismatch repair protein expression.** Among all of the analyzed samples the MMR stains were localized to the nucleus. The loss of expression of both *MLH1* and *PMS2* proteins was present in 6.3% of AD, 9.1% of AD with HGD and 9.4% of CRCs (Figure 1). However, no loss of expression of both *MLH1* and *PMS2* proteins was observed in tissue samples corresponding to non-neoplastic colonic mucosa, as it was expected. Negative nuclear staining for both *MSH2* and *MSH6* proteins was found in 2.4% of CRCs (Figure 2). Nonetheless, no loss of expression of both *MSH2* and *MSH6* proteins was observed in tissue samples of ADs, ADs with HGD and non-neoplastic colonic mucosa.

**Ancestry analysis of colon TMA samples.** The respective contribution of each ancestral population to the TMA samples was European 67.0% (SD 14.3%), African 19.0% (SD 13.5) and Native American 14.0% (SD 7.5%) (Table II). There were no significant differences in global ancestry

proportions after grouping the TMA samples by microsatellite instability status (Table III), or according to the presence or absence of *MSH1/PMS2* or *MSH2/MSH6* (Table IV). [There was a marked reduction in European ancestry and a consequent increase in the Native American component when compared to wild type tumors. Although statistical significance was not reached, this observation warrants further investigation in a larger cohort (Tables III and IV)].

## Discussion

In this study we assessed the MMR protein expression in both primary colorectal cancer and precursor lesions using the tissue microarray technology and immunohistochemistry. We used samples from colon resections from Puerto Rican patients with no family history of colorectal cancer or inflammatory bowel disease. Family history is the main component of the Amsterdam II criteria for Identifying individuals with Hereditary Non-Polyposis Colorectal Cancer (Lynch syndrome), thus individuals with Lynch syndrome are not likely included in our study (19, 20). We found that 6.3% of AD, 9.1% of HGDs and 9.4% of CAs showed loss of expression of both *MLH1* and *PMS2* proteins. Negative nuclear staining for both *MSH6* and *MSH2* proteins was found in 2.4% of CAs, while the expression of these two proteins was not lost in either ADs or HGDs. The increase of loss of expression according to

Table I. Clinicopathological characteristics of adenocarcinoma and high-grade dysplasia tissues.

Clinicopathological characteristics	Adenocarcinoma	High grade dysplasia adenoma
Total, n	84	11
Gender, n (%)		
Male	57 (67.9)	7 (63.6)
Female	27 (32.1)	4 (36.7)
Age, n (%)		
Average	69.4	69.2
<50 years	5 (5.95)	0 (0)
≥50 years	79 (94)	11 (100)
Location, n (%)		
Right colon	34 (40.4)	5 (45.4)
Transverse colon	3 (3.57)	1 (9.1)
Splenic flexure	1 (1.19)	0 (0)
Left colon	9 (10.7)	2 (18.2)
Sigmoid	12 (14.3)	1 (9.1)
Rectosigmoid	23 (27.4)	1 (9.1)
Rectum	2 (2.38)	1 (9.1)
Grade, n (%)		
Well differentiated	12 (14.3)	6 (54.5)
Well to moderately differentiated	5 (5.95)	1 (9.1)
Moderately differentiated	57 (67.9)	4 (36.4)
Moderately to poor differentiated	5 (5.95)	0 (0)
Poor differentiated	4 (4.8)	0 (0)
Undetermined	1 (1.2)	0 (0)
Stage, n (%)		
Stage 0	0 (0)	2 (18.2)
Stage I	14 (16.7)	7 (63.6)
Stage IIA	20 (23.8)	0 (0)
Stage IIB	2 (2.38)	0 (0)
Stage IIC	7 (8.33)	0 (0)
Stage IIIA	2 (2.38)	1 (9.1)
Stage IIIB	19 (22.6)	1 (9.1)
Stage IIIC	14 (16.7)	0 (0)
Stage IVA	2 (2.38)	0 (0)
Stage IVB	3 (3.6)	0 (0)
Undetermined	1 (1.2)	0 (0)

different stages of disease suggest the accumulation of mutations leading to gene silencing as the disease progresses. The data also suggest a higher prevalence of MLH1 and PMS2 mutations compared to MSH2 and MSH6.

Previous studies of MMR genetics in the Hispanic population including individuals from Puerto Rico and Dominican Republic with diagnosis of HPNCC demonstrated that MSH2 was the most commonly affected gene compared to MLH1 in non-Hispanic populations. Also, higher prevalence of MSH2 mutations has been reported among other Hispanic populations such as Argentinian while higher prevalence of MLH1 mutations has been reported

Table II. Mean (standard deviation, SD) ancestry proportions (%) for all samples from the colon TMA.

	EUR	AFR	NAT
Colon TMA samples	67.0 (14.3)	19.0 (13.5)	14.0 (7.5)

among Colombian, Uruguay and Brazilian populations. Therefore, our study correlated with the latter evidence. Regarding previous studies of mismatch repair protein among Caucasian populations, the study suggests a lower frequency among Puerto Rican population.

Ancestry analysis of the tissue microarray samples demonstrated that the majority of the patients had European ancestry (67%) followed by African (19%) and Native Puerto Rican (14%) (Figure 3).

CRC can arise from two main mechanisms, the first involving the chromosomal instability pathway, and second involving the microsatellite instability (MSI) pathway. The purpose of this paper is focused on assessing the presence of MSI pathway alterations in CRCs of Puerto Ricans. MSI occurs when a cell loses the ability to properly fix DNA mismatch errors that occur during cell replication via the DNA mismatch repair (MMR) mechanism. The MMR system is a DNA repair mechanism that prevents mutations from occurring in the cell. Without MMR, an increase in the frequency of mutations occurs and thus increases the potential for a cell to convert into a neoplastic process. The genes involved in the MMR system mainly include MLH1, PMS2, MSH2, and MSH6. MLH1 and PMS2 form dimers and loss of MLH1 on immunohistochemistry will manifest as loss of both MLH1 and PMS2 because PMS2 is not stable by itself. MSH2 and MSH6 form dimers and loss of MSH2 and MSH6 on immunohistochemistry will manifest as loss of both MSH2 and MSH6 because MSH6 is also not stable by itself (21).

CRCs with MSI as a result of sporadic or inherited MMR mutations have distinct clinicopathologic characteristics and therapeutic implications. DNA MMR deficient tumors are characteristically located in the proximal colon and usually exhibit prominent T-lymphocytes infiltration. MMR-deficient neoplasms have a correlation to a morphologic phenotype depending on the extent of neoplasm proliferation, for example, adenomas are usually sessile serrated and adenocarcinomas are usually of the mucinous phenotype (22). The tumors with MMR deficiency are said to be Microsatellite instability high (MSI-H) (22).

Management of colorectal adenocarcinomas depends on the stage of the disease. With local disease, surgery is the definitive treatment with adjuvant chemotherapy varying between stage. Adjuvant chemotherapy for resected stage II (node-negative) disease is not the standard of care for all

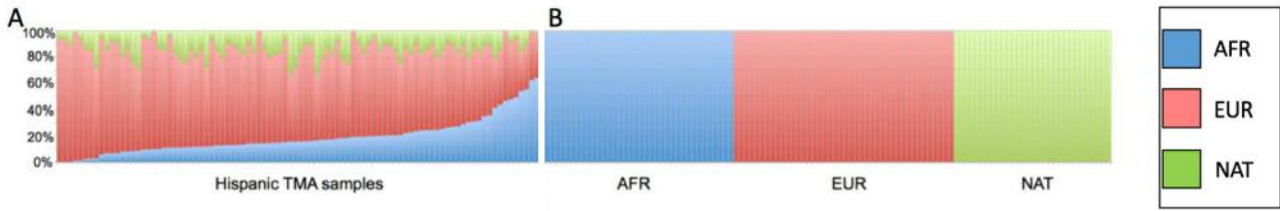


Figure 3. Genetic ancestry proportions of TMA samples from colonic adenoma and adenocarcinoma depicted by percentages of African (blue), European (red) and Native American ancestry (green) (A). Reference populations from African (AFR), European (EUR) and Native American (NAT) ancestry (B).

Table III. Mean (standard deviation, SD) ancestry proportions (%) for samples grouped by presence/absence of any protein (MSH2, MSH6, MLH1, PMS2). Normal tissues are excluded from the analysis (kept Ca, A and HGD).

	EUR	p-Value	AFR	p-Value	NAT	p-Value
Wild type	67.2 (15.3)		18.8 (14.6)		14.0 (7.9)	
MSI	66.1 (9.1)	0.7	19.0 (5.7)	0.9	14.8 (6.0)	0.3

Table IV. Mean (SD) ancestry proportions for samples grouped by presence/absence of MSH2/MSH6 and MLH1/PMS2.

	EUR	p-Value	AFR	p-Value	NAT	p-Value
Wild type	67.2 (15.3)		18.8 (14.6)		14.0 (7.9)	
MLH1/PMS2	66.8 (10.4)		19.7 (6.8)		13.5 (5.1)	
MSH2/MSH6	62.3 (9.0)	0.9	18.0 (3.2)	>0.9	19.7(12.2)	0.6

patients, however, patients with stage III (node-positive) disease can be considered for FOLFOX (folic acid, leucovorin, Fluro-Uracil, oxaliplatin) or XELOX (oxaliplatin plus capecitabine), with the latter combination being more toxic (23). Patients with MMR-deficient colorectal carcinomas have been documented to have resistance against fluorouracil-only treatment. However, patients with MMR-deficient colorectal carcinomas have a better prognosis compared to those with a proficient MMR system unless if the MMR deficiency was caused by a BRAF V600E mutation (24, 25). In the case of metastatic (stage 4) disease, immune checkpoint inhibitors are used as second-line treatment. Specifically, pembrolizumab was approved by the FDA in May of 2017 for the treatment of metastatic CRCs with MSI-high that is refractory to other conventional treatments. Pembrolizumab is a monoclonal antibody targeted against PD-1, and acts to activate the “shut down” immune response against the neoplasm. The programmed death-1 pathway is a negative feedback system that down-regulates T-helper cell activation via the programmed death-1 ligand (PDL-1) (26-29).

The ancestry analysis data presented herein describe similar ancestry proportions previously reported in genetic studies among the Puerto Rican population. MSI was considered high if any of the four proteins (MSH2, MSH6, MLH1 or PMS2) was absent in any of the cores. Since the study had a limited number of samples, the results may not be generalized to the entire Puerto Rican population. However, this is the first MMR study that reports frequency of protein expression in Puerto Ricans among the spectrum of colon cancer progression from non-neoplastic colon to pre-malignant ADs and to CRC. Furthermore, this is the first study done within a Puerto Rican region (Ponce) where the population was found to have a high percentage of Native Puerto Ricans as proven by the ancestry analysis. This study may be of interest when contemplating the application of the newer therapeutic approaches for CRC Puerto Rican patients and to decrease any cancer disparity it may exist for the diagnosis, therapy and surveillance of Puerto Ricans affected by this type of tumor.

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