Clinical and molecular characterization of colorectal cancer in young Moroccan patients

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Background/aims: *Early-onset colorectal cancers are relatively rare. About 20% of colorectal cancers are familial or hereditary. Two autosomal dominantly inherited cancer syndromes are more studied: Lynch syndrome accounts for 2-5% of colorectal cancers and familial adenomatous polyposis represents 1% of total colorectal cancers. Unlike the familial adenomatous polyposis syndrome, there are no clinical features that help in easily recognizing Lynch syndrome. The young age of cancer occurrence could be a criterion that should raise a suspicion of Lynch syndrome. In Morocco, the average age at diagnosis of colorectal cancers according to the register of cancers of Casablanca is 56 years, which is 10 years earlier than in European countries. Our study aimed to assess the frequency and molecular characteristics of the Lynch syndrome in Moroccan early-onset colorectal cancers patients.* **Materials and Methods:** *The population analyzed included 70 patients. The criteria for inclusion of patients in this study were a colorectal cancers before age 50 and the exclusion of familial adenomatous polyposis. We started by searching for microsatellite instability, first by immunohistochemistry of 3 mismatch repair proteins (MLH1, MSH2 and MSH6) and with second confirmation using 4 monomorphic markers (BAT25, BAT26, NR21, and CAT25).* **Results:** *We found instability in 10/70 (15%) of the cases. The loss of expression affects more often the MLH1 protein, with 8 cases, versus 2 cases of altered MSH2. None of the 70 patients of the series fulfilled the Amsterdam II criteria, indicative of Lynch syndrome.* **Conclusions:** *Further work needs to be done to discriminate hereditary cases from sporadic ones, but testing for microsatellite instability as a first step is important.*

Key words: Hereditary non-polyposis colorectal cancer, young Moroccan patients, microsatellite instability, MLH1, MSH2, MSH6

Genç Fas'lı kolorektal kanserli hastaların klinik ve moleküler karakterizasyonu

Giriş ve Amaç: *Kolorektal kanserin erken başlaması oldukça nadirdir. Kolorektal kanserlerin yaklaşık %20'si ailevi veya herediterdir. İki otozomal dominant kalıtılan kanser sendromu daha fazla çalışılmıştır: Lynch Sendromu kolorektal kanserin %2-5'inden sorumluyken, ailevi adenomatöz polipozis, bütün kolorektal kanserlerin %1'ini oluşturmaktadır. Ailevi adenomatöz polipozisin tersine, Lynch Sendromunu kolaylıkla tanımamıza yardımcı olan klinik özellik yoktur. Kanserin erken yaşta oluşması, Lynch sendromu şüphesini doğuran bir kriter olabilir. Fas'ta Kazablanka kanser kayıt sistemine göre ortalama kolorektal kanser tanı yaşı, 56'dır ki bu Avrupa ülkelerinden 10 yıl erkendir. Bu çalışmada Fas'lı erken başlangıçlı kolorektal kanser hastalarında Lynch Sendromu'nun sıklığı ve moleküler karakteristiklerini saptamayı amaçladık.* **Gereç ve Yöntem:** *Araştırılan popülasyon 70 hastadan oluşmaktaydı. Çalışmaya alınma kriterleri, 50 yaşından önce kolorektal kanser tanısı alan, ailevi adenomatöz polipozisin dışlandığı hastalardı. Önce mikrosatellit instabilitenin araştırılmasına başlandı, ilk olarak 3 MMR proteininin (MLH1, MSH2 ve MSH6) immünohistokimyası yapıldı, ikinci olarak 4 monomorfik belirteç (BAT25, BAT26, NR21 ve CAT25) kullanılarak konfirme edildi.* **Bulgular:** *Hastaların 10/70 (%15)'inde instabilite saptandı. Ekspresyon azalması en fazla 8 vakayla MLH1 proteinindeyken, 2 vakada MSH2 proteinindeydi. Serideki 70 hastanın hiçbirisi, Lynch Sendromu'nu gösteren Amsterdam II kriterlerini doldurmuyordu.* **Sonuç:** *Herediter vakaları sporadiklerden ayırmak için daha fazla çalışma yapılmalıdır, ilk basamak olarak mikrosatellit instabilite testi önemlidir.*

Anahtar kelimeler: Herediter nonpolipozis kolorektal kanser, genç Fas'lı hastalar, mikrosatellit instabilite, MLH1 MSH2 MSH6

INTRODUCTION

Early-onset colorectal cancers (CRC) are relatively rare. Several risk factors have been studied, but especially the genetic factors seem to be more associated in this cancer at a young age (1,2). About 20-25% of CRC are considered to be familial or hereditary, but germline mutations of known genes account for about 5% of the cases (3). Two autosomal dominantly inherited cancer syndromes are associated with this cancer: the Lynch syndrome, improperly called hereditary non-polyposis colorectal cancer (HNPCC), which accounts for 2-5% of total malignancies, and the familial adenomatous polyposis (FAP) (1% of total CRC) (2-4).

Two major pathways of carcinogenesis have been associated with sporadic CRC: The chromosomal instability (CIN) pathway, characterized by loss of heterozygosity and by chromosomal changes leading to alterations of tumor suppressor genes such as adenomatous polyposis coli (APC), and the microsatellite instability (MSI) pathway, associated with alterations of mismatch repair (MMR) genes such as *hMLH1, hMSH2* or *hMSH6* (2,4,5). Concerning hereditary forms, FAP and Lynch syndrome are of the CIN and MSI types, respectively. In cancers that follow the MSI pathway, instability of repeated sequences, called microsatellites, is observed and can be detected directly by amplifying monomorphic markers (6,7). Alternatively, immunohistochemical analysis of proteins corresponding to the MMR genes can be performed (6-8). Unlike the FAP syndrome, there are no clinical features that can easily distinguish Lynch syndrome. Several clinical criteria have been proposed to assist in making this diagnosis. In 1991, an international group established the Amsterdam criteria: Three or more family members with a confirmed diagnosis of CRC, one of whom is a first-degree relative (parent, child, sibling) of the other two; two successive affected generations (one of the patients is a first-degree family member of the other patients); one or more colon cancers diagnosed at younger than 50 years; and exclusion of FAP (9). These criteria are considered to be specific, but because of their low sensitivity, they have been revised several times. Nowadays, other criteria, called the Bethesda modified criteria, are used more generally (10). According to these new criteria, the young age alone is sufficient to raise a suspicion of Lynch syndrome.

In Morocco, the average age at diagnosis of CRC according to the register of cancers of Casablanca is 56 years (11). Our previous study confirmed the high rate of colon cancer among young people in Morocco (40.6%) (12). Our objective was to study the frequency and molecular characteristics of the putative Lynch cases in this Moroccan patient group.

MATERIALS AND METHODS

Patients

The criterion for inclusion of patients in this study was a CRC before 50 years of age with FAP excluded. A total of 70 patients were selected from the Department of Pathology in the University Hospital of Casablanca and a private laboratory (My Driss) in Casablanca. For each patient, pathological features were collected from pathology files.

Microdissection and DNA Extraction

Five sections (5 μm) from formalin-fixed paraffin-embedded tumor tissue were taken from all patients investigated. The DNA was extracted with MagneSil genomic fixed tissue system Promega kit.

Immunohistochemistry (IHC)

The immunohistochemical evaluation of MLH1, MSH2 and MSH6 protein expression was carried out on paraffin-embedded tissue sections of all tumors comprising adjacent normal mucosa. The antigen was revealed in 10 mM citrate buffer at 350 w for 30 minutes (min) in a microwave.

Mouse monoclonal antibodies against MLH1 protein (clone 168-15) and MSH2 protein (clone G129-1129, Pharmingen, San Diego, CA) at 1:100 dilution and the MSH6 protein (Transduction labs, BD Biosciences, Milano) at 1:2.000 dilution were incubated overnight. Immunoperoxidase staining, using diaminobenzidine as chromogen, was run with the NEXES Automatic Staining. Lack of expression of MLH1, MSH2 or MSH6 proteins was defined as complete absence of nuclear staining in tumor cells, while normal cells were stained.

Microsatellites Analysis

The MSI status was evaluated using 4 mononucleotide markers (BAT25, BAT26, NR24 and CAT25) (13). Using this panel of monomorphic markers, a tumor was defined as MSI-positive when at least 2 markers showed instability. Only cases with lack of expression of one of the MMR proteins (MLH1, MSH2 or MSH6) were analyzed for MSI. DNA from tumor tissue was amplified in a volume of 10 μl containing 50 ng of DNA, 0.15 pmol of dye labe-

led forward and unlabeled reverse primers, 2 mM of dNTP, 1.5 mM MgCl2, 2µl of Promega buffer (5x) and 0.125 µl of Gotaq. Thermocycling conditions were: a first denaturation at 94°C for 10 min followed by 30 cycles of a denaturation at 94°C for 45 seconds (s), an annealing step at 55°C for the markers: BAT25, BAT26, NR24 and 64°C for the CAT25 marker, during 45 s, and an extension step at 72°C for 45 s. A final extension at 72°C for 7 min was performed.

One µl of polymerase chain reaction (PCR) products of each marker was analyzed separately by adding 40 µl of deionized formamide and 0.5 µl of CEQ DNA size standard-400. Then, samples were run on CEQ 8000 sequencer and analyzed using the fragment analysis system by Beckman Coulter.

Statistical Analysis

The χ^2 test was used for assessing the statistical significance of differences between MSI+ patients and patients with stable tumors. A p value of <0.05 was considered significant.

RESULTS

Clinicopathological Results

The average age of these patients was 41.5 years (18-50 years); 62% of the patients were female. The mean age of the female and male patients was 41 and 42 years, respectively.

The cancers were located in the rectum in 42% of the cases. The right colon was affected in 16 patients (26%) and the left colon in approximately 32%. All cancers were adenocarcinoma in histologic examination and poorly differentiated in 15%, and mucinous adenocarcinomas and signet ring cells in 16.4%.

Immunohistochemistry (IHC) Results

Loss of MMR protein expression was detected in 10 cases (15%). In detail, 8 tumors showed complete loss of MLH1 expression (80%) and 2 carcinomas demonstrated complete loss of MSH2 expression (20%). From the 70 tumors, no tumors showed lack of MSH6.

Microsatellite Status

All the 10 colorectal carcinomas with loss of one of the MMR proteins showed instability of microsatellites (MSI+).

Clinicopathological Results

The mean age of the MSI+ patients was 39 years versus 41 years for the microsatellite stable (MSS) patients. The frequency of female gender in the MSI+ patients was 70%, while in the MSI- patients, 61.42% were women. 10% of adenocarcinomas with MSI+ phenotype were poorly differentiated versus 15.8% in MSI- patients. Mucinous adenocarcinomas and signet ring cells represented 30% in the MSI+ patients versus only 14% in the MSI- group (Table 1).

DISCUSSION

Our study aimed to assess the frequency and molecular characteristics of the Lynch syndrome in Moroccan early-onset CRC patients. We started by searching for MSI, because it is known that this is the carcinogenesis pathway of Lynch. However, this instability can also be found in a small percentage of sporadic CRC. Actually, about 15% of sporadic CRC shows MSI, which is not due to germline mutations of MMR genes, as in the case of cancers developed in the context of Lynch syndrome but by epigenetic modifications (14,15), more precisely, by hypermethylation of the MLH1 gene promoter, causing loss of protein function and then the same phenotype of MSI. To search for MSI, we chose to use IHC as the primary screening, because recent studies suggest similar effectiveness of this method compared to MSI (16) and especially because, with this technique, we can detect directly the gene most likely to be affected. Another principal reason is that MSH6 is associated with very low instability that cannot be detected with MSI (17).

Our results showed that the immunohistochemical analysis of MMR proteins and confirmation by using four monomorphic markers (BAT25, BAT26, NR21, and CAT25) revealed a rate of 15% instability (10/70). The loss of expression affects protein

Table 1. Clinicopathological features

MSI status	MSI +	MSI -	P value
Mean age (years)	39	41	0.86
Sex-ratio	0.42	0.66	0.62
Localization			
Rectum	50%	45.7%	0.76
Right colon	20%	24.28%	0.62
Left colon	30%	30%	-
Frequency of mucinous adenocarcinomas and signet ring cells phenotype	30%	14.28%	0.07

MLH1 more, with 8 cases, versus 2 cases of altered MSH2. This result is very important and confirms the interest of our study. Other similar studies reported similar frequencies (18). Whatever the origin of this MSI, testing for MSI is important. Several studies report that MSI is significantly related to improved prognosis independent of the other prognostic factors such as the stage and site of the tumor. The mortality is decreased in MSI+ patients (19-21). Many studies have found that Lynch patients have a more favorable prognosis than MSI+ patients with sporadic CRC (21-23). The MSI status is also related to sensitivity to the chemotherapy. It is shown that MSI+ patients are less responsive to the fluorouracil than those who are MSI-. Thus, fluorouracil adjuvant chemotherapy shows not only no benefit in MSI+ patients but may also decrease the survival of these patients (24).

With regard to the clinicopathological features,

the mean age of MSI+ patients was less than the mean age in the MSI- group (39 versus 41 years). Similarly, the frequency of female patients was more important in the MSI+ group (70% versus 60.3%). The mucinous adenocarcinomas and signet ring cells phenotype were associated with MSI+ phenotype (30% versus only 14.28%), but these differences were not significant (25).

In conclusion, none of the 70 patients fulfilled the Amsterdam criteria II and thus all were considered as sporadic cancers. However, the MSI rate of 15% is too much, and allows us to consider that the young age of patients with CRC is strongly associated with heredity. We intend to focus in a future work on the identification of germline mutations specific to the Moroccan population. Patients with MSI should benefit directly from genetic counseling. This will help us to provide better management of these patients and also to prevent other cancers in the family.

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