Approach to early-onset colorectal cancer: Clinicopathological, familial, molecular and immunohistochemical characteristics

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Abstract

AIM: To characterize clinicopathological and familial features of early-onset colorectal cancer (CRC) and compare features of tumors with and without microsatellite instability (MSI).

METHODS: Forty-five patients with CRC aged 45 or younger were included in the study. Clinical information, a three-generation family history, and tumor samples were obtained. MSI status was analyzed and mismatch repair genes were examined in the MSI families. Tumors were included in a tissue microarray and an immunohistochemical study was carried out with a panel of selected antibodies.

RESULTS: Early onset CRC is characterized by advanced stage at diagnosis, right colon location, low-grade of differentiation, mucin production, and presence of polyps. Hereditary forms represent at least 21% of cases. Eighty-one percent of patients who died during follow-up showed a lack of expression of cyclin E, which could be a marker of poor prognosis. β-catenin expression was normal in a high percentage of tumors.

CONCLUSION: Early-onset CRC has an important familial component, with a high proportion of tumors showing microsatellite stable. Cyclin E might be a poor prognosis factor.

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Key words: Early onset colorectal cancer; Microsatellite instability; Lynch syndrome; Microsatellite stable colorectal cancer

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INTRODUCTION

The prevalence of colorectal cancer (CRC) has been increasing during recent years. In 2004, it had the second highest incidence of all cancers and was the second most common cause of cancer-related death in the European Union[3]. Early onset CRC is infrequent, with an incidence of 2%-8% of all CRCs. In the United States, between 1992 and 2005, the incidence of CRC in young individuals (ages 20-49 years) increased at a rate of 1.5% per year in men and 1.6% per year in women[9].

Early onset of cancer is an indicator that a hereditary component is more likely. The most frequent hereditary form of CRC is Lynch syndrome. It is estimated to represent about 2%-5% of all CRCs, and is characterized by the development of CRC (and other types of cancer) at a mean age of 43 years[3,10]. Its molecular basis is a DNA mismatch repair (MMR) gene defect, which leads to changes in the length of repetitive DNA sequences, known as microsatellite instability (MSI).

The proportion of MSI tumors found in young patients varies between 19.7% and 41%, depending on the age of onset[10]. On the other hand, Lynch syndrome is estimated to cause about 1/3 of the CRC cases occurring at a young age[4,11,12]. There are some controversial aspects to the natural history and prognosis of early onset CRC, and some clinical and pathological differences compared to CRC in elderly patients[8]. Early onset CRCs are localized mostly in the right colon, are frequently poorly differentiated, show mucin production, and can develop synchronous and metachronous tumors[12]. These differences are more marked in cases with a family history suggestive of Lynch syndrome, or with molecular characteristics like MSI[13].

There is little information about microsatellite stable (MSS) forms of CRC in young adults, not only regarding their anatomoclinical features but also regarding their molecular characteristics. For example, there is an increased proportion of MSS tumors in young patients with rectal cancer[13]. Furthermore, several studies show that some alterations in molecular markers typical of MSS early onset CRCs also occur in sporadic cases of CRC, such as modified expression of APC, β-catenin and p53[9].

The aim of our study was to characterize early onset CRC by analyzing its clinical, pathological, familial, molecular, and immunohistochemical (IHC) features. We have determined the proportion of Lynch syndrome in our series, and have compared the characteristics of the MSS and MSI groups.

MATERIALS AND METHODS

Families, samples and data collection

A total of 45 individuals diagnosed with CRC at an age of 45 or younger were collected from two different Spanish institutions (Gregorio Marañón Hospital in Madrid, and Segovia General Hospital). All patients, or a first degree relative in case of death of the index case, provided written consent.

A full three-generation family medical history and colorectal paraffin-embedded tumors were obtained from each proband.

Personal and tumor clinicopathological information was obtained regarding age of onset, gender, location of the CRC (right/left colon or rectum), grade of cell differentiation (low, medium, or high), mucin production, modified Astler-Coller stage, the existence of polyps, and the presence of synchronous or metachronous CRCs. Mean follow-up was 60 mo.

To analyze the familial cancer history of each index case, we divided the neoplasms into two groups: Lynch syndrome-related tumors, and Lynch syndrome-unrelated tumors.

DNA extraction

A tissue specimen was obtained from the index case. Prior to DNA extraction, tumor and normal areas of the paraffin-embedded samples were selected via microscopic inspection. The proportion of tumor cells in the material used for DNA extraction exceeded 70% in all cases. DNA was extracted using proteinase K digestion, phenol-chloroform extraction, Phase Lock Gel™ Light (Eppendorf AG, Germany), and EtOH protocol precipitation.

MSI and MMR immunohistochemistry analyses

Microsatellite status was assessed using the BAT-26 mononucleotide marker, based on its high sensitivity[16-18]. However, in order to discard false negative results, all BAT-26 MSI cases fulfilling the Amsterdam I criteria[19], were analyzed using the Bethesda panel. BAT-26 was PCR amplified and fragments were evaluated using an ABI automated sequencer and GeneScan Software. For analysis of the Bethesda panel, we used the HNPPCC Microsatellite Instability Test kit (Roche, Mannheim, Germany).

IHC staining for markers of the following processes was performed: MMR; apoptosis; cell adhesion; cell cycle; proliferation, and others. Markers and clones used are shown in Table 1. All samples were fixed onto a tissue microarray, on which the IHC analysis was carried out.

Scoring of tumor staining was done without any knowledge of the patients’ family history or results of mutation analyses.

Detection of mutations and large deletions

Cases showing MSI and/or lack of expression of MMR proteins in tumors were screened for germline mutations in the DNA MMR genes MLH1 and MSH2, by denaturing gradient-gel electrophoresis (DGGE) on a DCode System (BioRad), using primers and denaturing conditions previously reported, with slight modifications[20]. Some samples were analyzed by denaturing high-performance liquid chromatography (dHPLC) (http://insertion.stanford.edu/melt.html). When an anomalous band pattern was observed by DGGE or dHPLC, a new PCR product of the fragment was sequenced, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed with a Genetic Analyzer ABI Prism 3130 (Applied Biosystems).
### Statistical analysis
Continuous variables were expressed as the median values plus/minus SD, and categorical variables were expressed as number of cases and their percentage. Differences were considered significant when $P < 0.05$. For associations between clinicopathological, familial and molecular features, and MSI status, statistical analyses were performed using Pearson’s $\chi^2$ test for parametric variables, and Fisher’s exact test for non-parametric variables. When those features were continuous variables, Student’s $t$-test was used, as well as for some familial features, to compare the differences between both groups. The SPSS v.11.5 for Windows (SPSS, Inc., Chicago, IL) statistical package was used.

### RESULTS

#### Clinicopathological features
We studied a total of 45 subjects diagnosed with CRC at an age of 45 years old or younger. Clinicopathological characteristics of all cases are shown in Table 2. A high proportion of tumors were located in the right side of the colon (45%). The proportion of poorly differentiated tumors was also notable (16%). The proportion of mucin-producing tumors (mucinous tumors and “signet ring” cell tumors) was 33% of all cases. More than 50% of all tumors were in an advanced stage when diagnosed, with lymph-node involvement and/or distant metastasis. Other remarkable features were incidence of polyps found during follow-up (33%) and three cases (7%) of synchronous and metachronous CRCs. Adenomatous polyps were the most frequently observed type of polyp (13/15).

Median overall survival was 60 mo, while Median Free-Disease Survival was 48 mo. Twenty-three point nine percent of the patients showed recurrence during follow-up, and overall mortality was 38% (17 patients).

#### Familial features
Table 2 shows the familial cancer history data. One patient was a case with familial adenomatous polyposis (FAP); 27 (60%) were sporadic cases with Lynch syndrome (without Lynch syndrome-associated neoplasms in their families), while 20% showed familial aggregation, and eight fulfilled Amsterdam II criteria. Apart from CRC, the other most frequent tumors were endometrial adenocarcinoma (six families) and gastric cancer (four families). Twelve families (27%) had other neoplasms not related to Lynch syndrome; the most frequent ones being breast cancer (four cases), larynx cancer (three cases), and leukemia (three cases).

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**Table 2 Clinical, pathological, and familial characteristics of the global group and the microsatellite stable and microsatellite instability groups n (%)**

<table>
<thead>
<tr>
<th></th>
<th>Global</th>
<th>MSS</th>
<th>MSI</th>
<th>$P (\chi^2)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>45 (100)</td>
<td>29 (69)</td>
<td>13 (31)</td>
<td></td>
</tr>
<tr>
<td>Median age of onset (range, yr)</td>
<td>39 (25-45)</td>
<td>41 (25-45)</td>
<td>37 (32-42)</td>
<td>0.03$^2$</td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23 (51)</td>
<td>13 (45)</td>
<td>7 (54)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>22 (49)</td>
<td>16 (55)</td>
<td>6 (46)</td>
<td>NS</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>20 (45)</td>
<td>11 (38)</td>
<td>9 (69)</td>
<td></td>
</tr>
<tr>
<td>Left colon</td>
<td>15 (33)</td>
<td>11 (38)</td>
<td>2 (16)</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>10 (22)</td>
<td>7 (24)</td>
<td>2 (15)</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>2 (4)</td>
<td>2 (7)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>36 (80)</td>
<td>23 (79)</td>
<td>10 (77)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>7 (16)</td>
<td>4 (14)</td>
<td>3 (23)</td>
<td>NS</td>
</tr>
<tr>
<td>Mucin production</td>
<td>15 (33)</td>
<td>5 (17)</td>
<td>5 (39)</td>
<td>NS</td>
</tr>
<tr>
<td>“Signet ring” cells</td>
<td>4 (9)</td>
<td>0/29 (0)</td>
<td>4/13 (30)</td>
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<td>Modified Astler</td>
<td></td>
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<td>Coller stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3 (7)</td>
<td>2 (7)</td>
<td>0 (0)</td>
<td></td>
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<tr>
<td>B</td>
<td>17 (38)</td>
<td>12 (41)</td>
<td>5 (39)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>15 (33)</td>
<td>7 (24)</td>
<td>6 (46)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>10 (22)</td>
<td>8 (28)</td>
<td>2 (15)</td>
<td>NS</td>
</tr>
<tr>
<td>Associated polyps during follow-up</td>
<td>15 (33)</td>
<td>10 (35)</td>
<td>4 (31)</td>
<td>NS</td>
</tr>
<tr>
<td>Synchronous or metachronous CRCs</td>
<td>3 (7)</td>
<td>1 (3)</td>
<td>1 (8)</td>
<td>NS</td>
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<tr>
<td>Familial history of cancer</td>
<td></td>
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<tr>
<td>Lynch-related neoplasms in family</td>
<td>18 (40)</td>
<td>5 (17)</td>
<td>11 (85)</td>
<td>&lt; 0.01</td>
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<td>Lynch unrelated neoplasms</td>
<td>12 (27)</td>
<td>8 (28)</td>
<td>7 (54)</td>
<td>0.02</td>
</tr>
<tr>
<td>Sporadic cases</td>
<td>27 (60)</td>
<td>24 (83)</td>
<td>2 (15)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

$^1$Proportions were calculated for 42 tumors; $^2$Statistical comparison between microsatellite stable (MSS) and microsatellite instability (MSI) groups was performed using Student’s $t$ test. CRC: Colorectal cancer; NS: Not significant.
Comparison between MSI and MSS tumors

Clinicopathological features: The comparison of the clinicopathological characteristics of the two defined groups (MSS and MSI) is shown in Table 2. Statistically significant differences were observed in the age of onset, which was earlier in the MSI cases, and in the presence of “signet-ring cell” tumors, which were absent in the MSS group. No statistically significant differences were found in the other variables analyzed, probably due to the small sample size. However, it is important to underline that the MSI CRCs were more frequently located proximally (69%), were poorly differentiated with higher mucin production, and were associated with other CRCs. The frequency of polyps during follow-up was the same in MSS and MSI tumors (35% and 31%, respectively). Another remarkable feature was that more than a half of all cases were diagnosed at an advanced stage (with lymph node involvement and/or distant metastasis, stages C or D): 52% for MSS and 61% for MSI.

There might be a trend towards a better prognosis for the MSI group when compared with the MSS group, but without reaching statistical significance: 62 mo vs 48 mo for median overall survival, and 62 mo vs 29 mo in terms of median disease-free survival. Mortality was higher in the MSS group (41%) than in the MSI group (31%).

Familial features: Table 2 shows familial cancer information and results obtained from the comparison of the MSS and MSI families. MSI cases are more frequently associated with Lynch-related neoplasia. On the other hand, the proportion of sporadic cases is very high in MSS tumors (83%).

Molecular features: The comparison of the IHC study in MSI and MSS tumors is shown in Table 4. None of the MSS tumors demonstrated lack of expression of MMR proteins in the IHC study. There was, as mentioned above, a good correlation between MSI and the IHC study of the MMR proteins.

Normal expression of β-catenin reached the same proportion (86%) in both types of tumors, indicative of the integrity of the Wnt signalling pathway in early age-onset CRCs. The high rate of MSI tumors with lack of expression of CK20 and RAD50 was also remarkable.

DISCUSSION

Early onset of CRC in young adults used to be considered to be rare, but many recent reports suggest not only that early onset CRC reaches 8% of all CRCs, but also that it might be increasing[1-2]. Similarly, it is a common belief that early onset CRC is mainly related to hereditary forms, especially to Lynch syndrome. In our study, the presence of a familial background of CRC is confirmed in 38% of the cases, 18% fulfilling Amsterdam criteria type II for Lynch syndrome. Hereditary forms of CRC were confirmed in nine patients of the present series. One was a FAP case with an APC germline mutation, and the other eight cases were Lynch syndrome. The rate of MSI in

Molecular features

Forty-two of the 45 cases were studied for MSI. The three excluded cases were: the FAP case, with an APC gene germline mutation (c.916delCT), and two additional cases, due to lack of paraffin embedded tumor tissue. Thirteen tumors (31%) showed MSI and the remaining 29 were therefore defined as MSS.

Blood samples were taken from the MSI index cases for MLH1 and MSH2 mutation screening. MSH6 was not studied because none of the tumors showed lack of Msh6 protein alone in IHC analysis. Eight of the 13 analyzed cases (62%) showed a pathogenic germline mutation in one of the MMR genes: three cases had a mutation in the MMR proteins. Eight of the 13 analyzed cases (62%) showed a pathogenic germline mutation in one of the MMR genes: three cases had a mutation in the MSS group (41%) than in the MSI group (31%).

Table 3 shows familial cancer information and results obtained from the comparison of the MSS and MSI families. MSI cases are more frequently associated with Lynch-related neoplasia. On the other hand, the proportion of sporadic cases is very high in MSS tumors (83%).

DISCUSSION

Early onset of CRC in young adults used to be considered to be rare, but many recent reports suggest not only that early onset CRC reaches 8% of all CRCs, but also that it might be increasing[1-2]. Similarly, it is a common belief that early onset CRC is mainly related to hereditary forms, especially to Lynch syndrome. In our study, the presence of a familial background of CRC is confirmed in 38% of the cases, 18% fulfilling Amsterdam criteria type II for Lynch syndrome. Hereditary forms of CRC were confirmed in nine patients of the present series. One was a FAP case with an APC germline mutation, and the other eight cases were Lynch syndrome. The rate of MSI in
our series is similar to that described in previous studies, ranging between 19.7% and 41% [7,8,9]. Twenty-nine cases showed MSS. This group showed a predominance of sporadic cases. Nevertheless, there were cases that showed a positive family history. Some of them might be associated to Familial CRC type X, namely cases with MSS tumors but fulfilling Amsterdam criteria for Lynch syndrome [21,22]. These findings, however, underline the important, but not unique role, of the known hereditary factors in this age group, prompting further searches for additional causative genes for inherited CRC.

The total sample of early age-of-onset CRCs is characterized by an important proportion of tumors that are localized in the right colon (44%), have a low-grade of differentiation (16%), produce mucin (33%), and have associated polyps (33%). Regarding pathological features, the strong trend towards low-grade differentiation and mucin production of tumors in this age group is described in the literature [7]. Other characteristics, such as synchronous and metachronous CRCs (7%) and the appearance of predominantly adenomatous polyps during the follow-up in a third of the cases, have rarely been studied previously [5,13].

Another finding is the advanced stage of the tumors at diagnosis, with more than half of the cases presenting with lymph node and/or distant metastasis in the pathological exam. This is the consequence of a delay in diagnosis, which is a characteristic of these early age-of-onset CRCs reported repeatedly in the literature [7,8,13].

The results obtained with the cyclin E antibody in the IHC study are quite remarkable. High levels of cyclin E, a G1/S phase transition controller, are found in many different tumors, including CRCs [23-25]. Cyclin E expression has only been evaluated in sporadic forms of CRC, with a variable value as a prognostic factor. Some publications suggest that lack of expression of cyclin E is associated with a faster growth of CRC, but others suggest the opposite [24,26,27]. Seventy per cent of our tumors showed lack of expression of this marker, especially the group of MSS CRCs, in which the proportion reached 86%, while the opposite occurred in the MSI group. The lack of expression of cyclin E might be associated with a poor prognosis, because expression was absent in most patients who died during follow-up (9/11), and all six stage D CRCs included in the IHC study also showed lack of expression of cyclin E. This was observed in both the MSS and the MSI groups, and in the latter group, the only case that showed lack of expression also died during follow-up. Although the sample size is small and the results must therefore be taken with caution, data related to cyclin E as a factor of poor prognosis must be validated in a larger series. It would be interesting to see if lack of expression of cyclin E corresponds with a subgroup of patients with apparently stable, near-diploid chromosomes and MSS (MACS); CRC in these patients shows an aggressive pattern and the MACS phenotype appears to be overrepresented in early-onset tumors [26,28].

Another important finding of the present study is related to the Wnt pathway. We found a high proportion of normal expression of β-catenin. This protein is an indicator of Wnt pathway dynamics [24], and the expression of β-catenin, mainly in the nucleus, is considered a good marker of the activity of the Wnt pathway [21,31,32]. The activation of this pathway often occurs in MSS tumors, but also in MSI tumors, independent of the age of onset [29]. Our findings, however, might indicate that the Wnt pathway is not involved in a substantial proportion of our early onset CRCs.

Early onset CRC is a heterogeneous group. To classify different subtypes with common etiology, the use of tools, such as MSI or IHC for MMR proteins, to identify an MMR system deficiency or an intact system (MSS) might be an appropriate and useful approach. All clinicopathological features analyzed in the global series tend to be more marked when the series is divided into MSI and MSS groups. MSI early onset CRCs showed characteristics similar to Lynch syndrome: earlier age of onset, predominant location in the right colon, and a high proportion of poorly differentiated tumors, in accordance with previous reports [8,13]. The same occurs with an elevated proportion of mucin-producing tumors (39%), and “signet-ring” cell tumors. MSS, on the contrary, are tumors that have an
older age of onset, are mainly located in the left colon, and have a low proportion of mucinous or “signet ring” cell tumors.

From the molecular point of view, our data must be confirmed in a larger series to reach more reliable conclusions. Nevertheless, some results should be emphasized. There is a good correlation between the lack of expression of MMR proteins and MSI. This is in agreement with published data, showing a positive prospective value of the IHC of 88%-100%[18,19]. Cyclin E is expressed in most of the MSI tumors, and the opposite occurs in the MSS, as published for sporadic CRC[20]. We found a normal expression of β-catenin in most MSI and MSS tumors. Our results contradict published findings for sporadic CRC, in which a high proportion of MSS tumors show an abnormal expression of β-catenin (90%), which decreases to 65% in MSI tumors[21,22]. There are few published studies focused on early onset CRC but in these the proportion of abnormal β-catenin expression is still significant (77.2% and 42.9% for MSS and MSI, respectively)[23]. Our results, however, should be confirmed by comparing them with control groups of CRCs, to exclude the possibility that technical differences or differences in interpretation of the staining patterns, might explain these contradictory findings.

Early onset CRC has an important proportion of hereditary forms of CRC. The apparent lack of involvement of the Wnt pathway is important, as is the possible value of cyclin E as a poor prognosis factor in early onset CRC. The advanced stage at diagnosis, as well as the still not fully understood group of MSS tumors, should promote a strong effort to diagnose these tumors at an earlier stage, providing a better understanding of MSS early onset CRC.

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APPLICATIONS

This is the first step towards a deeper understanding of early-onset CRC, an entity that is increasing in an especially sensitive group of population.

Peers review

The authors present an in depth analysis of young patients (< 45 years) presenting with CRC. Though the sample is small (n = 45) they perform a comparative analysis between those cases that are microsatellite stable and those that show microsatellite instability.

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