Original Article

# Cancerogenesis in colorectal neoplasms: Evidence from early onset colorectal cancer

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## ABSTRACT

**Objective:** Majority of colorectal cancers (CRC) happen via two distinct mechanisms of genomic instability: chromosomal and microsatellite instability. The proportion to which colorectal cancers belong to these pathways is well addressed in literature. However, there is much paucity and controversy regarding this proportion in early onset CRC; therefore, in the present study, major proteins involved in chromosomal and microsatellite instability pathways were determined in 104 early-onset CRC specimens. **Materials and Methods:** Outcome measures comprised expression of 4 mismatch repair (MMR) proteins (MLH1, MSH2, MSH6, PMS2), and two representative proteins of chromosomal instability pathway (P53 and β-catenin), which were determined by immunohistochemistry. **Results:** Twenty-nine cases (27.9%) had loss of expression of MMR proteins, of which 17 belonged to MutSα pathway and 12 to MutLα. Four tumors had solitary loss of PMS2. Tumors with abnormal MMR status were more likely to be right sided, and occurred mainly in familial setting ($P<0.05$). Seventy-four specimens (71.2%) had abnormal expression of P53 or β-catenin, of which 58 had P53 over-expression and 32 had abnormal β-catenin expression. There was an inverse association between P53 over-expression and abnormal MMR status ($P<0.05$). **Conclusions:** Taken together, our study demonstrated that loss of expression of MMR proteins happens more frequently in early-onset CRC, and on the contrary, the role of CIN pathway is less highlighted at the same time. Moreover, because of its ability to track the losses of expression of PMS2, IHC is recommended for determining the eligibility of mutation analysis of MMR genes, especially in younger ages.

**Keywords:** Chromosomal instability, colorectal neoplasms, DNA Mismatch Repair, early-onset

## INTRODUCTION

Two distinct mechanisms of genomic instability can give rise to colorectal cancers (CRCs): microsatellite instability (MSI) pathway and chromosomal instability (CIN).[1,2] MSI is a well-defined phenomenon that results from defects in DNA mismatch repair (MMR) system, and is responsible for 15% of CRCs.[3] These groups of tumors demonstrate high levels of microsatellite instability (MSI-H), and show defects in expression of certain proteins that take part in the MMR system such as MLH1, MSH2, MSH6, and PMS2.[3,4] MSI is the hallmark of hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome,[5] which is clinically defined by familial clustering of early-onset CRC and some other associated tumors. HNPCC is an inherited autosomal dominant disorder that constitutes 1–6% of CRCs;[6] however, this proportion can rise up to about 30% as the onset age of cancer decreases.[7] Accordingly, MSI has been reported to increase to more than 50% in early-onset CRCs, with peak incidence in the onset ages of less than 35 years.[8]

CIN, on the other hand, is believed to be responsible for 85% of CRCs, and is derived from a number of events at DNA level including mutations in mitotic checkpoint genes, microtubule spindle defects, and telomere dysfunction.[9] As a result, despite MSI-H tumors that barely have karyotype abnormalities, CIN tumors are in general aneuploid (or polyploid), have an abnormal karyotype, and are most often microsatellite stable (MSS).[10] CIN pathway is characterized by somatic mutations in tumor suppressor genes including adenomatous polyposis coli (APC) gene, and p53, and oncogenes like K-ras.[11] APC protein binds normally to β-catenin, and forms a complex with

Access this article online

Quick Response Code:

Website: www.ccij-online.org

DOI: 10.4103/2278-0513.99562

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axin and glycogen synthase kinase 3β (GSK-3β), which becomes degraded during process of ubiquitylation. This results in accumulation of β-catenin, which subsequently translocates from cell membrane to nucleus, and enhances the transcription of multiple genes that take part in tumor growth and invasion.[12] Mutations of β-catenin, however, can be found in 50% of sporadic tumors with intact APC.[13]

Studies on mutations and extent of expression of P53 in human cancers have improved our insight into their etiology and pathogenesis.[14] Mutations of p53 are identified in 40–50% of sporadic CRCs.[12] P53 level is normally kept below traceable quantities (by immunohistochemical staining) by some negative feedback mechanisms. Breakdown of these mechanisms during the process of cancerogenesis leads to accumulation of P53 to the traceable amounts inside tumor cells.[14] The frequency of positive P53 staining ranges from 45%–60% according to the reported data.[15-17]

Meanwhile, the genetic mechanism through which MSH-H tumors develop is different from that of CIN+ tumors; clinical and histopathologic features of these two types of CRCs differ to a large extent as well. MSI-H tumors express characteristic histopathology features including poor differentiation, marked lymphoid reaction, and mucinous histology.[18] Moreover, MSI-H tumors, in contrast to CIN+ tumors, tend more to be of proximal colon origin, are less aggressive, have better clinical outcome, show a rapid adenoma-to-carcinoma transformation, and prevail more in younger ages.[3,11,19]

Some of the below-mentioned markers are used in practice and some of them in new researches. These markers are categorized into three groups: 1) Diagnostic markers (less used in CRCs), 2) Prognostic markers: that can independently forecast clinical outcome, for example: high level of TS (Thymidylate Synthase),[20,21] loss of expression of DCC protein (Deleted in Colon Cancer gene),[22,23] expression of EGFR (Epidermal Growth Factor) Kras mutation, and BRAF mutation,[24-27] have been shown to convey poor prognosis in various subtypes of CRCs, 3) Predictive markers that can independently predict response to particular therapy: high level of TS shows lack of response to 5-FU both in primary and metastatic setting, EGFR mutation for eligibility for Erbitux (Cetuximab) and BRAF mutation for necessity of treatment with BRAF inhibitors like Sorafenib are among examples.[24,26]

It merits to be considered that the process of malignant transformation is quite different in a minority of colorectal cancers. These alternative pathways include TGF-β, serrated, and epigenetic pathways.[9,11,12] A body of evidence advocates the idea that these alternative pathways of carcinogenesis occur predominantly in younger patients,[28,29] or in a hereditary setting,[30] albeit some recent data contradict these findings.[31] So, there is the possibility that the extent to which MSI or CIN take part in the carcinogenesis of colorectal tumors will vary in early-onset CRC. But the fact is that data addressing this issue is so controversial. The traditional idea that MSI becomes more prominent in CRC as the age decreases is denied by some recent evidence.[32] In addition, the role of DNA aneuploidy, and increased expression of p53 and β-catenin, has been more highlighted in this setting.[33]

In populations with high prevalence of familial CRC, like where this study was undertaken,[34] institution of screening and surveillance programs needs a thorough understanding of ongoing mechanisms by which malignant transformation takes place at younger ages. Therefore, the present study was carried out aiming at determining the profile of MSI and CIN in early-onset CRC (onset ≤50 years of age). In this respect, we employed immunohistochemical staining for MLH1, MSH2, PMS2, and MSH6 as indicators of MMR status, and β-catenin and P53 as representatives of CIN pathway.

## MATERIALS AND METHODS

### Patients

Records of all early-onset CRC patients who were registered in the CRC Registry of Research Institute for Gastroenterology and Liver Disease (RIGLD, Shahid beheshti University of Medical Sciences, Tehran, Iran) from January 2004 to December 2008 were reviewed. Patients whose pathology slides and blocks were available at archives of pathology department of mentioned Institute were selected. Data regarding the age at diagnosis, gender, survival status, histopathology report, current medical condition, and location, grading, and staging of the tumors was abstracted from available RIGLD and hospital records of the patients, and was completed by telephone interview with them or their close relatives if necessary.

According to records, tumors were originally staged according to TNM system,[35] were graded according to the criteria of World Health Organization,[36] and were classified as proximal or distal in reference to the splenic flexure of colon. Patients with history of presurgical radiation therapy and inflammatory bowel disease were excluded, and finally, 104 patients were included in the study. Hematoxylin and eosin (HandE) slides were reviewed afterwards to complete some missing data such as tumor grading, and to confirm the original diagnoses.

### Immunohistochemical staining

One tumor specimen from each patient was used for immunohistochemical staining. Six sections (4 microns

thick) were obtained from each formalin-fixed paraffin-embedded tissue block. Sections were deparaffinized in xylene and were rehydrated in descending alcohol gradient. Blocking solution was used to block the endogenous peroxidase activity of samples. For antigen retrieval, samples that were due to be stained for p53 and β-catenin were boiled in citrate buffer (pH 6.0) in microwave oven. In case of samples assigned for MMR immunostaining, EDTA buffer (pH 9.0) was used. Slides were coded at this stage in order to preserve the anonymity of specimens and blindness of operating pathologist.

Sections were incubated in advance with primary antibodies against MLH1 (BD Biosciences Pharmingen, clone:G168-15, dilution 1:100), MSH2 (Calbiochem, Oncogene sciences, clone FE11, dilution 1:100), MSH6 (BD Trasduction Laboratory, clone: 44, dilution 1:1000), PMS2 (BD Pharmingen, clone:A16-4, dilution 1:500), P53 (DAKO, clone:DO-7), and β-catenin (DAKO, clone:β-catenin-1). After each step, slides were rinsed with TBS buffer for 3 minutes. Then, slides were treated with a streptavidin biotin immunoperoxidase complex (DAKO for p53 and β-catenin staining, REAL Envision for MMR IHC) for 20 minutes. To visualize immunoreactivity, 3,2′-diaminobenzidine was used and samples were counterstained with hematoxylin. Finally, specimens were dehydrated in ascending alcohol gradient.

All of the slides were examined blindly (two times) by one pathologist (MM). Normal epithelial cells, stromal cells, or intramucosal lymphocytes in the same slide were used as internal control for evaluation of immunohistochemical staining. Complete nuclear absence of any of MMR gene products was reported as abnormal MMR or MMR+ status. The whole IHC procedure was repeated exactly for all of MMR+ specimens. Expression of P53 was evaluated through a semi-quantitative method: samples were considered negative (normal) for P53 if less than an average of 10% of cells were stained for P53 in four high-power fields (40x), and positive (P53 over-expression) if more than this cutoff percent of cells were stained. Immunostaining for β-catenin was reported as normal if there was no nuclear staining and positive (abnormal) in case there was positive nuclear staining [Figure 1]. Specimens with abnormal staining for P53 or β-catenin will be referred to as CIN+ hereafter.

### Statistical analysis

Differences of distribution between the categorical variables were examined with Chi-square test and Fisher's exact test in case of need. For quantitative variables, Student's t-test was employed. Binary logistic regression analysis (with covariates of age, site, gender and family history included) was performed to determine if the rate of P53 over-expression in deficient MMR specimens was independent

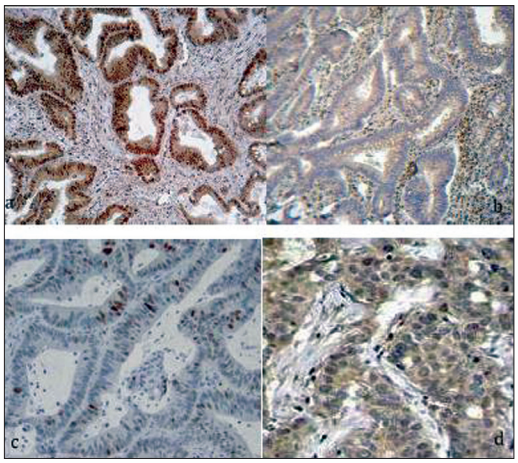


**Figure 1:** Immunohistochemical staining of tumors: a) normal nuclear staining for MSH2 in the tumor as well as in stromal tissue, b) loss of nuclear expression of MLH1 in tumor tissue but retained staining in stromal cells, c) nuclear staining for p53, and d) nuclear and cytoplasmic staining for β-catenin

form their site. Reported *P* values of less than 0.05 were considered to represent the statistical significance.

### Ethical considerations

This study was supervised by the Ethics Committee of Research Institute of Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences. The tissue samples and pathology slides were accessed with permission of the mentioned committee, and according to the original consent that was taken from each patient at the time of tissue sampling. Extreme care was taken to preserve the anonymity of patients during this study.

## RESULTS

### Clinical data

The study population comprised 104 CRC patients with mean age of 39.06 years (range 20–50, median 40.50, mode 39). Fifty patients were female (mean age: 38.76), and 54 were male (mean age: 39.33). A positive family history of CRC was recorded in 45 of the subjects. Thirty-seven cases (35.6%) had right-sided tumors, and the rest had left-sided tumors (rectum and left colon). Majority of patients had fulfilled 2 years of follow-up, but only seven were followed more than 3 years, of whom only two were followed up to 5 years. According to follow-up data, 21 patients had deceased as a consequence of CRC (distant metastasis or local recurrence).

### Staining patterns of mismatch repair proteins (MMRP)

Abnormal MMR staining was observed in 29 specimens (27.9%). MMR+ tumors tended more to be right sided, showed lower rates of P53 expression, and were likely to occur predominantly in the setting of a positive family history of CRC [Table 1]. Among tumors with abnormal MMR status, 12 specimens showed isolated or complex

**Table 1: Comparison of clinical data between normal and abnormal MMR groups**

| | Normal MMR (n=75) | Abnormal MMR (n=29) | *P* value |
|---|---|---|---|
| Mean age at diagnosis: year±SD | 39.17±7.53 | 38.76±7.43 | 0.801 |
| Age at diagnosis | | | 0.593 |
| ≤40 | 37 | 16 | |
| >40 | 38 | 13 | |
| Male:Female ratio | 0.92:1 | 1.6:1 | 0.198 |
| Family history of cancer | | | 0.004 |
| Positive | 26 | 19 | |
| Negative | 49 | 10 | |
| Location of the tumor | | | 0.002 |
| Proximal of colon | 20 | 17 | |
| Distal of colon and rectum | 55 | 12 | |
| TNM stage | | | 0.870 |
| Stage I | 5 | 3 | |
| Stage II | 31 | 10 | |
| Stage III | 31 | 13 | |
| Stage IV | 8 | 3 | |
| Grade of differentiation | | | 0.325 |
| Low | 29 | 15 | |
| Intermediate | 30 | 11 | |
| High | 16 | 3 | |
| P53 over-expression | 62.7% | 37.9% | 0.023 |
| Abnormal β-catenin | 33.3% | 24.1% | 0.362 |

staining abnormalities of MutLα complex (MLH1-PMS2 heterodimers): one solitary MLH1 defect, seven defects of both MLH1 and PMS2, and four isolated defects in staining for PMS2. Seventeen tumors had expression defects of MutSα (MSH2/MSH6) pathway, of which all had abnormal MSH2 expression and nine showed absence of MSH6 as well.

Among abnormal MMR+ tumors, the ones with defects in MutLα pathway showed differences from those with defects in MutSα complex. In comparison to the latter group, former tumors tended more to occur in male patients (91.7% vs. 41.2%, *P* 0.006, OR 0.064, 95% CI 0.007–0.612), and in ages over 40 (66.7% vs. 29.4%, *P* 0.047, OR 0.208, 95% CI 0.042–1.022). Moreover, tumors with abnormalcy of MutSα complex were more likely to express simultaneous over-expression of P53 (52.9% vs. 16.7%, *P* 0.047, OR 0.178, 95% CI 0.030–1.67). No statistically significant difference could be found between MutLα and MutSα pathways in terms of vital status, tumor site, staging and grading, and family history of CRC.

### Staining patterns of P53 and β-catenin

In general, 74 tumors (71.2%) showed staining abnormalities of P53 or β-catenin (CIN+). Over-expression of P53 was present in 58 tumors (55.8%), and 32 specimens (30.7%) showed abnormal expression of β-catenin. There was no statistically significant difference between tumors with and without CIN abnormality, P53 over-expression, and abnormal β-catenin regarding the age at diagnosis, gender, tumor site, grading, staging, family history of CRC, and vital status (data not presented).

Seventeen cases showed abnormal expression of both the MMR proteins and CIN pathway (CIN+/MMR+). The mentioned overlap area between two pathways mainly comprised specimens with a positive family history of CRC. Majority of these cases showed abnormal expression of P53, and some had defects in β-catenin [Table 2]. On the other hand, immunostaining was completely normal in 18 specimens (CIN-/MMR-). The only significant difference that could be found between these specimens and the rest of tumors was that CIN-/MMR- tumors were more likely to arise from right colon (70.6% vs. 29.4%, *P* 0.001, OR 5.952, 95% CI 1.900–18.647).

## DISCUSSION

The present study aspired to depict an overview of the quota of different pathways of carcinogenesis in early-onset CRC. In this respect, it could have been helpful to run same methodology on a sample of late-onset CRC patients to generate a reference point for comparison, but this was not feasible because of limitation of our resources. So, we have alluded to data of a recent study with similar methodology that was undertaken on general population of CRC patients[37] in order to generate an intelligible illustration of these differences.

As the reader may notice in the presented graph, the difference of share of various carcinogenesis pathways is the most significant in case of MMR pathway, which is more frequent in early-onset CRC. The proportion of alternative pathways (CIN-/MMR-), which are previously shown to comprise a distinct pathway in carcinogenesis of colorectal tumors,[36,38-40] is not different between CRC patients (as a whole) and early-onset CRC. While this finding is advocated by some studies,[38,41] some others have reported a much greater rate of CIN-/MMR- tumors in early-onset CRC.[36] Moreover, similar to some previous reports.[42] we could find no familial basis for this category of colorectal cancers.

Similar to MMR+ specimens, CIN-/MMR- tumors were shown in this study to be mainly of proximal colon origin. This is consistent with studies that address similar features for these two types of colorectal cancers.[43] Moreover, it highlights the importance of proximal colon in implementation of screening programs at younger ages. On the other hand, CIN-/MMR- tumors did not show any difference with MMR+ or CIN+ tumors regarding their level of differentiation and staging. This might imply that CIN-/MMR- tumors do not occupy a place in the succession of malignant transformation of other colorectal tumors;[37] in other words, they comprise a separate pathway.[42,44,45]

**Table 2: Characteristics of 17 specimens with abnormal immunostaining for both the CIN and MMR pathways**

| MutLα | MutSα | P53 | β-catenin | Family History | Tumor Site | Age at Diagnosis | Gender |
|---|---|---|---|---|---|---|---|
| Abnormal | - | Abnormal | - | Negative | Right | 26 | Male |
| Abnormal | - | Abnormal | - | Positive | Left | 45 | Female |
| Abnormal | - | - | Abnormal | Positive | Right | 38 | Male |
| Abnormal | - | - | Abnormal | Positive | Left | 48 | Male |
| Abnormal | - | - | Abnormal | Negative | Right | 45 | Male |
| Abnormal | - | - | Abnormal | Positive | Right | 41 | Male |
| - | Abnormal | Abnormal | - | Positive | Right | 39 | Male |
| - | Abnormal | Abnormal | - | Negative | Left | 43 | Female |
| - | Abnormal | Abnormal | - | Negative | Right | 25 | Female |
| - | Abnormal | Abnormal | - | Negative | Right | 40 | Male |
| - | Abnormal | Abnormal | - | Positive | Right | 34 | Female |
| - | Abnormal | Abnormal | - | Negative | Right | 20 | Male |
| - | Abnormal | Abnormal | - | Positive | Right | 40 | Female |
| - | Abnormal | Abnormal | - | Negative | Right | 43 | Female |
| - | Abnormal | Abnormal | Abnormal | Positive | Left | 26 | Female |
| - | Abnormal | - | Abnormal | Positive | Right | 49 | Female |
| - | Abnormal | - | Abnormal | Positive | Left | 45 | Female |

Data regarding the over-expression of P53 in early-onset CRC is scarce. In the present study, rate of over-expression of P53 was consistent with reports of CRC patients in general (45%–60%).[14-16] Moreover, this study showed an inverse relationship between MMR+ status and P53 over-expression, which was in accordance with previous studies that show lower rates of P53 over-expression in MSI+ CRCs in comparison to MSS tumors.[46-48] These studies consider the probability that lower rates of P53 expression in MSI+ tumors, might arise from right-sidedness of majority of MSI+ tumors.[46,47] This is not attributable to our results because on one hand, we could find no difference between proximal and distal tumors regarding P53 over-expression; on the other hand, according to logistic regression analysis, lower expression of P53 in MMR+ tumors was independent from their location [Table 3].

**Table 3: Logistic regression analysis indicated that difference of P53 over-expression between MMR+ and MMR-tumors was independent form their site**

| Covariates | *P* value | Odds Ratio | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Tumor Site | | | | |
| Right vs. Left colon | 0.218 | 0.244 | 0.026 | 2.301 |
| Gender | | | | |
| Female vs. Male | 0.024 | 0.077 | 0.008 | 0.714 |
| Family history of CRC | | | | |
| Positive vs. Negative | 0.063 | 0.119 | 0.013 | 1.119 |
| Age of onset | | | | |
| ≤40 vs. >40 | 0.206 | 0.252 | 0.030 | 2.132 |

It merits to be considered that the rate of P53 immunoreactivity would vary depending on ethnicity, type of underlying P53 mutation, technique of immunostaining, antibodies used, or cutoff values assigned for definition of over-expression.[38] This might partially explain the higher rates that we have reported for P53 over-expression in MMR-deficient tumors in comparison to average of 20%–28% reported by previous studies.[38,48] In addition, the concordance between P53 IHC and TP53 mutation analysis is a matter of debate as well. An association of about 70% has been recorded for these procedures.[39] This might explain why our results were closer to the studies that have used IHC for detection of abnormal expression of P53.[38]

But does simultaneous over-expression of P53 imply concurrent chromosomal instability in these MMR+ cases? Several studies have stated that MSI and CIN can take part in cancerogenesis of the same tumor.[44,49] Westra *et al*., reported high rates of TP53 mutation in MSI+ tumors; however, they also showed that majority of TP53 mutations were not accompanied by chromosomal aberrations in MSI+ colorectal tumors.[49] Given these data, the role of CIN is less highlighted in our CIN+/MMR+ specimens. This rationale puts extra emphasis on the role of MMR defects in cancerogenesis of early-onset CRC.

According to our data, defects of immunostaining for MMR gene products could be traced in up to 28% of early-onset CRC patients, which was much higher than reports for CRC patients in general.[3,40] Some recent evidence advocates such difference between early-onset CRC and the whole population of CRC patients;[41,43] besides, like the present study, the difference is more prominent with IHC of PMS2 also included.[41] But apart from the higher rates, our MMR-deficient specimens showed differences from previous reports in two other main aspects: higher rates of abnormal staining for MutSα heterodimers and higher rates of isolated loss of PMS2 immunostaining.

Normally, about 55% of MMR abnormalities are attributable to MutLα pathway, while defects of MutSα pathway include 45% of cases.[41,50] In the present study, deficient MutSα cases comprised 17/29 of specimens with abnormal MMR

status (58.6%). Because in this study all of MMR-deficient specimens were IHC double checked, the inversion of proportion of MutLα and MutSα can hardly be related to staining problems. Moreover, it has already been shown that germline mutations are usually responsible for defects in MutSα pathway,[51] whereas in case of MLH1, somatic hypermethylation of its promoter (which happens mainly in sporadic setting) can take part in addition to the germline mutations.[52,53] Thus, a possible explanation for the predominance of MutSα pathway in this study is the higher prevalence of familial CRC in early-onset CRC patients[40] and Iranian population in general.[34]

Solitary loss of PMS2 immunostaining was reported for 4/29 of MMR-deficient specimens in this study, which raises the notion of a possible added value of about 14% for IHC over MSI analysis in the present study.[40] Isolated loss of PMS2 expression can occur via germline mutations or somatic inactivation of PMS2.[54] In addition, mutations in MLH1 can lead to secondary loss of PMS2 expression while retaining MLH1 immunoreactivity;[55] therefore, PMS2 may help to identify families with subtle hereditary MLH1 mutations.[40,54-56] Gill *et al*., have shown that rate of isolated loss of PMS2 can reach up to 14% of MSI-H tumors as the diagnosis age of CRC falls below 60 years of age;[56] however, they have not determined the extent to which PMS2 defects can be missed by MSI analysis.

A recent review has stated that detection of PMS2 defects is an advantage for IHC over MSI analysis.[56] While immunostaining for PMS2 was recommended previously only in case of high suspicion for HNPCC in the absence of MSI, given an extra benefit of 23% in detecting the MLH1 mutations, a recent study recommended the inclusion of PMS2 staining in the panel of antibodies to identify families eligible for mutation analysis.[55] According to these benefits, Niessen *et al*., in a study on 281 early-onset CRC patients, concluded that IHC for MMR proteins is the best single method for determining the eligibility of CRC patients for mutation analysis of MMR genes.[41]

To conclude, early-onset CRC is more frequently associated with defects in MMR system. According to our results, the association of defects in proteins involved in CIN pathway (P53 and β-catenin) is less highlighted in early-onset CRC, while the quota of alternative carcinogenesis pathways remains similar to the population-based results.[57] Given our findings, IHC is recommended for tracking the eligibility of early-onset CRC patients for mutation analysis. In this respect, PMS2 should be entered into the panel of antibodies used for IHC of MMR proteins. In addition, according to the right-sidedness of majority of tumors with deficient MMR proteins and alternative pathways, extra emphasis should be put on proximal colon when screening programs are implemented at younger ages.

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**Cite this article as:** Molaei M, Mansoori BK, Ghiasi S, Nemati F, Almasi S, Fatemi SR, *et al*. Cancerogenesis in colorectal neoplasms: Evidence from early onset colorectal cancer. Clin Cancer Investig J 2012;1:57-64.

**Source of Support:** Nil, **Conflict of Interest:** None declared.