RESEARCH ARTICLE

Sporadic Early Onset Colorectal Cancer in Pakistan: a Case-Control Analysis of Microsatellite Instability

Sabeehuddin Siddique[1&], Kanwal Tariq[2&], Sobia Rafiq[2], Ahmed Raheem[1], Rashida Ahmed[1], Munira Shabbir-Moosajee[3], Kulsoom Ghias[2*]

Abstract

Background: Early onset sporadic colorectal cancer (CRC) is a biologically and clinically distinct entity hypothesized to exhibit differences in histological features and microsatellite instability (MSI) as compared to typical onset CRC. This study compared the MSI status, mismatch repair enzyme deficiency and clinicopathological features of early onset (aged ≤45 years) with controls (>45 years). Materials and Methods: A total of 30 cases and 30 controls were analyzed for MSI status using the Bethesda marker panel. Using antibodies against hMLH1, hMSH2 and hMSH6, mismatch repair protein expression was assessed by immunohistochemistry. Molecular characteristics were correlated with clinicopathological features. Results: The early onset sporadic CRCs were significantly more poorly differentiated tumors, with higher N2 nodal involvement and greater frequency of signet ring phenotype than the typical onset cases. MSI was observed in 18/30 cases, with 12/18 designated as MSI-high (MSI-H) and 6/18 designated as MSI-low (MSI-L). In the control group, 14 patients exhibited MSI, with 7 MSI-H and 7 MSI-L. MSI tumors in both cases and controls exhibited loss of hMLH1, hMSH2 and hMSH6. MSS tumors did not exhibit loss of expression of MMR proteins, except hMLH1 protein in 3 controls. No statistically significant difference was noted in MSI status or expression of MMR proteins in cases versus controls. Conclusions: Microsatellite status is comparable between early and typical onset sporadic CRC patients in Pakistan suggesting that differences in clinicopathological features between these two subsets are attributable to other molecular mechanisms.

Keywords: Colorectal cancer - early onset - microsatellite instability - mismatch repair

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Introduction

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in females and the third most commonly diagnosed cancer in males globally (Ferlay et al., 2015). Epidemiological studies indicate that the incidence and mortality due to CRC vary with the highest incidence of CRC reported in developed countries, but a higher mortality in the developing ones (Ferlay et al., 2015). A peak increase in incidence has been noted in economically transitioning countries (Center et al., 2009). This leads to the hypothesis that increase in CRC can be attributed to the environmental and dietary factors superimposed on genetic predisposition.

Age is one of the major risk factors for colon cancer with as many as 90% of cases being diagnosed in patients above 50 years of age (Jemal et al., 2010). Diagnosis under the age of 40-50 years is uncommon. Thereafter, the incidence increases sharply with each decade of life. An analysis by the Karachi Cancer Registry found Pakistan to be a low risk region with a crude incidence rate of 3.2 – 4.5% (Bhurgri et al., 2011). However, the ratio of patients diagnosed under the age of 40-50 years is much higher than the international average. While early onset cases account for only 7% of CRCs in more developed countries (O'Connell et al., 2004), they account for ~50% of cases in Pakistan (Bhurgri et al., 2011). The reason for this difference is yet to be elucidated.

Early onset of CRC is often attributable to a hereditary component, with Lynch syndrome being the most common hereditary form. This syndrome is characterized by germline mutations in DNA mismatch repair (MMR) genes, such as hMLH1, hMSH2, hMSH6, and hPMS2 (Peltomäki, 2005) that lead to changes in length of DNA fragments known as microsatellite instability (MSI) (Boland, 2005). However, 10% to 15% of sporadic tumors also exhibit MSI (Yoon et al., 2011).

In 1997, the National Cancer Institute validated a five marker panel for determination of MSI. This Bethesda marker panel includes two mononucleotide markers;

*[1]Department of Pathology and Laboratory Medicine, [2]Department of Biological and Biomedical Sciences, [3]Department of Oncology, Aga Khan University, Karachi, Pakistan [&]Equal contributors *For correspondence: kulsoom.ghias@aku.edu*

BAT25 and BAT26, and three dinucleotide markers; D5S346, D2S123 and D17S250 (Boland et al., 1998). Using this panel, a tumor is designated MSI high (MSI-H) if instability is observed in 30% or more of the five markers. Cases are designated as MSI low (MSI-L) and microsatellite stable (MSS) if less than 30% or no marker instability is found, respectively (Boland et al., 1998).

It has been hypothesized that early onset sporadic colon cancer is a biologically and clinically distinct entity from typical onset sporadic colon cancer. Given the noted increased incidence of early onset CRC patients in Pakistan, we compared the clinicopathological features and molecular characteristics, including microsatellite instability, of sporadic early onset and typical onset colon cancer cases in Pakistan.

Materials and Methods

Subjects: This was a single center retrospective case-control study approved by the institutional Ethical Review Committee. Cases and controls were defined as patients 45 years or younger and older than 45 years, respectively. Medical records and clinical details spanning 2003 – 2011 were reviewed for patients who had undergone surgical resection of histologically verified primary adenocarcinoma of the colon and rectum at The Aga Khan University Hospital in Karachi, Pakistan. Clinical data for 131 patients was retrieved, the details of which have been published previously (Zahir et al., 2014). Of this cohort, a total of 30 subjects each were selected for the case and control group depending on the following inclusion criteria: patients ≤45 years of age (cases) or >45 years (controls), without family history of CRC or suspicion of Lynch syndrome or inflammatory bowel syndrome, and enough tumor and adjacent normal tissue samples for molecular and immunohistochemical analysis. Formalin fixed paraffin embedded (FFPE) blocks of identified and consented cases and controls were obtained from the Department of Pathology and Laboratory Medicine.

Histopathologic assessment: Sections from the selected FFPE blocks were reviewed by histopathologists and assessed for tumor location, grade and histological sub-type. Tumors located before the splenic flexure were considered as right-sided.

Immunohistochemical analysis: For each case, a single FFPE block with tumor tissue and normal colonic tissue was selected for the detection of hMLH1, hMSH2 and hMSH6 proteins. In cases where normal and tumor tissue were not available in the same block, two separate blocks from the same patient were utilized. Tissue sections of 4μm thickness were incubated with concentrated monoclonal mouse anti-human antibodies against hMLH1 and hMSH2 and monoclonal rabbit anti-human antibody against hMSH6 (Dako, Denmark; dilutions 1:50, 1:600 and 1:100, respectively) on Dako Autostainer Link 48 (Agilent Technologies Company, Denmark) using EnVisionFLEX kit (Dako, Denmark). A positive reaction showed unequivocal nuclear staining of normal epithelial cells and neoplastic cells. Tumor cells without nuclear staining in the presence of a positive internal control were considered deficient for the antigen.

MSI analysis: For MSI analysis, histopathologists identified and marked areas with normal and tumor content on the relevant FFPE blocks. After micro-dissection, DNA was extracted from tumor tissue and from normal mucosa using protocol as described by Pikor et al. (2011). Extracted DNA was amplified using the 5 microsatellite primer sets for MSI markers Table 1) as outlined in the Bethesda panel. Tumor samples were classified as MSI-H, MSI-L and MSS accordingly. The forward primers were chemically labeled at 5' end with fluorescent dyes. PCR was performed in a total volume of 20μl using 100ng of genomic DNA, 1X PCR buffer (Promega, Madison, WI, United States), 250μM each deoxynucleotide triphosphate (Thermo Fisher Scientific Inc.), 0.5μM each primer, 1 unit of GoTaq polymerase (Promega, Madison, WI, United States) and 2.75nM MgCl2. PCR cycles for each marker were as in Table 2. Fluorescent PCR products were analyzed by capillary electrophoresis using an ABI 3730xl DNA analyzer (Applied Biosystems) and Peak Scanner Software 2.

Statistical analysis: Clinical data was collected using a retrospective chart review with a pre-designed and coded questionnaire. The Cox proportional hazard model was employed to compute prevalence ratios and 95% confidence intervals. Significance was set at the $P < 0.05$ level.

Table 1. PCR primers for MSI analysis

Marker	Forward primer	Reverse primer
BAT25	TCGCCTCCAAGAATGTAAGT	TCTGGATTTTAACTATGGCTC
BAT26	TGACTACTTTTGACTTCAGCC	AACCATTCAACATTTTTAACC
D2S123	AAACAGGATGCCTGCCTTTA	GGACTTTCCACCTATGGGAC
D5S346	ACTCACTCTAGTGATAAATCGGG	GCAGATAAGACAAGTATTACTAG
D17S250	GGAAGAATCAAATAGACAAT	GCTGGCCATATATATATTTAAACC

Table 2. PCR conditions for MSI analysis

Marker	Initial denaturation	PCR cycles	Final extension
BAT25	95° for 10 mins	35 cycle of 95°C for 1 min, 44°C for 1 min, 72°C for 1 min	72° for 10 mins
BAT26	95° for 10 mins	35 cycle of 95°C for 1 min, 42°C for 1 min, 72°C for 1 min	72° for 10 mins
D2S123	95° for 10 mins	35 cycle of 95°C for 45 s, 50°C for 45 s, 72°C for 45 s	72° for 10 mins
D5S346	95° for 10 mins	30 cycle of 95°C for 1 min, 44°C for 1 min, 72°C for 1 min	72° for 10 mins
D17S250	95° for 10 mins	35 cycle of 95°C for 45 s, 40°C for 45 s, 72°C for 45 s	72° for 10 mins

Table 3. Major clinicopathological features and patient characteristics of CRC cases and controls

	Cases (n = 30)	Controls (n = 30)	P value
Average age	36.93 (21-45)	63.7 (47-85)	
Gender			
Male	21 (70%)	24 (80%)	0.37
Female	9 (30%)	6 (20%)	0.37
Site			
Left	18 (60%)	16 (53.3%)	0.53
Right	12 (40%)	14 (46.6%)	0.46
Grade			
Well differentiated	3 (10%)	1 (3.3%)	0.7
Moderately differentiated	21 (70%)	29 (96.6%)	<0.001*
Poorly differentiated	6 (20%)	0 (0)	0.005*
Signet ring morphology	5 (16.6%)	2 (6.6%)	0.043*
Nodal status			
N0	15 (39.5%)	23 (60.5%)	0.06
N1	7 (58.3%)	5 (41.7%)	0.41
N2	7 (77.8%)	2 (22.2%)	0.017*
Nx	1 (100%)	0 (0%)	-
Clinical stage			
I	1 (3.3%)	5 (16.6%)	0.03*
II	15 (50%)	13 (43.3%)	0.61
III	10 (33.3%)	8 (26.6%)	0.67
IV	4 (13.3%)	3 (10%)	0.86

*Significant at the P < 0.05 level for 95% confidence interval

Table 4. Microsatellite status in CRC cases and controls

	Cases (n = 30)	Controls (n = 30)	P value
High (MSI-H)	12 (40.0%)	7 (23.3%)	0.16
Low (MSI-L)	6 (20.0%)	7 (23.3%)	0.75
Stable (MSS)	12 (40.0%)	16 (53.3%)	0.3

Table 5. Loss of mismatch repair protein expression with respect to microsatellite status in CRC cases and controls

	Loss of MMR protein expression			Overall P value
	hMLH1 (%)	hMSH2 (%)	hMSH6 (%)	
MSI-H tumors				
Case (n = 12)	9/12 (75)	10/12 (83.3)	10/12 (83.3)	
Control (n = 7)	6/7 (85.7)	6/7 (85.7)	6/7 (85.7)	
MSI-L tumors				
Case (n = 6)	5/6 (83.3)	2/6 (27.7)	2/6 (27.7)	0.21
Control (n = 7)	3/7 (42.8)	4/7 (57.1)	5/7 (71.4)	
MSS tumors				
Case (n = 12)	0/12 (0)	0/12 (0)	0/12 (0)	
Control (n = 16)	3/16 (18.7)	0/16 (0)	0/16 (0)	

Results

A cohort of 30 samples each was identified for the case (≤45 years at diagnosis) and control (>45 years at diagnosis) group (Table 3). The mean age at the time of diagnosis for the cases is 36.9 (range: 21-45) years and for controls is 63.7 (range: 47-85) years. A 3:1 male to female incidence ratio was observed in both cases and controls. Significantly more controls than cases had moderately differentiated tumors (P <0.001). No poorly differentiated tumors were found in controls as compared to 6 in the case cohort (P=0.005). Signet cell morphology was observed more in cases (16.6%) than controls (6.6%) (P=0.043). A significant proportion of cases had N2 nodal status as compared to controls. In cases and controls, the majority of patients had stage II and stage III disease at the time of diagnosis, however, more controls presented with stage I disease (P=0.03).

MSI was observed in 32 (53.3%) of the total patients with 19 (31.7%) classified as MSI-H and 13 (21.7%) as MSI-L tumors. Remaining 28 (46.7%) tumors were microsatellite stable. Equal numbers of cases (40% each) were MSI-H and MSS, while 53.3% of controls were designated as MSS tumors (Table 4). While more cases (40%) than controls (23.3%) were designated MSI-H, the difference was not statistically significant.

No differences were seen in major clinicopathological features and patient characteristics, including gender, tumor site, grade, morphology, nodal status and clinical stage, of cases and controls with respect to MSI status. The overall P value for each variable category (for e.g. nodal status) indicates that the differences were not statistically significant. Individual variables (for e.g. N0) also did not exhibit any statistically significant differences between cases and controls with respect to MSI status (data not shown).

Loss of MMR proteins (hMLH1, hMSH2 and/or HMSH6) was observed in all MSI tumors. MSI-H tumors more frequently showed loss of expression of these proteins than MSI-L tumors, irrespective of case or control cohort. Only 18.7% of MSS controls had loss of hMLH1 expression. No other loss of MMR protein expression was seen in either MSS cases or controls. However, there were no significant differences between protein expression in cases and controls categorized by MSI status (Table 5).

The overall sensitivity and specificity of immunohistochemical analysis for detection of MSI colorectal cancers was 93.7% and 89.2% for cases and controls, respectively. Positive predictive value was 90.9% and negative predictive value was 92.5%. The overall accuracy was 91.6% (P=0.001).

Discussion

Colorectal cancers can be stratified into two groups on the basis of molecular aberrations. The first group includes tumors with chromosomal instability and the second tumors with microsatellite instability. MSI is more frequently observed in patients with Hereditary Non-polyposis Colorectal Cancer Syndrome (HNPCC) or Lynch syndrome. However, approximately 15 to 20%

of sporadic CRC cases also demonstrate MSI.

Although the overall incidence of CRC is lower in developing countries, there is a significantly higher ratio of early onset to typical onset disease. Studies in the literature on Asian populations suggest that early onset CRC is a distinct entity characterized by presentation of advanced stage, poorly differentiated tumors, often found in the rectum. These characteristics have been reported in studies from India, Pakistan, Bangladesh, Nepal, Egypt, Jordan, Saudi Arabia, Morocco, Israel, Taiwan and Singapore (Singh et al., 2002; Al-Jaberi et al., 2003; Chiang et al., 2003; El-Hennawy et al., 2003; Ahmed et al., 2005; Guraya and Eltinay, 2006; Chew et al., 2009; Gupta et al., 2010; Shemesh-Bar et al., 2010; Bhurgri et al., 2011; Amini et al., 2013; Sekal et al., 2015). We also found significantly more poorly differentiated tumors and fewer moderately differentiated tumors in early onset CRC patients as compared to typical onset. While the majority of early onset CRC patients in this study were categorized as stage II or III at the time of diagnosis, the same trend was also observed in typical onset CRC patients. In the sporadic cancer cohort reported here, a 3:1 male to female ratio was observed irrespective of the age at the time of diagnosis. This is considerably higher that global average of approximately 1:1, although some studies have reported a higher proportion of incidence in males as compared to females (Center et al., 2009; Haggar and Boushey, 2009). In the current study, this ratio may be due to the limited sample size, as we have previously reported a 2:1 male to female ratio in a larger CRC cohort (Zahir et al. 2014).

This study aimed to determine the status of microsatellite instability and mismatch repair protein expression in sporadic early onset CRC as compared to typical onset CRC in the Pakistani population. We did not find any significant differences between the two groups. Earlier studies have reported variable results with respect to MSI in early onset CRCs, with early onset also inconsistently defined as age at diagnosis less than 40 – 50 years. Gryfe et al. (2000) reported that 17% of early onset cases (defined as <50 years of age) were designated as MSI-H tumors. In another study, 29.4% patients less than 40 years of age demonstrated MSI compared to only 6.3% patients of 60 years or more of age (Liang et al., 2003). Giraldez et al. (2010) reported MSI in 11.4% of early onset (<50 years) cases. Other studies have found no MSI or MMR protein loss in sporadic, early onset CRC (Dieumegard et al., 2000; Magnani et al., 2015) or differences in MSI status in early onset CRC as compared to typical onset, including a study from India (Raman et al., 2014).

MSI status was correlated with clinicopathological features, including tumor site, grade, stage and histological type. MSI tumors have been previously associated with certain predictive and prognostic features (Saridaki et al., 2014; Phipps et al., 2015; Andersen et al., 2016; Gatalica et al., 2016). No significant differences were observed with regards to gender in early or typical onset CRC exhibiting MSI in accordance with earlier published reports (Raman et al., 2014). However, other studies have found that more females have MSI tumors (Moghbeli et al., 2011). MSI-H colorectal cancers are usually located in the right or proximal colon and have particular phenotypic characteristics, including poor differentiation, mucinous type, Crohn's like lymphoid reaction and increased intraepithelial lymphocytes with an advanced clinical stage (Perea et al., 2010; Whitehall and Leggett, 2011; Michailidi et al., 2012; Kanth et al., 2014; Musulén et al., 2014). Albasri et al. (2014) described a predominance of stage B and C tumors and tumors in rectosigmoid region in the Tunisian population. Moghbeli et al. (2011) reported that MSI tumors were mostly proximally located and associated with lower stage in the Iranian population. Similarly, Giraldez et al. (2010) found MMR deficiency in proximal, well to moderately differentiated tumors and Karahan et al. (2015) reported that loss of MMR protein expression is associated with proximal, poorly differentiated tumors and mucinous phenotype. In the study reported here, MSI-H tumors were found predominantly on the left side, but this was not statistically significant. In addition, MSI-H was found to be associated with both lower and higher staged colorectal tumors. Tumors with poor differentiation and mucinous features were not limited to MSI-H tumors, and were seen in MSI-L and MSS tumors in both early and typical onset CRCs. This is similar to another earlier study that did not find any significant differences in tumor differentiation and presence of mucinous type between the MSI and MSS early onset tumors (Perea et al., 2010). Supporting the results of our study, Ziadi et al. (2014) also did not find any significant differences in tumor location, nodal involvement and stage.

Studies reported in the literature have shown that mononucleotide repeat instability is more sensitive and specific for mismatch repair deficiency and recent studies have advocated the use of five mononucleotide repeats as markers of MSI. In this study, using the Bethesda panel of five markers, including two mononucleotide and three dinucleotide markers, we found that the dinucleotide markers performed better in detecting the MSI-H tumors in both early and typical onset CRCs.

We also report high sensitivity and specificity of immunohistochemistry for MSI tumor detection. Previous studies have reported sensitivity ranging from 62%-89% for MSI detection (Hendriks et al., 2003; Shia et al., 2005; Barnetson et al., 2006; Niessen et al., 2006). Loss of hMLH1 is usually observed in CRC in an older age group (Herman et al., 1998; Veigl et al., 1998; Cheah et al., 2014). In contrast, in our study, loss of hMLH1 protein was frequently observed not only in typical onset CRC, but also in early onset CRC. Of note, a proportion of cases and controls in our study that showed loss of staining with hMLH1 antibody also had negative internal controls. Under such circumstances it is not possible to reliably comment on the mismatch enzyme deficiency. This is a drawback with immunohistochemical analysis for MMR, a fact that has been highlighted previously in a multicenter study (Müller et al., 2001). Furthermore, hMLH1 has a very low sensitivity (74%) due to a low rate of detection (Shia, 2008). Since hMLH1 forms dimers with hPMS2 and hMSH2 dimerizes with hMSH6, loss of these proteins usually occur in pairs. We found isolated loss of hMSH2 without loss of hMSH6 in both early and typical

onset CRCs, which has been reported previously in the literature (Giraldez et al., 2010; Ziadi et al., 2014). The cause for this observation needs to be evaluated further. However, it could be due to the fact that while there are mutations in the mismatch repair enzymes in MSI tumors, the expressed proteins still retain their antigenicity and react with the respective antibody giving false positive results. We also observed loss of expression of hMLH1 in three MSS tumors from the control typical onset CRC group. Giraldez et al. (2010) have similarly reported loss of hMLH1 and hMSH6 in MSS tumors.

Sample size was a limitation of this retrospective study due to availability of tissue samples. Another limitation is that we only investigated loss of hMLH1, hMSH2 and hMSH6 expression for mismatch repair. Even though isolated loss of hPMS2 is rarely reported (Gill et al., 2005; Mojtahed et al., 2011; Cheah et al., 2014), a lower hMLH1 immunohistochemistry sensitivity may necessitate hPMS2 detection.

In conclusion, although early onset sporadic colorectal cancer has been studied previously, there have been very few studies that have compared it to typical onset sporadic disease. In addition, molecular differences have not been previously profiled in the Pakistani population. In this study, we have shown that early onset sporadic CRCs present significantly with more poorly differentiated tumors, N2 nodal involvement and higher frequency of signet ring phenotype than typical onset sporadic CRC patients. Microsatellite instability and MMR protein expression patterns were similar in both early onset and typical onset sporadic CRCs and no significant clinicopathological differences were correlated with MSI status. This indicates that the comparative histological tumor profile observed in the early onset cohort could be a result of molecular features other than microsatellite instability. Future studies are needed to identify role of other molecular mechanisms in this population, such as chromosomal instability and the mutator phenotype, to identify causative and mechanistic differences between early onset and typical onset sporadic disease. Mutational profiling of frequently mutated genes in CRC may also provide further insight into the increased incidence of early onset colorectal cancers in Pakistan.

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