Identification of colorectal cancers with defective DNA damage repair by immunohistochemical profiling of mismatch repair proteins, CDX2 and BRCA1

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Received January 27, 2020; Accepted July 9, 2020

DOI: 10.3892/mco.2020.2128

Abstract. Colorectal cancer (CRC) is a complex disease as shown by consensus classification. The present study attempted to identify subtypes with known prognostic markers for better clinical management. A total of 72 CRC tumors were examined for the expression of mismatch repair (MMR) proteins, along with caudal-type homeobox protein 2 (CDX2) and BRCA1, by immunohistochemistry. Tumors were assigned based on the presence or loss of MMR proteins as proficient or deficient. Correlations were examined with CDX2 and BRCA1 along with clinico-pathological features. Expressional pattern of microRNAs (miRs/miRNAs), such as miR-183-96-182, known to be associated with defective DNA damage repair were evaluated by reverse transcription-quantitative PCR. A total of 22% of the CRC tumors were assigned as deficient in mismatch repair. 71% of the tumors expressed CDX2 while only 21% had nuclear expression of BRCA1. Loss of CDX2 protein was higher in the deficient subtype compared with the proficient subtype. A total of 14% of the tumors had dual loss of MMR and BRCA1 proteins and showed aggressive clinical features in addition to elevated expression of DNA damage repair microRNAs. The present study shows the presence of a small proportion of colorectal tumors with dual loss of key proteins involved in DNA damage repair which may be amenable to specific therapy. The implication of the present observations warrants investigation in a larger patient cohort with prognostic information.

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Key words: colorectal cancer, mismatch repair proteins, BRCA1, caudal-type homeobox protein 2, DNA damage repair, microRNA

Introduction

With 1.8 million new cases and almost 861,000 deaths in 2018 globally, colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females according to the World Health Organization (1). Improvements in earlier cancer detection and management, in combination with an increased understanding of the molecular and genetic basis of the disease will aid in better treatment decision and may also guide future therapeutic approaches (2).

The microsatellite instability (MSI) pathway is amongst the most important molecular pathways identified to cause CRC, along with other pathways, such as the chromosomal instability, CpG island methylator phenotype and serrated pathways (3). MSI is the accumulation of repeat length mutations in short DNA sequences and arises from defects in the DNA mismatch repair (MMR) system, which corrects any errors made by DNA polymerases during DNA replication (4). Tumours with MSI have a better prognosis than microsatellite stable CRC (5). Detection of MSI status performed using PCR-based methods or deficiency status of MMR proteins, including DNA mismatch repair protein Mlh1 (MLH1), DNA mismatch repair protein Msh2 (MSH2), DNA mismatch repair protein Msh6 (MSH6) and mismatch repair endonuclease PMS2 (PMS2), detected using immunohistochemistry (IHC) is routinely advocated to detect hereditary cancer, such as in Lynch syndrome, and also to predict the prognosis/chemotherapy response including the response to most recent immunotherapies (6).

CRC is known to be a heterogenous disease characterized by different molecular subtypes. Consensus molecular subtyping is most beneficial in the identification of a specific targeted therapy for both initial treatments and in metastatic settings (7-9). Similar attempts to classify CRC have been made by evaluating multiple markers, such as caudal-type homeobox protein 2 (CDX2), BRCA1, p53, adenomatous polyposis coli, β-catenin and other DNA repair proteins such as MMR proteins, O^6-methylguanine DNA methyltransferase and excision repair cross-complementing 1, and correlate them with survival and response to therapy (10). CDX2 has been well-established as a diagnostic marker for CRC and

its downregulation is associated with poor differentiation and MMR deficiency (11). However, less is known about the utility of BRCA1 in CRC, except in a few studies where the expression of BRCA1 predicting a better survival rate has been reported (12,13). The role of BRCA1 as a tumour suppressor gene was confirmed by its action in DNA damage repair (DDR), mediated mainly via homologous recombination-based pathways (14). Apart from mutations, epigenetic mechanisms, such as those mediated by miRNAs, are also known to mediate loss of BRCA1 function (15). miRNAs are small noncoding RNAs of 19-22 base pairs that are generated by a series of enzymatic processes in the nucleus and cytoplasm. Apart from affecting multiple cellular processes, several miRs, such as miR-16, miR-24, miR-188 and the miR-183-96-182 cluster have been implicated in DNA damage response and DNA repair (16,17). Defects in DDR drive cancer development by fostering DNA mutations, but also provide cancer-specific vulnerabilities that can be therapeutically exploited. The recent approval and use of multiple newer modalities of cancer therapy, such as using poly ADP ribose polymerase inhibitors through the approach of synthetic lethality, or checkpoint inhibitors and immunomodulatory drugs, have made them promising candidates for the treatment of multiple types of cancers (18-20).

Although the incidence of CRC in India is much lower than in the West, a higher proportion of right sided and grade III tumours has been reported and is associated with significant mortality and morbidity (21). With an interest to evaluate and correlate clinical features with known prognostic markers, the present study was performed on a retrospective series from the pathology archives. The present study analysed the association between MMR proteins, CDX2 and BRCA1 to determine the utility of their inter-relationship in the identification of subclasses amenable to specific therapies.

Materials and methods

Selection of primary colorectal cancer samples. Seventy-six colorectal cancer tumour blocks were identified and selected for the study. These cases were examined and reported by the Department of Pathology, St. John's Medical College and Hospital, Bangalore, India between 2013 and 2017. The present retrospective study was approved by the Institutional Ethical Committee, St John's Medical College and Hospital. Representative formalin-fixed and paraffin-embedded tumour blocks from each of the selected cases were obtained for the study. All tumours with a >50% tumour content, as estimated by a pathologist from the consecutive series, were chosen for analysis. Clinico-pathological characteristics, such as age, sex, grade, pathological and lymph node stage, histological type, lymphocytic response, lymphovascular invasion and tumour site were obtained from the clinical and histopathological records from the hospital.

Tissue microarray (TMA) construction and IHC of CDX2, BRCA1 and mismatch repair proteins MSH2, MSH6, MLH1 and PMS2. TMA was constructed using the Quick Ray manual tissue microarrayer (Unitma Co., Ltd.). A master block grid plan was made with adequate precautions to ensure an accurate orientation and unambiguous specimen identification. Block construction was performed according to the manufacturer's instructions. Two cores of 1.5 mm each were taken from each tumour block. Sections were cut and stained with hematoxylin and eosin (H&E) to confirm the adequate representation of each tumour. Cores with <100 interpretable tumour cells were excluded from the analysis.

IHC was performed for CDX2, BRCA1 and MMR proteins (MLH1, PMS2, MSH2 and MSH6), according to standard procedures. Briefly, 5-μm thick sections were placed on poly-L-lysine-coated slides and subjected to deparaffinization in xylene and rehydrated in graded alcohol. Following blocking endogenous peroxidase with a 3% hydrogen peroxide solution, antigen retrieval was performed in 0.01 mol/l EDTA buffer at pH 8 using a heat triggered multi-epitope retrieval system (PathnSitu Biotechnologies Pvt. Ltd.) for 15 min at 90˚C. Primary blocking was done with 1% BSA (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Details of the primary antibody clone, source and the dilutions are shown in Table SI. Primary antibodies were applied for 1 h at room temperature. Sections were further incubated with secondary antibody (cat. no. K5007; EnVision Detection System; Dako; Agilent Technologies, Inc.) for 20 min as per the kit instructions, followed by colour development using 3, 3-diaminobenzidine for 10 min. Sections were counterstained with haematoxylin for 5 min at room temperature and mounted after dehydration in graded alcohol and xylene. Appropriate positive and negative controls were run for each batch.

Evaluation of CDX2, BRCA1 and MMR proteins. For CDX2 and BRCA1 scoring, each tumour core was scored for intensity as follows: 0=no staining; 1=weak staining; 2=moderate staining; 3=strong staining. The percentage of cells stained was estimated from 0-100%. The histochemical score (H score range, 0-300) was calculated by multiplying the intensity and the percentage of staining. Although BRCA1 staining was observed in both the nucleus and the cytoplasm, only its nuclear presence was evaluated, indicating BRCA1 functional ability. A nuclear H score of ≥10 was considered as positive BRCA1 and CDX2 expression. The H score was used for the analysis of both proteins to obtain a quantitative estimate with a broad dynamic range from 0-300 (22). In the absence of any standard diagnostic criteria for proteins such as BRCA1 and CDX2, the H score method was followed for quantitation.

MMR proteins were scored only on the percentage of stained tumour cells, irrespective of staining intensity. Standard guidelines were followed where any presence of MMR proteins in the nucleus is considered adequate and acceptable to record as intact expression (23). Intact normal staining of non-tumour cells was considered as an internal positive control. Cases with ≥10% tumour cells showing nuclear staining were considered positive (intact expression). Weak focal nuclear stain in <10% of tumour cells was considered focal expression and a complete absence of nuclear stain in the presence of the positive internal control (lymphocytes and stromal cells) was considered negative (loss of expression). The dual loss of either MSH2 and MSH6 or MLH1 and PMS2, or the isolated loss of PMS2 was considered as an MMR-deficient group (dMMR). Intact/focal protein presence of either MSH2/MSH6 or MLH1/PMS2 was considered as an MMR-proficient group (pMMR).

H&E-stained microscopic sections of tumors were simultaneously evaluated by two trained pathologists to confirm the presence of above proteins in the tumor cells in comparison to normal cells (24). Each tissue microarray core was examined microscopically by the same pathologists independently to count ~1,500 cells in three different areas on the tissue section. The mean expression levels of both the observers were taken as final scores. In case of disagreement, the final score was determined by consensus after re-examination.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) of miRNAs. The methods used for nucleic acid extraction and RT-qPCR have been described in detail in our previous publication (25). In brief, two 20-μm sections taken from each patient's tumour block were deparaffinized using heat, and then subjected to overnight digestion using proteinase K (cat. no. 19133; Qiagen GmbH). Total RNA was then extracted using TRI reagent according to manufacturer's instructions (cat. no. T9424; Sigma-Aldrich; Merck KGaA). RNA quantification was performed using the Qubit RNA BR Assay kit (cat. no. Q10210; Invitrogen; Thermo Fisher Scientific, Inc.) on a Qubit 2.0 Fluorometer (cat. no. Q32866; Invitrogen; Thermo Fisher Scientific, Inc). Samples with a yield of ≥500 ng RNA and adequate transcript preservation to show amplification by RT-qPCR were used for subsequent experiments.

miRNA present in total RNA extracted as described above was converted to cDNA using stem-loop primers specific for the chosen miR, according to published protocols. Detailed methodology for quantification of miR using qPCR is provided in our previous publication (26). Briefly, 50 ng of total RNA was used for cDNA conversion using the TaqMan microRNA Reverse Transcription kit (cat. no. 4366596; Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression levels of a selected set of DDR miRs (miR183, miR96 and miR182) were determined, along with the control miR (RNU48). The assay IDs (cat. no. 4427975; Applied Biosystems; Thermo Fisher Scientific, Inc,) and sequences of the miRs are shown in Tables SII and SIII. Cycle threshold (Ct) values for the test miRs were normalized relative to the mean Ct value of the control miR for each sample as ΔCt. The relative normalized units of expression of the test miRs were calculated as 15-ΔCt, representing the dynamic range of the assay as being 15 Cts.

Statistical analysis. Descriptive analysis was used to tabulate the clinical variables amongst the various groups segregated based on the presence or absence of the chosen protein markers. All markers were expressed as the proportion of cases noted as positive or negative. Parametric or non-parametric tests of significance, such as paired Student's t-test or the Mann Whitney U test, based on the normality of distribution, were applied to determine the levels of significance in the distribution of chosen variables between the groups. The correlation between protein expression levels was calculated using Pearson's correlation coefficient. In the absence of prior available data for combined loss of BRCA1 and MMR proteins, sample size estimates were not attempted. Analysis was performed on XLStat software (version 2019.4.2; Addinsoft). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics of the cohort. The mean and median age of the CRC patients was 54.9 and 55 years, respectively. Of the 76 tumours studied, two were uninterpretable in CDX2 and BRCA1 IHC, one tumour yielded insufficient RNA and one tumour had loss of all MMR proteins, and therefore, only 72 (95%) tumours could be satisfactorily interpreted. A slight male preponderance was noted with 58% (42/72) in the group of tumours selected. The right and left sided tumours were equal in ratio. Most tumours belonged to stage II (38%) and stage III (43%). Details on the tumour grade were available for 70% (51/72) of tumours, and most (84%) of them were grade II tumours. Approximately 40% (29/72) of the tumours had a high lymphocytic response and lympho-vascular invasion was present in 43% (31/72) of tumours.

Of the 36 right sided tumors, 12 (33%) were mucinous tumors. The presence of the CDX2, BRCA1 proteins and the MMR status were further examined among mucinous and non-mucinous tumours. No statistically significant difference was observed between the two groups (data not shown).

Staining pattern and distribution of all proteins. CDX2 is known to be widely expressed in the large intestine (11) and showed nuclear staining with bright intensity. BRCA1 staining intensity was lower compared with CDX2 expression and varied across tumours. The intensity of MMR protein staining also varied widely.

Expression of MMR proteins. Among the four MMR proteins studied, MSH2 and PMS2 had intact/focal expression in 89% of the tumours. Table I details the expression levels of all the MMR proteins. MLH1 protein was detected in a small proportion of tumours (42%). MSH6 was always intact (60%) in the presence of MSH2, while the presence of PMS2 was observed in 34/42 tumours when MLH1 was absent. Tumours with the dual loss of MSH2 and MSH6 (11%) had intact protein status for either MLH1 and/or PMS2, and vice versa (10%). The dual presence of MLH1 and PMS2 was observed in 29% of the tumours, while only one tumour had isolated loss of PMS2. A total of 22% (16/72) of the tumours were categorized as dMMR, as they had a dual loss of MSH2/MSH6 or MLH1/PMS2, or the isolated loss of PMS2. Representative microscopic IHC images of all the MMR proteins showing positive nuclear stain are shown in Fig. 1A-D. As shown in Fig. 1, MLH1 presented with the lowest staining intensity compared to other MMR proteins. Table II shows the association of MMR status with clinical variables. None of the clinical variables were significantly different between the MMR proficient and deficient subtypes, aside from dMMR tumours, which had a higher lymph node spread (P-0.02). Among the dMMR tumours, 75% of them belonged to males and almost 70% of them belonged to stage III, although this was not statistically significant.

CDX2 and BRCA1 expression. An intense nuclear staining of CDX2 protein in >10% of the tumour nuclei was considered positive and observed in 71% (51/72) of tumours (Fig. 1E; H score, 100x3-300). Clinical variables, such as age, sex, grade or stage did not differ between CDX2-positive and -negative

Table I. Distribution of MMR protein expression in colorectal tumours.

IHC marker	Loss of expression	Intact expression	Focal expression
MLH1	42 (58)	22 (31)	8 (11)
PMS2	8 (11)	49 (68)	15 (21)
MSH2	8 (11)	57 (79)	7 (10)
MSH6	29 (40)	30 (42)	13 (18)
MLH1 and PMS2 dual loss	7 (10)	-	-
MSH2 andMSH6 dual loss	8 (11)	-	-
Isolated PMS2 loss	1 (1)	-	-
dMMR	16 (22)	-	-
pMMR	56 (78)	-	-

Data are presented as n (%). MMR, mismatch repair; dMMR, MMR deficient; pMMR, MMR proficient; MSH2, DNA mismatch repair protein Msh2; MSH6, DNA mismatch repair protein Msh6; MLH1, DNA mismatch repair protein Mlh1; PMS2, mismatch repair endonuclease PMS2.

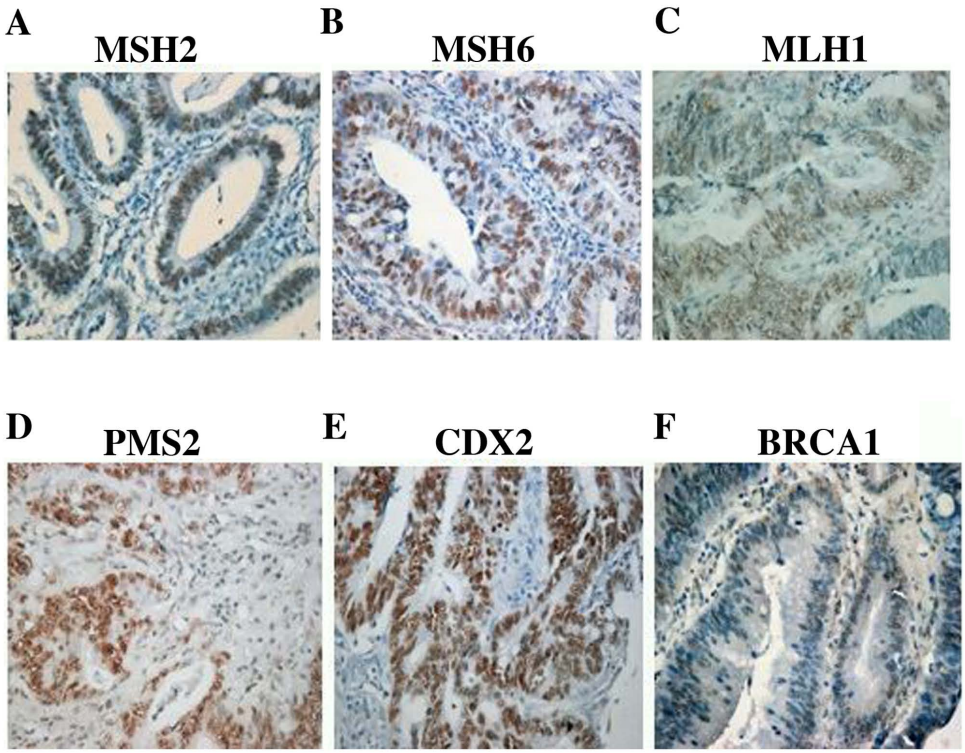

Figure 1. Representative microscopic images of MMR proteins, CDX2 and BRCA1. Nuclear staining of MMR proteins (A) MSH2, (B) MSH6, (C) MLH1 and (D) PMS2 in the presence of internal positive controls (lymphocytes and stromal cells). Nuclear staining of (E) CDX2 (H score, 100x3-300) and (F) BRCA1 (H score, 50x2-100) in tumour cells. Magnification, x20. MMR, mismatch repair; DNA mismatch repair protein Msh2; MSH6, DNA mismatch repair protein Msh6; MLH1, DNA mismatch repair protein Mlh1; PMS2, mismatch repair endonuclease PMS2; CDX2, caudal- type homeobox protein 2.

tumours (Table II), although a higher proportion of right sided tumours was CDX2-negative (62% in right tumours vs. 45% in left tumours; P-0.2). Two-thirds (67%) of the CDX2-negative tumours belonged to males and more than half of the CDX-positive tumours were lymph node-negative.

BRCA1 nuclear expression was observed in only 21% (15/72) of the tumour samples (Fig. 1F; H score, 50x2-100). Cytoplasmic expression of BRCA1 was observed in an additional 14% (10/72) of tumours; however, they were considered negative. No significant differences were seen for any clinical variables between BRCA1-positive or -negative tumours, although a higher proportion of BRCA1-positive tumours (60%) was lymph-node negative (Table II).

Association of MMR proteins with CDX2 and BRCA1 expression. Association of CDX2 loss is often reported to be high in MMR-deficient tumours (10,11). In the present study, 44% (7/16) of dMMR tumours showed loss of CDX2, compared with 25% (14/56) observed in pMMR tumours (P-0.1). However, BRCA1 and MMR were inversely correlated, with 38% (6/16) of the dMMR group expressing BRCA1 protein, compared with only 16% (9/56) in the pMMR

Table II. Distribution of CDX2, BRCA1 and MMR proteins amongst clinical variables.

Marker	CDX2		BRCA1		MMR	
Staining	Positive, n-51 (71%)	Negative, n-21 (29%)	Positive, n-15 (21%)	Negative, n-57 (79%)	Proficient, n-56 (78%)	Deficient, n-16 (22%)
Variable						
Age						
Mean	55.8	52.9	55.6	54.8	54.7	55.7
Median	56	55	60	55	55	58
Sex						
Male	28 (55)	14 (67)	8 (53)	34 (60)	30 (54)	12 (75)
Female	23 (45)	7 (33)	7 (47)	23 (40)	26 (46)	4 (25)
Tumor site						
Right	23 (45)	13 (62)	8 (53)	28 (49)	27 (48)	9 (56)
Left	28 (55)	8 (38)	7 (47)	29 (51)	29 (52)	7 (44)
Grade						
I	2 (5)	1 (10)	0 (0)	3 (7)	2 (5)	1 (10)
II	36 (88)	7 (70)	9 (90)	34 (83)	37 (90)	6 (60)
III	3 (7)	2 (20)	1 (10)	4 (10)	2 (5)	3 (30)
LN status						
N0	29 (57)	10 (48)	9 (60)	30 (53)	34 (61)	5 (31)[a]
N1	14 (27)	4 (19)	4 (27)	14 (25)	14 (25)	4 (25)
N2	8 (16)	7 (33)	2 (13)	13 (23)	8 (14)	7 (44)
Stage						
I	7 (14)	3 (14)	2 (13)	8 (14)	8 (14)	2 (13)
II	20 (39)	7 (33)	7 (47)	20 (35)	24 (43)	3 (19)
III	21 (41)	10 (48)	6 (40)	25 (44)	20 (36)	11 (69)
IV	3 (6)	1 (5)	0 (0)	4 (7)	4 (7)	0 (0)

Data are presented as n (%). [a]P<0.05, derived from paired two-tailed Student's t-test. LN status, lymph node status; MMR, mismatch repair; CDX2, caudal- type homeobox protein 2.

subgroup (P-0.06).When the expressional pattern of BRCA1 and CDX2 were compared as H scores, a negative correlation was found between the two proteins (Pearson's correlation coefficient, -0.13; P-0.2), as expected.

Combined loss of BRCA1 and MMR proteins show a higher expression of DDR miRNAs. Subsequently, the tumours were divided into two separate classes based on the dual presence and absence of BRCA1 and MMR status. While two-thirds of the tumours (53/72) showed either BRCA1 or MMR protein expression, only a small proportion of tumours (12.5%) had the combined presence of both. A small subset (10/72; 14%) of tumours was both BRCA1 negative and MMR deficient. When the clinical variables were compared between these groups (Table III), tumours with the combined loss of BRCA1 and MMR proteins showed aggressive features, such as a younger age, male preponderance and a higher proportion of grade III and stage III tumours. There was no difference in CDX2 expression between the two groups.

When the distribution of the miRs implicated in defective DDR (miR-183, miR-96 and miR-182) was examined in the two groups, tumours with the combined loss of BRCA1 and MMR showed trends of higher expression of all the three miRs (P-0.01, miR-182), as shown in Table III, indicating defective pathways for DNA damage repair in these tumours.

Discussion

CRC is the most common malignancy in the western world and is associated with a significant morbidity. Among the different molecular pathways involved, although MSI contributes to less than one quarter of CRC cases, defects in MMR proteins have been implicated both in carcinogenesis and CRC progression (27). Although 90% of hereditary CRC present with MSI, it is limited to 15-20% of sporadic tumours (28). The present study showed that 22% of tumours presented with MMR deficiency, consistent with previously published studies (21,29). An earlier study on a cohort from a similar setting reported a deficient MMR prevalence of 22.9% (21). The present study observed loss of MLH1 protein in more than half of tumours of the cohort. Weaker staining patterns of MLH1, patchy and heterogenous staining pattern of MMR proteins, inactivation by promoter methylation and mutations are considered as multiple reasons for loss of MLH1 (30). The

Table III. Comparison of clinical variables between tumors with dual loss of BRCA1 and MMR with other tumors.

Variable	Dual loss of BRCA1 and MMR, n-10 (14%)	Others[a], n-62 (86%)	P-value
Age			NS
Mean	50.9	55.6	
Median	50.5	55.5	
Sex			NS
Male	8 (80)	34 (55)	
Female	2 (20)	28 (45)	
Tumor site			NS
Right	5 (50)	31 (50)	
Left	5 (50)	31 (50)	
Grade			0.072
I	1 (14)	2 (5)	
II	3 (43)	40 (91)	
III	3 (43)	2 (5)	
LN status			0.036[b]
N0	3 (30)	36 (58)	
N1	2 (20)	16 (26)	
N2	5 (50)	10 (16)	
Stage			NS
I	2 (20)	8 (13)	
II	1 (10)	26 (42)	
III	7 (70)	24 (39)	
IV	0 (0)	4 (6)	
CDX2			NS
Positive	4 (40)	17 (27)	
Negative	6 (60)	45 (73)	
miR-183			
Mean	8.3	7.7	
miR-182			0.01[b]
Mean	10.2	9.3	
miR-96			
Mean	7.4	6.8	

Data are presented as n (%). [a]Others, BRCA1 positive with/without MMR expression and BRCA1 negative with MMR expression. [b]P<0.05, derived from paired two-tailed Student's t-test or Mann-Whitney U test. MMR, mismatch repair; dMMR, MMR deficient; pMMR, MMR proficient; CDX2, caudal-type homeobox protein 2; miR, microRNA; NS, not significant.

results of the present study are consistent with observations found in previous studies (30,31).

CDX2, a nuclear transcription factor implicated in CRC prognosis, was also found to be lowly expressed (56%) in MMR deficient tumours and a higher proportion of CDX2 negative tumours was right-sided. While some reports (32) have shown similar presence of CDX2 positivity in 50% of dMMR tumours, other reports indicated that the low/loss of CDX2 expression was significantly associated with MMR deficiency and with right-sided tumours (10,33).

With the advent of therapeutics that can target tumours with BRCA1/2 mutations , CRC tumours are also investigated for mutations in these genes. Although MMR and BRCA1 status is investigated independently, their combined loss has been reported in very few studies. A recent study (34) has shown that BRCA1 mutations are present in 1.1% of CRC tumours using next-generation sequencing on a 592-gene panel, which was the first study to show that BRCA1/2 mutations are more frequent in MSI high (MSI-H) tumours, and independently associated with higher tumour mutational burden. However, other studies have performed combined testing for BRCA1/2 mutational screening, along with MMR proteins, while screening for hereditary cancers (35). Other studies have reported IHC detection of BRCA1 in CRC (12,13). A higher proportion of BRCA1 staining in these reports may be due to both cytoplasmic and nuclear staining being considered

as positive. The present study considered nuclear presence in >10% of cells as BRCA1 positive, which perhaps is the reason for the low BRCA1 positivity observed.

The tumours in the present study, with dual loss of BRCA1 and MMR, showed a high expression of miRs (miR-183-96-182 cluster) implicated in DDR, in addition to other aggressive features. This cluster is proven to be highly expressed in CRC tumours, promoting tumorigenesis, cancer progression and metastasis (36). These DDR miRs impair the homologous recombination mediated DNA repair by acting as negative regulators of the genes involved in the DDR pathway and may serve as predictive biomarkers for prognosis and potential therapeutic targets in CRC treatment (37). The present study, a retrospective CRC collected from pathology archives, has several limitations. A small sample size and TMA design restricting the evaluation of MMR proteins, which are known to be heterogenous in expression, may have an impact on the MMR and BRCA1 status. Moreover, although BRCA1 antibody (clone-MS110) is the most widely used, it has issues with specificity and sensitivity (38). While other studies have shown association of favourable prognostic factors, such as young age, well-differentiated tumours and low lymph node positivity with dMMR status, the present study observed a significant association with a higher lymph node positive status, which could be due to a higher proportion of stage III disease (43%) in the present cohort. Although the present study showed that the incidence of MSI differs between stage II and stage III diseases, stage-specific analysis was not performed due to the small sample size. A lack of prognostic information on the present CRC series has limited ability of validating the effect of dual loss on clinical progression and requires validation in a larger series with a clinical outcome.

Newer modalities of treatment with immune checkpoint inhibitors are approved for treatment of colorectal cancers with MSI-H (6). However, the approval is limited to refractory mismatch deficient colorectal tumours in a metastatic setting (20). Although extensive efforts to explore additional combination therapies are ongoing, not all patients uniformly benefit from newer therapeutic modalities, which showcases the need to identify predictive biomarkers for a better selection of the patients (2). The present approach of using IHC-based markers to identify prognostic subtypes shows an easy, novel, and adaptable approach for subtyping colorectal cancer and should be further investigated in a larger series to confirm its relevance.

Acknowledgements

Not applicable.

Funding

This study was supported by the Department of Biotechnology (DBT), India (grant no. BT/MED/30/SP11263/2015).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SR performed miRNA experiments and data analysis. ACE was involved in the construction of TMA, performing IHC and sample collection. MC reported the histological details of tumors. BJ identified the tumor blocks and collated clinical information. SS conceived and designed the study. JSP was involved in interpretation of all markers performing histological examination, analysis of data, conception and design of the study and drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Institutional Ethical Committee, St John's Medical College and Hospital, Bangalore and approved by the same institute. Waiver of consent has been approved for this study since this was a retrospective study that was planned on specimens received for routine diagnostic purpose in the Department of Pathology, St John's Medical College and Hospital, Bangalore. All details of personal information regarding patients were blinded to the investigators. There was no direct contact between the researcher and participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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