RESEARCH ARTICLE

Colorectal Carcinoma in Malaysians: DNA Mismatch Repair Pattern in a Multiethnic Population

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Abstract

Background: The interesting preponderance of Chinese with colorectal carcinoma (CRC) amongst the three major ethnic groups in Malaysia prompted a study to determine DNA mismatch repair (MMR) status in our CRC and attempt correlation with patient age, gender and ethnicity as well as location, grade, histological type and stage of tumour. Histologically re-confirmed CRC, diagnosed between 1st January 2005 and 31st December 2007 at the Department of Pathology, University of Malaya Medical Centre, were immunohistochemically stained with monoclonal antibodies to MMR proteins, MLH1, MSH2, MSH6 and PMS2 on the Ventana Benchmark XT autostainer. Of the 142 CRC cases entered into the study, there were 82 males and 60 females (M:F=1.4:1). Ethnically, 81 (57.0%) were Chinese, 32 (22.5%) Malays and 29 (20.4%) Indians. The patient ages ranged between 15-87 years (mean=62.4 years) with 21 cases <50-years and 121 ≥50-years of age. 14 (9.9%) CRC showed deficient MMR (dMMR). Concurrent loss of MLH1 and PMS2 occurred in 10, MSH2 and MSH6 in 2 with isolated loss of MSH6 in 1 and PMS2 in 1. dMMR was noted less frequently amongst the Chinese (6.2%) in comparison with their combined Malay and Indian counterparts (14.8%), and was associated with right sided and poorly differentiated tumours (p<0.05). 3 of the 5 (60.0%) dMMR CRC cases amongst the Chinese and 1 of 9 cases (11.1%) amongst the combined Malay and Indian group were <50-years of age. No significant association of dMMR was noted with patient age and gender, tumour stage or mucinous type.

Keywords: DNA mismatch repair - microsatellite instability - ethnic - colorectal carcinoma - Malaysia

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Introduction

Colorectal carcinoma (CRC) is the most common malignancy in Malaysian males and third most common in females (Lim et al., 2008). Malaysia shows a unique racial distribution of its population with multiple ethnic groups, the three major being Malays, Chinese and Indians. The Malays constitute about 63%, Chinese 25% and Indians 7% of the total population (Population and Housing Census, Malaysia, 2010). Interestingly, age-standardised incidence per 100,000 population for cases of CRC diagnosed in 2003-2005 demonstrated a preponderance of Chinese (31.5, 15.7 and 12.3 for Chinese, Indian and Malay males respectively and 26.2, 12.9, and 9.7 for Chinese, Indian, and Malay females). Whilst the Indians and Malays shared a seemingly close age-standardised incidence per 100,000 population, the Chinese had almost double the rate of CRC compared with the other two races. Nonetheless, the age-standardised incidence rate of CRC for the Malaysian Chinese corresponded closely with their counterparts in Hongkong and Taiwan. A similar trend of the age-standardised incidence rates between Chinese, Indians and Malays, is also seen in neighbouring Singapore with one minor variation viz Singaporean Malay males had a higher incidence rate compared with Singaporean Indian males but both remained below Singaporean Chinese males (Lim et al., 2008). Ethnicity in Malaysia also plays a role in knowledge about colorectal cancer and screening behaviour (Yusoff et al., 2012; Al-Naggar and Bobryshev, 2013; Loh et al., 2013)

Apart from the well-recognised chromosomal instability pathway of colorectal carcinogenesis involving key oncogenes and tumour suppressor genes e.g. adenomatous polyposis coli (APC), KRAS, BRAF and TP53 genes (Fearon, 2011), the identification of widespread length variations of short repeat segments of DNA in some familial and sporadic CRC (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993) has further evolved understanding in this field. Short segments of DNA (microsatellites) made up of 1-5 nucleotides in tandem repeats, which are stably inherited, unique to an individual and widely scattered in the introns, exons and untranslated terminal regions throughout the genome (Weber and May, 1989; Hearne et al., 1992) become "unstable" resulting in microsatellite instability (MSI) consequent upon failure of the DNA mismatch repair

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system (Parsons et al., 1993). Various insertions or deletions of the microsatellites without effective repair result in a "mutator" phenotype with effect on legions of genes eg PTEN, BAX, EGFR, TGFβRII, that have critical functions in cell signalling, apoptosis and proliferation etc (Iacopetta et al., 2010). Based on the evidently different rates of CRC amongst our major ethnic groups and in the light of hitherto conflicting reports of possible MSI differences in CRC of various ethnic populations (Ashktorab et al., 2003; Vilkin et al., 2009; De Jesus-Monge et al., 2010; Sylvester et al., 2012) we initiated a study to determine the status of DNA mismatch repair in our CRC cases via immunohistochemical detection of the mismatch repair (MMR) gene products, MLH1, MSH2, MSH6 and PMS2, known to fairly reflect MSI status (Pinol et al., 2005; Niessen et al., 2006). Apart from attempting to determine whether MSI status, as represented by proficient or deficient MMR protein expression, differs between our major ethnic groups, we also attempted to correlate MMR status with age and gender of patients and location, grade, histological type and stage of the CRC.

Materials and Methods

All colorectal carcinomas, histologically diagnosed for the first time between 1st January 2005 and 31st December 2007 were retrieved from the archives of the Department of Pathology, University of Malaya Medical Centre. Of these, only cases which were surgically resected, irrespective of whether prior preoperative chemo and/or radiotherapy were instituted, were considered for the study. All cases were histologically reviewed and graded as well, moderately and poorly differentiated, depending on the degree of glandular formation identified (Blenkinsopp et al., 1981) and classified as mucinous or non-mucinous phenotype, without further consideration of the histological type as per WHO Classification. Mucinous phenotype was defined as tumours with neoplastic cells in large lakes of mucin constituting at least 50% of the tumour mass. Staging was according to the 7th edition of the International Union Against Cancer TNM Classification (Sobin et al., 2009). For the case to be permitted entry, the selected paraffin block should have sufficient tissue remaining in the block for future review following sectioning for immunohistochemical staining. For this study, demographic data and relevant clinical information were obtained from the histopathology request forms. Patients' demographic data were captured from the hospital's computerised patient database as a barcoded label on the histopathology request forms. All cases where the racial origin of the patient was uncertain were deleted from the study. The study was approved by the Institutional Review Board (IRB) of the University of Malaya Medical Centre (MEC 794.75). Informed consent was provided during submission for interventional treatment at the University of Malaya Medical Centre.

Four 4um sections were cut from the formalin-fixed, paraffin-embedded tumour tissue block selected during histopathological review of the case and placed on platinum coated slides (Matsunami Glass Industries, Japan). Antigen retrieval using a tris based alkaline buffer (CC1: Ventana Medical Systems Inc., Tucson, Arizona), immunohistochemical staining with monoclonal antibodies to MLH1 (BD Pharmingen, clone G168-728 at 1:100 dilution), MSH2 (BD Pharmingen, clone G219-1129 at 1:800 dilution), MSH6 (BD Transduction Laboratories, clone 44/MSH6 at 1:500 dilution) and PMS2 (BD Pharmingen, clone A16-4 at 1:100 dilution) and detection by the ultraView universal DAB detection kit (Ventana Medical Systems Inc., Tucson, Arizona) was carried out on the Ventana Benchmark XT autostainer (Ventana Medical Systems Inc., Tucson, Arizona). Proficient MMR (pMMR) protein expression by the tumour was defined as unequivocal tumour nuclear immunostaining in the presence of immunopositivity in the internal positive controls (lymphocytes, fibroblasts or normal enterocytes in the vicinity of the tumour). The tumour was classified as having deficient MMR (dMMR) when there was unequivocal loss of tumour nuclear staining (Figure 1) for one or more of the MMR proteins, MLH1, MSH2, MSH6 or PMS2, in the presence of immunoreactivity in the internal positive controls. The case would be withdrawn whenever the internal control failed.

Statistical analysis was carried out by chi-square test or Fisher' exact test with a significance value at $p<0.05$.

Results

A total of 158 cases of colorectal carcinoma had undergone surgical resection during the period of study. In total, 16 of the 158 cases were excluded from this study (11 due to uncertainty of the patient's ethnic origin, 1 re-classified as anal squamous carcinoma, 1 was recurrent and 3 were synchronous right and left CRC). Finally 142 cases were entered into the study.

The patients' ages ranged between 15-87 years (mean =62.4 years). As per revised Bethesda guideline for hereditary non-polyposis colorectal cancer (HNPCC) (Umar et al., 2004) or Lynch syndrome and also taking into account Yearsley et al. (2006)'s observation that in comparison with histological parameters, age was the only significant factor which differentiated germline from sporadic dMMR CRC (Yearsley et al., 2006), a cut-off of 50-years was used to categorise the cases into 2 groups. 21 of all the CRC patients in the study were <50-years of age and 121≥50-years of age at the time of diagnosis. There were 82 males and 60 females (M:F=1.4:1). Ethnically, there were 81 (57.0%) Chinese, 32 (22.5%) Malays and

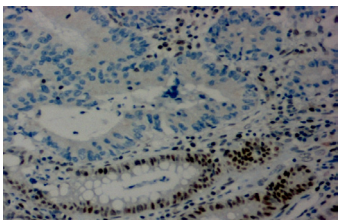

Figure 1. Colorectal Carcinoma with Deficient Mismatch Repair Protein (MLH1) Expression Where the Tumour (Arrow) is Immunonegative in the Presence of Immunopositive Internal Controls Made Up in This Case of Normal Enterocytes and Lymphoid Cells

29 (20.4%) Indians. 107 (75.4%) tumours were located on the left (distal to the splenic flexure) and 35 (24.6%) right (proximal to the splenic flexure). Histologically, 101 (71.1%) were well to moderately differentiated (6 well-differentiated and 95 moderately differentiated) and 41 (28.9%) poorly differentiated. 22 (15.5%) were of the mucinous type and 120 (84.5%) non-mucinous. 69 (48.6%) tumours were confined to the intestine (18 in stage I and 51 stage II) and 73 (51.4%) had extended beyond the intestine (62 in stage III and 11 stage IV).

Failure of the internal positive control for immunostaining of the MMR protein was not observed in any case. 128 (90.1%) of the CRC were pMMR while 14 (9.9%) were dMMR. Of the dMMR, concurrent loss of MLH1 and PMS2 occurred in 10 cases, concurrent loss of MSH2 and MSH6 in 2 and isolated loss of MSH6 in 1 and PMS2 in 1. Table 1 shows the correlation between pMMR and dMMR with age, gender and patient ethnicity and location, grade, histological type and stage of the CRC. Although in comparison with pMMR, there appears to be a trend for dMMR to be more prevalent in patients <50-years of age, females, tumours on the right side and for the tumors to be less differentiated, more mucinous and more extensively spread, statistically significant difference ($p<0.05$) was only reached for tumour location and tumour grade. Ethnically, dMMR was noted in 5 (6.2%) Chinese, 5 (15.6%) Malays and 4 (13.8%) Indians. MMR status did not differ significantly when the Chinese CRC cases were compared with Malays ($p=0.11$) and Indians ($p=0.19$) respectively. However, taking into account the established higher age-standardised incidence per 100,000 population rate for CRC amongst the Chinese compared with the Malays and Indians who have fairly similar rates, MMR status when compared between Chinese and combined Malay and Indian CRCs demonstrated a lower ($p=0.045$) prevalence of dMMR (6.2%) amongst the Chinese compared with the latter 2 races (14.8%).

Table 1. Association of DNA Mismatch Repair Protein Status viz MMR Proficient (pMMR) and MMR Deficient (dMMR) with Age, Gender and Ethnicity of Patients and Location, Grade, Histological Type and Stage of the Colorectal Carcinoma (n=142)

	pMMR (n=128) No. of cases (%)	dMMR (n=14) No. of cases (%)	p-value
Age <50-years (n=21)	17 (13.3)	4 (28.6)	0.130
≥50-years (n=121)	111 (86.7)	10 (71.4)	
Gender			
Male (n=82)	76 (59.4)	6 (42.9)	0.118
Female (n=60)	52 (40.6)	8 (57.1)	
Ethnicity			
Chinese (n=81)	76 (59.4)	5 (35.7)	0.045
Malay+Indian (n=61)	52 (40.6)	9 (64.3)	
Tumour location			
Right (n=35)	24 (18.8)	11 (78.6)	0.0001
Left (n=107)	104 (81.3)	3 (21.4)	
Tumour grade			
Well+moderately differentiated (n=101)	95 (74.2)	6 (42.9)	0.019
Poorly differentiated (n=41)	33 (25.8)	8 (57.1)	
Histological type			
Mucinous (n=22)	19 (14.8)	3 (21.4)	0.372
Non-mucinous (n=120)	109 (85.2)	11 (78.6)	
Tumour stage			
I+II (n=69)	64 (50.0)	5 (35.7)	0.372
III+IV (n=73)	64 (50.0)	9 (64.3)	

Table 2. Profile of MMR Deficient (dMMR) Colorectal Carcinoma Cases with Reference to Age Versus Gender and Ethnicity of Patients and Location, Grade, Histological Type and Stage of the Tumours (n=14)

Age		<50-years (n=4)	≥50-years (n=10)
Gender	Male (n=6)	2	4
	Female (n=8)	2	6
Ethnicity	Chinese (n=5)	3	2
	Malay+Indian (n=9)	1	8
Tumour location	Right (n=11)	3	8
	Left (n=3)	1	2
Histological type	Mucinous (n=3)	2	1
	Non-mucinous (n=11)	2	9
Tumour stage	I+II (n=5)	0	5
	III+IV (n=9)	4	5
dMMR	MLH1+PMS2 (n=10)	2	8
	MSH2+MSH6 (n=2)	1	1
	MHS6 (n=1)	1	0
	PMS2 (n=1)	0	1

Table 2 illustrates the profile of the 14 CRC with dMMR as categorised by patient's age. 4 cases were <50-years of age while 10 were ≥50-years. In the <50-years of age group, 2 (50.0%) cases had concurrent loss of MLH1 and PMS2, 1 concurrent MSH2 and MSH6 loss and 1 isolated loss of MSH6. In patients ≥50-years of age, combined MLH1 and PMS2 loss occurred in 8 of 10 (80.0%), combined MSH2 and MSH6 in 1 and isolated loss of PMS2 in 1. Considering that MMR status of the CRCs in this study appears to be influenced by ethnicity, tumour location and grade, 3 of 5 cases (60.0%) of dMMR amongst the Chinese and 1 of 9 cases (11.1%) amongst the combined Malay and Indian group were <50-years of age with the inverse noted in those cases ≥50-years of age. Location wise, 75.0% (3/4) dMMR CRC were right sided in patients <50-years and 80.0% (8/10) in patients ≥50-years of age. 75.0% of the tumours were poorly differentiated in patients <50-years and 50.0% in patients ≥50-years of age.

Discussion

50-85% of CRC develop through the chromosomal instability pathway while deficient DNA mismatch repair resulting in MSI accounts for about 10-20% of colorectal carcinoma (Koopman et al., 2009; Kanthan et al., 2012 ; Legolvan et al., 2012). Of the latter, germline mutation, followed by subsequent somatic knock-out of one of the mismatch repair genes, most frequently MLH1 or MSH2, resulting in HNPCC makes up less than 5% of all CRCs (Koopman et al., 2009). More frequently, MSI is due to sporadic hypermethylation silencing of the MLH1 promoter. dMMR was noted in 9.9% (14/142) of CRC in this study. This rate is comparable to those generally observed, except for being on the lower end of the range (Koopman et al., 2009; Kanthan et al., 2012; Legolvan et al., 2012). This could be due to inclusion of rectal carcinoma, which have been observed to show

lower incidence of MSI (Hoogerbrugge et al., 2003), in this study. That dMMR was significantly associated with right sided location and poor differentiation of the CRC has been demonstrated by others (Yearsley et al., 2006; Iacopetta et al., 2010). More interestingly, this study showed a lower prevalence of dMMR amongst the Chinese with CRC (6.2%) compared with their combined Malay and Indian counterparts (14.8%), although this was not statistically evident when comparing the Chinese versus Malays and Indians independently. It is notable that an earlier study on a smaller cohort of Malaysian CRC had shown no racial difference in MMR defects (Tan et al., 2007). The difference between our observations and that of Tan et al. (2007)'s needs further clarification. This study also shows that 60.0% of Chinese and only 11.1% of the combined Malay and Indian dMMR CRC were <50-years of age at presentation indicating a possibility for a more important role of MSI through mismatch repair gene germline mutations in development of CRC amongst the Chinese, much like their Hongkong counterparts, compared with the Malays and Indians (Chan et al., 1999). A possibility of lower frequency of developing MSI via the sporadic MLH1 promoter methylation process amongst the Chinese, a finding which may be in keeping with Jin et al. (2008)'s suggestion of distinct clinicopathological and germline mutational differences between Chinese and Western populations, should also be investigated further (Jin et al., 2008).

The pattern of loss of the different MMR proteins in this study is akin to that reported elsewhere (Truninger et al., 2005; Shia et al., 2009; Mojtahed et al., 2011). Concurrent MLH1 and PMS2 loss was the most commonly encountered and formed 71.4% of dMMR. This rate is similar to rates reported by other workers, in particular when there was no selection for HNPCC (Truninger et al., 2005). Unlike other variations of dMMR protein loss, which are more likely to be associated with germline mutations of the respective DNA mismatch repair genes, the combined loss of MLH1 and PMS2 can be attributed to either germline mutational loss of MLH1 or hypermethylation silencing of the MLH1 promoter. In the interpretation of MMR status, it is also imperative to bear in mind the obligatory dimeric partnerships of MMR proteins. Unlike bacterial MMR which function as homodimers, eukaryotic homologues function as heterodimers with the most important pairs being MutLα (MLH1+PMS2) and MutSα (MSH2+MSH6). MLH1 and MSH2 are obligatory while PMS2 and MSH6 are secondary partners in the MutLα and MutSα pairs respectively. An abnormality of the obligatory partner leads to degradation of the heterodimeric pair thus making it unlikely for loss of PMS2 or MSH6 when in combination with loss of MLH1 or MSH2 respectively to be independent events. In contrast, abnormality of secondary partners do not affect the obligatory proteins as the latter can heterodimerize with alternative secondary partners eg MLH1 with PMS1 or MLH3 to form MutLβ and MutLγ respectively while MSH2 can partner with MSH3 to form MutSβ (Sinicrope and Sargent, 2012). Thus, the cases with isolated loss of PMS2 and MSH6 observed here are most likely due to mutational loss of the respective DNA mismatch repair gene.

In conclusion, the general trend of CRCs with dMMR in Malaysians to be right sided and poorly differentiated is very much to be expected. However, the implication of an ethnic association with MMR status of CRC is interesting, in particular the possibility of the Chinese having a lower dMMR prevalence and a higher predisposition for germline mutations of the MMR gene. This would be of particular importance as CRC shows a definite predilection for the Malaysian Chinese compared with the two other major ethnic groups. Sequence and methylation analyses on an increased population size are required to provide further elucidation.

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