RESEARCH ARTICLE

Analysis of Hereditary Nonpolyposis Colorectal Cancer in Malay Cohorts using Immunohistochemical Screening

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Abstract

    <u>Background</u>: Lynch syndrome (LS) is an inherited predisposition to colorectal, endometrial (uterine) and other cancers. Although most cancers are not inherited, about 5 percent (%) of people who have colorectal or endometrial cancer have the Lynch syndrome. It involves the alteration of mismatch repair (MMR) genes; MLH1, MSH2, MSH6 or PMS2. In this study, we analyzed the expression of MMR proteins in colorectal cancer in a Malay cohort by immunohistochemistry. <u>Materials and Methods</u>: A total of 17 patients were selected fulfilling one of the Bethesda criteria: colorectal cancer diagnosed in a patient aged less than 50 years old, having synchronous and metachronous colorectal cancer or with a strong family history. Immunohistochemical staining was performed on paraffin embedded tumour tissue samples using four antibodies: MLH1, MSH2, MSH6 and PMS2. <u>Results</u>: Twelve out of 17 patients (70.6%) were noted to have a family history. A total of 41% (n=7) of the patients had abnormal immunohistochemical staining with one or more of the four antibodies. Loss of expression were noted in 13 tumour tissues with a negative staining score <4. Of 13 tumour tissues, four showed loss expression of MLH1. For PMS2, loss of expression were noted in five cases. Both MSH2 and MSH6 showed loss of expression in two tumour tissues respectively. <u>Conclusions</u>: Revised Bethesda criteria and immunohistochemical analysis constituted a convenient approach and is recommended to be a first-line screening for Lynch syndrome in Malay cohorts.

Keywords: Colorectal carcinoma - HNPCC - Lynch syndrome - immunohistochemistry - MMR

Introduction

    Cancers of the colon and rectum accounted for almost 1 million new cases in 2002, 9.4% of the world total reports (Parkin et al., 2005). Lynch syndrome (LS) is the most common hereditary cancer and account for 5-10% of total colorectal cancer (Cai et al., 2001). It is caused by mutations in mismatch repair (MMR) genes, MLH1, MSH2, MSH6 and PMS2 (Lynch et al., 2003). Being an autosomal dominant genetic variation, LS can be differentiated from sporadic colorectal cancer by the assessment of family history and based on young age at onset of malignancy (Haghighi et al., 2009). Individuals with LS are also predisposed to the development of cancer in extracolonic organs such as endometrial, stomach, ovary, small bowel, hepatobiliary tract, renal pelvis, ureter, skin, and brain (Lynch et al., 1999; Lynch et al., 2003). HNPCC can be differentiated from the sporadic colorectal cancer based on young age at onset of

malignancy (Haghighi et al., 2009). Mutations in MLH1 and MSH2 genes account for almost 90% of the identified cases (Jasperson et al., 2010; Gala & Chung, 2011), while 7% of mutations occurred in MSH6 gene, and 3% for the remaining genes (Peltomaki et al., 2004). Single base pair mismatches and single nucleotide insertion/deletion loops are recognized by hMutSα (a heterodimer complex formed by the MSH1 and PMS2 proteins), whereas hMutSβ (a heterodimer complex formed by the MSH2 and MSH6 proteins) recognizes larger insertion deletion loops of two to eight nucleotides (Barrow et al., 2009). Malfunction in the coordination of the MMR genes results in the inability to repair error during DNA replication.

    The gold standard for diagnostic of LS is germline mutation in MMR genes (Gala and Chung, 2011). Direct DNA sequencing (Sanger sequencing) is believed to be the key procedure for detecting mutations in the MMR genes. However, immunohistochemistry (IHC) is used to show abnormal MMR proteins expression in tumours

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as an abnormal seen for LS guiding the selection of the appropriate gene for sequencing. The use of four MMR antibodies (MLH1, MSH2, MSH6 and PMS2) are recommended over the use of only two MMR antibodies (Arends et al., 2008). The sensitivity and specifity for MLH1 is 100% and 91.5% respectively. For the MSH2 gene mutation carrier, the sensitivity and specificity is 87.5% and 88.5% respectively (Barrow et al., 2010). This is the first LS study focusing on the Malay individuals. There are several reports on germline mutations and IHC analysis in Malaysian Lynch Syndrome patients in two LS genes (Murad et al., 2012; Zahary et al., 2012).

However, there were no reports on the Malay Lynch Syndrome patients. Therefore, this study aims to demonstrate the immunohistochemical staining analysis patterns from tumours in a group of Malay Lynch syndrome patients.

Materials and Methods

Patient assessment

Informed consent was obtained from the patients after the approval from the Universiti Sains Malaysia (USM) Research Ethics Committee and Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia. Patients were recruited from three hospitals, Hospital Universiti Sains Malaysia, Kelantan, Hospital Sultanah Bahiyah, Kedah and Hospital Raja Perempuan Zainab 2, Kelantan. The samples recruitment was only be the Malay patients due to the demographic pattern with a high prevalence of Malay subjects in this three selected hospitals.

The patients were selected fulfilling one of the Bethesda criteria (Vasen et al., 2007) in which colorectal cancer diagnosed in a patient with the age of less than 50 years old, having synchronous and metachronous colorectal cancer or with a strong family history (Table 1). Informed consent was obtained from the patients.

The family history was assessed by personal interview and the detailed cancer diagnosis of the patients and their respective relatives were then validated against the hospital registry records and pathology reports.

Sample collection and immunohistochemical staining

For each patient's sample, a formalin-fixed paraffin embedded (FFPE) tissue block from biopsy or resected bowel specimen with 4 µm thick was sectioned and mounted on to glass slide. The sections were then be dewaxed in xylene and rehydrated in graded alcohol concentrations to distilled water. The slides were then placed in Tris-EDTA buffer (pH 9.0) in a pressure cooker for antigen retrieval for 3 minutes (121°C, 15 lb). Slides were subsequently incubated at 40C overnight with 200μl of primary antibodies to MutL Protein Homolog 1 (MLH1), MutS Protein Homolog 2 (MSH2), MutS Protein Homolog 6 (MSH6) and Postmeiotic Segregation Increased 2 (PMS2). The detail of the antibodies are as in the Table 2.

Endogenous peroxidase activity was blocked by incubation with peroxidase blocking reagent for 5 minutes. The slides were then washed thoroughly with Tris-buffered saline (TBS) to remove excess reagents after overnight incubation. Two drops of Horseradish-peroxidase (HRP) labelled polymer conjugated to secondary antibody were applied to the slides and were incubated for 45 minutes. Subsequent washing by TBS buffer was performed followed by incubating 200μl of DAB substrate chromogen for 3 minutes. The slides were counterstained with hematoxylin, dehydrated and mounted.

Normal appendix tissue was used as the external positive control while the lymphocytes and benign colonocytes served as an internal positive control. Loss of expression was recorded when nuclear staining is observed in normal appendix tissue but not in adjacent malignant cells.

Table 1. Features of HNPCC-related CRC Patient with their Respective Age of Onset and Family History

Patient ID	Age of onset	Family history
F1	26	Two second degree relatives had ovarian cancer, one second degree relative had breast cancer and one first degree relative (mother) had ovarian cancer at the age of 40 years old
F2	50	No family history
F3	39	One second degree relative had breast cancer at the age of >50 years old
F4	27	One first degree relative (mother) had colorectal cancer at the 53 years old, two second degree relatives had colorectal cancer at >50 years old
F5	43	One first degree relative (sister) had colorectal cancer at the age of 58 years old
F7	53	One first degree relative (daughter) had colorectal cancer at the age of 27 years old, two first degree relatives had colorectal cancer at the age of >50 years old
F8	58	One first degree relative (sister) had colorectal cancer at the age of 43 years old
F10	29	One first degree relative (mother) had colorectal cancer at the age of 53 years old
F11	23	No family history
F12	53	One first degree relative (daughter) had colorectal cancer at the age of 29 years old
F13	37	Second degree relative had endometrial cancer at the age of >50 years old
F14	54	One first degree relative (brother) had colorectal cancer at the age of 39 years old, one first degree relative (sister) had uterus cancer at the age of 51 years old and one first degree relative (mother) had brain cancer at the age of 60 years old
F16	44	Unknown family history
F18	25	Unknown family history
F19	48	Unknown family history
F20	29	Two second degree relatives had colorectal cancer at <50 years old
F21	26	One first degree relative (mother) had ovarian cancer at the age of 59 years old

Table 2. Primary Antibodies Used in the Study

Antibody	Source	Type	Pretreatment	Dilution
MLH1	Dako®	monoclonal mouse	pressure cooking 3 min	1 : 200
MSH2	Dako®	monoclonal mouse	pressure cooking 3 min	1 : 200
MSH6	Dako®	monoclonal rabbit	pressure cooking 3 min	Ready-to-use
PMS2	Dako®	monoclonal rabbit	pressure cooking 3 min	Ready-to-use
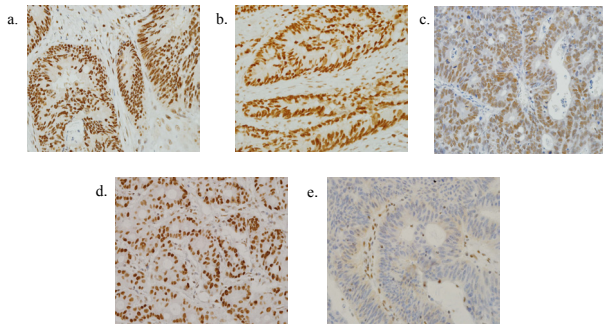
Figure 1. Immunohistochemical Staining (x200 magnification) for MMR Protein Expressions of a) Normal MLH1 b) Normal MSH2 c) Normal PMS2 d) Normal MSH6 e) Abnormal MLH1
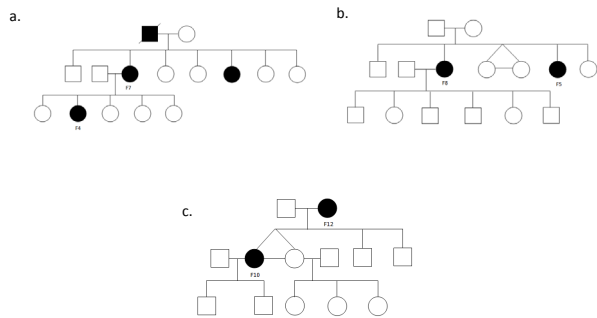
Figure 2. a) F4 is a First Degree Relative to F7, b) F5 is a First Degree Relative to F8, c) F10 have a First Degree Relationship with F12

Staining assessment and scoring

The immunohistochemical scoring analysis was performed by the pathologist based on the following semiquantitative scoring method. The presence of internal control immunopositivity (lymphocytes and benign colonocytes) was necessary for a valid result. The intensity (I) of immunoreactivity of the nuclear compartment of the malignant epithelial cells was measured on 0-3 scale. This scale was based on comparison of intensity of reactivity of the tumour cells with the positive control cells. A score of 0 indicated no reactivity, a score of 2 if moderate intensity and a score of 3 denoted an intensity of of tumour cell reactivity equivalent to positive control cells (Barrow et al., 2010).

The score of percentage (%) of tumour cells staining were as follows; score 0 indicated no tumour cell immunopositivity, score 1 for 1-10% positive tumour cells, score 2 for 11-50% positive tumour cells, score 3 for 51-80% positive tumour cells and score 4 for >80% positive tumour cells. The tumour cells were differentiated from tumour infiltrating lymphocytes which were also immunopositive (Muller et al., 2001).

The score of percentage and intensity were multiplied. The total scores of ≥4 were considered positive and <4 as negative (Barrow et al., 2010).

Table 3. Immunohistochemical Staining Score of HNPCC-related CRC Patients.

Patient ID	Immuhistochemical staining			
	(percentage immunopositivity score x staining intensity score)			
	MLH1	MSH2	MSH6	PMS2
F1	8 (4x2)	12 (4x3)	12 (4x3)	12 (4x3)
F2	8 (4x2)	8 (4x2)	12 (4x3)	12 (4x3)
F3	1 (1x1)	12 (4x3)	12 (4x3)	1 (1x1)
F4	2 (2x1)	12 (4x3)	12 (4x3)	1 (1x1)
F5	12 (4x3)	12 (4x3)	12 (4x3)	6 (3x2)
F7	1 (1x1)	8 (4x2)	12 (4x3)	1 (1x1)
F8	12 (4x3)	12 (4x3)	12 (4x3)	12 (4x3)
F10	12 (4x3)	12 (4x3)	12 (4x3)	12 (4x3)
F11	2 (2x1)	12 (4x3)	12 (4x3)	0 (0x0)
F12	8 (4x2)	12 (4x3)	12 (4x3)	12 (4x3)
F13	6 (3x2)	3 (3x1)	12 (4x3)	12 (4x3)
F15	12 (4x3)	2 (2x1)	2 (2x1)	12 (4x3)
F16	6 (3x2)	4 (2x2)	6 (3x2)	4 (2x2)
F18	12 (4x3)	12 (4x3)	12 (4x3)	12 (4x3)
F19	12 (4x3)	12 (4x3)	12 (4x3)	12 (4x3)
F20	12 (4x3)	12 (4x3)	6 (2x3)	4 (2x2)
F21	12 (4x3)	8 (4x2)	0 (0x0)	0 (0x0)

Results

Paraffin embedded tumour tissue samples were examined using 4 antibodies, MLH1, MSH2, MSH6 and PMS2. Table 3 showed the immunohistochemical scores for four antibodies tested on the tumour samples. Eleven out of 17 patients (64.7%) were noted to have a family history. A total of 41% (n=7) of the patients had abnormal immunohistochemical staining in either four antibodies. As each patient was tested with four MMR antibodies, 13 tumour tissues demonstrated loss of expression with the negative stain of score <4. Of 13 tumour tissues, 4 out of 13 showed loss expression for MLH1, 5 out of 13 showed loss of expression for PMS2 and 2 out of 13 showed loss of expression for MSH2 and MSH6 respectively (Table 3).

Interestingly, six patients who had a family history satisfying the Bethesda criteria (Figure 2) also presented similar pattern of immunohistochemical staining with their corresponding relatives. The similar immunohistochemical staining pattern were acclaimed by the similar prominent immunohistochemical staining between each patient with their corresponding relative as both patients in the family (Figure 2a) demonstrated loss of immunohistochemical staining for MLH1 and PMS2. However, two patients in the two respective families (Figure 2b and Figure 2c) showed normal protein expression in these tested four antibodies; MLH1, MSH2, MSH6 and PMS2.

Discussion

Revised Bethesda criteria was known to have a sensitivity and specificity of 91% and 77% respectively (Pinol et al., 2005). The incidence of HNPCC in the Malay population is still difficult to estimate. A reason to this factor might be due to lack of participation and awareness of those individuals with colorectal cancer risk to perform screening in Malaysia as compared to other countries (Yusoff et al., 2012). In contrast, a study revealed that increasing awareness on the importance of evaluating the family history among LS patient have increased the survival rate than sporadic colorectal cancer (Haghighi et al., 2009). In Malaysia and Singapore, where three major Asian races; Malay, Chinese and Indian live together, the incidence rate of CRC observed in Chinese have higher rates Indians and Malays (Goh et al., 2005). This is concordant with other study which stated that Malaysian Chinese have a clear predilection for CRC as compared to the Malaysian Indians and Malays (Cheah et al., 2014). CRC is often diagnosed in these patients at the mean age of 45 years old and is located in the proximal part of the colon in two thirds of cases. We have identified distinct features in family history of the individuals. The strong family association would probably be a strong risk factor in these individuals as there is an evidence that close relatives of individuals with CRC are at risk of developing extracolonic cancer (Coggins et al., 2005).

IHC staining is the appropriate way to detect the MMR dysfunction as by using IHC, the expression pattern of MLH1, MSH2, MSH6 and PMS2 was examined in primary tumours and in the corresponding normal mucosa from patients with CRC (Amira et al., 2014). Immunohistochemical analysis of colorectal tumours in HNPCC families has shown that the gene product from the mutated MMR gene is absent in tumour tissue and at the same time present in adjacent normal tissue (Thibodeau et al., 1996).

In this study, we found a noticeable loss of expression in four and five cases for MLH1 and PMS2 respectively as the abnormality of MLH1 is accompanied by abnormality of PMS2 due to heterodimeric association of proteins (Arends et al., 2008). Abnormal MMR protein expression can be detected in two patterns: either complete loss of expression (when there is no expression of that MMR protein or only expression of a truncated protein to which the antibody does not bind or the mutated protein has lost the epitope recognized by the antibody), or patchy/

weak expression (if the mutation generates a prematurely truncated but variably stable protein, or a protein with alterations to the epitope recognized by the antibody, such as may be the case with some missense mutations) (Poulogiannis et al., 2009). A deleterious mutation resulting in an absent or truncated protein will usually result in a complete loss of reactivity. However, certain pathogenic mutations (including frame shift mutations) may leave protein product that retains antigenicity, resulting in false-positive immunoreactivity (normal protein staining persists). This is dependant on the location of the antibody binding site on the protein, the site of mutation, and the effect of the mutation on normal gene expression and protein maturation (Barrow et al., 2010). Mutations may also have destroyed the enzymatic activity while preserving the immunoreactivity and stability of proteins (Southey et al., 2005). MMR plays a vital role in the control of processes occurring during oncogenesis in the majority of HNPCC cases (Marra et al., 2005). However, in a small proportion of 5 to 20% of HNPCC-related tumours do not demonstrate any abnormality or loss of protein expression on analysis by IHC even though they have lost MMR function. This may due to the mutations that functionally inactivate the MMR protein but allow its expression as a stable protein with nuclear localisation and intact epitope (Arends et al., 2008).

However, in this study, although we noted six cases of CRC with strong family history, the immunohistochemical analysis done only showed two cases with absence of immunohistochemical staining among these families. The familial risk may not be entirely due to LS but the MMR abnormalities may be associated to the role of susceptibility. The abnormalities of the MLH1 deficient CRC may be not expressed due to early diagnosis as the MLH1 deficient tend to occur at a later age of 75 years old for a family history of colorectal cancer or HNPCC spectrum and also in the absence of family history (Coggins et al., 2005).

In conclusion, revised Bethesda criteria and immunohistochemical analysis constituted a convenient approach and is recommended to be a first-line screening for Lynch syndrome in Malay cohorts. It is also able to add significant information to support the increased risk of developing colorectal or other cancers in most families with HNPCC.

Acknowledgement

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