EVALUATION OF MISMATCH REPAIR AND MICROSATELLITE INSTABILITY IN COLORECTAL CANCER PATIENTS IN KUANTAN, PAHANG

BY

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ABSTRACT

Colorectal cancer (CRC) is the second most common tumour in Malaysia. Universal screening for the identification of microsatellite instability/mismatch repair (MSI/MMR) status in CRC patients is recommended by several guidelines. The detection of MSI/MMR status in CRC patients is not only essential to identify Lynch Syndrome (LS), but it also has predictive and prognostic values. The study aimed to investigate the MMR and MSI status among CRC patients in Kuantan, Pahang as well as to assess the consistency between immunohistochemistry (IHC) and MSI analysis. Formalin-fixed paraffin-embedded (FFPE) tissue blocks of 123 CRC patients were retrieved for the years 2017-2018. For IHC and MSI analysis, EnVision^{TM} FLEX, Mini Kit, High PH, and MSI Analysis System 1.2 (Promega) were utilized, respectively. MSI analysis was performed on selected deficient mismatch repair (dMMR) and proficient mismatch repair (pMMR) cases. IHC detected 11.4% (14 out of 123) patients as dMMR and 88.6% (109 out of 123) as pMMR. MSI analysis identified 26% (13 out of 50) patients as MSI-H, 6% (3 out of 50) patients as MSI-L, and 64% (32 out of 50) patients as MSS. Both the IHC and MSI analysis showed perfect agreement (0.896, Kappa value) for the recognition of dMMR or MSI-H CRC patients while demonstrating only 4% (2 out of 50) discordant results. Almost all dMMR patients detected by IHC were recognized by MSI analysis as MSI-H except one. The significant prevalence of dMMR in current cohort support the recommendation that the assessment of MSI/MMR status should be addressed in CRC patients. The selection of the choice method may be based on the availability of the expertise and equipment. Since IHC is an affordable, a reproducible and readily available in most histopathological laboratories, it can be used as a primary screening test to detect MSI/MMR status in CRC patients.

خلاصة البحث

يعتبر سرطان القولون والمستقيم ثاني اكثر الاورام شيوعا في ماليزيا. يوصى بالعديد من الاختبارات لتحديد حاله عدم استقرار/اصلاح عدم التطابق (MSI/MMR) في مرضى سرطان القولون والمستقيم. ان الكشف عن حاله (MSI/MMR) في مرضى سرطان القولون والمستقيم ليس ضروريا فقط لتحديد متلازمه لينش (Lynch Syndrome), ولكنه يحتوي ايضا على قيم تنبؤيه و تحذيريه. هدفت هذه الدراسه الى التحقيق في(MMR and MSI) بين مرضى سرطان القولون والمستقيم في كونتان,باهانج, وكذلك لتقييم الاتساق بين طريقتي الكيمياء المناعيه (IHC) , و (MSI). تم وضع عينات الانسجه المجمعه بالفورمالين لعدد١٢٣ حاله مرضيه بسرطان القولون والمستقيم للسنوات ٢٠١٧ و٢٠١٨. لتحليل(IHC and MSI Analysis), تم استخدام (EnVisionTM FLEX, Mini Kit, High PH, and MSI Analysis System 1.2 ((Promega)) على التوالي. تم اجراء تحليل(MSI)على حالات اصلاح عدم تطابق غير محدده (dMMR) و حالات عدم التوافق الدقيق (pMMR). طريقه الكيمياء المناعيه (IHC) اكتشفت١١,٤ (١٤ من اصل١٢٣) مريضآ على انها (dMMR), و ٨٨,٦ ٪ (١٠٩ من اصل١٢٣) على انها (pMMR). حدد تحليل (MSI) ٢٦٪ (١٣ من اصل ٥٠) على انهم (MSI-H), و٦٪ (٣ من اصل٥٠) مريضا على انهم (MSI-L), و ٦٤٪ (٣٢ من اصل ٥٠) مريضا على انهم (MSS). أظهر كل من تحليل (IHC and MSI) اتفاقا مثاليا (0.896, kappa value) للاعتراف بمرضى (dMMR or MSI-H) مع اظهار ٤٪ فقط (٢من اصل٥٠) نتائج متناقضه. تم التعرف على جميع مرضى (dMMR) الذين اكتشفهم (IHC) من خلال تحليل (MSI) على انه (MSI-H) باستثناء واحد. يدعم الانتشار الكبير ل(dMMR) في الدراسه الحاليه التوصيه بأن تقييم حاله (MSI/MMR) يجب ان يعالج في مرضى سرطان القولون والمستقيم. قد يعتمد اختيار طريقه الاختيار على توفر الخبره والمعدات. نظرا لان (IHC) هو باسعار معقوله,وقابله للتكرار,ومتوفر بسهوله في معظم مختبرات علم الانسجه,يمكن استخدامه كاختيار فحص اولي للكشف عن حاله (MSI/MMR) في مرضى سرطان القولون والمستقيم.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Medical Sciences

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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DEDICATION

TO MY PARENTS

TO MY TEACHERS AND

TO MY BELOVED WIFE AND KIDS

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B	Beta
%	Percentage
≤	Lesser or equal to
≥	Greater or equal to
°C	Centigrade
µg	Microgram
µl	Microlitre
5FU	5-Fluorouracil
APC	Adenomatous polyposis coli
APC	Antigen presenting cell
APCCWG	Asian Pacific Colorectal Cancer Working Group
ASCO	American Society of Clinical Oncology
ASCP	American Society for Clinical Pathology
ASRi	Age-standardized incidence rate
ASRm	Age-standardized mortality rate
bp	base pair
CAP	College of American Pathologists
CE	Capillary Electrophoresis
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
CMMRD	Constitutional Mismatch Repair deficiency
CRC	Colorectal cancer
CT	Computer Tomography
CT	Chemotherapy
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte associated antigen 4
CXR	Carboxy-X-rhodamine
DAB	3.3’-diaminobenzidine tetrahydrochloride
DFS	Disease free survival

dH2O	Distilled Water
dMMR	Deficient Mismatch Repair
DPX	Dibutylphthalate polystyrene xylene
EGAPP	Evaluation of genomic applications in practice and prevention group
EpCAM	Epithelial cell adhesion molecule
FAP	Familial Adenomatous Polyposis
FCCTX	Familial colorectal cancer type X
FDA	Food and drug administration
FFPE	Formalin fixed paraffin embedded
FIT	Fecal immunohistochemical test
FOLFIRI	Folinic acid 5FU irinotecan
FOLFOX	Folinic acid, 5FU, and Oxaliplatin
gDNA	Genomic Deoxyribonucleic acid
gFOBT	Guaiac-based fecal occult blood test
GLOBOCAN	Global Cancer Observatory
H&E	Hematoxylin and Eosin
HR	Hazard ratio
HRP	Horseradish peroxidase
HTAA	Hospital Tengku Ampuan Afzan
ICI	Immune Checkpoint Inhibitor
IFL	Irinotecan
iFOBT	Immunochemical fecal occult blood test
IHC	Immunohistochemistry
IIUMMC	International Islamic University Malaysia Medical Center
ILS 600	Internal Lane Standard 600
IMT	Immunotherapy
LAG-3	Lymphocyte activation gene 3
LLS	Lynch-like syndrome
LOH	Loss of Heterozygosity
mAb	Monoclonal antibodies
MANA	Mutation associated neoantigens
MAPK	Mitogen-activated protein kinase

Mg	Milligram
MHC	Major histocompatibility complex
miRNA	MicroRNA
Ml	Millilitre
Mm	Millimeter
MMR	Mismatch Repair
mMSI	Metastatic Microsatellite Instability
MOSAIC	Multicenter International Study of Oxaliplatin, 5FU, and Leucovorin (FOLFOX) in adjuvant treatment of colon cancer
mRNA	Messenger RNA
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability High
MSI-L	Microsatellite Instability Low
MSS	Microsatellite stable
NCCN	National Comprehensive Cancer Network
NCI	National Cancer Institute
NGS	Next-generation sequencing
NICE	National Institute for Health and Care Excellence
NSAID	Nonsteroidal anti-inflammatory drug
ORR	Objective response rate
OS	Overall survival
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PD-1	Programmed death 1
PFS	Progress free survival
pMMR	Proficient Mismatch Repair
RFS	Relapse free survival
SUR	Surgery
T	Temperature
TACSTD1	Tumour-associated calcium signal transducer 1
TIL	Tumour infiltrating lymphocyte
TKI	Tyrosine kinase inhibitor
TMB	Tumour mutation burden

CHAPTER ONE

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Cancer is a general term used for several diseases characterized by the abnormal cells that have uncontrolled growth and cross their normal boundaries (American Cancer Society, 2018). Globally, colorectal cancer (CRC) is the third most frequent cancer in males (10.9%) and second in females (9.5%) (Bray et al., 2018). According to a Global Cancer Observatory (GLOBOCAN) database, CRC ranked fourth in males (9%) and third in females (9.5%) in mortality. The estimated incidence of new CRC cases and related deaths in 2018 was more than 1.8 million and 881,000, respectively worldwide (Bray et al., 2018). CRC is the third common cause of tumour-associated deaths in the USA (Ashktorab et al., 2019). According to the American Cancer Society, 140250 estimated new cases, and nearly 50630 deaths were caused by CRC (American Cancer Society, 2018).

The incidence of CRC is increasing in Asia and Southeastern Asian nations (Bray et al., 2018). In 2018, the incidence and mortality of CRC cases were 51.8% and 52.4% per 100,000 persons (including both sexes and all ages), respectively in Asia which was the highest in Asia among the others (Onyoh et al., 2019). A higher incidence of CRC is reported in developed Asian nations like Singapore, Japan, South Korea than developing Asian nations including Malaysia (Cancer & Organization, 2017; J. J. Y. Sung et al., 2015). In Malaysia, CRC is the second most common tumour in both sexes with an estimation of approximately 3342 (16.2%) and 2795 (12%) new cases in males and females, respectively in 2018 (Bray et al., 2018). The incidence is reported to be the

highest in Chinese followed by Indians and Malays, respectively (Wendy & Radzi, 2008). Men are more likely to develop CRC compared to women (S.-E. Kim et al., 2015).

Several factors are attributed to be involved in this trend. An unhealthy lifestyle, consumption of westernized diet, aging population, smoking, and physical inactivity could be contributing factors to the increasing incidence of CRC cases in Asians in the last few decades (Onyoh et al., 2019; Pang et al., 2020). The percentage of aged population is increasing in Malaysia (Ramely, Ahmad, & Harith, 2016), with growing affluence and an enhanced incidence of risk factors such as obesity, smoking and westernized diet for CRC (Center, Jemal, Smith, & Ward, 2009; J. J. Y. Sung et al., 2015). People aged more than 50 years old constitute nearly 80% of CRC cases in Malaysia (Wendy & Radzi, 2008).

CRC initiates as a benign adenomatous polyp, then progresses into a developed adenoma possessing high-grade dysplasia and finally advances to an invasive tumour (Vogelstein & Kinzler, 2002). Most cases of the tumour that arise in the form of adenomatous polyps stay asymptomatic for a long time. Thus, screening is helpful in this period to detect adenomas or early tumors that are most treatable and highly considered to prevent their transformation into last stage disorder with great fatality rates (Winawer et al., 1993; Zauber et al., 2012). An organized national screening programme is recommended by the Asian Pacific Colorectal Cancer Working Group (APCCWG) in nations with maximum incidence (> 30 per 100,000) of CRC (J. Sung et al., 2015). Most European, North American and developed Asian countries are practicing an organized national screening programme while a majority of Southern and Central American, African and developing Asian countries including Malaysia have not yet

implemented such-national programmes (Fuzi, Hassan, Sabirin, & Bakri, 2015; Navarro, Nicolas, Ferrandez, & Lanas, 2017; Schreuders et al., 2015). Findings from the studies in western countries have revealed that screening decreases CRC deaths by up to 53% (Schoen et al., 2012). Generally, majority of CRC patients (65%) in Malaysia are presented late and diagnosed with late-stage CRC, and most of them are left-sided cancers (75%), whereas the prognosis of right-sided cancer is poorer than left-sided tumor (GOH et al., 2005; G. Lee et al., 2015).

Three major pathways are involved in the pathogenesis of CRC. Chromosomal instability (CIN) accounts for 75% of cases, CpG island methylator phenotype (CIMP) for 20% of cases, and defective DNA mismatch repair (MMR) system or microsatellite instability (MSI) is causing nearly 15% of CRC cases (Tariq & Ghias, 2016).

Microsatellite instability-high/deficient mismatch repair (MSI-H/dMMR) tumours are a subtype of CRC which shows the inability of mismatch repair genes (MLH1, MSH2, MSH6, and PMS2) to repair faults in microsatellites (R. Gupta, Sinha, & Paul, 2018). The human genome has >100,000 regions of microsatellites (V. Lee, Murphy, Le, & Diaz, 2016). Microsatellites are short DNA chains positioned throughout the human genome (coding and non-coding regions) consisting of a single, di, tri, or tetranucleotide repeat sequences. These repetitive sequences are susceptible to replication defects if the MMR system is functionally deficient. The defects that accumulate in these repetitive structures (microsatellites), known as MSI, reflects the non-functional MMR system. Tumours with non-functional MMR systems are named as MSI-H/dMMR tumours. In contrast, the Microsatellite stable/proficient mismatch repair (MSS/pMMR) tumours highlight the functional MMR system.

The MMR system comprises of four main proteins known as MLH1, MSH2, MSH6, and PMS2. During the replication of DNA, these MMR proteins recognize and repair DNA mismatches in the microsatellite regions that are triggered by DNA polymerase. MMR proteins function in the form of heterodimers, MLH1/PMS2, and MSH2/MSH6, structuring MutLα and MutSa, respectively. MutSa identifies single base pair mismatch, generates a sliding clamp around DNA, and later attaches the second compound, MutLα (Fishel, 2015). This grouping of complexes interact with several enzymes, including DNA polymerase, to achieve excision of the single mismatch and synthesize the DNA strand again (Kawasoe, Tsurimoto, Nakagawa, Masukata, & Takahashi, 2016; Frank A Sinicrope & Sargent, 2012). Hence, a non-functional MMR system is caused by the inactivation of one or more of the MMR proteins, leading to the accumulation of mutations and subsequently resulting in MSI-H/dMMR CRC phenotype (Hampel et al., 2008). The role of the MMR system to protect genetic fidelity is well established (Tutlewska, Lubinski, & Kurzawski, 2013). The MMR system also causes programmed cell death and/or cell cycle arrest when DNA damage occurs (Stojic, Brun, & Jiricny, 2004). Thus, mutation accumulation and cancer progression occur when non-functional MMR shows the inability to remove badly damaged cells (T.-M. Kim, Laird, & Park, 2013). MSI is a molecular biomarker for a deficient DNA MMR system (C. R. Boland et al., 1998) and reflects the deficiency of DNA MMR system (Ryan, Sheahan, Creavin, Mohan, & Winter, 2017).

MSI-H/dMMR tumours which constitute 15% of the total CRC, out of which 12% are acquired due to methylation-related silencing of a gene which codes for DNA MMR protein, in contrast only 3% of MSI-H/dMMR tumours are caused by germline mutations in one of the MMR genes (Lynch syndrome) (R. Gupta et al., 2018). Lynch

syndrome (LS), previously known as hereditary nonpolyposis colorectal cancer (HNPCC), is the most prevalent familial hereditary syndrome which results from autosomal dominant germline mutations in any of the DNA MMR genes (MLH1, MSH2, MSH6, and PMS2) which cause MSI (Baretti & Le, 2018; Ryan et al., 2017).

In 1993, MSI was found to be present in CRC. Additionally, it was further discovered in colon tumours from most of the LS patients (Aaltonen et al., 1998). The identification of LS is essential as MSI-H/dMMR status is well known to be a genetic marker for LS patients. Such patients profit from prophylactic aspirin (Burn et al., 2011), enhanced surveillance (Järvinen et al., 2000), and severe radical surgery (Heneghan, Martin, & Winter, 2015), and may need various strategies to adjuvant therapy (Le et al., 2015). Also, the risk of developing several extracolonic cancers is higher in the LS patients which include endometrial cancer, ovary, small bowel, stomach, bladder, brain, kidney, biliary tract, gallbladder cancers, and skin sebaceous tumors (Aarnio et al., 1999; Bansidhar, 2012; Williams & Huang, 2013). Individuals affected with LS also have a higher lifetime risk of developing CRC which ranges from 30% to 70% compared to 5.5% in common population (Giardiello et al., 2014; Lynch, Snyder, Shaw, Heinen, & Hitchins, 2015).

Previous studies have demonstrated that MSI-H/dMMR CRC has better overall survival and an improved prognosis compared to MSS/pMMR CRC (Guastadisegni, Colafranceschi, Ottini, & Dogliotti, 2010; Klingbiel et al., 2015; Popat, Hubner, & Houlston, 2005; F. A. Sinicrope et al., 2015). Such cases are less susceptible to the synchronous liver (Nordholm-Carstensen, Krarup, Morton, Harling, & Group, 2015) and lymph node metastasis (Mohan et al., 2016). Furthermore, MSI-H/dMMR could be a predictive marker of response to 5-Fluorouracil (5-FU) as studies have shown that

stage-II MSI-H/dMMR CRC cases show resistance to 5-FU based chemotherapy (Ryan et al., 2017). MSI-H/dMMR phenotype is observed in approximately 20% of stage II and III CRCs with comparatively improved prognosis (Yahagi, Okabayashi, Hasegawa, Tsuruta, & Kitagawa, 2016). National Comprehensive Cancer Network (NCCN) recommended the detection of MSI-H/dMMR status in all stage-II CRC patients since stage-II MSI-H/dMMR CRC cases have shown to have a better prognosis and show resistance to chemotherapy (A. B. Benson, 3rd et al., 2017).

A study had reported the upregulation of immunoregulatory genes in MSI-H/dMMR CRCs, indicating the increased response of the immune system (Banerjea et al., 2004). MSI leads to enhanced somatic mutations in cancer cells, causing biological and molecular alterations, that include plenty of tumour-infiltrating lymphocytes, increased tumour mutational load, and enhanced demonstration of neoantigens. Such molecular and biological changes increase the sensitivity of MSI-H/dMMR tumours to immune checkpoint inhibitors (ICIs) (Gargiulo et al., 2016; Gelsomino, Barbolini, Spallanzani, Pugliese, & Cascinu, 2016; Llosa et al., 2015; Overman et al., 2017). Recent findings have also demonstrated the success of immunotherapy in metastatic MSI cancers (Gupta et al., 2018). American Society for Clinical Pathology (ASCP), the College of American Pathologists (CAP), the American Society of Clinical Oncology (ASCO), the National Institute for Health and Care Excellence (NICE) and National Comprehensive Cancer Network (NCCN) have recommended universal screening for all individuals with CRC (ASCO, 2017; S. Gupta et al., 2019; Health & Excellence, 2017).

Various methods are available to assess the MSI-H/dMMR status of CRC patients with the consideration of the advantages, limitations, applicability, and