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RELATIONSHIP BETWEEN TUMOUR-ASSOCIATED MACROPHAGES, CD8+ and IL17+ Cells, TGF- β, TGF-βRII, IL-17R and WNT SIGNALING IN COLORECTAL CARCINOMA

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By

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Colorectal carcinoma is one of the most common cancers and is a heterogeneous disease. The inflammatory tumour microenvironment plays a critical role in colorectal carcinoma development. Many researchers have extensively studied tumour-associated macrophages (TAMs), $CD8^+$ cells, $IL\text{-}17^+$ cells, TGF-β and IL-17 expressions as well as Wnt signaling in the tumour microenvironment of colorectal carcinoma. However the relationship between TAMs, $CD8^+$ cells, $IL\text{-}17^+$ cells, expression of TGF-β and IL-17 receptors and ligands and Wnt signaling are poorly understood.

The main objective of this study is to study interrelationship of M1 TAMs and M2 TAMs with $CD8^+$ cells, $IL\text{-}17^+$ cells TGF-βRII, TGF-β, IL-17R, IL-17, and biomolecules of Wnt signaling in the progression of colorectal carcinoma.

Double immunohistochemical staining was performed to evaluate densities of $CD68^+$/iNOS M1 TAMs and $CD68^+/CD163^+$ M2 TAMs in colorectal carcinoma. Single immunohistochemical staining was performed to determine the density of $CD8^+$ cell and $IL\text{-}17^+$ cells and expression of TGF-β and IL-17 receptors and ligands.

Using Spearman's correlation, a positive correlation were observed between $CD8^+$ T cells and $IL\text{-}17^+$ cells ($p=0.031$); M1 TAMs and TGF-βRII expression ($p<0.001$); M1 TAMs and IL-17 expression ($p=0.003$); $IL\text{-}17^+$ cells and IL-17 expression ($p=0.031$); TGF-βRII expression and IL-17R expression ($p=0.011$); M2 TAMs and loss of nucleus APC ($p=0.013$); M2 TAMs and loss of cytoplasmic

APC (p=0.011); M1/M2 and loss of cytoplasmic APC (p=0.001); M1 TAMs and p-GSK3β (p=0.003); IL-17$^+$ cells and p-GSK3β (p=0.014); TGF-βRII expression and p-GSK3β (p=0.020). A negative correlation were found between M1 TAMs and CD8$^+$ T cells/IL-17$^+$ cells (p=0.007); M2 TAMs and TGF-β expression (p=0.0007); M2 TAMs and TGF-βRII expression (p=0.049); M1/M2 and TGF-β expression (p=0.031); M1/M2 and TGF-βRII expression (p=0.001).

Densities of immune cells and biomolecules in this study were associated with various demographical and clinicopathological features using chi-square test. Early TMN stage was associated with high density of M2 TAMs (p<0.001) and IL-17$^+$ cells (p<0.001), low CD8$^+$ T cell/IL-17$^+$ cells (p<0.001), high expression of TGF-βRII (p<0.001) and IL-17R (p=0.027). Low histological grade of tumour was associated with high density of IL-17$^+$ cells (p=0.035), high expression of IL-17R (p=0.019) and low CD8$^+$ cell/IL-17$^+$ cells (p=0.030). Decreased depth of tumour invasion (T) was associated with high density of IL-17$^+$ cells (<0.001) and IL-17R (p=0.023). Low lymph nodes metastasis (N) was associated with high density of M2 TAMs (p=0.005) and IL-17$^+$ cells (p<0.001).

In conclusion, this study demonstrated that high density of M2 TAMs and IL-17$^+$ cells, high expression of TGF-βRII and IL-17R, and low CD8$^+$ cells/IL-17$^+$ cells are associated with early TNM stage, low histological grade, decreased depth of tumour invasion, low lymph nodes metastasis indicating a favourable prognosis of colorectal carcinoma. M1 TAMs and IL-17 expression are positively correlated TGF-βRII and IL-17$^+$ cells respectively. This implied high density of M1 TAMs and high IL-17 expression also indicating a favourable prognosis. Hence, evaluation of immune cells, ligands and receptors of cytokines may serve as a potential biomarker for assessing colorectal carcinoma progression and developing immune cells/cytokine targeted treatment.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

HUBUNGAN ANTARA MAKROFAJ BERKAITAN DENGAN TUMOR, SEL CD8$^+$, SEL IL-17$^+$, TGF-β, TGF-βRII,IL-17R SERTA LALUAN ISYARAT WNT BARAH KOLOREKTAL

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Barah Kolorektal adalah salah satu barah yang paling biasa dan merupakan sejenis penyakit heterogenus. Inflamatori mikrosekitaran tumor memainkan peranan yang kritikal dalam perkembangan barah kolorektal. Kebanyakan penyelik telah menkaji secara meluas mengenai makrofaj berkaitan dengan tumor (TAMs), sel CD8$^+$, sel IL-17$^+$, pengekspresan reseptor and ligan TGF-β and IL-17 serta laluan isyarat Wnt dalam mikrosekitaran tumor barah kolorektal. Tetapi hubungan antara TAMs, sel CD8$^+$, sel IL-17$^+$, pengekspresan reseptor dan ligan bagi TGF-β dan IL-17 serta laluan isyarat Wnt kurang difahami.

Objektif utama kajian ini adalah untuk mengkaji hubungan antara M1 TAMs dan M2 TAMs dengan sel CD8$^+$, sel IL-17$^+$, TGF- βRII, TGF-β, IL-17R, IL-17 serta biomolekul laluan isyarat Wnt dalam perkembangan barah kolorektal.

Pewarnaan imunohistokimia berganda telah dijalankan untuk menilai kepadatan CD68$^+$/iNOS M1 TAMs dan CD68$^+$/CD163$^+$ M2 TAMs dalam barah kolorektal. Pewarnaan imunohistokimia tunggal telah dijalankan untuk menilai kepadatan sel CD8$^+$, sel IL-17$^+$, dan pengekspresan reseptor and ligan bagi TGF-β dan IL-17.

Dengan penggunaan ujian kolerasi Spearman's, kolerasi positif telah diperhatikan antara sel CD8$^+$ dan sel IL-17$^+$ ($p=0.031$); M1 TAMs dan pengekspresan TGF- β RII ($p<0.001$); M1 TAMs dan pengekspresan IL-17 ($p=0.003$); sel IL-17$^+$ dan pengekspresan IL-17 ($p=0.031$); pengekspresan TGF- RII dan pengekspresan IL-17R ($p=0.011$); M2 TAMs dan kehilangan pengekspresan APC nucleus ($p=0.013$); M2 TAMs dan kehilangan

pengekspresan APC sitoplasma (p=0.011); M1/M2 TAMs dan kehilangan pengekspresan APC sitoplasma (p=0.001); M1 TAMs dan p-GSK3β (p=0.003); sel IL-17^{+} dan p-GSK3□ □ (p=0.014); pengekspresan TGFβRII dan p-GSK3β (p=0.020). Kolerasi yang negative didapati antara M1 TAMs dan sel CD8^{+}/sel IL-17^{+} (p=0.007); M2 TAMs dan pengekspresan TGF-β (p=0.007); M2 TAMs dan pengekspresan TGF-βRII (p=0.049); M1/M2 TAMs dan pengekspresan TGF-β(p=0.005); M1/M2 TAMs dan pengekspresan TGF-βRII (p=0.001).

Kepadatan sel-sel keimunan dan biomolekul dalam kajian ini berhubungkait dengan pelbagai ciri-ciri demografik dan klinikal patologi melalui penggunaan ujian chi-square. Peringkat awal TMN berhubungkait dengan kepadatan M2 TAMs (p<0.001) dan sel IL-17^{+} (p<0.001) yang tinggi, sel CD8^{+}/sel IL-17^{+} (p<0.001) yang rendah, pengekspresan TGF-βRII (p<0.001) dan IL-17R (p=0.027) yang tinggi. Grad histologi yang rendah berhubungkait dengan kepadatan sel IL-17^{+} (p=0.035) yang tinggi, pengekspresan IL-17R (p=0.019) yang tinggi, dan sel CD8^{+}/sel IL-17^{+} (p=0.030) yang rendah. Peringkat rendah penyusup tumor (T) berhubungkait dengan kepadatan sel IL-17^{+} (p<0.001) yang tinggi, sel CD8^{+}/sel IL-17^{+} (p<0.001) yang tinggi, serta pengekspresan TGF-βRII (p<0.001) dan IL-17R (p=0.023) yang tinggi. Metastasis nodus limfa yang rendah berhubungkait dengan kepadatan M2 TAMs (p=0.023) dan sel IL-17^{+} (p<0.001) yang tinggi.

Kesimpulannnya, kajian ini menunjukkan kepadatan M2 TAMs dan sel IL-17^{+} yang tinggi, pengekspresan TGF-βRII dan IL-17R yang tinggi, dan sel CD8^{+}/sel IL-17^{+} yang rendah berhubung kait dengan preingakt awal TMN, grad histologi yang rendah, peringkat rendah penyusup tumor dan metastasis nodus limfa yang rendah. Ini menunjukkan prognosis barah kolorektal yang menggalakkan. M1 TAMs dan pengekspresan IL-17 berkolerasi secara positif masing-masing dengan TGF-βRII dan sel-sel IL-17^{+}. Ini mengimplikasikan bahawa, kepadatan M1 TAMs yang tinggi dan pengekspresan IL-17 yang tinggi juga menunjukkan prognosis barah kolorektal yang menggalakkan. Maka, penaksiran sel-sel keimunan bersama ligan and reseptor sitokin mungkin boleh berfungsi sebagai biomarker berpotensi untuk menilai perkembangan barah kolorektal dan pembangunan rawatan sasaran.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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µl	microliter
µM	microMolar
•NO	Nitric Oxide
•O2-	Superoxide
AJCC	American Joint Committee on Cancer
AHR	Aryl hydrocarbon receptor
AKT	Protein Kinase B
ANA	Apparently Normal Adjacent
APC	Adenomatous Polyposis Coli
APES	3-Aminopropyltriethoxysilane
BATF	Basic leucine zipper transcription factor
BMPs	Bone Morphogenetic Proteins
BRAF	Serine/Threonine-Protein Kinase B-Raf
BSA	Bovine Serum Albumin
CIMP	Cpg Island Methylator Phenotype Pathways
CIN	Chromosomal Instability
CRC	Colorectal Carcinoma
CSC	Cancer Stem Cells
c-Src	Proto-Oncogene Tyrosine-Protein Kinase Src
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
CTLs	Cytotoxic T Lymphocytes
DAB	3,3'-Diaminobenzidine
DC	Dendritic Cells
DFS	Disease Free Survival
DSS	Disease Specific Survival
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EMT	Epithelial Mesenchymal Transition
FFPE	Formalin Fixed Paraffin Embedded
FGF	Fibroblast Growth Factor
G-CSF	Granulocyte colony-stimulating factor
GDFs	Growth And Differentiation Factors
GF	Growth Factor
GM-CSF	Granulocyte/Macrophage Colony-Stimulating Factor
GSK3	Glycogen Synthase Kinase 3
H&E	Hematoxylin And Eosin
H_2O_2	Hydrogen Peroxide
HIER	Heat-Induced Epitope Retrieval
IFN	Interferon
IL	Interleukin
IL-1ra	IL-1 Receptor Antagonist
IRF4	Interferon regulatory factor 4
JNK	C-Jun N-Terminal Kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
LTBP	Latent TGF-B Binding Protein
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein 1

MDSCs	Myeloid-derived Suppressor Cells
MET	Mesenchymal-To-Epithelial Transition
MHC	Major Histocompatibility Complex
MIF	Müllerian Inhibitory Factor
miRs	MicroRNAs
mM	miliMolar
MMP	Matrix Metalloproteinase
MSCs	Mesenchymal Stem Cells
MSH2	Muts Homolog 2
MSH6	Muts Protein Homolog 6
MSI	Microsatellite Instability
m-TOR	The Mammalian Target Of Rapamycin
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NKT	Natural Killer T Cells
OS	Overall Survival
pCR	Pathological Complete Response
PD-1	Programmed Cell Death Protein-1
PDGF	Platelet-Derived Growth Factor
PI3K	Phosphoinositide 3-Kinase
RORC	RAR-related orphan receptor C
RORγ	RAR-related orphan receptor gamma
RAS	Receptor Tyrosine Kinases
RGM	Repulsive Guidance Molecule
RNOS	Reactive Nitrogen And Oxygen Species
STAT	Signal transducer and activator of transcription
TAAs	Tumour-Associated Antigens
TAMs	Tumor-Associated Macrophages
TBS	Tris-Buffered Saline
TCR	T-Cell Receptor
TCR	T-Cell Receptor
TGF-β	Transforming Growth Factor- β
TGF-βRII	Transforming Growth Factor- β type II receptor
TNF	Tumor Necrosis Factor
TR1	Type 1 Regulatory Cells
TRAF6	TNF Receptor-Associated Factor 6
Tregs	Regulatory T Lymphocytes
VEGF	Vascular Endothelial Growth Factor
β2m	Beta-2-Microglobulin

CHAPTER 1

INTRODUCTION

Colorectal carcinoma (CRC) is the 3rd most common human cancer (Erreni *et al.*, 2011) with approximately 1 million new cases every year (Pernot *et al.*, 2014). It is one of the most common cancer deaths around the world (Erreni *et al.*, 2011) causing more than 500,000 deaths yearly (Pernot *et al.*, 2014). Some patients respond well with the classic therapy while other remains ineffective for this aggressive cancer (Sadanandam *et al.*, 2013). Although early detection is possible through screening strategies with the advance of technology, colorectal carcinoma development and mortality persist. Challenges in colorectal carcinoma includes reduced risk of recurrence after surgery, prolong survival of patients with metastasis of colorectal carcinoma (Pernot *et al.*, 2014). Furthermore, around 50% of colorectal carcinoma patients develop liver metastasis during their lifetime and rarely survived longer than 3 years despite current improvement of therapies (Misiakos *et al.*, 2011).

This disease occurs sporadically mainly due to genetic predisposition (eg. APC, MSH2, MSH6) and prior intestinal inflammation (Norton *et al.*, 2015) not to mention changes of diet and lifestyles (Mao *et al.*, 2017). The pathogenesis of colorectal carcinoma is a highly complex process that involves many factors including accumulation of sequential genetic alteration and formation of an inflammatory microenvironment. The increased infiltration of leukocytes, a hallmark of an inflammatory microenvironment, in colorectal carcinoma consists a primarily of T lymphocytes and macrophages although other immune cells such as eosinophils, mast cells, NK cells and rare Dendritic Cells (DC) were seen (Erreni *et al.*, 2011). There are various therapeutic approaches in colorectal carcinoma treatment including surgery, chemotherapy, radiotherapy (Oki *et al.*, 2015) and immunotherapy (Mao *et al.*, 2017; Sievers *et al.*, 2016). Nevertheless, approximately 45% of the patients die from this disease (Norton *et al.*, 2015).

Tumour associated macrophages (TAMs) are the most abundant tumour infiltrating cells in tumour. TAMs assume a central role in the modulation of the tumour microenvironment in colorectal carcinoma. TAMs are highly plastic cells (Vogel *et al.*, 2014) and their phenotypes and functions change depending on various endogenous and exogenous stimuli within the tumor microenvironment (Hao *et al.*, 2012). In brief, two distinct functional phenotypes of macrophages have been categorized, M1 and M2 macrophages. M1 macrophages expressed proinflammatory cytokines and have tumoricidal capacity whereas M2 macrophages secrete a wide array of anti-inflammatory molecules and facilitate tumour progression and invasion (Vogel *et al.*, 2014). The balance between two distinct phenotype of TAMs, M1 macrophage and M2 macrophage was speculated in skewing the tumour microenvironment toward tumour prevention or tumour promotion (Edin *et al.*, 2012).

Macrophages are a crucial part of the innate immune system and are involved in many aspects of immunity and contribute to mediated tumour-immunity depending on their phenotype (Ostrand-Rosenberg, 2010). Tumour-associated macrophages (TAMs) refer to macrophages infiltrating solid tumours. TAMs are essential in tumour progression reflected through its promotion of cancer cell proliferation, invasion, migration, metastasis and angiogenesis (Laoui *et al.*, 2014). In colorectal carcinoma, dense $CD68^+$TAMs infiltration is associated with improved prognosis (Forssell *et al.*, 2007; Koelzer *et al.*, 2016) and the phenotype of TAMs regulate prognostic impact of TAMs (Koelzer *et al.*, 2016).

$CD8^+$cytotoxic T cells are one of the major effector cells of adaptive cellular immunity and are a crucial part of immune system. They are vital players in eliminating viral pathogen, foreign antigens (Wood *et al.*, 2009) and against cancer cells (Ostrand-Rosenberg, 2010). $CD8^+$T cells are vital players in antitumor immunity in colorectal carcinoma (Deschoolmeester *et al.*, 2011; Naito *et al.*, 1998) and have positive correlation with prognosis of colorectal carcinoma (Funada *et al.*, 2003; Ling *et al.*, 2014). Furthermore, combined analysis of $CD8^+$T cells with other T cell subpopulation gave an improved prognosis in colorectal carcinoma (Tosolini *et al.*, 2011). Colocalization of $CD8^+$T cells with also $CD20^+$ B cells indicates favourable prognosis in cancer (Whiteside, 2013).

TGF-β is a multifunctional cellular factor. It regulates growth, differentiation, adhesion, migration, and apoptosis of diverse cells via binding to its receptor TGF-βRI and TGF-βRII. TGF-β suppresses colorectal epithelial cells growth by inhibition of cell proliferation and promotion of apoptosis. Many tumours have the capability to bypass this mechanism and excessive TGF-βin tumours facilitate invasion and migration of tumour (Li *et al.*, 2011). Although TGF-β function as tumour suppressor in normal epithelium (Gulubova *et al.*, 2010), TGF-β also has a multifunctional role in tumour development including modulating the biological activity of both the tumour and TAMs (Peng *et al.*, 2013). TGF-β promotes tumourigenesis via stimulation of angiogenesis and induction of epithelial-mesenchymal transition (EMT) (Ikushima & Miyazono, 2010) concurrently suppresss the tumoricidal ability of macrophages (Pardali & Moustakas, 2007). In addition, TGF-β secretion by tumour contributes to macrophages recruitment enable effective tumour evasion (Byrne *et al.*, 2008).

In general, binding of TGF-β to the TGF-β type II receptor (TGF-βRII) initiates the cascade of TGF-β signaling pathway (Meng *et al.*, 2011). The common cause of TGF-β signalling pathway aberration in colorectal carcinoma is the loss of TGF-βRII expression (Kalkhoven *et al.*, 1995). Loss of TGF-βRII expression inactivates TGF-β signalling and its function hence render TGF-β resistance in tumour (Takenoshita *et al.*, 1996). Loss of TGF-βRII expression are caused by either lack of detectable receptor types or low expression of receptor (Laiho *et al.*, 1990). Subsequently, tumour progression was promoted (Busch *et al.*, 2015; Lu *et al.*, 2006; Malkoski *et al.*, 2012).

Interleukin-17 (IL-17) is a pro-inflammatory cytokine which links innate and adaptive immunity (Wu *et al.*, 2013) by participating in both innate and adaptive immunity during the physiological or pathological process of autoimmunity, allergy, host defence and cancer (Gu *et al.*, 2013; Song & Qian, 2013). IL-17 either displays protumour or antitumour role in various cancers (Murugaiyan & Saha, 2009). Recent studies have shown that IL-17 plays a critical role in modulating angiogenesis and production of a variety of proangiogenetic factors in cancer (Yang *et al.*, 2014). Majority studies in colorectal carcinoma regard IL-17 as promoter in tumour initiation and progression (Wu *et al.*, 2013). Deregulation of IL-17 contributes to the pathogenesis of inflammatory and autoimmune diseases as well as cancer progression (Yang *et al.*, 2014).

IL-17 mediates its function via its receptor to promote the generation of pro-inflammatory cytokines and chemokines (Jin & Dong, 2013) which recruit monocytes and neutrophils in to the inflammatory site (Punt *et al.*, 2015; Wu *et al.*, 2013). Albeit IL-17 is the hallmark of Th17 T cells, innate immune cells including $\gamma\delta$ T cells, invariant natural killer T cells, neutrophils, macrophages, and mast cells also serve as source of IL-17 (Punt *et al.*, 2015). In colorectal carcinoma, CRC-infiltrating IL-17$^+$ cells have been observed to recruit highly cytotoxic CD8$^+$ T cells (Amicarella *et al.*, 2015) and associated with infiltration of M2 TAMs (Mao *et al.*, 2016).

Wnt signaling is one of the major signaling pathways involved in embryogenesis, regulation of cell growth, motility and differentiation, and wound healing (Behrens & Lustig, 2004; Krausova & Korinek, 2014). Wnt signaling transduces signals through β-catenin dependent (canonical) and β-catenin independent (non-canonical) signaling. Wnt signaling is association with various cancers (Zhan *et al.*, 2017). Aberrants in Wnt signaling is the most studied in colorectal carcinoma. The hallmark of aberrated Wnt signaling is overexpression of β-catenin. Loss of Adenomatous Polyposis Coli (APC) expression due to gene mutation is one of the main causes (Behrens & Lustig, 2004; Zhan *et al.*, 2017).

Both diversity of tumour infiltrating immune cells and functional involvement of biomolecules are part of the complex system that exerts a significant influence on tumour development, clinical outcome and treatment of cancer. Their roles in colorectal carcinoma have been studied separately in various studies. Tumour-associated macrophages (TAMs) are the major population of innate immune cells in the tumour microenvironment and are the critical player in modulating molecular events in the tumour microenvironment that lead to disease progression. However, the interrelationships between TAMs, other immune cells and biomolecules are poorly understood. Hence, a better understanding of the relationship between these immune cells and biomolecules and their relationship between severity of colorectal carcinoma may produce potential biomarkers of prognosis as well as personalized therapy in colorectal carcinoma.

The hypotheses of this study are:

1. M1 TAMs are positively correlated with the density of IL-17$^+$ cells and CD8$^+$ cells
2. M1 TAMs are positively correlated with TGF-βRII and IL-17
3. M2 TAMs are negatively correlated with TGF-βRII and TGF-β
4. TGF-βRII, TGF-β, IL-17R, and IL-17 are positively correlated to one and another
5. Density of IL-17$^+$ cells and CD8$^+$cells are positively correlated with TGF-βRII, TGF-β, IL-17R and IL-17
6. M1 TAMs, M2 TAMs, CD8$^+$cells, IL-17$^+$ cells, TGF-βRII, TGF-β, IL-17R, and IL-17 in colorectal carcinoma are associated with Wnt signaling pathway

General objective:

To study interrelationship of M1 TAMs and M2 TAMs with CD8$^+$ cells, IL-17$^+$ cells, TGF-βRII, TGF-β, IL-17R and IL-17 in the progression of colorectal carcinoma.

Specific Objectives:

1. To evaluate density of tumour infiltrating immune cells, M1 TAMs, M2 TAMs, CD8$^+$ cells and IL-17$^+$ cells in colorectal carcinoma
2. To evaluate the expression of biomolecules, TGF-βRII, TGF-β, IL-17R and IL-17 in colorectal carcinoma
3. To correlate tumour infiltrating immune cells with the expression of biomolecules in colorectal carcinoma in this study
4. To determine the possible relationship between tumour infiltrating immune cells and the expression of biomolecules with Wnt signaling pathways in colorectal carcinoma.
5. To associate tumour infiltrating immune cells and the expression of biomolecules in colorectal carcinoma in this study with clinicopathological features in colorectal carcinoma

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