VALIDATION OF PASD1 PROTEIN AS AN IMMUNOTHERAPEUTIC TARGET IN COLORECTAL CANCER

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VALIDASI PROTEIN PASD1 SEBAGAI SASARAN IMMUNOTERAPI DALAM KANSER KOLOREKTAL

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DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged.

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ABSTRAK

Kanser kolorektal (CRC) merupakan penyebab kematian ketiga tertinggi di Asia termasuk Malaysia. Cancer Testis Antigen (CTA) diekpreskan dalam pelbagai jenis kanser namun dengan ekspresi terhad di dalam tisu normal kecuali testis, merupakan suatu pendekatan yang baharu bagi meningkatkan pilihan rawatan CRC. PAS (Per ARNT Sim) Domain 1 merupakan salah satu CTA yang banyak dilaporkan terekspresi dalam kanser hematopoitik tetapi sangat terhad dalam tumor pepejal. Tujuan kajian ini dilakukan adalah untuk mengkaji pengekspresan PASD1 di dalam CRC dan kesan tindakbalas sel T CD8 serta profil sitokinnya selepas distimulasi dengan peptida PASD1. Pengekspressan mRNA PASD1 telah ditentukan menggunakan PCR masa nyata pada tisu kanser dan tisu normal bagi 25 sampel pesakit CRC. Bagi penentuan pengekspresan tisu PASD1 pula, kaedah imunohistokimia telah digunakan untuk 23 sampel pesakit CRC. Sebanyak empat imunogenik peptida PASD1 yang spesifik kepada HLA-A*24:02 telah dikenalpasti menggunakan platform algoritma berasaskan web. Sel-sel darah periferal mononuklear daripada pesakit CRC telah digunakan untuk mengkaji secara *in vitro* kesan imunogenesiti peptida-peptida ini dengan menggunakan *IFN-y ELISpot*. Kesan efektor sel T $CD8^+$ dikultur bersama peptida PASD1 terhadap sel kanser telah dikesan menggunakan asai *cytolytic and granzyme-B release ELISpot cytotoxicity*. Profil sitokin pada sampel ko-kultur antara sel efektor dan target juga turut dinilai. Melalui kajian ini, peratusan ekspresi PASD1 menunjukkan sebanyak 20% dalam gen dan 17.3% dalam protin sampel CRC. Di samping itu, PASD1(4) menunjukkan kesan imunogenik secara selektif dalam sampel CRC. Sel T sitotoksik spesifik kepada PASD1 yang dihasilkan daripada ketiga-tiga pesakit CRC berjaya melisiskan sel selanjar SW480. Selain daripada itu, kajian ini menunjukkan bahawa berlakunya peningkatan kebergantungan dos bagi IL-10, IFN-γ, TNF-α, sFas, sFasL, granzyme A, granzyme B dan perforin dalam media sel T CD8 yang dikultur bersama sel selanjar SW480. Secara keseluruhannya, kajian ini telah menunjukkan bahawa peptida PASD1 (4) dengan urutan amino asid TYCSSTVFL pada kedudukan 276-273, telah dikenalpasti menjadi lebih imunogenik berbanding peptide lain dan ia berpotensi untuk menjadi sasaran berasaskan peptida dalam imunoterapi HLA-A*24:02 CRC.

ABSTRACT

Colorectal cancer (CRC) is the third commonest malignancy in Asia including Malaysia. Cancer testis antigens (CTA) which are expressed in a variety of cancers but with limited expression in normal tissues except the testis, represent an attractive approach to improve treatment options for CRC. PAS (Per ARNT Sim) domain containing 1 (PASD1) is a CTA reported to be expressed mostly in hematopoietic malignancies but has a limited expression in solid tumours. The aim of the study was to investigate the expression of PASD1 in CRC samples and assess the CD8 T cell responses, as well as the cytokine profiles upon induction with PASD1 peptides. PASD1 mRNA expression was determined via RT-PCR on paired tumour and normal tissue samples from 25 CRC patients. Tissue expression of PASD1 was assessed via immunohistochemistry in 23 CRC samples. Four immunogenic PASD1 peptides specific to HLA-A*24:02 were identified using web-based algorithms. Peripheral blood mononuclear cells from CRC patients were used to investigate the immunogenicity of these peptides *in vitro* using IFN-y ELISpot assay. The effects of the CD8$^+$ T cells pulsed with PASD1 peptides against cancer cells were performed using the cytolytic and granzyme-B ELISpot cytotoxicity assays. The cytokine profiles of the co-culture between effector and target cells were also assessed. From this study, the gene and protein expression of PASD1 were detected in 20% and 17.3% of the CRC samples respectively. PASD1(4), was shown to be selectively immunogenic in CRC samples. The generated PASD1 specific cytotoxic T cells from the three CRC patients were able to lyse the SW480 cell line. Moreover, this study also demonstrated the dose-dependent increase of IL-10, IFN-y, TNF-α, sFas, sFasL, granzyme A, granzyme B and perforin in the co-culture media of CD8 T cells with SW480 cell line. Overall, the PASD1 (4) peptide with the amino acids sequence of TYCSSTVFL at the position 276-273 was identified to be relatively more immunogenic as compared to the other peptides and it represents a potential target for peptide-based immunotherapy in HLA-A*24:02 CRC.

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LIST OF SYMBOLS

˚	degree
˚C	degree Celcius
µL	microlitres
mL	millilitre
L	litre
g	gram
ng	nanogram
µg	microgram
mg	milligram
min	minutes
s	seconds
h	hour
U	Unit
µM	microMolar
µm	micrometre
E/T	Effector/Target cells
Ct	Cycle threshold
kb	kilobase
bp	base pair
α	alpha
β	beta
γ	gamma
®	registered sign
P	significant
× g	g force
rpm	revolutions per minute

LIST OF ABBREVIATIONS

ABL	Abelson murine leukaemia
AFND	Allele Frequencies Network Database
AKAP-4	A-kinase anchoring protein 4
ALP	Alkaline phosphatase
AML	Acute myeloid leukaemia
ANOVA	Analysis of Variance
APCs	Antigen-presenting cells
APC dye	Allophycocyanin dye
ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guérin
β-hCG	β-human chorionic gonadotropin
bHLH	Basic helix loop helix
BCR	Breakpoint cluster region
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
CA-125	Cancer antigen 125
CAR	Chimeric antigen receptor
5CD	5’-aza-2’-deoxycytidine
CEA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CRC	Colorectal cancer
CRT	Chaperone calreticulin
CNX	Chaperones calnexin
CTA	Cancer testis antigen

CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CO_2	Carbon dioxide
COX-2	Celecoxib
CXC9/10	Chemokine (C-X-C motif) ligand 9 or 10
CX3CL1	C-X3-C motif chemokine ligand 1
DAB	3,3-diaminobenzidine
DAPI	4,6-diamidino-2-phenylindole
DBLCL	Diffuse large B cell lymphoma
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase inhibitors
DR	Dioxin (aryl hydrocarbon) receptor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
EMEM	Eagle's minimum essential medium
ER	Endoplasmic reticulum
ERAAP	Endoplasmic reticulum-associated aminopeptidase
ERAP1	Endoplasmic reticulum aminopeptidase 1
EST	Expressed sequence tag
FACS	Fluorescence-activated cell sorting
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FF	Fresh frozen
FFPE	Fresh frozen formalin-fixed paraffin-embedded
FITC	Fluorescein isothiocyanate
FS	Flexible sigmoidoscopy

FOBT	Fecal occult blood test
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GB	Granzyme B
GM2	Ganglioside activator 2
GM-CSF	Granulocyte macrophages colony stimulating factor
Gln	Glutamine
GVAX	Allogeneic GM-CSF secreting tumour vaccine
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER-2	Human epidermal growth factor receptor 2
HIF-α	Hypoxia-inducible factor-alpha
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HNPCC	Hereditary nonpolyposis colorectal cancer
HUKM	Hospital UKM
ICAM1	Intercellular adhesion molecule 1
IFN-α	Interferon alpha
IFN-γ	Interferon-gamma
IHC	Immunohistochemistry
IgG	Immunoglobulins G
IF	Immunofluorescence
IL	Interleukin
Kras	Kristen-ras
LAGE-1	Also as known as cancer-testis antigen 2
MADCAM1	Mucosal addressin cell adhesion molecule 1
MAGE	Melanoma-associated antigen
MCAK	Mitotic centromere-associated kinesin

MHC	Major Histocompatibility Complex
MM	Multiple myeloid
MMA-1A	Malignant melanoma associated protein 1
MLH1	mutL homolog 1
MSH2	mutS homolog 2
MSH6	mutS homolog 6
MSI	Microsatellite instability
mRNA	Messenger ribonucleic acid
MRI	Magnetic resonance imaging
NCBI	National Center for Biotechnology Information
NBT	Nitro-blue tetrazolium chloride
NK	Natural killer
NSAID	Nonsteroidal anti-inflammatory drugs
NY-ESO-1	New York oesophagal squamous cell carcinoma 1
OCT	Optimal cutting temperature
OY-TES-1	Also known as acrosin-binding protein
PASD1	Per ARNT SIM domain-containing 1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
Pen-Strep	Penicillin/streptomycin
PCR	Polymerase chain reaction
PD1	Programmed cell death protein 1
PE	Phycoerythrin
PET	Positron emission tomography
PHA	Phytohaemagglutinin
PMS1	PMS homolog 1
PMS2	PMS homolog 2
PRAME	Preferentially expressed antigen in melanoma
PSA	Prostate-specific antigen

PVDF	Polyvinylidene fluoride
rIL	Recombinant interleukin
RT-PCR	Real-time polymerase chain reaction
SAGE	Serial analysis of gene expression
SAGE-1	Sarcoma-associated antigen 1
SART3	Squamous cell carcinoma antigen T cells 3
SEREX	Serological identification of antigens by recombinant expression cloning
SERPA	Serological proteome analysis
SPAG-9	Sperm-associated antigen 1
SPAN-X-C1	Sperm protein associated nucleus X chromosome 1
SSX	Synovial sarcoma, X breakpoint 1
TAA	Tumour-associated antigens
TAE	Tris acetate-EDTA
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	Tumour growth factor-beta
TIL	Tumour infiltrating lymphocytes
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumour necrosis factor-alpha
TP53	Tumour protein 53
TPTE	Tyrosine-protein phosphatase
T-VEC	Talimogene laherparepvec
UKM	Universiti Kebangsaan Malaysia
UMBI	UKM Medical Molecular Biology Institute
USA	United States of America
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

1.1 RESEARCH BACKGROUND

Colorectal cancer is known as the third commonest malignancy in the world affecting 1.4 million cases and 700,000 death worldwide has become a major public health problem across the globe (Arnold et al. 2017; Ferlay et al. 2013). With the rising rate of colorectal cancer (CRC) in young and middle-aged adults, this disease is also ranked as the second most frequent cancer in Asian men and third in women (Faltejskova et al. 2012; Marley & Nan 2016; Siegel et al. 2017). Approximately 96% of CRC are adenocarcinomas while the rest include mucinous carcinomas and adenosquamous carcinomas (Ferlay et al. 2013). In the United States, the five-year survival rate of CRC patients who undergo surgical resection, starting with stage I is (91.1%), followed by stage II and III (71.7%) and stage IV (13.3%) respectively (Siegel et al. 2017). On the other hand, the standard treatment option for CRC is commonly by surgical resection for (stage I and II), followed by surgical resection with chemotherapy for higher risk (stage II and III) and lastly a combination of surgical with chemotherapy or targeted therapies for metastatic (stage IV) CRC patients (Siegel et al. 2017).

In Malaysia, the treatment response of CRC remains poor, with 51.1% of overall five-year survival rate for cases diagnosed in 2007-2011 and followed up to 2016 (National Cancer Registry 2018). Although conventional chemotherapy and radiotherapy have had some clinical success, a subset of CRC patients still harbours residual disease due to disseminated tumour cells (Augestad et al. 2015; Homayounfar et al. 2013). The mortality rates for each representing CRC stage are still on the rise, with 8-13% reported for stage I and II, followed by 11%-47% for stage III and 89% for stage IV CRC (Koi & Carethers 2017). About 20–30% stage II and 50–60% stage III CRC patients experience relapse within the five years after chemo or radical therapy with poor prognosis (Morgan et al. 2004). A subpopulation of resistant cancer stem cells, residing within the tumour bulk is believed to be responsible for the recurrence of the disease (Hu & Fu 2012). Hence, innovative and effective alternative therapies are urgently needed to target and combat against CRC. A key paradigm shift from traditional chemotherapy to focus on immunology-cancer interface and microenvironment has been a surge in cancer immunotherapy for alternative therapies. The understanding between the interaction between cancer and the immune system has provided many insights into approaches that can be exploited to trigger anti-tumour immune responses.

Cancer immunotherapy, a more-recent approach has centred on the equal importance on the tumour and tumour microenvironment plays a pivotal role in cancer progression and remission (Cheever & Higano 2011). It emphases on provoking the immune system and triggering the body defence system to combat against cancer rather than administrating a toxin that directly causes damages to both normal and cancer cells (Koi & Carethers 2017). This integrative strategy has predicted to improve the outcomes of all stages of CRC. Cancer-testis antigens, a group of testicular-restricted proteins are chosen as the interest targets due to the restricted expression in normal tissues being predominantly expressed only in testis but are aberrantly expressed in a wide range of tumours (Shao et al. 2008). The testis is an immunopurified site with low or even absent human leukocyte antigen (HLA) expression in the cell surface (Shao et al. 2008). Such pattern of expression raises the possibility of targeted treatment while minimizing any potential autoimmune problems for cancer patients (Caballero & Chen 2009). In this study, Per ARNT SIM (PAS) domain-containing 1 (PASD1) protein represents one of the cancer-testis antigens is selected in treating CRC. The objectives of this project are to investigate the expression and T cell responses of PASD1 as well as to assess the cytokine profiles of CRC patients upon induction to PASD1.

1.2 PROBLEM STATEMENT

According to the National Cancer Registry Report in 2007, a total of 2,246 CRC cases were registered nationwide and it represented 12.3% of all registered cancer cases (Zainal Ariffin & Nor Saleha 2011). Despite the conventional surgical resection or/and in combination with chemotherapy or radiotherapy, nearly 50% of CRC patients still experience a recurrence (Morgan et al. 2004). The five-year survival rate for patients with advanced colon cancer is as low as 13% (Siegel et al. 2017). It is understood that some of the conventional treatments for CRC are not efficient and have resulted in multiple unwanted side effects.

In Malaysia, CRC ranks to be the third most diagnosed malignancy in the population after lung and breast adenocarcinomas (Siegel et al. 2017). This malignancy has certainly imposed a great financial burden on the Malaysian health system and warrants better approaches for treatment option aimed at improving its overall survival rate. Cancer immunotherapy may serve to be the next line targeted approach for CRC but there is still a need to find a potential immunotherapeutic target in CRC in Malaysia population.

1.3 OBJECTIVE OF RESEARCH AND SCOPE OF WORKS

1.3.1 General Objective

This research focuses on the role of Per ARNT SIM (PAS) domain-containing 1 (PASD1) proteins and its potential as an immunotherapeutic target in CRC. The scopes of this study are to determine the expression and T cell responses of PASD1 as well as to assess the cytokine profiles in CRC patients.

1.3.2 Specific Objectives

1) To determine the mRNA and protein expression of PASD1 in CRC cell lines and primary CRC tissues

2) To identify a potential immunogenic PASD1 peptide for *in vitro* expansion of cytotoxic T lymphocytes from CRC patients

3) To determine the specific cytolytic T cell responses in CRC patients upon stimulation with PASD1 peptide

4) To determine the cytokine profiles of CRC samples upon induction of PASD1

1.4 HYPOTHESIS

1) The mRNA and protein of PASD1 are predicted to be moderately expressed and localized at the nucleus and cytoplasm region of SW480 and CRC primary cells.

2) The designed PASD1 peptides are immunogenic in HLA-A*24:02 positive CRC patients.

3) *In vitro* culture PASD1-positive CTLs from CRC patients able to lyse PASD1-positive cell lines in CD8-mediated T cell lysis manner.

4) Cytokines such as IFN-γ, TNF-α and IL-10 are expected to be elevated in CRC upon induction of PASD1.

CHAPTER II

LITERATURE REVIEW

2.1 COLORECTAL CANCER

2.1.1 Epidemiology of Colorectal Cancer

Colorectal cancer (CRC) is one of the leading causes of mortality affecting both men and women in the world (Favoriti et al. 2016). It is the third most common malignancy and the second leading cause of cancer-related deaths among Asian and Pacific Islander population (Siegel et al. 2017). The incidence rate was reported with an estimated total of 490,000 cases up to 1,361,000 newly diagnosed CRC cases in 1992 to 2012 (De Martel et al. 2012; Ferlay et al. 2015; Rafiemanesh et al. 2016). On the other hand, the new cases of CRC are estimated to elevate up to 1,400,000 cases and expected to have 700,000 cases of CRC deaths in the coming years (Arnold et al. 2017). In terms of geographical distribution, the newly diagnosed cases of CRC in some of the listed countries such as Eastern Europe, Latin America, and Asia are reported to have a higher rate of increase (Center et al. 2009). On that account, the global burden of CRC is anticipated to grow by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030 in the world (Arnold et al. 2017).

Colorectal cancer was primarily considered as a Western disease, but its incidence has increased in many developing Asian countries such as Malaysia over the years (Azeem et al. 2016). In Malaysia, CRC is ranked to be the second most frequently diagnosed cancer (746,000 cases, 10% of the total reported cancers) in men after lung cancer and third most frequent diagnosed cancer (614,000 cases, 9.2% of the total reported cancers) in women after breast cancer and cervical cancer (Ferlay et al. 2013). In Malaysia, the incidence of CRC was reported to have 21.32 cases per

100,000 in 2012, with Chinese (27.35 per 100,000) as the highest, followed by Malay (18.95 per 100,000) and Indian (17.55 per 100,000) population (Abu Hassan et al. 2016). However, the mortality rate of CRC in Malaysia was reported to have 9.79 cases per 100,000 Malaysia population, with Chinese as the highest (11.85 per 100,000), followed by Malay (9.56 per 100,000) and Indian (7.08 per 100,000) population (Abu Hassan et al. 2016). The overall incidence and mortality rate of CRC was 1.33 and 1.42 times higher in males as compared to females (Abu Hassan et al. 2016).

Furthermore, in terms CRC incidence rate in the aspect of CRC tumour subsite distribution, 41% was identified in the proximal colon, followed by rectum (28%), distal colon (22%) and other parts of the colon (8%) (Siegel et al. 2017). The risk of CRC localized in the proximal proportion of colon was reported to increase with age and to be found higher in women than men (Siegel et al. 2017). The CRC risk is known to be affected by the acceptance of westernized diets, tobacco intake, alcohol consumptions and the unhealthy lifestyles among the Asians (Ferlay et al. 2013; Pericleous et al. 2013). Other factors such as the availability of diagnostic and preventive measures in some rural areas, the limited access to healthier diets and the environments can also be the contributing risks (Faltejskova et al. 2012).

2.1.2 Risk Factors of Colorectal Cancer

Approximately 76% of CRC patients experience a sporadic risk of CRC whereas the rest is believed to have genetically inherited the disease (Askling et al. 2001; Haggar & Boushey 2009; Siegel et al. 2017). In Malaysia, the Chinese population is reported to have a higher burden for CRC as compared to Malay and Indian population (Ghee 2014). The common risk factors are the low nutritional intake, high consumption of red meat and saturated fats, high excessive alcohol consumption, followed by cigarette smoking, lack of physical activity and high tobacco consumption (Ghee 2014). Besides, the high dietary content of heme in red processed meat and heterocyclic amines production in high temperature blackened meat is reported to be one of the causes of CRC (Ishikawa et al. 2010; Martínez et al. 2007). Nevertheless, the habitual consumption of alcohol leads to the formation of reactive oxygen species that obstruct metabolic processes and creating changes in bile acid compositions (Schernhammer et al. 2010; Stickel & Hampe 2012). It was believed to cause destruction in DNA and the proliferation of colonic epithelial cells (Cross et al. 2003; Ishikawa et al. 2010).

Inevitably, a small proportion of CRC risk can be inherited through family history. Two most common forms of inherited CRC syndromes are Hereditary Non-Polyposis CRC (HNPCC) and Familial Adenomatous Polyposis (FAP) (Ghee 2014). HNPCC also called as Lynch syndrome, is an autosomal dominant inheritance causing 2-3% cases of CRC caused by the HNPCC related mutation in the DNA mismatch repair genes such as MLH1, MSH2, MSH6, PMS1 and PMS2 (Zahary et al. 2017). On the other hand, FAP is a rare autosomal inherited disorder accounting for less than 1% of all colorectal cancer cases (Burke et al. 2016; Haggar & Boushey 2009). As such, this genetic disease is reported to cause by germline mutation with the loss of heterozygosity at chromosome 5q arm in the *adenomatous polyposis coli* gene which attributes to the formation of multiple adenomatous polyps in the lining of colon or rectum (Haggar & Boushey 2009).

2.1.3 Diagnosis of Colorectal Cancer

The tumorigenesis of CRC is identified in two forms, as benign or adenocarcinoma (Bardhan & Liu 2013). About 96% of CRC is estimated to be adenocarcinomas, while 2% are the other specified carcinomas such as carcinoid tumours, 0.4% are epidermoid carcinomas and 0.08% are sarcomas (Stewart et al. 2006). To date, the advancement in CRC preventive screening has nonetheless contributed to the expansion of many available yet affordable diagnosis for CRC. The diagnostic measures for CRC include the use of carcinoembryonic antigen (CEA) test, faecal occult blood tests, stool DNA test and digital rectal examination (Shrestha et al. 2017). Moreover, for people who have the higher risk of CRC, more precise diagnostic options such as visual colonography, colonoscopy, sigmoidoscopy, and double-contrast barium enema are encouraged (Atkin et al. 2010; De Martel et al. 2012; Halligan et al. 2013). Furthermore, other imaging tests such as Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) scans may be beneficial in certain cases of CRC (Schaefer & Langer 2007).

2.1.4 Molecular Mechanisms of Colorectal Carcinogenesis

Colorectal cancer is a multifactorial disease and many conditions may influence the likelihood of CRC occurrence. According to the previous study, approximately 15% of CRC is identified as hypermutated CRC while 85% is classified as non-hypermutated CRC (Koi & Carethers 2017). The heterogeneity nature of CRC can be influenced by several genetic and epigenetic factors, ranging from the involvement of chromosomal instability (CIN) pathway, CpG island methylator phenotype (CIMP), mutation of oncogenes, microsatellite instability (MSI) phenotype to DNA hypomethylation (Grizzi et al. 2013). The molecular mechanisms of CRC are also reported to be influenced by the role of CpG island methylator phenotype, the instability of chromosome and microsatellite in the intestinal crypt stem cells of CRC (Tariq & Ghias 2016). Furthermore, the tumorigenesis of CRC can be caused by the accumulation of the genetically altered genes such as the oncogenes, tumour suppressor genes, mismatch repair genes or defective genes in Wnt signalling pathway (Armaghany et al. 2012; Segditsas & Tomlinson 2006). The disruptions in the genetic profiles of the colonic cells are known to clonal expansion and irregular cell proliferation contributing to the pathogenesis of CRC (Luebeck & Moolgavkar 2002).

In term of chromosomal instability, the assimilation of mutated *adenomatous polyposis coli* gene, the mutational activation of oncogenes such as *Kirsten-ras* (Kras) and the silencing of tumour suppressor gene, *TP53* are reported to be the reasons of chromosomal instability in CRC (Fearon & Vogelstein 1990; Tariq & Ghias 2016). The chromosomal instability associated with sporadic CRC is influenced by the mutation in the chromosome segregation, loss of heterozygosity, disruption in the DNA damage pathway, tumour suppressor genes, aneuploidy and excess telomere breakage (Smith et al. 2002; Tariq & Ghias 2016). Followed by, the mutations in the *adenomatous polyposis coli* gene in the modulation of the Wnt signalling pathway in CRC stem cell differentiation and renewal (Fodde 2002). Moreover, the point mutations in codons 12,13 and 31 of the *Kras* gene was known to promote the growth of the tumour and lymph node metastasis of CRC (Li et al. 2015). The defective in *TP53* gene resulted in a mutant isoform of P53 protein can increase inflammation and upregulate tumorigenesis of CRC (Cooks et al. 2013).

Furthermore, the defective modifications in the DNA mismatch repair genes such as *hMLH1*, *hMLH3*, *hMSH2*, *hMSH3* and *hMSH6* have also been described to affect DNA repair mechanisms and impair cellular pathways in CRC tumorigenesis (Bronner et al. 1994; East et al. 2008; Nicolaides et al. 1994; Zhang et al. 2005). Similarly to the epigenetic mutations such as hypermethylation or hypomethylation of CpG island at the promoter region of the gene results in the silence of tumour suppressive genes in regulating cellular transformation and cell cycle of CRC (Weisenberger et al. 2006). Lastly, non-coding RNAs which comprises of 2% of the human genomes such as microRNAs, small interfering RNAs, and long non-coding RNAs tend to play a role in the cell proliferation and invasion of CRC as well (Bartel 2009; Kamatani et al. 2013; Tariq & Ghias 2016). Overall, the impact of non-coding RNAs has become increasingly crucial in the area of CRC pathogenesis and metastasis due to their roles in chromatin remodelling, transcriptional and post-transcriptional gene expression regulations (Hauptman & Glavač 2013).

2.1.5 Current Treatments of Colorectal Cancer

Surgical removal of CRC with post-surgical therapies such as adjuvant chemotherapy or radiotherapy is known to be the standard line of treatments for CRC (Morgan et al. 2004; Veettil et al. 2016). It is estimated about 84% of stage I and II of CRC patients receive partial or total colectomy while 67% of stage II and III of CRC patients receive colectomy in addition to chemotherapy to reduce the risk of CRC occurrence (Miller et al. 2016). On the other hand, stage II and III of CRC cases are commonly given neoadjuvant chemotherapy in combination with radiation (Miller et al. 2016). Nevertheless, cytotoxic chemotherapy is commonly given to treat all stage IV cases of CRC (Miller et al. 2016). Some of the well-known chemotherapy drugs used in treating CRC are capecitabine (Xeloda) (Twelves et al. 2001; Van Cutsem et al. 2001; Walko & Lindley 2005), 5-fluorouracil (Adrucil) with leucovorin (folinic acid) (Qazi et al. 2007; Robinson et al. 2011), FOLFOX: leucovorin (folinic acid), 5-fluorouracil (Adrucil, 5-FU) and oxaliplatin (Eloxatin) (Bokemeyer et al. 2011; Van Cutsem et al. 2009) and CAPOX (also called XELOX): capecitabine (Xeloda) and oxaliplatin (Eloxatin) (Porschen et al. 2007; Souglakos et al. 2006).

Furthermore, combination therapy of either preoperatively or postoperatively with chemotherapy or radiotherapy can be coupled at the same time to achieve better remission of CRC. It is also reported that a combination of chemo drugs after surgery is shown to have some positive feedback evidence of the reduced possibility of local reappearance of CRC when compared to only surgical removal of CRC alone (Wolpin & Mayer 2008). In spite of the clinical success of the adjuvant drug in treating CRC, it is often linked to adverse high level of cytotoxicity side effects against the healthy cells (Urruticoechea et al. 2010). Due to the lack of selectively, there is a tendency to cause drug resistance these cancer cells (Urruticoechea et al. 2010).

As such, some common set of adverse effects of chemotherapy and radiotherapy are the loss of cognition such as difficulty with memory, coordination, mood; nausea; dehydration; anaemia; peripheral neuropathy is frequently be related to general fatigue, numbness or pain (Winocur et al. 2016). Besides, neutropenia caused by the presence of low white blood cells leads to the increased susceptibility to infections (Chen et al. 2016). Thrombocytopenia that is affected by the cause of bleeding in the digestive tract, complications with menstruations or even manifests in vomits, stools or nosebleeds (Heianna et al. 2016). Cardiomyopathy that implicates arrhythmia; infertility in men and women as well as osteoporosis (Miller et al. 2016). Other obvious effects such as hair loss, rashes, dryness and burning sensations at the palms; alopecia that causes the fingernails and toenails to be brittle and turns colours (Beijers et al. 2016; Miller et al. 2016).

Based on a study reported on the inclusive use of cytotoxic chemotherapy to CRC patients in 2011 predicted that the five-year survival rate in early stages of CRC to be 2.3% in Australia population and 2.1% in USA population (Morgan et al. 2004). In addition, for Duke's B colon carcinoma, it has been reported to be 1.8% and 1.0% of CRC patients in Australia and the USA have actually benefited from chemotherapy respectively. Similarly, for Duke's B and C rectal cancer patients in Australia was 5.4% and the USA was 3.4% respectively (Morgan et al. 2004). Taken together, most of the CRC patients generally had low survival rates when only chemotherapy alone was given. Hence, the use of cytotoxic chemotherapy as the alternative palliative treatments in CRC may only partly explain the 20% improvement in the five-year survival of CRC (Slater 2001). More effective alternative treatment is urgently desired to improve the quality of life and prolong the survival rate.

2.2 CANCER IMMUNOTHERAPY

Cancer immunotherapy, also known as targeted cancer therapy, is a way to eliminate cancer cells by stimulating the patient's own immune system rather than the passive means of conventional approaches (Palucka & Banchereau 2012). It is an emergent approach that focuses on enhancing the immune system to destroy cancer cells prophylactically and therapeutically (Palucka & Banchereau 2012). It also aims to halt the activation of defective genes in promoting tumour growth (Koi & Carethers 2017). Historically, the concept of immunotherapy first emerged when William Coley, a New York leading surgical oncologist, administered toxins from *Streptococcus pyrogenes and Serratia marscescens* to his sarcoma patient in 1890s (Coley 1893; Richardson et al. 1999; Wiemann & Starnes 1994). The vaccine was later named as Coley's toxins (Cann et al. 2003).

With his discovery in days where the advances in cancer treatment option were still in scarcity, the idea was first accepted with positive responses by the community and some patients with inoperable sarcomas benefited from the treatment with more than 10% of the cure rate (Parish 2003). However, his approach in cancer immunotherapy was abandoned later due to the short-lived effects of the therapy and the success was rather sporadic and difficult to replicate scientifically (Cann et al. 2003). Until 1976, when one notable exception trial of using tuberculosis vaccine *Bacille Calmette-Guérin* (BCG) with Coley's approach as cancer therapy to treat the recurrence of non-muscle invasive bladder cancer resurfaced and proved to be effective until today (Morales et al. 2017). Since then, numerous immunologists and oncologists worldwide have centred to discover more immunotherapeutic target hoping to elicit a more precise and durable immune response towards cancer.

Our immune system comprises of both innate immunity and adaptive immunity that act as an integral part of our body defence system (Iwasaki & Medzhitov 2015). Several elements in the immune system known to play significant roles in cancer prevention and protection (Simpson et al. 2005). The innate immune system functions to mediate immediate and short-lived responses (monocytes, macrophages, dendritic cells, natural killer cells, cytokines, and the complement proteins) while adaptive immunity develops long-lived and memory immune responses (T cells and B cells) (Borghaei et al. 2009). These immunities work together to provide rapid response in defending against malignant cells that are detrimental to the host.

More importantly, the vital feature in cancer immunotherapy is the regulation of different types of immune cells in tumour immunity. These immune cells work together as the regular surveillance checkers to ensure a delicate balance in the tumour microenvironment (Kimball & Webb 2013). With this precise approach, only a targeted group of specific antigens will be recognized as harmful and to be destroyed (Simpson et al. 2005). Moreover, the ability of the immune system to respond effectively and rapidly against previously encountered antigens may be a plus point to outcompete the conventional cancer treatment in preventing cancer relapse (Fuertes Marraco et al. 2015). Therefore, the understanding between the immune system to the tumour milieu is essential for the development of diverse strategies and breakthrough in cancer immunotherapy.

Cancer immunotherapy is generally divided into active and passive forms (Table 2.1). It is functionality falls into the various category, ranging from stimulating effector mechanisms to countering inhibitory and suppressive mechanisms of the immune cells (Papaioannou et al. 2016). Generally, active cancer immunotherapy defines as the introduction of anti-cancer targets into the host to stimulate and enhance the endogenous responses of the host's immune system. The examples may include the use of peptide-based, dendritic cells based, immune checkpoint inhibitors, administration of oncolytic viruses or allogeneic whole-cell administered *in vivo* in the activation of the body effector cells against malignancy (Chodon et al. 2015; Papaioannou et al. 2016). On the other hand, passive immunotherapy substitutes the host deficient immune systems by repeatedly administrating large quantities immune molecules externally to the host who do not produce them on their own (Bertrand et al. 2014; Motta 1971; Papaioannou et al. 2016). The examples of passive cancer immunotherapy is the adoptive T cell transfer, monoclonal antibodies and the cytokines-based therapies (Rusch et al. 2017).

In an effort to accelerate translation developments in cancer immunotherapy, some of the hurdles have to be acknowledged. Immunoediting is one of the key players in immunosurveillance and tumour escape leading to the appearance of immune-resistant variants through the three major sequential trials: elimination, equilibrium, and escape (Koebel et al. 2007). Secondly, the anti-tumour responses towards a therapeutic intervention across different individual may be unique and distinct due to the complexity of cancer and its tumour heterogeneity (Junttila & De Sauvage 2013). Thirdly, variables such as the age, diet, immune status, and the epigenetic involvement can influence the overall immune response of a patient (Fox et al. 2011). Lastly, the economic burden and potential side effects into translating this therapy to cancer patients can be the next challenge (Fox et al. 2011).

However, despite its substantial long clinical success, the field of cancer immunotherapy has renowned some noteworthy breakthrough. The approval of autologous cellular immunotherapy, Sipuleucel-T (metastatic prostate cancer) (Topalian et al. 2011), followed by the approval of the anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4) antibody, ipilimumab and anti-programmed cell death protein (PD1) antibodies to treat melanoma in 2011 and 2014 with the success of recent proof-of-concept in clinical trials (Sharma & Allison 2015). With all the successful trials have gained more attention and provide a better alternative for cancer immunotherapeutic approaches in the future.

Table 2.1 Active cancer immunotherapies

Active Cancer Immunotherapies			
Category	Mechanism	Examples	References
a) Peptide vaccine	Comprises of immunogenic epitopes from tumour-specific or tumour associated antigens either as monotherapy or combination therapies to stimulate and elicit either humoral or adaptive responses in cancer patients.	Mutated oncogenes (KRAS, BCR/ABL), oncogenic viruses (HPV, HBV, EBV), oncofetal proteins (CEA, PSA), tumour suppressor genes (p53), aberrantly expressed self-proteins (HER-2/neu), or altered glycolipids and glycoproteins (e. MUC-1, CA-125, GM2) and CTA.	(Disis et al. 1999; Durrant et al. 2012; Gabrilovich et al. 1996; Khleif et al. 1999; Marshall et al. 2000; Scanlan et al. 2002b; Simpson et al. 2005; Van Kaer et al. 2011)
b) Dendritic cells (DCs)	Acts as ideal vehicles or adjuvant for anti-cancer vaccine delivery with its high potency to initiate and augment in the immune response. By directing specific antigens to DC receptors i*n vivo* and *ex vivo* generated antigen-loaded immune cells.	Sipuleucel-T (Provenge, Dendreon Corporation)	(Banchereau & Steinman 1998; Obeid et al. 2015; Ophir et al. 2016)
c) Allogenic whole-cell vaccines	Consists of the administration of irradiated or inactivated tumour cells that do not proliferate that express wide ranges of TAAs to produce cytokines or co-stimulatory molecules in orchestrating anti-cancer effects from the patient.	*in vitro* cultured immortalized cell lines, GM-CSF gene-transduced tumour cells (GVAX)	(De Gruijl et al. 2008; Obeid et al. 2015; Ophir et al. 2016)
d) Checkpoint inhibitors	Works by binding and blocking interaction T cell coinhibitory pathways that lead to T cells susceptibility to apoptosis and programmed cells death, T cells exhaustion, T cells suppression and downregulation in the anti-tumour function and efficacy against cancer.	Ipilimumab (Yervoy, Bristol-Myers Squibb), Pembrolizumab (Keytruda, Merck), Nivolumab (Opdivo, Bristol-Myers Squibb)	(Bour‐Jordan et al. 2011; Buchbinder & Hodi 2015; Hamid et al. 2013; Hodi et al. 2010; Larkin et al. 2015; Sharpe & Freeman 2002; Weber et al. 2016)
e) Oncolytic viruses	It is innocuous viral strains that selectively lyse tumour cells (directly) or involves transfecting attenuated viruses to cancer cells to promote a systemic T cell immune response (indirectly). However, some issues have been linked to the toxicity effects.	*Talimogene laherparepvec* (T-VEC)	(Andtbacka et al. 2015; Ferguson et al. 2012; Qiao et al. 2008a; Qiao et al. 2008b; Rojas et al. 2015)

to be continued…

…continuation

Passive Cancer Immunotherapies

Category	Mechanism	Examples	References
a) Monoclonal antibodies (mAbs)	Uses engineered chimeric, humanized or fully human mAbs to block specific downstream signalling pathways upon binding to cancer-specific tumour targets, involved in antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC) via varies mechanism.	Alemtuzumab, Bevacizumab, Cetuximab, Denosumab, Gemtuzumab ozogamicin, Nimotuzumab, Ofatumumab, Panitumumab, Pertuzumab, Rituximab, Tositumomab and I-tositumomab and Trastuzumab	(Bross et al. 2001; Ellis & Hicklin 2008; Lundin et al. 2002; Rech & Vonderheide 2009)
b) Cytokines	Preferably administered in combination with standard treatments such as chemotherapy, peptides vaccines or mAbs. Serves as signalling molecules to trigger the host's immune system. It can be acted pleiotropically or exert immunomodulatory effects on immune cells. Some have a suppressive effect on regulatory T cells in the tumour milieu.	Interferon alpha (IFN-α), interleukin-2 (IL-2) and interleukin-12 (IL-12)	(Belardelli et al. 2002; Gaffen & Liu 2004; Kilinc et al. 2006; Little et al. 2006; Rook et al. 1999; Younes et al. 2004; Zhan et al. 2012)
c) Adoptive cell transfer	Exploits the expansion of *in vitro* autologous peripheral blood monocytes or T lymphocytes of a cancer patient to recognize specific antigens before it is infused back to the patient to mount anti-tumour responses. It is tailored based immunotherapy that produces a high avidity in effector T cells.	Tumour-infiltrating lymphocytes (TILs), Cytokine-induced killer (CIK) cells, Cascade primed (CARPI) cells, T cell receptor (TCR) transduced T cells and chimeric antigen receptor (CAR)-modified T cells	(Chmielewski et al. 2011; Haji-Fatahaliha et al. 2016; Laumbacher et al. 2012; Rosenberg et al. 2011; Tran et al. 2014)

2.2.1 Peptide-based Cancer Vaccines

Peptide-based cancer vaccine is an active based therapeutic approach to trigger immune cells to act against the tumour antigens (Farkona et al. 2016). Since our understanding of basic immune mechanisms has expanded substantially, a wide array of immune pathways has been identified as attractive targets to promote anti-tumour responses in cancer patients including the use of peptides derived from tumour-associated antigens (Markman & Shiao 2015). To date, numerous successful clinical trials using hepatitis B virus and human papillomavirus (HPV) vaccines have proven its efficacy as of prophylactic vaccines in combating cancer-causing infectious diseases (Jemal et al. 2013). The ability of the immune system in stimulating the immune cells specifically CD4$^+$ and CD8$^+$ T lymphocytes against immunogenic peptide antigens have stipulated many insights into developing a peptide-based cancer vaccine (Mellman et al. 2011; Pardoll & Topalian 1998). It functions by stimulating the immune system for the cytotoxic T cells recognition and activation against proliferating tumour cells (Iwasaki & Medzhitov 2015). It is also designed to eradicate cancers by destroying the growth of neoplastic cells and inducing the shrinkage of tumour cells (Farkona et al. 2016; Schumacher & Schreiber 2015).

This concept of therapy exploits the use of tumour-associated antigens present in the form of protein or macromolecules to trigger the immune system to react against tumours (Markman & Shiao 2015; Moulton et al. 2002). One or more antigens can be combined and administrated as a single peptide-based vaccine to elicit T cell activation and the antibody production (Van Der Burg et al. 2016). The peptide-based vaccine can be designed as either direct vaccination method using tumour associated antigen or as an augmentation boost to the host cells (Simpson et al. 2005). Having said that, several considerations should be taken in the investigation of using peptide-based cancer vaccine as the potential immunological target. One of the major hindrances to the development of a successful vaccine target is the identification of a suitable antigen that can provoke immune responses efficiently (Fox et al. 2011). A correct antigen must be chosen with sufficiently high quantities to achieve clinical effectiveness for cancer vaccines to overcome tolerance (Cheever et al. 2009; Fenoglio et al. 2013). This can be further attributed to higher pharmacokinetic properties without leading to the clearance of the antigens (Boon et al. 2006; Segal et al. 2008). Besides, the co-administration together with other immune stimulants such as IL-2 and IL-7 or with certain immune checkpoint blockade have been reported to increase the therapeutic efficacy of a cancer vaccine (Fehniger et al. 2002). On the other hand, an ideal tumour antigen should have high expression in target tumour populations while pertaining low level or even no expression in all normal tissue in order to ensure the specificity of the tumour antigen (Fenoglio et al. 2013). It is also crucial to monitor the immunosurveillance escape mechanisms in warranting a complete clearance of the cancer cells (Fenoglio et al. 2013; Zur Hausen 2009).

Currently, researchers are still on the investigation to search for a potential immunogenic antigen target that fulfils the criteria of maintaining a long-lasting T cell responses (Greiner et al. 2008; Thomas et al. 2004; Zur Hausen 2009). Genome sequencing of tumour antigen alone may be insufficient to ensure the immunogenicity of the peptide and to validate the expression of the antigen on the tumour cells (Farkona et al. 2016). Even if there is an expression, it may not necessarily be recognized and processed by Major Histocompatibility Complex (MHC) class I complexes to evoke immunological responses *in vivo* (Farkona et al. 2016).

At present, immunologists opt to use bioinformatics prediction tool prior to the wet lab experiment for an advance predicament (Kasuga 2013; Rammensee et al. 2002; Segal et al. 2008). To date, some of the well-recognised antigens reported in CRC are carcinoembryonic antigen (CEA) (Bilusic et al. 2014), mucin-1 (Kimura et al. 2012), squamous cell carcinoma antigen recognized by T cells 3 (SART3) (Miyagi et al. 2001), β-human chorionic gonadotropin (β-hCG) (Moulton et al. 2002), Survivin-2B (Idenoue et al. 2005) or p53 (Speetjens et al. 2009). However, efforts are urgently needed to assess the limitations in order to illuminate the best path forward in developing an immunogenic vaccine target in CRC.

2.2.2 Cancer Testis Antigen

Cancer-testis antigen (CTA) is a group of testicular-restricted proteins which are encoded by a cluster of cancer-testis genes (Caballero & Chen 2009; Chen et al. 2009). Initially, CTA was recognized to be encoded by chromosome X, however, with the increased research in CTA for the recent years, some CTA is also identified to be encoded by somatic chromosomes (Simpson et al. 2005). To date, approximately 250 genes encoding CTA can be distinctly classified into classical (located on the X chromosome) and non-classical (located on non-X chromosome) CTA (Simpson et al. 2005; Suri 2006). About 10% of CTA that is found on X-chromosomes (Cho et al. 2006; Simpson et al. 2005; Stevenson et al. 2007). Classical CTA is commonly encoded on the X chromosome but their biological function is poorly understood while non-classical CTA is autosomal encoded and their biological functions are often characterized (Tarnowski et al. 2016).

The testis is an immune-privileged site due to the blood-testis barrier that has restricted the interaction between testicular and immune cells (Kaur et al. 2014). The deficiency of human leukocyte antigen (HLA) class I expression on the surface of germ cells is known to be the uniqueness of CTA (Dadabayev et al. 2005). The antigen is unable to present onto T cells for its killing effects due to the lack of HLA expression of normal testicular germ cells (Janitz et al. 1994). Hence, any unintended autoimmune responses may not be triggered when a CTA is administered. Other than their restricted distribution, CTA explicit high immunogenicity *in vivo* (Li et al. 2010). This makes this group of CTA be a unique target for cancer immunotherapy.

Several high throughout CTA screening techniques such as cDNA microarray analysis, differential display, representational difference analysis, Serial Analysis of Gene Expression (SAGE), Expressed Sequence Tag (EST) sequencing with bioinformatics analysis, Serological Identification of Antigens by Recombinant Expression Cloning (SEREX), Serological Proteome Analysis (SERPA), are used to search for neoantigen with expression profiles restricted to cancer and testis in the design of a potential target (Khan et al. 2013; Klade et al. 2001; Sahin et al. 1995; Scanlan et al. 2004). As such, a few transcripts have been cloned through the techniques, such as LAGE-1/CT2 (Lucas et al. 1998), CTp11/SPAN-X-C1 (Zendman et al. 1999), SAGE (Martelange et al. 2000), MAGE (Güre et al. 2000) , BRDT/CT9 (Scanlan et al. 2000), PAGE5/CT16 (Scanlan et al. 2002a), MMA-1A (De Wit et al. 2002) and TPTE (Dong et al. 2003).

A growing list of 44 CTA has been characterized in several types of malignancies up until now (Scanlan et al. 2004; Simpson et al. 2005; Whitehurst 2014). With 17/23 (53%) melanoma, 17/33 (51%), non-small cell lung cancer and 11/20 (55%) bladder cancer classified as the high expressers of CTA genes, while 23/32 (37%) breast and 6/20 (30%) prostate cancer categorized as the moderate CTA gene expressers, and 3/33 (9%) renal as well as 4/25 (16%) colon cancer as low CTA gene expressers correspondingly (Scanlan et al. 2004). The classical CTA were found to be expressed in CRC are as NY-ESO-1, MAGE-A, MAGE-D, GAGE, and PRAME while the non-classical CTA are SPAG-9, MCAK, AKAP-4 and OY-TES-1 (Alves et al. 2007; Chi Soh et al. 2018; Zhang et al. 2014).

However, most of the classical CTA identified in other malignancies have not been reported to be expressed in CRC. Only a small subset of CRC patients has shown clinical benefits after immunization with well-recognized CTA such as NY-ESO-1 and MAGE-A3 (Brichard & Lejeune 2007; Kruit et al. 2005). Studies on NY-ESO-1 and MAGE-A3 immune responses in CRC reported measurable responses of circulating lymphocytes reactive against the individual peptide in their respective findings (Alves et al. 2007; Kumara et al. 2012; Long et al. 2014). Meanwhile, synthetic MAGE-A4 peptide coupled with OK432 and Montanide ISA-51 has been tested as immunotherapy targets in phase II clinical trials in metastatic CRC patients (Takahashi et al. 2012). Despite all the effort into comprehending CTA in CRC, our knowledge pertaining to CTA expression and their immune responses in CRC is still in the early stages of clinical translation. More research is needed to discover new CTA in CRC and to determine its efficacy as a suitable target for cancer immunotherapy.

2.2.3 The *PASD1* Gene

PASD1, known as Per ARNT SIM (PAS) domain-containing 1, is a member of CTA which is encoded by chromosome X at q28 (Zendman et al. 2003). It comprises of two isoform transcripts which are known as PASD1_v1 and PASD1_v2 (Cooper et al. 2006). PASD1_v1 transcript encodes for 639 amino acid protein products whereas PASD1_v2 encodes for another set of 773 amino acid with an additional unique region (Liggins et al. 2004a). Furthermore, both isoforms of PASD1 contain two individual domains in the N-terminal region between amino acids 32-94 and amino acids 41-137 (Liggins et al. 2004a). Besides, the basic helix-loop-helix (bHLH) sequences in PASD1 are believed to have some associations in the signal transduction pathway of several numbers of organisms (Vreede et al. 2003). Referring to Entrez Gene, there are a couple of the PASD1 homologs have been determined in sixteen additional species such as chicken, cow, mouse, and gorilla (Khan et al. 2013).

Consequently, PASD1 genes show the most similarity to the CLOCK gene in mice and were found to have a role in blocking circadian rhythms in human cancer cells (Michael et al. 2015). Furthermore, the domain of PASD1 is predicted to functionally work as a regulatory protein in controlling metabolic and development processes in human (Chapman-Smith et al. 2004). The PASD1 gene is majorly expressed in the testis. This gene shares certain common phenotypes as spermatogonia in the global promoter hypomethylation and rapid proliferation pathway (Simpson et al. 2005). However, the role and function of PASD1 in tumorigenesis is yet to be known and should be elucidated. Meanwhile, the PASD1 peptide has been reported to recognize host cytotoxic T lymphocytes (CTL) in a methodological manner using SEREX, followed by SERPA, peptide elution mass spectrometry and validation in Cancer Immunome database (Khan et al. 2013; Sahin et al. 1995; Türeci et al. 1999; Türeci et al. 2005). Alternatively, the identification of peptide is also known by the immunoscreening of cDNA using immunoglobulins G (IgG) from peripheral blood sera of the patients (Sahin et al. 1995; Türeci et al. 1999). This research flow has led to the discovery of a couple of neoantigens such as cTAGE-1, MAGE-A3, NY-ESO-1 as well as PASD1 as the potential immunotherapeutic target (Eichmüller et al. 2001; Guinn et al. 2005; Jäger et al. 1999; Liggins et al. 2004b).

2.2.4 PASD1 Expression in Normal Cells and Neoplasms

The expression of PASD1 is highly restricted to the nuclei of spermatogonia in normal germ cells. The expression is also reported to reduce with the maturity of spermatogonia over time (Scanlan et al. 2004). Interestingly, since testis is an immunologically protected site with no expression of MHC class 1 protein, PASD1 should not lead to any autoimmune responses against the healthy normal tissue (Ait-Tahar et al. 2011; Whitehurst 2014). To date, PASD1 is found to express majorly in hematopoietic malignancies such as in diffuse large B cell lymphoma (DBLCL), acute myeloid leukaemia (AML) and multiple myeloid (MM) and subset of solid tumours such as testicular cancer, ovarian cancer, cervical cancer, pancreatic cancer and non-small cell lung cancer (Ait-Tahar et al. 2011; Ait-Tahar et al. 2009; Cooper et al. 2006; Sahota et al. 2006). Several techniques of analysis are used to determine the expression of PASD1 includes the use of immunostaining procedures as the gold standard in protein expression analysis (Cooper et al. 2006), protein tissue array (Liggins et al. 2004a), real-time polymerase chain reaction (Guinn et al. 2005; Hardwick et al. 2013), immunohistochemistry and immunocytochemistry staining (Sahota et al. 2006), Western blot for protein identification and detection (Khan et al. 2013) and flow cytometry (Liggins et al. 2004a) in the last decades.

According to the previous study, 29 out of 40 recruited DBLCL patients were identified to express PASD1 protein in the cytoplasm of tumour cells using the immunohistochemistry method (Ait-Tahar et al. 2011). However, it was found that the heterogeneous expression of PASD1 varied with the intensities of the staining (Ait-Tahar et al. 2011). Additionally, 20% of AML patients were detected to have a positive expression of PASD1 in 12 of 60 of studied cases (Baghdady et al. 2013). Similarly, to the previous finding, differential expression of PASD1 was illustrated in these 12 positive cases of AML patients. The differential expression of PASD1 is predicted to be affected by the presence of PASD1 isoforms (Nakagawa et al. 2005), DNA hypermethylation of PASD1 (Simpson et al. 2005) and post-translational modifications (Ait-Tahar et al. 2011). Hence, there is still a gap of interest to in-depth investigate the expression of PASD1 in different types of cancer.

2.2.5 CD8 T Cell Responses

In the early 1900s, Mac Carty and his teams found the idea to improve cancer patient's survival rate through the understanding of host defence mechanism (Maccarty 1922). A transformed tumour microenvironment is composed of a group of phenotypically distinct stromal cells from the healthy microenvironment (Koi & Carethers 2017; Peddareddigari et al. 2010). There is a variety of tumour-infiltrating cells such as (tumour-associating macrophages, myeloid-derived suppressor cells, monocytes, dendritic cells, natural killer cells, $CD4^+$ and $CD8^+$ T lymphocytes, granulocytes, mast cells, endothelial progenitor cells, mesenchymal stem cells and platelets), vasculature, extracellular matrix such as (laminin, hyaluronan, collagens, fibronectins, integrins, proteoglycans, metalloproteases, mucin, galectin and osteopenia) and other matrix-associated molecules (periostin and betaig-h3) in association with each other in a variable proportion (Murdoch et al. 2008). These cells interact with other each through the cell to cell contact mechanism to directly or indirectly contribute to the inflammation of CRC and the production of cytokines (Koi & Carethers 2017).

Several components in the microenvironment are interrelated to influence the development of adenomatous colonic polyp to CRC (Peddareddigari et al. 2010). The adaptive immunity comprises of the interaction of between T and B lymphocytes, natural killer cells, macrophages and dendritic cells for a tumoricidal effect on the infected cell (Brunner & Cerottini 1971). An inimitable feature of CD8 T cells is known to be the main players in mounting T cell-mediated immunities in the body (Feinerman et al. 2008). At the molecular level, the activation of naive T cells by antigens requires the specific recognition of MHC Class 1 molecule and co-stimulatory signal to antigen presenting cells (APCs) (Lanzavecchia & Sallusto 2001). The antigen presentation through MHC molecules is a cumulative process involving the biochemical effects of a vast array of signalling molecules and lymphokines resulting in the induction of gene and production of protein for the T cells to function (Voskoboinik et al. 2015).

The cytotoxic potential of killer T cells is extensively studied over the years and remains to be a very crucial part in adaptive T cell-mediated immunity. In this case, the biology of MHC class I and II presentation is essential in the evaluation of the T cell lysis to achieve successful antigen presentation to the immune system (Neefjes et al. 2011). In general, endogenous peptides are commonly first presented by MHC molecules to the T lymphocytes prior to the lysis (Vyas et al. 2008). Tentatively, the cross-priming of exogenous peptides begins with the degradation of peptides into smaller peptide fragments by the cytosolic proteasome after being phagocytosed by the dendritic cells (Kurts et al. 2010). The peptide complex will then ATP-dependently be translocated to the endoplasmic reticulum (ER) (Neefjes et al. 2011). It is followed by the gradually chaperoned of peptides into the Golgi apparatus before it is bound by the nascent MHC molecules and presented externally for CD8 T cells to elicit an immune response (Cresswell et al. 2005; Neefjes et al. 2011).

This is followed by the association of the assembly of the complex with tapasin to stabilize the transporter associated with antigen processing (TAP1 and TAP2) before binding to the soluble ER chaperone calreticulin (CRT) and the soluble thiol oxidoreductase ERp57 protein complex via its transmembrane domain (Bangia et al. 1999; Momburg & Tan 2002). The chaperone calreticulin is a lectin specific N-linked glycan, with its carboxylate side chain adheres to MHC class 1 molecule (Helenius & Aebi 2004). On the other hand, ERp57 is a disulfide isomerase that cooperates with CRT or CNX in peptide folding and makes transient disulfide linkages with its active sites (Helenius & Aebi 2004). Upon dissociation from the complex, it is transported into ER to be further trimmed by ER-associated aminopeptidase (ERAAP or ERAP1) if required (Neefjes et al. 2011). Peptides with appropriate sequences are gradually chaperoned into the Golgi apparatus before it is bound by the nascent MHC class 1 molecule and presented externally for accurate CD8 T cells recognition to initiate an immune response (Cresswell et al. 2005; Neefjes et al. 2011). However, the proposed interaction may be intertwined with Hence, the MHC presentation is essential in the evaluation of the T cell lysis to achieve successful antigen presentation to the immune system (Neefjes et al. 2011).

Cytotoxicity is a cell-specific multifactorial event modulated by a variety of immune cells. Attaining physiological levels of CTLs may require the assistance of the T cell receptor, other co-receptors, adhesion and mediator molecules to trigger an efficient cell-mediated immune response. Several multifaceted mechanisms that have been hypothesized for the cytolytic activity via MHC class 1 recognition in CRC (Barry & Bleackley 2002). The complex CD8 T cell lysis consists of a combination of pathways such as 1) the calcium-dependent granule exocytosis (Chavez-Galan et al. 2009; De Saint Basile et al. 2010), 2) calcium-independent pathway (perforin, granzyme and a family of serine proteinases-mediated lysis) (Trapani & Smyth 2002; Voskoboinik et al. 2006) and 3) FAS dependent interaction (Ostergaard et al. 1987; Rouvier et al. 1993; Waring & Müllbacher 1999) that act synergistically for efficient cytotoxic lymphocyte function (Figure 2.1). Calcium-dependent granule cytolysis is one of the important events in assessing CD8 T cell cytolytic pathway and mediating apoptosis of cells (Zhang et al. 2001).

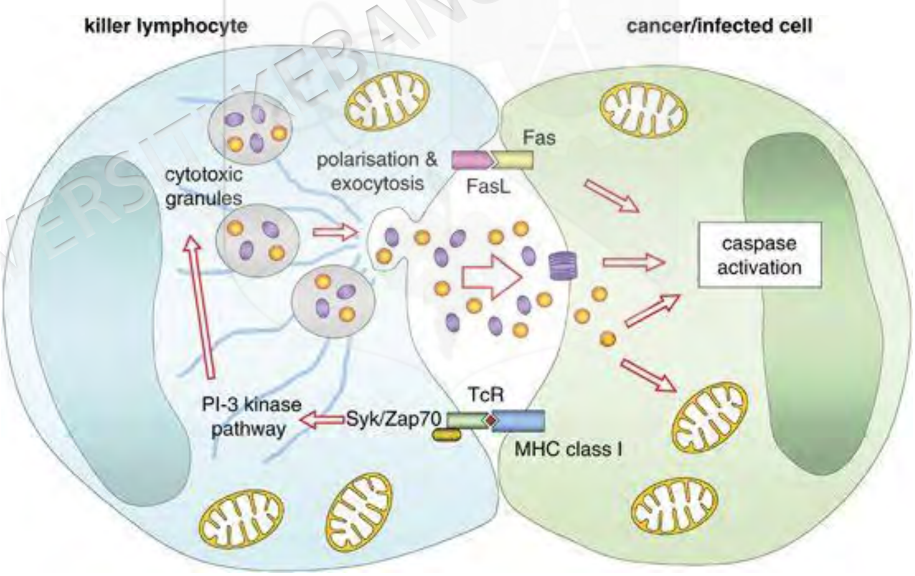

Figure 2.1 The basic illustration of proposed molecular mechanisms of cytotoxic T cell lysis which consist of calcium-dependent granule exocytosis, calcium independent pathway (perforin, granzyme and a family of cysteine proteinases-mediated lysis) and FAS dependent interaction.

Source: (Trapani 2012)

Cytotoxic T cells are known to eradicate cancerous cells by exocytosing the granular contents of CD8 T lytic granules through the calcium-dependent mechanism (De Saint Basile et al. 2010). Besides, CTLs can also eliminate the cells, in the absence of calcium, involving a mixture of enzymatic components including granzymes, perforin, and proteinases (Trapani & Smyth 2002; Voskoboinik et al. 2006). Nonetheless, another pathway consisting of Fas-FasL can also be linked to the CTL cytotoxic killing mechanism (Waring & Müllbacher 1999). Taken together, these pathways will eventually lead to the suicide of the target cells through inherent apoptosis, necrotic death or programmed cell death resulting in the later fragmentation of DNA or the accumulation of lytic proteins accountable for cell perforation (Cohen et al. 1985; Cohen et al. 1992).

Additionally, cytotoxic T lymphocytes are known to lyse the infected cells using the granule exocytosis pathway rather than the Fas-mediated cytotoxic route (Kreuwel et al. 1999). One possibility might be that the CD8 effector T cells come preloaded with cytolytic granular proteins in a more rapid and efficient manner than for the Fas protein interaction to occur (Barry & Bleackley 2002). Nevertheless, some may argue that Fas signalling is more crucial in regulating a balance in CTL homeostasis than the primary cytotoxic function of CTLs (Nagata & Golstein 1995). It is important to note that the nature of CD8 T cells specific lysis phenomena *in vivo* may not only differ from *in vitro* studies but is also more complex and often outcomes have been contradictory (Barry & Bleackley 2002; Lancki & Fitch 1992). Further studies are required to elucidate the molecular pathways involved in the killing of CRC cells by CTLs upon PASD1 induction.

2.2.6 Cytokines

The roles of anti-inflammatory and pro-inflammatory cytokines in the tumour milieu is another critical feature of immune system (De Simone et al. 2013; Kantola et al. 2012; Krzystek-Korpacka et al. 2013). Anti-inflammatory cytokines such as IFN-γ, IL-12, IL-15, IL-17, IL-18, granzyme A, granzyme B, perforin and granulysin are known to suppress the tumour growth whereas pro-inflammatory cytokines such as IL-2, IL-4, IL-6, IL-8, IL-11, IL-17A, IL-22, IL-23, IL-33, TNF-α, TGF-β and VEGF work together in promoting the CRC tumorigenesis (Figure 2.2) (Reissfelder et al. 2015). It has been reported in previous study that both interferons and interleukins do play pivotal roles in driving the communication of the cellular T cell immunity in dampening immunopathogenic responses of CRC (Cooper & Khader 2008; Lin & Karin 2007).

At the molecular level, IFN-γ acts on CRC cells by hindering CRC, promoting NK cell activity and JAK/STAT phosphorylation, stimulating the production of NF-κB, interrupting the catenin signalling routes as well as inducing adhesion and leukocyte migration (Schroder et al. 2004; Wang et al. 2015). Moreover, IFN-γ has also been known to regulate the expansion of $CD8^+$ T cells and memory T immunity in encountering intracellular antigens (Tewari et al. 2007). Furthermore, a deficiency of IFN-γ in CRC is reported to promote CRC carcinogenesis and reduce survival rate (Wang et al. 2015). The importance of IFN-γ and TNF-α in the modulation CTL-mediated lysis have also been reported previously (Barry & Bleackley 2002; Peddareddigari et al. 2010).

In CRC, TNF-α is known to be a pro-inflammatory cytokine due to the involvement of TNF-α in promoting cancer cells proliferation (Al Obeed et al. 2014; Stanilov et al. 2014). The release of TNF-α through STAT3 signalling has been demonstrated to lead to the propagation of malignant cells in CRC cell lines (Hamilton et al. 2011; Mizoguchi et al. 2002). Consequently, the signalling of TNF-α can indirectly result in the activation of NK-κB and MAPKs signalling pathways, contributing to the cell survival and proliferation of CRC in a caspase-dependent manner (Brenner et al. 2015). TNF-α can also trigger necrotic cell death by mediating reactive oxygen species as a result to inhibit tumorigenesis of cells (Degterev et al. 2008; Kim et al. 2007).

Numerous reports on granzyme A and B have also shown that their functions as the pro-inflammatory cytokines within the CRC tumour microenvironment (Buzza et al. 2005; Lieberman 2010; Waterhouse et al. 2006). Upon cleavage, the release of granzyme B will cause the degradation of pro-apoptotic protein Bid and dissipate mitochondrial potential in mediating permeabilization of the outer membrane of mitochondria (Jacquemin et al. 2015). Subsequently, granzyme B further amplify downstream several caspases related pathway with the release with Cytochrome C (Kadam & Abhang 2015). On the other hand, granzyme B has also been demonstrated to be involved in caspase-independent actions such as directly interfering caspases substrates including DNA protein kinase (Sebbagh et al. 2005), nuclear mitotic apparatus protein (Zeng 2000) and inhibitor of caspase-activated DNase (Thomas et al. 2000).

A considerable amount of studies have been published on the mechanisms of action of perforin in various types of malignancies, bacterial and viral infections, autoimmune diseases as well as in immune surveillance (Trapani & Smyth 2002; Van Den Broek & Hengartner 2000; Voskoboinik et al. 2015). Both CTLs and NK cells are the two main producers of perforin (Law et al. 2010; Liu et al. 1995; Podack et al. 1991), while minute amounts of perforin have been found to be secreted by other subsets of T cells occasionally (Williams & Engelhard 1997). Perforin monomers can create pores in the presence of calcium ions by forming a channel through the perforated bilayer of cells to enable the influx and efflux of ions, cytolytic molecules, enzymes and polypeptides (Voskoboinik et al. 2015). Ultimately, disrupting the integrity of the intact bilayers and damaging the protective barrier of the cell membrane (Osińska et al. 2014). Furthermore, the CD8$^+$ T cells are also capable of inducing calcium-independent Fas-Fas ligand system for an apoptotic response. The cellular features of apoptotic death as a result of Fas-Fas ligand interactions begins with the trimerization of Fas receptor by Fas ligand, followed by the activation of caspases pathways, cleavage of caspases substrates, association with DNA fragmentation and dismantling of the cell (Boldin et al. 1995; Chinnaiyan et al. 1995; Nicholson & Thornberry 1997).

In terms of CD8 T cells cytotoxicity, IL-10 exerts dual proliferative and inhibitory effects in various types of malignancies (Del Prete et al. 1993; Groux et al. 1996; Ouyang et al. 2011; Pestka et al. 2004). As reported, IL-10 stimulates the proliferation of T cells in gastric cancers (Xi et al. 2017) but suppresses the T cells responses in breast cancer (Ruffell et al. 2014) and chronic liver disease (Zhang &

Wang 2006). Mechanistically, the engagement of the IL-10 receptor has also been proposed to be involved in the activation of STAT1, STAT3 and STAT5 the phosphorylation. Inappropriate activation of STAT signalling pathway is known to deregulate genes that are involved in cell maintenance and homeostasis (Ouyang et al. 2011; Thomas et al. 2015). Taken together, these studies have highlighted the differential effects of IL-10 on CRC development depending on the tumour microenvironment. Future investigation of the role of IL-10 might be helpful in further evaluating its role as either a promising diagnostic or therapeutic target for CRC. In the last decade, therapeutic drugs such as bevacizumab, anti-VEGF agents, selective COX-2 inhibitor and NSAID have been used as an alternative therapy in synergy with conventional treatments CRC (Peddareddigari et al. 2010).

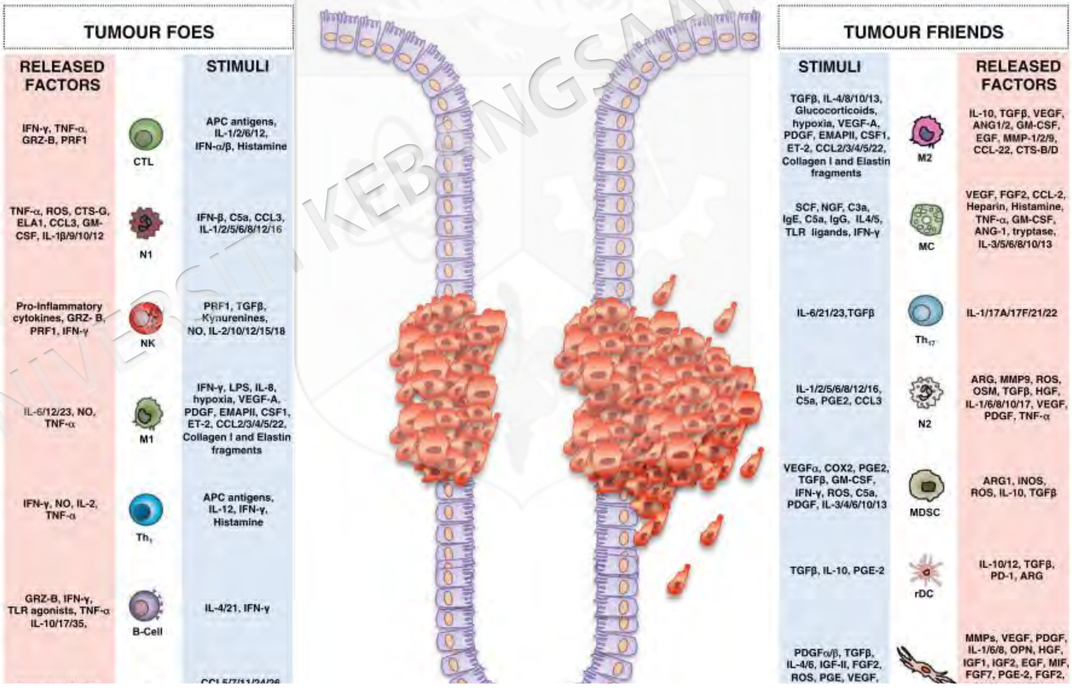

Figure 2.2 Cellular components of tumour microenvironment (tumour foes and tumour friends) in CRC. Distinct types of CRC are composed of a variety differential molecular expression profiles of cytokine.

Source: (Colangelo et al. 2017)

CHAPTER III

METHODOLOGY

3.1 MATERIALS

3.1.1 Reagents

The listed reagents are RNA*later* RNA Stabilization Reagent (Qiagen, Germany), Roswell Park Memorial Institute 1640 medium (RPMI-1640) (Nacalai Tesque, USA) and Eagle's Minimum Essential Medium (EMEM) (Nacalai Tesque, USA), phosphate buffered saline (Hyclone, USA), trypsin-EDTA (Gibco, USA), Tryphan blue stain (Sigma Aldrich, USA), AllPrep® DNA/RNA/miRNA Universal Kit (Qiagen, Germany), β-mercaptoethanol (Sigma Aldrich, USA), Rnase-free water (Thermo Fisher Scientific, USA), NanoDrop 2000 (Thermo Fisher Scientific, USA), iSciptTM cDNA Synthesis Kit (Biorad Laboratories, USA), iTaq^{TM} Universal SYBR Green Supermix (Biorad Laboratories, USA), primers (First Base Laboratories Sdn Bhd, Malaysia), xylene (Leica, Germany), absolute ethanol (Leica, Germany) hematoxylin (Richard Allan Scientific, USA), Bluing solution (Leica, Germany), eosin (Leica, Germany), Sub-X Surgipath mounting medium (Leica Biosystems, Germany), rabbit anti-human PASD1 polyclonal antibody (Thermo Fisher Scientific, USA), Dako Envision Plus Kit (Abcam, UK), antigen retrieval buffer (Abcam, UK), anti-rabbit IgG linked FITC (Thermo Fisher Scientific, USA), gelatin (Sigma Aldrich, USA), DAPI dye (Invitrogen, USA), formaldehyde (Sigma Aldrich, USA), glycerol (Thermo Fisher Scientifc, USA). Peptides (Think Peptides, UK), QIAamp® DNA Blood Mini Kit (Qiagen, Germany), GoTaq® DNA Polymerase (Promega, USA), agarose powder (Sigma Aldrich, USA), TAE buffer (Thermo Fisher Scientific, USA), GelStar™ Nucleic Acid Gel Stain (Lonza, USA), blue loading dye (Promega, USA), GeneRuler

100 bp DNA ladders and 1 kb DNA ladder (Thermo Fisher Scientific, USA), Ficoll-Pague PLUS (GE Healthcare Life Sciences, USA), fetal calf serum (Hyclone, USA), phytohaemagglutinin (Sigma Aldrich, USA), rIL-2 (R&D Systems, USA), rIL-7 (R&D Systems, USA), Human Interferon-γ (IFN- γ) ELISpot^{BASIC} Kit (Mabtech, Sweden), TMB substrate solution (Mabtech, Sweden), rGM-CSF (Cell Guidance Systems Ltd, UK), rIL-4 (Cell Guidance Systems Ltd, UK), rIL-1β (Cell Guidance Systems Ltd, UK), rTNF-α (Cell Guidance Systems Ltd, UK), rIL-6 (Cell Guidance Systems Ltd, UK), PGE_2 (Sigma Aldrich, USA), 1% of penicillin/streptomycin (Gibco, USA), Versene solution (Thermo Fisher Scientific, USA), β2 microglobulin (BD Pharmingen, USA), EasySep™ Human CD8 Positive Selection Kit II (Stemcell, Canada), DNase I solution (Thermo Fisher Scientific, USA), PE-conjugated mouse anti-CD4 (BD Pharmingen, USA), FITC-conjugated mouse anti-CD3 (BD Pharmingen, USA), APC-conjugated mouse anti-CD8 (BD Pharmingen, USA), CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes, UK), Propidium Iodide Nucleic Acid Stain (Invitrogen, UK), Human Granzyme B ELISpot^{BASIC} Kit (Mabtech, Sweden), BCIP/NBT substrate solution (Sigma Aldrich, USA) and Human CD8/NK Panel LEGENDplex™ Kit (BioLegend, USA).

3.1.2 Apparatus

The listed apparatuses are Galaxy® 170 R incubator (Eppendorf, Germany), Centrifuge 5810 R (Eppendorf, Germany), haemocytometer (Resistance, Germany), Nikon Eclipse TS100 Inverted Microscope (Nikon, Japan), CFX96™ Real-Time PCR Detection System (Biorad Laboratories, USA), high-profile microtome blade (Sakura, Japan), polysine microscope slide (Menzel, Germany), decloacking chamber (Biocare Medical, USA), DeltaVision™ Ultra widefield microscope (GE Healthcare Life Sciences, USA), Centrifuge 5424 R (Eppendorf, Germany), Veriti 96 Well Thermal Cycler (Applied Biosystems, USA), Shandon Finesse ME+ (Thermo Scientific, USA), Leica EG 1150 C Ice Block (Leica, Germany), Section Flotation Bath (Thermo Scientic, USA), Olympus CX31 Compound Microscope (Olympus, Japan), Wide Mini-Sub Cell GT Systems (Biorad, USA), Power Supply-PowerPac (Biorad, USA), Gel Documentation System Quantum-ST4 (Fisher Thermo Scientific, USA), vortex (Heidolph, Germany), CTL S6 Universal ELISpot Analyzer (Cellular Technology

Limited, USA), EasySep™ magnet (Stemcell, Canada), Rotamax 120 orbital shaker (Heidolph, Germany), BD FACSVerse™ (BD Biosciences, USA) and Tissue-Tek (Sakura Finetek, USA).

3.2 CELL CULTURE

3.2.1 Cell Lines & Subculture Conditions

The following established human normal colon, colorectal adenocarcinoma, and lung adenocarcinoma cell lines were obtained from the UKM Medical Molecular Biology Institute (UMBI) bioban and were previously purchased from the American Typr Culture Collection, as detailed in Table 3.1. Seven cell lines namely CCD112CoN, COLO320DM, HT29, HCT116, SW48, SW480 and H1299 were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) and Eagle's Minimum Essential Medium (EMEM) with L-Glutamine and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) (Nacalai Tesque, USA) respectively. Both culture media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Hyclone, USA). All the cell lines were purchased from the American Type Culture Collection (ATCC) (Table 3.1). Cells were cultured and maintained in 5% carbon dioxide (CO_2) with 95% humidity at 37˚C and pH (7.2-7.4) in Galaxy® 170 R incubator (Eppendorf, Germany).

Table 3.1 Information regarding cell lines used in this study

Cell line	Organism	Tissue	Disease	ATCC Catalog
CCD112CoN	Human	Colon	Normal	ATCC® CRL-1541™
COLO320DM	Human	Colon	Dukes' type C, Colorectal carcinoma	ATCC® CCL-220™
HT29	Human	Colon	Colorectal adenocarcinoma	ATCC® HTB-38™
HCT116	Human	Colon	Colorectal carcinoma	ATCC® CCL-247™
SW48	Human	Colon	Dukes' type C, Colorectal carcinoma	ATCC® CCL-231™
SW480	Human	Colon	Dukes's type B, Colorectal carcinoma	ATCC® CCL-228™
H1299	Human	Lung	Non-small cell lung carcinoma	ATCC® CRL-5803™

Cell lines were split every two to four days respectively when 85% - 90% confluency was achieved depending on the type of cells. Spent culture medium was discarded using a 3 mL or 10 mL sterile serological pipette. Sterile 1X Phosphate Buffered Saline (1X PBS) (Hyclone, USA) was used to rinse the monolayer of cells before 3.0 to 5.0 mL of 0.25% of trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, USA) was added to detach the adherent cultures for 3 minutes in a 37˚C CO_2 incubator. An equal volume of fresh cell culture medium was added into the culture flask to inactivate trypsin activity and centrifuged at 100 × g for 5 minutes in room temperature in Centrifuge 5810 R (Eppendorf, Germany). The supernatant was discarded, and the cell suspension was used for further sub-cultures in either T-25 or T-75 cell culture flask.

3.2.2 Cell Counting

The number of cells in a specific population was determined by a dye exclusion viability-based assay using a hemocytometer. Equal volume of 0.08% (w/v) Trypan blue stain (Sigma Aldrich, USA) was added into 10 µL of cell culture in 1:1 ratio and mixed well before loaded on to a clean hemocytometer (Resistance, Germany) chamber to be counted at 100× magnification under Nikon Eclipse TS100 Inverted Microscope (Nikon, Japan). The stained cells (dead cells) appeared blue incolour while the unstained cells (viable cells) remained as bright clear spheres under the microscope. The number of cells (NC) in the population was determined by the following formula.

$$NC = \frac{Total\ no.of\ cells\ counted}{4} \times 10^4 \times Dilution\ factor\ x\ 1mL\ of\ cell\ suspension$$

3.3 PRIMARY SAMPLE SELECTION

3.3.1 Sample Collection for Fresh Frozen and Formalin Fixed Paraffin Embedded CRC Tissues

CRC tumour specimens were retrieved after consultation with collaborated surgeon and pathologist from Hospital UKM (HUKM), Kuala Lumpur, Malaysia. Ethics application with the reference number: UKM PPI/111/8/JEP-2017-584 was approved by HUKM Research Ethics Committee for all collected specimens with reference to Appendix A. Informed consent was obtained from all patients for the use of their specimens. Fixed and frozen tumour together with the adjacent non-tumour tissue specimens were obtained from CRC patients during surgery. Pathology verified primary tissues from CRC were used. All CRC tissues were collected and dissected under stringent sterile conditions and stored in RNA*later* RNA Stabilization Reagent (Qiagen, USA) to prevent RNA contamination. Fresh frozen (FF) CRC tissue specimens (n=25) obtained from surgery were immediately processed for its total RNA to be used in PASD1 mRNA detection. On the other hand, formalin-fixed paraffin-embedded (FFPE) CRC tissue blocks (n=23) were obtained from the Department of Pathology in HUKM for PASD1 protein detection. Demographic data of CRC patients for PASD1 mRNA expression are listed in (Table 3.2) while demographic data of CRC patients for PASD1 protein expression are listed in (Table 3.3).

3.3.2 Sample Collection for Peripheral Blood Mononuclear Cells Isolation

Peripheral blood was obtained from 40 CRC patients and 23 healthy controls in heparin vacutainer tubes (Becton Dickinson, USA) after colonoscopy or prior to surgery in HUKM, Kuala Lumpur, Malaysia. Ethical approval and written consent were obtained from HUKM Research Ethics Committee. The clinical details of recruited PBMC population are displayed in (Table 3.4) and all the samples were screened for HLA-A*24:02 allele prior to being used in the selection of PASD1 peptide. Only HLA-A*24:02 positive patients were assessed for CD8 T cell responses against PASD1 peptides.

Table 3.2 Demographic data of CRC FF for PASD1 mRNA expression

Characteristics	CRC	Paired Normal
Total number of samples	25	25
Gender		
Male	20	20
Female	5	5
Age		
Range	(33-84)	(33-84)
Race		
Chinese	9	9
Malay	15	15
Indian	1	1
Anatomical site		
Sigmoid	5	5
Rectum	11	11
Rectosigmoid	9	9
Modified Dukes Classification		
A	6	-
B	11	-
C	7	-
D	1	-
Lymph node metastasis		
Positive	8	-
Negative	17	-
Histological grades		
Well-differentiated	15	-
Moderately differentiated	10	-
Poor differentiated	-	-

Table 3.3 Demographic data of CRC FFPE for PASD1 protein expression

Characteristics	CRC
Total number of samples	23
Gender	
Male	16
Female	7
Age	
Range	(35-84)
Race	
Chinese	10
Malay	9
Indian	4
Anatomical site	
Sigmoid	5
Rectum	15
Rectosigmoid	3
Modified Dukes Classification	
A	1
B	9
C	12
D	1
Lymph node metastasis	
Positive	13
Negative	10
Histological grades	
Well-differentiated	13
Moderately differentiated	10
Poor differentiated	-

Table 3.4 Clinical details of the recruited population for PBMCs isolation

Characteristics	CRC	Control
Total number of cases from HUKM	40	23
Gender		
Male	35/40	16/23
Female	5/40	7/23
Age		
Range	(35-82)	(37-82)
Race		
Chinese	18/40	10/23
Malay	15/40	11/23
Indian	7/40	2/23
Anatomical site		
Rectum	29/40	-
Sigmoid	9/40	-
Rectosigmoid	2/40	-
Modified Dukes Classification		
A	1/40	-
B	6/40	-
C	12/40	-
D	2/40	-
*High grade dysplasia / inflammatory polyp)	19/40	-
Lymph node metastasis		
Positive	14/40	-
Negative	7/40	-
*High grade dysplasia / inflammatory polyp)	19/40	-
Histological grades		
Well-differentiated	9/40	-
Moderately differentiated	11/40	-
Poor differentiated	1/40	
*High grade dysplasia / inflammatory polyp)	19/40	-

3.4 SECTIONING OF FRESH FROZEN AND FORMALIN FIXED PARAFFIN EMBEDDED CRC TISSUES

Cryosectioning of fresh frozen CRC tissues was performed using Tissue-Tek (Sakura Finetek, USA) based on optimal cutting temperature (OCT) in a suitable mold. The tissues were frozen onto a specialized metal grid on a cryostat to be cut at -20˚C. Alternatively, FFPE CRC tissues were trimmed and sectioned by using a microtome, Shandon Finesse ME+ (Thermo Scientific, USA). All the tissue blocks to be sectioned were placed on Leica EG 1150 C Ice Block (Leica, Germany) to be cooled for 10 min. The FFPE blocks were inserted onto the microtome chuck and aligned in a vertical plane. The thickness of the FFPE tissues was set to 4 µM and the high-profile microtome blade (Sakura, Japan) was angled at 5˚. The layer of cut FFPE tissues was immediately floated in a Section Flotation Bath (Thermo Scientific, USA) and coated onto a polysine microscope slide (Menzel, Germany). The slides were stored overnight at room temperature prior to H&E and IHC staining.

3.5 HEMATOXYLIN AND EOSIN STAINING

The FFPE of CRC tissues were subjected to H&E staining (Table 3.5) to confirm that more than 80% of tissue malignancy was present. The slides were mounted using Sub-X Surgipath mounting medium (Leica Biosystems, Germany) with a coverslip. All the slides were viewed and examined under Olympus CX31 Compound Microscope (Olympus, Japan) by a pathologist.

Table 3.5 Protocol for H&E staining

Reagent	Duration
Xylene I	Immerse, 5 min
Xylene II	Immerse, 5 min
Absolute ethanol	Immerse, 3 min
95% ethanol	Immerse, 3 min
80% ethanol	Immerse, 3 min
75% ethanol	Immerse, 3 min
Deionized water	Rinse, 3 min
Hematoxylin	2-3 dips
Running tap water	1 min
Deionized water	4-5 dips
Bluing solution	Immerse,1 min
Running tap water	Rinse, 3 min
Eosin	2-3 dips
Deionized water	Rinse, 3 min
80% ethanol	Immerse, 3 min
90% ethanol	Immerse, 3 min
Absolute ethanol I	Immerse, 3 min
Absolute ethanol II	Immerse, 3 min
Xylene I	Immerse, 3 min
Xylene II	Immerse, 3 min

3.6 RNA ISOLATION

3.6.1 Isolation of Total RNA

Total RNA from CRC cell lines and histologically confirmed CRC tissue samples were harvested using the AllPrep® DNA/RNA/miRNA Universal Kit (Qiagen, Germany) according to the manufacturer's instructions. CRC tissue samples were disrupted using a mortar and pestle then homogenized using a QIAshredder homogenizer. Frozen samples (10 - 20 mg) were ground into fine powder under liquid nitrogen and 600 µL of Buffer RLT Plus was added into a 1.5 mL tube cooled with liquid nitrogen respectively before it was loaded onto QIAshredder spin column. β-mercaptoethanol (Sigma Aldrich, USA) was added to Buffer RLT Plus prior to the lysis. The tube was centrifuged at 14,000 × g for 2 min at 4˚C using Centrifuge 5424 R (Eppendorf, Germany). The lysate was transferred to an AllPrep DNA Mini spin column and further centrifuged at 14,000 × g for another 30 s. The supernatant was aspirated to a new 2 mL collection tube for RNA purification. A volume of 80 µL of Proteinase K and 350 µL of absolute ethanol was added and mixed thoroughly prior to incubation for 10 min at room temperature. Next, a total of 700 µL was of absolute ethanol was added into the mixture and mixed well before a total of 700 µL of the required sample was transferred to an RNeasy Mini spin column with a new collection tube. The column tube was centrifuged for 15 s at 14,000 × g and the flow-through was discarded. The column was transferred to a new collection tube and 500 µL of Buffer RPE was added and centrifuged for 15 s at 14,000 × g. A total of 80 µL of DNase 1 incubation mixture (10 µL of DNase 1 and 70 µL Buffer RDD) was added onto the column and incubated at room temperature for 15 min prior to the addition of 500 µL of Buffer FRN to the same column and centrifuged for 15 s at 14,000 × g. This step was repeated for another time before 500 µL of Buffer RPE was added to the column to be centrifuged at for 15 s at 14,000 × g. The flow-through was discarded and 500 µL of absolute ethanol was added into the spin column and centrifuged for 15 s at 14,000 × g. Alternatively, the spin column was transferred to a clean collection to be centrifuged for another 2 min to dry the membrane. Next, a total of 40 µL of RNase-free water (Thermo Fisher Scientific, USA) was then pipetted directly to the spin column and the tube was centrifuged for 1 min at 12,000 × g to elute the RNA.

3.6.2 RNA Quantitation and Purity Check

The concentration and purity of extracted RNA were analyzed by using NanoDrop 2000 (Thermo Fisher Scientific, USA). One µL of RNase-free water was pipetted onto the pedestal and calibrated as a blank. Next, 1 µL of the respective RNA sample were pipetted and measured in OD_{260}, OD_{280}, $A_{260/280}$, and $A_{260/230}$ ratio.

3.7 REAL-TIME POLYMERASE CHAIN REACTION

3.7.1 Synthesis of cDNA

Complementary DNA (cDNA) of the samples were synthesized by using Real-Time Polymerase Chain Reaction (RT-PCR) technique. One µg of each sample was subjected to cDNA synthesis using the iScipt^{TM} cDNA Synthesis Kit (Biorad Laboratories, USA). The preparation of the reaction mix (Table 3.6) was performed on ice.

Table 3.6 Kit component of iScript cDNA synthesis kit

Component	Volume per Reaction, μL
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	14
RNA template	1
Total volume	20

The complete reaction mix of each sample was gently mixed and completed in Veriti 96 Well Thermal Cycler (Applied Biosystems, USA) using the protocol outlined in (Table 3.7). The synthesized cDNA was used in RT-PCR.

Table 3.7 The reaction protocol in iScript cDNA synthesis

Protocol Step	Time, Temperature
1. Priming	5 min at 25˚C
2. Reverse Transcription (RT)	20 min at 46˚C
3. RT Inactivation	1 min at 95˚C
Optional Step	Hold at 4˚C

3.7.2 Primer Design

Forward and reverse primers of the housekeeping gene, GAPDH and the gene of interest, PASD1 gene (Integrated DNA Technologies, USA) was designed using National Center for Biotechnology Information (NCBI) RefSeq RNA database and blasted against Nucleotide Basic Local Alignment Search Tool (BLAST) (NCBI, USA) (Table 3.8).

Table 3.8 Primers used for RT-PCR determination of PASD1 expression

Primer	Sequence	Length
PASD1 Forward	5’-CAG CAA CTT TAC ACT TCA AAG GC-3’	23
PASD1 Reverse	5’-ACG TAC ACG GCT TTC ATG CTA-3’	21
GAPDH Forward	5’-GGA TTT GGT CGT ATT GGG C -3’	19
GAPDH Reverse	5’-TGG AAG ATG GTG ATG GGA TT -3’	20

3.7.3 Real-Time Polymerase Chain Reaction

The expression of PASD1 in CRC cell lines and tumour tissues was assessed by RT-PCR using iTaq^{TM} Universal SYBR Green Supermix (Biorad Laboratories, USA). GAPDH was used as the internal control. Tubes without the cDNA template were used as non-template control. Each RT-PCR reaction was prepared in a 0.2 mL of microcentrifuge tube. The preparation of the reaction mix (Table 3.9) was performed on ice and mixed gently.

Table 3.9 Kit components used to prepare RT-PCR samples

Component	Volume per Reaction, µL	Final Concentration
iTaq™ Universal SYBR® Green Supermix (2x)	10	1X
Forward primer	2	5 µM
Reverse primer	2	5 µM
cDNA template	1	1 µg
Nuclease-free water	5	-
Total Volume	20	

The reaction tubes were loaded onto RT-PCR instrument, CFX96™ RT-PCR Detection System (Biorad Laboratories, USA) and programmed accordingly (Table 3.10). Relative quantification of the target transcript relates to the RT-PCR signal in Ct values was normalized against a control group using a double delta Ct method (Livak & Schmittgen 2001). The relative mRNA expression levels were evaluated using the CFX Manager (Biorad Laboratories, USA). All PCR reactions were performed in triplicates.

Table 3.10 Thermal cycling protocol for RT-PCR

Protocol Step (SYBR$^{®}$)		Temperature	Time	Cycle
1. Polymerase Activation & DNA Denaturation		95˚C	30 s	1X
	Denaturation	95˚C	5 s	
2. Amplification	Annealing	58 ˚C	30 s	40X
	Extension	58 ˚C	30 s	
3. Melt-Curve Analysis		4˚C	Indefinite	-

3.8 IMMUNOHISTOCHEMISTRY STAINING

The FFPE of CRC samples that were cut into 4-µm thick slices were used in immunohistochemical analyses using rabbit anti-human PASD1 polyclonal primary antibody at a dilution of 1:500 (Thermo Fisher Scientific, USA) and 3,3-diaminobenzidine (DAB) Dako Envision Plus Kit (Abcam, UK). The antigen was retrieved by using antigen retrieval buffer (100X Tris-EDTA Buffer at pH 9.0) (Abcam, UK) in a decloaking chamber (Biocare Medical, USA) for 30 min at 110˚C. The slides were placed at room temperature to be cooled down prior to IHC staining

(Table 3.11) per reaction. Normal adult testis tissue was used as a positive tissue control and normal tonsil tissue was used as negative tissue control. Incubation of CRC sample of same procedures omitting the primary PASD1 antibody was used as the experimental negative control.

Table 3.11 Protocol for IHC staining

Reagent	Duration	Volume	Cycle
1X PBST	5 min	1 mL	3X
3% of hydrogen peroxide	10 min	3 drops	-
1X PBST	5 min	1 mL	3X
Rabbit anti-human PASD1 polyclonal antibody (1:500)	30 min	100 µL	-
1X PBST	5 min	1 mL	3X
Horseradish Peroxidase (HRP)	30 min	200 µL	-
DAB Chromogen	5 min	2 drops	-
1X PBST	5 min	1 mL	5X
Counterstain with hematoxylin	-	3-4 dips	-
Running tap water	1 min	Rinse	-
80% ethanol	3 min	Immerse	-
90% ethanol	3 min	Immerse	-
Absolute ethanol	3 min	Immerse	-
Xylene I	5 min	Immerse	-
Xylene II	5 min	Immerse	-

The slides were subsequently counterstained with hematoxylin and mounted using Sub X Surgipath mounting medium (Leica Biosystems, Germany) with a coverslip (Menzel, Germany). All the slides were viewed and examined under the compound microscope by a pathologist. The extent of immunohistochemical reactivity was graded as follows: weak (<25% of cells stained), moderate (25% to 75% of cells stained) and strong (>75% of cells stained) of a focal single cell or small clusters of cells verified by a pathologist.

3.9 IMMUNOFLUORESCENCE STAINING

Protein expression of PASD1-positive cell lines, SW480 and H1299 were determined by using IF technique. The rabbit anti-human PASD1 polyclonal primary antibody at a dilution of 1:500 (Thermo Fisher Scientific, USA) and anti-rabbit IgG linked FITC antibody at a dilution of 1:500 (Thermo Fisher Scientific, USA) were used. The coverslip was coated with 0.1% of sterile gelatin (Sigma Aldrich, USA) for 30 min prior to the culture. Cells were grown on a sterile coverslip (Menzel, Germany) in a 24 well plate for 3 days in 37˚C until 70-80% confluency was achieved. Culture medium was discarded and IF staining (Table 3.12) was performed in each of the respective wells in a 24-well plate. About 1.0 µg/mL of 4,6-diamidino-2-phenylindole (DAPI) dye (Invitrogen, USA) was used as a counterstain dye.

Table 3.12 Protocol for IF staining

Reagent	Duration	Volume	Cycle
1X PBST	5 min	2 mL	3X
4.0% % (w/v) paraformaldehyde	5 min	1 mL	-
0.25% Triton X-100 in 1X PBS	10 min	1mL	-
1X PBST	5 min	2 mL	3X
1.0% (w/v) Bovine Serum Albumin (BSA) in 1X PBST	30 min	1 mL	-
Rabbit anti-human PASD1 polyclonal antibody (1:500)	Overnight in dark at 4˚C	1 mL	-
1X PBST	5 min	2 mL	3X
Anti-rabbit IgG–FITC antibody (1:500)	1 hour	1 mL	-
1X PBST	5 min	2 mL	5X
1.0 µg/mL DAPI dye	1 min	1 mL	-
1X PBST	5 min	2 mL	5X

Incubation samples using the same procedure by omitting the primary antibody and were used as negative controls. The coverslip was then carefully removed from 24 well plates and placed onto a clean microscopic slide to be mounted with 10% glycerol (Thermo Fisher Scientific, USA) mounting medium in dark. The coverslip on the slide was sealed by using nail polish and placed in the dark at room temperature overnight to dry. All the slides were viewed and examined under DeltaVision™ Ultra widefield microscope (GE Healthcare Life Sciences, USA).

3.10 PEPTIDE GENERATION

BIMAS prediction algorithm and SYFPEITHI programs were used to select PASD1 CTL peptides predicted to be immunogenic in the context of HLA-A*24:02 (the most prevalent HLA allele among Malaysia population). The following peptides were synthesized as shown in Table 3.13. The PASD1 peptides were synthesized by standard chemistry and were more than 90% pure (Think Peptides, UK). The PASD1 peptides were a gift from the University of Oxford as a collaborative project.

Table 3.13 HLA-A*24:02 Nanomer PASD1 CTL Peptides

Peptides	Amino Acid Sequence	Position	BIMAS Score	SYFPEITHI Score	Purity
PASD1 (1)	DYIRLWQEL	688-696	475.2	22	96.33%
PASD1 (2)	AYDIISQEL	366-374	369.6	21	96.84%
PASD1 (3)	VYQKIILKF	89-97	231.0	27	96.80%
PASD1 (4)	TYCSSTVFL	265-273	200.0	21	92.35%

3.11 HUMAN LEUKOCYTE ANTIGEN ALLELE TYPING

3.11.1 Isolation of Genomic DNA

Genomic DNA from 200 mL of peripheral blood of CRC patients (n=40) and healthy controls (n=23) was isolated using QIAamp® DNA Blood Mini Kit (Qiagen, Germany) as described in the manufacturer's protocol. 20 µL Proteinase K and 200 µL of whole blood sample were added into a 1.5 mL microcentrifuge prior to the addition of 200 µL of Buffer AL into the same tube. The mixture was mixed by pulse-vortexing using a vortex (Heidolph, Germany) for 15 s and incubated for 10 min at 56˚C. The tube was briefly centrifuged and another 200 µL of absolute ethanol was added and mixed thoroughly before it was incubated for another 15 s. The mixture was pipetted into a new QIAamp Mini column and centrifuged at 6000 × g for 3 min using Centrifuge 5424 R (Eppendorf, USA). The column was transferred to a new collection tube and 750 µL of Buffer AW1 was added before it was centrifuged at 6000 × g for 3 min. The lysate was discarded and 750 µL of Buffer AW1 was added and the tube was centrifuged at 6000 × g for 3 min. Alternatively, the spin column was further centrifuged at 6000 × g for 1 min to dry membrane completely. 200 µL of Buffer AE was then pipetted directly to the spin column in a 1.5 mL microcentrifuge tube and the tube was centrifuged for 1 min at 12,000 × g to elute the DNA.

3.11.2 DNA Quantitation and Purity Check

The concentration and purity of extracted DNA were analyzed using NanoDrop 2000 (Thermo Fisher Scientific, USA). One µL of Buffer AE was pipetted onto the pedestal and calibrated as a blank. Next, 1 µL of the respective DNA sample were pipetted and measured in OD_{260}, OD_{280}, $A_{260/280}$, and $A_{260/230}$ ratio.

3.11.3 Polymerase Chain Reaction

HLA-A*24:02 allele typing in SW480 cell line, COLO320DM cell line, CRC patients and healthy controls was assessed by PCR using GoTaq® DNA Polymerase (Promega, USA) (Nakatsugawa et al. 2011). The HLA primers (Integrated DNA Technologies, USA) used for PCR (Table 3.14) with an expected PCR product size of 546 base pairs (Nakatsugawa et al. 2011). GAPDH was used as the internal control. Tubes without the cDNA template were used as non-template control.

Table 3.14 Primers used for PCR determination of HLA allele typing

Primer	Sequence	Length
HLA-A*24:02 Forward	5'- ACT GAC CGA GAG AAC CTG CGG AT -3'	23
HLA-A*24:02 Reverse	5'- ACT TGC GCT TGG TGA GAG CC -3'	20
GAPDH Forward	5'-GGA TTT GGT CGT ATT GGG C -3'	19
GAPDH Reverse	5'-TGG AAG ATG GTG ATG GGA TT -3'	20

Each PCR reaction was prepared in a 0.2 mL of the clear microcentrifuge tube. The preparation of the reaction mix (Table 3.15) was performed on ice and mixed gently. The reaction tubes were loaded onto the PCR instrument, Veriti 96 Well Thermal Cycler (Applied Biosystems, USA) and programmed accordingly (Table 3.16). All PCR reactions were performed in triplicates. The PCR products were then visualized using gel electrophoresis.

Table 3.15 Kit components used to prepare PCR samples

Component	Volume per Reaction, µL	Final Concentration
GoTaq® Flexi Buffer	10	1X
$MgCl_2$ solution, 25 mM	2	1 mM per reaction
PCR nucleotide mix, 10 mM each	2	200 µM each
HLA-A*24:02 forward	2.5	0.1 µM
HLA-A*24:02 reverse	2.5	0.1 µM
GoTaq® DNA polymerase (5 U/µL)	0.25	1.25 U
Template DNA	1.5	100 ng per reaction
Nuclease-free water	29.25	-
Total Volume	50	-

Table 3.16 Optimized Thermal Cycling Conditions for PCR

Protocol Step		Temperature	Time	Cycle
1. Initial Denaturation		94˚C	2 min	1X
2. Amplification	Denaturation	94˚C	15 s	40X
	Annealing	70˚C	30 s	
	Extension	72˚C	30 s	
3. Final Extension		72˚C	5 min	1X
4. Soak		4˚C	Indefinite	1X

3.11.4 Agarose Gel Electrophoresis

The PCR products were visualized using agarose gel electrophoresis on 1.2% agarose gel. Around 0.9 g of agarose powder (Sigma Aldrich, USA) was added into a conical flask with 60 mL of 1X Tris base/Acetic acid/EDTA (TAE) buffer (Thermo Fisher Scientific, USA). The flask was microwaved for 1 min until agarose powder was dissolved completely. The gel mixture was cooled with running tap water for 3 min and 2 µL of GelStar™ Nucleic Acid Gel Stain (Lonza, USA) was added and swirled thoroughly. The molten gel was poured into the casting tray with a well comb in place and left it at room temperature to be solidified. Once the gel was solidified, the gel was transferred into the Wide Mini-Sub Cell GT Systems (Biorad, USA) filled with 1X TAE buffer. 10 µL to 15 µL of DNA sample was mixed with 1.0 µL of blue loading dye (Promega, USA) and loaded into respective wells.

A total volume of 5.0 µL of GeneRuler 100 bp DNA ladders and 1 kb DNA ladder (Thermo Fisher Scientific, USA) were added to additional wells as indicators. The cover lid was assembled onto the chamber tank after all the samples were loaded and connected to a Power Supply-PowerPac (Biorad, USA). The electrophoresis was set to run at 80.0 volts and 400.0 mA for 60 min. The gel with DNA band was visualized under a UV transillumination and captured by Gel Documentation System Quantum-ST4 (Fisher Thermo Scientific, USA). Optionally, nucleotide sequences of the above PCR products could be confirmed and validated by direct sequencing using an ABI Genetic Analyzer PRISM 310 and an AmpliCycle sequencing kit (Perkin-Elmer, USA).

3.12 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood samples collected from HUKM in vacutainer tubes containing sodium citrate were processed immediately using Ficoll-Paque PLUS (GE Healthcare Life Sciences, USA) for *in vitro* isolation of lymphocytes. Peripheral blood was diluted with 1X PBS in 1:1 ratio and mixed well prior to the isolation. By using aseptic technique, 6.75 mL of Ficoll-Paque PLUS was added into a sterile 50 mL falcon tube and layered carefully with 9 mL of the diluted blood sample in a 3:4 ratio (Ficoll-Paque PLUS to the diluted blood). The tube was centrifuged at 400 × g for 30 min at 20˚C. The upper plasma layer was discarded by using a 3 mL sterile Pasteur pipette and lymphocyte layer was isolated to a clean 15 mL centrifuge tube. At least 3 volumes of sterile 1X PBS were added into the tube containing lymphocytes and resuspended gently in and out of the Pasteur pipette. The tube was then centrifuged at 100 × g for 10 min at 20˚C. The supernatant was discarded and replaced with 13.5 mL of sterile 1X PBS and centrifuged. The lymphocytes were isolated and counted before cryopreserved in liquid nitrogen.

3.12.1 Cryopreservation Method

Approximately 1×10^6 of the lymphocytes were resuspended with 1 mL of freezing media containing 95%FCS/5% DMSO into a 1.8 mL of cryovial. The cryovial containing the lymphocytes was placed in Nalgene Mr Frosty Freezing container (Thermo Fisher Scientific, USA) filled with isopropyl alcohol at -80˚C overnight. The frozen lymphocytes in the cryovial was then transferred to a vapour phase of a liquid nitrogen storage tank and stored in the UMBI's biobank until further analysis. Similarly, the same protocol was used to cryopreserve all the cell lines in this study.

3.13 HUMAN INTERFERON-γ ELISPOT^{BASIC} ASSAY

Peripheral blood mononuclear cells (PBMCs) in RPMI 1640 medium containing 10% fetal calf serum (RPMI1640/FCS) (Hyclone, USA) were prepared. PBMCs in 200 µL of cells (5×10^4 cells) were then added to each well of a 96-well round-bottomed plate and incubated for 10 days with 10 µM of one of the followings: one of the PASD1 peptides (Table 3.13), the irrelevant HIV peptide (ILKEPVHGV), 10 µg/mL phytohaemagglutinin (PHA) (Sigma Aldrich, USA) and RPMI 1640/FCS only. Recombinant human interleukin-2 (rIL-2) (20 IU/mL) and recombinant human interleukin-7 (rIL-7) (25 ng/mL) (R&D Systems, USA) were added on days 2, 5 and 7. PBMCs were co-cultured with PASD1 CTL epitopes for 8 days in 37˚C CO_2 incubator before being used in Human Interferon-γ (IFN- γ) ELISpot^{BASIC} assay.

After the 8 days of culture, new RPMI1640/FCS media was added to the PBMCs culture for 24 h at 37ºC in 5% CO_2 pulsed with respective PASD1 peptides (10 µM), irrelevant HIV control peptide (ILKEPVHGV) (10 µM), phytohaemagglutinin (10 µg/mL) and medium only. Human Interferon-γ (IFN- γ) ELISpot^{BASIC} Kit (Mabtech, Sweden) assay was performed according to the manufacturer's instructions. PVDF ELISpot plate (Merck Millipore, USA) was pretreated with 50 µL of 70% of sterile ethanol for 2 min and washed off immediately with 200 µL of 1X PBS per well for 5 times. Without drying the PVDF membrane, 100 µL of 15 µg/mL diluted coating monoclonal antibody (1-DIK) in sterile 1X PBS was carefully pipetted into each well and incubated in dark at 4˚C for 24 h. On the next day, the excess antibody 1-DIK was washed off with 200 µL of 1X PBS for 5 times and replaced with 200 µL of RPMI1640/FCS for 30 min in room temperature. After incubation, the medium was removed. The cell suspension from the 8 days culture in 96 well plates was transferred into ELISpot plate, wrapped with aluminium foil and placed in a 37˚C CO_2 incubator for another 24 h.

On the following day, the cells were removed by washing with 200 µL of 1X PBS for 5 times. 100 µL of 1 µg/mL diluted detection antibody (7-B6-1-biotin) in sterile 1X PBS/0.5%FCS was carefully pipetted into each well and incubated for 2 h at room temperature. The plate was washed with 200 µL of 1X PBS for 5 times and 100 µL of 1:1000 of streptavidin-horseradish peroxidase (HRP) in 1X PBS/0.5%FCS was added into each well and incubated for 1 h at room temperature. After 1 h of incubation, the plate was washed with 200 µL of 1X PBS for 5 times and 100 µL of 1:1000 of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Mabtech, Sweden) was added until obvious spot blue spots were seen. Deionized water was used to stop the colour development and the plate was left to dry in dark at room temperature. The number of spots was counted and examined by using CTL Immunoqualify^{TM} software in CTL S6 Universal ELISpot Analyzer (Cellular Technology Limited, USA). The assay was performed in triplicates. Results were considered positive if the number of spots in the test wells was at least twice the number present in the control cultures (media only or in the presence of the HIV irrelevant peptide) and assays were excluded if there were more than 25 spots per well in the absence of peptides.

3.14 *IN VITRO* EXPANSION OF ANTIGEN-SPECIFIC CYTOTOXIC T CELLS

Cryopreserved PBMCs was thawed at 37˚C water bath and resuspended in 7 volumes of RPMI+HEPES Medium (RPMI-1640 medium containing 10% of heat-inactivated fetal calf serum (FCS), 1% of glutamine (Gln), 1% of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Nacalai Tesque, USA), 1% of penicillin/streptomycin (Pen-Strep) (Gibco, USA) immediately using a sterile 10 mL serological pipette. It was then centrifuged at 100 × g for 5 min at 20˚C. The supernatant was discarded, and the cells were resuspended with 1 mL of RPMI+HEPES Medium and the number of cells was counted using hemocytometer. Approximate 20 to 30 x 10^6 PBMCs in a final volume of 5 mL of RPMI+HEPES Medium was transferred to a T-25 flask. The cells were incubated for 4 h in a 37˚C CO_2 incubator for the adhesion of monocytes to the bottom surface of the flask. At this stage, two populations of that constitute PBMCs: the adherent population (monocytes, dendritic cells, macrophages) and suspension population (lymphocytes) were separated using plastic adherence method (Bender et al. 1996).

After 4 h, the supernatant containing T lymphocytes was collected and centrifuged at 450 × g for 5 min at 20˚C. The cell pellet (1x10^6 cells/mL) was resuspended gently in 95%FCS/5%DMSO and frozen at liquid nitrogen. At the same time, 5mL of fresh RPMI+HEPES Medium supplemented with 1000 U/mL of rGM-CSF and 1000 U/mL of the reconstituted rIL-4 was added carefully to the adherent monocyte flask. The monocytes were incubated in a 37˚C CO_2 incubator for 6 days. The cells in the flask were checked from time to time under the inverted microscope within the 6 days culture to ensure no contamination was seen. Alternatively, 1 to 3 mL of fresh RPMI+HEPES Medium could be added or replaced based on the confluency of the cells. At day 6, the medium from the flask was discarded and washed twice with 5 mL of prewarmed sterile 1X PBS. 10 mL of RPMI+HEPES medium containing 25 ng/mL of rIL-1β, 50 ng/mL of rTNF-α, 1000 U/mL of rIL-6 and 10^{-6} mol/L of PGE_2 was added gently into the flask and incubated for additional 2 days (Gigante & Ranieri 2014).

After 2 days of culture with maturation cytokines, the medium was removed and the adherent cells (monocytes derived-dendritic cells) were detached gently using 5 mL Versene solution (Thermo Fisher Scientific, USA) twice and transferred to a 50 mL tube and centrifuged 450 × g for 5 min at 20˚C prior subjected to cell count. Every 1 x 10^6 of monocytes derived-dendritic cells (mDCs) was frozen in 1 mL aliquot of 95%FCS/5%DMSO for further T cell stimulation. Optionally, 5 x 10^4 of detached cells could be used for cytometric analyses of DCs population. The first aliquot of mDCs containing 1 x 10^6 of cells were used in the first loading with PASD1(4) peptide. mDCs was grown in a 6-well culture plate with 1.5 mL of RPMI+HEPES Medium. 10 µg/mL of PASD1(4) peptide and 3 µg/mL of recombinant human β2 microglobulin (BD Pharmingen, USA) were added and incubated for 4 h in a 37˚C CO_2 incubator with occasional agitation. After 4 h, the cells were washed with RMPI/10%FCS and resuspended in 2 mL of RPMI+HEPES Medium. Cryopreserved T lymphocytes were thawed and resuspended at 10 to 20 x 10^6 cells/mL in 20:1 ratio in RPMI+HEPES Medium and incubated for subsequent 7 days in a 37˚C CO_2 incubator.

Alternatively, 3 mL of sterile 1X PBS was added to the empty wells to equilibrate the system. The T cells in the flask were checked from time to time under an inverted microscope within the 7 days culture to ensure no contamination was seen. After one week of culture, the second aliquot of T cells was thawed and stimulated with (PASD1(4) and β2 microglobulin) and supplemented with (5 ng/mL of rIL-2 and 20 U/mL of rIL-7) for another 7 days as described for first stimulation (Gigante & Ranieri 2014). The cells were re-stimulated for third stimulation with rIL-2 and of rIL-7. At the third week, CD8 positive PASD1-specific cytotoxic T lymphocytes (PASD1-CTL) were isolated by positive selection using immunomagnetic bead technique before being used for the immunofunctional assays.

3.15 POSITIVE ISOLATION OF CD8 PASD1-SPECIFIC CYTOTOXIC T LYMPHOCYTES

CD8 positive PASD1-specific cytotoxic T lymphocytes (PASD1-CTL) from the 21-days culture was isolated using EasySep™ Human CD8 Positive Selection Kit II (Stemcell, Canada) according to the manufacturer's instructions. T cell pellet from the above culture was immediately resuspended in 100 µg/mL of DNase I solution (Thermo Fisher Scientific, USA) for 15 min at room temperature prior to labelling and separation. The aggregated suspension was filtered through a 40 µM cell strainer. The flow-through sample was then resuspended at 1 x 10^8 cells/mL of EasySep™ Buffer in a 5 mL sterile FACS tube. 100 µL of Selection Cocktail was added to 1 mL of sample.

The RapidSpheres™ beads were vortexed for 30 s before adding to the sample at 50 µL per 1 mL of sample and incubated for 3 min. EasySep™ Buffer was added to top up 2.5 mL and the tube was placed into the EasySep™ magnet (Stemcell, Canada) and incubated for 3 min at room temperature. After the incubation, the tube together with the magnet was picked up to pour off the supernatant. The tube was then removed from the magnet and resuspended twice in 2.5 mL of EasySep™ Buffer for 3 min each as indicated. At the final step, the CD8 positive T cells were resuspended in RPMI+HEPES Medium for downstream analysis. Besides, 5 to 10 µL of sample was labelled with 10 µL of PE-conjugated mouse anti-CD4, FITC-conjugated mouse anti-CD3 and APC-conjugated mouse anti-CD8 markers each (BD Pharmingen, US) and added with 200 µL of sterile 1X PBS/10%FCS for flow cytometry analysis for 30 min at room temperature on Rotamax 120 orbital shaker (Heidolph, Germany). T cell fractions were confirmed to contain > 90% CD8 T lymphocytes using BD FACSVerse™ (BD Biosciences, USA).

3.16 CYTOLYTIC ASSAY

On the day of the assay, four CRC cell lines and one lung adenocarcinoma cell line (as the target cells), SW480 (HLA-A*24:02+, PASD1+), H1299 (HLA-A*24:02–, PASD1+), COLO320DM (HLA-A*24:02+, PASD1–), CCD112CoN (HLA-A*24:02–, PASD1–) target cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) fluorescent-based dye using CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes, UK). Cells were stained with 1 µL of 5 mM of CFSE dye and suspended to 1 x 10^6 cells per mL in prewarmed 1X PBS/1%FCS media for 20 min in 37˚C CO_2 incubator in the dark. Without discarding any medium, 1 mL of RPMI+HEPES Medium was added into the tube and centrifuged at 100 × g for 5 min at room temperature. The supernatant was removed and replaced with 1 mL of fresh RPMI+HEPES Medium to be further incubated for another 10 min in a 37˚C CO_2 incubator. After the incubation, 5 x 10^4 cells CFSE-stained target cells were resuspended and various number of the isolated PASD1-CTLs (as the effector cells) were added into a round-bottomed 96-well plate respectively at a final volume of 200 µL of RPMI+HEPES Medium to yield 3:1, 5:1 and 10:1 of effector/target (E/T) rations. The effector and CFSE-stained target cells were co-cultured for 6 h in a 37˚C CO_2 incubator for the lysis to occur. After the 6 h of co-culture, the 96-well plate was centrifuged at 400 × g for 5 min at 20 ˚C using Centrifuge 5810 R (Eppendorf, Germany). The supernatant of the co-culture from each sample was transferred to a 1.5 mL microcentrifuge tube and frozen at -80˚C for cytokine profiling. Concurrently, CFSE-stained target cells were isolated and stained with 2 µg/mL of Propidium Iodide Nucleic Acid Stain (Invitrogen, UK) for 15 min at room temperature in dark. The cells were then added with 200 µL of 1X PBS/1%FCS/0.1% sodium azide in 5 mL FACS tubes to be analyzed by using BD FACSVerse™ (BD Biosciences, USA). Tubes with CFSE-stained target cells and PI-stained target cells only were the control tubes.

3.17 HUMAN GRANZYME B ELISPOT^{BASIC} ASSAY

Human Granzyme B ELISpot^{BASIC} kit assay was performed according to the manufacturer's instructions (Mabtech, Sweden). PVDF ELISpot plate (Merck Millipore, USA) was pretreated with 50 µL of 70% of sterile ethanol for 2 min and washed off immediately with 200 µL of 1X PBS per well for 5 times. Without drying the PVDF membrane, 100 µL of 15 µg/mL diluted human granzyme-B capture Abs (GB10) coating antibody in sterile 1X PBS was carefully pipetted into each well of ELISpot plate and incubated in dark at 4˚C for 24 h. On the following day, the excess antibody GB10 was washed off with 200 µL of sterile 1X PBS for 5 times and replaced with 200 µL of RPMI+HEPES Medium for 30 min in room temperature. After incubation, the medium was discarded. The isolated effector PASD1-CTLs in 3:1, 5:1 and 10:1 of effector/target (E/T) ratios were seeded to duplicate wells of pre-coated with antibody GB10, together with respective target cells (5 x 10^4 cells /well) into the ELISpot plate respectively at a final volume of 200 µL of RPMI+HEPES Medium. The cancerous target cells were: SW480 (HLA-A*24:02+, PASD1+), H1299 (HLA-A*24:02–, PASD1+), COLO320DM (HLA-A*24:02+, PASD1–), CCD112CoN (HLA-A*24:02–, PASD1–). The ELISpot plate containing both effector and target cells was incubated in a 37˚C CO_2 incubator for 6 h of co-culture and wrapped with aluminum foil. After the 6 h of co-culture, the cells were removed by washing with 200 µL of 1X PBS for 5 times. 100 µL of 1 µg/mL diluted biotinylated monoclonal (GB11) detection antibody in sterile 1X PBS/0.5%FCS was carefully pipetted into each well and incubated for 2 h at room temperature. The plate was washed with 200 µL of 1X PBS for 5 times and 100 µL of 1:1000 of streptavidin-alkaline phosphatase (ALP) in 1X PBS/0.5%FCS was added into each well and incubated for 1 h at room temperature. After 1 h of incubation, the plate was washed with 200 µL of 1X PBS for 5 times and 100 µL of 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) (Sigma Aldrich, USA) was added into each well until distinct spots were seen. Deionized water was used to stop the color development and the plate was left to dry in dark at room temperature. The number of spots was counted and examined by using CTL ImmunoqualifyTM software in CTL S6 Universal ELISpot Analyzer (Cellular Technology Limited, USA). The assay was performed in duplicates. Results were considered positive if the number of spots in the test wells was at least twice the number present in the control cultures (media only) and assays were excluded if there were more than 25 spots per well in the absence of peptides.

3.18 CYTOKINE PROFILING

The cytokine profiles of the colorectal cancer patients (n=3) from the supernatant of 6 h co-cultures (from cytolytic assay) were analyzed using Human CD8/NK Panel LEGENDplex™ Multi-Analyte Flow Assay Kit platform (BioLegend, USA) according to the manufacturer's procedure. The selected cytokines were interleukin-10 (IL-10), interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), soluble FAS cell surface death receptor (sFAS), soluble FAS cell surface death receptor ligand (sFASL), Granzyme A, Granzyme B, and perforin. The assay started with the preparation of the standards containing the selected cytokines. 250 μL of LEGENDplex™ Assay Buffer from the kit was reconstituted and mixed well with the lyophilized Human CD8/NK Panel Standard Cocktail at room temperature for 10 min before being used. Eight different standard concentrations following a serial dilution of 1:4 was performed (Table 3.17) by transferring 25 μL of the standard (C7) to a new 1.5 mL microcentrifuge tube to make C6 (1:4) and mixed well. In the same manner, serial dilution in 1:4 was carried out to obtain other C5 (1:16), C4 (1:64), C3 (1:256), C2 (1:1024), C1 (1:4096) and C0 (Assay Buffer only). All the standards were prepared in 1.5 mL of microcentrifuge tubes.

Table 3.17 The standard preparation

Tube	Serial Dilution	Assay Buffer to add (μL)	Standard to add (μL)	Final Con. (pg/mL) *	Final Con. (pg/mL) **
C7	-	-	-	10000	50000
C6	1:4	75	25 μL of C7	2500	12500
C5	1:16	75	25 μL of C6	625	3125
C4	1:64	75	25 μL of C5	156.3	781.3
C3	1:256	75	25 μL of C4	39.1	195.3
C2	1:1024	75	25 μL of C3	9.8	48.8
C1	1:4096	75	25 μL of C2	2.4	12.2
C0	-	75	-	0	0

* Standard concentration of IL-10, IFN-γ, TNF-α, Granzyme A, sFASL and perforin is 10 ng/mL
** Standard concentration of sFAS and Granzyme B is 50 ng/mL

The reagents in the kit were placed at room temperature prior to performing the assay. At the same time, the supernatant from the 6 h of co-cultures was thawed to room temperature and mixed well by flicking the tubes. By using a sterile 96-well round bottom microplate, 25 μL of RPMI+HEPES Medium and 25 μL of each standard (Table 3.17) was added to respective standard wells. Next, 25 µL of LEGENDplex™ Assay Buffer and 25 µL of from each sample (the supernatant in 1:2 dilution) was added to the sample wells accordingly. After all, 25 µL of Human CD8/NK Panel Mixed Beads and 25 µL of Human CD8/NK Panel Detection Antibodies were then added to all the wells and mixed thoroughly. The plate was placed at room temperature on Rotamax 120 orbital shaker (Heidolph, Germany) at 150 rpm for 2 h and wrapped with aluminum foil. After 2 h of incubation, without washing the plate, 25 µL of LEGENDplex™ SA-PE was added into each well and placed back on the orbital shaker for 30 min at room temperature in dark.

The plate was then centrifuged at 1000 × g for 5 min at 20˚C using Centrifuge 5810 R (Eppendorf, Germany). The supernatant after the centrifugation was discarded using a multichannel pipette. The beads were added with 200 µL of 1x Wash Buffer to be centrifuged at 1000 × g for 5 min at 20˚C. Another total volume of 200 µL of 1x Wash Buffer was added to resuspend the beads and transferred to 5 mL FACS tubes respectively. All the standard and the samples were analyzed by using BD FACSVerse™ (BD Biosciences, USA) on the same day. The flow cytometry was set up properly prior to reading all the samples. All the samples were vortexed for 5 s before analysis. The flow rate was set to “low” and the number of beads was set to 4000 beads per acquisition. The FCS files generated on the BD FACSVerse™ (BD Biosciences, USA) were analyzed using BioLegend’s LEGENDplex™ Data Analysis Software and Software Dongle provided in the kit.

3.19 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS v.18.0 software (IBM SPSS Statistics, USA). Experimental data were presented as a mean±standard deviation. Statistical differences of results between studied groups were assessed using One-way Analysis of Variance (ANOVA). P-value $* \leq 0.05$, $** \leq 0.01$, $*** \leq 0.001$ and $**** \leq 0.0001$ were considered as significant.

CHAPTER IV

RESULTS

4.1 EXPRESSION OF PASD1

4.1.1 mRNA Expression of *PASD1* in Cell Lines

Analysis of the gene expression of *PASD1* was carried out in seven of the cell lines, namely CCD112CoN, COLO320DM, SW48, HT29, HCT116, SW480 and H1299. Only the SW480 and H1299 cell lines showed positive expression of *PASD1* (Figure 4.1).

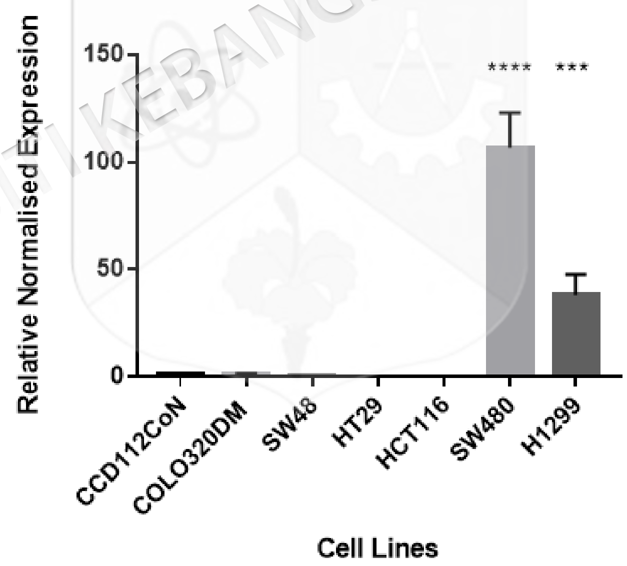

Figure 4.1 RT-PCR analysis of *PASD1* mRNA expression in the cell lines namely CCD112CoN, COLO320DM, SW48, HT29, HCT116, SW480 and H1299. Normalization was performed using the internal control, *GAPDH*. All the experiments were in triplicates. P-value: *≤ 0.05, ** ≤ 0.01, ***≤ 0.001 and ****≤ 0.0001 were statistically significant against the CCD112CoN normal colon cell line.

4.1.2 mRNA Expression of *PASD1* in CRC Tissues

In CRC tissues, gene expression of *PASD1* was determined in 25 primary CRC tissues and the respective paired normal tissue samples. Among the 25 CRC tissue samples, 5 (20%) showed positive expression of *PASD1* whereas none of the 25 paired normal colonic tissue samples were positive as shown in Table 4.1. The mRNA expression of PASD1 was highest in CRC samples derived from the rectum (16%) compared to samples from the rectosigmoid (4%) and sigmoid (0%) regions.

Table 4.1 Positive PASD1 gene expression in CRC tissues

Characteristics	CRC (n=25)
	No. of Positive Cases
Total number of samples	5/25 (20%)
Gender	
Male	5/20 (20%)
Female	0/5 (0%)
Age	
Range	(59-84)
Race	
Chinese	3/9 (12%)
Malay	2/15 (8%)
Indian	0/1 (0%)
Anatomical site	
Sigmoid	0/5 (0%)
Rectum	4/11 (16%)
Rectosigmoid	1/9 (4%)
Modified Dukes Classification	
A	1/6 (4%)
B	2/11 (8%)
C	1/7 (4%)
D	1/1 (4%)
Lymph node metastasis	
Positive	2/8 (8%)
Negative	3/17 (12%)
Histological grades	
Well differentiated	5/15 (20%)
Moderately differentiated	0/10 (0%)
Poorly differentiated	-

Normalization was performed using internal control, *GAPDH.* All the experiments were in triplicates.

4.1.3 Protein Expression of PASD1 in Human Testis Tissues and Cell Lines

Two PASD1-positive cell lines and one human testis control were examined for PASD1 protein expression and localization using immunofluorescence staining. The two cell lines, SW480 and H1299, were both positive for PASD1 mRNA expression (Figure 4.1). Some positivity of PASD1 (in green) was exhibited in the nucleus of the primary spermatogonia of the testicular tissues (Figure 4.2). Additionally, moderate staining (in green) was also observed in the cytoplasmic region of the SW480 and H1299 cell lines (Figure 4.3).

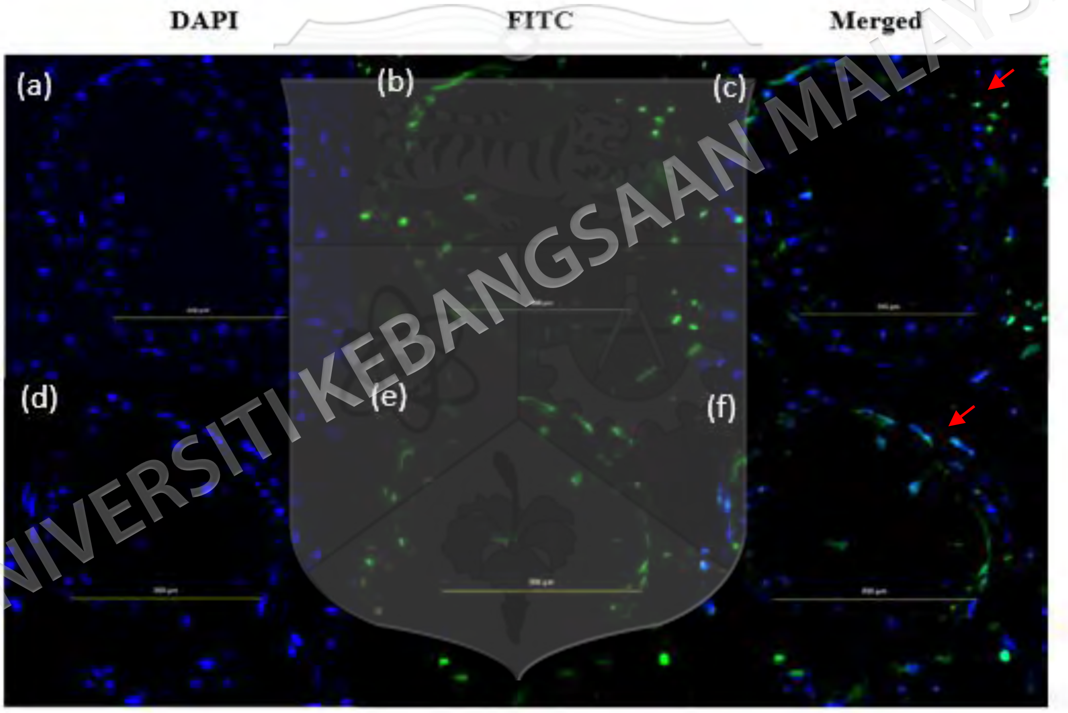

Figure 4.2 Immunofluorescence of PASD1 in human testis controls. PASD1 expression was shown to localize at the nucleus of the primary spermatogonia region (indicated with red arrows) of the testis tissue. Representative fluorescence and merged images of human testis control in duplicates (a,d) DAPI (DNA binding dye), (b,e) FITC-tagged PASD1 and (c,f) Merged. All images were taken under DeltaVision™ Ultra widefield microscope at 200× magnification.

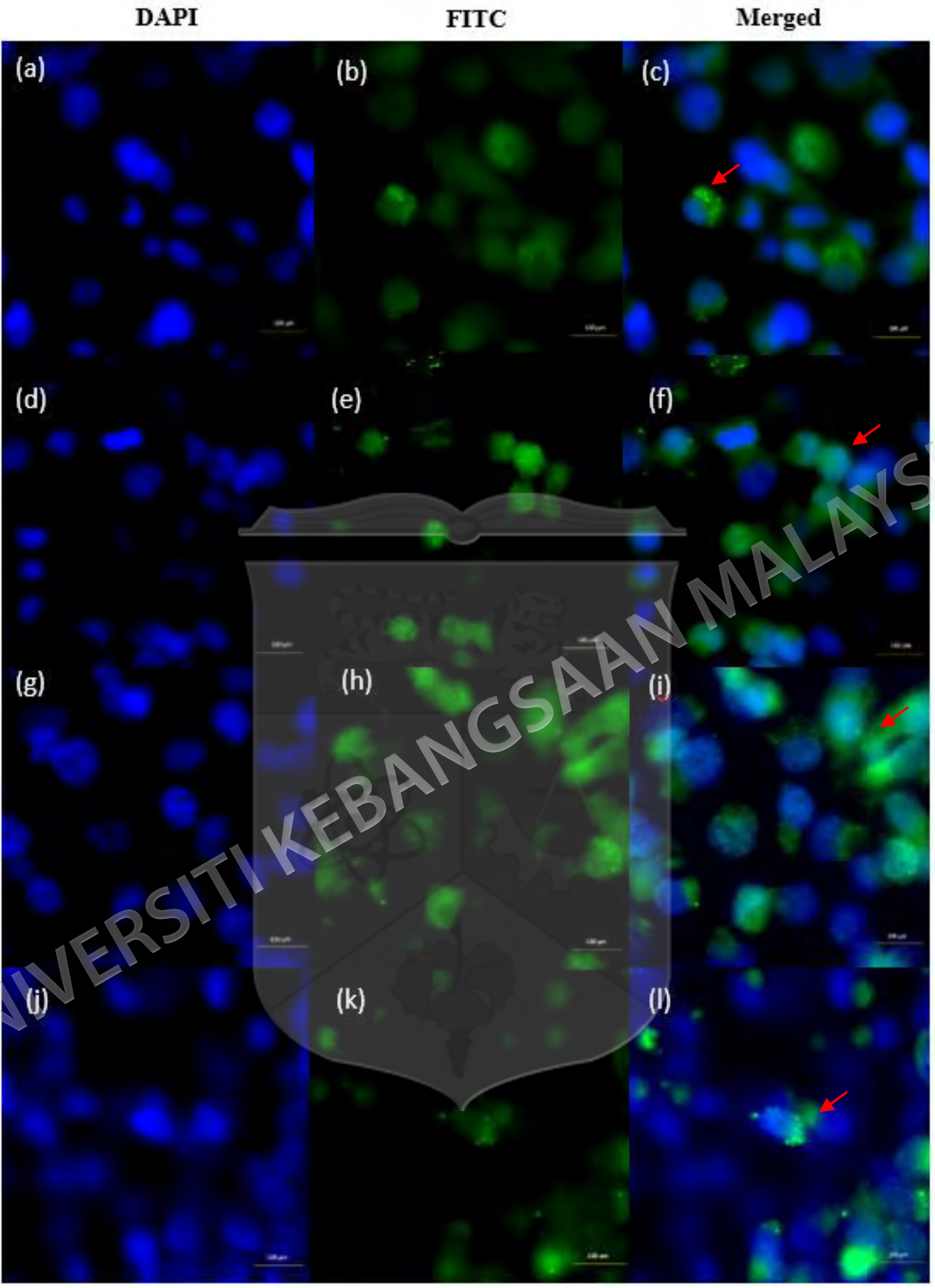

Figure 4.3 Immunofluorescence of PASD1 in SW480 and H1299. PASD1 expression was localized at the cytoplasmic and nucleus region (indicated with red arrows) of SW480 and H1299 cells. Representative fluorescence and merged images of SW480 cells in duplicates (a, d) DAPI (DNA binding dye); (b, e) FITC-tagged PASD1; (c, f) Merged; H1299 cells in duplicates (g, j) DAPI; (h, k) FITC-tagged PASD1; (i, l) Merged. All images were taken under DeltaVision™ Ultra widefield microscope at 600× magnification.

4.1.4 Protein Expression of PASD1 in CRC

Protein expression of PASD1 was determined in 23 FFPE CRC tissue samples (Table 4.2). Immunohistochemistry staining was performed with optimized dilution (1:500) of rabbit anti-human PASD1 polyclonal primary antibody (Thermo Fisher Scientific, USA). Similarly, a human testicular tissue (the origin of PASD1) was used as a positive control. Tonsil tissue was used as a negative control. Notably, only 4 out of 23 cases (17.4%) was shown to have weak to moderate cytoplasmic staining of PASD1, as verified by a pathologist (Figure 4.4). All microscopic images of immunohistochemistry results were taken at equivalent microscope settings under 100× magnification. The extent of immunohistochemical reactivity was graded as follows: weak (<25% of cells stained), moderate (25% to 75% of cells stained) and strong (>75% of cells stained) of a focal single cell or small clusters of cells.

Table 4.2 Positive PASD1 protein expression in CRC FFPE tissues

Characteristics	No of positive cases in CRC
Total number of samples	4/23 (17.4%)
Gender	
Male	4/16 (17.4%)
Female	0/7 (0%)
Age	
Range	(59-84)
Race	
Chinese	2/10 (8.7%)
Malay	2/9 (8.7%)
Indian	0/4 (0%)
Anatomical site	
Sigmoid	0/5 (0%)
Rectum	3/15 (13.0%)
Rectosigmoid	1/3 (4.4%)
Modified Dukes Classification	
A	0/1 (0%)
B	2/9 (8.7%)
C	1/12 (4.4%)
D	1/1 (4.4%)
Lymph node metastasis	
Positive	2/13 (8.7%)
Negative	2/10 (8.7%)
Histological grades	
Well differentiated	4/13 (17.4%)
Moderately differentiated	0/10 (0%)

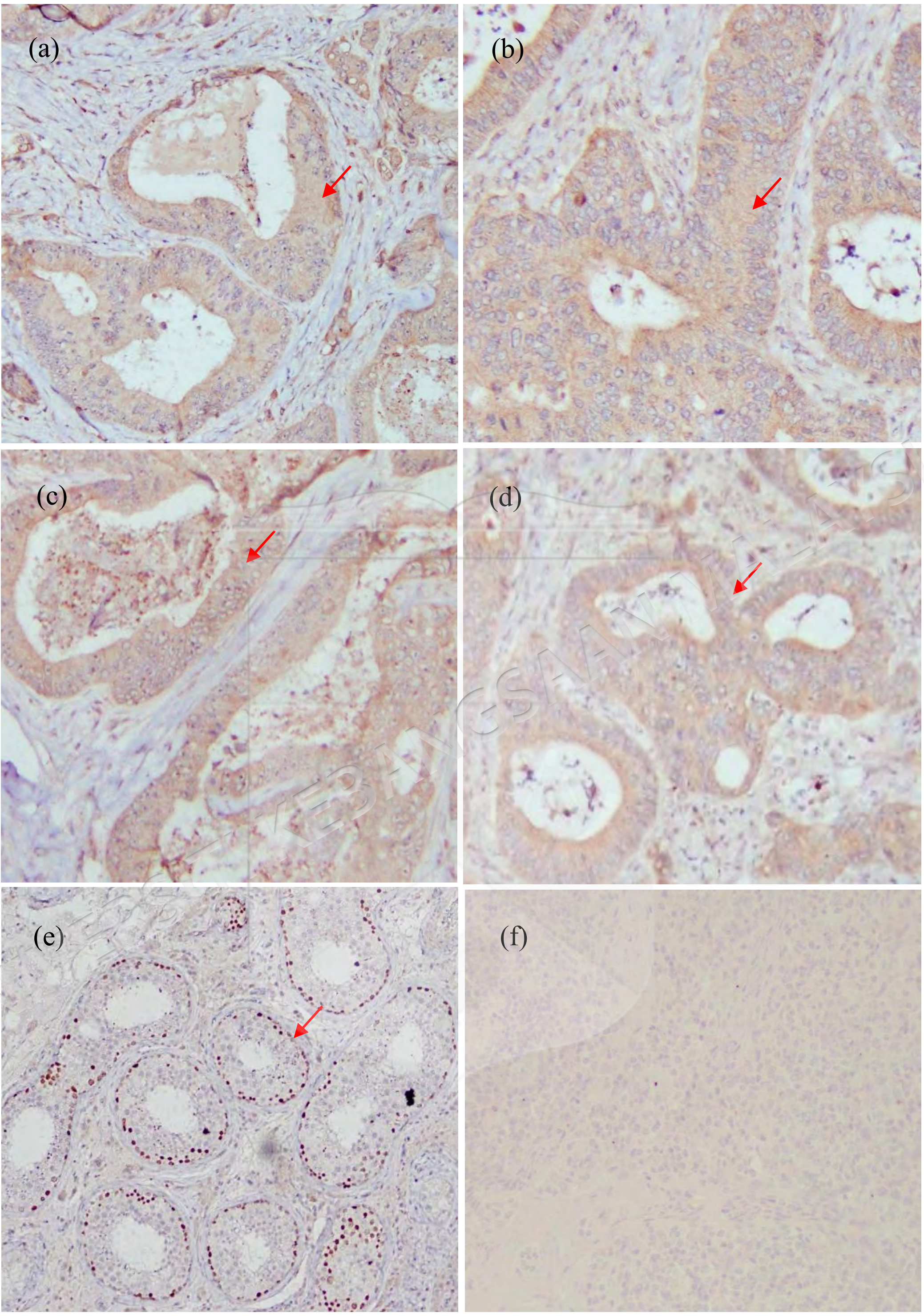

Figure 4.4 Immunohistochemistry staining of PASD1 in CRC FFPE tissues specimens. Representative IHC images of different CRC tumour blocks (a, b, c and d) with weak to moderate PASD1 expression was localized at the cytoplasmic region (*indicated with red arrows)* of CRC tumour cells were taken under at 200× magnification. The positive control (e) human testicular tissues and negative control (f) tonsil tissues were taken at 4× magnification.

4.2 SELECTION OF PASD1 PEPTIDE

4.2.1 HLA Allele Typing

HLA allele typing was performed in 40 recruited CRC samples and 23 healthy controls to screen for HLA-A*24:02 allele expression. This study showed that 15 out of 40 cases (37.5%) CRC and 5 out of 23 cases (21.7%) healthy controls expressed the HLA-A*24:02 allele (Table 4.3). The identification of HLA status was determined by the detection of a band size at 546 base pairs on the agarose gel (Figure 4.5). HLA allele typing in SW480 and COLO320DM cell lines was also determined. The rest of the samples were in Appendix H. The HLA-A*24:02 allele was chosen because it is the most common allele found in the Malaysian population generally. According to Allele Frequencies Network Database (AFND), the HLA-A*24:02 allele represents the highest and second-highest frequency alleles found in the Peninsular Malaysia Malay and Chinese population respectively according to AFND.

Table 4.3 Demographic data of the HLA typing in CRC and healthy samples

Characteristics	CRC		Healthy Control	
	Positive	Negative	Positive	Negative
Total number of cases	15/40 (37.5%)	25/40 (62.5%)	5/23 (21.7%)	18/23 (78.3%)
Gender				
Male	12/35 (30.0%)	23/35 (57.5%)	3/16 (13.0%)	13/16 (56.5%)
Female	3/5 (7.5%)	2/5 (5.0%)	2/7 (8.7%)	5/7 (21.8%)
Age				
Range	35-82	49-82	37-76	40-82
Race				
Chinese	4/18 (10.0%)	14/18 (35.0%)	2/10 (8.7%)	8/10 (34.9%)
Malay	7/15 (17.5%)	8/15 (20.0%)	2/11 (8.7%)	9/11 (39.1%)
Indian	4/7 (10.0%)	3/7 (12.0%)	1/2 (4.3%)	1/2 (4.3%)
Anatomical site				
Rectum	10/29 (25.0%)	19/29 (47.5%)	-	-
Sigmoid	5/9 (12.5%)	4/9 (10.0%)	-	-
Rectosigmoid	0/2 (0%)	2/2 (5.0%)	-	-
Modified Dukes Classification				
A	0/1 (0%)	1/1 (2.5%)	-	-
B	3/6 (7.5%)	3/6 (12.0%)	-	-
C	5/12 (12.5%)	7/12 (17.5%)	-	-
D	2/2 (5.0%)	0/2 (0%)	-	-
*High grade dysplasia / inflammatory polyp	5/19 (12.5%)	14/19 (35.0%)	-	-
Lymph node metastasis				
Positive	4/14 (10.0%)	10/14 (25.0%)	-	-
Negative	6/7 (15.0%)	1/7 (2.5%)	-	-
*High grade dysplasia / inflammatory polyp	5/19 (12.5%)	14/19 (35.0%)	-	-
Histological grades				
Well differentiated	5/9 (12.5%)	4/9 (10.0%)	-	-
Moderately differentiated	5/11 (12.5%)	6/11 (15.0%)	-	-
Poor differentiated	0/1 (0%)	1/1 (2.5%)	-	-
*High grade dysplasia / inflammatory polyp	5/19 (12.5%)	14/19 (35.0%)	-	-

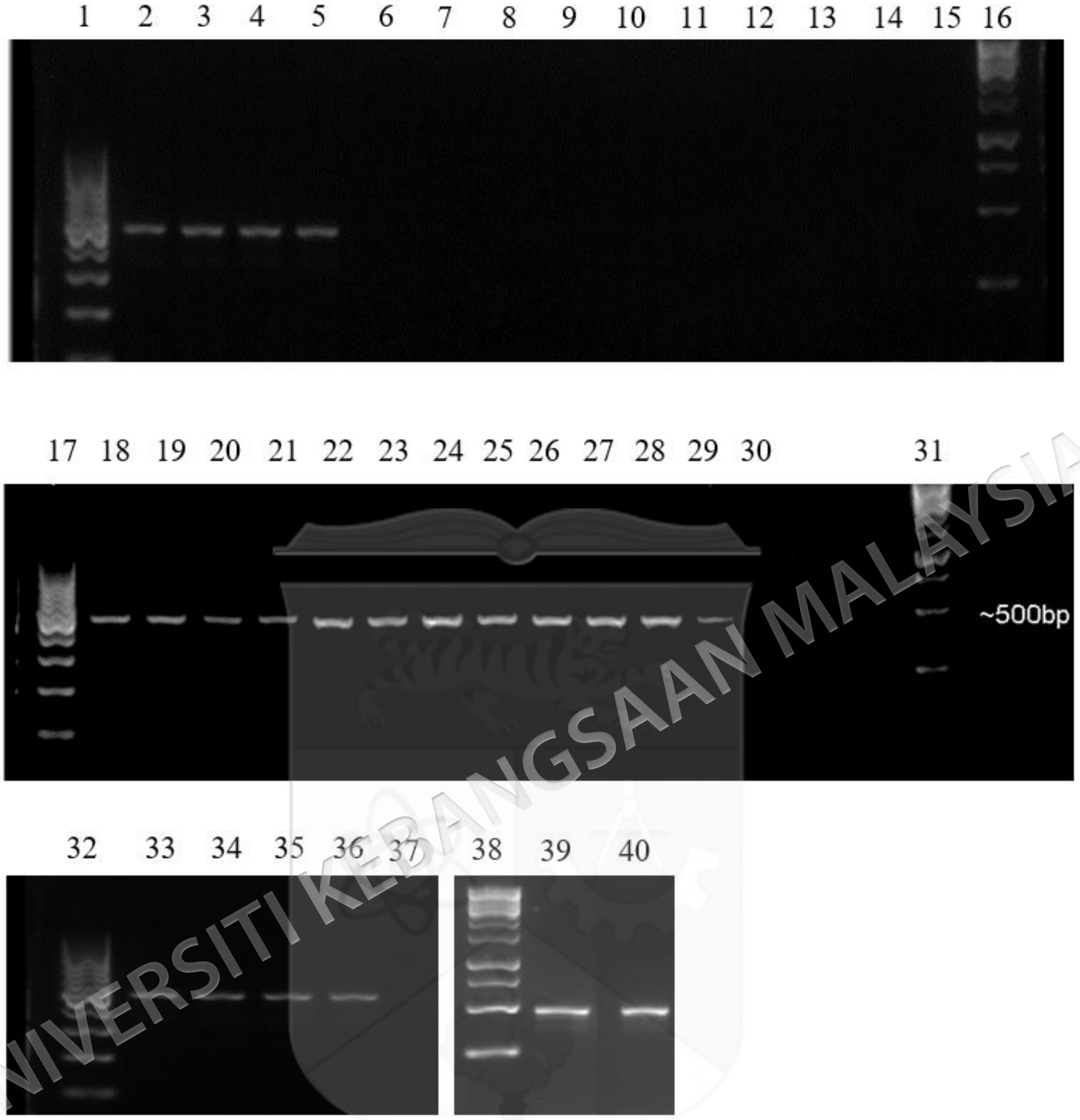

Figure 4.5 Agarose gel analysis of the total DNA extracted from the studied samples. The amplified PCR products at 546 base pairs were detected in the 4 positive CRC samples (Lane 2-5); 10 negative representative CRC samples (Lane 6-15), Lane 1: DNA Ladder 1 kb, Lane 16: DNA Ladder 100 bp, Lane 17: DNA Ladder 1 kb, Lane 18-22: 5 positive healthy controls, Lane 23-29: 7 positive CRC samples, Lane 30: NTC, Lane 31: DNA Ladder 1 kb, Lane 32: DNA Ladder 100bp, Lane 33-36: 4 positive CRC samples, Lane 37: NTC, Lane 38: DNA Ladder 1 kb, Lane 39: SW480 and Lane 40: COLO320DM.

4.2.2 Detection of Human Interferon-γ Activity

Selection of an immunogenic PASD1 peptide for downstream analysis was done using the Human Interferon-γ (IFN-γ) ELISpotBASIC Assay by measuring PASD1-specific IFN-γ production. Samples from four different screening groups: (a) HLA-A*24:02 positive CRC patients, (b) HLA-A*24:02 negative CRC patients, (c) HLA-A*24:02 positive healthy controls and (d) HLA-A*24:02 negative healthy controls were pulsed with corresponding PASD1 peptides: PASD1(1), PASD1(2), PASD1(3) and PASD1(4). The IFN-γ responses to all PASD1 peptides from each screening groups were different as observed from the varied count spots. From Figure 4.6, the healthy control samples generally had lower IFN-γ responses to all PASD1 peptides as compared to the CRC samples regardless of their HLA status. The IFN-γ responses from wells with PHA positive control were significantly higher (P ≤ 0.0001) when compared to the HIV negative peptide control and the wells pulsed with PASD1 peptides. Interestingly, significant IFN-γ response was detected in HLA-A*24:02 positive CRC samples after short culture with PASD1(4) peptide in relation to PASD1(2) peptide (Figure 4.6). PASD1(4) showed the highest IFN-y response with the highest selectivity in HLA-A*24:02 positive CRC samples. P-value *≤ 0.05, **≤ 0.01, ***≤ 0.001 and ****≤ 0.0001 were considered as significant.

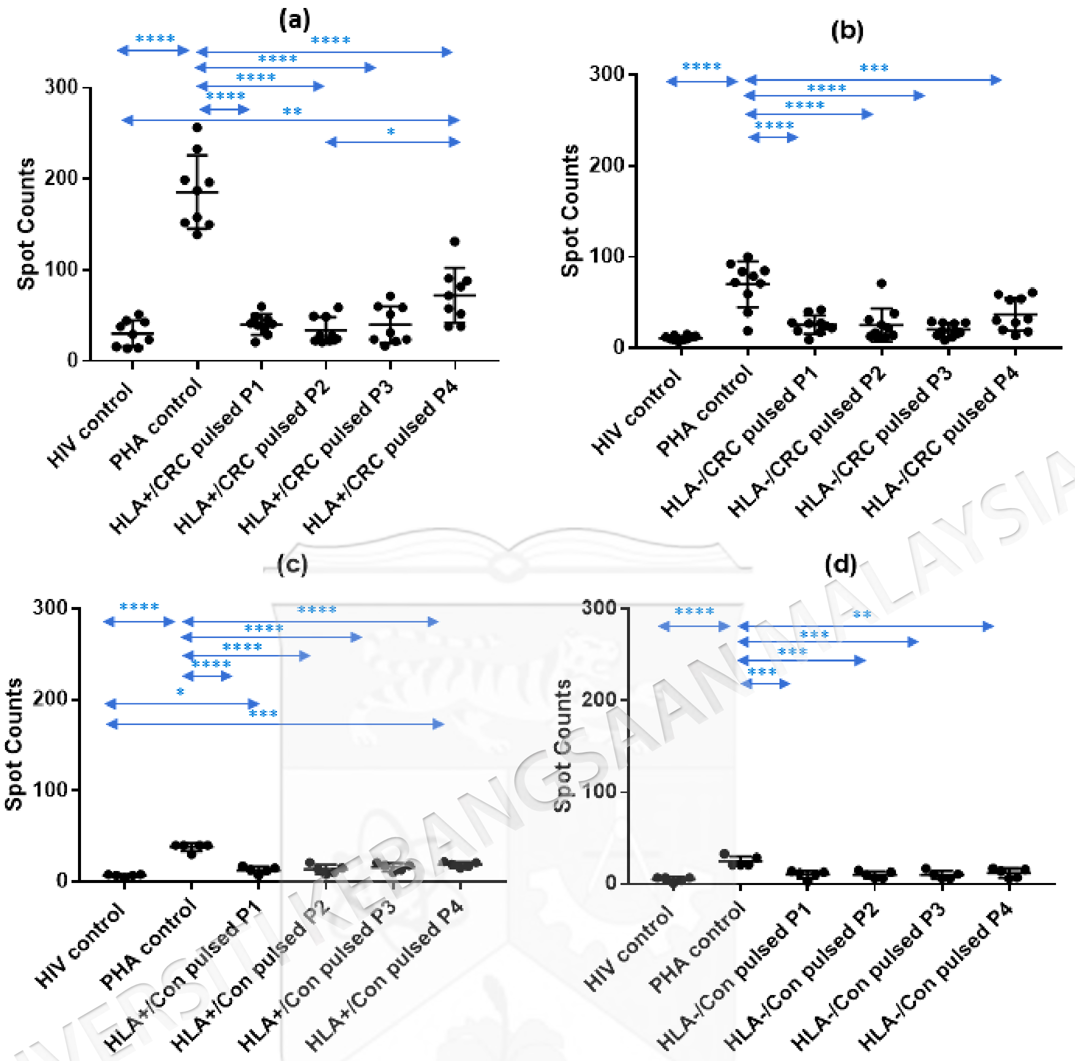

Figure 4.6 Human Interferon-γ ELISpot results for all four different screening samples with (a) HLA-A*24:02 positive CRC patients, n=9; (b) HLA-A*24:02 negative CRC patients, n=10; (c) HLA-A*24:02 positive healthy controls, n=5; (d) HLA-A*24:02 negative healthy controls, n=5 respectively who had variable positive immune responses to the corresponding PASD1 peptides (P1=PASD1(1), P2=PASD1(2), P3=PASD1(3) and P4=PASD1(4). Well with phytohaemagglutinin (PHA) was used as positive control and well with HIV peptide was used the negative control. P-value: *≤ 0.05, **≤ 0.01, ***≤ 0.001 and **** ≤ 0.0001 were statistically significant analyzed using one way-ANOVA.

4.3 CD8 T CELL RESPONSES TO PASD1

4.3.1 *In vitro* Generation of Antigen-specific Cytotoxic T Cells

In vitro generation of human cytotoxic T lymphocytes (CTL) was performed to assess intrinsic CD8 T cell responses in HLA-A*24:02 positive CRC patients (n=3). The expansion of PASD1 specific CTLs required the nurturing of naive T cells with antigen presenting cells (APCs) as stimulator cells that express PASD1(4) peptide as antigens (Figure 4.7). Peripheral blood mononuclear cells (PBMCs) from CRC patients were expanded in cultures and CTLs were then isolated for further assessment. Phenotypic characterization of expanded CTLs (expressing CD8 and CD3 surface markers) were analyzed weekly using flow cytometry with CRC 1 (5.25X fold), CRC 2 (5.81X fold) and CRC 3 (5.33X fold) (Figure 4.8). Generated PASD1 specific CTLs from the cultures was then isolated using immunomagnetic beads with purity more than 90% from all three cultures respectively prior to cytotoxic functional assays (Figure 4.9).

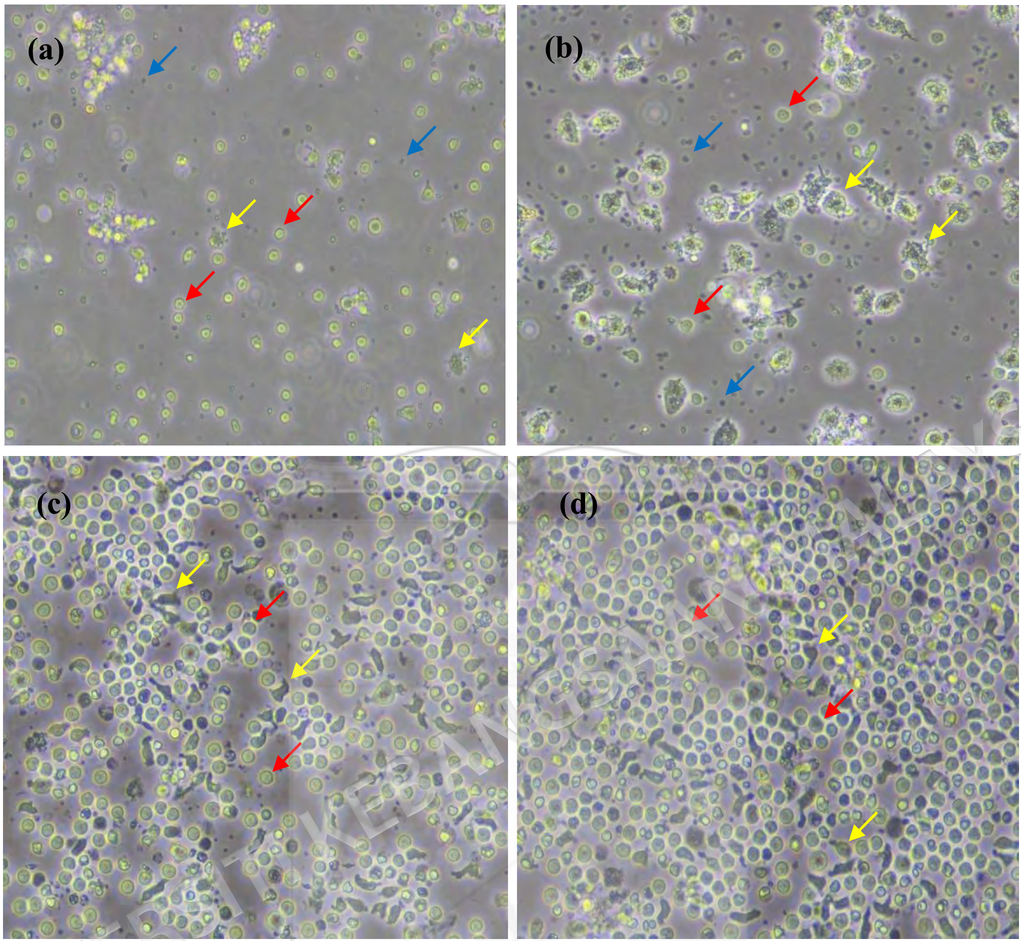

Figure 4.7 Representative microscopy images of *in vitro* expansion of PASD1 specific cytotoxic T lymphocytes (CTL) of CRC sample in (a) Week 1; (b) Week 2; (c) Week 3 and (d) Week 4. CTL with antigen presenting cells (APCs) loaded with P4 peptide over 4 weeks. *Red arrows* pointed at T lymphocytes, *yellow arrows* pointed at APCs and *blue arrows* pointed at dying platelets. The average of generated T lymphocytes is approximately 8 µm in diameter. All images were taken under an inverted microscope at 400× magnification.

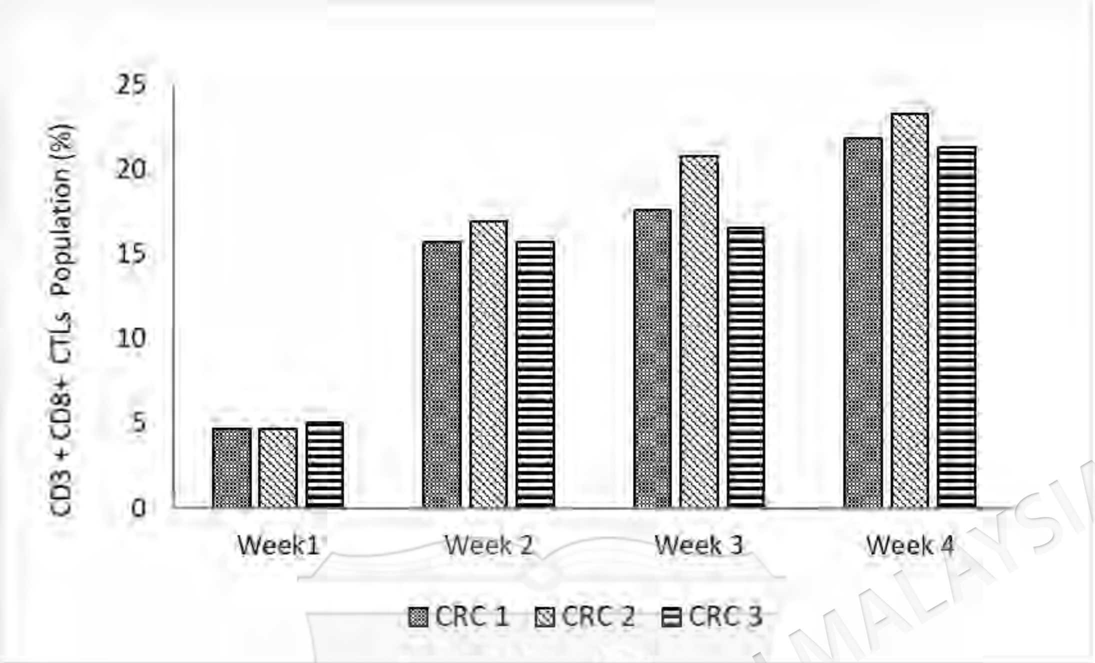

Figure 4.8 Immunophenotypic CTL characterization (CD3 and CD8 cell surface markers) from each of the HLA-A*24:02 positive CRC samples (n=3) by flow cytometry. The data presented in percentage of the expansion.

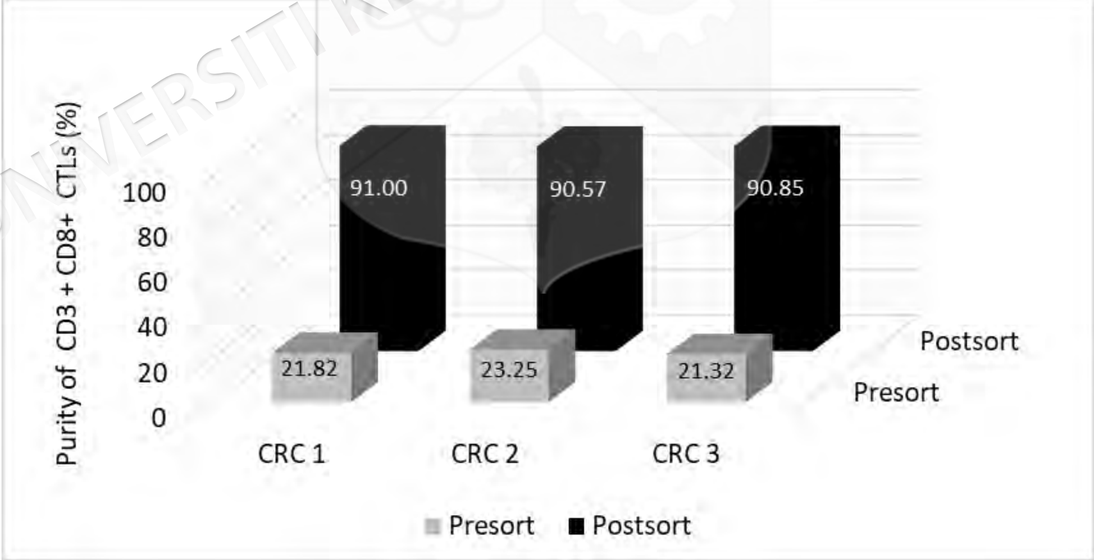

Figure 4.9 The purity of presort and postsort cultures of CTL from HLA-A*24:02 CRC samples (n=3) analyzed using flow cytometry before and after the isolation. The data presented in percentage of isolation T cells purity.

4.3.2 Cytolytic Activity

Generated PASD1(4) specific CTLs derived from HLA-A*24:02 positive CRC patients were used as the effector cells while four different cell lines with PASD1 and HLA-A*24:02 expressions were used as the target cells. The ability of PASD1(4) specific CTLs in recognizing the endogenous expression of PASD1 was studied in four different cell lines which were SW480 (PASD1+, HLA-A24+), H1299 (PASD1+, HLA-A24–), COLO320DM (PASD1–, HLA-A24+) and CCD112CoN (PASD1–, HLA-A24–).In all experiments, PASD1-specific CTLs effector cells were able to lyse only SW480 target cells expressing PASD1 and HLA-A*24:02 molecules. The co-cultured cells (50,000 cells per well) (E/T) in 3:1, 5:1 and 10:1 ratio were incubated for 6 hours for lysis to occur. All the CFSE-target cells were counterstained with propidium iodide (PI) dye and flow cytometry was performed immediately.

PASD1-specific CTLs effector cells significantly lysed SW480 at 10:1 (2.96±0.59%, P=0.0236) for CRC 1. Furthermore, for CRC 2, the specific lysis was at 10:1 (28.39±2.03%, P=0.018), 5:1 (16.05±0.75%, P=0.0123) and 3:1 (4.31±0.02%, P=0.0141). Followed by CRC 3, the lysis was at 10:1 (9.00±0.87%, P=0.0036) and 5:1 (3.35±0.33%, P=0.0103) respectively. Significant cytolytic activities of PASD1-specific CTLs against SW480 raised from CRC 2 showed to be more potent, followed by CRC 3 and CRC 1 (Figure 4.10). Flow cytometric results were recorded as the mean values ± SD from duplicate wells. Wells without effector cells were used as the negative controls. Statistical differences of results between studied groups were assessed using one-way ANOVA. P-values *≤ 0.05 and **≤ 0.01 were considered as significant.

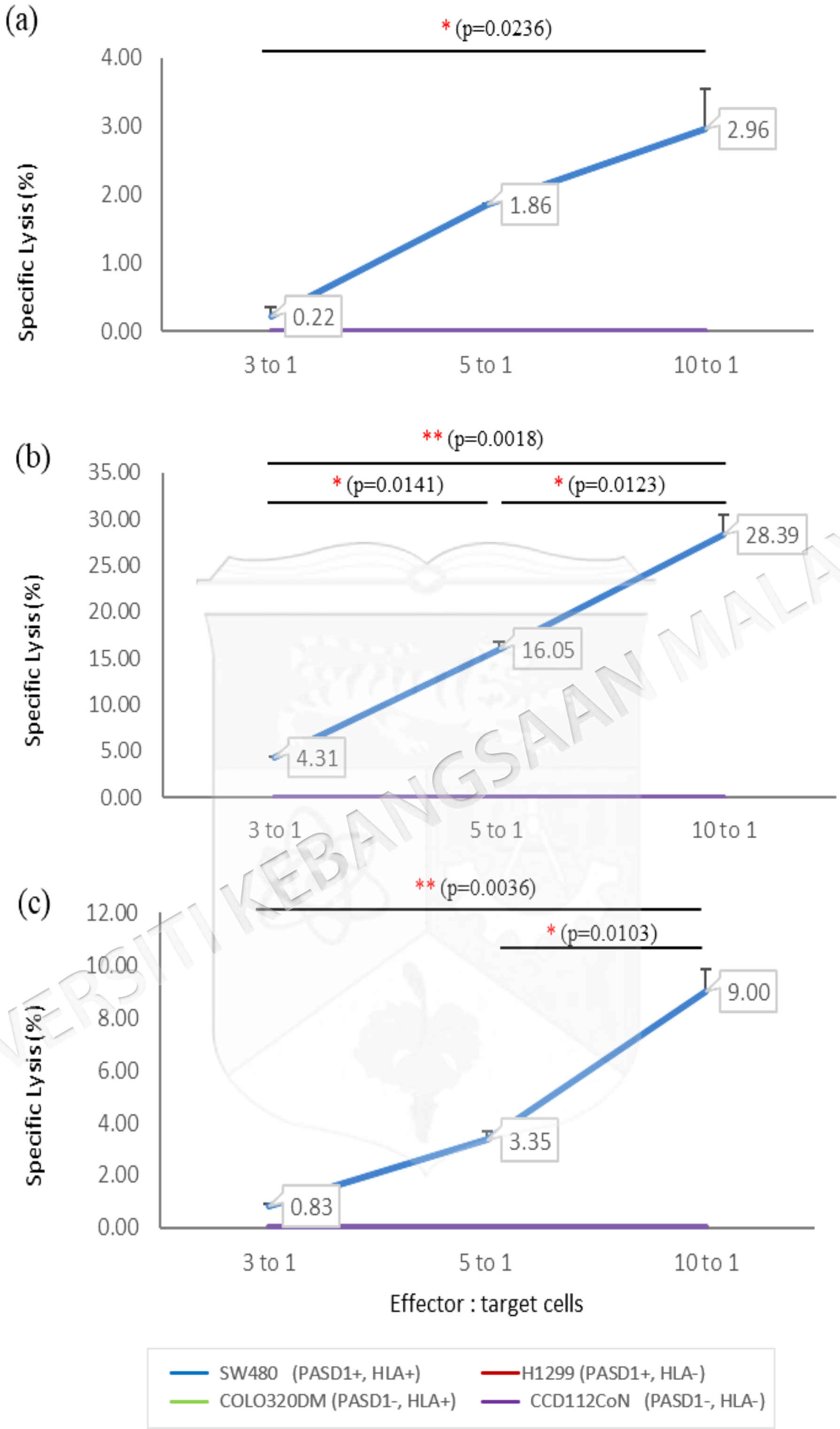

Figure 4.10 Cytolytic activity of PASD1-CTLs in three HLA-A*24:02 positive CRC samples: (a) CRC sample 1, (b) CRC sample 2 and (c) CRC sample 3 against the target cells.

4.3.3 Granzyme B Enzymatic Activity

Besides IFN-γ, we also measured the presence of granzyme B upon co-culture of the effector-target cells. Granzyme B activities of PASD1-specific CTLs against SW480 cells were diverse across all three CRC patients. The granzyme B spot counts were detected to be significantly higher at 10:1 ratio in all three samples. For CRC 1, the spot counts for 10:1 ratio were 127.5±25.5 (P=0.0189). Meanwhile for CRC 2, the spot counts at 10:1 ratio were 586.0±42.0 (P=0.0095), 308.5±14.5 (P=0.0208) at 5:1, and 98.5±0.5 (P=0.0019) at 3:1. For CRC 3, the spot counts at 10:1 and 5:1 were 374.0±36.0 (P=0.0078) and 120.0±12.0 (P=0.0031) respectively. Generally, the production of granzyme B in CRC sample 2 showed the highest activity of granzyme B compared to CRC 1 and CRC 3 (Figure 4.11). ELISpot results were recorded as the mean ± SD from duplicate wells. Wells without effector cells were used as the negative controls. Statistical differences of results between studied groups were assessed using one-way ANOVA. P-value $* \leq 0.05$ and $** \leq 0.01$ were considered as significant.

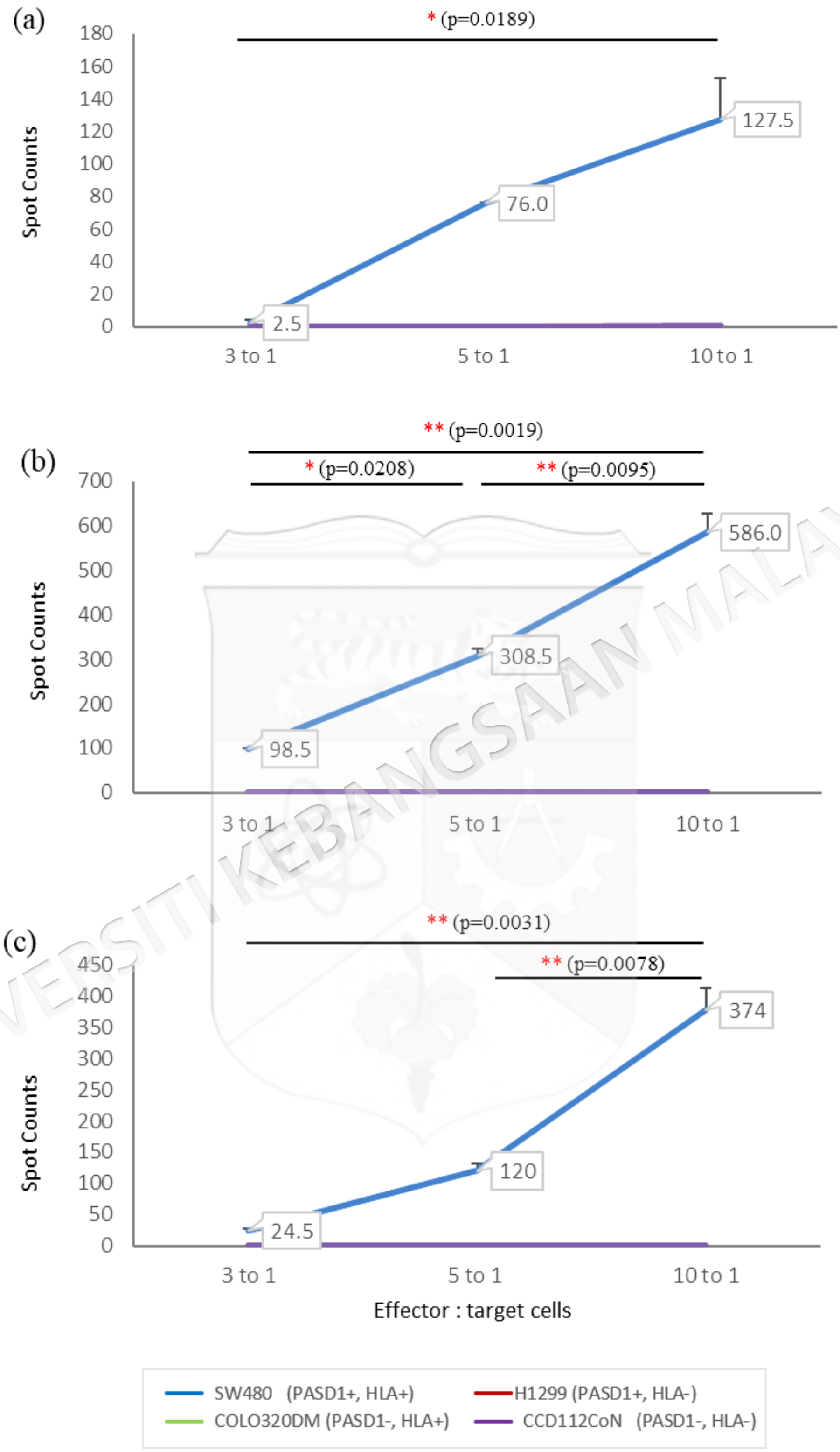

Figure 4.11 Granzyme B enzymatic activities released from PASD1-CTLs in three HLA-A*24:02 positive CRC samples (a) CRC sample 1, (b) CRC sample 2 and (c) CRC sample 3 against the target cells.

4.4 CYTOKINE PROFILES OF CO-CULTURE BETWEEN EFFECTOR AND TARGET CELLS

We also determined the expression of cytokines in the co-culture analysis of generated CD8 T cells upon induction with PASD1 peptide. Supernatant from the co-culture of PASD1-specific CTLs against respective cell lines was collected and assessed. Differences in expression patterns of the individual cytokines (IL-10, TNF-α, sFas, sFasL, IFN-γ, granzyme A, granzyme B, and perforin) from the CRC sample 1 (Figure 4.12), CRC sample 2 (Figure 4.13) and CRC sample 3 (Figure 4.14) were observed. The results showed that the expression profiles of the cytokines, namely interleukin-10 (IL-10), interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), soluble surface Fas protein (sFas), soluble surface Fas ligand (sFasL), granzyme A, granzyme B and perforin in CRC upon stimulation with PASD1(4) peptide were elevated gradually from 3:1 to 5:1 to 10:1 against SW480 (PASD1+, HLA-A*24:02+) cell line (Figure 4.15). Nevertheless, it was not significant between all three ratios. While for the other cell lines, fluctuated expression patterns were observed distinctly at all ratios. Cytokine results were reported as the mean values ± SD from duplicate wells. Statistical differences of results between studied groups were assessed using one-way ANOVA.

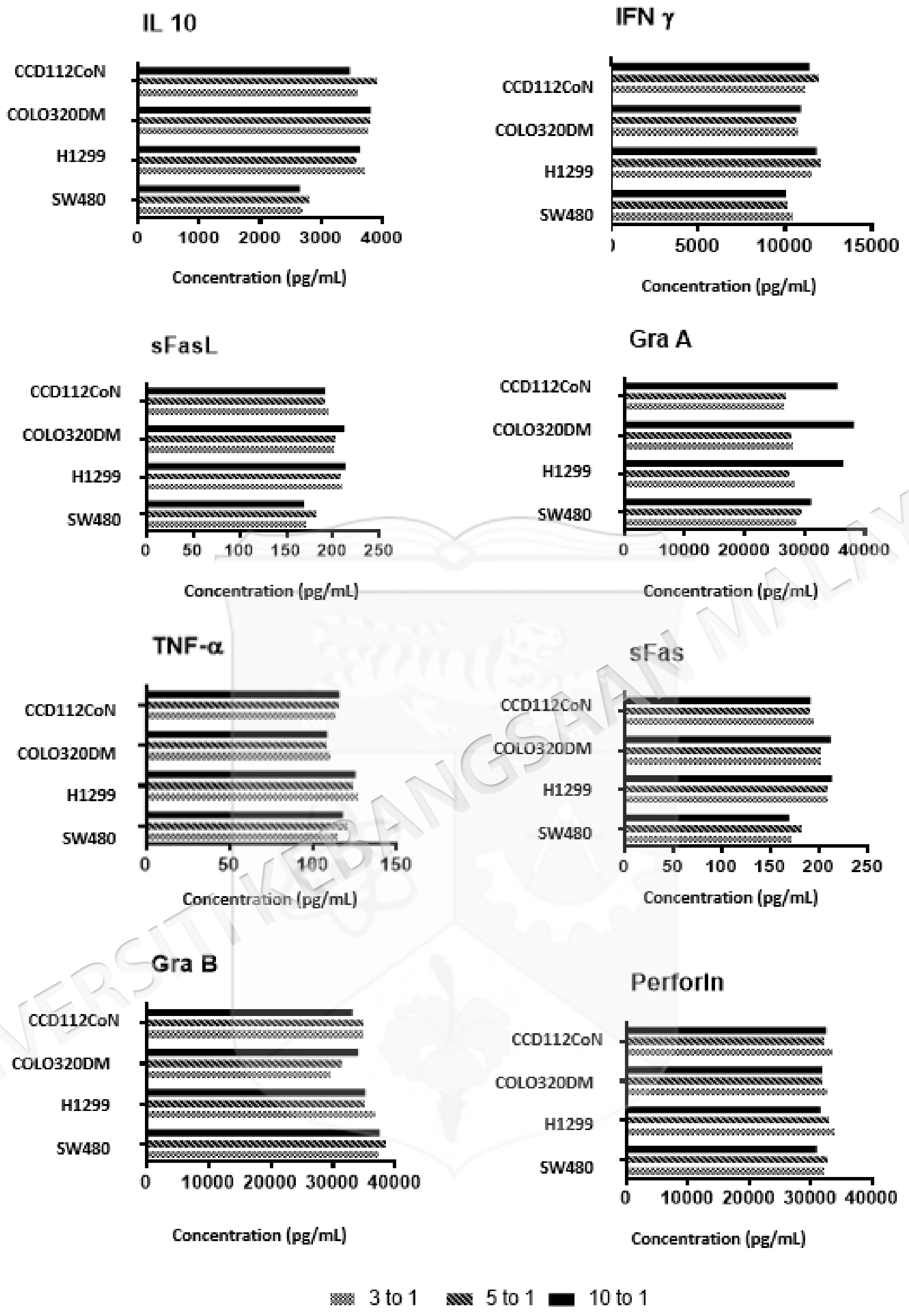

Figure 4.12 The individual cytokine profiles of CRC sample 1 upon PASD1 (4) peptide stimulation in the respective cell lines: CCD112CoN (PASD1–, HLA-A24-), COLO320DM (PASD1–, HLA-A24+), H1299 (PASD1+, HLA-A24–) and SW480 (PASD1+, HLA-A24+) in 3:1, 5:1 and 10:1 ratio.

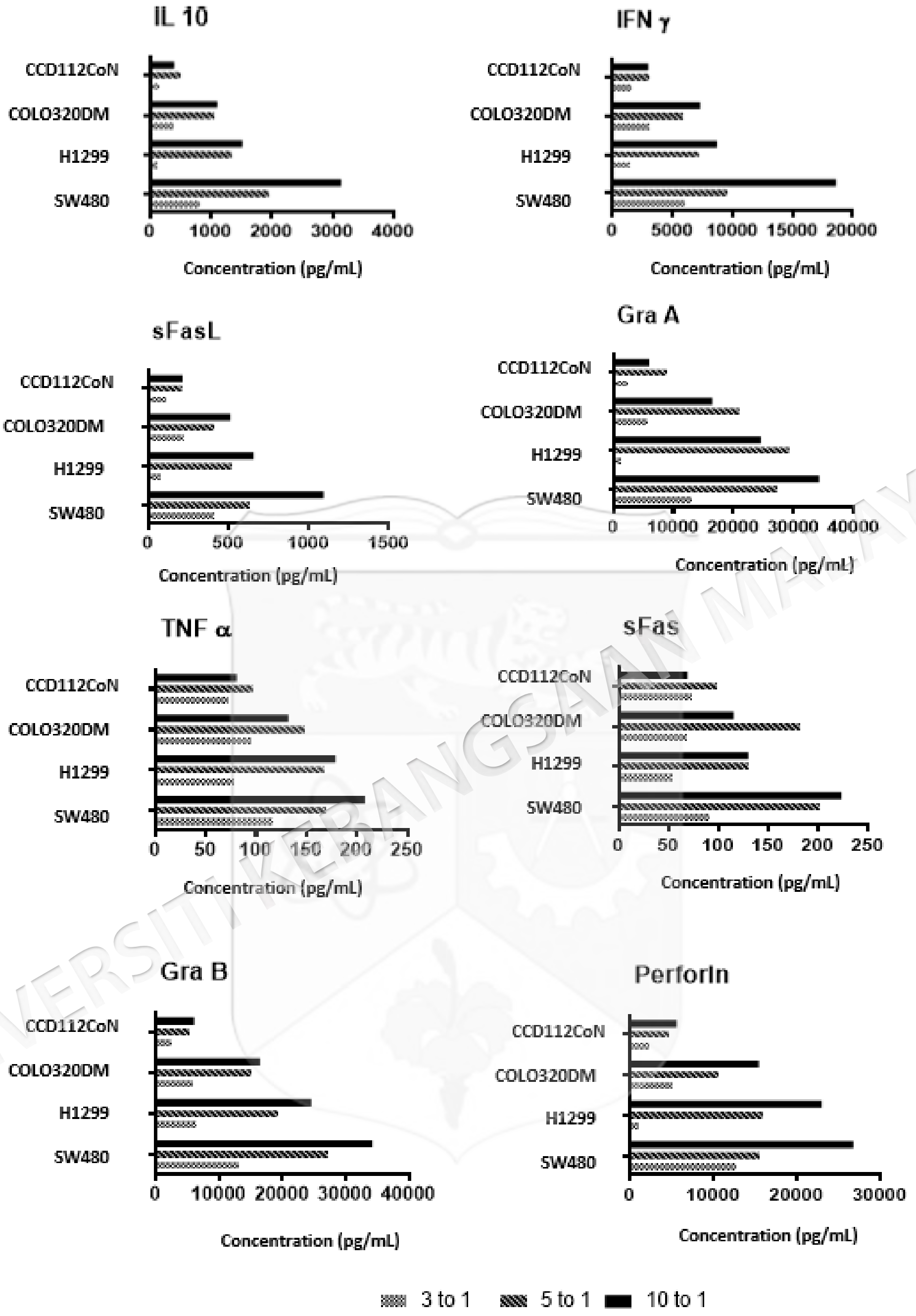

Figure 4.13 The individual cytokine profiles of CRC sample 2 upon PASD1 (4) peptide stimulation in the respective cell lines: CCD112CoN (PASD1–, HLA-A24-), COLO320DM (PASD1–, HLA-A24+), H1299 (PASD1+, HLA-A24–) and SW480 (PASD1+, HLA-A24+) in 3:1, 5:1 and 10:1 ratio.

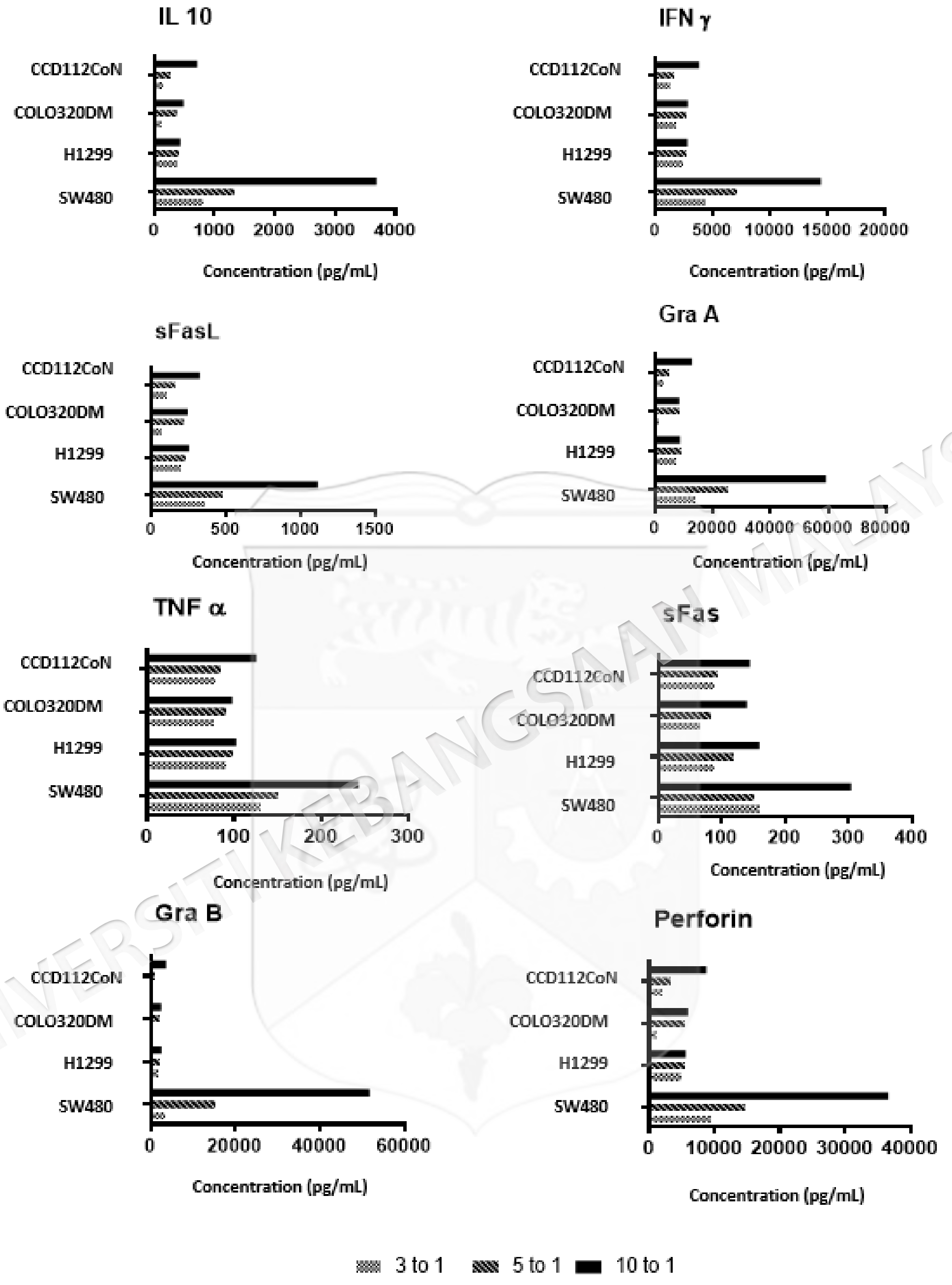

Figure 4.14 The individual cytokine profiles of CRC sample 3 upon PASD1 (4) peptide stimulation in the respective cell lines: CCD112CoN (PASD1–, HLA-A24-), COLO320DM (PASD1–, HLA-A24+), H1299 (PASD1+, HLA-A24–) and SW480 (PASD1+, HLA-A24+) in 3:1, 5:1 and 10:1 (effector to target cells) ratio.

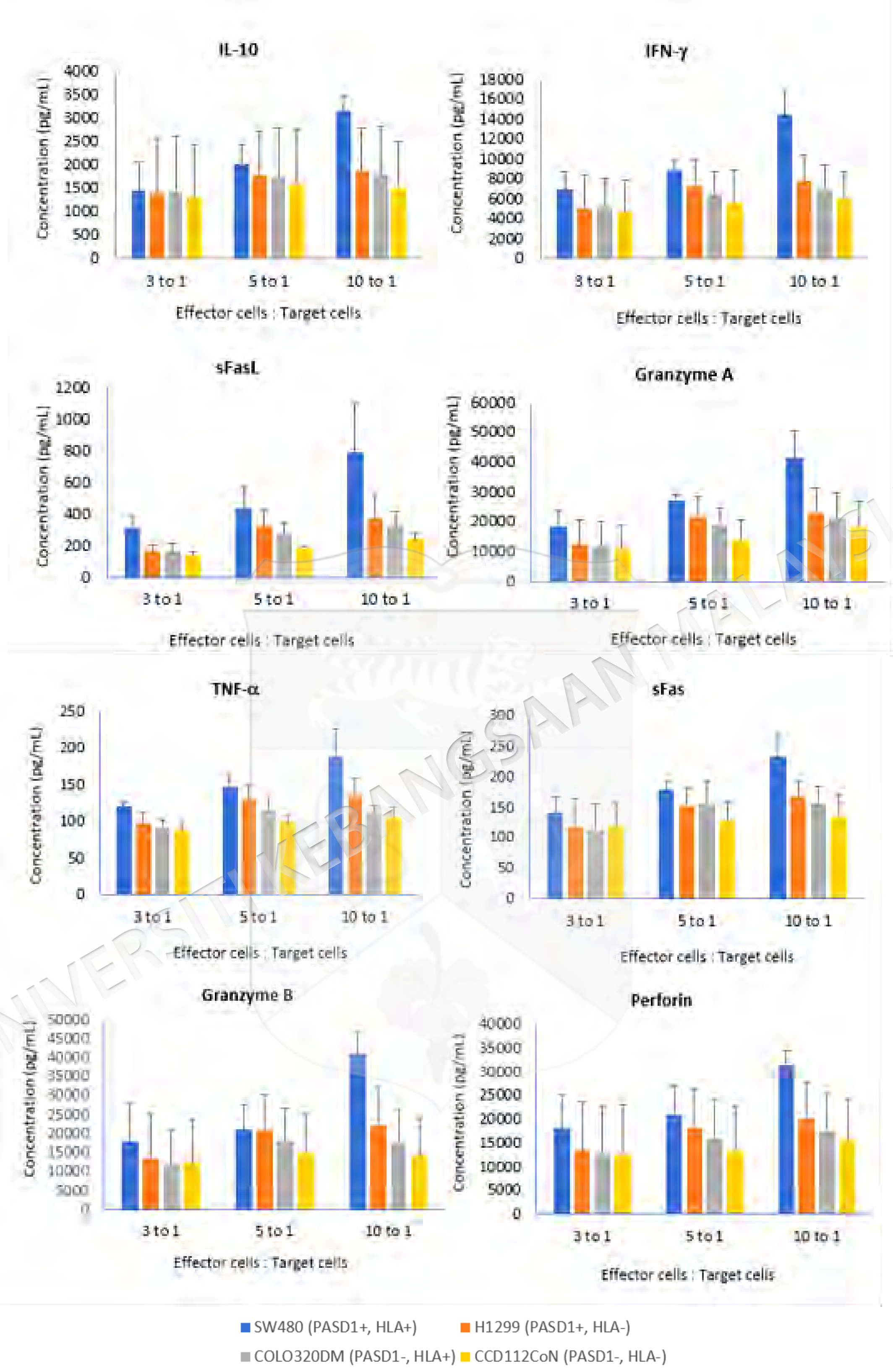

Figure 4.15 The average cytokine profiles of all the CRC patients (n=3) from the co-culture of PASD1 (4) peptide in the respective cell lines: SW480, represented in *blue* (PASD1+, HLA-A24+), H1299, represented in *orange* (PASD1+, HLA-A24–), COLO320DM, represented in *grey* (PASD1–, HLA-A24+) and CCD112CoN, represented in *yellow* (PASD1–, HLA-A24-) in 3:1, 5:1 and 10:1 ratio.

CHAPTER V

DISCUSSION

PASD1 is an X-linked gene located at q28 on chromosome X. Its expression is restricted primarily to normal cells derived from germ cells, but it is aberrantly expressed in various types of cancer (Khan et al. 2013; Zendman et al. 2003). Studies on PASD1 have suggested that it functions as a critical transcriptional regulator involved in metabolic and developmental processes (Chapman-Smith et al. 2004; Vreede et al. 2003). However, the exact role of PASD1 in human tumorigenesis at large remains unknown. The expression of PASD1 in CRC is generally uncertain, therefore we aimed to study PASD1's pattern of expression in various CRC cell lines and primary CRC tissues. Analysis of PASD1 expression using RT-PCR, immunohistochemistry, and immunofluorescence showed that 20.0% of our samples expressed the PASD1 gene and 17.4% of our samples expressed the PASD1 protein. Our results showed that the overall expression of PASD1 was found to be weak to moderate at the cytoplasmic region of CRC cells. Correspondingly, the infrequent expression of PASD1 in this study is in agreement with the expression profiles of other well recognized CTA such as MAGE-A3 and NY-ESO-1 in CRC previously (Li et al. 2017; Zhan et al. 2016).

As reported in a different study, the expression of CTA in CRC ranges between 5% to 40% (Li et al. 2005). The percentage mRNA expression of 10 selected CTA reported in the study were *MAGE-A3* (27.3%), *MAGE-A4* (22.3%), *LAGE-1* (15.7%), *MAGE-A1* (11.6%), *NY-ESO-1* (9.9%), *CT10* (6.6%), *SSX-1* (5.0%), *SSX-4* (2.5%), *SSX-2* (2.5%) and *SCP-1* (1.7%) (Li et al. 2005). However, the study did not detect the level of protein expression of the 10 selected CTAs. This is probably because the gene expression of the CTAs were very low. To address this gap of knowledge, an independent IHC analysis evaluating the protein expression of two promising CTAs in CRC was performed. As expected, the results of the study indicated that MAGE-A3 (28.0%) and CTAG2 (17.0%) were moderately expressed in 82 cases of CRC patients (Kumara et al. 2012). Similarly, in another study, it was reported that different CTAs have different frequencies of mRNA expression in CRC such as *MAGE-A10* (2%), *MAGE-A4* (15%), *MAGE-A3* (13%), *NY-ESO-1* (4%) and *SSX2* (4%) while low level of protein expression of MAGE-A3 (8%), MAGE-A4 (14%) and NY-ESO-1 (10%) respectively (Alves et al. 2007). Nonetheless, the previous investigation of PASD1 in AML reported 20% of the PASD1 gene was expressed in the 12 of the AML patients but not in the controls (Baghdady et al. 2013). The study of PASD1 in acute myeloid leukaemia (AML) also found no significant relation between PASD1 expression to any of the clinical parameters (Baghdady et al. 2013). In contrast in ovarian cancer, it was reported that no expression of PASD1 was found in both of the ovarian cancer cell lines and endometrium tumour samples (Khan et al. 2015). As discussed, the expression of other CTAs including PASD1 was relatively modest in CRC. Therefore, our results were in concordance with other CTA-related studies in CRC.

An expression study in CRC had also shown that the overall expression of CTA genes was less than 20% in CRC (Scanlan et al. 2004). They also mentioned that there is a low transcript copy number of CTA genes in CRC (Scanlan et al. 2004). Epigenetic events such as DNA methylation and histone acetylation have been reported to regulate the expression of CTAs (De Smet et al. 1995; Weiser et al. 2001). More importantly, the global methylation of DNA in CRC has also been reported to have contributed to the low gene expression in CRC (Goelz et al. 1985). Henceforth, the expression of PASD1 in cancer may be further investigated by studying the modulation of epigenetic agents such as DNA-methyl-transferase I inhibitors such as 5’-aza-2’-deoxycytidine (5CD) or histone deacetylase (HDAC) inhibitors which may play a role in upregulating its expression (De Smet et al. 1999; Wischnewski et al. 2006). Furthermore, the evidence from studies on *MAGE-A1* and *MAGE-D4* has demonstrated that DNA demethylation of CpG dinucleotides at the 5’ end of the *MAGE-A1* genes provided access to the activators and consequently led to the induction of promoter activity (De Smet et al. 1995; Zhang et al. 2014). It is also suggested that histone acetylation is also a determining factor in re-activating *MAGE-A* gene regulation in cancer (Sang et al. 2011). Moreover, it was recently reported, that by inducing colon and ovarian cancer cells with DNA methyltransferase inhibitors (DNMTs) for demethylation, the level of expression of several CTAs were upregulated (Siebenkäs et al. 2017).

Besides epigenetic events, the differential expression of PASD1 in CRC could be explained by the presence of other possible PASD1 isoforms and the post-translational modification of genes expressing PASD1 (Ait-Tahar et al. 2009; Nakagawa et al. 2005). Another reason could be the clonal evolution of aggressive cancer cells in CRC that no longer express PASD1 that could be detected by RT-PCR and IHC (Caballero & Chen 2009). Moreover, the PASD1 expression was believed to be inhibited through immunoediting as cancer progressed (Dunn et al. 2004). Therefore, with the heterogeneous nature of PASD1, the antibodies used in this study may not be able to cross-react with specific PASD1 epitopes. The affinity of different antibodies binding to different isoforms of PASD1 may not provide a comprehensive picture of the expression in CRC tissues. Other suggestive screening tools such as using tissue microarray, RNA *in situ* hybridization and western blot can be coupled to further characterize and verify PASD1 expression in CRC more extensively (Sang et al. 2011).

There is a considerable number of studies on the use of peptide-based vaccines in cancer immunotherapy (Banchereau & Palucka 2018; Melief 2018). In vaccines development, the specificity of HLA ligands and the binding capability to antigens are among the crucial criteria in determining a candidate peptide in cancer immunotherapy (Ott et al. 2017; Sahin et al. 2017). Traditionally, a number of bioinformatics prediction algorithms for the identification of prospective peptides that can bind to major histocompatibility complex (MHC) molecules have been used for most epitope discovery (Ait-Tahar et al. 2009; Pan et al. 2017; Qu et al. 2018; Zhang et al. 2018). It is also important to assess the abundance of the specific HLA allele type within a study population in designing a peptide of interest due to the polymorphic nature of HLA genes in the human genome (Parham et al. 1988). Thousands of different composition of HLA alleles at several loci are known to encode different variants of MHC class 1 molecule with variety peptide binding specificities which differ individually (Lund et al. 2013). Thus, without proper evaluation of the HLA allele type, it will lead to difficulty in developing a more generalized peptide target to cater to a larger population of subjects in the epitope-specific immunotherapy approach. In fact, the HLA binding of antigen-derived epitopes is where the immunotherapy instigates the specific immune response (Provenzano et al. 2006).

As such, our current study found that HLA-A*24:02 is the most common expressed HLA allele with an expression rate of around 28.9%-35.3% in the Malaysian population as reported on Allele Frequency Network Database (Middleton et al. 2003). It has also been demonstrated by Lin et al. that HLA-A*24:02 has a moderate classification accuracy using publicly available immune-informatics tools compared to other allelic types (Lin et al. 2008). Furthermore, Nagorsen and Thiel reported that HLA-A*24:02 and HLA-A*02:01 are most prevalently used for the peptide-based vaccine in cancers including CRC in Asian countries (Nagorsen & Thiel 2008). BIMAS (matrix-based) algorithm predicts the HLA-peptide binding interaction as the half-time dissociation (Lin et al. 2008) while SYFPEITHI (SVM-based) is a database that evaluates the possibility of epitope predictions for peptide motifs to the publicly available MHC allelic products (Rammensee et al. 1999). Nonetheless, Pearson correlation coefficient analysis indicated that BIMAS and SYFPEITHI presented equal predictions (r =1), stating that the chosen prediction algorithms have a close fit (Lin et al. 2008). The computational prediction applications rank peptides with respective prediction scores to guide the experimental investigation in a direction that increases the likelihood of discovering immunologically-relevant peptides.

These findings further support our analysis on the design of four different sequences of PASD1 HLA-A*24:02 9-mers in screening for an immunogenic target for downstream CD8 T cell responses in CRC (Lund et al. 2013). Therefore, four potential MHC class 1 restricted CTL PASD1 peptides recognizing HLA-A*24:02 allele were designed and characterized by two highly reliable *in silico*-based epitopes method using the BIMAS and SYFPEITHI programs (Guinn et al. 2007; Lund et al.

2013). Our results showed that the prediction scores for PASD1(1)$_{688\text{-}696}$ was BIMAS: 475.2, SYFPEITHI: 22; PASD1(2)$_{366\text{-}374}$ was BIMAS: 369.6, SYFPEITHI: 21; PASD1(3)$_{89\text{-}98}$ was BIMAS: 231.0, SYFPEITHI: 27 and PASD1(4)$_{265\text{-}273}$ was BIMAS: 200.0, SYFPEITHI: 21 respectively. However, the bioinformatics prediction value does not guarantee the bioactivity, thus further experimental validation is needed.

The designed PASD1 CTL peptides were further validated using Enzyme-Linked ImmunoSpot (ELISpot) assay (Lund et al. 2013). The relevance of wet-lab experiment validation is important as shown in a study reported previously (Lin et al. 2008). For instance, it was reported that the CTL peptides (with the amino acids sequence of AINPELLQL and FFEIYNGKL) derived from the mitotic centromere associated kinesin (MCAK) CTA revealed higher cytotoxicity activities against the colon cancer cell line DLD-1 and the chronic myelocytic leukaemia cell line K562 although these peptides were predicted with lower BIMAS and SYFPEITHI scores than the other peptides (Kawamoto et al. 2011). This shows that although the BIMAS and SYFPEITHI scores may offer guidance on the selection of peptides, biological validation is still needed to verify the immunogenicity.

Of these four HLA-A*24:02 restricted PASD1 CTL peptides, significant IFN-γ response was detected with the PASD1(4) peptide as compared to the other groups. This indicates that the epitope sequence of PASD1(4) peptide was specific to HLA-A*24:02 allele and could elicit the IFN-γ response in peripheral blood mononuclear cells of CRC samples that are HLA-A*24:02 positive. To further understand the immunogenicity, the T cell responses in three HLA-A*24:02 positive CRC samples upon PASD1(4) stimulation were further evaluated. From Figure 4.10, the results showed that the PASD1 specific CTLs in the CRC samples were able to lyse PASD1 positive SW480 cell line distinctly in a dose-dependent manner. However, individually, the CD8 T cell responses of CRC sample 1 was comparatively lower than CRC sample 2 and 3. A similar study also demonstrated that the CD8^{+} T cell responses in diffuse large B-cell lymphoma (DLBCL) samples were also patient-dependent (Ait-Tahar et al. 2009). It was reported that the functional activity of the CTL lines obtained from the studied patients pulsed with the PASD1 peptide against a range of haematological cell lines were distinct depending on the individual it came from. Hence, this could possibly explain why there were differences in the responses between all the CRC samples. Moreover, this observation could also explain that certain individuals have a higher tolerance and certain individuals have a lower tolerance to the PASD1 peptides (Ait-Tahar et al. 2009).

Furthermore, this study also demonstrated that the PASD1-specific CTLs were able to lyse the PASD1+ HLA-A*2402+ (SW480) cell line and not the other three cell lines (PASD1+ HLA−, PASD1−HLA+ and PASD1−HLA−). This finding was in concordance to a similar study conducted by Ait-Tahar et al. (2009), where the CTL lines generated against PASD1 peptides were only able to lyse the PASD1+ HLA+ cell line, Thiel. They also showed that the CTLs were not able to lyse the remaining negative cell lines. This shows that the generated CTLs were successfully educated to recognise PASD1 specifically. Apart from the cytolysis assay, the granzyme B ELISpot assay was also conducted to complement the cytolysis results. Previous studies have shown that there is a correlation between CD8 CTL cytolysis and the release of granzyme B (Yang et al. 2019). It was reported that there was a significant correlation between the level of granzyme B enzymatic activities in the cell lysates to the level of T cell killing of the infected cells (Yang et al. 2019). The killing of target cells by CD8$^+$ T cells was achieved by the release of the contents of secretory lysosomes such as granzyme B, at the immunological synapse formed upon recognition of MHC class I-bound antigenic peptides by the T cell receptors (Hlongwane et al. 2018; Yang et al. 2019).

In this study, the specific lysis and granzyme B spot counts in CRC sample 1 against SW480 were lower as compared to the other two CRC samples. An association was observed between the cytolytic and the granzyme B ELISpot results at 10 to 1 ratio for CRC sample 1. Nevertheless, for CRC sample 2 and 3, there was a positive correlation between the specific lysis (Figure 4.10) and the granzyme B ELIspot enzymatic activities (Figure 4.11) respectively. The specific lysis in CRC sample 2 and 3 was coherent to their respective granzyme B productions. Hence, this study showed that granzyme B could be a dominant effector molecule in CD8$^+$ T cell-mediating killing (Yang et al. 2019). Furthermore, this study also performed granzyme B profiling using the supernatant of the conditioned media after the coculture experiment. It is worth noting that, the granzyme B enzymatic activities in the ELISpot did not correlate to the granzyme B profile in the multiplex cytokine profiling of CRC sample 1. In contrast to CRC sample 1, this current study also observed a positive correlation in the granzyme B detection in CRC sample 2 between the granzyme B ELISpot assay (Figure 4.11) and the cytokine profile of granzyme B in SW480 cell line (Figure 4.13). Moreover, the dose-dependent increase of granzyme B in SW480 cell in CRC sample 2 was also observed. Similar findings were also seen in CRC sample 3 (Figure 4.14). Based on a previous study on the cytotoxic effects of another CTA, MAGE-A3, they reported that the CD8$^+$ T cells recognising MAGE-A3 could release cytotoxic molecules such as granzyme B after restimulation with natural MAGE-A3 peptide in the MAGE-A3+ HLA-A2+ human hepatocellular carcinoma patient samples (Zhang et al. 2007). A sufficient number of granzyme B releasing effector CD8$^+$ T cells were reported to exhibit killing capacity to the MAGE-A3 peptide-pulsed with TAP-deficient T2 cell line (Zhang et al. 2007). Similarly, another study has shown the increase in the cytotoxic potential (granzyme B production) and also cytokine production (IFN-γ) of T cells in response to NY-ESO-1 peptide-loaded targets (Rapoport et al. 2015).

The CD8-associated cytokine/immune-related molecule profiles other than granzyme B such as interleukin 10 (IL-10), interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), granzyme A and perforin were also studied. From our study, we observed the gradual elevation of cytokines from 3:1 to 5:1 and 10:1 (effector: target cells) even though there was no significance observed (Figure 4.15). This could be due to the variation between human biological samples. The biological variables, however, can be overcome by increasing the number of study samples or further validation using other analytic approach (Fichorova et al. 2008). Moreover, based on the lysis assay, CRC sample 1 showed the lowest lysis percentage as compared to CRC sample 2 and 3. The cytokine profile of CRC sample 1 was also fluctuating and inconsistent, thus could contribute to the no significant differences. As in Figure 4.13 and Figure 4.14, the dose-dependent increase of granzyme A was also found to be similar in the SW480 cell lines of CRC sample 2 and 3. In contrast to the SW480 cell line, the granzyme A of CRC sample 2 and 3 were found to fluctuate between CCD112, COLO320DM and H1299 cell lines. Traditionally, it has been reported that granzyme A disintegrates the mitochondrial membrane of the cell and driving the caspase-independent cell death (Martinvalet et al. 2008; Martinvalet et al. 2005; Zhu et al. 2006). However, a recent paper published has reported that granzyme A able to mediate toll-like receptor (TLR) signalling and the release of granzyme A was able to synergistically increase the secretion of other proinflammatory cytokines such as TNF-α, IL-6, and IL-8 from human monocytes (Van Eck et al. 2017).

Similarly, in this study, there was also a dose-dependent increase of TNF-α in the CRC samples. Tumour necrosis factor-alpha (TNF-α) is known to be an important determinant in the regulation of anti-cancer immunity in the context of CRC (Balkwill 2009). In this study, a positive trend of increase in the TNF-α secretion against the two PASD1+ (SW480 and H1299) cell lines in CRC sample 2 was observed. In comparison to CRC sample 2, CRC sample 3 was found to have an increase in the TNF-α secretion in the SW480 cell line. There was no significant difference between the TNF-α level detected in CRC sample 1. The elevated TNF-α in CRC sample 2 and 3 against PASD1+ HLA:A*2402+ cell line is in agreement with the study conducted by Chiriva Internati et al. (2012) where there was a significant increase in the optical density (OD) ratio in four out of the six prostate cancer patients upon stimulation with AKAP-4 peptides (Chiriva Internati et al. 2012). The ELISA results revealed that TNF-α to be the highest cytokine secreted, followed by IFN-γ, IL-4, IL-5 and IL-10 (Chiriva Internati et al. 2012).

Perforin, on the other hand, is a glycoprotein responsible for polymerizing and forming pores in the membrane of a target cell as part of the immune function (Osińska et al. 2014). In general, a correlation increase in the perforin was found in CRC sample 2 and 3 against PASD1+ HLA: A*2402+ cell line but distinctly in the other three cell lines. In contrast to the perforin expression in CRC sample 1, there was no significant difference found between the four cell lines. Evidently, a study conducted using cancer-testis antigen MAGE-C2/CT10 has reported to produce perforin as a result of the spontaneous $CD4^+$ and $CD8^+$ T cell responses in multiple myeloma patients (Reinhard et al. 2014). The study has also reported that the isolated $CD4^+$ T-cell clones from multiple myeloma patients were able to react with perforin secretion upon encountering with the MAGE-C2/CT10 antigen, suggesting the cytolytic potential of the T cell clones in response to perforin (Reinhard et al. 2014). Moreover, a similar study on AKAP-4, a novel cancer testis antigen in prostate cancer reported that there was no significant change in the IL-10 detected against all the cancerous prostate cells (Chiriva Internati et al. 2012). The absence of IL-10 expression in the prostate cancer samples was discussed previously to be the Th1/Th2 polarizing effects modulating the immune responses of the prostate cancer patients (Chiriva Internati et al. 2012). Interestingly, in this study, it was discovered that IL-10 was elevated in CRC sample 2 and 3. This shows that the release of cytokines could be dependent on the type of cancer and CTAs being studied.

Furthermore, the elevated IFN-γ expression found in CRC sample 2 and 3 showed comparable findings to a study using AKAP-4 peptide against autologous prostate cancerous cells (Chiriva Internati et al. 2012). The IFN-γ responses of AKAP-4 was also reported to show high specificity in hampering cytotoxicity against the autologous prostate cells (Chiriva Internati et al. 2012). Besides, IFN-γ has been also known to increase the expression of MHC class 1 molecule on dendritic cells in the human CTL responses against prostate cancer (Pantuck et al. 2004). Likewise, another study reported on the increased IFN-γ responses in the patients bearing MAGE-A1/A3 positive tumours (Toungouz et al. 2001). This study demonstrated an expansion of peptide-specific lymphocytes that produced the IFN-γ in six out of the eight patients whose dendritic cells were pulsed with MAGE peptides (Toungouz et al. 2001). This observation indicates that the peptide-loaded dendritic cells were able to trigger a clear and specific IFN-γ secretion in the MHC class I-restricted manner in the recognising the immunogenic peptide antigen (Toungouz et al. 2001).

Aside from the granule-mediated exocytosis, the CD8$^+$ T cells are also capable of inducing calcium-independent Fas-Fas ligand system for an apoptotic response (Waring & Müllbacher 1999). The expression of Fas and FasL were upregulated across the ratio groups in the SW480 cell line of CRC sample 2 and 3. However, a minimal amount of Fas and FasL were observed in the other three cell lines. According to a comparative study on the assessment of Fas-Fas ligand has reported that CD8$^+$ T cell lines raised by *in vitro* stimulation with the self-peptide were was shown to kill self peptide-coated targets via Fas-Fas ligand-mediated pathway

(Brossart & Bevan 1996). In this study however, we did not thoroughly investigated the effects of Fas/FasL in regards to the cytolysis results.

Overall, this study has demonstrated that the cytolysis of CTLs against PASD1+ cells could result in the release of important cytokines and immune-related players. Nevertheless, further understanding of how these molecules interact with each other and affect the downstream activity of CTLs should be further studied. Up till now, the immune responses of PASD1 in solid tumours is still insufficiently studied. Hence, it would be interesting for future research to further elucidate the molecular pathway involved in the cytolytic pathway of CTLs upon PASD1 induction.

CHAPTER VI

CONCLUSION

6.1 SUMMARY

This is the first study on the expression of PASD1 in CRC primary adenocarcinomas and the CD8 T cell responses in the CRC samples with the HLA-A*24:02 allele. The PASD1 (4) peptide with the amino acids sequence of TYCSSTVFL at the position 276-273 identified to be relatively more immunogenic than the rest of the peptides. The PASD1 (4) peptide represents to be a potential vaccine candidate for peptide-based immunotherapeutic strategies in the treatment of HLA-A*24:02 specific CRC and is worthy of further studies. The overall study extends our knowledge into a better understanding of the interaction between the CD8 T cell responses of PASD1 and the tumour microenvironment in the advancement of cancer immunotherapy in treating HLA-A*24:02 specific CRC.

6.2 LIMITATIONS

Several limitations to this pilot study need to be acknowledged. Firstly, this thesis was unable to show any significance in the tested cytokines among these three CRC samples. A possible explanation for the insignificant differences in the cytokine profiles of CRC patients may be due to the individual genetic differences among these CRC samples. Besides, the sample size of CRC patients used in PASD1 expression and peptide screening was also small. Secondly, the correlation of PASD1 protein expression of CRC tissues to its corresponding IFN-γ production using the sera of CRC patients was not performed in our study. Therefore, we are unable to predict the presence of anti-PASD1 antibodies in the blood sera of CRC patients prior to the in vitro peptide pulsing. Of note, we were unable to determine in-depth the correlation expression profile of PASD1 to its humoral immune responses and memory T cell responses in CRC patients. Thirdly, the PASD1 peptide motif binding affinity to other HLA subtypes found in Malaysia population was not determined in our study. Hence, there has been a limitation in designing a multivalent peptide target of PASD1 for a wider group of CRC patients, not restricting to only HLA-A*24:02 positive CRC patients. We believe that these limitations may provide a basis for further research on PASD1-based cancer vaccine in the future.

6.3 FUTURE RECOMMENDATIONS

This thesis has indeed provided valuable insights for future research. It would be interesting to demonstrate the overexpressed of PASD1 for the enhancement of PASD1 related immune responses in CRC. It is also recommended that the functional assays such as *in vitro* cell proliferation, migration, and chemosensitivity assays and *in vivo* knockout mice models may be helpful in further evaluating the role of PASD1 in CRC. Additionally, the impact of PASD1 in other subsets of immune cells such as B cells, $CD4^+$ T cells, memory T cells, infiltrate T lymphocytes in the tumour milieu is equally important in assessing the value of PASD1 as the potential immunogenic peptide in CRC. A further investigation of the molecular framework of CTL-mediated cytolysis and the caspase activation pathways of PASD1 in the CRC microenvironment also remains to be elucidated. The roles of other pro-inflammatory and anti-inflammatory cytokines such as IL-2, IL-12, and TNF-β concerning PASD1 in CRC also need to be further demonstrated. As a summary, the characterization of PASD1 as the potential CTA in CRC will need to be further pursued comprehensively.

REFERENCES

Abu Hassan, M. R., Ismail, I., Mohd Suan, M. A., Ahmad, F., Wan Khazim, W. K., Othman, Z., Mat Said, R., Tan, W. L., Mohammed, S. R. N. S., Soelar, S. A. & Nik Mustapha, N. R. 2016. Incidence and mortality rates of colorectal cancer in malaysia. *Epidemiology and Health* 38(1): 1-5.

Ait-Tahar, K., Liggins, A. P., Collins, G. P., Campbell, A., Barnardo, M., Cabes, M., Lawrie, C. H., Moir, D., Hatton, C. & Banham, A. H. 2011. CD4-positive T-helper cell responses to the PASD1 protein in patients with diffuse large b-cell lymphoma. *Haematologica* 96(1): 78-86.

Ait-Tahar, K., Liggins, A. P., Collins, G. P., Campbell, A., Barnardo, M., Lawrie, C., Moir, D., Hatton, C., Banham, A. H. & Pulford, K. 2009. Cytolytic T cell response to the PASD1 cancer testis antigen in patients with diffuse large B cell lymphoma. *British Journal of Haematology* 146(4): 396-407.

Al Obeed, O. A., Alkhayal, K. A., Al Sheikh, A., Zubaidi, A. M., Vaali-Mohammed, M. A., Boushey, R., Mckerrow, J. H. & Abdulla, M. H. 2014. Increased expression of tumor necrosis factor-alpha is associated with advanced colorectal cancer stages. *World Journal of Gastroenterology* 20(48): 18390-18396.

Alves, P. M., Lévy, N., Bouzourene, H., Viatte, S., Bricard, G., Ayyoub, M., Vuilleumier, H., Givel, J. C. R., Halkic, N. & Speiser, D. E. 2007. Molecular and immunological evaluation of the expression of cancer/testis gene products in human colorectal cancer. *Cancer Immunology* 56(6): 839-847.

Andtbacka, R. H., Kaufman, H. L., Collichio, F., Amatruda, T., Senzer, N., Chesney, J., Delman, K. A., Spitler, L. E., Puzanov, I. & Agarwala, S. S. 2015. Talimogene laherparepvec improves durable response rate in patients with advanced melanoma. *Journal of Clinical Oncology* 33(25): 2780-2788.

Armaghany, T., Wilson, J. D., Chu, Q. & Mills, G. 2012. Genetic alterations in colorectal cancer. *Gastrointestinal Cancer Research* 5(1): 19-27.

Arnold, M., Sierra, M. S., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. 2017. Global patterns and trends in colorectal cancer incidence and mortality. *Gut* 66(4): 683-691.

Askling, J., Dickman, P. W., Ekbom, A., Karlén, P., Broström, O., Lapidus, A. & Löfberg, R. 2001. Family history as a risk factor for colorectal cancer in inflammatory bowel disease. *Gastroenterology* 120(6): 1356-1362.

Atkin, W. S., Edwards, R., Kralj-Hans, I., Wooldrage, K., Hart, A. R., Northover, J. M. A., Parkin, D. M., Wardle, J., Duffy, S. W. & Cuzick, J. 2010. Once only flexible sigmoidoscopy screening in prevention of colorectal cancer: A multicentre randomised controlled trial. *The Lancet* 375(9726): 1624-1633.

Augestad, K., Bakaki, P., Rose, J., Crawshaw, B., Lindsetmo, R., Dørum, L., Koroukian, S. & Delaney, C. 2015. Metastatic spread pattern after curative colorectal cancer surgery. A retrospective, longitudinal analysis. *Cancer Epidemiology* 39(5): 734-744.

Azeem, E., Gillani, S. W., Poh, V., Sulaiman, S. a. S. & Baig, M. R. 2016. Barriers to colorectal cancer screening in Asia: A systematic review. *Tropical Journal of Pharmaceutical Research* 15(7): 1543-1548.

Baghdady, I. M., Glal, A. Z., Shoeab, S. A., Ahmed, T. M., Essa, E. S., Ragheb, A. & El Hafez, M. A. 2013. PASD1 gene expression in acute myeloid leukemia patients. *Menoufia Medical Journal* 26(1): 1-6.

Balkwill, F. 2009. Tumour necrosis factor and cancer. *Nature Reviews Cancer* 9(5): 361-371.

Banchereau, J. & Palucka, K. 2018. Immunotherapy: Cancer vaccines on the move. *Nature Reviews Clinical Oncology* 15(1): 9-10.

Banchereau, J. & Steinman, R. M. 1998. Dendritic cells and the control of immunity. *Nature* 392(6673): 245-252.

Bangia, N., Lehner, P. J., Hughes, E. A., Surman, M. & Cresswell, P. 1999. The N terminal region of tapasin is required to stabilize the MHC class I loading complex. *European Journal of Immunology* 29(6): 1858-1870.

Bardhan, K. & Liu, K. 2013. Epigenetics and colorectal cancer pathogenesis. *Cancers* 5(2): 676-713.

Barry, M. & Bleackley, R. C. 2002. Cytotoxic T lymphocytes: All roads lead to death. *Nature Reviews Immunology* 2(6): 401-409.

Bartel, D. P. 2009. MicroRNAs: Target recognition and regulatory functions. *Cell* 136(2): 215-233.

Beijers, A. J., Mols, F., Van Den Hurk, C. J. & Vreugdenhil, A. 2016. Are chemotherapy-associated symptoms underestimated? A view beyond common toxicity criteria. *Acta Oncologica* 55(4): 516-518.

Belardelli, F., Ferrantini, M., Proietti, E. & Kirkwood, J. M. 2002. Interferon-alpha in tumor immunity and immunotherapy. *Cytokine & Growth Factor Reviews* 13(2): 119-134.

Bender, A., Sapp, M., Schuler, G., Steinman, R. M. & Bhardwaj, N. 1996. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *Journal of Immunological Methods* 196(2): 121-135.

Bertrand, N., Wu, J., Xu, X., Kamaly, N. & Farokhzad, O. C. 2014. Cancer nanotechnology: The impact of passive and active targeting in the era of modern cancer biology. *Advanced Drug Delivery Reviews* 66(1): 2-25.

Bilusic, M., Heery, C. R., Arlen, P. M., Rauckhorst, M., Apelian, D., Tsang, K. Y., Tucker, J. A., Jochems, C., Schlom, J. & Gulley, J. L. 2014. Phase I trial of a recombinant yeast-CEA vaccine (GI-6207) in adults with metastatic CEA-expressing carcinoma. *Cancer Immunology, Immunotherapy* 63(3): 225-234.

Bokemeyer, C., Bondarenko, I., Hartmann, J., De Braud, F., Schuch, G., Zubel, A., Celik, I., Schlichting, M. & Koralewski, P. 2011. Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: The OPUS study. *Annals of Oncology* 22(7): 1535-1546.

Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H. & Wallach, D. 1995. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *Journal of Biological Chemistry* 270(14): 7795-7798.

Boon, T., Coulie, P. G., Eynde, B. J. V. D. & Bruggen, P. V. D. 2006. Human T cell responses against melanoma. *Annual Review of Immunology* 24(1): 175-208.

Borghaei, H., Smith, M. R. & Campbell, K. S. 2009. Immunotherapy of cancer. *European Journal of Pharmacology* 625(1-3): 41-54.

Bour‐Jordan, H., Esensten, J. H., Martinez‐Llordella, M., Penaranda, C., Stumpf, M. & Bluestone, J. A. 2011. Intrinsic and extrinsic control of peripheral T cell tolerance by costimulatory molecules of the CD28/B7 family. *Immunological Reviews* 241(1): 180-205.

Brenner, D., Blaser, H. & Mak, T. W. 2015. Regulation of tumour necrosis factor signalling: Live or let die. *Nature Reviews Immunology* 15(6): 362-374.

Brichard, V. G. & Lejeune, D. 2007. GSK's antigen-specific cancer immunotherapy programme: Pilot results leading to phase III clinical development. *Vaccine* 25(1): B61-B71.

Bronner, C. E., Baker, S. M., Morrison, P. T., Warren, G., Smith, L. G., Lescoe, M. K., Kane, M., Earabino, C., Lipford, J. & Lindblom, A. 1994. Mutation in the DNA mismatch repair gene homologue hMLH 1 is associated with hereditary non-polyposis colon cancer. *Nature* 368(6468): 258-261.

Bross, P. F., Beitz, J., Chen, G., Chen, X. H., Duffy, E., Kieffer, L., Roy, S., Sridhara, R., Rahman, A. & Williams, G. 2001. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clinical Cancer Research* 7(6): 1490-1496.

Brossart, P. & Bevan, M. J. 1996. Selective activation of Fas/Fas ligand-mediated cytotoxicity by a self peptide. *Journal of Experimental Medicine* 183(6): 2449-2458.

Brunner, K. T. & Cerottini, J. C. 1971. Cytotoxic lymphocytes as effector cells of cell-mediated immunity supported by the swiss national foundation for scientific research. Dlm. Amos, B. (pnyt.). *Progress in immunology,* hlm. 385-398. Academic Press.

Buchbinder, E. & Hodi, F. S. 2015. Cytotoxic T lymphocyte antigen-4 and immune checkpoint blockade. *The Journal of Clinical Investigation* 125(9): 3377-3383.

Burke, C. A., Dekker, E., Samadder, N. J., Stoffel, E. & Cohen, A. 2016. Efficacy and safety of Eflornithine (CPP-1X)/sulindac combination therapy versus each as monotherapy in patients with familial adenomatous polyposis (FAP): Design and rationale of a randomized, double-blind, phase III trial. *BMC Gastroenterology* 16(1): 87-95.

Buzza, M. S., Zamurs, L., Sun, J., Bird, C. H., Smith, A. I., Trapani, J. A., Froelich, C. J., Nice, E. C. & Bird, P. I. 2005. Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin and laminin. *Journal of Biological Chemistry* 280(1): 23549-23558.

Caballero, O. L. & Chen, Y. T. 2009. Cancer/testis (CT) antigens: Potential targets for immunotherapy. *Cancer Science* 100(11): 2014-2021.

Cann, S. H., Van Netten, J. & Van Netten, C. 2003. Dr William Coley and tumour regression: A place in history or in the future. *Postgraduate Medical Journal* 79(938): 672-680.

Center, M. M., Jemal, A. & Ward, E. 2009. International trends in colorectal cancer incidence rates. *Cancer Epidemiology and Prevention Biomarkers* 18(6): 1688-1694.

Chapman-Smith, A., Lutwyche, J. K. & Whitelaw, M. L. 2004. Contribution of the Per/Arnt/Sim (PAS) domains to DNA binding by the basic helix-loop-helix PAS transcriptional regulators. *Journal of Biological Chemistry* 279(7): 5353-5362.

Chavez-Galan, L., Arenas-Del Angel, M., Zenteno, E., Chavez, R. & Lascurain, R. 2009. Cell death mechanisms induced by cytotoxic lymphocytes. *Cellular & Molecular Immunology* 6(1): 15-25.

Cheever, M. A., Allison, J. P., Ferris, A. S., Finn, O. J., Hastings, B. M., Hecht, T. T., Mellman, I., Prindiville, S. A., Viner, J. L. & Weiner, L. M. 2009. The prioritization of cancer antigens: A national cancer institute pilot project for the acceleration of translational research. *Clinical Cancer Research* 15(17): 5323-5337.

Cheever, M. A. & Higano, C. S. 2011. PROVENGE (Sipuleucel-T) in prostate cancer: The first FDA-approved therapeutic cancer vaccine. *Clinical Cancer Research* 17(11): 3520-3526.

Chen, M., May, B. H., Zhou, I. W., Sze, D. M.-Y., Xue, C. C. & Zhang, A. L. 2016. Oxaliplatin-based chemotherapy combined with traditional medicines for neutropenia in colorectal cancer: A meta-analysis of the contributions of specific plants. *Critical Reviews in Oncology/Hematology* 105(1): 18-34.

Chen, Y. T., Hsu, M., Lee, P., Shin, S. J., Mhawech Fauceglia, P., Odunsi, K., Altorki, N. K., Song, C. J., Jin, B. Q. & Simpson, A. J. 2009. Cancer/testis antigen CT45: Analysis of mRNA and protein expression in human cancer. *International Journal of Cancer* 124(12): 2893-2898.

Chi Soh, J. E., Abu, N. & Jamal, R. 2018. The potential immune-eliciting cancer testis antigens in colorectal cancer. *Immunotherapy* 10(12): 1093-1104.

Chinnaiyan, A. M., O'rourke, K., Tewari, M. & Dixit, V. M. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of fas and initiates apoptosis. *Cell* 81(4): 505-512.

Chiriva Internati, M., Yu, Y., Mirandola, L., D'cunha, N., Hardwicke, F., Cannon, M. J., Cobos, E. & Kast, W. M. 2012. Identification of AKAP-4 as a new cancer/testis antigen for detection and immunotherapy of prostate cancer. *The Prostate* 72(1): 12-23.

Chmielewski, M., Kopecky, C., Hombach, A. A. & Abken, H. 2011. IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively Muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression. *Cancer Research* 71(17): 5697-5706.

Cho, H. J., Caballero, O. L., Gnjatic, S., Andrade, V. C., Colleoni, G. W., Vettore, A. L., Outtz, H. H., Fortunato, S., Altorki, N. & Ferrera, C. A. 2006. Physical interaction of two cancer-testis antigens, MAGE-C1 (CT7) and NY-ESO-1 (CT6). *Cancer Immunity Archive* 6(1): 1-12.

Chodon, T., Koya, R. C. & Odunsi, K. 2015. Active immunotherapy of cancer. *Immunological Investigations* 44(8): 817-836.

Cohen, J. J., Duke, R. C., Chervenak, R., Sellins, K. S. & Olson, L. K. 1985. DNA fragmentation in targets of CTL: An example of programmed cell death in the immune system. Dlm. (pnyt.). *Mechanisms of cell-mediated cytotoxicity ii,* hlm. 493-508. Springer.

Cohen, J. J., Duke, R. C., Fadok, V. A. & Sellins, K. S. 1992. Apoptosis and programmed cell death in immunity. *Annual Review of Immunology* 10(1): 267-293.

Colangelo, T., Polcaro, G., Muccillo, L., D'agostino, G., Rosato, V., Ziccardi, P., Lupo, A., Mazzoccoli, G., Sabatino, L. & Colantuoni, V. 2017. Friend or foe?: The tumour microenvironment dilemma in colorectal cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 1867(1): 1-18.

Coley, W. B. 1893. The treatment of malignant tumours by repeated inoculations of erysipelas with a report of ten original cases. *American Journal of Medical Sciences* 105(6): 487.

Cooks, T., Pateras, I. S., Tarcic, O., Solomon, H., Schetter, A. J., Wilder, S., Lozano, G., Pikarsky, E., Forshew, T. & Rozenfeld, N. 2013. Mutant p53 prolongs NF-κb activation and promotes chronic inflammation and inflammation-associated colorectal cancer. *Cancer Cell* 23(5): 634-646.

Cooper, A. M. & Khader, S. A. 2008. The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis. *Immunological Reviews* 226(1): 191-204.

Cooper, C., Liggins, A., Ait-Tahar, K., Roncador, G., Banham, A. & Pulford, K. 2006. PASD1, a DLCBL-associated cancer testis antigen and candidate for lymphoma immunotherapy. *Leukemia* 20(12): 2172-2174.

Cresswell, P., Ackerman, A. L., Giodini, A., Peaper, D. R. & Wearsch, P. A. 2005. Mechanisms of MHC class I restricted antigen processing and cross presentation. *Immunological Reviews* 207(1): 145-157.

Cross, A. J., Pollock, J. R. & Bingham, S. A. 2003. Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer Research* 63(10): 2358-2360.

Dadabayev, A. R., Wang, Z., Zhang, Y., Zhang, J., Robinson, W. R. & Lim, S. H. 2005. Cancer immunotherapy targeting Sp17: When should the laboratory findings be translated to the clinics? *American Journal of Hematology* 80(1): 6-11.

De Gruijl, T. D., Van Den Eertwegh, A. J., Pinedo, H. M. & Scheper, R. J. 2008. Whole-cell cancer vaccination: From autologous to allogeneic tumor and dendritic cell-based vaccines. *Cancer Immunology, Immunotherapy* 57(10): 1569-1577.

De Martel, C., Ferlay, J., Franceschi, S., Vignat, J., Bray, F., Forman, D. & Plummer, M. 2012. Global burden of cancers attributable to infections in 2008: A review and synthetic analysis. *The Lancet Oncology* 13(6): 607-615.

De Saint Basile, G., Ménasché, G. & Fischer, A. 2010. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nature Reviews Immunology* 10(8): 568-579.

De Simone, V., Pallone, F., Monteleone, G. & Stolfi, C. 2013. Role of TH17 cytokines in the control of colorectal cancer. *Oncoimmunology* 2(12): 1-5.

De Smet, C., Courtois, S. J., Faraoni, I., Lurquin, C., Szikora, J.-P., De Backer, O. & Boon, T. 1995. Involvement of two Ets binding sites in the transcriptional activation of the MAGE-1 gene. *Immunogenetics* 42(4): 282-290.

De Smet, C., Lurquin, C., Lethé, B., Martelange, V. & Boon, T. 1999. DNA methylation is the primary silencing mechanism for a set of germ line-and tumor-specific genes with a Cpg-rich promoter. *Molecular and Cellular Biology* 19(11): 7327-7335.

De Wit, N. J., Weidle, U. H., Ruiter, D. J. & Van Muijen, G. N. 2002. Expression profiling of MMA-1a and splice variant MMA-1b: New cancer/testis antigens identified in human melanoma. *International Journal of Cancer* 98(4): 547-553.

Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I. L., Korkina, O., Teng, X., Abbott, D., Cuny, G. D., Yuan, C. & Wagner, G. 2008. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nature Chemical Biology* 4(5): 313-321.

Del Prete, G., De Carli, M., Almerigogna, F., Giudizi, M. G., Biagiotti, R. & Romagnani, S. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *The Journal of Immunology* 150(2): 353-360.

Disis, M. L., Grabstein, K. H., Sleath, P. R. & Cheever, M. A. 1999. Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. *Clinical Cancer Research* 5(6): 1289-1297.

Dong, X., Su, Y., Qian, X., Yang, X., Pang, X., Wu, H. & Chen, W. 2003. Identification of two novel CT antigens and their capacity to elicit antibody response in hepatocellular carcinoma patients. *British Journal of Cancer* 89(2): 291-297.

Dunn, G. P., Old, L. J. & Schreiber, R. D. 2004. The three Es of cancer immunoediting. *Annual Review of Immunology* 22(1): 329-360.

Durrant, L., Noble, P. & Spendlove, I. 2012. Immunology in the clinic review series; focus on cancer: Glycolipids as targets for tumour immunotherapy. *Clinical & Experimental Immunology* 167(2): 206-215.

East, J. E., Saunders, B. P. & Jass, J. R. 2008. Sporadic and syndromic hyperplastic polyps and serrated adenomas of the colon: Classification, molecular genetics, natural history, and clinical management. *Gastroenterology Clinics* 37(1): 25-46.

Eichmüller, S., Usener, D., Dummer, R., Stein, A., Thiel, D. & Schadendorf, D. 2001. Serological detection of cutaneous T cell lymphoma-associated antigens. *Proceedings of the National Academy of Sciences* 98(2): 629-634.

Ellis, L. M. & Hicklin, D. J. 2008. VEFG-targeted therapy: Mechanisms of anti-tumour activity. *Nature Reviews Cancer* 8(8): 579-591.

Faltejskova, P., Svoboda, M., Srutova, K., Mlcochova, J., Besse, A., Nekvindova, J., Radova, L., Fabian, P., Slaba, K. & Kiss, I. 2012. Identification and functional screening of microRNAs highly deregulated in colorectal cancer. *Journal of Cellular and Molecular Medicine* 16(11): 2655-2666.

Farkona, S., Diamandis, E. P. & Blasutig, I. M. 2016. Cancer immunotherapy: The beginning of the end of cancer? *BMC Medicine* 14(1): 73-90.

Favoriti, P., Carbone, G., Greco, M., Pirozzi, F., Pirozzi, R. E. M. & Corcione, F. 2016. Worldwide burden of colorectal cancer: A review. *Updates in Surgery* 68(1): 7-11.

Fearon, E. R. & Vogelstein, B. 1990. A genetic model for colorectal tumorigenesis. *Cell* 61(5): 759-767.

Fehniger, T. A., Cooper, M. A. & Caligiuri, M. A. 2002. Interleukin-2 and interleukin-15: Immunotherapy for cancer. *Cytokine & Growth Factor Reviews* 13(2): 169-183.

Feinerman, O., Germain, R. N. & Altan-Bonnet, G. 2008. Quantitative challenges in understanding ligand discrimination by αβ T cells. *Molecular Immunology* 45(3): 619-631.

Fenoglio, D., Traverso, P., Parodi, A., Kalli, F., Zanetti, M. & Filaci, G. 2013. Generation of more effective cancer vaccines. *Human Vaccines & Immunotherapeutics* 9(12): 2543-2547.

Ferguson, M. S., Lemoine, N. R. & Wang, Y. 2012. Systemic delivery of oncolytic viruses: Hopes and hurdles. *Advances in Virology* 2012(1): 1-14.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D. & Bray, F. 2015. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBACAN 2012. *International Journal of Cancer* 136(5): E359-E386.

Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D., Forman, D. & Bray, F. 2013. Cancer incidence and mortality worldwide: IARC CancerBase no. 11. Lyon, France: International Agency for Research on Cancer. GLOBACAN 2012 v1. 0, 2013.

Fichorova, R. N., Richardson-Harman, N., Alfano, M., Belec, L., Carbonneil, C., Chen, S., Cosentino, L., Curtis, K., Dezzutti, C. S. & Donoval, B. 2008. Biological and technical variables affecting immunoassay recovery of cytokines from human serum and simulated vaginal fluid: A multicenter study. *Analytical Chemistry* 80(12): 4741-4751.

Fodde, R. 2002. The APC gene in colorectal cancer. *European Journal of Cancer* 38(7): 867-871.

Fox, B. A., Schendel, D. J., Butterfield, L. H., Aamdal, S., Allison, J. P., Ascierto, P. A., Atkins, M. B., Bartunkova, J., Bergmann, L. & Berinstein, N. 2011. Defining the critical hurdles in cancer immunotherapy. *Journal of Translational Medicine* 9(1): 214-227.

Fuertes Marraco, S. A., Soneson, C., Cagnon, L., Gannon, P. O., Allard, M., Abed Maillard, S., Montandon, N., Rufer, N., Waldvogel, S., Delorenzi, M. &

Speiser, D. E. 2015. Long-lasting stem cell-like memory CD8+ T cells with a naive-like profile upon yellow fever vaccination. *Science Translational Medicine* 7(282): 248-251.

Gabrilovich, D. I., Cunningham, H. T. & Carbone, D. P. 1996. IL-12 and mutant p53 peptide-pulsed dendritic cells for the specific immunotherapy of cancer. *Journal of Immunotherapy with Emphasis on Tumor Immunology: Official Journal of The Society for Biological Therapy* 19(6): 414-418.

Gaffen, S. L. & Liu, K. D. 2004. Overview of interleukin-2 function, production and clinical applications. *Cytokine* 28(3): 109-123.

Ghee, L. K. 2014. A review of colorectal cancer research in malaysia. *Medical Journal of Malaysia* 69(1): 23-32.

Gigante, M. & Ranieri, E. 2014. *In vitro\ex vivo* generation of cytotoxic T lymphocytes. Dlm. (pnyt.). *Cytotoxic t cells,* hlm. 13-20. Springer.

Goelz, S. E., Vogelstein, B. & Feinberg, A. 1985. Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science* 228(4696): 187-190.

Greiner, J., Bullinger, L., Guinn, B.-A., Döhner, H. & Schmitt, M. 2008. Leukemia-associated antigens are critical for the proliferation of acute myeloid leukemia cells. *Clinical Cancer Research* 14(22): 7161-7166.

Grizzi, F., Bianchi, P., Malesci, A. & Laghi, L. 2013. Prognostic value of innate and adaptive immunity in colorectal cancer. *World Journal of Gastroenterology : WJG* 19(2): 174-187.

Groux, H., Bigler, M., De Vries, J. E. & Roncarolo, M.-G. 1996. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *Journal of Experimental Medicine* 184(1): 19-29.

Guinn, B.-A., Bland, E. A., Lodi, U., Liggins, A. P., Tobal, K., Petters, S., Wells, J. W., Banham, A. H. & Mufti, G. J. 2005. Humoral detection of leukaemia-associated antigens in presentation acute myeloid leukaemia. *Biochemical and Biophysical Research Communications* 335(4): 1293-1304.

Guinn, B.-A., Kasahara, N., Farzaneh, F., Habib, N. A., Norris, J. S. & Deisseroth, A. B. 2007. Recent advances and current challenges in tumor immunology and immunotherapy. *Molecular Therapy* 15(6): 1065-1071.

Güre, A. O., Stockert, E., Arden, K. C., Boyer, A. D., Viars, C. S., Scanlan, M. J., Old, L. J. & Chen, Y. T. 2000. CT10: A new cancer testis (CT) antigen homologous to CT7 and the MAGE family, identified by representational difference analysis. *International Journal of Cancer* 85(5): 726-732.

Haggar, F. A. & Boushey, R. P. 2009. Colorectal cancer epidemiology: Incidence, mortality, survival, and risk factors. *Clinics in Colon and Rectal Surgery* 22(4): 191-197.

Haji-Fatahaliha, M., Hosseini, M., Akbarian, A., Sadreddini, S., Jadidi-Niaragh, F. & Yousefi, M. 2016. CAR-modified T cell therapy for cancer: An updated review. *Artificial Cells, Nanomedicine and Biotechnology* 44(6): 1339-1349.

Halligan, S., Wooldrage, K., Dadswell, E., Kralj-Hans, I., Von Wagner, C., Edwards, R., Yao, G., Kay, C., Burling, D., Faiz, O., Teare, J., Lilford, R. J., Morton, D., Wardle, J. & Atkin, W. 2013. Computed tomographic colonography versus barium enema for diagnosis of colorectal cancer or large polyps in symptomatic patients (SIGGAR): A multicentre randomised trial. *The Lancet* 381(9873): 1185-1193.

Hamid, O., Robert, C., Daud, A., Hodi, F. S., Hwu, W.-J., Kefford, R., Wolchok, J. D., Hersey, P., Joseph, R. W. & Weber, J. S. 2013. Safety and tumor responses with lambrolizumab (anti–PD-1) in melanoma. *New England Journal of Medicine* 369(2): 134-144.

Hamilton, K. E., Simmons, J. G., Ding, S., Van Landeghem, L. & Lund, P. K. 2011. Cytokine induction of tumor necrosis factor receptor 2 is mediated by STAT3 in colon cancer cells. *Molecular Cancer Research* 9(12): 1718-1731.

Hardwick, N., Buchan, S., Ingram, W., Khan, G., Vittes, G., Rice, J., Pulford, K., Mufti, G., Stevenson, F. & Guinn, B.-A. 2013. An analogue peptide from the cancer/testis antigen PASD1 induces CD8+ T cell responses against naturally processed peptide. *Cancer Immunity Archive* 13(3): 16-25.

Hauptman, N. & Glavač, D. 2013. Long non-coding RNA in cancer. *International Journal of Molecular Sciences* 14(3): 4655-4669.

Heianna, J., Muto, O., Miyauchi, T., Endo, W., Togashi, A., Azama, K. & Murayama, S. 2016. Successful treatment continuation with a single mild partial splenic embolization for thrombocytopenia caused by oxaliplatin-based chemotherapy for advanced colon cancer. *Indian Journal of Gastroenterology* 35(3): 245-247.

Helenius, A. & Aebi, M. 2004. Roles of N-linked glycans in the endoplasmic reticulum. *Annual Review of Biochemistry* 73(1): 1019-1049.

Hlongwane, P., Mungra, N., Madheswaran, S., Akinrinmade, O., Chetty, S. & Barth, S. 2018. Human granzyme B based targeted cytolytic fusion proteins. *Biomedicines* 6(2): 72-82.

Hodi, F. S., O'day, S. J., Mcdermott, D. F., Weber, R. W., Sosman, J. A., Haanen, J. B., Gonzalez, R., Robert, C., Schadendorf, D. & Hassel, J. C. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *New England Journal of Medicine* 363(8): 711-723.

Homayounfar, K., Bleckmann, A., Conradi, L., Sprenger, T., Lorf, T., Niessner, M., Sahlmann, C., Meller, J., Liersch, T. & Ghadimi, B. 2013. Metastatic recurrence after complete resection of colorectal liver metastases: Impact of surgery and chemotherapy on survival. *International Journal of Colorectal Disease* 28(7): 1009-1017.

Hu, Y. & Fu, L. 2012. Targeting cancer stem cells: A new therapy to cure cancer patients. *American Journal of Cancer Research* 2(3): 340-356.

Idenoue, S., Hirohashi, Y., Torigoe, T., Sato, Y., Tamura, Y., Hariu, H., Yamamoto, M., Kurotaki, T., Tsuruma, T. & Asanuma, H. 2005. A potent immunogenic general cancer vaccine that targets survivin, an inhibitor of apoptosis proteins. *Clinical Cancer Research* 11(4): 1474-1482.

Ishikawa, S. I., Tamaki, S., Ohata, M., Arihara, K. & Itoh, M. 2010. Heme induces DNA damage and hyperproliferation of colonic epithelial cells via hydrogen peroxide produced by heme oxygenase: A possible mechanism of heme ‐ induced colon cancer. *Molecular Nutrition and Food Research* 54(8): 1182-1191.

Iwasaki, A. & Medzhitov, R. 2015. Control of adaptive immunity by the innate immune system. *Nature Immunology* 16(4): 343-353.

Jacquemin, G., Margiotta, D., Kasahara, A., Bassoy, E., Walch, M., Thiery, J., Lieberman, J. & Martinvalet, D. 2015. Granzyme B-induced mitochondrial ROS are required for apoptosis. *Cell Death and Differentiation* 22(5): 862-874.

Jäger, D., Stockert, E., Scanlan, M. J., Güre, A. O., Jäger, E., Knuth, A., Old, L. J. & Chen, Y.-T. 1999. Cancer-testis antigens and ING1 tumor suppressor gene product are breast cancer antigens. *Cancer Research* 59(24): 6197-6204.

Janitz, M., Fiszer, D., Michalczak ‐ Janitz, K., Lukaszyk, A., Fernandez, N., Skorupski, W. & Kurpisz, M. 1994. Analysis of mRNA for class I HLA on human gametogenic cells. *Molecular Reproduction and Development* 38(2): 231-237.

Jemal, A., Simard, E. P., Dorell, C., Noone, A.-M., Markowitz, L. E., Kohler, B., Eheman, C., Saraiya, M., Bandi, P., Saslow, D., Cronin, K. A., Watson, M., Schiffman, M., Henley, S. J., Schymura, M. J., Anderson, R. N., Yankey, D. & Edwards, B. K. 2013. Annual report to the nation on the status of cancer, 1975–2009, featuring the burden and trends in uman Papillomavirus (HPV)–associated cancers and HPV vaccination coverage levels. *JNCI: Journal of the National Cancer Institute* 105(3): 175-201.

Junttila, M. R. & De Sauvage, F. J. 2013. Influence of tumour microenvironment heterogeneity on therapeutic response. *Nature* 501(7467): 346-354.

Kadam, C. Y. & Abhang, S A. 2015. Serum levels of soluble Fas ligand, granzyme B and cytochrome c during adjuvant chemotherapy of breast cancer. *Clinica Chimica Acta* 438(1): 98-102.

Kamatani, A., Nakagawa, Y., Akao, Y., Maruyama, N., Nagasaka, M., Shibata, T., Tahara, T. & Hirata, I. 2013. Downregulation of anti-oncomirs miR-143/145 cluster occurs before APC gene aberration in the development of colorectal tumors. *Medical Molecular Morphology* 46(3): 166-171.

Kantola, T., Klintrup, K., Väyrynen, J., Vornanen, J., Bloigu, R., Karhu, T., Herzig, K., Näpänkangas, J., Mäkelä, J. & Karttunen, T. 2012. Stage-dependent alterations of the serum cytokine pattern in colorectal carcinoma. *British Journal of Cancer* 107(10): 1729-1736.

Kasuga, K. 2013. Comprehensive analysis of MHC ligands in clinical material by immunoaffinity mass spectrometry. *Methods in Molecular Biology* 1023(1): 203-218.

Kaur, G., Thompson, L. A. & Dufour, J. M. 2014. Sertoli cells-immunological sentinels of spermatogenesis. *Seminars in Cell and Developmental Biology*, hlm. 36-44.

Kawamoto, M., Tanaka, F., Mimori, K., Inoue, H., Kamohara, Y. & Mori, M. 2011. Identification of HLA-A*0201/-A*2402-restricted CTL epitope-peptides derived from a novel cancer/testis antigen, MCAK, and induction of a specific antitumor immune response. *Oncology Reports* 25(2): 469-476.

Khan, G., Brooks, S. E., Mills, K. I. & Guinn, B.-A. 2015. Infrequent expression of the cancer-testis antigen, PASD1, in ovarian cancer. *Biomarkers in Cancer* 7(1): 31-38.

Khan, G., Denniss, F., Mills, K., Pulford, K. & Guinn, B.-A. 2013. PASD1: A promising target for the immunotherapy of haematological malignancies. *Journal of Genetic Syndromes & Gene Therapy* 4(9): 1-8.

Khleif, S. N., Abrams, S. I., Hamilton, J. M., Bergmann-Leitner, E., Chen, A., Bastian, A., Bernstein, S., Chung, Y., Allegra, C. J. & Schlom, J. 1999. A phase I vaccine trial with peptides reflecting ras oncogene mutations of solid tumors. *Journal of Immunotherapy* 22(2): 155-165.

Kilinc, M. O., Aulakh, K. S., Nair, R. E., Jones, S. A., Alard, P., Kosiewicz, M. M. & Egilmez, N. K. 2006. Reversing tumor immune suppression with intratumoral IL-12: Activation of tumor-associated T effector/memory cells, induction of T suppressor apoptosis, and infiltration of CD8+ T effectors. *The Journal of Immunology* 177(10): 6962-6973.

Kim, Y.-S., Morgan, M. J., Choksi, S. & Liu, Z.-G. 2007. TNF-induced activation of the Nox1 NADPH oxidase and its role in the induction of necrotic cell death. *Molecular Cell* 26(5): 675-687.

Kimball, A. S. & Webb, T. J. 2013. The roles of radiotherapy and immunotherapy for the treatment of lymphoma. *Molecular and Cellular Pharmacology* 5(1): 27-38.

Kimura, T., Mckolanis, J. R., Dzubinski, L. A., Islam, K., Potter, D. M., Salazar, A. M., Schoen, R. E. & Finn, O. J. 2012. MUC1 vaccine for individuals with advanced adenoma of the colon: A cancer immunoprevention feasibility study. *Cancer Prevention Research* 6(1): 18-26.

Klade, C. S., Voss, T., Krystek, E., Ahorn, H., Zatloukal, K., Pummer, K. & Adolf, G. R. 2001. Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. *Proteomics* 1(7): 890-898.

Koebel, C. M., Vermi, W., Swann, J. B., Zerafa, N., Rodig, S. J., Old, L. J., Smyth, M. J. & Schreiber, R. D. 2007. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450(7171): 903-907.

Koi, M. & Carethers, J. M. 2017. The colorectal cancer immune microenvironment and approach to immunotherapies. *Future Oncology* 13(18): 1633-1647.

Kreuwel, H. T., Morgan, D. J., Krahl, T., Ko, A., Sarvetnick, N. & Sherman, L. A. 1999. Comparing the relative role of perforin/granzyme versus Fas/Fas ligand cytotoxic pathways in CD8+ T cell-mediated insulin-dependent diabetes mellitus. *The Journal of Immunology* 163(8): 4335-4341.

Kruit, W. H., Van Ojik, H. H., Brichard, V. G., Escudier, B., Dorval, T., Dréno, B., Patel, P., Van Baren, N., Avril, M. F. & Piperno, S. 2005. Phase 1/2 study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable metastatic melanoma. *International Journal of Cancer* 117(4): 596-604.

Krzystek-Korpacka, M., Diakowska, D., Kapturkiewicz, B., Bębenek, M. & Gamian, A. 2013. Profiles of circulating inflammatory cytokines in colorectal cancer (CRC), high cancer risk conditions, and health are distinct. Possible implications for CRC screening and surveillance. *Cancer Letters* 337(1): 107-114.

Kumara, H. S., Grieco, M. J., Caballero, O. L., Su, T., Ahmed, A., Ritter, E., Gnjatic, S., Cekic, V., Old, L. J. & Simpson, A. J. 2012. MAGE-A3 is highly expressed in a subset of colorectal cancer patients. *Cancer Immunity Archive* 12(2): 9-24.

Kurts, C., Robinson, B. W. & Knolle, P. A. 2010. Cross-priming in health and disease. *Nature Reviews Immunology* 10(6): 403-414.

Lancki, D. W. & Fitch, F. W. 1992. Cytolytic T lymphocytes: An overview of their characteristics. *Biotherapy* 5(1): 71-81.

Lanzavecchia, A. & Sallusto, F. 2001. Regulation of T cell immunity by dendritic cells. *Cell* 106(3): 263-266.

Larkin, J., Chiarion Sileni, V., Gonzalez, R., Grob, J. J., Cowey, C. L., Lao, C. D., Schadendorf, D., Dummer, R., Smylie, M. & Rutkowski, P. 2015. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *New England Journal of Medicine* 373(1): 23-34.

Laumbacher, B., Gu, S. & Wank, R. 2012. Activated monocytes prime naïve T cells against autologous cancer: Vigorous cancer destruction *in vitro* and *in vivo*. *Scandinavian Journal of Immunology* 75(3): 314-328.

Law, R. H., Lukoyanova, N., Voskoboinik, I., Caradoc-Davies, T. T., Baran, K., Dunstone, M. A., D'angelo, M. E., Orlova, E. V., Coulibaly, F. & Verschoor, S. 2010. The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature* 468(7322): 447-451.

Li, F., Liu, Q., Han, Y., Wu, B. & Yin, H. 2010. Sperm protein 17 is highly expressed in endometrial and cervical cancers. *BMC Cancer* 10(1): 429-436.

Li, M., Yuan, Y., Han, Y., Liu, Y., Yan, L., Wang, Y. & Gu, J. 2005. Expression profile of cancer-testis genes in 121 human colorectal cancer tissue and adjacent normal tissue. *Clinical Cancer Research* 11(5): 1809-1814.

Li, W., Qiu, T., Zhi, W., Shi, S., Zou, S., Ling, Y., Shan, L., Ying, J. & Lu, N. 2015. Colorectal carcinomas with KRAS codon 12 mutation are associated with more advanced tumor stages. *BMC Cancer* 15(1): 340-348.

Li, Y., Song, R., Li, X. & Xu, F. 2017. Expression and immunogenicity of NY-ESO-1 in colorectal cancer. *Experimental and Therapeutic Medicine* 13(6): 3581-3585.

Lieberman, J. 2010. Granzyme A activates another way to die. *Immunological Reviews* 235(1): 93-104.

Liggins, A., Brown, P., Asker, K., Pulford, K. & Banham, A. 2004a. A novel diffuse large B-cell lymphoma-associated cancer testis antigen encoding a PAS domain protein. *British Journal of Cancer* 91(1): 141-149.

Liggins, A., Guinn, B., Hatton, C., Pulford, K. & Banham, A. 2004b. Erratum: Serologic detection of diffuse large B-cell lymphoma-associated antigens *International Journal of Cancer* 110(6): 934-934.

Lin, H. H., Ray, S., Tongchusak, S., Reinherz, E. L. & Brusic, V. 2008. Evaluation of MHC class I peptide binding prediction servers: Applications for vaccine research. *BMC Immunology* 9(1): 8-20.

Lin, W. W. & Karin, M. 2007. A cytokine-mediated link between innate immunity, inflammation, and cancer. *The Journal of Clinical Investigation* 117(5): 1175-1183.

Little, R. F., Pluda, J. M., Wyvill, K. M., Rodriguez-Chavez, I. R., Tosato, G., Catanzaro, A. T., Steinberg, S. M. & Yarchoan, R. 2006. Activity of subcutaneous interleukin-12 in AIDS-related Kaposi sarcoma. *Blood* 107(12): 4650-4657.

Liu, C. C., Walsh, C. M. & Young, J. D. E. 1995. Perforin: Structure and function. *Immunology Today* 16(4): 194-201.

Livak, K. J. & Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods* 25(4): 402-408.

Long, Y. Y., Wang, Y., Huang, Q. R., Zheng, G. S. & Jiao, S. C. 2014. Measurement of serum antibodies against NY-ESO-1 by ELISA: A guide for the treatment of specific immunotherapy for patients with advanced colorectal cancer. *Experimental and Therapeutic Medicine* 8(4): 1279-1284.

Lucas, S., De Smet, C., Arden, K. C., Viars, C. S., Lethé, B., Lurquin, C. & Boon, T. 1998. Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. *Cancer Research* 58(4): 743-752.

Luebeck, E. G. & Moolgavkar, S. H. 2002. Multistage carcinogenesis and the incidence of colorectal cancer. *Proceedings of the National Academy of Sciences* 99(23): 15095-15100.

Lund, O., Karosiene, E., Lundegaard, C., Larsen, M. V. & Nielsen, M. 2013. Bioinformatics identification of antigenic peptide: Predicting the specificity of major MHC class I and II pathway players. Dlm. (pnyt.). *Antigen processing,* hlm. 247-260. Springer.

Lundin, J., Kimby, E., Björkholm, M., Broliden, P.-A., Celsing, F., Hjalmar, V., Möllgård, L., Rebello, P., Hale, G. & Waldmann, H. 2002. Phase II trial of subcutaneous anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) as first-line treatment for patients with b-cell chronic lymphocytic leukemia (B-CLL). *Blood* 100(3): 768-773.

Maccarty, W. C. 1922. Longevity in cancer: A study of 293 cases. *Annals of Surgery* 76(1): 9-12.

Markman, J. L. & Shiao, S. L. 2015. Impact of the immune system and immunotherapy in colorectal cancer. *Journal of Gastrointestinal Oncology* 6(2): 208-223.

Marley, A. R. & Nan, H. 2016. Epidemiology of colorectal cancer. *International Journal of Molecular Epidemiology and Genetics* 7(3): 105-114.

Marshall, J. L., Hoyer, R. J., Toomey, M. A., Faraguna, K., Chang, P., Richmond, E., Pedicano, J. E., Gehan, E., Peck, R. A. & Arlen, P. 2000. Phase i study in advanced cancer patients of a diversified prime-and-boost vaccination protocol using recombinant vaccinia virus and recombinant nonreplicating avipox virus to elicit anti–carcinoembryonic antigen immune responses. *Journal of Clinical Oncology* 18(23): 3964-3973.

Martelange, V., De Smet, C., De Plaen, E., Lurquin, C. & Boon, T. 2000. Identification on a human sarcoma of two new genes with tumor-specific expression. *Cancer Research* 60(14): 3848-3855.

Martínez, M. E., Jacobs, E. T., Ashbeck, E. L., Sinha, R., Lance, P., Alberts, D. S. & Thompson, P. A. 2007. Meat intake, preparation methods, mutagens and colorectal adenoma recurrence. *Carcinogenesis* 28(9): 2019-2027.

Martinvalet, D., Dykxhoorn, D. M., Ferrini, R. & Lieberman, J. 2008. Granzyme A cleaves a mitochondrial complex i protein to initiate caspase-independent cell death. *Cell* 133(4): 681-692.

Martinvalet, D., Zhu, P. & Lieberman, J. 2005. Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. *Immunity* 22(3): 355-370.

Melief, C. J. 2018. Peptide-based therapeutic cancer vaccines. Dlm. (pnyt.). *Oncoimmunology,* hlm. 249-261. Springer.

Mellman, I., Coukos, G. & Dranoff, G. 2011. Cancer immunotherapy comes of age. *Nature* 480(7378): 480-189.

Michael, Alicia k., Harvey, Stacy l., Sammons, Patrick j., Anderson, Amanda p., Kopalle, Hema m., Banham, Alison h. & Partch, Carrie l. 2015. Cancer/testis antigen PASD1 silences the circadian clock. *Molecular Cell* 58(5): 743-754.

Middleton, D., Menchaca, L., Rood, H. & Komerofsky, R. 2003. New allele frequency database. *Tissue Antigens* 61(5): 403-407.

Miller, K. D., Siegel, R. L., Lin, C. C., Mariotto, A. B., Kramer, J. L., Rowland, J. H., Stein, K. D., Alteri, R. & Jemal, A. 2016. Cancer treatment and survivorship statistics, 2016. *CA: A Cancer Journal for Clinicians* 66(4): 271-289.

Miyagi, Y., Imai, N., Sasatomi, T., Yamada, A., Mine, T., Katagiri, K., Nakagawa, M., Muto, A., Okouchi, S. & Isomoto, H. 2001. Induction of cellular immune responses to tumor cells and peptides in colorectal cancer patients by vaccination with SART3 peptides. *Clinical Cancer Research* 7(12): 3950-3962.

Mizoguchi, E., Mizoguchi, A., Takedatsu, H., Cario, E., De Jong, Y. P., Ooi, C. J., Xavier, R. J., Terhorst, C., Podolsky, D. K. & Bhan, A. K. 2002. Role of tumor necrosis factor receptor 2 (TNFR2) in colonic epithelial hyperplasia and chronic intestinal inflammation in mice. *Gastroenterology* 122(1): 134-144.

Momburg, F. & Tan, P. 2002. Tapasin—the keystone of the loading complex optimizing peptide binding by MHC class I molecules in the endoplasmic reticulum. *Molecular Immunology* 39(3-4): 217-233.

Morales, A., Eidinger, D. & Bruce, A. 2017. Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. *The Journal of Urology* 197(2): S142-S145.

Morgan, G., Ward, R. & Barton, M. 2004. The contribution of cytotoxic chemotherapy to 5-year survival in adult malignancies. *Clinical Oncology* 16(8): 549-560.

Motta, R. 1971. Passive immunotherapy of leukemia and other cancer. Dlm. (pnyt.). *Advances in cancer research,* 14. hlm. 161-179. Elsevier.

Moulton, H. M., Yoshihara, P. H., Mason, D. H., Iversen, P. L. & Triozzi, P. L. 2002. Active specific immunotherapy with a β-human chorionic gonadotropin peptide vaccine in patients with metastatic colorectal cancer: Antibody response is associated with improved survival. *Clinical Cancer Research* 8(7): 2044-2051.

Murdoch, C., Muthana, M., Coffelt, S. B. & Lewis, C. E. 2008. The role of myeloid cells in the promotion of tumour angiogenesis. *Nature Reviews Cancer* 8(8): 618-631.

Nagata, S. & Golstein, P. 1995. The Fas death factor. *Science* 267(5203): 1449-1456.

Nagorsen, D. & Thiel, E. 2008. HLA typing demands for peptide-based anti-cancer vaccine. *Cancer Immunology, Immunotherapy* 57(12): 1903-1910.

Nakagawa, K., Noguchi, Y., Uenaka, A., Sato, S., Okumura, H., Tanaka, M., Shimono, M., Eldib, A. M. A., Ono, T. & Ohara, N. 2005. XAGE-1 expression in non–small cell lung cancer and antibody response in patients. *Clinical Cancer Research* 11(15): 5496-5503.

Nakatsugawa, M., Hirohashi, Y., Torigoe, T., Inoda, S., Kiriyama, K., Tamura, Y., Sato, E., Takahashi, H. & Sato, N. 2011. Comparison of speedy PCR-ssp method and serological typing of HLA-A24 for japanese cancer patients. *Journal of Immunoassay and Immunochemistry* 32(2): 93-102.

National Cancer Registry, N. C. I. 2018. Malaysian study on cancer survival (MyScan) Malaysia, M. O. H.

Neefjes, J., Jongsma, M. L., Paul, P. & Bakke, O. 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature Reviews Immunology* 11(12): 823-836.

Nicholson, D. W. & Thornberry, N. A. 1997. Caspases: Killer proteases. *Trends in Biochemical Sciences* 22(8): 299-306.

Nicolaides, N. C., Papadopoulos, N., Liu, B., Weit, Y.-F., Carter, K. C., Ruben, S. M., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D. & Fraser, C. M. 1994. Mutations of two P/WS homologues in hereditary nonpolyposis colon cancer. *Nature* 371(6492): 75-80.

Obeid, J., Hu, Y. & Slingluff, C. L. 2015. Vaccines, adjuvants, and dendritic cell activators—current status and future challenges. *Seminars in Oncology*, hlm. 549-561.

Ophir, E., Bobisse, S., Coukos, G., Harari, A. & Kandalaft, L. E. 2016. Personalized approaches to active immunotherapy in cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 1865(1): 72-82.

Osińska, I., Popko, K. & Demkow, U. 2014. Perforin: An important player in immune response. *European Journal of Immunology* 39(1): 109-115.

Ostergaard, H. L., Kane, K. P., Mescher, M. F. & Clark, W. R. 1987. Cytotoxic t lymphocyte mediated lysis without release of serine esterase. *Nature* 330(6143): 71-72.

Ott, P. A., Hu, Z., Keskin, D. B., Shukla, S. A., Sun, J., Bozym, D. J., Zhang, W., Luoma, A., Giobbie-Hurder, A. & Peter, L. 2017. An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* 547(7662): 217-221.

Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. A. & Hymowitz, S. G. 2011. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual Review of Immunology* 29(1): 71-109.

Palucka, K. & Banchereau, J. 2012. Cancer immunotherapy via dendritic cells. *Nature Reviews Cancer* 12(4): 265-277.

Pan, W., Chen, D. S., Lu, Y. J., Sun, F. F., Xu, H. W., Zhang, Y. W., Yan, C., Fu, L. L., Zheng, K. Y. & Tang, R. X. 2017. Bioinformatic prediction of the epitopes of Echinococcus granulosus antigen 5. *Biomedical Reports* 6(2): 181-187.

Pantuck, A. J., Van Ophoven, A., Gitlitz, B. J., Tso, C.-L., Acres, B., Squiban, P., Ross, M. E., Belldegrun, A. S. & Figlin, R. A. 2004. Phase I trial of antigen-specific gene therapy using a recombinant vaccinia virus encoding MUC-1 and IL-2 in MUC-1-positive patients with advanced prostate cancer. *Journal of Immunotherapy* 27(3): 240-253.

Papaioannou, N. E., Beniata, O. V., Vitsos, P., Tsitsilonis, O. & Samara, P. 2016. Harnessing the immune system to improve cancer therapy. *Annals of Translational Medicine* 4(14): 261-275.

Pardoll, D. M. & Topalian, S. L. 1998. The role of CD4+ T cell responses in antitumor immunity. *Current Opinion in Immunology* 10(5): 588-594.

Parham, P., Lomen, C., Lawlor, D., Ways, J., Holmes, N., Coppin, H., Salter, R., Wan, A. & Ennis, P. 1988. Nature of polymorphism in HLA-A,-B and-C molecules. *Proceedings of the National Academy of Sciences* 85(11): 4005-4009.

Parish, C. R. 2003. Cancer immunotherapy: The past, the present and the future. *Immunology and Cell Biology* 81(2): 106-113.

Peddareddigari, V. G., Wang, D. & Dubois, R. N. 2010. The tumor microenvironment in colorectal carcinogenesis. *Cancer Microenvironment* 3(1): 149-166.

Pericleous, M., Mandair, D. & Caplin, M. E. 2013. Diet and supplements and their impact on colorectal cancer. *Journal of Gastrointestinal Oncology* 4(4): 409-423.

Pestka, S., Krause, C. D., Sarkar, D., Walter, M. R., Shi, Y. & Fisher, P. B. 2004. Interleukin-10 and related cytokines and receptors. *Annual Review of Immunology* 22(1): 929-979.

Podack, E. R., Hengartner, H. & Lichtenheld, M. G. 1991. A central role of perforin in cytolysis? *Annual Review of Immunology* 9(1): 129-157.

Porschen, R., Arkenau, H.-T., Kubicka, S., Greil, R., Seufferlein, T., Freier, W., Kretzschmar, A., Graeven, U., Grothey, A. & Hinke, A. 2007. Phase iii study of capecitabine plus oxaliplatin compared with fluorouracil and leucovorin plus oxaliplatin in metastatic colorectal cancer: A final report of the AIO Colorectal Study Group. *Journal of Clinical Oncology* 25(27): 4217-4223.

Provenzano, M., Panelli, M. C., Mocellin, S., Bracci, L., Sais, G., Stroncek, D. F., Spagnoli, G. C. & Marincola, F. M. 2006. MHC–peptide specificity and T-cell epitope mapping: Where immunotherapy starts. *Trends in Molecular Medicine* 12(10): 465-472.

Qazi, S., Dumez, D. & Uckun, F. 2007. Meta analysis of advanced cancer survival data using lognormal parametric fitting: A statistical method to identify effective treatment protocols. *Current Pharmaceutical Design* 13(15): 1533-1544.

Qiao, J., Kottke, T., Willmon, C., Galivo, F., Wongthida, P., Diaz, R. M., Thompson, J., Ryno, P., Barber, G. N. & Chester, J. 2008a. Purging metastases in lymphoid organs using a combination of antigen-nonspecific adoptive t cell therapy, oncolytic virotherapy and immunotherapy. *Nature Medicine* 14(1): 37-44.

Qiao, J., Wang, H., Kottke, T., Diaz, R., Willmon, C., Hudacek, A., Thompson, J., Parato, K., Bell, J. & Naik, J. 2008b. Loading of oncolytic vesicular stomatitis virus onto antigen-specific t cells enhances the efficacy of adoptive t-cell therapy of tumors. *Gene Therapy* 15(8): 604-616.

Qu, C., Gao, S., Shao, H., Zhang, W., Bo, H., Lu, X., Chen, T., Kou, J., Wang, Y. & Chen, G. S. 2018. Identification of an HLA-A2-restricted CD147 epitope that can induce specific ctl cytotoxicity against drug resistant MCF-7/Adr cells. *Oncology Letters* 15(4): 6050-6056.

Rafiemanesh, H., Pakzad, R., Abedi, M., Kor, Y., Moludi, J., Towhidi, F., Makhsosi, B. R. & Salehiniya, H. 2016. Colorectal cancer in Iran: Epidemiology and morphology trends. *EXCLI Journal* 15(1): 738-744.

Rammensee, H. G., Bachmann, J., Emmerich, N. P. N., Bachor, O. A. & Stevanović, S. 1999. SYFPEITHI: Database for MHC ligands and peptide motifs. *Immunogenetics* 50(3-4): 213-219.

Rammensee, H. G., Weinschenk, T., Gouttefangeas, C. & Stevanovi Eq, S. 2002. Towards patient-specific tumor antigen selection for vaccination. *Immunological Reviews* 188(1): 164-176.

Rapoport, A. P., Stadtmauer, E. A., Binder Scholl, G. K., Goloubeva, O., Vogl, D. T., Lacey, S. F., Badros, A. Z., Garfall, A., Weiss, B. & Finklestein, J. 2015. NY-ESO-1–specific TCR–engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nature Medicine* 21(8): 914-921.

Rech, A. J. & Vonderheide, R. H. 2009. Clinical use of anti-CD25 antibody daclizumab to enhance immune responses to tumor antigen vaccination by targeting regulatory T cells. *Annals of the New York Academy of Sciences* 1174(1): 99-106.

Reinhard, H., Yousef, S., Luetkens, T., Fehse, B., Berdien, B., Kröger, N. & Atanackovic, D. 2014. Cancer-testis antigen MAGE-C2/CT10 induces spontaneous CD4+ and CD8+ T cell responses in multiple myeloma patients. *Blood Cancer Journal* 4(5): e212.

Reissfelder, C., Stamova, S., Gossmann, C., Braun, M., Bonertz, A., Walliczek, U., Grimm, M., Rahbari, N. N., Koch, M. & Saadati, M. 2015. Tumor-specific cytotoxic T lymphocyte activity determines colorectal cancer patient prognosis. *The Journal of Clinical Investigation* 125(2): 739-751.

Richardson, M. A., Ramirez, T., Russell, N. C. & Moye, L. A. 1999. Coley toxins immunotherapy: A retrospective review. *Alternative Therapies in Health and Medicine* 5(3): 42-47.

Robinson, S., Manas, D., Pedley, I., Mann, D. & White, S. 2011. Systemic chemotherapy and its implications for resection of colorectal liver metastasis. *Surgical Oncology* 20(2): 57-72.

Rojas, J. J., Sampath, P., Hou, W. & Thorne, S. H. 2015. Defining effective combinations of immune checkpoint blockade and oncolytic virotherapy. *Clinical Cancer Research* 21(24): 5543-5551.

Rook, A. H., Wood, G. S., Yoo, E. K., Elenitsas, R., Kao, D. M., Sherman, M. L., Witmer, W. K., Rockwell, K. A., Shane, R. B. & Lessin, S. R. 1999. Interleukin-12 therapy of cutaneous T-cell lymphoma induces lesion regression and cytotoxic t-cell responses. *Blood* 94(3): 902-908.

Rosenberg, S. A., Yang, J. C., Sherry, R. M., Kammula, U. S., Hughes, M. S., Phan, G. Q., Citrin, D. E., Restifo, N. P., Robbins, P. F. & Wunderlich, J. R. 2011. Durable complete responses in heavily pretreated patients with metastatic melanoma using T cell transfer immunotherapy. *Clinical Cancer Research* 17(13): 4550-4557.

Rouvier, E., Luciani, M. & Golstein, P. 1993. Fas involvement in Ca (2+)-independent T cell-mediated cytotoxicity. *Journal of Experimental Medicine* 177(1): 195-200.

Ruffell, B., Chang-Strachan, D., Chan, V., Rosenbusch, A., Ho, C. M., Pryer, N., Daniel, D., Hwang, E. S., Rugo, H. S. & Coussens, L. M. 2014. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell* 26(5): 623-637.

Rusch, T., Bayry, J., Werner, J., Shevchenko, I. & Bazhin, A. V. 2017. Immunotherapy as an option for cancer treatment. *Archivum Immunologiae et Therapiae Experimentalis* 66(2): 89-96.

Sahin, U., Derhovanessian, E., Miller, M., Kloke, B.-P., Simon, P., Löwer, M., Bukur, V., Tadmor, A. D., Luxemburger, U. & Schrörs, B. 2017. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* 547(7662): 222-226.

Sahin, U., Türeci, O., Schmitt, H., Cochlovius, B., Johannes, T., Schmits, R., Stenner, F., Luo, G., Schobert, I. & Pfreundschuh, M. 1995. Human neoplasms elicit multiple specific immune responses in the autologous host. *Proceedings of the National Academy of Sciences* 92(25): 11810-11813.

Sahota, S. S., Goonewardena, C. M., Cooper, C. D., Liggins, A. P., Ait-Tahar, K., Zojer, N., Stevenson, F. K., Banham, A. H. & Pulford, K. 2006. PASD1 is a potential multiple myeloma–associated antigen. *Blood* 108(12): 3953-3955.

Sang, M., Lian, Y., Zhou, X. & Shan, B. 2011. MAGE-A family: Attractive targets for cancer immunotherapy. *Vaccine* 29(47): 8496-8500.

Scanlan, M. J., Altorki, N. K., Gure, A. O., Williamson, B., Jungbluth, A., Chen, Y.-T. & Old, L. J. 2000. Expression of cancer-testis antigens in lung cancer: Definition of bromodomain testis-specific gene (BRDT) as a new CT gene, CT9. *Cancer Letters* 150(2): 155-164.

Scanlan, M. J., Gordon, C. M., Williamson, B., Lee, S. Y., Chen, Y. T., Stockert, E., Jungbluth, A., Ritter, G., Jäger, D. & Jäger, E. 2002a. Identification of cancer/testis genes by database mining and mRNA expression analysis. *International Journal of Cancer* 98(4): 485-492.

Scanlan, M. J., Gure, A. O., Jungbluth, A. A., Old, L. J. & Chen, Y. T. 2002b. Cancer/testis antigens: An expanding family of targets for cancer immunotherapy. *Immunological Reviews* 188(1): 22-32.

Scanlan, M. J., Simpson, A. & Old, L. J. 2004. The cancer/testis genes: Review, standardization, and commentary. *Cancer Immunity* 4(1): 1-15.

Schaefer, O. & Langer, M. 2007. Detection of recurrent rectal cancer with CT, MRI and PET/CT. *European Radiology* 17(8): 2044-2054.

Schernhammer, E. S., Giovannucci, E., Kawasaki, T., Rosner, B., Fuchs, C. S. & Ogino, S. 2010. Dietary folate, alcohol and B vitamins in relation to LINE-1 hypomethylation in colon cancer. *Gut* 59(6): 794-799.

Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. 2004. Interferon gamma: An overview of signals, mechanisms and functions. *Journal of Leukocyte Biology* 75(2): 163-189.

Schumacher, T. N. & Schreiber, R. D. 2015. Neoantigens in cancer immunotherapy. *Science* 348(6230): 69-74.

Sebbagh, M., Hamelin, J., Bertoglio, J., Solary, E. & Bréard, J. 2005. Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase-independent manner. *Journal of Experimental Medicine* 201(3): 465-471.

Segal, N. H., Parsons, D. W., Peggs, K. S., Velculescu, V., Kinzler, K. W., Vogelstein, B. & Allison, J. P. 2008. Epitope landscape in breast and colorectal cancer. *Cancer Research* 68(3): 889-892.

Segditsas, S. & Tomlinson, I. 2006. Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* 25(57): 7531-7537.

Shao, Y., Sun, Z. Y., Sun, S. W., Zhao, Y., Sin, W. Y., Yuan, Y. H., Simpson, A. J., Old, L. J., Sang, X. T. & Mao, Y. L. 2008. Identification and expression analysis of novel LAGE-1 alleles with single nucleotide polymorphisms in cancer patients. *Journal of Cancer Research and Clinical Oncology* 134(4): 495-502.

Sharma, P. & Allison, J. P. 2015. Immune checkpoint targeting in cancer therapy: Toward combination strategies with curative potential. *Cell* 161(2): 205-214.

Sharpe, A. H. & Freeman, G. J. 2002. The B7–CD28 superfamily. *Nature Reviews Immunology* 2(2): 116-126.

Shrestha, M. P., Borgstrom, M. & Trowers, E. 2017. Digital rectal examination reduces hospital admissions, endoscopies, and medical therapy in patients with acute gastrointestinal bleeding. *The American Journal of Medicine* 130(7): 819-825.

Siebenkäs, C., Chiappinelli, K. B., Guzzetta, A. A., Sharma, A., Jeschke, J., Vatapalli, R., Baylin, S. B. & Ahuja, N. 2017. Inhibiting DNA methylation activates cancer testis antigens and expression of the antigen processing and presentation machinery in colon and ovarian cancer cells. *PloS one* 12(6): e0179501.

Siegel, R. L., Miller, K. D., Fedewa, S. A., Ahnen, D. J., Meester, R. G., Barzi, A. & Jemal, A. 2017. Colorectal cancer statistics, 2017. *CA: A Cancer Journal for Clinicians* 67(3): 177-193.

Simpson, A. J., Caballero, O. L., Jungbluth, A., Chen, Y.-T. & Old, L. J. 2005. Cancer/testis antigens, gametogenesis and cancer. *Nature Reviews Cancer* 5(8): 615-625.

Slater, S. 2001. Non-curative chemotherapy for cancer–is it worth it? *Clinical Medicine* 1(3): 220-222.

Smith, G., Carey, F. A., Beattie, J., Wilkie, M. J., Lightfoot, T. J., Coxhead, J., Garner, R. C., Steele, R. J. & Wolf, C. R. 2002. Mutations in APC, Kirsten-ras, and p53—alternative genetic pathways to colorectal cancer. *Proceedings of the National Academy of Sciences* 99(14): 9433-9438.

Souglakos, J., Kalykaki, A., Vamvakas, L., Androulakis, N., Kalbakis, K., Agelaki, S., Vardakis, N., Tzardi, M., Kotsakis, A. & Gioulbasanis, J. 2006. Phase II trial of capecitabine and oxaliplatin (CAPOX) plus cetuximab in patients with metastatic colorectal cancer who progressed after oxaliplatin-based chemotherapy. *Annals of Oncology* 18(2): 305-310.

Speetjens, F. M., Kuppen, P. J., Welters, M. J., Essahsah, F., Lantrua, M. G. K., Valentijn, A. R. P., Oostendorp, J., Fathers, L. M., Nijman, H. W. & Drijfhout, J. W. 2009. Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer. *Clinical Cancer Research* 15(3): 1086-1095.

Stanilov, N., Miteva, L., Dobreva, Z. & Stanilova, S. 2014. Colorectal cancer severity and survival in correlation with tumour necrosis factor-alpha. *Biotechnology & Biotechnological Equipment* 28(5): 911-917.

Stevenson, B. J., Iseli, C., Panji, S., Zahn-Zabal, M., Hide, W., Old, L. J., Simpson, A. J. & Jongeneel, C. V. 2007. Rapid evolution of cancer/testis genes on the X chromosome. *BMC Genomics* 8(1): 129-139.

Stewart, S. L., Wike, J. M., Kato, I., Lewis, D. R. & Michaud, F. 2006. A population ‐ based study of colorectal cancer histology in the United States, 1998–2001. *Cancer* 107(S5): 1128-1141.

Stickel, F. & Hampe, J. 2012. Genetic determinants of alcoholic liver disease. *Gut* 61(1): 150-159.

Suri, A. 2006. Cancer testis antigens–their importance in immunotherapy and in the early detection of cancer. *Expert Opinion on Biological Therapy* 6(4): 379-389.

Takahashi, N., Ohkuri, T., Homma, S., Ohtake, J., Wakita, D., Togashi, Y., Kitamura, H., Todo, S. & Nishimura, T. 2012. First clinical trial of cancer vaccine therapy with artificially synthesized helper/killeR hybrid epitope long peptide of MAGE-A4 cancer antigen. *Cancer Science* 103(1): 150-153.

Tariq, K. & Ghias, K. 2016. Colorectal cancer carcinogenesis: A review of mechanisms. *Cancer Biology & Medicine* 13(1): 120-135.

Tarnowski, M., Czerewaty, M., Deskur, A., Safranow, K., Marlicz, W., Urasińska, E., Ratajczak, M. Z. & Starzyńska, T. 2016. Expression of cancer testis antigens in colorectal cancer: New prognostic and therapeutic implications. *Disease Markers* 2016(1): 1-9.

Tewari, K., Nakayama, Y. & Suresh, M. 2007. Role of direct effects of IFN-γ on t cells in the regulation of CD8 T cell homeostasis. *The Journal of Immunology* 179(4): 2115-2125.

Thomas, A. M., Santarsiero, L. M., Lutz, E. R., Armstrong, T. D., Chen, Y.-C., Huang, L.-Q., Laheru, D. A., Goggins, M., Hruban, R. H. & Jaffee, E. M. 2004. Mesothelin-specific CD8+ T cell responses provide evidence of *in vivo* cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. *Journal of Experimental Medicine* 200(3): 297-306.

Thomas, D. A., Du, C., Xu, M., Wang, X. & Ley, T. J. 2000. DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. *Immunity* 12(6): 621-632.

Thomas, S., Snowden, J., Zeidler, M. & Danson, S. 2015. The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. *British Journal of Cancer* 113(3): 365-371.

Topalian, S. L., Weiner, G. J. & Pardoll, D. M. 2011. Cancer immunotherapy comes of age. *Journal of Clinical Oncology* 29(36): 4828-4836.

Toungouz, M., Libin, M., Bulté, F., Faid, L., Lehmann, F., Duriau, D., Laporte, M., Gangji, D., Bruyns, C. & Lambermont, M. 2001. Transient expansion of peptide-specific lymphocytes producing IFN-γ after vaccination with dendritic cells pulsed with MAGE peptides in patients with MAGE-A1/A3 positive tumors. *Journal of Leukocyte Biology* 69(6): 937-943.

Tran, E., Turcotte, S., Gros, A., Robbins, P. F., Lu, Y.-C., Dudley, M. E., Wunderlich, J. R., Somerville, R. P., Hogan, K. & Hinrichs, C. S. 2014. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science* 344(6184): 641-645.

Trapani, J. 2012. Granzymes, cytotoxic granules and cell death: The early work of Dr. Jurg Tschopp. *Cell Death and Differentiation* 19(1): 21-27.

Trapani, J. A. & Smyth, M. J. 2002. Functional significance of the perforin/granzyme cell death pathway. *Nature Reviews Immunology* 2(10): 735-747.

Türeci, Ö., Sahin, U., Zwick, C., Neumann, F. & Pfreundschuh, M. 1999. Exploitation of the antibody repertoire of cancer patients for the identification of human tumor antigens. *Hybridoma* 18(1): 23-28.

Türeci, Ö., Usener, D., Schneider, S. & Sahin, U. 2005. Identification of tumor-associated autoantigens with SEREX. *Adoptive Immunotherapy: Methods and Protocols* 137-154.

Twelves, C., Boyer, M., Findlay, M., Cassidy, J., Weitzel, C., Barker, C., Osterwalder, B., Jamieson, C., Hieke, K. & Group, X. C. C. S. 2001. Capecitabine (Xeloda™) improves medical resource use compared with 5-fluorouracil plus leucovorin in a phase III trial conducted in patients with advanced colorectal carcinoma. *European Journal of Cancer* 37(5): 597-604.

Urruticoechea, A., Alemany, R., Balart, J., Villanueva, A., Vinals, F. & Capella, G. 2010. Recent advances in cancer therapy: An overview. *Current Pharmaceutical Design* 16(1): 3-10.

Van Cutsem, E., Rivera, F., Berry, S., Kretzschmar, A., Michael, M., Dibartolomeo, M., Mazier, M.-A., Canon, J.-L., Georgoulias, V. & Peeters, M. 2009. Safety and efficacy of first line bevacizumab with FOLFOX, XELOX, FOLFIRI and fluoropyrimidines in metastatic colorectal cancer: The BEAT study. *Annals of Oncology* 20(11): 1842-1847.

Van Cutsem, E., Twelves, C., Cassidy, J., Allman, D., Bajetta, E., Boyer, M., Bugat, R., Findlay, M., Frings, S. & Jahn, M. 2001. Oral capecitabine compared with intravenous fluorouracil plus leucovorin in patients with metastatic colorectal cancer: Results of a large phase III study. *Journal of Clinical Oncology* 19(21): 4097-4106.

Van Den Broek, M. F. & Hengartner, H. 2000. The role of perforin in infections and tumour surveillance. *Experimental Physiology* 85(6): 681-685.

Van Der Burg, S. H., Arens, R., Ossendorp, F., Van Hall, T. & Melief, C. J. 2016. Vaccines for established cancer: Overcoming the challenges posed by immune evasion. *Nature Reviews Cancer* 16(4): 219-233.

Van Eck, J. A., Shan, L., Meeldijk, J., Hack, C. E. & Bovenschen, N. 2017. A novel proinflammatory role for granzyme A. *Cell Death & Disease* 8(1): e2630.

Van Kaer, L., Parekh, V. V. & Wu, L. 2011. Invariant NK T cells: Potential for immunotherapeutic targeting with glycolipid antigens. *Immunotherapy* 3(1): 59-75.

Veettil, S. K., Lim, K. G., Chaiyakunapruk, N., Ching, S. M. & Abu Hassan, M. R. 2016. Colorectal cancer in Malaysia: Its burden and implications for a multiethnic country. *Asian Journal of Surgery* 40(6): 481-489.

Voskoboinik, I., Smyth, M. J. & Trapani, J. A. 2006. Perforin-mediated target-cell death and immune homeostasis. *Nature Reviews Immunology* 6(12): 940-952.

Voskoboinik, I., Whisstock, J. C. & Trapani, J. A. 2015. Perforin and granzymes: Function, dysfunction and human pathology. *Nature Reviews Immunology* 15(6): 388-400.

Vreede, J., Van Der Horst, M. A., Hellingwerf, K. J., Crielaard, W. & Van Aalten, D. M. 2003. PAS domains common structure and common flexibility. *Journal of Biological Chemistry* 278(20): 18434-18439.

Vyas, J. M., Van Der Veen, A. G. & Ploegh, H. L. 2008. The known unknowns of antigen processing and presentation. *Nature Reviews Immunology* 8(8): 607-618.

Walko, C. M. & Lindley, C. 2005. Capecitabine: A review. *Clinical Therapeutics* 27(1): 23-44.

Wang, L., Wang, Y., Song, Z., Chu, J. & Qu, X. 2015. Deficiency of interferon-gamma or its receptor promotes colorectal cancer development. *Journal of Interferon & Cytokine Research* 35(4): 273-280.

Waring, P. & Müllbacher, A. 1999. Cell death induced by the Fas/Fas ligand pathway and its role in pathology. *Immunology and Cell Biology* 77(4): 312-317.

Waterhouse, N. J., Sedelies, K. A. & Trapani, J. A. 2006. Role of Bid-induced mitochondrial outer membrane permeabilization in granzyme B-induced apoptosis. *Immunology and Cell Biology* 84(1): 72-78.

Weber, J., Gibney, G., Kudchadkar, R., Yu, B., Cheng, P., Martinez, A. J., Kroeger, J., Richards, A., Mccormick, L. & Moberg, V. 2016. Phase I/II study of metastatic melanoma patients treated with nivolumab who had progressed after ipilimumab. *Cancer Immunology Research* 4(4): 345-353.

Weisenberger, D. J., Siegmund, K. D., Campan, M., Young, J., Long, T. I., Faasse, M. A., Kang, G. H., Widschwendter, M., Weener, D. & Buchanan, D. 2006. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nature Genetics* 38(7): 787-793.

Weiser, T. S., Guo, Z. S., Ohnmacht, G. A., Parkhurst, M. L., Tong-On, P., Marincola, F. M., Fischette, M. R., Yu, X., Chen, G. A. & Hong, J. A. 2001. Sequential 5-aza-2-deoxycytidine-depsipeptide FR901228 treatment induces apoptosis preferentially in cancer cells and facilitates their recognition by cytolytic T lymphocytes specific for NY-ESO-1. *Journal of Immunotherapy* 24(2): 151-161.

Whitehurst, A. W. 2014. Cause and consequence of cancer/testis antigen activation in cancer. *Annual Review of Pharmacology and Toxicology* 54(1): 251-272.

Wiemann, B. & Starnes, C. O. 1994. Coley's toxins, tumor necrosis factor and cancer research: A historical perspective. *Pharmacology & Therapeutics* 64(3): 529-564.

Williams, N. S. & Engelhard, V. H. 1997. Perforin-dependent cytotoxic activity and lymphokine secretion by CD4+ T cells are regulated by CD8+ T cells. *The Journal of Immunology* 159(5): 2091-2099.

Winocur, G., Wojtowicz, J. M., Merkley, C. M. & Tannock, I. F. 2016. Environmental enrichment protects against cognitive impairment following chemotherapy in an animal model. *Behavioral Neuroscience* 130(4): 428-436.

Wischnewski, F., Pantel, K. & Schwarzenbach, H. 2006. Promoter demethylation and histone acetylation mediate gene expression of MAGE-A1,-A2,-A3, and-A12 in human cancer cells. *Molecular Cancer Research* 4(5): 339-349.

Wolpin, B. M. & Mayer, R. J. 2008. Systemic treatment of colorectal cancer. *Gastroenterology* 134(5): 1296-1310.e1291.

Xi, J., Xu, M., Song, Z., Li, H., Xu, S., Wang, C., Song, H. & Bai, J. 2017. Stimulatory role of interleukin 10 in CD8+ T cells through STATs in gastric cancer. *Tumor Biology* 39(5): 1-9.

Yang, J., Pemberton, A., Morrison, W. I. & Connelley, T. 2019. Granzyme B is an essential mediator in CD8+ T cell killing of Theileria parva-infected cells. *Infection and Immunity* 87(1): e00386-00318.

Younes, A., Pro, B., Robertson, M. J., Flinn, I. W., Romaguera, J. E., Hagemeister, F., Dang, N. H., Fiumara, P., Loyer, E. M. & Cabanillas, F. F. 2004. Phase II clinical trial of interleukin-12 in patients with relapsed and refractory non-Hodgkin's lymphoma and Hodgkin's disease. *Clinical Cancer Research* 10(16): 5432-5438.

Zahary, M. N., Ankathil, R., Yahaya, M. M., Tuan Shariff, S. E. & Kaur, G. 2017. Protein expression profiles of deoxyribonucleic acid mismatch repair genes: Association with clinicopathological characteristics of Malaysian Lynch syndrome patients. *Journal of Histotechnology* 40(1): 13-20.

Zainal Ariffin, O. & Nor Saleha, I. 2011. National cancer registry report 2007. Malaysia: Ministry of Health.

Zendman, A. J., Cornelissen, I. M., Weidle, U. H., Ruiter, D. J. & Van Muijen, G. N. 1999. CTp11, a novel member of the family of human cancer/testis antigens. *Cancer Research* 59(24): 6223-6229.

Zendman, A. J., Ruiter, D. J. & Van Muijen, G. N. 2003. Cancer/testis ‐ associated genes: Identification, expression profile, and putative function. *Journal of Cellular Physiology* 194(3): 272-288.

Zeng, C. 2000. NuMA: A nuclear protein involved in mitotic centrosome function. *Microscopy Research and Technique* 49(5): 467-477.

Zhan, H., Gao, X., Pu, X., Li, W., Li, Z., Zhou, X. & Qiu, J. 2012. A randomized controlled trial of postoperative tumor lysate-pulsed dendritic cells and cytokine-induced killer cells immunotherapy in patients with localized and locally advanced renal cell carcinoma. *Chinese Medical Journal* 125(21): 3771-3777.

Zhan, W., Zhang, Z., Zhang, Y., Ma, J., Wu, T., Gu, Y., Li, Y. & Yang, J. 2016. Prognostic value of MAGE-A9 expression in patients with colorectal cancer. *Clinics and Research in Hepatology and Gastroenterology* 40(2): 239-245.

Zhang, D., Beresford, P. J., Greenberg, A. H. & Lieberman, J. 2001. Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granule-mediated cytolysis. *Proceedings of the National Academy of Sciences* 98(10): 5746-5751.

Zhang, H.-G., Chen, H.-S., Peng, J.-R., Shang, X.-Y., Zhang, J., Xing, Q., Pang, X.-W., Qin, L.-L., Fei, R. & Mei, M.-H. 2007. Specific CD8+ T cell responses to HLA-A2 restricted MAGE-A3 p271–279 peptide in hepatocellular carcinoma patients without vaccination. *Cancer Immunology, Immunotherapy* 56(12): 1945-1954.

Zhang, L. J. & Wang, X. Z. 2006. Interleukin-10 and chronic liver disease. *World Journal of Gastroenterology* 12(11): 1681-1685.

Zhang, M., Lu, G., Meng, F., Li, S., Li, X. & Gong, X. 2018. Identification of HLA-A2-restricted immunogenic peptides derived from vitamin D-binding protein. *Cellular Immunology* 328(1): 18-23.

Zhang, Q. M., He, S. J., Shen, N., Luo, B., Fan, R., Fu, J., Luo, G. R., Zhou, S. F., Xiao, S. W. & Xie, X. X. 2014. Overexpression of MAGE-D4 in colorectal cancer is a potentially prognostic biomarker and immunotherapy target. *International Journal of Clinical and Experimental Pathology* 7(7): 3918-3927.

Zhang, Y., Yuan, F., Presnell, S. R., Tian, K., Gao, Y., Tomkinson, A. E., Gu, L. & Li, G.-M. 2005. Reconstitution of 5'-directed human mismatch repair in a purified system. *Cell* 122(5): 693-705.

Zhu, P., Zhang, D., Chowdhury, D., Martinvalet, D., Keefe, D., Shi, L. & Lieberman, J. 2006. Granzyme A, which causes single stranded DNA damage, targets the double strand break repair protein Ku70. *EMBO Reports* 7(4): 431-437.

Zur Hausen, H. 2009. Papillomaviruses in the causation of human cancers-a brief historical account. *Virology* 384(2): 260-265.

APPENDIX A ETHICS APPLICATION

FRGS/1/2016/SKK02/UKM/02/1

UNIVERSITI KEBANGSAAN MALAYSIA
The National University of Malaysia

Pusat Pengurusan Penyelidikan dan Instrumentasi ***Centre for Research and Instrumentation Management***

Rujukan : UKM PPI/111/8/JEP-2017-584
Tarikh : 11 Disember 2017

DITERIMA
[illegible] DEC 2017
Pejabat Pro-Naib Canselor
(Kampus Kuala Lumpur)
UKM

Profesor Datuk Dr. A Rahman A Jamal
Institut Perubatan Molekul UKM (UMBI)
Universiti Kebangsaan Malaysia

Y.Bhg. Profesor/Datuk/Dato'/Datin/Tuan/Puan,

KELULUSAN ETIKA MENJALANKAN PENYELIDIKAN DI UKM

Tajuk Penyelidikan : *Validation Of SP17 And PASD1 Protein As An Immunotherapeutic Target In Colorectal Cancer*

Perkara yang tersebut di atas adalah dirujuk.

2. Sukacita dimaklumkan, Jawatankuasa Etika Penyelidikan UKM meluluskan permohonan penyelidikan Y. Bhg Profesor/Datuk/Dato'/Datin/Tuan/Puan bagi tajuk diatas. Tempoh kelulusan penyelidikan adalah daripada **28 November 2017 - 27 November 2020.** Sila kemukakan sebarang Laporan **Kesan Sampingan, Laporan Kemajuan Setiap 6 Bulan** dan **Laporan Akhir** sebaik sahaja penyelidikan tamat kepada Jawatankuasa Etika Penyelidikan UKM.

3. Sukacita diingatkan projek penyelidikan ini hanya boleh dijalankan setelah mendapat **surat kelulusan menjalankan penyelidikan** dari Timbalan Dekan Penyelidikan Fakulti atau Pengarah Pusat/Institut.

Sekian, terima kasih.

Yang benar,

PROFESOR DATO' DR. FUAD ISMAIL
Pengerusi
Jawatankuasa Etika Penyelidikan
Universiti Kebangsaan Malaysia

12 DEC 2017

s.k. - **Pengarah**
Pusat Pengurusan Penyelidikan dan Instrumentasi (CRIM)
Universiti Kebangsaan Malaysia

- **Pengarah**
Institut Perubatan Molekul UKM (UMBI)
Universiti Kebangsaan Malaysia

- **Profesor Dr. Shamsul Azhar Shah**
- **Profesor Madya Dr. Neoh Hui Min**
- **Dr. Nadiah Abu**
- **Joanne Soh Ern Chi (P90450)**
Institut Perubatan Molekul UKM (UMBI)
Universiti Kebangsaan Malaysia

UNIVERSITI KEBANGSAAN MALAYSIA
The National University of Malaysia

Pusat Pengurusan Penyelidikan dan Instrumentasi ***Centre for Research and Instrumentation Management***

NAME OF ETHICS COMMITTEE/IRB: Research Ethics Committee, The National University of Malaysia	**ETHICS COMMITTEE/IRB REF NO :** **UKM PPI/111/8/JEP-2017-584**

PROTOCOL TITLE :
Validation Of SP17 And PASD1 Protein As An Immunotherapeutic Target In Colorectal Cancer

PRINCIPAL INVESTIGATOR :
Professor Datuk Dr. A Rahman A Jamal
UKM Medical Molecular Biology Institute (UMBI)
The National University of Malaysia

The following items ☒ have been received and reviewed in connection with the above study to be conducted by the above investigator.

Documents
☒ Research Application Form
☒ Research Proposal / Protocol
☒ Publication Policy
☒ Non-Disclosure Agreement
Information Sheet:-
 ☒ Malay ☒ English
Consent Form:-
 ☒ Malay ☒ English
Questionnaire:-
 ☐ Malay ☐ English
Curriculum Vitae of Researcher:-
 ☒ Principal ☒ Co-researcher ☒ Student
☒ Good Clinical Practice Certificate (GCP)
☐ Project Agreement

The Research Ethics Committee, The National University of Malaysia operates in accordance to the International Conference of Harmonization Good Clinical Practice Guidelines.

Comments (if any): Profesor Dr. Shamsul Azhar Shah is the co-investigator for this study and also member of UKM Research Ethics Committee. He strictly was not involved in the decision of UKM Research Ethics Committee to approve this study.

Date of Approval: 28 November 2017

PROFESSOR DATO' DR. FUAD ISMAIL
Chairman
Research Ethics Committee
The National University of Malaysia

Research Ethics Committee, The National University Of Malaysia, 1st Floor, Clinical Block, Hospital Canselor Tuanku Muhriz, UKM Medical Centre
Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia. **Telefon: +603-9145 5046 / 5048**

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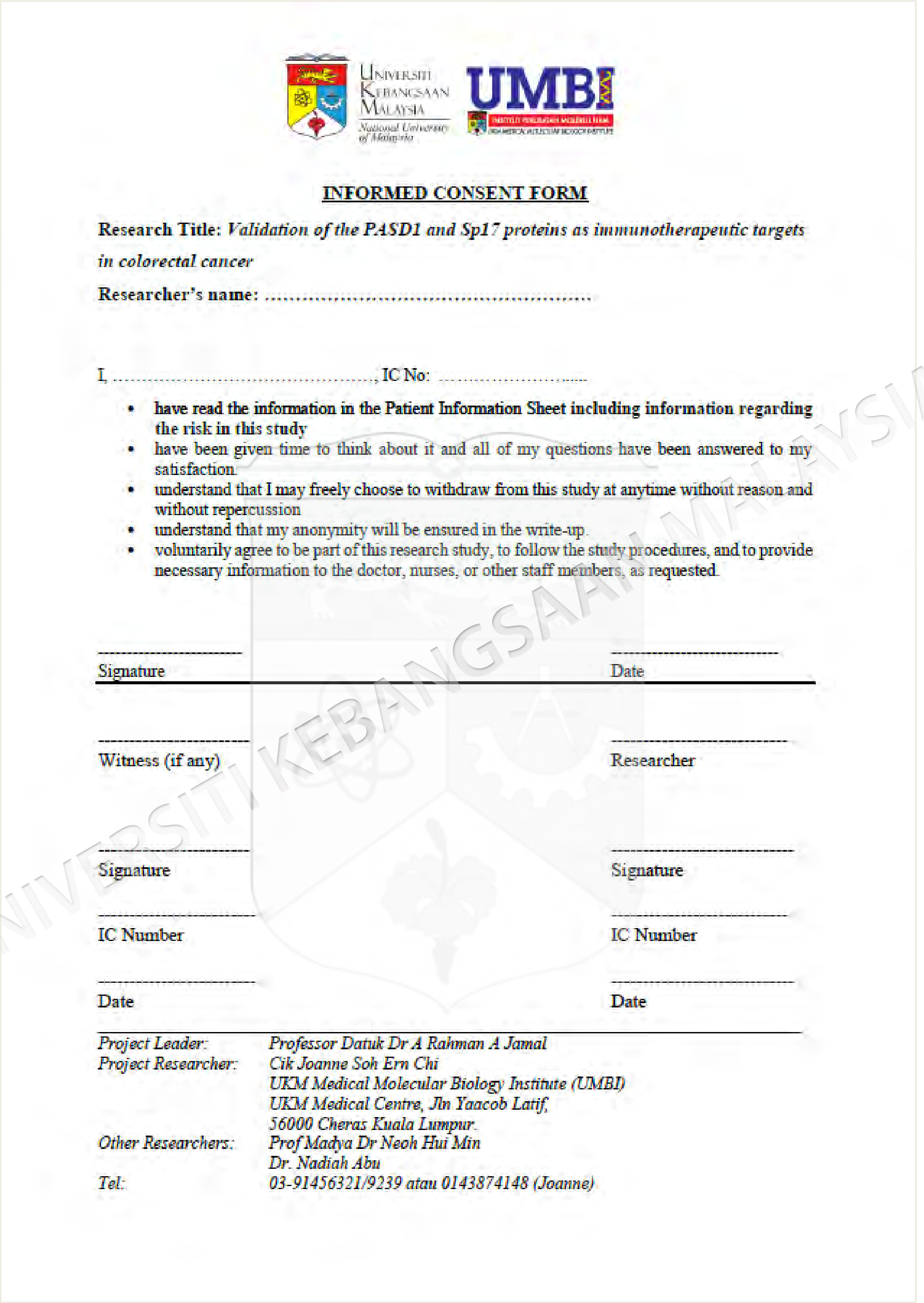
APPENDIX B INFORMED CONSENT FORM



INFORMED CONSENT FORM

Research Title: ***Validation of the PASD1 and Sp17 proteins as immunotherapeutic targets in colorectal cancer***

Researcher's name: ....

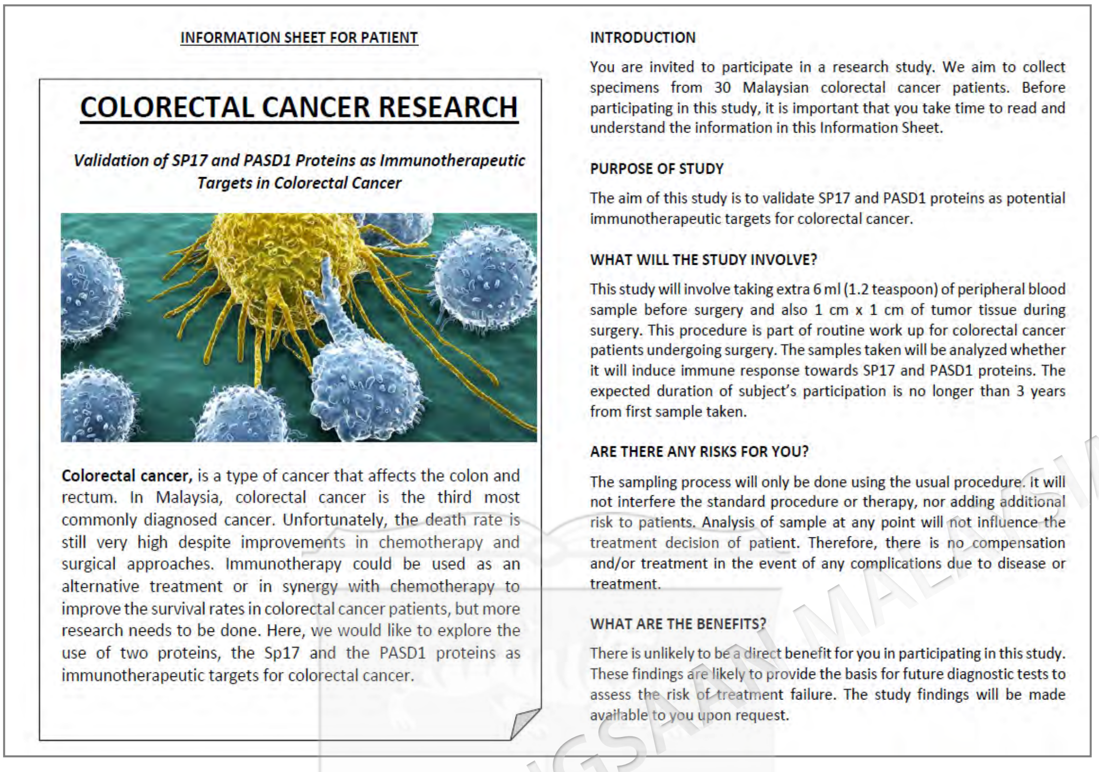
I, ..., IC No: ....

- have read the information in the Patient Information Sheet including information regarding the risk in this study
- have been given time to think about it and all of my questions have been answered to my satisfaction.
- understand that I may freely choose to withdraw from this study at anytime without reason and without repercussion
- understand that my anonymity will be ensured in the write-up.
- voluntarily agree to be part of this research study, to follow the study procedures, and to provide necessary information to the doctor, nurses, or other staff members, as requested.

------------------------ Signature	---------------------------- Date
------------------------ Witness (if any)	---------------------------- Researcher
------------------------ Signature	---------------------------- Signature
------------------------ IC Number	---------------------------- IC Number
------------------------ Date	---------------------------- Date

Project Leader:	*Professor Datuk Dr A Rahman A Jamal*
Project Researcher:	*Cik Joanne Soh Ern Chi* *UKM Medical Molecular Biology Institute (UMBI)* *UKM Medical Centre, Jln Yaacob Latif,* *56000 Cheras Kuala Lumpur.*
Other Researchers:	*Prof Madya Dr Neoh Hui Min* *Dr. Nadiah Abu*
Tel:	*03-91456321/9239 atau 0143874148 (Joanne)*

APPENDIX C INFORMATION SHEET FOR PATIENT



INFORMATION SHEET FOR PATIENT

COLORECTAL CANCER RESEARCH

Validation of SP17 and PASD1 Proteins as Immunotherapeutic Targets in Colorectal Cancer

Colorectal cancer, is a type of cancer that affects the colon and rectum. In Malaysia, colorectal cancer is the third most commonly diagnosed cancer. Unfortunately, the death rate is still very high despite improvements in chemotherapy and surgical approaches. Immunotherapy could be used as an alternative treatment or in synergy with chemotherapy to improve the survival rates in colorectal cancer patients, but more research needs to be done. Here, we would like to explore the use of two proteins, the Sp17 and the PASD1 proteins as immunotherapeutic targets for colorectal cancer.

INTRODUCTION

You are invited to participate in a research study. We aim to collect specimens from 30 Malaysian colorectal cancer patients. Before participating in this study, it is important that you take time to read and understand the information in this Information Sheet.

PURPOSE OF STUDY

The aim of this study is to validate SP17 and PASD1 proteins as potential immunotherapeutic targets for colorectal cancer.

WHAT WILL THE STUDY INVOLVE?

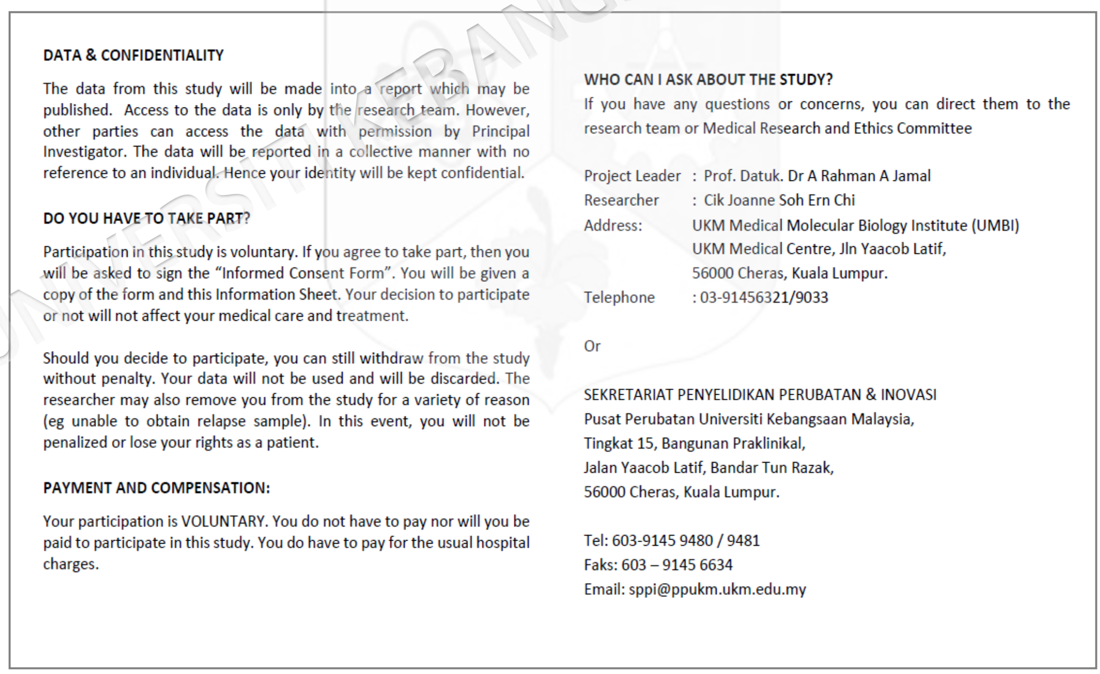
This study will involve taking extra 6 ml (1.2 teaspoon) of peripheral blood sample before surgery and also 1 cm x 1 cm of tumor tissue during surgery. This procedure is part of routine work up for colorectal cancer patients undergoing surgery. The samples taken will be analyzed whether it will induce immune response towards SP17 and PASD1 proteins. The expected duration of subject's participation is no longer than 3 years from first sample taken.

ARE THERE ANY RISKS FOR YOU?

The sampling process will only be done using the usual procedure. It will not interfere the standard procedure or therapy, nor adding additional risk to patients. Analysis of sample at any point will not influence the treatment decision of patient. Therefore, there is no compensation and/or treatment in the event of any complications due to disease or treatment.

WHAT ARE THE BENEFITS?

There is unlikely to be a direct benefit for you in participating in this study. These findings are likely to provide the basis for future diagnostic tests to assess the risk of treatment failure. The study findings will be made available to you upon request.



DATA & CONFIDENTIALITY

The data from this study will be made into a report which may be published. Access to the data is only by the research team. However, other parties can access the data with permission by Principal Investigator. The data will be reported in a collective manner with no reference to an individual. Hence your identity will be kept confidential.

DO YOU HAVE TO TAKE PART?

Participation in this study is voluntary. If you agree to take part, then you will be asked to sign the "Informed Consent Form". You will be given a copy of the form and this Information Sheet. Your decision to participate or not will not affect your medical care and treatment.

Should you decide to participate, you can still withdraw from the study without penalty. Your data will not be used and will be discarded. The researcher may also remove you from the study for a variety of reason (eg unable to obtain relapse sample). In this event, you will not be penalized or lose your rights as a patient.

PAYMENT AND COMPENSATION:

Your participation is VOLUNTARY. You do not have to pay nor will you be paid to participate in this study. You do have to pay for the usual hospital charges.

WHO CAN I ASK ABOUT THE STUDY?

If you have any questions or concerns, you can direct them to the research team or Medical Research and Ethics Committee

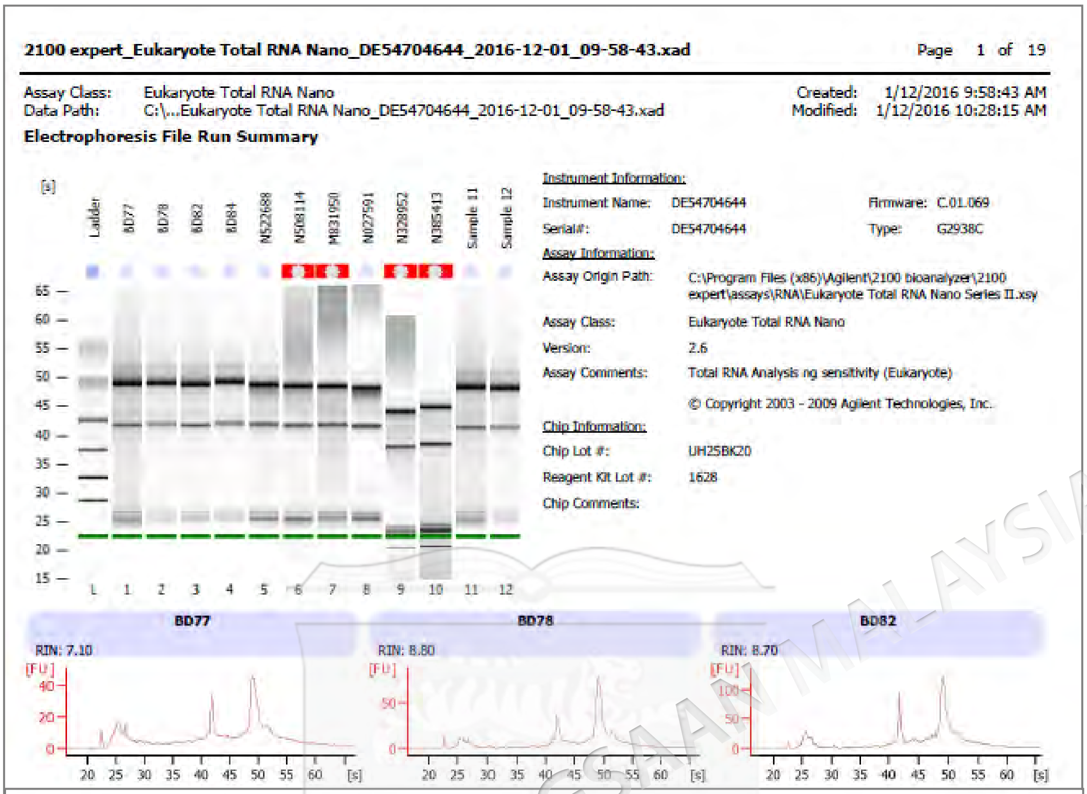
Project Leader : Prof. Datuk. Dr A Rahman A Jamal
Researcher : Cik Joanne Soh Ern Chi
Address: UKM Medical Molecular Biology Institute (UMBI)
UKM Medical Centre, Jln Yaacob Latif,
56000 Cheras, Kuala Lumpur.
Telephone : 03-91456321/9033

Or

SEKRETARIAT PENYELIDIKAN PERUBATAN & INOVASI
Pusat Perubatan Universiti Kebangsaan Malaysia,
Tingkat 15, Bangunan Praklinikal,
Jalan Yaacob Latif, Bandar Tun Razak,
56000 Cheras, Kuala Lumpur.

Tel: 603-9145 9480 / 9481
Faks: 603 – 9145 6634
Email: sppi@ppukm.ukm.edu.my

APPENDIX D RNA RIN NUMBER



2100 expert_Eukaryote Total RNA Nano_DE54704644_2016-12-01_09-58-43.xad Page 1 of 19

Assay Class: Eukaryote Total RNA Nano
Data Path: C:\...Eukaryote Total RNA Nano_DE54704644_2016-12-01_09-58-43.xad
Created: 1/12/2016 9:58:43 AM
Modified: 1/12/2016 10:28:15 AM

Electrophoresis File Run Summary

Instrument Information:
Instrument Name: DE54704644
Firmware: C.01.069
Serial#: DE54704644
Type: G2938C

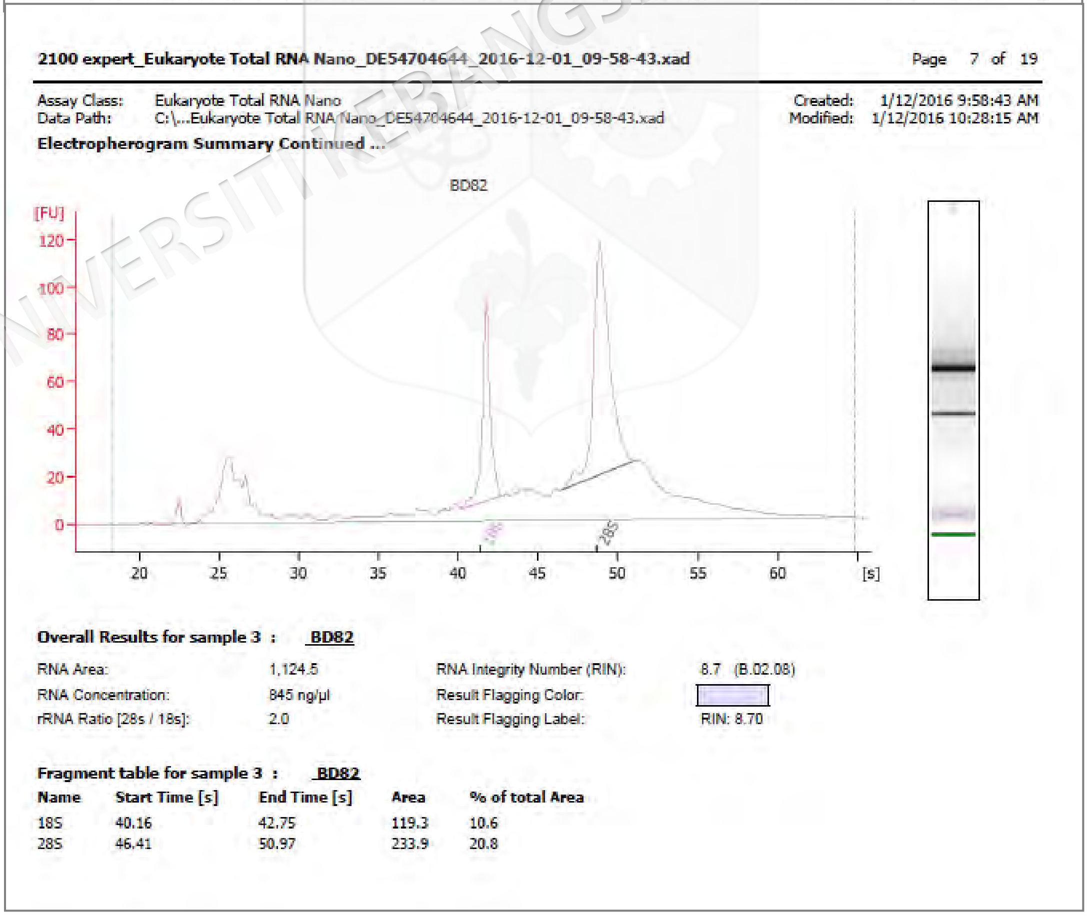
Assay Information:
Assay Origin Path: C:\Program Files (x86)\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\Eukaryote Total RNA Nano Series II.xsy
Assay Class: Eukaryote Total RNA Nano
Version: 2.6
Assay Comments: Total RNA Analysis ng sensitivity (Eukaryote)
© Copyright 2003 - 2009 Agilent Technologies, Inc.

Chip Information:
Chip Lot #: UH25BK20
Reagent Kit Lot #: 1628
Chip Comments:

BD77
RIN: 7.10

BD78
RIN: 8.80

BD82
RIN: 8.70



2100 expert_Eukaryote Total RNA Nano_DE54704644_2016-12-01_09-58-43.xad Page 7 of 19

Assay Class: Eukaryote Total RNA Nano
Data Path: C:\...Eukaryote Total RNA Nano_DE54704644_2016-12-01_09-58-43.xad
Created: 1/12/2016 9:58:43 AM
Modified: 1/12/2016 10:28:15 AM

Electropherogram Summary Continued ...

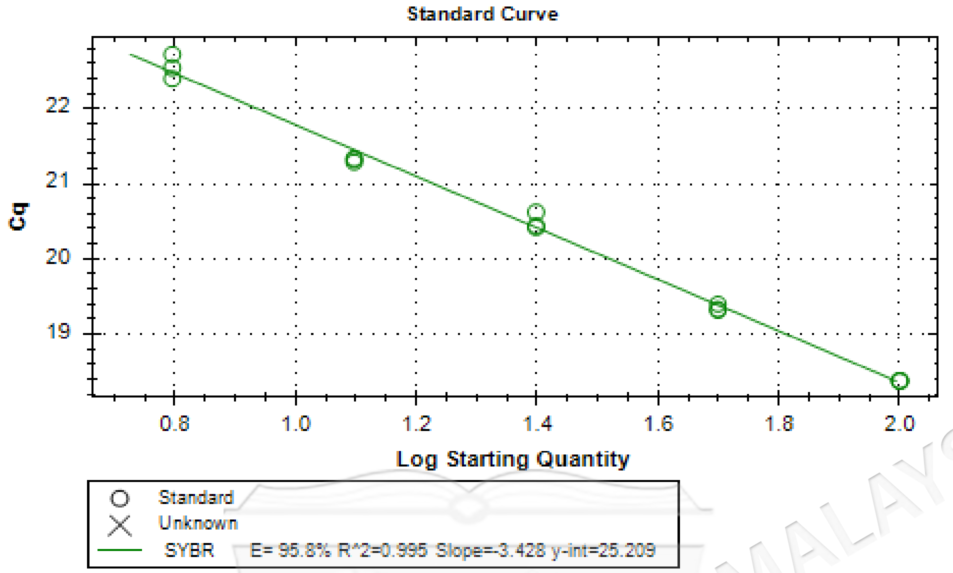
Overall Results for sample 3 : BD82

RNA Area:	1,124.5	RNA Integrity Number (RIN):	8.7 (B.02.08)
RNA Concentration:	845 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	2.0	Result Flagging Label:	RIN: 8.70

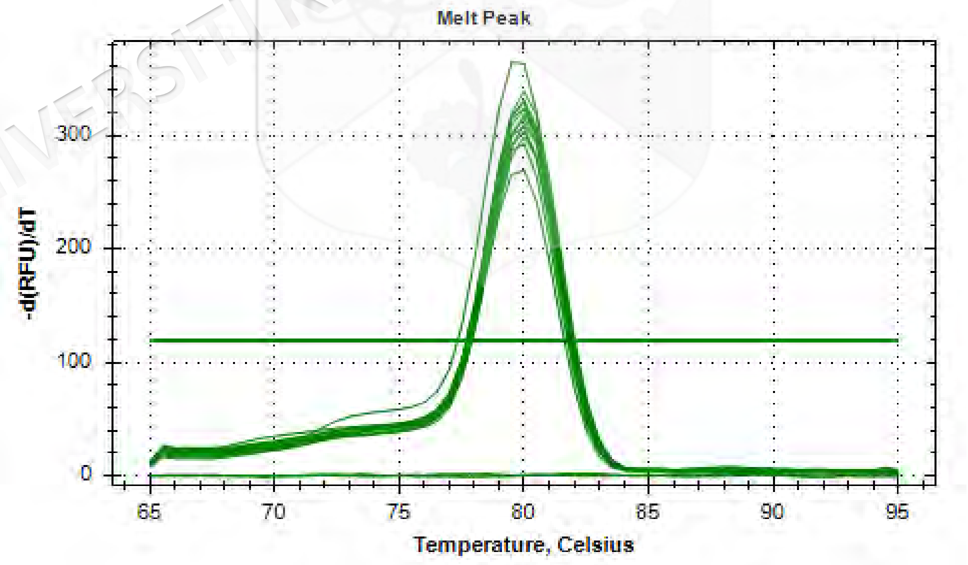
Fragment table for sample 3 : BD82

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.16	42.75	119.3	10.6
28S	46.41	50.97	233.9	20.8

APPENDIX E PRIMER: *GAPDH*

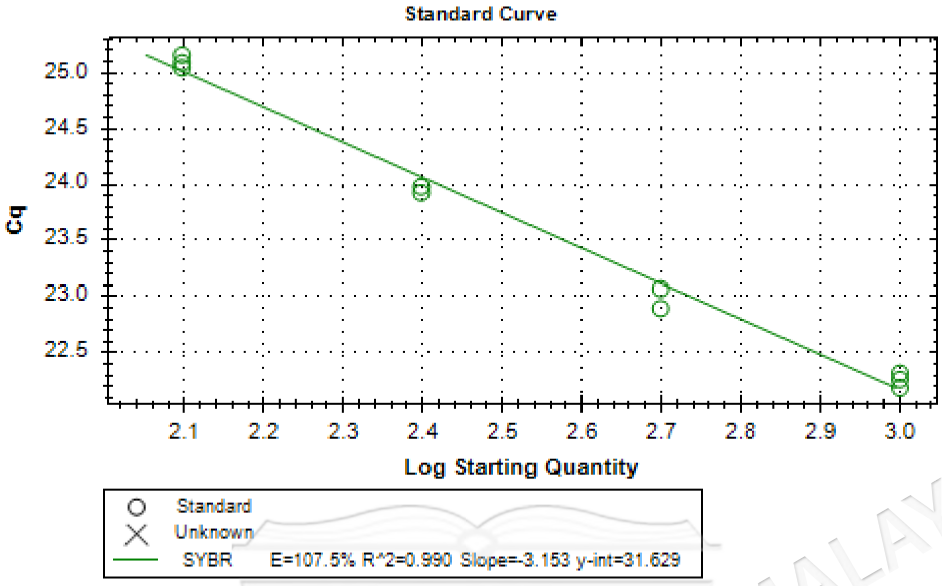


The standard curve for *GAPDH* primer with cDNA (100 ng) extracted from SW480. The data presented in triplicates with E=95.8%, R square value: 0.995 and slope = -3.4.

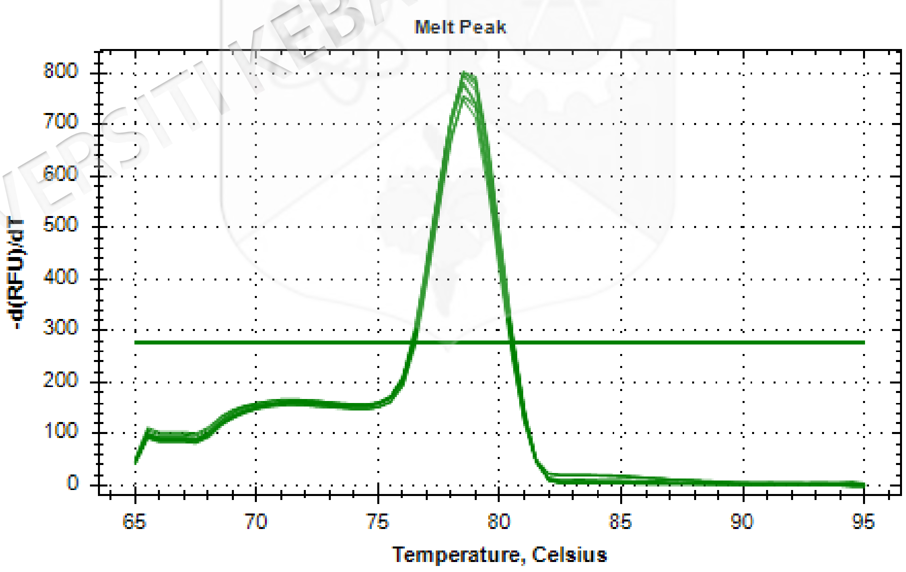


The melt peak for *GAPDH* primer with cDNA (100 ng) extracted from SW480. The data presented in triplicates with a melt peak at 80˚C.

APPENDIX F PRIMER: *PASD1*



The standard curve for *PASD1* primer with cDNA (100 ng) extracted from SW480. The data presented in triplicates with E=107.5%, R square value: 0.990 and slope = -3.153.

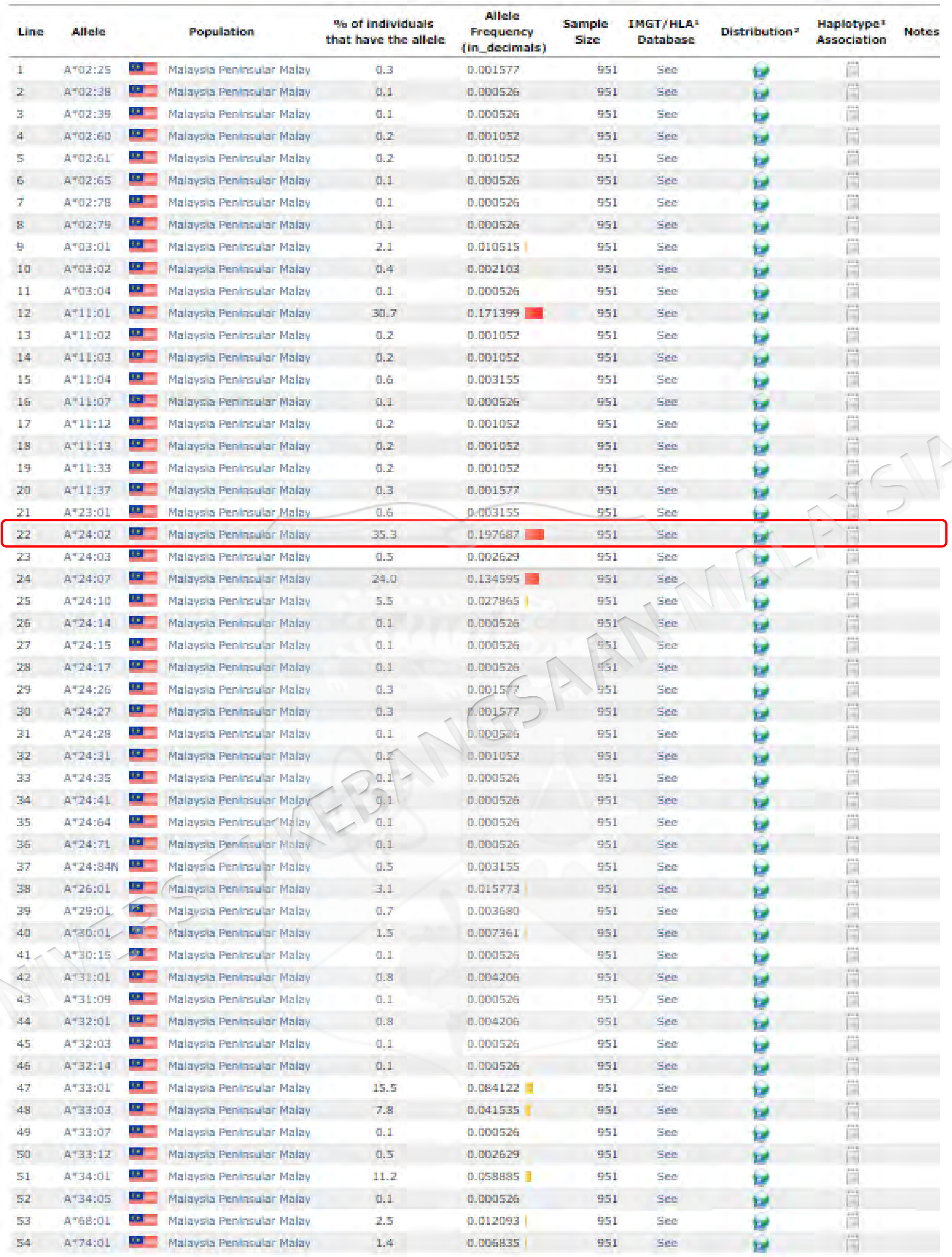


The melt peak for *PASD1* primer with cDNA (100 ng) extracted from SW480. The data presented in triplicates with a melting peak at 78.5˚C.

APPENDIX G ALLELE FREQUENCY NETWORK DATABASE

Line	Allele	Population	% of individuals that have the allele	Allele Frequency (in_decimals)	Sample Size	IMGT/HLA[1] Database	Distribution[2]	Haplotype[3] Association	Notes[4]
1	A*02:27	Malaysia Peninsular Chinese	0.5	0.002580	194	See			
2	A*02:68	Malaysia Peninsular Chinese	0.5	0.002580	194	See			
3	A*03:01	Malaysia Peninsular Chinese	0.5	0.002580	194	See			
4	A*11:01	Malaysia Peninsular Chinese	50.0	0.296390 🟥	194	See			
5	A*11:02	Malaysia Peninsular Chinese	1.6	0.007730	194	See			
6	A*11:09	Malaysia Peninsular Chinese	1.0	0.005150	194	See			
7	A*11:15	Malaysia Peninsular Chinese	0.5	0.002580	194	See			
8	A*11:23	Malaysia Peninsular Chinese	0.5	0.002580	194	See			
9	A*23:01	Malaysia Peninsular Chinese	1.0	0.005150	194	See			
10	A*24:02	Malaysia Peninsular Chinese	28.9	0.159790 🟥	194	See			
11	A*24:07	Malaysia Peninsular Chinese	2.1	0.010310	194	See			
12	A*26:01	Malaysia Peninsular Chinese	6.7	0.033510 🟨	194	See			
13	A*29:01	Malaysia Peninsular Chinese	0.5	0.002580	194	See			
14	A*30:01	Malaysia Peninsular Chinese	2.1	0.010310	194	See			
15	A*31:01	Malaysia Peninsular Chinese	2.6	0.012890	194	See			
16	A*32:03	Malaysia Peninsular Chinese	0.5	0.002580	194	See			
17	A*33:01	Malaysia Peninsular Chinese	18.0	0.097940 🟨	194	See			
18	A*33:03	Malaysia Peninsular Chinese	4.1	0.028350 🟨	194	See			
19	A*33:12	Malaysia Peninsular Chinese	1.0	0.005150	194	See			
20	A*69:01	Malaysia Peninsular Chinese	0.5	0.002580	194	See			

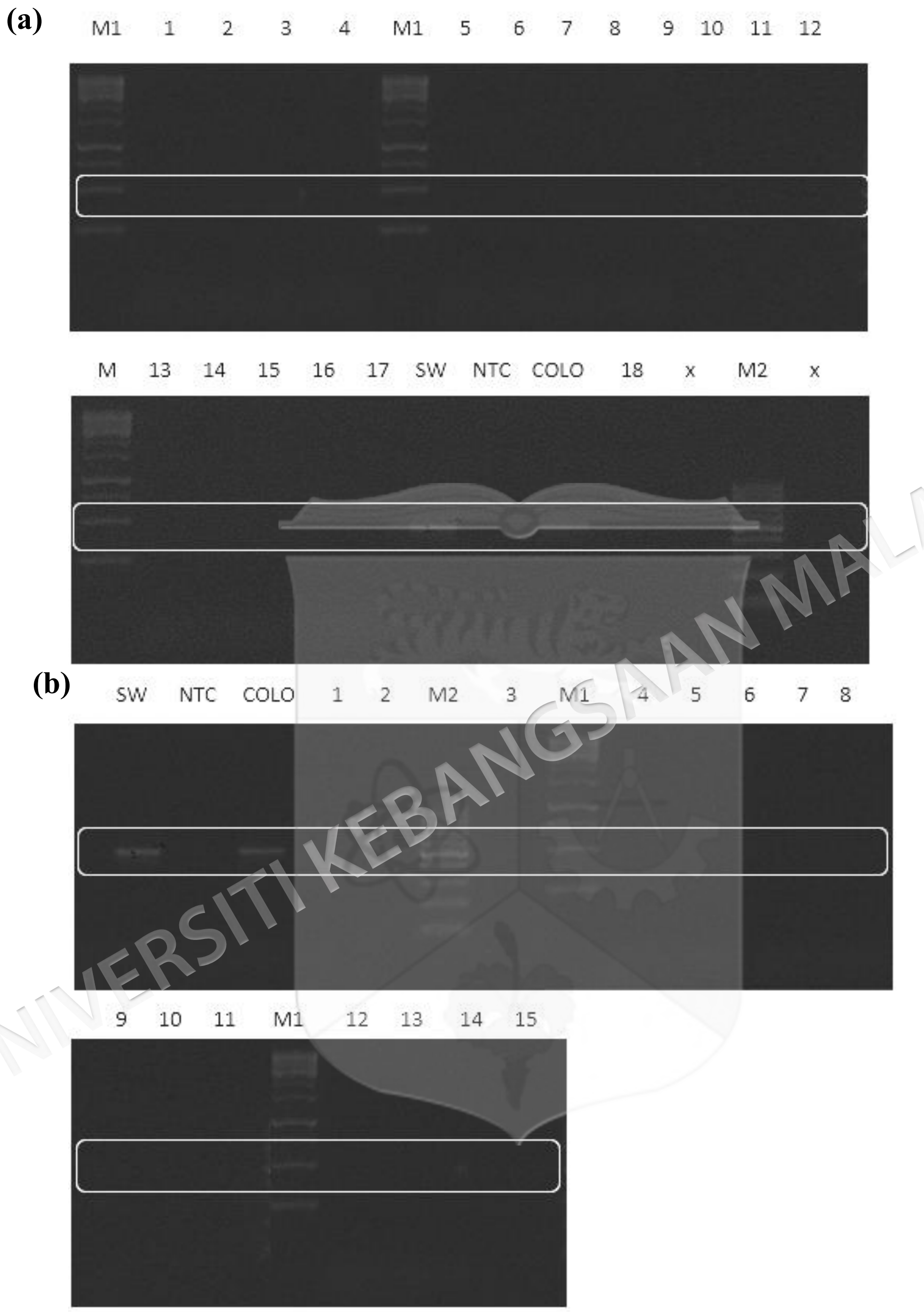
The HLA-A*24:02 allele frequency in Peninsular Malaysia Chinese population reported in AFND. The percentage of an individual with HLA-A*24:02 positive allele was identified to be 28.9% and denoted (with red bar) to be the second highest frequency allele in this population (http://www.allelefrequencies.net/default.asp).



Line	Allele	Population	% of individuals that have the allele	Allele Frequency (in_decimals)	Sample Size	IMGT/HLA[1] Database	Distribution[2]	Haplotype[3] Association	Notes
1	A*02:25	Malaysia Peninsular Malay	0.3	0.001577	951	See			
2	A*02:38	Malaysia Peninsular Malay	0.1	0.000526	951	See			
3	A*02:39	Malaysia Peninsular Malay	0.1	0.000526	951	See			
4	A*02:60	Malaysia Peninsular Malay	0.2	0.001052	951	See			
5	A*02:61	Malaysia Peninsular Malay	0.2	0.001052	951	See			
6	A*02:65	Malaysia Peninsular Malay	0.1	0.000526	951	See			
7	A*02:78	Malaysia Peninsular Malay	0.1	0.000526	951	See			
8	A*02:79	Malaysia Peninsular Malay	0.1	0.000526	951	See			
9	A*03:01	Malaysia Peninsular Malay	2.1	0.010515 🟨	951	See			
10	A*03:02	Malaysia Peninsular Malay	0.4	0.002103	951	See			
11	A*03:04	Malaysia Peninsular Malay	0.1	0.000526	951	See			
12	A*11:01	Malaysia Peninsular Malay	30.7	0.171399 🟥	951	See			
13	A*11:02	Malaysia Peninsular Malay	0.2	0.001052	951	See			
14	A*11:03	Malaysia Peninsular Malay	0.2	0.001052	951	See			
15	A*11:04	Malaysia Peninsular Malay	0.6	0.003155	951	See			
16	A*11:07	Malaysia Peninsular Malay	0.1	0.000526	951	See			
17	A*11:12	Malaysia Peninsular Malay	0.2	0.001052	951	See			
18	A*11:13	Malaysia Peninsular Malay	0.2	0.001052	951	See			
19	A*11:33	Malaysia Peninsular Malay	0.2	0.001052	951	See			
20	A*11:37	Malaysia Peninsular Malay	0.3	0.001577	951	See			
21	A*23:01	Malaysia Peninsular Malay	0.6	0.003155	951	See			
22	A*24:02	Malaysia Peninsular Malay	35.3	0.197687 🟥	951	See			
23	A*24:03	Malaysia Peninsular Malay	0.5	0.002629	951	See			
24	A*24:07	Malaysia Peninsular Malay	24.0	0.134595 🟥	951	See			
25	A*24:10	Malaysia Peninsular Malay	5.5	0.027865 🟨	951	See			
26	A*24:14	Malaysia Peninsular Malay	0.1	0.000526	951	See			
27	A*24:15	Malaysia Peninsular Malay	0.1	0.000526	951	See			
28	A*24:17	Malaysia Peninsular Malay	0.1	0.000526	951	See			
29	A*24:26	Malaysia Peninsular Malay	0.3	0.001577	951	See			
30	A*24:27	Malaysia Peninsular Malay	0.3	0.001577	951	See			
31	A*24:28	Malaysia Peninsular Malay	0.1	0.000526	951	See			
32	A*24:31	Malaysia Peninsular Malay	0.2	0.001052	951	See			
33	A*24:35	Malaysia Peninsular Malay	0.1	0.000526	951	See			
34	A*24:41	Malaysia Peninsular Malay	0.1	0.000526	951	See			
35	A*24:64	Malaysia Peninsular Malay	0.1	0.000526	951	See			
36	A*24:71	Malaysia Peninsular Malay	0.1	0.000526	951	See			
37	A*24:84N	Malaysia Peninsular Malay	0.5	0.003155	951	See			
38	A*26:01	Malaysia Peninsular Malay	3.1	0.015773 🟨	951	See			
39	A*29:01	Malaysia Peninsular Malay	0.7	0.003680	951	See			
40	A*30:01	Malaysia Peninsular Malay	1.5	0.007361 🟨	951	See			
41	A*30:15	Malaysia Peninsular Malay	0.1	0.000526	951	See			
42	A*31:01	Malaysia Peninsular Malay	0.8	0.004206	951	See			
43	A*31:09	Malaysia Peninsular Malay	0.1	0.000526	951	See			
44	A*32:01	Malaysia Peninsular Malay	0.8	0.004206	951	See			
45	A*32:03	Malaysia Peninsular Malay	0.1	0.000526	951	See			
46	A*32:14	Malaysia Peninsular Malay	0.1	0.000526	951	See			
47	A*33:01	Malaysia Peninsular Malay	15.5	0.084122 🟨	951	See			
48	A*33:03	Malaysia Peninsular Malay	7.8	0.041535 🟨	951	See			
49	A*33:07	Malaysia Peninsular Malay	0.1	0.000526	951	See			
50	A*33:12	Malaysia Peninsular Malay	0.5	0.002629	951	See			
51	A*34:01	Malaysia Peninsular Malay	11.2	0.058885 🟨	951	See			
52	A*34:05	Malaysia Peninsular Malay	0.1	0.000526	951	See			
53	A*68:01	Malaysia Peninsular Malay	2.5	0.012093 🟨	951	See			
54	A*74:01	Malaysia Peninsular Malay	1.4	0.006835 🟨	951	See			

The HLA-A*24:02 allele frequency in Peninsular Malaysia Malay population reported in AFND. The percentage of an individual with HLA-A*24:02 positive allele was identified to be 35.3% and denoted (with red bar) to be the highest frequency allele in this population (http://www.allelefrequencies.net/default.asp).

APPENDIX H AGAROSE GEL ANALYSIS

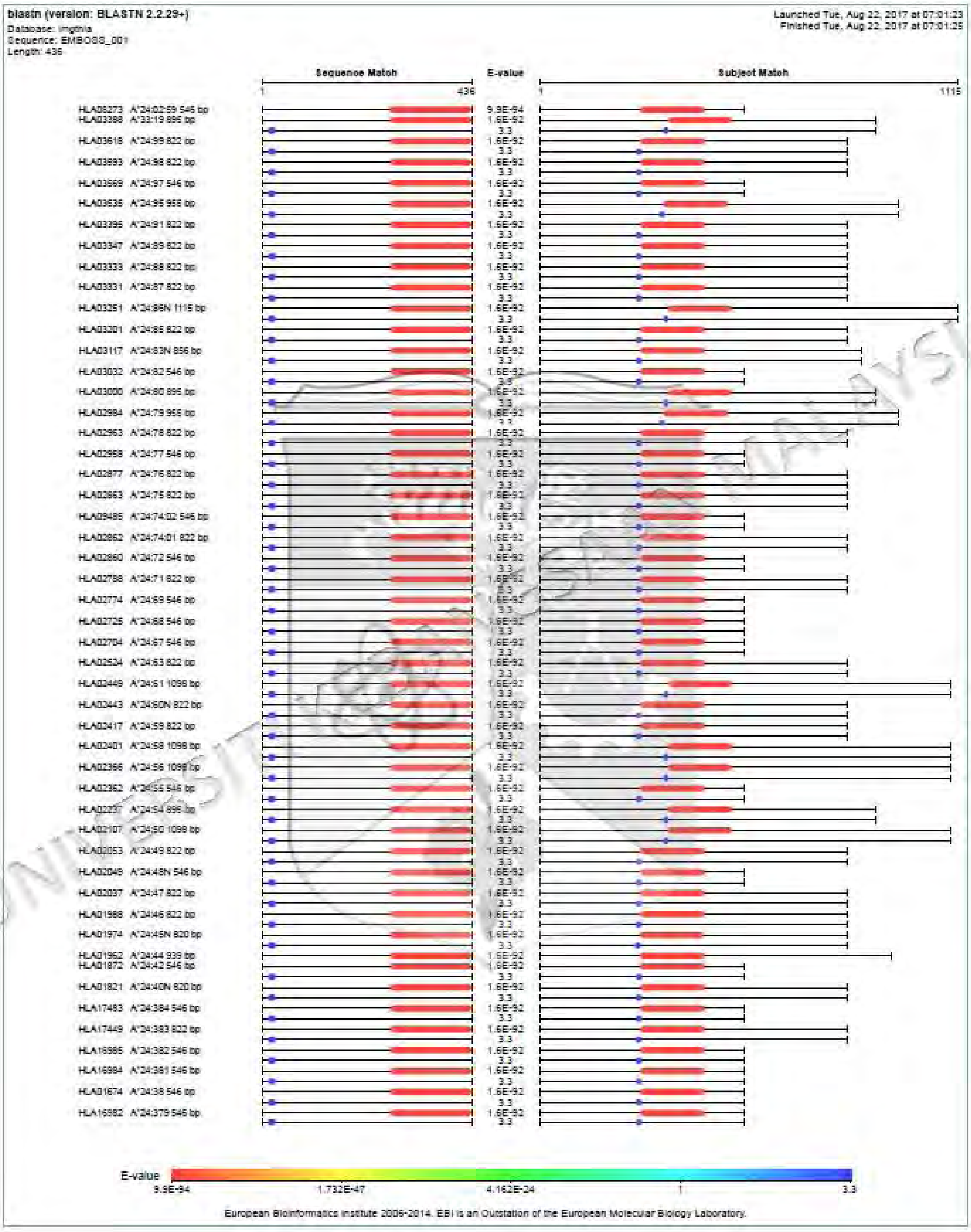


(a) The gel analysis showed the 18 cases of HLA-A*24:02 negative healthy samples.
(b) The gel analysis shown the rest 15 cases of HLA-A*24:02 negative CRC samples.

SW= SW480; COLO=COLO320; M1= DNA Ladder 1kb; M2= DNA Ladder 100bp; NTC= non template control; x = the unloaded

APPENDIX I SANGER SEQUENCING OF HLA PRIMERS

HLA-A24 Forward Primer



blastn (version: BLASTN 2.2.29+)
Database: imgthla
Sequence: EMBOSS_001
Length: 436

Launched Tue, Aug 22, 2017 at 07:01:23
Finished Tue, Aug 22, 2017 at 07:01:25

European Bioinformatics Institute 2006-2014. EBI is an Outstation of the European Molecular Biology Laboratory.

HLA-A24 Reverse Primer

APPENDIX J PUBLICATION

JOURNAL PUBLICATION

1. The Potential Immune-eliciting Cancer Testis Antigens in Colorectal Cancer Immunotherapy, September 2018
Joanne Ern Chi Soh, Nadiah Abu* & Rahman Jamal*

2. Validation of Immunogenic PASD1 Peptides against HLA-A*24:02 Colorectal Cancer
Accepted for publication, Immunotherapy
Joanne Ern Chi Soh, Nadiah Abu*, Ismail Sagap, Luqman Mazlan, Azyani Yahaya, Muaatamarulain Mustangin, Tze Sean Khoo, Sazuita Saidin, Muhiddin Ishak, Nurul Syakima Ab Mutalib and Rahman Jamal*

CONFERENCE PAPER

1. Validation of PASD1 as An Immunotherapeutic Target in Colorectal Cancer (CRC)
Abstract for 7th Regional Conference on Molecular Medicine (RCMM), 2017
Joanne Ern Chi Soh, Nadiah Abu, Hui-min Neoh, Luqman Mazlan and Rahman Jamal*

2. Validation of PASD1 as An Immunotherapeutic Target in Colorectal Cancer (CRC)
Abstract for 8th Regional Conference on Molecular Medicine (RCMM), 2018
Joanne Ern Chi Soh, Nadiah Abu*, Hui-min Neoh, Luqman Mazlan, Azyani Yahaya and Rahman Jamal*

3. Validation of PASD1 as An Immunotherapeutic Target in Colorectal Cancer
Abstract for International Conference on Drug Discovery and Translational Medicine 2018 (ICDDTM '18)
Joanne Ern Chi Soh, Nadiah Abu*, Hui-min Neoh, Luqman Mazlan, Azyani Yahaya and Rahman Jamal*