GENOME-WIDE ASSOCIATION STUDIES OF NASOPHARYNGEAL CARCINOMA IN THE MALAYSIAN CHINESE COHORT USING SINGLE GENES, META-ANALYSIS AND PATHWAY ANALYSIS APPROACHES

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GENOME-WIDE ASSOCIATION STUDIES OF NASOPHARYNGEAL CARCINOMA IN THE MALAYSIAN CHINESE COHORT USING SINGLE GENES, META-ANALYSIS AND PATHWAY ANALYSIS APPROACHES

ABSTRACT

Nasopharyngeal carcinoma (NPC) is an epithelial squamous cell carcinoma on the mucosal lining of the nasopharynx with high incidence in the southern Chinese population. NPC is constantly linked to Epstein Barr virus (EBV) infection but its etiology remains elusive considering many carriers of EBV never develop NPC. Genetic factors play an important role in NPC susceptibility. This study set out to identify genetic variants linked to NPC susceptibility using a genome-wide association (GWAS) approach in the Malaysian Chinese. The GWAS encompasses single genes, meta-analysis with NPC cohorts from Taiwan and southern China and a pathway/gene set approach. In the single gene approach, GWAS results confirmed the association of *HLA-A* to NPC with the strongest signal detected in rs3869062 (P=1.73x10^{-9}). Fine mapping revealed associations in the amino acid variants as well as its corresponding SNPs in the antigen peptide binding groove ($P_{\text{HLA-A-aa-site-99}}$=3.79x10^{-8}, $P_{\text{rs1136697}}$=3.79x10^{-8}) and T-cell receptor binding site ($P_{\text{HLA-A-aa-site-145}}$=1.41x10^{-4}, $P_{\text{rs1059520}}$=1.41x10^{-4}) of the *HLA-A*. HLA-A amino acid variants and SNPs were correlated with the effects of HLA-A*02:07. Results showed a protective trend towards NPC for *HLA-A* variants in the Malaysian Chinese, consistent with previous findings of *HLA-A* NPC association. Meta-analysis performed by combining results from NPC GWAS of Malaysia, Taiwan and southern China (2,152 cases; 3,740 controls) revealed 43 noteworthy findings outside the MHC region were identified and targeted for replication in a pooled analysis (4,716 cases; 5,379 controls). In the combined meta-analysis, rs31489, located within the *CLPTM1L/TERT* region on chromosome 5p15.33, was strongly associated with NPC (OR=0.81; P=6.3x10^{-13}). Associations of previously reported NPC GWAS were also replicated, namely rs6774494 (P=1.5x10^{-12}; located in

the *MECOM* gene region), rs9510787 (P=5.0x10^{-10}; located in the *TNFRSF19* gene region), and rs1412829/rs4977756/rs1063192 (P=2.8x10^{-8}, P=7.0x10^{-7}, and P=8.4x10^{-7}, respectively; located in the *CDKN2A/B* gene region). The *TERT* gene is important for telomere maintenance and is overexpressed in NPC. The EBV protein LMP1 has been reported to modulate *TERT* expression/telomerase activity in NPC. The findings suggest that factors involved in telomere length maintenance are involved in NPC pathogenesis. An integrated pathway approach was employed to identify dysregulated pathways linked to NPC. This approach combines imputation NPC GWAS data from a Malaysian Chinese cohort as well as published expression data GSE12452 from both NPC and healthy nasopharynx tissues. Results identified NPC association with the Gene Ontology (GO) axonemal dyenein complex pathway ($P_{GWAS\text{-}GSEA}$=1.98x10^{-2}; $P_{Expr\text{-}GSEA}$=1.27x10^{-24}; $P_{Bonf\text{-}Combined}$=4.15x10^{-21}). This association was replicated in a separate cohort using gene expression data from NPC and healthy nasopharynx tissues ($P_{AmpliSeq\text{-}GSEA}$=1.37x10^{-3}). Loss of function in the axonemal dyenein complex causes impaired cilia function, leading to poor mucociliary clearance and subsequently upper or lower respiratory tract infection, the former of which includes the nasopharynx. Our approach illustrates the potential use of integrated pathway analysis in detecting gene sets involved in the development of NPC in the Malaysian Chinese cohort.

Keywords: nasopharyngeal carcinoma, genome-wide association studies, imputation, meta-analysis, gene-set enrichment analysis

ABSTRAK

Karsinoma salur udara nasofarinks (NPC) adalah sejenis karsinoma sel skuamus epithelial pada lapisan mukosa salur udara nasofarinks dengan insiden yang tinggi di kalangan populasi masyarakat Cina selatan. NPC sentiasa dikaitkan dengan jangkitan virus Epstein Barr (EBV) tetapi etiologinya masih sukar difahami memandangkan kebanyakan pembawa virus EBV tidak akan menghidap NPC. Adalah dipercayai bahawa faktor genetik memainkan peranan penting dalam kerentanan NPC. Oleh itu, dalam penyelidikan ini, variasi genetik yang dikaitkan dengan NPC dikenalpasti dengan menggunakan kaedah kajian perkaitan menyeluruh genom (GWAS) dalam populasi berketurunan Cina Malaysia. Kajian GWAS ini merangkumi gen tunggal, analisis meta terhadap pesakit NPC dari Taiwan dan selatan Cina serta satu kaedah set laluan gen. Melalui kaedah gen tunggal, analisis GWAS yang dijalankan telah mengesahkan perkaitan antara *HLA-A* dengan NPC di mana isyarat paling tinggi dikesan pada rs3869062 (P=1.73x10^{-9}). Pemetaan terperinci mendedahkan perkaitan dalam varian-varian asid amino berserta SNP yang sepadan dalam alur pengikat peptida antigen ($P_{\text{HLA-A-aa-site-99}}$=3.79x10^{-8}, $P_{\text{rs1136697}}$=3.79x10^{-8}) dan tapak pengikat reseptor sel T ($P_{\text{HLA-A-aa-site-145}}$=1.41x10^{-4}, $P_{\text{rs1059520}}$=1.41x10^{-4}) *HLA-A*. Varian-varian asid amino dan SNP *HLA-A* menunjukkan korelasi tinggi dengan kesan *HLA-A*02:07*. Hasil kajian ini menunjukkan varian-varian asid amino *HLA-A* cenderung sebagai penghindar NPC dalam populasi keturunan Cina Malaysia, sejajar dengan penemuan terdahulu bagi perkaitan antara *HLA-A* dan NPC. Kaedah analisis meta menggabungkan data NPC GWAS Malaysia, Taiwan dan selatan China (2,152 kes; 3740 kawalan) yang dijalankan mendedahkan 43 penemuan berpotensi di luar rantau MHC yang telah dikenalpasti dan disasarkan untuk replikasi dalam analisis gabungan (4,716 kes; 5,379 kawalan). Dalam meta-analisis gabungan, rs31489 yang terletak dalam rantau *CLPTM1L/TERT* pada kromosom 5p15.33, dikaitkan dengan NPC (OR=0.81; P=6.3x10^{-13}). Perkaitan variasi

SNP NPC yang dilaporkan sebelum ini juga dikenalpasti, iaitu rs6774494 (P=1.5x10^{-12}; terletak di rantau gen *MECOM*), rs9510787 (P=5.0x10^{-10}; terletak di rantau gen *TNFRSF19*), dan rs1412829/rs4977756/rs1063192 (P=2.8x10^{-8}, P=7.0x10^{-7} dan P=8.4x10^{-7}, masing-masing terletak di rantau gen *CDKN2A/B*). Gen *TERT* adalah penting untuk penyelenggaraan telomer dan menunjukkan ekspresi tinggi dalam NPC. Protein EBV iaitu LMP1 telah dilaporkan mampu mengubah aktiviti TERT dalam NPC. Penemuan penyelidikan ini menunjukkan bahawa faktor-faktor yang terlibat dalam penyelenggaraan telomer terlibat dalam patogenesis NPC. Pendekatan set laluan gen untuk mengenal pasti set gen yang berkait dengan NPC. Pendekatan kami menggabungkan data imput NPC GWAS dari populasi Cina Malaysia dan data ekspresi gen sedia ada GSE12452 dari kedua-dua tisu NPC dan nasofarinks sihat. Kami mengenal pasti perkaitan NPC dengan Ontologi Gen (GO) kompleks "axonemal dynein" ($P_{\text{GWAS-GSEA}}$=1.98x10^{-2}; $P_{\text{Expr-GSEA}}$=1.27x10^{-24}; $P_{\text{Bonf-Combined}}$=4.15x10^{-21}). Penemuan ini telah disahkan dengan data ekspresi gen tisu NPC dan nasofarinks sihat ($P_{\text{AmpliSeq-GSEA}}$=1.37x10^{-3}). Kehilangan fungsi kompleks "axonemal dynein" menyebabkan rencatan fungsi silia, seterusnya mengakibatkan pengumpulan mukus dan seterusnya jangkitan di bahagian atas dan bawah saluran pernafasan, di mana jangkitan bahagian atas termasuklah salur udara nasofarinks. Pendekatan kami menunjukkan potensi kegunaan kaedah integrasi laluan dalam mengesan kumpulan gen yang terlibat dalam penularan NPC di kalangan masyarakat Cina di Malaysia.

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On a more personal note, I would like to dedicate this work to people whom I hold dear in my heart. To my family, my closest friends and my colleagues. Thank you for everything.

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λ_{gc} : lambda genomic control inflation factor

1 kG : 1000 genomes project

3'-UTR : 3'-untranslated region

5'-UTR : 5'-untranslated region

95% CI : 95% confidence interval

AJCC : The American Joint Committee on Cancer

ASN : Asians

ASP : affected sib-pair

ASR : age-standardized rate

BARF1 : Epstein-Barr virus BamHI-A rightward frame 1

CHB : Chinese Han Beijing

CHD : Chinese Han Denver

CNV : copy number variation

CT : computerized tomography

dbMHC : database for major histocompatibility complex

EA : early antigen

EBER : Epstein-Barr virus-encoded small RNAs

EBNA : Epstein-Barr virus nuclear antigen

EBV : Epstein-Barr virus

EGFR : epidermal growth factor receptor

ENCODE : Encyclopedia of DNA Elements

eQTL : expression quantitative trait loci

GEO : Gene Expression Omnibus

GO : Gene ontology

GSEA : gene-set enrichment analysis

GWAS : genome wide association study

HKL : Kuala Lumpur General Hospital

HPP : Penang General Hospital

HUS : Hospital University Sarawak

HWE : Hardy–Weinberg equilibrium

IARC : International Agency for Research on Cancer

IBS : identity-by-state

IgA : immunoglobulin A

ImmPort : Immunology Database and Analysis Portal

JPN : Japan

KEGG : Kyoto Encyclopedia of Genes and Genomes

LD : linkage disequilibrium

LMP : Epstein-Barr virus latent membrane protein

LOD : logarithm of odds

MAF : minor allele frequency

MHC : major histocompatibility complex

MRI : magnetic resonance imaging

NCBI : National Center for Biotechnology Information

NCCN : National Comprehensive Cancer Network

NDMA : N-nitrosodimethylamine

NPC : nasopharyngeal carcinoma

NPIP : N-nitrosopiperidine

NPYR : N-nitrosopyrrolidine

OR : odds ratio

PCA : principal component analysis

PET : positron emission tomography

PROVEAN : Protein Variation Effect Analyzer

QQ plot : quantile-quantile plot

QES : Queen Elizabeth Hospital Sabah

r^2 : Pearson's correlation coefficient

RIKEN : The Institutes of Physical and Chemical Research

SIFT : Sorting Intolerant from Tolerant

SNP : single nucleotide polymorphism

TNM : tumor node metastasis

UICC : International Union Against Cancer

UMMC : University Malaya Medical Centre

VCA : viral capsid antigen

VEGF : vascular endothelial growth factor

WHO : World Health Organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial cell carcinoma on the mucosal lining of the nasopharynx. It is rare in most parts of the world (Feng, 2013). However, in certain regions where NPC is prevalent, it is a major health issue that requires immediate attention. In Malaysia, it is the fifth most common cancer among Malaysians and third most common among men according to the latest Malaysian Cancer Registry Report, 2007-2011 (Azizah *et al.*, 2016). It is particular prevalent among the Chinese as well as the indigenous tribe of Bidayuh people. NPC displays an enigmatic and multifactorial etiology, making effective treatment challenging. It is consistently linked to Epstein-Barr virus (EBV) infection, making it a viral-induced cancer. However, almost 95% of the world population is infected and they are healthy carriers of the EBV virus (Kutok & Wang, 2006). Not all EBV carriers develop NPC. Diet also strongly influences the onset of NPC. Consumption of Chinese-style salted fish has been strongly linked to NPC risk (Jeannel *et al.*, 1999; Zheng *et al.*, 1994). However, for Chinese immigrants in non-endemic regions (e.g. North America), the risk of NPC remains though at a much lower rate compared to their ancestors (Chang & Adami, 2006). This proves that evolving lifestyles do not completely eradicate the risk of NPC and genetic factors could play an important role in NPC onset, especially for high-risk populations like the southern Chinese and its descendants.

Several NPC GWAS have been reported, with the *HLA-A* gene continously being identified. However, most GWAS studies have yet to identify causal SNPs or annotate the functional implication of these polymorphisms. A NPC GWAS was performed to fine-map the *HLA-A* region in high resolution with focus on variants that either influence peptide loading or expression of the *HLA-A*. *HLA-A* variants with potential enhancer and promoter activity could affect differential binding affinity for

nuclear factors, consequently influence eQTL expression trends. In view of the high number of variants reported in the *HLA-A* or even the MHC region, it is imperative to identify the key variants driving the associations in the *HLA-A* region, while the remaining variants are mainly proxy association signals due to presence of linkage disequilibrium (LD).

Genetic effects reported from single GWAS studies are generally small, causing suspicion of false positives (Chapman *et al.*, 2011; Ioannidis *et al.*, 2006; Moonesinghe *et al.*, 2008). Therefore meta-analysis, the statistical analysis of pooling genetic effects across different GWAS studies is performed to increase the power of variants association and reduce false-positive findings. Meta-analysis is able to utilize summary data without resorting to sharing individual genotype data, overcoming restrictions on sharing individual-level data (Evangelou & Ioannidis, 2013). Therefore meta-analysis has become a popular approach for the discovery of new genetic loci for common diseases and phenotypes. This study was a collaborative effort between 1) Sun Yat-sen University Cancer Center, Guangzhou, China; 2) Chang Gung University, Taiwan; 3) University of Malaya, Kuala Lumpur, Malaysia; 4) Genome Institute of Singapore, Singapore. The study was coordinated by the Infections and Immunoepidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland.

The search for NPC susceptibility genes have been limited to only single platform analysis, in particular GWAS or transcriptomics involving probe-based microarrays. Cross platform analysis has not been reported for NPC. There are limitations attached to single platform approaches. Firstly, GWAS and microarray expression studies report single gene associations, the former under very stringent multiple testing correction (Wang *et al.*, 2010). Thus, causal loci with moderate effects will be missed. Secondly, complex diseases like NPC arise due to the interplay of

several genes (Chou *et al.*, 2008). Single gene analysis would neglect the dynamics of gene sets or pathways leading to NPC development, thus ignoring potentially new insights pertaining to NPC etiology. By employing an integrated pathway approach combining GWAS SNP and gene expression data, dysregulated pathways with relevant function to nasopharyngeal carcinoma (NPC) can be identified. This approach moves us closer to identifying key pathways or genes affecting NPC.

This study aims to achieve the following: 1) A genome-wide sweep of 712,717 SNPs on the Illumina Human OmniExpress platform to identify NPC associated loci; 2) Imputation with HapMap, 1000 Genomes datasets and previously reported GWAS data (Ng *et al.*, 2009a) to fine-map the *HLA-A* region; 3) High resolution molecular *HLA-A* allele genotyping to identify *HLA-A* alleles as well as its corresponding SNP genotypes; 4) *In silico* prediction and amino acid variant analysis to identify functional associations; 5) Identify new loci outside of the major histocompatibility complex (MHC) region associated to NPC by pooling data across 4 NPC GWAS studies; 6) Identify dysregulated pathways associated with NPC through GWAS and gene expression data.

CHAPTER 2: LITERATURE REVIEW

2.1 NPC diagnosis and classification

Nasopharyngeal carcinoma (NPC) is an epithelial cell carcinoma on the mucosal lining of the nasopharynx. The nasopharynx is the uppermost part of the pharynx, behind the nasal cavity (Khoo & Pua, 2013). The epicenter of NPC begins at the Fossa of Rosenmüller and can spread in any direction to adjacent regions such as the nasal cavity, the oropharynx, the skull base, the parapahryngeal space or the retropharyngeal space (Figure 2.1). Spreading of NPC to different regions would manifest different symptoms. For example, when NPC spreads to the skull base, it leads to compression of cranial nerves, resulting in cranial nerve palsies. When NPC spreads to the cervical lymph nodes from the Fossa of Rosenmüller through the node of Rouvier, a neck lump appears (Khoo & Pua, 2013).

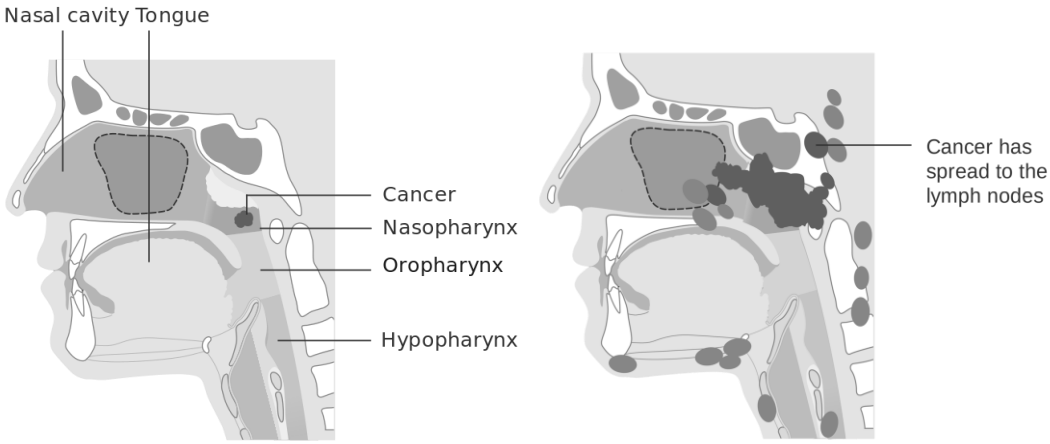


Figure 2.1: Anatomical view of the nasopharynx and possible routes of local spread of tumor to adjacent regions. Images adapted from Cancer Research UK. (http://www.cancerresearchuk.org)

2.2 NPC symptoms and method of diagnosis

NPC typically portray atypical symptoms at early stages, making it difficult for clinicians to detect. Among the more common symptoms of NPC include appearance of neck lumps, nasal symptoms (blood stained nasal discharge, blood stained saliva, or nasal blockage), aural symptoms (unilateral blocked ear, pressure in the ear, mild hearing loss or tinnitus) and facial neurologic symptoms (unilateral facial numbness, diplopia or unilateral headache).

The nasopharynx is located in an obscure position behind the nasal cavity, making visual examination challenging. Examination of this area is carried out with the aid of flexible fiber optic or rigid endoscope connected to a camera. NPC appears as a mass in the nasopharynx. Biopsies are taken to confirm the diagnosis of NPC. In cases where the NPC is a submucosal tumor, magnetic resonance imaging is used to locate the tumor and aid the biopsy.

Serological methods are also useful for early detection of endemic NPC due to its close association with EBV infection. Elevated levels of the EBV-IgA-VCA antibody preceded the onset of NPC, with a window of about 3 years (Hao *et al.*, 2003). Therefore, measuring the IgA antibody titers would be useful for early detection. IgA antibody titers to EBV viral capsid antigen (EBV-IgA-VCA) and EBV early antigen (EBV-EA) in immunofluorescent assays may be used for the serologic screening of NPC (Yi *et al.*, 1980; Zeng *et al.*, 1982). However, in recent years, immunofluorescent assays have largely been replaced with enzyme-linked immunosorbent assays (ELISA) employing purified recombinant EBV antigens (Nadala *et al.*, 1996). These tests serve as NPC tumor markers of remission and relapse (Chang *et al.*, 2008; De-Vathaire *et al.*, 1988)

NPC classification is achieved through histopathological examination of the biopsy. The current classification system follows the WHO classification of 2005 (Chan *et al.*, 2005) after many revisions from previous classification systems. The challenges of classifying NPC lie in lack of acceptance by pathologists because tumor gradations are not correlated with eventual treatment options. NPC is classified as keratinizing and non-keratinizing (Table 2.1). Non-keratinizing NPC is the pre-dominant type in endemic areas (Nicholls, 1997; Wei & Sham, 2005). A third type of NPC introduced by the WHO classification of 2005 is basaloid squamous cell carcinoma. As most NPC is detected at later stages where neck lumps appear, fine needle aspiration cytology of enlarged lymph nodes is performed to detect nodal spread.

NPC staging is performed to evaluate extent of the cancer, determine prognosis and recommend treatment. Staging is deduced from information obtained from symptoms, physical examination, endoscopy, imaging of the tumor, lymph node spread and metastases (Edge *et al*., 2010). Imaging techniques employed in NPC staging include computerized tomography (CT), magnetic resonance imaging (MRI), chest X-ray, ultrasound, bone scintigraphy and positron emission tomography (PET) scan (Khoo & Pua, 2013). Combination of techniques employed for NPC staging is dependent on cost, availability as well as the extent of NPC. As per recommendation by the National Comprehensive Cancer Network (NCCN), CT with contrast or MRI with gadolinium is performed for NPC imaging. For more advanced stages of NPC (Stage III-IV or distant metastasis) PET or CT is preferred (National Comprehensive Cancer Network, 2011). MRI is superior to PET/CT scan in imaging tumor spread in the areas adjacent to the epicenter, namely parapharyngeal space, base of the skull, intracranial area, sphenoid sinus and retropharyngeal lymph nodes (Liao *et al.*, 2008). PET/CT scan is more superior in capturing distant metastatis (Lin *et al.*, 2008; Ng *et al.*, 2009b) to extent of replacing more conventional methods such as chest radiography, abdominal ultrasound

and bone scintigraphy (Liu *et al.*, 2007). As such, using inferior imaging equipment would result in understaging of the disease.

The various symptoms that manifests during NPC progression is a consequence of the spread of the tumor. For example, nasal and aural symptoms suggest the tumor is still confined to the primary site of the nasopharynx (T1), neck mass suggests tumor spread to cervical lymph nodes (N1-3) while facial neurologic symptoms imply spread to the skull base (T4).

NPC staging follows the "tumor node metastasis" (TNM) staging system, jointly developed by The American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) (Edge *et al*., 2010). The TNM staging describes the anatomy of the tumor, in which, T refers to the local extent of the primary tumor, N refers to the extent of regional nodes involvement and M refers to the distant spread (metastasis) of the tumor. The TNM scores are then combined to determine the overall stage (Table 2.2).

Table 2.1: WHO histopathological classification of NPC (Edge *et al.*, 2010).

WHO Classification (2005)	Former Terminology (WHO 1998)
Keratinizing carcinoma	WHO Type 1
Nonkeratinizing carcinoma	
- differentiated	WHO Type 2
- undifferentiated	WHO Type 3
Basaloid squamous cell carcinoma	(no former terminology)

Table 2.2: TNM clinical classification for tumors of the nasopharynx (Edge *et al*., 2010).

Primary Tumor (T)

- T1 - Tumor confined to nasopharynx, or extends to oropharynx and/or nasal cavity without parapharyngeal extension
- T2 - Tumor with parapharyngeal extension (posterolateral infiltration of tumor)
- T3 - Tumor involves bony structures and/or paranasal sinuses
- T4 - Tumor with intracranial extension and/or involvement of cranial nerves, hypopharynx, orbit, or with extension to the infratemporal fossa/masticator space

Regional Lymph Nodes (N)

- N0 - No regional lymph node metastasis
- N1 - Unilateral metastasis in cervical lymph node(s), 6 cm or less in greatest dimension, above the supraclavicular fossa, and/or unilateral or bilateral, retropharyngeal lymph nodes, 6 cm or less, in greatest dimension
- N2 - Bilateral metastasis in cervial lymph node(s), 6 cm or less in greatest dimension, above the supraclavicular fossa
- N3 - Metastasis in a lymph node(s) greater than 6 cm and/or to supraclavicular fossa
- N3a - Greater than 6 cm in dimension
- N3b - Extension to the supraclavicular fossa

Distant Metastasis (M)

- M0 - No distant metastasis
- M1 - Distant metastasis

Clinical Stage Groups (Anatomic Stage/Prognostic Groups)

Stage I: T1, N0, M0
Stage II: T1, N1, M0; T2, N0, M0; T2, N1, M0
Stage III: T1, N2, M0; T2, N2, M0; T3, N0, M0; T3, N2, M0
Stage IVA: T4, N0, M0; T4, N1, M0; T4, N2, M0
Stage IVB: Any T, N3, M0
Stage IVC: Any T, any N, M1

2.3 NPC treatment

Radiotherapy remains the mainstay treatment for NPC. Radiation is typically concentrated on the adjacent skull base and nasopharynx. The control rate on conventional radiotherapy is 75 to 90% in T1 and T2 tumors, and 50 to 75% in T3 and T4 tumors (Wei & Kwong, 2010). The control of cervical nodal regions is achieved in 90% of N0 and N1 cases, and about 70% of N2 and N3 cases (Wei & Kwong, 2010). It is mandatory to keep the treatment schedule because interrupted or prolonged treatment reduces the benefits of radiotherapy (Kwong *et al.*, 1997).

Recent studies conclude that the inclusion of chemotherapy to radiotherapy improves treatment outcome of NPC patients. Phase III randomized intergroup study 0099 showed that patients treated with radiation alone had a significantly lower 3-year

survival rate than those receiving radiation with cisplatin and 5-fluorouracil chemotherapy (Al-Sarraf *et al.*, 1998). Several meta-analysis reported a definite improvement of the 5-year survival rate due to the addition of chemotherapy (56% with radiotherapy alone versus 62% with chemoradiotherapy) (Baujat *et al.*, 2006).

Studies and clinical trials are ongoing to find the optimal treatment strategy for NPC. These include molecular targeted therapies in NPC, including the epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), epigenetic therapy, Epstein-Barr virus (EBV) directed immunotherapy and gene therapy (Hui & Chan, 2013).

2.4 NPC epidemiology: Geographic origin

NPC is a rare cancer in most parts of the world with the exception of certain endemic regions (Figure 2.2). In 2012, 87,000 cases of NPC were reported. However, this only made up 0.6% of all cancers reported that year (Ferlay *et al.*, 2015). A whopping 71% of the 87,000 NPC cases came from highly prevalent regions in southern China, Hong Kong, Taiwan, Southeast Asia, Maghrebian countries in northern Africa (Algeria, Morocco and Tunisia) and the arctic and sub-arctic region of North America and Greenland (Ferlay *et al*., 2015).

Figure 2.2: Endemic regions of nasopharyngeal carcinoma occurence. Incidence rates at these regions record ASR of > 10 per 10^5 person-years. In non-endemic regions, NPC incidence is 0.5 per 10^5 person-years.

The populations most affected by NPC are the southern Chinese, Amazigh- and Arabic-speaking North Africans and the Inuits (Feng, 2013). Zhongshan city of the Guangdong province in southern China reported one of the highest incidence rates of NPC, with age-standardized rates (ASR) of 26.9 per 10^5 person-years for males and 10.1 for females (Curado *et al.*, 2013). Similar ASRs were observed in neighboring cities like Hong Kong and Guangzhou (Curado *et al*., 2013). The majority of the population in these cities is Cantonese. Taiwan recorded a moderate NPC incidence rate of ASR 6.96 (Chiang *et al.*, 2016). The inuits in Alaska, Canada and Greenland also show high rates of NPC with ASR was 12.1 for males and 7.3 for females (Kelly *et al.*, 2008). Magrebian countries of Tunisia and Algeria show moderate rates of NPC with ASRs of 5.4 and 4.6 per 105 person-years for males and ASRs of 1.7 and 1.9 for females (Curado *et al*., 2013). The incidence rate in other non-endemic countries is 0.5 per 10^5 person-years with a general trend of higher incidence in males compared to females (incidence ratio 2-3:1) (Curado *et al*., 2013).

The population of Southeast Asia is an admixed population of major southern Chinese dialect groups, namely the Cantonese, Hakka and Teochew people from Guangdong province and the Hokkien from Fujian province (Feng, 2013). The Cantonese has an NPC risk twice those of other dialect groups in China (Yu & Yuan, 2002). Other populations showing moderate to high NPC incidence include the Thais, Vietnamese and Filipinos with ASRs ranging from 2.5 to 15 for males (Yu & Yuan, 2002).

In Malaysia, NPC is the fifth most common cancer among Malaysians and third most common among men (Azizah *et al*., 2016). A general trend is observed where NPC incidence is higher in males (ASR 6.4) than in females (ASR 2.2). The disease is particularly prevalent in the Chinese, with low incidences in the Malays and Indians. Malaysian Chinese record the highest incidence of NPC with ASRs of 11.0 in Chinese males and 3.5 in Chinese females (Azizah *et al*., 2016). The Malays record NPC incidence ASRs of 3.3 in males and 1.3 in females while the Indians show low incidence rates of ASR 1.1 in males and 0.6 in females (Azizah *et al*., 2016). A high risk of NPC has also been observed in Sarawak, particularly the indigenous tribe of Bidayuh people, with ASRs of 31.5 for males and 11.8 for females (Devi *et al.*, 2004). The incidence rate for Bidayuhs is much higher than other ethnic groups living in Sarawak. Overall, the incidence rate of NPC in Malaysian Chinese, Malays and Indians is similar to that of neighboring Singapore (Azizah *et al*., 2016). NPC incidences in Malaysian males are elevated from age 25 years and peaks at age 65 years (Azizah *et al*., 2016). This is somewhat late compared to other high-risk Asian populations with peak incidence reported at the age of 45-55 years old (Bray *et al.*, 2008). Most nasopharyngeal carcinoma cases detected in Malaysia were of Stage III and IV (Azizah *et al*., 2016), highlighting once again the difficulties in diagnosis due to its non-distinct symptoms.

Migration does influence the NPC incidence rate for people belonging to high-risk areas. Immigrants from high-risk regions show higher incidence of NPC than the local population after moving to low-risk countries. This has been observed for Chinese immigrants in the USA, UK, Canada and Australia (Chang & Adami, 2006), Inuit in Denmark (Boysen *et al.*, 2008) and North Africans in several European countries (Jeannel *et al*., 1999). However, the incidence rate tends to decrease with successive generations. The disparate NPC incidence rates among pioneering immigrants and successive generations points to the influence of environmental factors in NPC development. However, in multi-racial countries such as Malaysia and Singapore populated by successive generations of immigrants, NPC incidence rates remain high with little difference in incidence rates among the different ethnic groups, pointing to the probable influence of genetic factors as well as the continued practise of our ancestors' lifestyle. NPC incidence from different populations is summarized in Figure 2.3.

2.5 Environmental factors

2.5.1 Diet

Consumption of preserved food has been constantly linked to NPC. A common factor that links NPC to its endemic regions is the low socioeconomic status, leading to high consumption of preserved food as a cheap form of sustenance (Jeannel *et al*., 1999). Chinese-style salted fish is constantly linked to NPC among the Cantonese as well as Thais in South and South East Asia (Jeannel *et al*., 1999). Chinese-style salted fish is prepared by curing the fish in salt and then sun-dried. Other methods of food preservation are not linked to NPC. An example would be in among the Eskimos in Greenland where consumption of wind-dried and fermented fish is not associated with NPC (Jeannel *et al*., 1999). In Japan, salted fish in consumed frequently and yet, NPC is

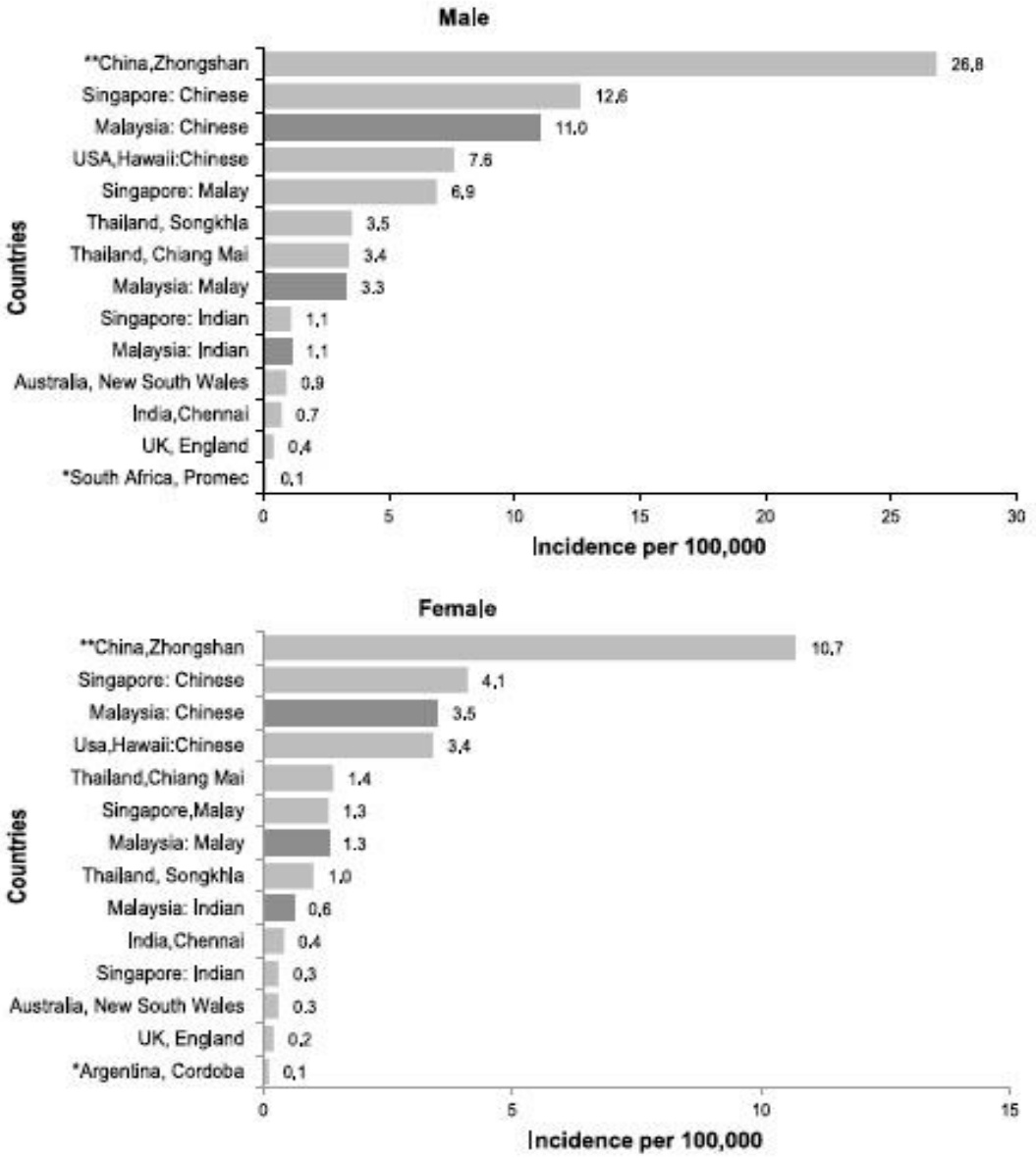


Figure 2.3: Nasopharyngeal carcinoma incidence worldwide. A comparison of NPC incidence between Malaysian Chinese and Malay against other populations. Incidence reported as age standardized rates (ASR). Image taken from Azizah *et al*. (2016). NPC incidence data taken from Azizah *et al*. (2016) and Curado *et al*. (2007).

rare (Jeannel *et al*., 1999). In Thailand, 3 types of salted fish are consumed but only Chinese-style salted fish is linked to NPC (Sriamporn *et al.*, 1992). Therefore, the consumption of salted-fish leading to risk of NPC is reliant upon the type of fish (salt water fish), method of preparation (salt-cured then sun-dried) and method of cooking (steamed salted fish) (Jeannel *et al*., 1999).

Other types of preserved food posing NPC risks are fermented fish sauce, salted shrimp paste, moldy bean curd, preserved plums, salted duck eggs, salted mustard green, dried fish, and fermented soy bean paste (Jeannel *et al*., 1999). The age of consumption of preserved food is also critical for NPC risks. Studies have shown that consumption of preserved food during weaning or childhood increases NPC risks as opposed to consumption only in adulthood (Jeannel *et al*., 1999). Preserved food elevates the risk of NPC because it contains volatile N-nitrosamines, namely N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP), compounds that are classified as probably or possibly carcinogenic to humans by the International Agency for Research on Cancer, better known as IARC (International Agency for Research on Cancer, 1978).

The link between smoking and risks of NPC is less established. Smoking is linked to NPC in low-risk areas like North America (Chow *et al.*, 1993; Mabuchi *et al.*, 1985; Nam *et al.*, 1992; Vaughan *et al.*, 1996; Zhu *et al.*, 1997). It is linked to a particular histological type, namely differentiated NPC (Ou *et al.*, 2007). However, in endemic regions, smoking is not linked or only confers moderate risk to NPC (Chen *et al.*, 1990; Cheng *et al.*, 1999; Friborg *et al.*, 2007; Yuan *et al.*, 2000). Alcohol consumption also shows mixed results when correlated with NPC onset. Most studies reveal no association between alcohol consumption and NPC with the exception of Malaysia (Armstrong *et al.*, 1983) and the United States (Nam *et al*., 1992; Vaughan *et al*., 1996). The discrepant observation could be due to the small sample sizes of these studies, leading to conflicting results. Therefore, the relationship between smoking and alcohol towards NPC risk warrants further investigation.

2.5.2 Occupational exposures

Occupational exposures can also elevate the risk of NPC. In a study evaluating occupational risk factors and its link to NPC in Hong Kong, exposure to cotton dust (OR=1.93; 95% CI=1.13-3.28), chemical fumes (OR=13.11; 95% CI=1.53-112.17), and welding fumes (OR=9.18; 95% CI=1.05-80.35) increased the risk of NPC (Xie *et al.*, 2017). Interestingly, formaldehyde was not linked to elevated NPC risk despite reports linking its exposure to NPC risks in non-endemic regions (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006; Xie *et al*., 2017). Inhalation of domestic fumes due to poor ventilation in kitchen or cooking with wood fire has also been linked with NPC (Chen & Huang, 1997; Yu *et al.*, 1986; Zheng *et al*., 1994).

2.5.3 Epstein-Barr virus (EBV)

Epstein-Barr virus is a double-stranded DNA human herpes virus. Epstein-Barr virus has been repeatedly linked with NPC through clinical, epidemiological and experimental data (Gourzones *et al.*, 2013). EBV infection is constantly linked with non-keratinizing NPC, the more prevalent form of NPC in endemic regions (Andersson-Anvret *et al.*, 1977; Nicholls *et al.*, 1997) and this association transcends geographic origins. The EBV genome is constantly detected in epithelial cells but not lymphoid cells. It is interesting to note that despite repeated studies and clinical data reaffirming this association, EBV infection and NPC onset are not mutually exclusive occurrences. More than 95% adults in all ethnic groups through the world are healthy carriers of EBV (Kutok & Wang, 2006) and not all go on to develop NPC. Thus, interaction of EBV infection and host genetic factors play an important role in NPC onset.

Once a subject has been primo-infected, EBV virus enters latency for long-term persistence. EBV in latent phase undergoes restricted expression of limited viral genes and production of viral particles is ceased. Circular viral genomes co-exist with the host

genome. In its latent state, EBV expresses several viral proteins or noncoding RNAs, most of them with the potential to contribute to apoptosis resistance or proliferation of the host cells. Latent proteins are either nuclear (called Epstein-Barr nuclear antigens: EBNA1, EBNA2, EBNA 3a,b,c, EBNA-LP) or associated to the cell membrane network (called latent membrane proteins : LMP1, LMP2 a and b) (Kutok & Wang, 2006). The oncogenic function of key EBV latent proteins is discussed below.

LMP1 is able to modulate the expression of key tumor suppressor genes by repressing p53-mediated apoptosis. This is achieved by inducing TNFAIP3/A20 that blocks p53-mediated apoptosis (Fries *et al.*, 1996), activating MAPK/SAPK complex to modulate p53 expression through phosphorylation (Li *et al.*, 2007) or by working together with Bcl-2 to override the growth suppression induced by wild-type p53 (Sheu *et al.*, 2004). LMP1 is also capable of modulating the G1-S cell-cycle checkpoint. In epithelial cells, LMP1 down regulate p16INK4a and p21, and induces Cdk2 and cyclin D1, resulting in progression from G1 phase to S phase (Huang & Huang, 2003; Lo *et al.*, 2004).

There is little evidence to illustrate the oncogenic role of LMP2 in epithelial cells. The most prominent being its ability to activate the PI3-K/Akt pathway, which, in turn, affects cell survival, apoptosis, proliferation and genomic instability via its downstream target proteins to cause cancer (Allen *et al.*, 2005; Fukuda & Longnecker, 2007; Lu *et al.*, 2006; Morrison & Raab-Traub, 2005; Scholle *et al.*, 2000).

EBNA1 is a EBV nuclear protein, consistently expressed in NPC cells and it was the first viral protein to be detected in this tumor (Huang *et al.*, 1978). It is required in proliferating latently infected cells as a critical factor for the replication of the viral episomes and their balanced segregation in dividing cells (Sivachandran *et al.*, 2011). BARF1 is an EBV oncoprotein that has oncogenic and anti-apototic effects in various types of epithelial cells (Seto *et al.*, 2008; Wang *et al.*, 2006; Wei *et al.*, 1997). It is

speculated to be a decoy receptor for the m-CSF (Strockbine *et al.*, 1998). M-CSF affects macrophages and monocytes in several ways, including stimulating increased phagocytic and chemotactic activity, and increased tumor cell cytotoxicity (Cohen & Lekstrom, 1999).

Epstein-Barr encoded RNA or EBERs are small nuclear untranslated RNAs and they are the most abundant viral RNAs in NPC cells. EBERs have been linked to malignant phenotypes leading to oncogenesis. *In vitro* studies in B-lymphocyte (BL)-derived Akata cells and immune-deficient mice have shown that EBER genes expression inhibit apoptosis (Komano *et al.*, 1999; Ruf *et al.*, 2000; Shimizu *et al.*, 1994; Yamamoto *et al.*, 2000), induce IGF1, which acts as an autocrine growth factor for NPC cells (Iwakiri *et al.*, 2003; Iwakiri *et al.*, 2005). EBERs are also known to mediate pathogenesis by modulating innate immune signals. Through constitutive activation of RIG-I by EBERs, NFκB and IRF-3 are activated, subsequently induction of type-I IFN (Samanta *et al.*, 2006). The induction of IFN induction appears disadvantageous for EBV, though it enables the virus to maintain a latent infection state because of resistance to IFN, which is provided by EBER-mediated PKR inhibition (Nanbo *et al.*, 2002).

Based on data and information currently available, a hypothesis is put forth suggesting EBV as a tumor promoting agent rather than an initiator. Nasopharynx epithelial cells with pre-existing molecular aberrations come in contact with EBV, establishing a latent infection. Over time, the EBV infected cells progress to severe dysplasia or pre-invasive carcinomas or even full malignancy.

2.6 Genetic factors

2.6.1 Familial studies of NPC

Familial studies or linkage analysis entails genotyping relatives and family members with a set or panel of genetic markers, calculating the linkage statistics and identifying the inherited genes predisposing to NPC among the affected relatives (Lander & Kruglyak, 1995). Two important designs of familial studies is affected sib-pair (ASP) and extended pedigree analysis (Freimer & Sabatti, 2004). To date, four linkage studies of NPC have been reported where the linkage of 3p21 (Xiong *et al.*, 2004), 4p15-q12 (Feng *et al.*, 2002), 5p13 (Hu *et al.*, 2008), and 6p21 (Lu *et al.*, 1990) were identified.

The earliest linkage analysis was reported by Lu *et al*. (1990) using an affected sib-pair design, focusing on the HLA region due to previous reports linking HLA antigens to NPC risk in the Chinese (Chan *et al.*, 1983; Simons *et al.*, 1974; Simons *et al.*, 1975). The study recruited 30 sibships from Guangxi and Hong Kong in China, Singapore and Malaysia and performed *HLA* typing using allelic typing antisera corresponding to *HLA* alleles (Lu *et al*., 1990). Results indicate a recessive susceptibility gene(s) with logarithm of odds for linkage (LOD) score +2.39 and *P*-values 0.004. The *HLA* region confers an increased risk of 20.9 (95% CI = 5.1 to infinite) for NPC, which is around 10-fold greater than previously reported associations with *HLA Bw46* or *B17*, suggesting the genetic lesion is *HLA*-linked but might be distinct from *HLA Bw46* or *B17* (Lu *et al*., 1990).

The first extensive linkage study of NPC was done by Feng *et al*. (2002). The study utilized an extended pedigree design; with genome-wide microsatellite markers placed 10 cM apart covering 22 autosomes. This study examined members among 20 high-risk Cantonese-speaking families in the Guangdong region. The study identified strong linkage to the D4S405 marker on chromosome 4p15–q12 with a logarithm of

odds (LOD) score of 3.06 (Feng *et al*., 2002). Fine mapping using denser microsatellite markers and SNPs pinpointed a smaller region of 8.29 cM in genetic length at 4p11–p14 (Feng *et al*., 2002).

Another linkage study using the extended pedigree design was reported by Xiong *et al*. (2004). The study recruited 18 high-risk NPC families of southern Chinese descent from the Hunan province and genotyping was performed using a less extensive panel covering only microsatellite markers on the short arms of chromosomes 3, 9, and 4p15.1–q12. Locus 3p21.31–21.2 showed strong linkage to NPC through adjacent markers D3S3624 (LOD=4.177) and D3S1568 (LOD=3.922) (Xiong *et al*., 2004). Linkage of locus 4p15–q12 as reported by Feng *et al*. (2002) in the Guangdong Chinese families was not detected.

The most recent linkage study was carried out by Hu *et al*. (2008) utilizing a pedigree design with a denser microsatellite marker distribution of 5cM apart. The study recruited 15 families from the Guangdong province. Initial analysis identified four loci on chromosomes 2q, 5p, 12p, and 18p showing LOD scores for linkage above 1.5. Fine-mapping with additional markers only identified suggestive linkage at 5p13.1 with its corresponding marker D5S2021 showing a LOD score of 2.1 (Hu *et al*., 2008).

2.6.2 Candidate gene approach

Most candidate gene approaches employ a case-control study design to detect association to NPC. A case-control design is more suited to detect genes or loci prediposed to sporadic NPC cases rather than familial NPC. In addition, recruitment of samples is easier when extended pedigrees are unavailable. Association is determined if a variant, be it an allele, genotype or haplotype, shows a statistically significant bias or difference between the affected (in this case NPC patients) and unaffected (healthy controls). To rule out false associations, affected and unaffected samples are matched as

much as possible in terms of ethnicity, gender, age and other related covariates to minimize confounders. Candidate approach studies have identified many susceptible genes to NPC, many of which were driven by prior knowledge related to NPC carcinogenesis. Many genes associated with NPC are related to immune-related genes (*HLA* class I and II), carcinogenic metabolism (*CYP2E1, CYP2A6, GSTM1, NAT2*), DNA repair (*XRCC1* and *hOGG1, ERCC1, RAD51L1*), cell cycle regulation (*TP53, CCND1*), immune response (*TLRs, PLUNC, interleukins, FAS131*), or EBV receptors (*PIGR, TCR*).

2.6.3 Immune-related genes

It is sensible to target immune-related genes as possible NPC susceptibility genes considering its EBV etiology. Studies were focused on the HLA antigen presenting molecules given its ability to elicit an immune response against viral infection. The first study reported NPC susceptible associations of *HLA-A2* and *-A11* in Singapore Chinese (Simons *et al*., 1974). Subsequent studies on the *HLA-A2* have been consistent in detecting increased NPC risk in Chinese populations from Taiwan, southern China, and Singapore (Hu *et al.*, 2005; Lu *et al.*, 2003; Wu *et al.*, 1989) though the same association was not detected in non-Chinese populations (Betuel *et al.*, 1975; Burt *et al.*, 1996; Herait *et al.*, 1983; Mokni-Baizig *et al.*, 2001; Moore *et al.*, 1983; Zervas *et al.*, 1983). The association of *HLA-A11* confers protective effect towards NPC and this has been observed in Taiwan (Lu *et al*., 2003; Wu *et al*., 1989), Singapore (Chan *et al*., 1983; Ooi *et al.*, 1997) and southern China (Hu *et al*., 2005). Another class I HLA antigen studied was the *HLA-B. HLA-B13* conferred a protective effect in the Chinese of Taiwan (Hildesheim *et al.*, 2002), Singapore (Chan *et al*., 1983) and southern China (Hu *et al*., 2005) but not in Caucasian (Moore *et al*., 1983) and Maghrebian populations (Herait *et al*., 1983). *HLA-B46* conferred a risk effect towards

NPC in the Chinese of Singapore (Chan *et al*., 1983) and Taiwan (Hu *et al*., 2005). These early studies utilized the serological typing approach for the HLA allele typing.

Subsequent HLA typing studies evolved with the emergence of polymerase chain reaction and sequencing technologies, giving rise to higher resolution HLA typing. *HLA-A*0201* (Ren *et al.*, 1995), *HLA-A*0203* (Lu *et al*., 2003) and *HLA*0207* (Hildesheim *et al*., 2002) were linked to higher risk of NPC in the Chinese. *HLA-A*1101* confers a protective effect against NPC in Taiwanese Chinese (Hildesheim *et al*., 2002). *HLA-B*4601* showed a protective effect to NPC in Thais (Pimtanothai *et al.*, 2002) and Taiwanese (Hildesheim *et al*., 2002). *HLA-DRB1*03* and *-DRB1*0301* were associated with NPC risk in Tunisian (Mokni-Baizig *et al*., 2001) and Taiwan Chinese (Hildesheim *et al*., 2002).

Apart from the antigen presenting molecules, researchers also looked at immune-related cytokines and surface proteins on immune cells for NPC susceptibility genes. In the southern Chinese, certain interleukin variants conferred risk to NPC, such as *IL1A* (rs3783553, deletion allele) (Yang *et al.*, 2011), *IL1B* (−511T) (Zhu *et al.*, 2008), *IL2* (−330G) (Wei *et al.*, 2010), *IL8* (−251A) (Wei *et al.*, 2007b), *IL10* (−1082G) (Wei *et al.*, 2007a), *IL12* (rs3212227, C allele) (Wei *et al.*, 2009), *IL16* (rs11556218, G allele) (Gao *et al.*, 2009), and *IL18* (−137C) (Nong *et al.*, 2009). In addition, toll-like receptors, a critical part of the innate immune surveillance also harbored variants that confer risk to NPC. For example *TLR3* (829A>C) (He *et al.*, 2007), *TLR4* (11350G>C) (Song *et al.*, 2006), *TLR10* (haplotype GCGTGGC for rs10856837, rs11466651, rs11466652, rs11466653, rs11096956, rs11096955 and rs11466655) (Zhou *et al.*, 2006), *DC-SIGN* (−139A>G and −939G>A) (Xu *et al.*, 2010), and *CTLA-4* (+49A>G) (Xiao *et al.*, 2010).

Tumorigenesis encompass many genes and cascades of processes, culminating in uncontrolled cell division and growth, forming a malignant mass. Some genes related to the process were examined for the association with NPC. A case-control study in Nanning city, southern China evaluated associations of SNPs along Phosphatase and tensin homolog (*PTEN*) (rs11202592), v-akt murine thymoma viral oncogene homolog 1 (*AKT1*) (rs3803300, rs1130214, rs3730358, rs1130233 and rs2494732), mouse double minute 2 (*MDM2*) and *p53* (rs1042522) (Zhang *et al.*, 2014). None of the single SNPs were associated with NPC risk. However, haplotype analyses indicated that a two-SNP core haplotype (rs1130233-A-rs2494732-A) in *AKT1* was associated with a significantly increased susceptibility to NPC risk (adjusted OR = 3.87, 95% CI = 1.96-7.65; $P<0.001$). Combined risk genotypes from 3-4 SNPs gave significantly increased susceptibility to NPC risk (adjusted OR = 1.67, 95% CI = 1.12-2.50; $P = 0.012$)

In Malaysia, Yew *et al*. (2012) conducted a case-control study on 447 NPC cases and 487 controls of Chinese descent. Results found association to NPC risk at SNP rs2752903 of *SPLUNC1* (P=0.00032, odds ratio = 1.62, 95% confidence interval = 1.25-2.11) (Yew *et al*., 2012). Functional analysis identified rs1407019 located in intron 3 (r^2=0.994 with rs2752903) caused allelic difference in the binding of specificity protein 1 (Sp1) transcription factor and affected luciferase activity (Yew *et al*., 2012). SPLUNC1 is believed to play a role in innate immune defense in the airway because of its ability to regulate ENaC, influencing airway pathology (Garcia-Caballero *et al.*, 2009).

2.6.4 Metabolic genes

NPC is heavily influenced by diet, particularly the consumption of salted fish containing high amounts of carcinogenic nitrosamines. Thus, research was focused on identifying variants in metabolic enzymes that function in metabolizing carcinogens. The CYP superfamily has been constantly studied for its relation to NPC susceptibility. The earliest report was carried out by Hildesheim *et al*. (1995) comparing *CYP2E1* RFLP digestion sites between 50 NPC cases and matched control samples in Taiwan. Homozygous carriers of the *CYP2E1 Dra*I digestion showed a 5-fold risk of NPC (95% CI= 0.95-16) while homozygous carriers of *CYP2E1 Rsa*I digestion conferred a 7.7-fold risk of NPC (95% CI=0.87-68) (Hildesheim *et al.*, 1995). An extended study using 364 NPC cases and 364 controls in a Taiwanese population linked *CYP2E1 Rsa*I digestion to elevated NPC risk (relative risk [RR] = 2.6; 95% CI = 1.2-5.7) (Hildesheim *et al.*, 1997). Similar results were reported by a study investigating *CYP2E1* polymorphism in Thailand where *CYP2E1 Rsa*I polymorphism elevating NPC risk in the Thais (RR = 1.51; 95% CI = 0.08-90.06) and Thai Chinese (RR = 1.99; 95% CI = 0.39-10.87). When combined, the ethnicity-adjusted odds ratio is 2.39 with 95% CI, 0.72-7.89 (Kongruttanachok *et al.*, 2001). A separate study from Thailand also reported elevated NPC risk for carriers of *CYP2A6* mutants (*1B and *4C) when compared to wild type *1A/*1A (OR=2.37, 95% CI=1.27-4.46) (Tiwawech *et al.*, 2006).

More recent *CYP* typing utilizes high-resolution genotyping. A study in Guangdong, southern China employed both a family-based and case-control design to investigate *CYP2E1* association between *CYP2E1* and NPC susceptibility (Jia *et al.*, 2009). In the case-control analysis, 755 NPC cases and 755 controls were compared and SNP rs9418990, rs3813865, rs915906, rs2249695, rs8192780, rs1536826, rs3827688 (OR=1.88-2.99; P<0.015) and haplotypes h2 with OR = 1.65 (*P* = 0.026), h5 (CCCGTTAA) with OR = 2.58 (*P* = 0.007) were found to increase NPC risk (Jia *et al.*,

2009). Recently, a meta-analysis combining previous case-control *CYP2E1* NPC studies (Ben Chaaben *et al.*, 2015; Guo *et al.*, 2010; Hildesheim *et al*., 1995; Hildesheim *et al*., 1997; Kongruttanachok *et al*., 2001; Lourembam *et al.*, 2015) found association of *Rsa*I/*Pst*I polymorphism with NPC, however only under recessive and homozygote genetic models (OR = 2.72, 95% CI 1.73–4.25; OR = 2.64, 95% CI=1.68–4.16, respectively) (Yao *et al.*, 2017).

2.6.5 DNA repair genes

Due to the notion that both EBV and environmental carcinogens may promote DNA damage (Frenkel, 1992; Gruhne *et al.*, 2009), subsequently contributing to NPC carcinogenesis, association of the genetic variants of genes related to DNA repair and damage was widely studied. Cho *et al*. (2003) conducted a case-control study to investigate the genotypes of 334 NPC patients and 283 healthy controls in a Taiwanese population and found increased NPC risk for *hOGG1* codon 326 genotypes of Ser/Cys and Cys/Cys compared with the Ser/Ser genotype (OR=1.6; 95% CI=1.0-2.6). For *XRCC1* codon 280 genotypes of Arg/His and His/His compared with the Arg/Arg genotype, the OR was 0.64 (95% CI=0.43-0.96) (Cho *et al.*, 2003). When high-risk genotypes for both *hOGG1* and *XRCC1* were combined, the OR was 3.0 (95% CI=1.0-8.8) (Cho *et al*., 2003). Another study by Cao *et al*. (2006) found reduced risk of developing NPC in individuals with the Trp194Trp genotype (OR=0.48; 95% CI=0.27-0.86). Further analysis stratified by gender and smoking status revealed a significantly reduced risk of NPC among males (OR = 0.32; 95% CI, 0.14-0.70) and smokers (OR = 0.34; 95% CI=0.14-0.82) carrying the *XRCC1* 194Trp/Trp genotype (Cao *et al.*, 2006). *XRCC1* and *hOGG1* associations were not replicated in the Maghrebian population of north Africa (Laantri *et al.*, 2011). The *XRCC1* Codon399 Gln/Gln allele may also be associated with better tumor regression (Zhai *et al.*, 2016). A large scale case-control

study of *XRCC3* in the southern Chinese found association of rs861539 and NPC risk under the recessive model (TT vs. CT + CC) (OR = 2.72; 95 % CI=1.10-6.72; *P* = 0.03) (Cui *et al.*, 2016b).

Yang *et al*. (2008) performed a case-control study in Sichuan province, southern China. The results identified *ERCC1* SNP 8092 C>A, with 8092 C allele showing 1.411-fold (OR = 1.411, 95% CI, 1.076–1.850, P = 0.014) increased risk of developing NPC (Yang *et al.*, 2009). However, a separate study arrived at a contradictory conclusion. Patients with the *ERCC1* SNP 8092 C/A or A/A genotype had an increased risk of disease progression on cisplatin-based chemotherapy (7.9 vs. 9.3 months; HR 1.61; 95 % CI 1.08-2.61; *P* = 0.047) (Chen *et al.*, 2013).

Lye *et al*. (2015) reported association of xeroderma pigmentosum group D (XPD) K751Q polymorphism to NPC risk. Subjects with homozygous Lys/Lys (wildtype) genotype have 1.58 times higher odds of developing NPC compared to subjects with recessive combination of heterozygous Lys/Gln and homozygous Gln/Gln genotypes (OR = 1.58, 95% CI = 1.05-2.38 p = 0.028) (Lye *et al*., 2015).

2.7 Association studies in the age of genomics

The widespread adoption of genomics and bioinformatics started after the completion of the first human genome draft in 2001 (Lander *et al.*, 2001). Since then, the reference genome has gone through several revisions, and at the time of writing, it is currently on build GRCh38.p7. With the emergence of detailed genetic and physical maps, scientists are able to pinpoint location of plausible disease genes.

2.7.1 The HapMap and 1000 genomes project

The completion of the human genome project spurred the establishment of the HapMap (International HapMap Consortium, 2003, 2005) and 1000 genomes (1000 Genomes Project Consortium, 2015; Sudmant *et al.*, 2015) databases to catalog distribution of genomic variants in major populations in the world. The International HapMap project was established with the aim of mapping haplotypes and SNPs due to the presence of linkage disequilibrium (LD)- the co-inheritance of SNP alleles in haplotypes. This strong correlation between SNPs enables the genotyping of tag SNPs, representative SNPs that are able to provide enough information to predict much of the information about the remainder of the common SNPs in that region (Carlson *et al.*, 2003; Daly *et al.*, 2001; Johnson *et al.*, 2001). The pilot study recruited 270 DNA samples: 90 samples from a US Utah population with Northern and Western European ancestry (samples collected in 1980 by the Centre d'Etude du Polymorphisme Humain (CEPH) (49) and used for other human genetic maps, 30 trios of two parents and an adult child), and new samples collected from 90 Yoruba people in Ibadan, Nigeria (30 trios), 45 unrelated Japanese in Tokyo, Japan, and 45 unrelated Han Chinese in Beijing, China (International HapMap Consortium, 2003). Genotyping was performed across different centers in Japan, China, Europe and the United States using various platforms, namely Thirdwave Invader Asssay, Illumina Beadarrays, Sequenom MassExtend, ParAllele MIP and PerkinElmer AcycloPrime-FP. This pilot effort identified 2.8 million SNPs (International HapMap Consortium, 2003). However, the HapMap project is currently superseded by the 1000 genomes project (1000 Genomes Project Consortium, 2015; Sudmant *et al.*, 2015), which remains the current reference database for SNPs, CNVs and structural variants data.

The 1000 genomes project set out to comprehensively catalog all forms of genomic variants using low-coverage whole-genome sequencing, deep exome sequencing, and dense microarray genotyping (1000 Genomes Project Consortium, 2015; Sudmant *et al*., 2015). The project surveys the genomes of 2,504 individuals from 26 populations. The 1000 genomes project improved upon the HapMap initiative by profiling a larger number of SNPs (80 million) in addition to structural variants such as deletion, insertion, CNV (1000 Genomes Project Consortium, 2015; Sudmant *et al*., 2015). The larger number of samples also enable discovery of rare variants with frequency <0.5%. With the availability of the 1000 genomes haplotypes, a detailed and more comprehensive investigation of disease genes is made possible either through physical genotyping or *in silico* imputation methods (Browning & Browning, 2007; Browning & Browning, 2013, 2016; Howie *et al.*, 2009; Howie *et al.*, 2011; Howie *et al.*, 2012; Marchini *et al.*, 2007).

2.7.2 Imputation

Imputation is an *in silico* method to predict or impute genotypes that are not directly assayed in samples (Figure 2.4). This method takes into consideration the reference dataset in use, LD structure and the recombination rate (Marchini & Howie, 2010; Marchini *et al*., 2007). The current recommended reference phased haplotypes are from the 1000 genomes project phase 3 data (1000 Genomes Project Consortium, 2015; Sudmant *et al*., 2015), with all phased haplotypes used for imputation rather than samples of the population in study (Delaneau *et al.*, 2013a; Howie *et al*., 2011). Imputation is done using different algorithms, though more common methods in use currently are IMPUTE2 (Howie *et al*., 2009; Howie *et al*., 2011; Howie *et al*., 2012; Marchini *et al*., 2007) and BEAGLE v4.1 (Browning & Browning, 2007; Browning & Browning, 2013, 2016). Imputation is especially useful for fine mapping of a particular

region or for meta-analysis. In fine mapping, imputation allows a high-resolution view of an associated region with the possibility of finding the causal variant. As in the case of meta-analysis, when combining studies using different genotyping chips, very rarely do the variants overlap. To avoid direct genotyping of the missing variants, imputation is able to "fill-in" missing genotypes, thus combining analysis across different studies. This method not only can boost up the power of association but also uncover new genotypes. Though promising, the accuracy of imputation varies and is affected by factors such as variant density, frequency of variants (imputation fares poorly for rare alleles), recombination hotspots and LD structure. Therefore, it is imperative to evaluate imputation accuracy prior to selection for association analysis. IMPUTE2 uses an information measure (Howie *et al*., 2009) to assess post imputation accuracy while BEAGLE v4.1 relies on the R^2 correlation (Browning & Browning, 2013). Variants that are genotyped in the GWAS can be masked and imputed to evaluate imputation accuracy (Marchini & Howie, 2010).

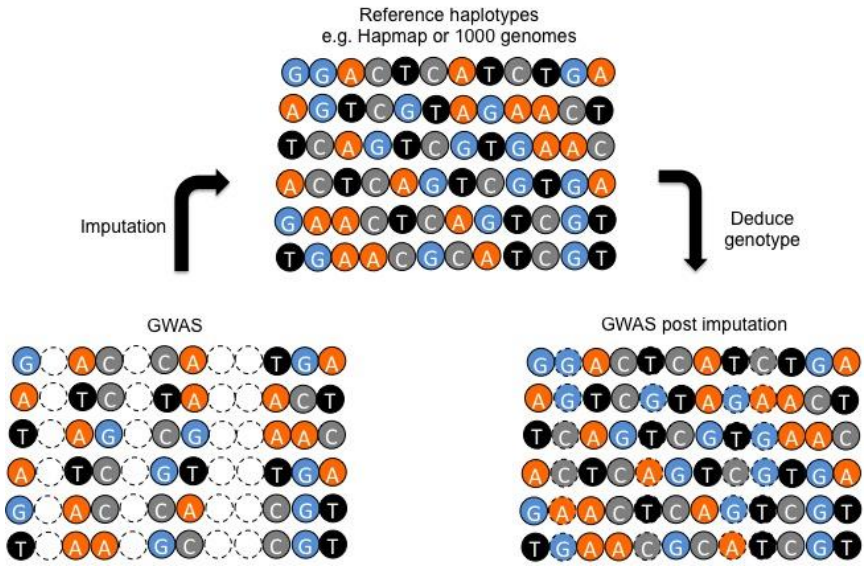


Figure 2.4: Imputation work flow for genome-wide association studies (GWAS). Missing genotypes in the GWAS data set are 'filled' in by matching with the reference data set (e.g. the Hapmap or 1000 Genomes reference haplotypes). The existing genotypes on the GWAS data set act as reference points or markers to match the data set. The subsequent step of 'filling in' the missing genotypes is performed, taking into account the linkage disequilibrium (LD) and recombination rate of the target region. Each row represents a haplotype from a single individual while each row represents a SNP.

2.7.3 Genome-wide association studies (GWAS)

GWAS is a large-scale association study, typically using a case-control approach, testing hundreds or even millions of SNPs concurrently to identify disease risk (Figure 2.5). The difference between GWAS and candidate gene approach is: 1) Candidate gene approach is hypothesis driven while GWAS is not - variants encompassing the whole genome is analyzed simultaneously; 2) Magnitude of variants - candidate approach usually studies limited variants on the genes of choice while GWAS screening can include up to millions of variants. GWAS screening can be performed either using a 2-tier or 3-tier design. The preliminary screening involves GWAS genotyping and association analysis followed either 1 or 2 separate replication cohorts. Replication is essential to identify variants showing a true association rather than by chance due to small sample size. Typically the selection of case and control subjects is crucial to avoid false positive associations. The case and control subjects ideally should be matched in terms of ethnicity, age and gender (McCarthy *et al.,* 2008). Subjects showing population stratification or cryptic relatedness should be removed to avoid inflation of the type I error with the remaining spurious population structure to be corrected using genomic control (Zheng *et al.,* 2006) or principal components (Price *et al.*, 2006). Genotyping is performed using proprietary microarray platforms, with varying configuration of SNPs to best capture variants of a particular ethnicity or across different populations. Marker selection is based on data generated from the 1000 genomes project, with tag SNPs selected to maximize coverage of all available variants.

Association analysis adopts the frequentist method (McCarthy *et al*., 2008), sometimes including covariates such as age, gender, populations structure and lifestyle habits. Frequentists methods can also account for various genetic models: additive, dominant, recessive or even co-dominant models. Though accounting for different genetic models could enhance the association of some variants (Lettre *et al.*, 2007), the

use of multiple genetic models also complicates computation of type 1 error rates by increasing the stringency of the multiple-testing threshold. This in turn reduces the number of potential disease loci and subsequent follow-up efforts. In addition, without confirmation of the causal function of SNPs, the adoption of genetic models at the genomics stage is academic at best.

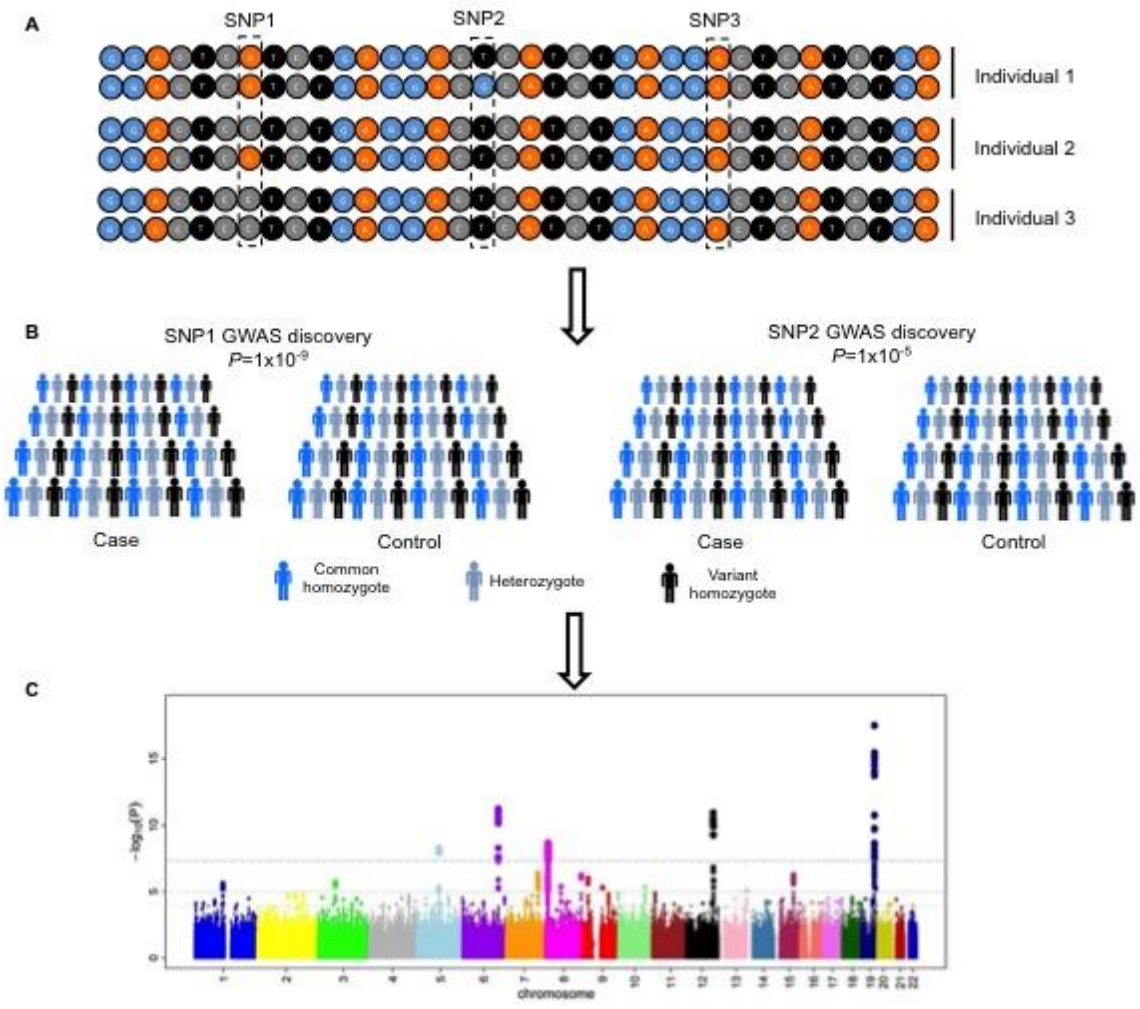


Figure 2.5: The case-control genome-wide association study (GWAS) design. A. Polymorphic SNP sites in the genome. B. Association analysis is done using a case control design testing for association of SNPs on a genome-wide scale. C. Manhattan plot shows an overview of all association signals from a GWAS study.

To date, there are 2838 GWAS publications and 33353 unique SNP-trait associations reported (MacArthur *et al.*, 2017), surpassing the genome-wide threshold of $P \leq 5.0 \times 10^{-8}$ (Hoggart *et al.*, 2008). The introduction of GWAS has improved the understanding of genetic basis of particular diseases, for example type 1 (Hakonarson *et al.*, 2007; Todd *et al.*, 2007) and type 2 diabetes (Saxena *et al.*, 2007; Scott *et al.*, 2007; Sladek *et al.*, 2007; Steinthorsdottir *et al.*, 2007; Zeggini *et al.*, 2007; Zeggini *et al.*, 2008), inflammatory bowel disease (Duerr *et al.*, 2006; Hampe *et al.*, 2007; Libioulle *et al.*, 2007; Parkes *et al.*, 2007; Rioux *et al.*, 2007), prostate cancer (Eeles *et al.*, 2008; Gudmundsson *et al.*, 2007a; Gudmundsson, *et al.*, 2007b; Gudmundsson *et al.*, 2008; Thomas *et al.*, 2008; Yeager *et al.*, 2007) and breast cancer (Easton *et al.*, 2007; Hunter *et al.*, 2007; Stacey *et al.*, 2007). Results from GWAS studies have confirmed previously hypothesized risk gene, though there are also instances whereby genes originally not thought to have a role in a given disease showed consistent association (Helgadottir *et al.*, 2007; Klein *et al.*, 2005; Moffatt *et al.*, 2007; Rioux *et al.*, 2007; Scott *et al.*, 2007). The bulk of variants identified through GWAS falls in the low penetrance, high prevalence category (Manolio, 2010; McCarthy *et al.*, 2008). In addition, most variants have modest effect sizes, with a median of 1.33 (Hindorff *et al.*, 2009). Many of the variants are situated in non-coding regions (Manolio, 2010), making functional interpretation difficult. It is suggested these GWAS signals could perhaps tag nearby causal variants. Perhaps, with the emergence of epigenetic initiatives like the ENCODE project (ENCODE Project Consortium, 2004; Kellis *et al.*, 2014) and the Roadmap Epigenomics project (Bernstein *et al.*, 2010), potential roles of intronic, and particularly intergenic regions in regulating gene expression can be identified.

The transition from identifying genetic variants to understanding its functional relevance remains elusive. However, with the emergence of new and more affordable

technologies, it is hoped this gap can be bridged, paving the way for widespread adoption of genomics in a clinical setting.

2.7.4 GWAS in NPC

To date, there are 6 GWAS studies conducted on NPC (Bei *et al.*, 2010; Chin *et al.*, 2015; Cui *et al.*, 2016a; Ng *et al.*, 2009a; Tang *et al.*, 2012; Tse *et al.*, 2009). The details of the Malaysian GWAS published in 2015 (Chin *et al.*, 2015) will be discussed in detail in subsequent chapters of this thesis. Thus it will be omitted from discussion at this point. All the GWAS studies are concentrated in the Chinese population, be it from southern China, Taiwan or Malaysian Chinese. Previous studies have indicated the sporadic nature of NPC, involving multiple genetic variants, each with low penetrance.

The first GWAS of NPC was carried out in the Malaysian Chinese using a 2-tier case-control design (Ng *et al.*, 2009a). At the discovery stage, 111 NPC cases and 260 unrelated controls were genotyped at the genome-wide scale of 533,048 SNPs. The top 200 GWAS SNPs were replicated in a separate set of 168 cases and 252 controls. The combined analysis identified a SNP in intron 3 of the *ITGA9* (integrin-alpha 9) gene, rs2212020, is strongly associated with NPC (P=8.27x10^{-7}, OR=2.24, 95% CI=1.59-3.15). Fine mapping of the surrounding region was also done, genotyping an additional 19 tag SNPs within a 40-kb linkage disequilibrium (LD) block surrounding rs2212020. SNP rs189897 was identified, showing improved an improved association to NPC risk (P=6.85x10^{-8}, OR=3.18, 95% CI=1.94-5.21). *ITGA9* encodes a subunit of integrin, which mediates many biological processes such as cell-cell adhesion, proliferation, apoptosis, and differentiation (Birkenbach *et al.*, 1992; Lin *et al.*, 1997; Stewart & Nemerow, 2007; Young *et al.*, 1989).

At about the same time, a separate GWAS study was conducted in Taiwan, using a 3-tier case-control design (Tse *et al*., 2009). The discovery stage recruited 277 NPC patients and 285 healthy controls, analyzing 480,365 single-nucleotide polymorphisms (SNPs). This identified 12 statistically significant SNPs at the 6p21.3 region. The GWAS signals were replicated in 2 independent sets of case-controls (replication 1=339 NPC-696 controls; replication 2=296 NPC-944 controls). The results identified SNPs in the following genes: 1) rs2517713 (P=3.9x10^{-20}) and rs2975042 (P=1.6x10^{-19}), located downstream of the *HLA-A* gene; 2) rs29232 (P=8.97x10^{-17}) gamma aminobutyric acid b receptor 1 (*GABBR1*); 3) rs3129055 (P=7.36x10^{-11}) and rs9258122 (P=3.33x10^{-10}) on *HLA-F*. rs29232 retained its association (P<5x10^{-4}) after adjustment with HLA-related SNPs, proving its association to be independent and not correlated with HLA SNPs. Immunohistochemical staining of NPC biopsies showed that the expression of GABBR1 protein in tumor cells was significantly higher than that in adjacent normal epithelial cells (P<0.001). GABA may affect cancer growth through activation GABA receptors (Zhang *et al.*, 2013).

Bei *et al*. (2010) reported a large NPC GWAS from populations of southern Chinese descent. A 2-tier case control desgin was employed, supported by a trio linkage study. The discovery stage recruited 1,583 NPC cases and 1,894 controls, analyzing 464,328 autosomal SNPs. The top 49 SNPs from the GWAS were replicated in 3,507 cases and 3,063 controls of southern Chinese descent from Guangdong and Guangxi. The seven SNPs showing genome-wide association significance ($P\leq$5.0x10^{-8}) were further confirmed by transmission disequilibrium test analysis in 279 trios from Guangdong. This study identified three new susceptibility loci, *TNFRSF19* on 13q12 (rs9510787, P=1.53x10^{-9}, OR=1.20), *MDS1-EVI1* on 3q26 (rs6774494, P=1.34x10^{-8}, OR=0.84) and the *CDKN2A-CDKN2B* gene cluster on 9p21 (rs1412829, P=4.84x10^{-7}, OR=0.78) (Bei *et al*., 2010). The study also confirmed the association of HLA by

revealing independent associations at rs2860580 (P=4.88x10^{-67}, OR=0.58), rs2894207 (P=3.42x10^{-33}, OR=0.61) and rs28421666 (P= 2.49x10^{-18}, OR=0.67) (Bei *et al*., 2010). *TNFRSF19* encodes a member of the TNF receptor superfamily (Hu *et al.*, 1999), and when overexpressed, activates the c-Jun N-terminal kinase (JNK) pathway and induces caspase-independent cell death (Eby *et al.*, 2000). EVI1 can suppress the effect of transforming growth factor (TGF)-β on growth inhibition, which in turn promotes tumor growth. EVI1 can also protect cells from stress-induced cell death by inhibiting c-JNK (Kurokawa *et al.*, 2000; Nitta *et al.*, 2005). Fusion of EVI1 with MDS1 impairs its capacity to repress TGF-β signaling (Nitta *et al*., 2005). Presence of SNPs in the *MDS1-EVI1* might interrupt the balance between EVI1 and MDS1, and subsequently the regulation of (TGF)-β. *CDKN2A* and *CDKN2B* are known tumor suppressor genes (Krimpenfort *et al.*, 2007; Sharpless *et al.*, 2001).

A separate GWAS was conducted by Tang *et al*. (2012) from Guangxi Zhuang Autonomous Region and Guangdong province of southern China. In this study, the authors focused on the amino acid variants of the HLA class I genes, complementing GWAS results with HLA class I typing. The results identified strong association signals involving SNPs, HLA alleles, and amino acid (aa) variants across the major histocompatibility complex-*HLA-A* ($P_{\text{HLA-A-aa-site-62}}$=7.4x10^{-29}), *HLA-B* ($P_{\text{HLA-B-aa-site-116}}$=6.5x10^{-19}), and *HLA-C* class I genes ($P_{\text{HLA-C-aa-site-156}}$=6.8x10^{-8}) (Tang *et al*., 2012). Multivariate logistic regression of adjacent regions found that all association signals were driven by effects of *HLA- A*11:01, HLA-B*13:01, B*38:02* and *B*55:02* (Tang *et al*., 2012). The strong association of amino acid variants implicate specific class I peptide recognition motifs in *HLA-A* and *-B* peptide binding groove as conferring strong genetic influence on the development of NPC in China.

The latest NPC GWAS study by Cui *et al*. (2016a) is an extension of the GWAS reported by Bei *et al*. (2010). The study recruited samples of southern Chinese ancestry, and the GWAS was done using a 3-tier case-control design. The discovery stage consisting of 463,250 SNPs in 1,583 NPC cases and 2,979 controls of southern Chinese ancestry revealed 1,257 top SNPs to be associated with NPC, which were validated in a separate case-control set of 1,925 NPC cases and 1,947 controls of southern Chinese (Cui *et al*., 2016a). Further 11 SNPs were selected for another independent validation in 3,538 NPC cases and 3,644 controls of southern Chinese giving a total set of 7,046 NPC cases and 8,570 controls. This study found associations with genome-wide significance ($P \leq 5.0 \times 10^{-8}$) at *TERT-CLPTM1L* at chromosome 5p15 (rs401681; $P = 2.65 \times 10^{-14}$; OR = 0.82) and *CIITA* at chromosome 16p13 (rs6498114; $P = 4.01 \times 10^{-9}$; OR=0.87) (Cui *et al*., 2016a). *CLPTM1L* and *TERT* have been implicated in cancers (Blasco, 2005; James *et al.*, 2014) and CIITA is considered as the "master control factor" for the expression of NPC-associated MHC class II genes (Steimle *et al.*, 1993). Table 2.3 summarizes all the NPC GWAS studies reported to date.

2.7.5 Meta-analysis of NPC GWAS studies

There are several approaches to GWAS meta-analysis (Cooper *et al.*, 2009), the most commonly adopted method being the fixed effects (Pfeiffer *et al.*, 2009) or random effects method (Pereira *et al.*, 2009). Fixed effects meta-analysis assumes that the true effect of each risk allele is the same in each data set. In other words, fixed effects is used when there is no heterogeneity across meta-analysis studies. Fixed effects method employs inverse variance weighting (Kavvoura & Ioannidis, 2008) to weight genetic effects across different studies. Each study is weighted according to the inverse of its squared standard error (Zeggini & Ioannidis, 2009). Random effects method is used when there is probable between-study heterogeneity (Ioannidis *et al.*, 2007; Pereira *et*

al., 2009). The most popular estimator used is the DerSimonian and Laird estimator (DerSimonian & Laird, 1986), though many other approaches do exist. Weighting of genetic effects also use the inverse variance method. The bayesian method is another alternative method for meta-analysis. However, bayesian models may depend on assumptions made about the prior distributions of parameters of interest, and their genome-wide implementation can become computationally intensive (Evangelou & Ioannidis, 2013).

Nasopharyngeal carcinoma (NPC) is linked to Epstein-Barr virus (EBV) infection. While EBV infection is ubiquitous, NPC incidence varies considerably around the world (Chang & Adami, 2006). It is hypothesized that genetic differences across populations partly explain the predisposition of this cancer to individuals of Southeast Asian descent. Several lines of evidence support a role for genetic susceptibility in NPC. The disease clusters within families (Chang & Adami, 2006). Also, numerous studies have implicated polymorphisms in candidate genes in NPC (Bei *et al.*, 2012; Hildesheim & Wang, 2012). The most consistent evidence has been for an association between *HLA* and NPC, an association that is biologically plausible given the central role of EBV in NPC and of HLA in immune presentation or handling (Su *et al.*, 2013).

Many major GWAS meta-analyses have been published thus far, though not for NPC. Several NPC GWAS have recently been published (Bei *et al*., 2010; Chin *et al*., 2015; Ng *et al*., 2009a; Tang *et al*., 2012; Tse *et al*., 2009); all provided support for the importance of genetic factors and clearly confirmed the involvement of genes in the MHC region (region where *HLA* genes reside) in NPC. Associations outside the MHC were also reported from these GWAS but were not as strong or consistent, suggesting the need for pooling across studies and larger efforts to identify novel genes involved in this disease (Bei *et al*., 2010; Chin *et al*., 2015; Ng *et al*., 2009a; Tang *et al*., 2012; Tse *et al*., 2009).

2.7.6 Pathway analysis of NPC GWAS studies

Pathway or 'gene-set' analysis on GWAS has been gaining traction of late. However, this approach has not been popular for NPC, with only two candidate gene approaches implicating DNA repair (Qin *et al.*, 2011) and *TERT-CLPTM1L* (Yee Ko *et al*., 2014) pathways in NPC etiology. Pathway analysis is appealing as it tests the cumulative association for gene sets of related function without the constraints of stringent multiple testing threshold. The inclusion of genes or loci with moderate to small effects better represent the gene interaction dynamics of NPC. This study aims to identify aberrant pathways associated to NPC in both GWAS and gene expression platforms. Pathway analyses employ either a competitive or self-contained hypothesis testing method (Tian *et al.*, 2005).

Table 2.3: Summary of NPC association results based on GWAS studies.

Gene	SNP	Pos	Effect allele/ AA	Sample (case/ctrl)	*P*-value	OR (95% CI)	Reference
ITGA9	rs2212020	3p22-21.3	T	279/512	$8.27x10^{-7}$	2.24 (1.59-3.15)	(Ng *et al*., 2009a)
GABBR1	rs2267633	6p22.1	A	912/1925	$1.28x10^{-9}$	1.57 (1.36-1.82)	(Tse *et al*., 2009)
GABBR1	rs2076483		A		$1.49x10^{-9}$	1.57 (1.36-1.82)	
GABBR1	rs29230		A		$4.77x10^{-9}$	1.56 (1.34-1.80)	
GABBR1	rs29232		A		$8.87x10^{-17}$	1.67 (1.48-1.88)	
HLA-F	rs3129055		G		$7.36x10^{-11}$	1.51 (1.34-1.71)	
HLA-F	rs9258122		A		$3.33x10^{-10}$	1.49 (1.32-1.69)	
HLA-A	rs2517713		A		$3.65x10^{-38}$	1.88 (1.65-2.15)	
HLA-A	rs2975042		A		$3.90x10^{-20}$	1.86 (1.63-2.13)	
HCG9	rs9260734		G		$1.60x10^{-19}$	1.85 (1.61-2.12)	
HCG9	rs3869062		A		$6.77x10^{-18}$	1.78 (1.55-2.05)	
HCG9	rs5009448		G		$1.30x10^{-15}$	1.72 (1.51-1.96)	
HCG9	rs16896923		A		$2.49x10^{-10}$	1.66 (1.42-1.94)	
MECOM	rs6774494	3q26	G	5090/4957	$1.34x10^{-8}$	0.84 (0.79-0.89)	(Bei *et al*., 2010)
HLA-A	rs2860580	6p22.1	A		$4.88x10^{-67}$	0.58 (0.55-0.62)	
HLA-B/C	rs2894207	6p21.33	G		$3.42x10^{-33}$	0.61 (0.57-0.66)	
HLA-DQ/DR	rs28421666	6p21.32	G		$2.49x10^{-18}$	0.67 (0.61-0.73)	
CDKN2A/2B	rs1412829	9p21	G		$4.84x10^{-7}$	0.78 (0.71-0.85)	
TNFRSF19	rs9510787	13q12	G		$1.53x10^{-9}$	0.84 (0.79-0.90)	
TNFRSF19	rs1572072	13q12	A		$1.30x10^{-8}$	0.84 (0.79-0.90)	
HLA-A	11:01	6p22.1	-	1405/2650	$1.72x10^{-19}$	0.59 (0.53-0.66)	(Tang *et al*., 2012)
	AAsite 62		Q		$1.17x10^{-24}$	0.59	
	AAsite 70		Q		$1.47x10^{-21}$	0.61	
	AAsite 97		I		$3.05x10^{-20}$	0.62	
	AAsite 114		R		$5.74x10^{-22}$	0.61	
	AAsite 276		L		$3.29x10^{-23}$	0.58	
HLA-B	13:01	6p21.33	-		$4.52x10^{-7}$	0.66 (0.56-0.77)	
	38:02		-		$1.96x10^{-10}$	1.88 (1.55-2.28)	
	55:02		-		$1.57x10^{-10}$	0.27 (0.18-0.40)	
	AAsite -16		L		$1.70x10^{-13}$	0.65	
	AAsite 97		R		$4.41x10^{-11}$	0.66	
	AAsite 116		L		$2.37x10^{-13}$	0.63	
	AAsite 116	6p21.33	F		$1.58x10^{-7}$	1.45	
	AAsite 158		T		$5.96x10^{-8}$	1.48	
HLA-C	12:02		-		$4.28x10^{-5}$	0.41 (0.27-0.63)	
	AAsite 24		S		$3.88x10^{-6}$	1.24	
	AAsite 95		I		$4.48x10^{-6}$	0.76	
	AAsite 95		L		$1.50x10^{-5}$	0.77	
	AAsite 156		W		$1.35x10^{-9}$	0.47	
	AAsite 304		M		$6.15x10^{-5}$	0.78	
CLPTM1L	rs401681	5p15	T		$2.65x10^{-14}$	0.82 (0.78-0.87)	(Cui *et al*., 2016a)
CIITA	rs6498114	16p13	G		$4.01x10^{-9}$	0.87 (0.83-0.91)	

CHAPTER 3: METHODOLOGY

3.1 Methodology for NPC GWAS study

3.1.1 Study cohort

NPC patients were recruited from University Malaya Medical Centre (UMMC), Tung Shin Hospital, Kuala Lumpur General Hospital (HKL), Penang General Hospital (HPP), Nilai Cancer Institute Hospital (NCI), Hospital University Sarawak (HUS) and Queen Elizabeth Hospital Sabah (QES). All cases were histo-pathologically diagnosed according to the World Health Organization (WHO) classification. All healthy controls were blood donors recruited from the UMMC blood bank with no familial history of cancer. All NPC patients and healthy control samples were unrelated self-reported Malaysian Chinese of Southern Chinese descent. All participants gave their written informed consent. The study was approved by the ethics committee of RIKEN, Yokohama, Japan and UMMC as well as the Ministry of Health, Malaysia. The genome-wide analysis was performed using a GWAS cohort of 193 NPC patients and 260 healthy controls followed by a second replication cohort of 260 NPC patients and 245 healthy controls.

3.1.2 Genotyping of SNPs and statistical analysis

Genomic DNA was extracted from the peripheral leukocytes using well established phenol-chloroform methods (Ausubel *et al*., 1987). GWAS genotyping was performed by the Laboratory for Genotyping Development at RIKEN Center for Integrative Medical Sciences, Yokohama using the Illumina HumanOmniExpress_12 v1.1 Beadchips (San Diego, CA, USA). Quality control removed 9 NPC patients and 24 healthy controls due to gender mismatches, cryptic relatedness or were outliers of the study population. Principal component analysis (PCA) indicated that NPC patients and controls were genetically matched, with minimal evidence of population stratification.

For SNP quality control, 137,470 SNPs were excluded where 29,876 SNPs had call rates < 99%, 107,589 SNPs had minor allele frequency (MAF) < 1% (74,883 SNPs were mono-allelic) and 5 SNPs showed significant deviation from Hardy-Weinberg equilibrium (HWE) in controls ($P<1.0x10^{-6}$). A total of 575,247 autosomal SNPs passed quality control in a GWAS cohort of 184 NPC patients and 236 healthy controls. Validation of the GWAS genotyping results as well as genotyping of replication samples were performed using multiplex-PCR-based invader assay (Third Wave Technologies, Madison, WI, USA). The same quality control measures as the genome-wide analysis were applied but with more stringent HWE thresholds in controls ($P<0.05$). Genotyping of previously reported GWAS SNPs from Ng *et al*. (2009a), Tse *et al*. (2009), Bei *et al.* (2010), Tang *et al*. (2012) were performed in the same manner. The quantile-quantile plot (Q-Q plot) of trend test was generated using R (https://cran.r-project.org/) to evaluate overall association of GWAS and presence of population stratification inferred through genomic control inflation factor (λ_{gc}). The Manhattan plot of $-\log_{10}(P_{GWAS})$ for autosomal SNPs was generated using Haploview (Barrett *et al.*, 2005). The genome-wide association analysis was performed in PLINK (Purcell *et al.*, 2007) using multivariate logistic regression assuming an additive model (0, 1, 2 allele dosage coding for minor allele) adjusting for age, gender and the first principal component (PC1). Only PC1 was estimated to be significant by the Tracy-Widom statistic ($P<0.05$). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated per risk allele assuming an additive model. SNPs were ranked in order of the lowest *P*-value. Association of previously reported GWAS SNPs from Ng *et al*. (2009a), Tse *et al*. (2009), Bei *et al.* (2010) and Tang *et al*. (2012) was analyzed in the same manner.

3.1.3 Imputation

Imputation of all *HLA-A* region SNPs were performed using IMPUTE (Marchini *et al*., 2007) utilizing HapMap 2 (CHB & JPN), HapMap 3 Mixed Population, 1000 Genomes Asian population and previously reported GWAS data (Ng *et al*., 2009a). SNPs showing MAF ≥ 1%, imputation power (INFO) > 0.5 and imputation confidence (CERTAINTY) > 0.9 were selected for validation and replication through multiplex-PCR-based invader assay (Third Wave Technologies, Madison, WI, USA). Statistical analysis and quality control measures were performed similar to the GWAS analysis.

3.1.4 *HLA-A* SNP and amino acid variants analysis

HLA-A genotyping was carried out using a WAKFlow HLA typing kit and the data was analyzed using the WAKFlow HLA typing software (Wakunaga, Hiroshima, Japan). We mapped *HLA-A* SNPs and amino acid variants referencing the *HLA-A* sub-type sequences from dbMHC using the WAKFlow *HLA-A* typing data (www.ncbi.nlm.nih.gov/projects/gv/mhc/). Statistical analysis and quality control measures for *HLA-A* alleles and *HLA-A* single SNPs were performed in PLINK similar to the GWAS analysis. For multi-allelic SNPs and amino acid variants, statistical analysis was performed in R employing a Fisher's Exact Test in R x C contingency table. OR and 95% CI were calculated with reference to the major allele. To identify LD-driven associations, Multivariate logistic regression was used to adjust for the additive effects of both *HLA-A* single SNPs and *HLA-A* alleles, entered as 0, 1, 2 allele dosage coding. The coded allele was either the minor allele of a *HLA-A* SNP, a carrier allele of a *HLA-A* allele or the rare amino acid variant. Same approach was used to analyse multi-allelic SNPs by only considering the strongest bi-allelic or damaging/deleterious combination. All *P*-values were also adjusted for age, gender and PC1. Corresponding function of amino acid residues were annotated with reference to

the Immunology Database and Analysis Portal (ImmPort) system (immport.niaid.nih.gov/). Functional impact of *HLA-A* gene SNPs were annotated using PROVEAN (Choi *et al.*, 2012), SIFT (Kumar *et al.*, 2009) and Polyphen-2 (Adzhubei *et al.*, 2010) prediction scores. Variants were deemed damaging or deleterious if predicted in 2 out of 3 of the annotation methods.

3.1.5 Regulatory functions of NPC associated *HLA-A* SNP variants

HaploReg (Ward & Kellis, 2012) was used to predict 5'-UTR *HLA-A* SNP variants with function related to transcription regulation. SNP variants were annotated to locate epigenetic functions related to i) enhancer (H3K4me1 and H3K27ac histone modification) or promoter activity (H3K4me3 histone modification) ii) DNase I enzyme hypersensitivity (suggestive of a regulatory region) iii) Protein or transcription factor binding iv) Regulatory motif changes as predicted from Position Weight Matrices (PWM) of TRANSFAC (Matys *et al.*, 2003), JASPAR (Portales-Casamar *et al.*, 2010) and protein-binding microarray experiments (Badis *et al.*, 2009; Berger *et al.*, 2008). Strength of regulatory motif binding is inferred by log-of-odds (LOD) of reference and alternate alleles. We investigated effects of SNP genotypes on *HLA-A* expression through expression quantitative trait loci analysis (eQTL) data obtained from the Sanger Institute Genevar Database (Yang *et al.*, 2010) using fibroblasts, LCLs and T cells derived from the umbilical cords from the Geneva GenCord (Dimas *et al.*, 2009) dataset and adipose, LCLs and skin tissues from the MuTHER resource (Nica *et al.*, 2011) dataset. A *cis*-eQTL is defined as a SNP located within 2Mb region of the gene of interest, in our case the *HLA-A* gene, with significant Spearman-rank correlation between SNP genotype and gene expression ($P<1.0\text{x}10^{-3}$).

3.2 Methodology for meta-analysis of NPC GWAS

3.2.1 GWAS data for meta-analysis

The GWAS contributing to the meta-analysis included histologically confirmed NPC cases and region-specific controls restricted to individuals of Chinese ethnicity (Table 3.1) (Bei *et al*., 2010; Chin *et al*., 2015; Ng *et al*., 2009a; Tang *et al*., 2012; Tse *et al*., 2009). GWAS genotyping was performed by the Laboratory for Genotyping Development at RIKEN Center for Integrative Medical Sciences, Yokohama. Genotyping for the Malaysian GWAS was performed using Illumina Hap550v3 Beadchip and Illumina Human OmniExpress_12 v1.1 Beadchip. Both Malaysian GWAS were analyzed as one. All data included passed QC filtering criteria as described previously (Chin *et al*., 2015; Ng *et al*., 2009a).

Table 3.1: Summary of studies included in the meta-analysis (Bei *et al.*, 2016).

Phase	Reference	Location	Genotyping platform	Cases	Controls
GWAS	Bei *et al*., 2010	Southern China	Illumina Hap610	1,583	2,979
GWAS	Tse *et al*., 2009	Taiwan	Illumina 600 Hap550v3 Beadchips	277	285
GWAS	Ng *et al*., 2009	Malaysia 1	Illumina Hap550v3 Beadchip	108	240
GWAS	Chin *et al*., 2015	Malaysia 2	Illumina Human OmniExpress_12 v1.1 Beadchip	184	236
GWAS-SUM				2,152	3,740
Replication I		Southern China I	Sequenom custom array	3,525	4,121
Replication I		Malaysia	Sequenom custom array	335	405
Replication I		Taiwan I	Sequenom custom array	352	312
Replication I		Taiwan II	Sequenom custom array	504	541
Rep I - SUM				4,716	5,379
Total				6,868	9,119

3.2.2 Imputation to combine GWAS SNPs

To maximize coverage across studies, genome-wide imputations were performed for both Malaysian NPC GWAS studies using typed SNPs. SNPs with call rates >90%, minor allele frequencies >3%, and that had genotype distributions that did not deviate from the expected by Hardy-Weinberg equilibrium (in controls; $P>10^{-6}$) were retained for imputation using IMPUTE2 (Howie *et al*., 2009). HapMap reference data were used (HapMap phase III, CHB+CHD+JPN data). Imputated genotypes with information score <90%, MAF <3%, or missing >10% were excluded. GTOOL (http://www.well.ox.ac.uk/~cfreeman/software/gwas/gtool.html) was used for data conversions.

3.2.3 Statistical analysis

For each GWAS, SNPs were analyzed by logistic regression under a log additive model, adjusting for age and cryptic population stratification. To define population stratification adjustment factors, principal component analysis was performed using EIGENSTRAT (Price *et al*., 2006) with a pruned set of 30,956 SNPs defined based on pairwise linkage disequilibrium (r^2 < 0.05 among Chinese) and restricted to SNPs with MAF >3%. The top 10 eigenvectors were evaluated for their association with NPC (separately for each individual GWAS) and included in the final logistic models if *P*-value <0.05 by the Wald test.

Using results from individual GWAS, the 500 SNPs with the lowest *P*-values from each of the studies were identified after exclusion of SNPs that could not be imputed or failed QC filtering. These study-specific lists of top-SNPs were combined into a single list for consideration as part of the present meta-analysis. In total, 1,590 SNPs were identified through this process and these 1,590 SNPs comprised the basis for the present meta-analysis. Summary statistics (number cases/controls, genotype counts,

β-coefficients, and SDs) were obtained from individual studies for selected SNPs and a meta-analysis was performed using a fixed effects model in R (https://cran.r-project.org). The merging of post-imputation data from individual GWAS was performed by the Biostatistics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland.

3.2.4 Replication of meta-analysis targets

SNPs were selected for replication as follows. The top 200 hits (all with *P*-values <0.0167) from the meta-analysis for which the direction of the association was consistent across all individual studies were arbitrarily ranked by *P*-value. These selected SNPs with *P*-values $<1x10^{-5}$ were 250 kb+ from other selected SNPs. For SNPs within 250kb of another SNP on the list, the SNP with the smaller *P*-value when the r^2 between SNPs was >0.80 were retained (r^2 based on Chinese population from the 1000 genomes project). Thirty SNPs were selected based on these criteria. A further 14 SNPs nominated by consortium members were added based on results from individual GWAS and other information from candidate-based studies and other studies in the published literature. One SNP that qualified based on the criteria above but failed in the design of the custom array described below was excluded (rs11865086). A second SNP that qualified but failed in the custom array design (rs6931820) was replaced with a SNP in strong LD with the original SNP (rs1324103; r^2=0.88). In total, 43 SNPs were evaluated in the replication phase in the Malaysian samples.

Replication studies were restricted to studies among individuals of Chinese descent. All four studies were hospital-based, recruiting NPC cases from selected hospitals in their respective geographical area. From Malaysia, 335 cases and 405 controls were recruited for the replication phase. Cases were recruited from the University of Malaya Medical Center (Kuala Lumpur, Malaysia) and from a network of

additional hospitals across the country. For the southern China study, cases were recruited from the Sun Yat-Sen University Cancer Center (Guangzhou, China) and the Southern Medical University Hospital. For the Malaysia study, For the two Taiwan studies, cases were recruited form the National Taiwan University (Taipei, Taiwan) and MacKay Memorial hospitals and from the Chang Gung Memorial and Linkou hospitals, respectively. Cases were restricted to adults with histologically confirmed NPC. Geographically matched controls of Chinese descent were frequency (southern China, Malaysia, and Taiwan II studies) or individually (Taiwan I study) matched to cases on age and gender. Controls did not have a history of NPC diagnosis. Studies were reviewed/approved by ethical committees and informed consent was obtained from participants. In total, 4,716 cases and 5,379 controls across four case–control studies in Mainland China, Malaysia, and Taiwan were recruited for the meta-analysis (Table 3.1).

A custom designed array containing the 43 SNPs selected for replication was developed using the Sequenom MassARRAY iPlex assay. To ensure comparable quality across laboratories, a common QC panel consisting of 94 HapMap samples was tested. Percent agreement across laboratories for the 43 SNPs tested was 97% (range: 82%–100%; agreement was >85% for all but two SNPs: rs189897 and rs4714505).

To analyze the replication studies, individual genotyping results were pooled and an additive logistic regression model used to evaluate the effect of each SNP, adjusting for study. To summarize information across the GWAS and replication studies, a meta-analysis was conducted using the fixed effect model to integrate estimates from all studies. As for the meta-analysis merging both GWAS and replication association data across different study groups, the analysis was performed by the Biostatistics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland.

3.3 Methodology for integrated pathway analysis of NPC

3.3.1 NPC GWAS data

Samples for this study were from previous NPC GWAS studies generated on the Illumina HumanHap 550K and Illumina Human OmniExpress_12 v1.1 platform in a Malaysian Chinese cohort (Chin *et al*., 2015; Ng *et al*., 2009a). The details of sample recruitment, quality controls and patient consent have been described elsewhere (Chin *et al*., 2015; Ng *et al*., 2009a). Briefly, SNPs with call rates >99%, minor allele frequency (MAF) >1% and minimal Hardy–Weinberg equilibrium (HWE) deviation in controls (P>1.0x10^{-6}) were retained. Samples were retained after removing gender mismatches, cryptic relatedness and outliers in the study population. Principal component analysis (PCA) was performed to adjust for population structure and to identify outliers using EIGENSOFT (Patterson *et al.*, 2006) package in a pruned set of 76,181 overlapping SNPs (r^2<0.2) from both Illumina HumanHap 550K v3 and Human OmniExpress_12 v1.1. The study cohort after quality control are as follows: (1) Illumina HumanHap 550K v3 platform, 441,812 autosomal SNPs were retained in 108 NPC patients and 240 healthy controls; (2) Illumina Human OmniExpress_12 v1.1 platform, 575,247 autosomal SNPs were retained in 184 NPC patients and 236 healthy controls.

3.3.2 Imputation and combining GWAS datasets

Both Illumina HumanHap 550K and Illumina Human OmniExpress_12 v1.1 datasets were phased using SHAPEIT v2 release 7 (Delaneau *et al.*, 2013b) prior to imputation. Imputation was performed using IMPUTE2 (Howie *et al*., 2009) with phased haplotypes from all populations of the December 2013 release of the 1000 Genomes Project data. SNP genotypes were called from posterior probabilities in PLINK 1.9 beta (Chang *et al.*, 2015) using default parameters. Only overlapping SNPs found on both Illumina HumanHap 550K and Illumina Human OmniExpress_12 v1.1 datasets with MAF>1%, imputation confidence (INFO)>0.5 and best called genotype certainty (CERTAINTY)>0.9 were retained for pathway analysis. The association analysis was also performed in PLINK 1.9 beta using multivariate logistic regression assuming an additive model (0, 1, 2 allele dosage coding for minor allele) adjusting for age, gender, and the first principal component (PC1). Only PC1 was estimated to be statistically associated with case–control status by the Tracy–Widom statistic (P<0.05). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated per minor allele assuming an additive model. The quantile–quantile plot (QQ plot) of trend test was generated to evaluate overall association of GWAS and presence of population stratification inferred through genomic control inflation factor (λ_{gc}). The Manhattan plot of $-\log_{10}(P_{\text{imp-GWAS}})$ for autosomal SNPs was generated to plot post-imputation GWAS signals.

3.3.3 GWAS Pathway analysis

Pathway analysis was performed in MAGENTA (Segre *et al*., 2010). Details of MAGENTA analysis parameters have been described previously (Segre *et al*., 2010). Briefly, SNPs were mapped onto genes using several boundary settings following the gene's most extreme transcript start and end site: 110kb UTR-40kb DTR; 20kb UTR

DTR; 10kb UTR-2kb DTR. Gene scores were calculated adjusting for potential confounders using step-wise multiple linear regression and the best SNP (strongest adjusted *P*-value) will be retained to represent the said gene. The confounders include: (1) Gene size; (2) Number of SNPs per kb for each gene; (3) Number of independent SNPs per kb for each gene (pairwise $r^2<0.5$ based on Malaysian Chinese population); (4) Number of recombination hotspots per kb each gene; (5) Genetic distance of each gene; (6) Linkage equilibrium units (LDU) per kb for each gene. The MHC region was omitted from the analysis. Pathway analysis was performed on 2757 predefined pathways from BioCarta, GO, Ingenuity, KEGG, Panther and Reactome databases. Pathways were limited to 10-100 gene-sets size. Nominal P_{GSEA} was calculated through permutation via random resampling of 10,000 gene sets of identical size. MAGENTA sets two thresholds for gene-level significance, 95th percentile and 75th percentile with the latter capturing weaker gene-level association signals. In this study, the more stringent 95th percentile was set as threshold for gene-level significance. Gene sets with $P<0.05$ were selected for integrated pathway analysis.

3.3.4 GEO Gene expression pathway analysis

Microarray data from dataset GSE12452 (Sengupta *et al*., 2006) was chosen to corroborate the gene expression profiles of pathways identified by MAGENTA. GSE12452 consists of mRNA from laser-captured epithelium of 31 nasopharyngeal carcinomas and 10 non-NPC nasopharynx tissues from a Taiwanese case control cohort. The mRNA profiling was performed on Affymetrix Human Genome U133 Plus 2.0 Array. Normalization of gene expression values are as described previously (Sengupta *et al*., 2006). Differential gene expression analysis was performed in GEO2R employing the limma package. A t-test was performed, adjusting for $\log_2$ fold-change of expression in the direction of NPC cases vs healthy nasopharynx tissues. *P*-values were used as

gene-level input for GSEA in GSA-SNP (Nam *et al.*, 2010). Pathway sizes were limited to 10-100 gene-sets size. For purpose of consistency, gene sets used for GSEA in GSA-SNP were the same gene sets used for MAGENTA covering 2757 predefined pathways from BioCarta, GO, Ingenuity, KEGG, Panther and Reactome databases. Pathways showing nominal $P<0.05$ were selected for integrated pathway analysis.

3.3.5 Fisher's method for integrating GWAS and expression data

Fisher's method was used to combine FDR-corrected GWAS pathway and gene expression pathway *P*-values. Pathways selected for integrated pathway analysis must show nominal $P<0.05$ in both platforms. Fisher's method is a combined probability test of results from independent tests sharing the same null hypothesis H_0 (Fisher, 1925), generating a combined statistic, χ^2 using the formula below:

$$\chi^2_{2k} = -2\sum_{i=1}^{k} \ln(pi)$$

pi is the p-value for the i^{th} hypothesis test

χ^2 is the chi-square critical value with $2k$ degrees of freedom

3.3.6 Sample collection for gene expression analysis

A total of 10 NPC and 7 non-NPC nasopharynx tissues were collected from the Queen Elizabeth Hospital (QES), Kuala Lumpur General hospital (HKL), and Pulau Pinang General Hospital (HPP). Tissues were snap frozen and stored at -80^{o}C. All NPC cases were histologically diagnosed following the World Health Organization (WHO) classification. Non-NPC nasopharynx tissues were from patients initially suspected for NPC but histologically diagnosed otherwise. All patients provided written, informed

consent prior to tissue collection for the purposes of research. The Medical Review and Ethics Committee of the Ministry of Health, Malaysia, approved the study.

3.3.7 Tissue processing and RNA isolation for gene expression analysis

Snap frozen tissue samples were embedded into Optimal Cutting Temperature (OCT) compound for cryosectioning. For every 9 sections of 8 micron thick tissue sections, a H&E slide was made for histology review. For NPC, RNA was extracted from laser capture microdissection or whole tissue sections of tumour tissue with at least 80% of cancer cells. Non-NPC nasopharynx tissue sections were lymphoid tissues with 20% to 40% normal epithelial cells. Total RNA was isolated using AllPrep DNA/RNA/miRNA universal kit (Qiagen, Germany). RNA yield was assessed using Qubit® RNA HS Assay Kit (Thermo Fisher Scientific, USA) and RNA quality was assessed using Fragment Analyzer (Advanced Analytical, Germany). RNA samples with at least 10 ng of material and DV200 (percentage of RNA fragments larger than 200 nt) >30% were used for RNA sequencing.

Whole genome gene expression analysis was performed by Lotterywest State Biomedical Facility Genomics in the University of Western Australia using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific, USA). Briefly, 10 ng of total RNA is reverse transcribed to construct a barcoded cDNA library using the SuperScript® VILO™ cDNA Synthesis kit. Then, cDNA is amplified using Ion AmpliSeq™ technology to produce a single amplicon spanning 2 exons for each gene (20,813 genes). Library quality was evaluated using Agilent Bioanalyzer. Libraries were then amplified using emulsion PCR and enriched following manufacturer's instructions. The final mixture was then sequenced on a Ion PI™ chip using an Ion Torrent Proton™ sequencing system.

3.3.8 Read alignment and differential gene expression analysis

Primary analysis of Ion AmpliSeq™ Transcriptome data was done using the Torrent Suite™ software. Raw reads are mapped to a custom reference sequence set using the Torrent Mapping Alignment Program (TMAP) (Li *et al.*, 2015). The custom reference set contains all transcripts targeted by the AmpliSeq kit. This method greatly improves alignment time. The number of reads mapping to each target is counted using samtools (Li *et al.*, 2009), giving the raw total read counts. Differential gene expression analysis was performed in DESeq2 (Love *et al.*, 2014) with raw read counts from AmpliSeq. Raw counts were normalized and transformed using the regularized logarithm (rlog) method in DESeq2. Genes showing normalized read counts <10 were removed from analysis. Sample quality was evaluated using sample-to-sample distance heatmap and the PCA plot. Differential expression *P*-values calculated through FDR-corrected Wald test were used as gene-level input for GSEA of axonemal dynein complex and chromosomal centromeric region pathways in GSA-SNP following similar methods in the "GEO Gene expression pathway analysis" section.

CHAPTER 4: RESULTS

4.1 Results for NPC GWAS study

4.1.1 GWAS genotyping and validation

The PCA indicated that NPC patients and controls were genetically matched, with minimal evidence of population stratification (Figure 4.1). Quantile-quantile plot analysis detected a small inflation factor (λ_{gc}) of 1.04, indicating minimal inflation of the genome-wide association significance due to population stratification (Figure 4.2). Several loci showed potential association to NPC susceptibility, although not genome-wide significant ($P_{GWAS}<5.0x10^{-8}$) (Figure 4.3). A total of 45 SNPs showing $P_{SNP-GWAS}<1.0x10^{-4}$ were selected for validation and replication analysis through multiplex-PCR-based-Invader assay. Only the $P_{SNP-Combined}$ for the top 20 GWAS SNPs are shown (Appendix A). Strong association signals were detected in only the major histocompatibility complex *HLA-A* region. These 6 *HLA-A* region SNPs showed $P_{SNP-Combined}$ ranging from 10^{-8} to 10^{-9} and are located upstream or downstream of the *HLA-A* (Table 4.1, Figure 4.4). All 6 SNPs render a protective effect against NPC with rs3869062 showing the strongest association ($P_{SNP-Combined}=1.73x10^{-9}$; odds ratio (OR) =2.37; 95% confidence interval (CI) =1.79-3.14) and is located 13-kb downstream of the *HLA-A* gene. All 6 SNPs are in high linkage disequilibrium (LD) with each other (r^2=0.72-0.99) (Figure 4.4). Association signals of the 6 *HLA-A* region SNPs were lost after regressing against each other, suggesting highly correlated associations (Table 4.2).

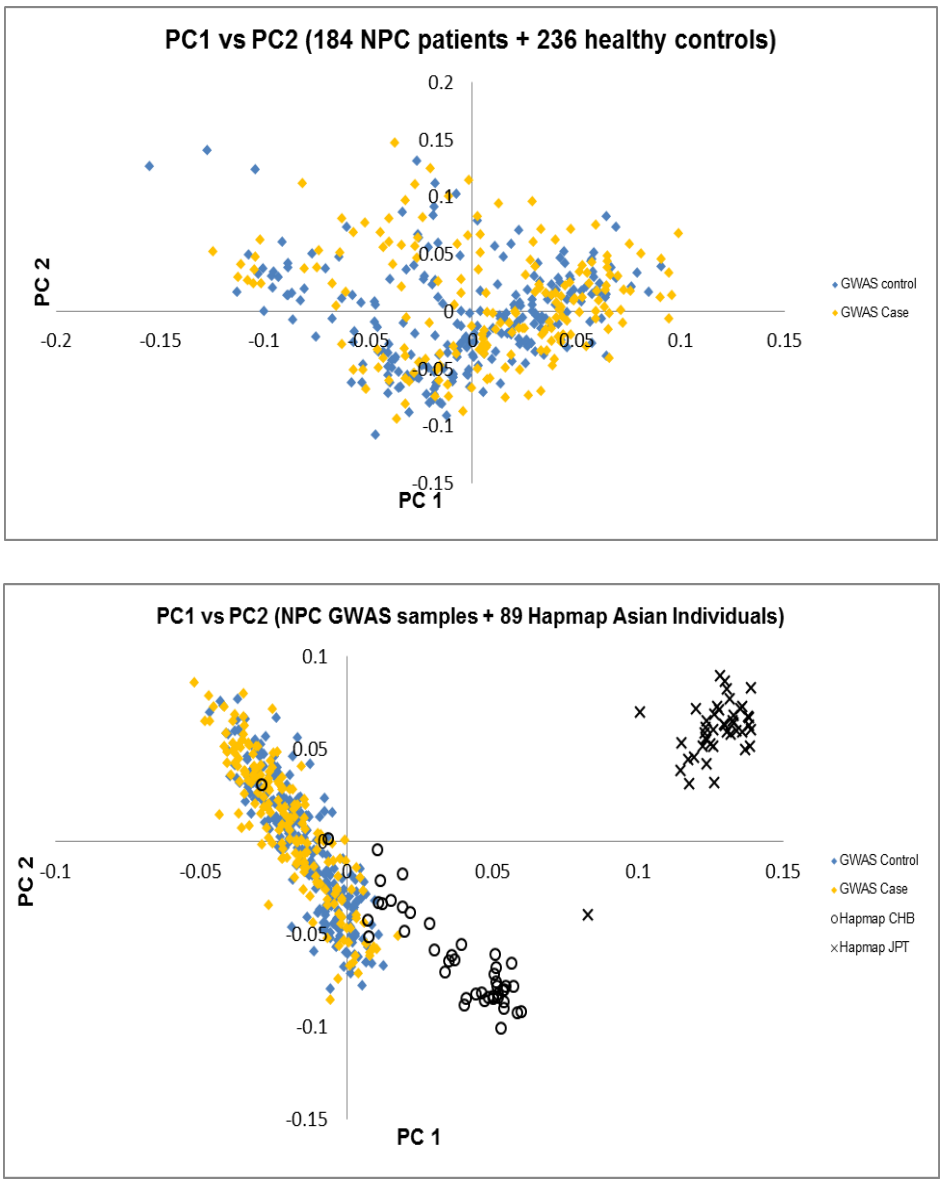


Figure 4.1: Plots of principal components from PCA analysis of NPC GWAS samples. (Top) Plot of PC1 and PC2 from the PCA of 184 NPC patients and 236 healthy controls. Orange markers signify GWAS cases (NPC patients) while purple markers signify GWAS controls (healthy controls). (Bottom) Plot of PC1 and PC2 from the PCA of 184 NPC patients and 236 healthy controls together with 44 Japanese in Tokyo, Japan (JPT), 45 Han Chinese in Beijing, China (CHB). Orange markers signify GWAS cases (NPC patients) while purple markers signify GWAS controls (healthy controls). Hapmap controls were designated with round circles for Han Chinese in Beijing (CHB) and 'X' for Japanese in Tokyo, Japan (JPT).

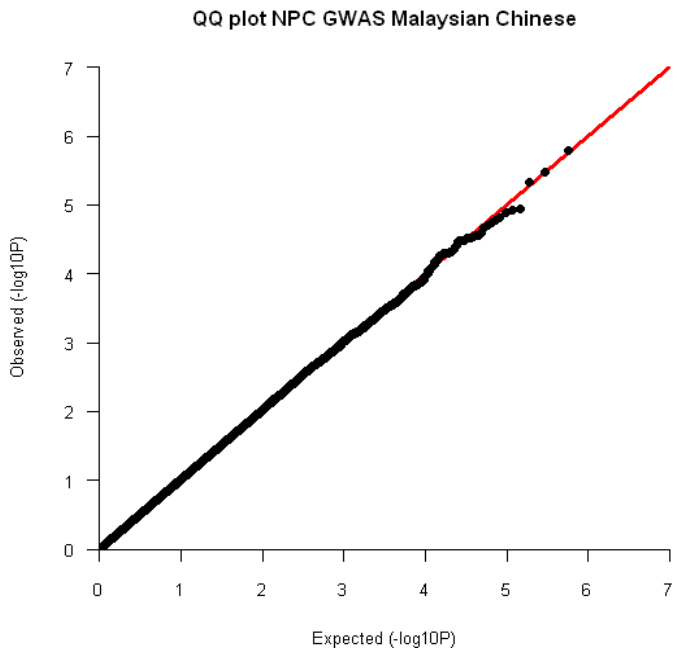


Figure 4.2: Log_{10} quantile-quantile (Q-Q) plot for all SNPs from Malaysian NPC GWAS. Q-Q plot showing the distribution of observed statistics by trend test for all 575,247 SNPs from our genome-wide study of 184 NPC patients and 236 healthy controls of a Malaysian Chinese population. The diagonal line shows the values expected under null hypothesis of a χ^2 distribution. Genomic control inflation factor (λ_{gc}) is 1.04, suggesting little or absence of stratification in our samples.

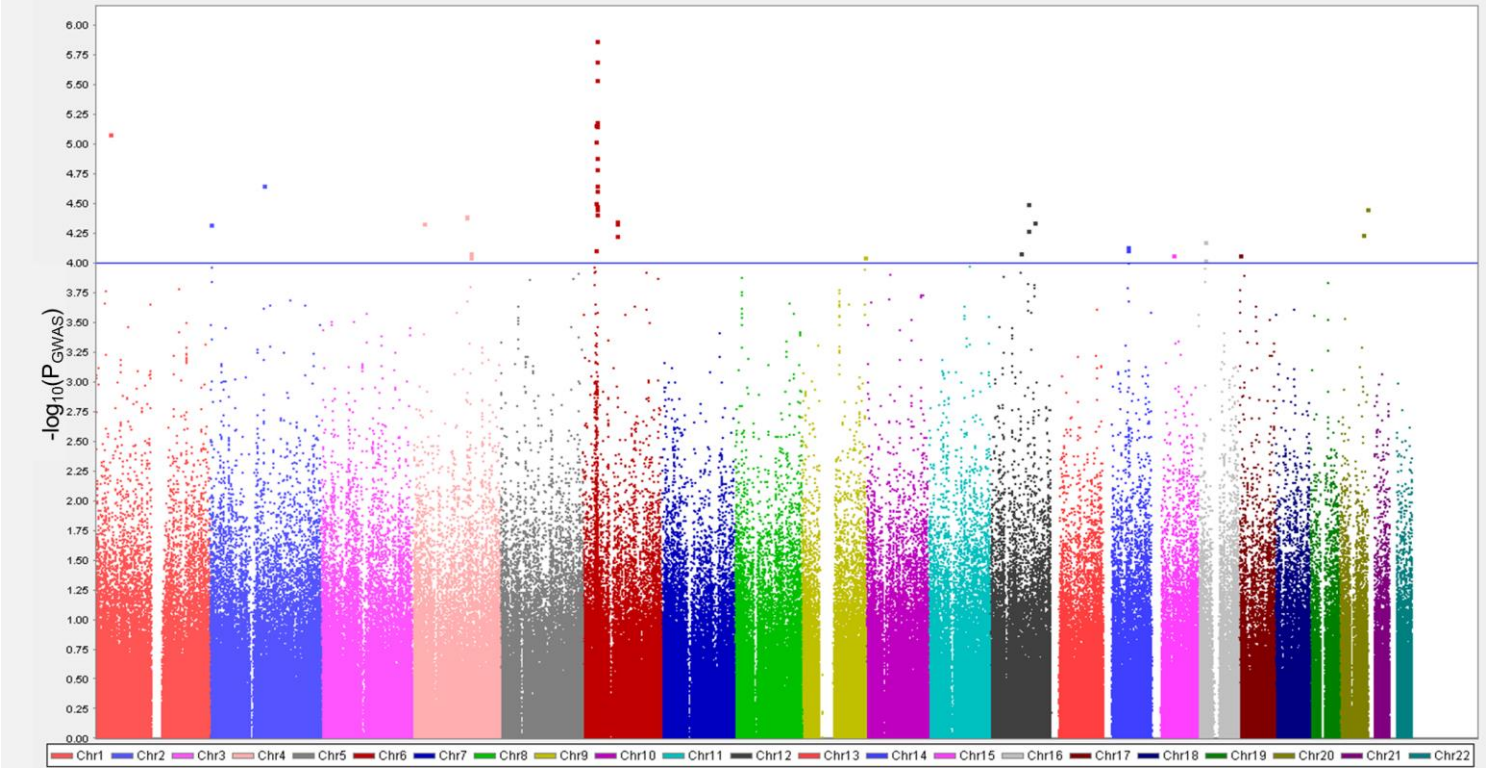


Figure 4.3: Manhattan plot of the genome wide *P*-values of association in NPC Malaysian Chinese. SNPs were ranked using logistic regression assuming an additive model adjusting for age, gender and principal component 1 (PC1). The $-\log_{10}(P_{GWAS})$ (y axis) of 575,247 SNPs in 184 NPC cases and 236 healthy controls are shown mapped against its corresponding chromosomal positions (x axis). Screening threshold was set at 1.0×10^{-4}.

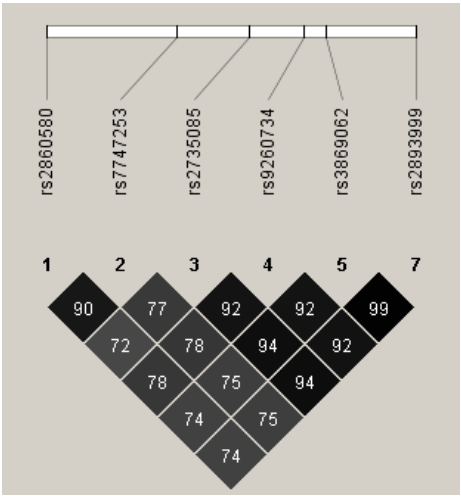


Figure 4.4: LD structure of GWAS SNPs with genome-wide significant association. LD structure of GWAS SNPs showing moderate to strong LD (r^2=0.72-0.99), suggesting all SNPs to be strongly linked in a single LD block.

Table 4.1: Association of GWAS SNPs to NPC in Malaysian Chinese.

No	SNP	CHR	Position b36	Position b37	Risk allele	Panel	Case, RAF	Control, RAF	*P*-val	OR (95% CI)	Nearby Gene
1	rs3869062 1/2=A/G	6	30042870	29934891	G	Cohort 1 (GWAS)	0.81	0.66	2.6×10^{-6}	2.57 (1.73-3.81)	3'-UTR *HLA-A*
						Cohort 2	0.80	0.68	3.51×10^{-4}	2.11 (1.4-3.17)	
						Combined	0.80	0.67	1.73×10^{-9}	2.37 (1.79-3.14)	
2	rs2735085 1/2=T/C	6	30035073	29927095	C	Cohort 1 (GWAS)	0.79	0.66	7.16×10^{-6}	2.41 (1.64-3.53)	3'-UTR *HLA-A*
						Cohort 2	0.79	0.65	1.95×10^{-4}	2.12 (1.43-3.14)	
						Combined	0.79	0.65	1.92×10^{-9}	2.31 (1.76-3.03)	
3	rs7747253 1/2=C/A	6	30027757	29919779	A	Cohort 1 (GWAS)	0.75	0.62	1.58×10^{-5}	2.2 (1.54-3.15)	3'-UTR *HLA-A*
						Cohort 2	0.75	0.60	8.44×10^{-5}	2.14 (1.47-3.14)	
						Combined	0.75	0.61	2.08×10^{-9}	2.21 (1.71-2.86)	
4	rs2893999 1/2=C/T	6	30051811	29943832	T	Cohort 1 (GWAS)	0.81	0.66	1.73×10^{-6}	2.63 (1.77-3.9)	3'-UTR *HLA-A*
						Cohort 2	0.79	0.67	8.12×10^{-4}	1.98 (1.33-2.95)	
						Combined	0.80	0.67	2.61×10^{-9}	2.32 (1.76-3.07)	
5	rs9260734 1/2=A/G	6	30040644	29932666	G	Cohort 1 (GWAS)	0.79	0.65	8.25×10^{-6}	2.39 (1.63-3.5)	3'-UTR *HLA-A*
						Cohort 2	0.79	0.66	2.35×10^{-4}	2.1 (1.41-3.12)	
						Combined	0.79	0.65	2.96×10^{-9}	2.29 (1.74-3)	
6	rs2860580 1/2=T/C	6	30014669	29906691	C	Cohort 1 (GWAS)	0.75	0.79	7.28×10^{-6}	2.23 (1.57-3.17)	5'-UTR *HLA-A*
						Cohort 2	0.73	0.79	1.29×10^{-3}	1.8 (1.26-2.58)	
						Combined	0.74	0.79	1.86×10^{-8}	2.04 (1.59-2.62)	

1=minor allele; 2=major allele; RAF=Risk allele frequency; *P*-val= Logistic regression *P*-value adjusting for age, gender and PC1; OR=Odds ratio; 95% CI= 95% confidence interval
Cohort 1 (GWAS): Cases, n=184; Controls, n=236; Total, n=420
Cohort 2: Cases, n=260; Controls, n=245; Total, n=505
Combined: Cases, n=444; Controls, n=481; Total, n=925

Table 4.2: Multivariate logistic regression for GWAS SNPs with genome-wide significant association. SNP1 is adjusted against effects of SNP2 assuming an additive model. Results are also adjusted for age and gender.

SNP1	**unadjusted**	**SNP2**								
	***P*-value**	**rs2860580**	**rs7747253**	**rs2735085**	**rs9260734**	**rs3869062**	**rs3202637**	**rs2893999**	**rs2286404**	**rs16896044**
rs2860580	$1.86x10^{-08}$	NA	$7.53x10^{-01}$	$3.02x10^{-01}$	$6.27x10^{-01}$	$3.89x10^{-01}$	$8.36x10^{-03}$	$3.97x10^{-01}$	$6.02x10^{-03}$	$6.02x10^{-03}$
rs7747253	$2.08x10^{-09}$	$3.30x10^{-02}$	NA	$1.23x10^{-01}$	$2.15x10^{-01}$	$1.26x10^{-01}$	$8.20x10^{-04}$	$1.28x10^{-01}$	$9.83x10^{-04}$	$9.83x10^{-04}$
rs2735085	$1.92x10^{-09}$	$1.06x10^{-02}$	$7.53x10^{-02}$	NA	$5.26x10^{-01}$	$6.24x10^{-01}$	$4.14x10^{-04}$	$5.75x10^{-01}$	$1.79x10^{-03}$	$1.79x10^{-03}$
rs9260734	$2.96x10^{-09}$	$1.16x10^{-02}$	$6.72x10^{-02}$	$2.60x10^{-01}$	NA	$3.83x10^{-01}$	$2.83x10^{-04}$	$3.39x10^{-01}$	$1.04x10^{-03}$	$1.04x10^{-03}$
rs3869062	$1.73x10^{-09}$	$1.06x10^{-02}$	$5.52x10^{-02}$	$3.02x10^{-01}$	$3.37x10^{-01}$	NA	$1.33x10^{-04}$	NA	$1.50x10^{-03}$	$1.50x10^{-03}$
rs2893999	$2.61x10^{-09}$	$9.82x10^{-03}$	$5.79x10^{-02}$	$3.58x10^{-01}$	$4.02x10^{-01}$	NA	$2.40x10^{-04}$	NA	$1.66x10^{-03}$	$1.66x10^{-03}$

4.1.2 Imputation to fine map the *HLA-A* gene

Fine mapping of the *HLA-A* gene region was performed using imputation in search of stronger association signals. Imputation was performed using the HapMap 2, HapMap 3 and 1000 Genomes reference datasets. Imputated SNPs with MAF ≥ 1%, INFO > 0.5, CERTAINTY > 0.9 and $P_{SNP\text{-}GWAS}<1.0x10^{-4}$ were retained. Total of 20 SNPs from HapMap 2, 1 SNP from HapMap 3 and 60 SNPs from 1000 Genomes imputation were detected. Upon validation of GWAS cohort samples and replication cohort samples, all but 4 SNPs (rs7754245, rs3873291, rs2275854, rs16896970) showed strong association signals with genome-wide significance ($P_{SNP\text{-}Combined}$ 10^{-8}-10^{-9}) (Figure 4.5; Appendix B). All imputation SNPs detected were also protective SNPs. The strongest imputation association signals (rs414215, rs9260029, rs9260033 $P_{SNP\text{-}Combined}=1.44x10^{-8}$; OR=2.21; 95% CI=1.71-2.86) did not improve upon the strongest association signal from the GWAS (rs3869062; $P_{SNP\text{-}Combined}=1.73x10^{-9}$). None of the imputated SNPs were located on exonic or intronic regions of the *HLA-A* gene. These imputated SNPs were all flanking SNPs of the *HLA-A* gene.

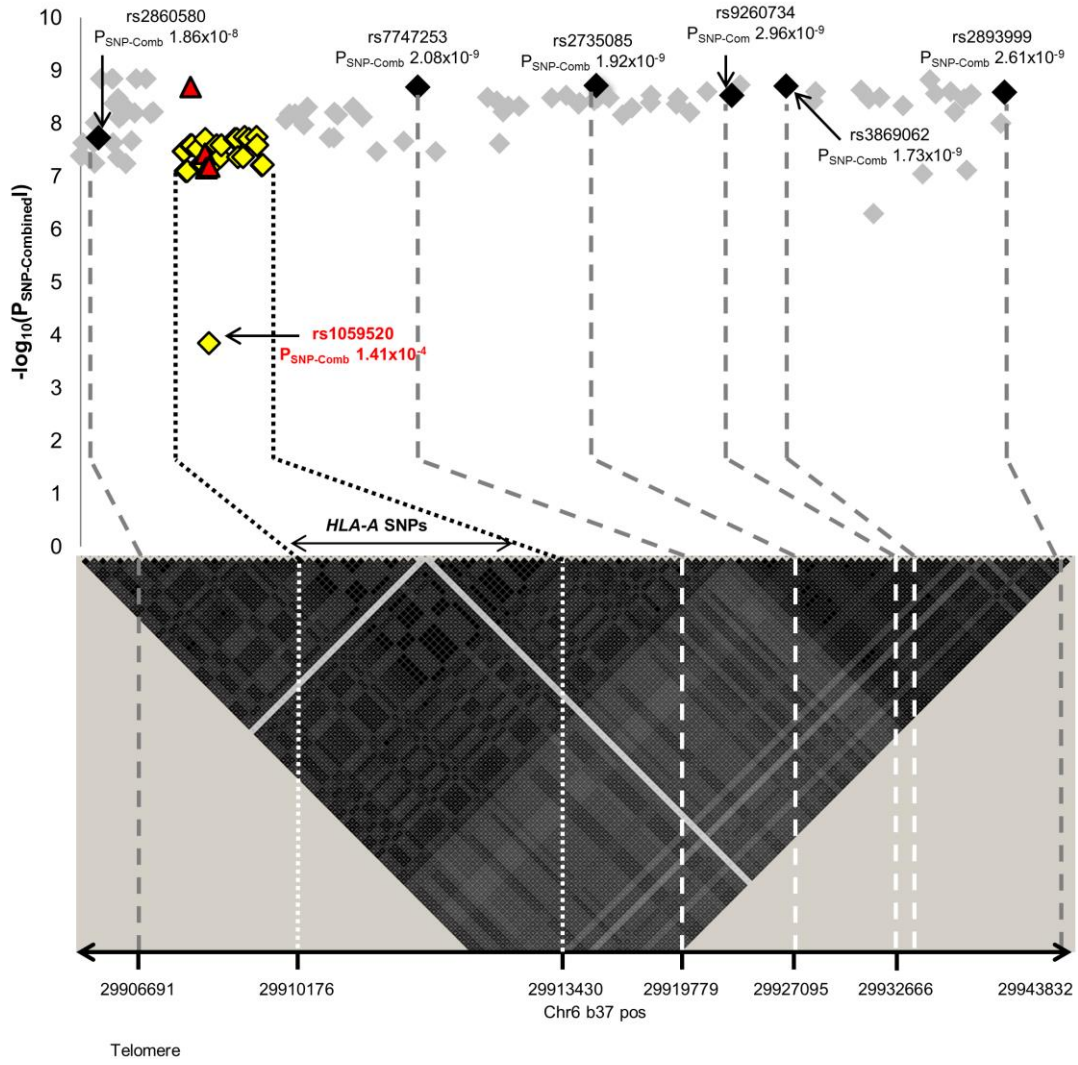


Figure 4.5: NPC associated SNPs spanning the *HLA-A* gene and its adjacent regions. Black markers signify GWAS SNPs; Grey markers signify imputation obtained SNPs; Yellow markers signify *HLA-A* alleles mapped SNPs; Red triangle markers signify multi-allelic SNPs for *HLA-A* region. Pair-wise LD values are shown as r^2. The colour intensity of the squares in the LD triangle correlates with LD strength.

4.1.3 Molecular *HLA-A* alleles genotyping

HLA-A alleles genotyping identified 23 *HLA-A* alleles in the Malaysian Chinese cohort (Table 4.3). Only 3 *HLA-A* alleles were associated or showed suggestive association to NPC, namely *HLA-A*02:06* ($P_{HLA\text{-}A\text{-}Combined}$=6.22x$10^{-3}$; OR=3.14; 95% CI= 1.38-7.14), *HLA-A*02:07* ($P_{HLA\text{-}A\text{-}Combined}$=7.71x$10^{-5}$; OR=1.94; 95% CI=1.40-2.69) and *HLA-A*11:01* ($P_{HLA\text{-}A\text{-}Combined}$=4.84x$10^{-7}$; OR=0.48; 95% CI=0.36-0.64). The association did not reach genome-wide significance. All *HLA-A* SNPs were derived from our *HLA-A* genotyping data referencing *HLA-A* allele sequences of the NCBI dbMHC database. A total of 187 bi-allelic and 18 multi-allelic SNPs were found on the *HLA-A* gene, with 31 bi-allelic SNPs and 5 multi-allelic SNPs reaching genome-wide thresholds ($P_{SNP\text{-}Combined}$ <5.0x10^{-8}) (Figure 4.5; Appendix C-D).

In order to test the independence of *HLA-A* single SNPs from the effects of *HLA-A* alleles, association signals of both *HLA-A* significant single SNPs and the 3 most prominent *HLA-A* alleles (*HLA-A*02:06*, *HLA-A*02:07* and *HLA-A*11:01*) were conditioned against each other (Appendix E-F). Controlling for *HLA-A*02:06* and *HLA-A*02:07* only saw marginal reduction in strength of association for *HLA-A* significant single SNPs ($P_{adjusted\_A*02:06}$ range from 10^{-9}-10^{-4}; $P_{adjusted\_A*02:07}$ range from 10^{-7}-10^{-2}) but controlling for *HLA-A*11:01* saw a distinct reduction ($P_{adjusted\_A*11:01}$ range from 10^{-4}-10^{-1}) (Appendix E). A similar trend was observed when *HLA-A*11:01* was designated as the index allele, conditioned against all *HLA-A* significant single SNPs ($P_{A*11:01}$ 4.84x10^{-7}; extreme $P_{A*11:01\_index}$ 10^{-3}-10^{-1}) showing a reduction in association strength (Appendix F). The results indicate that *HLA-A* single SNP is correlated to *HLA-A*11:01*, and to a lesser extent *HLA-A*02:06* and *HLA-A*02:07*.

Table 4.3: Association of *HLA-A* alleles to NPC in Malaysian Chinese.

No	*HLA-A* alleles	CHR	Sample set	Case Carrier Frequency	Control Carrier Frequency	*P*-val	OR (95% CI)
1	*A*01:01*	6	Cohort 1 (GWAS)	0.003	0.011	2.11E-01	0.24 (0.03-2.23)
			Cohort 2	0.008	0.006	1.87E-01	3.47 (0.55-21.99)
			Combined	0.006	0.008	8.16E-01	0.86 (0.23-3.17)
2	*A*02:01*	6	Cohort 1 (GWAS)	0.099	0.069	2.11E-01	1.49 (0.8-2.79)
			Cohort 2	0.068	0.088	5.48E-01	0.83 (0.45-1.52)
			Combined	0.081	0.079	7.47E-01	1.07 (0.7-1.65)
3	*A*02:03*	6	Cohort 1 (GWAS)	0.082	0.088	9.44E-01	1.02 (0.55-1.91)
			Cohort 2	0.076	0.074	5.14E-01	0.81 (0.43-1.53)
			Combined	0.079	0.081	6.99E-01	0.92 (0.59-1.43)
4	*A*02:06*	6	Cohort 1 (GWAS)	0.031	0.022	1.66E-01	2.15 (0.73-6.34)
			Cohort 2	0.039	0.008	2.02E-02	4.98 (1.29-19.3)
			Combined	0.036	0.015	6.22E-03	3.14 (1.38-7.14)
5	*A*02:07*	6	Cohort 1 (GWAS)	0.165	0.093	4.28E-03	2.11 (1.26-3.51)
			Cohort 2	0.206	0.113	1.60E-02	1.71 (1.11-2.63)
			Combined	0.189	0.103	7.71E-05	1.94 (1.4-2.69)
6	*A*02:10*	6	Cohort 1 (GWAS)	0.000	0.000	1.00E+00	#N/A
			Cohort 2	0.000	0.006	9.99E-01	#N/A
			Combined	0.000	0.003	9.99E-01	#N/A
7	*A*02:18*	6	Cohort 1 (GWAS)	0.006	0.000	9.99E-01	#N/A
			Cohort 2	0.000	0.000	1.00E+00	#N/A
			Combined	0.002	0.000	9.99E-01	#N/A
8	*A*03:01*	6	Cohort 1 (GWAS)	0.003	0.002	4.35E-01	4.19 (0.12-152.4)
			Cohort 2	0.004	0.006	6.41E-01	0.57 (0.05-6.2)
			Combined	0.004	0.004	9.29E-01	1.09 (0.17-6.9)
9	*A*03:02*	6	Cohort 1 (GWAS)	0.000	0.000	1.00E+00	#N/A
			Cohort 2	0.002	0.000	9.99E-01	#N/A
			Combined	0.001	0.000	9.99E-01	#N/A
10	*A*11:01*	6	Cohort 1 (GWAS)	0.190	0.300	1.20E-04	0.46 (0.3-0.68)
			Cohort 2	0.202	0.297	1.32E-03	0.51 (0.34-0.77)
			Combined	0.197	0.298	4.84E-07	0.48 (0.36-0.64)
11	*A*11:02*	6	Cohort 1 (GWAS)	0.023	0.052	5.92E-02	0.39 (0.15-1.04)
			Cohort 2	0.041	0.043	7.69E-01	1.15 (0.45-2.9)
			Combined	0.033	0.047	2.05E-01	0.66 (0.35-1.26)
12	*A*24:02*	6	Cohort 1 (GWAS)	0.170	0.149	7.65E-01	1.08 (0.67-1.73)
			Cohort 2	0.158	0.121	5.40E-01	1.16 (0.72-1.89)
			Combined	0.163	0.134	5.10E-01	1.12 (0.8-1.57)
13	*A*24:03*	6	Cohort 1 (GWAS)	0.000	0.000	1.00E+00	#N/A
			Cohort 2	0.002	0.000	9.99E-01	#N/A
			Combined	0.001	0.000	9.99E-01	#N/A
14	*A*24:07*	6	Cohort 1 (GWAS)	0.009	0.009	9.49E-01	0.94 (0.14-6.36)
			Cohort 2	0.002	0.000	9.99E-01	#N/A
			Combined	0.005	0.004	8.10E-01	1.24 (0.22-7.1)
15	*A*24:08*	6	Cohort 1 (GWAS)	0.003	0.000	9.99E-01	#N/A
			Cohort 2	0.000	0.000	NA	#N/A
			Combined	0.001	0.000	9.99E-01	#N/A
16	*A*24:10*	6	Cohort 1 (GWAS)	0.000	0.002	9.99E-01	#N/A
			Cohort 2	0.000	0.004	9.99E-01	#N/A
			Combined	0.000	0.003	9.99E-01	#N/A
17	*A*24:20*	6	Cohort 1 (GWAS)	0.006	0.004	9.75E-01	0.96 (0.06-14.59)
			Cohort 2	0.004	0.002	3.82E-01	9.06 (0.06-1267)
			Combined	0.005	0.003	6.37E-01	1.7 (0.19-15.3)
18	*A*26:01*	6	Cohort 1 (GWAS)	0.023	0.024	6.06E-01	0.76 (0.26-2.18)
			Cohort 2	0.033	0.016	3.25E-02	3.14 (1.1-8.95)
			Combined	0.029	0.020	2.20E-01	1.58 (0.76-3.26)
19	*A*29:01*	6	Cohort 1 (GWAS)	0.009	0.002	4.65E-01	3.03 (0.16-58.96)
			Cohort 2	0.000	0.018	9.98E-01	#N/A
			Combined	0.004	0.011	3.37E-01	0.44 (0.08-2.34)
20	*A*30:01*	6	Cohort 1 (GWAS)	0.009	0.009	7.71E-01	0.78 (0.15-4.09)
			Cohort 2	0.004	0.014	1.26E-01	0.2 (0.02-1.58)
			Combined	0.006	0.012	1.79E-01	0.42 (0.12-1.49)
21	*A*31:01*	6	Cohort 1 (GWAS)	0.003	0.022	1.59E-01	0.2 (0.02-1.86)
			Cohort 2	0.006	0.008	6.56E-01	1.52 (0.24-9.53)
			Combined	0.005	0.015	3.27E-01	0.52 (0.14-1.93)
22	*A*32:01*	6	Cohort 1 (GWAS)	0.003	0.000	9.99E-01	#N/A
			Cohort 2	0.000	0.002	9.99E-01	#N/A
			Combined	0.001	0.001	8.19E-01	1.44 (0.06-32.48)
23	*A*33:03*	6	Cohort 1 (GWAS)	0.148	0.121	9.37E-03	2.36 (1.24-4.51)
			Cohort 2	0.134	0.141	3.48E-01	1.25 (0.78-2)
			Combined	0.140	0.131	2.79E-02	1.51 (1.05-2.18)

P-val=Logistic regression additive model *P*-value adjusting for age, gender and PC1; OR=Odds ratio; 95% CI= 95% confidence interval

Example coding of *HLA-A* alleles, e.g. A*02:06/A*02:06=1/1; A*02:06/0=1/2; 0/0=2/2

Multiple testing threshold = 2.17×10^{-03}

4.1.4 Amino acid variants

This study aimed to identify amino acid variants that are also functional variants coded by the corresponding *HLA-A* SNP. *HLA-A* gene sequence was translated to identify amino acid variants using NCBI dbMHC database. Corresponding function of amino acid residues were identified using the Immunology Database and Analysis Portal (ImmPort) database while functional impact of amino acid/SNP substitutions were predicted by PROVEAN (Choi *et al*., 2012), SIFT (Kumar *et al*., 2009) and Polyphen-2 (Adzhubei *et al*., 2010). Out of 364 amino acid residues identified (Figure 4.6, Appendix G-H), 27 amino acid variants were statistically significant after multiple testing correction ($P_{\text{aa-Combined}}<1.37\text{x}10^{-4}$) (Table 4.4, Appendix I). These amino acid variants have corresponding bi-allelic and multi-allelic SNPs. PROVEAN (Choi *et al*., 2012), SIFT (Kumar *et al*., 2009) and Polyphen-2 (Adzhubei *et al*., 2010) only predicted HLA-A-aa-site-99 ($P_{\text{aa-Combined}}=3.79\text{x}10^{-8}$; $\text{OR}_{\text{AA99-Tyr/Cys}}=2.19$; 95% $\text{CI}_{\text{AA99-Tyr/Cys}}=1.66\text{-}2.89$; $\text{OR}_{\text{AA99-Tyr/Phe}}=1.40$; 95% $\text{CI}_{\text{AA99-Tyr/Phe}}=1.09\text{-}1.82$) and HLA-A-aa-site-145 ($P_{\text{aa-Combined}}=1.41\text{x}10^{-4}$; $\text{OR}_{\text{AA145-Arg/His}}=1.64$; 95% $\text{CI}_{\text{AA145-Arg/His}}=1.27\text{-}2.12$) to be deleterious or damaging variants (Table 4.4, Appendix G-I). The corresponding SNP for HLA-A-aa-site-99 is rs1136697 ($P_{\text{SNP-Combined}}=3.79\text{x}10^{-8}$; $\text{OR}_{\text{rs1136697-A/G}}=2.19$; 95% $\text{CI}_{\text{rs1136697-A/G}}=1.66\text{-}2.89$; $\text{OR}_{\text{rs1136697-A/T}}=1.40$; 95% $\text{CI}_{\text{rs1136697-A/T}}=1.09\text{-}1.82$) and HLA-A-aa-site-145 is rs1059520 ($P_{\text{SNP-Combined}}=1.41\text{x}10^{-4}$; OR=1.64; 95% CI=1.27-2.12). The HLA-A-99Tyr reference amino acid tyrosine (Tyr, Y) is substituted for cysteine (Cys, C) when rs1136697-A is substituted for G. The HLA-A-145Arg reference amino acid arginine (Arg, R) is substituted for histidine when rs1059520-G is substituted for –A. The remaining amino acid variants, though showing association signals in both amino acid and SNP variants were benign or tolerated variants (Table 4.4, Appendix I). To establish if the association of HLA-A-99 and HLA-A-145 is independent or due to LD with *HLA-A* alleles, logistic regression controlling for effects of *HLA-A*02:07* was

done. This is because HLA-A-99, HLA-A-145 and *HLA-A*02:07* show a susceptible genetic effect. Controlling for *HLA-A*02:07* reduced the effects of HLA-A-99Tyr/Cys and rs1136697-A/G ($P_{adjusted}$=1) and HLA-A-145Arg/His and rs1059520 ($P_{adjusted}$=8.04x10^{-2}) and vice versa (Appendix E-F, Appendix J-K). These results indicated that effects of HLA-A-99Tyr/Cys and HLA-A-145Arg/His to be driven by and correlated with *HLA-A*02:07*.

4.1.5 Regulatory functions of NPC associated *HLA-A* SNP variants

HaploReg was used to predict potential epigenetic signatures in the NPC associated *HLA-A* SNP variants. The search only focused on 5'-UTR region of the *HLA-A*. Epigenetic data was limited to those of the B-cell lymphoblastoid cell lines as we focus on epigenetic signatures related to immune-response function. HaploReg data indicated that rs9260067, rs9260072, rs9260077, rs41545520, rs9260120 and rs114945359 may regulate transcription of *HLA-A* as the SNP variants intersect histone modification sites and/or DNase I hypersensitivity sites, suggesting promoter or enhancer activity. The variants also influence binding of transcription factors and/or a regulatory motif (Table 4.5). The 6 SNPs with potential epigenetic function are correlated with *HLA-A*11:01*. Controlling for effects of *HLA-A*11:01* diminished the association of the 6 SNPs (extreme $P_{adjusted\_A*11:01}$ 10^{-3}-10^{-2}) (Appendix E). The association of *HLA-A*11:01* also diminished after controlling against effects of the 6 SNPs ($P_{A*11:01\_unadjusted}$ 4.63x10^{-7}; extreme $P_{A*11:01\_index}$ 10^{-1}) (Appendix F).

SNP rs41545520 is of particular interest as it influence change in strength of motif binding for activating transcription factor 3 (ATF3). ATF3 binds the cAMP response element (CRE), repressing transcription by stabilizing the binding of inhibitory cofactors at the promoter (Gilchrist *et al.,* 2006). Genotype data of rs41545520 was corroborated with eQTL data from Genevar Database to investigate effects of SNP

genotypes on *HLA-A* expression. Though not listed in the Genevar Database, rs41545520 is tightly linked to rs2860580 (r^2=0.84). The eQTL expression profiles of rs2860580 is more consistent with HaploReg predictions of strengthened motif binding for repressor ATF3 in reference allele rs41545520-G (rs2860580-G), resulting in lower *HLA-A* expression. The alternate allele rs41545520-T (rs2860580-A) shows weakened binding of ATF3, resulting in higher *HLA-A* expression (Table 4.5; Figure 4.7). rs41545520-T also defines *HLA-A*11:01*, with the higher *HLA-A*11:01* expression implying a protective effect towards NPC. The epigenetic influence of rs9260077 is not discussed as the predicted transcription factor, HDAC2, does not directly bind DNA but is tethered against other transcription factors (Ropero & Esteller, 2010), thus strength of motif binding is not directly influenced by change in rs9260077 alleles. Further examination in NPC cell lines to replicate effects of *HLA-A* 5'-UTR SNP variants on transcriptional regulation and gene expression would be necessary.

4.1.6 Association signals from previous NPC GWAS studies

Previously reported association signals from Ng *et al*. 2009a, Tse *et al*. 2009, Bei *et al*. 2010 and Tang *et al*. 2012 were genotyped (Appendix L). Our results replicated the associations reported in the *HLA-A* region (including adjacent *GABBR1*, *HLA-F* and *HCG9*). Controlling for *HLA-A*11:01* reduced the association strength of *GABBR1*, *HLA-F*, *HLA-A* and *HCG9* where the extreme NPC association is reduced from $1.73\text{x}10^{-9}$-0.01 to $1.0\text{x}10^{-4}$-0.51 (Appendix E). The association of *HLA-A*11:01* also diminished after controlling against effects of the NPC SNPs ($P_{A*11:01}$ $4.84\text{x}10^{-7}$;extreme $P_{A*11:01\_\text{index}}$ 10^{-1}) (Appendix F). The results suggest the *HLA-A* region association signals from previous NPC reports to be correlated with the effects of *HLA-A*11:01*.

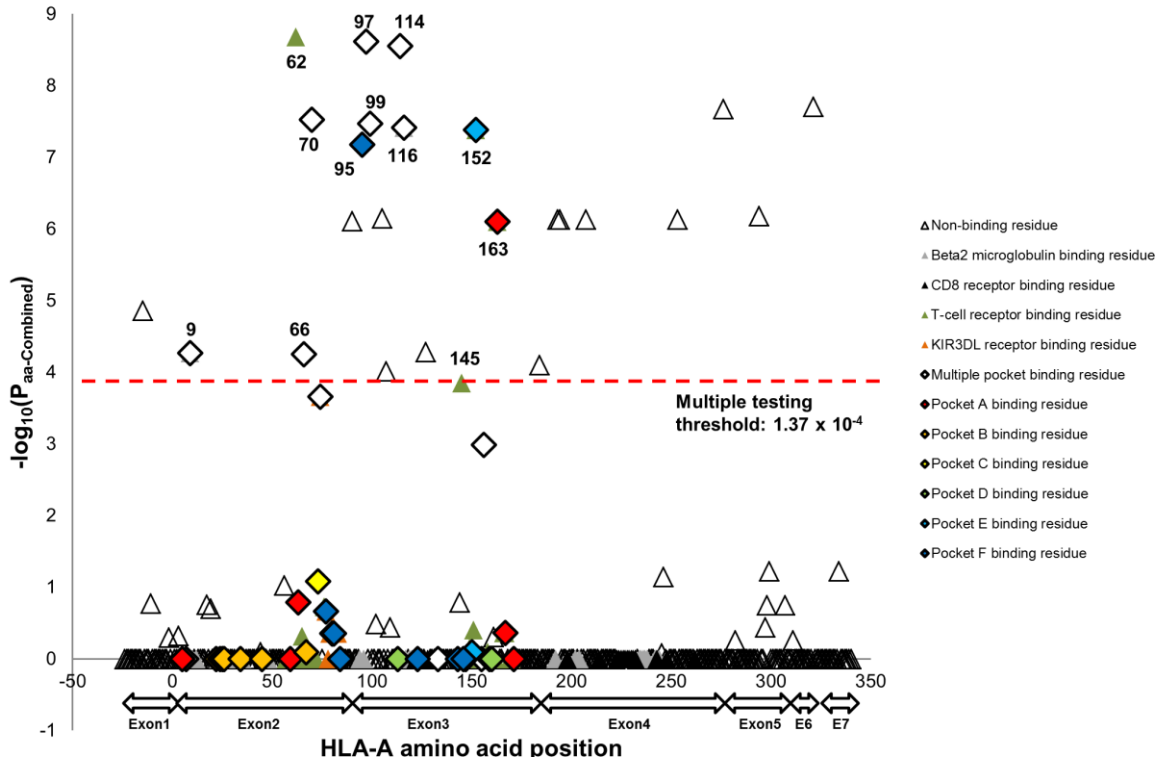
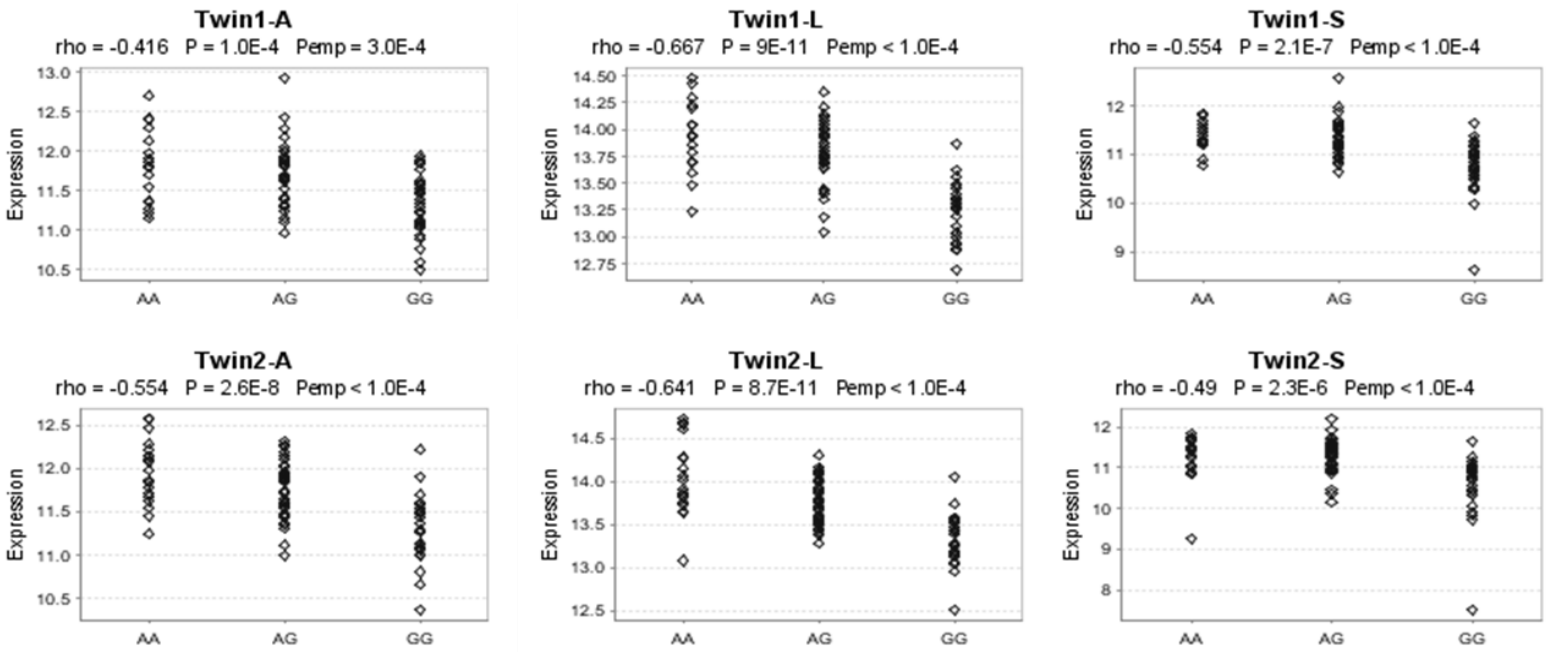


Figure 4.6: NPC association plot of amino acid variants mapped from *HLA-A* alleles. The false positive rates are expressed in $-\log_{10} P_{\text{aa-Combined}}$ (y-axis) according to the HLA-A amino acid residue position (x-axis). Coloured markers signify corresponding function of amino acid positions, be it for β_2-microglobulin binding, CD8 receptor binding, T-cell receptor binding, Natural killer cell receptor binding KIR3DL or antigenic peptide binding pocket A-F; red line signifies multiple testing threshold 1.37×10^{-4}.



SNP rs2860580
Reference allele : G
Variant allele : A
Major allele : G LD r^2=0.84 with rs41545520-G ATF3 LOD 11.6
Minor allele : A LD r^2=0.84 with rs41545520-T ATF3 LOD 8.6
Ensemble Gene ID : ENSG00000206503
Gene Name : *HLA-A*

Figure 4.7: GeneVar eQTL analysis of *HLA-A* flanking SNP rs41545520 using MuTHER resource datasets (Nica *et al*., 2011) as well as corresponding nuclear factor binding affinity.

Table 4.4: *HLA-A* amino acid variants association and function prediction by PROVEAN, SIFT and Polyphen-2.

No	Amino acid	$P_{aa\text{-}Combined}$	SNP	$P_{SNP\text{-}Combined}$	Codon Change	Residue Change	PROVEAN	SIFT	Polyphen-2
1	AA-15	1.37×10^{-5}	rs1143146	1.37×10^{-5}	C/G	Leu>Val	Neutral	Tol	Benign
2	AA9	5.36×10^{-5}	rs1136659	1.46×10^{-1}	T/A	Phe>Ile	Neutral	Dmg	Benign
			rs2075684	6.98×10^{-6}	T/A/C	Phe>Tyr/Ser	Neutral/Neutral	Tol/Tol	Benign/Benign
3	AA62	2.10×10^{-9}	rs1059455	3.74×10^{-5}	C/G	Gln>Glu	Neutral	Tol	Benign
			rs1064588	1.29×10^{-6}	A/G/T	Gln>Arg/Leu	Neutral/Del	Tol/Tol	Benign/Benign
4	AA66	5.60×10^{-5}	rs199474436	5.60×10^{-5}	T/A	Asn>Lys	Neutral	Tol	Benign
5	AA70	3.01×10^{-8}	rs78306866	3.01×10^{-8}	G/C	Gln>His	Neutral	Tol	Benign
6	AA74	2.20×10^{-4}	rs1136683	2.20×10^{-4}	G/C	Asp>His	Neutral	Tol	Pos Dmg
7	AA90	7.73×10^{-7}	rs1136692	7.73×10^{-7}	C/A	Ala>Asp	Del	Tol	Benign
8	AA95	1.21E-07	rs1071743	1.21×10^{-7}	A/C/G	Ile>Leu/Val	Neutral/Neutral	Tol/Tol	Benign/Benign
9	AA97	2.44×10^{-9}	rs199474485	4.65×10^{-5}	T/G	Ile>Arg	Neutral	Tol	Benign
			rs1136695	1.95×10^{-8}	A/G	Ile>Met	Neutral	Tol	Benign
10	AA99	3.79×10^{-8}	rs1136697	3.79×10^{-8}	A/G/T	Tyr>Cys/Phe	Del/Del	Dmg/Tol	Prob Dmg/Benign
11	AA105	7.06×10^{-7}	rs1136700	7.06×10^{-7}	T/C	Ser>Pro	Neutral	Tol	Benign
12	AA107	9.61×10^{-5}	rs1136702	9.61×10^{-5}	G/T	Gly>Trp	Del	Tol	Prob Dmg
13	AA114	2.78×10^{-9}	rs3173420	4.60×10^{-8}	G/A	Arg>Gln	Neutral	Tol	Pos Dmg
			rs12721717	7.00×10^{-8}	G/A/C	Arg	Neutral/Neutral	Tol/Tol	N/A
14	AA116	7.00×10^{-8}	rs3173419	7.00×10^{-8}	G/C/T	Asp>His/Tyr	Neutral/Neutral	Tol/Tol	Benign/Benign
15	AA127	5.24×10^{-5}	rs1059509	5.24×10^{-5}	C/A	Asn>Lys	Del	Tol	Benign
16	AA142	1.41×10^{-4}	rs1059516	1.41×10^{-4}	T/C	Ile>Thr	Del	Tol	Prob Dmg
17	AA145	1.41×10^{-4}	rs1059520	1.41×10^{-4}	G/A	Arg>His	Del	Tol	Prob Dmg
18	AA152	6.64×10^{-8}	rs9256983	6.64×10^{-8}	A/T/C/G	Glu>Val/Ala/Gly	Neutral/Neutral/Neutral	Tol/Tol/Dmg	Benign/Benign/Benign
19	AA163	1.66×10^{-7}	rs3129017	2.82×10^{-7}	A/C	Thr>Pro	Neutral	Tol	Pos Dmg
			rs3129018	2.82×10^{-7}	C/G	Thr>Arg	Neutral	Tol	Benign
20	AA184	7.94×10^{-5}	rs1136741	7.94×10^{-5}	C/G	Pro>Ala	Del	Tol	Prob Dmg
21	AA193	7.42×10^{-7}	rs1059563	1.09×10^{-6}	C/G	Pro>Ala	Del	Tol	Prob Dmg
			rs1059564	8.14×10^{-5}	C/G/T	Pro	Neutral/Neutral	Tol/Tol	N/A
22	AA194	7.42×10^{-7}	rs9260179	7.42×10^{-7}	A/G	Ile>Val	Neutral	Tol	Benign
23	AA207	7.42×10^{-7}	rs9260180	7.42×10^{-7}	G/A	Gly>Ser	Del	Tol	Pos Dmg
24	AA253	7.42×10^{-7}	rs2231095	7.42×10^{-7}	G/C	Glu>Gln	Neutral	Tol	Pos Dmg
25	AA276	2.11×10^{-8}	rs1136903	1.95×10^{-8}	T/C	Leu>Pro	Neutral	Tol	Benign
			rs1136917	5.28×10^{-1}	G/A	Leu	Neutral	Tol	N/A
26	AA294	6.58×10^{-7}	rs3179982	6.58×10^{-7}	C/T	Leu>Phe	Neutral	Tol	Prob Dmg

Table 4.4, continued.

No	Amino acid	$P_{aa\text{-}Combined}$	SNP	$P_{SNP\text{-}Combined}$	Codon Change	Residue Change	PROVEAN	SIFT	Polyphen-2
27	AA321	1.95×10^{-8}	rs2231119	1.95×10^{-8}	A/T	Thr>Ser	Neutral	Tol	Benign

$P_{aa\text{-}Combined}$=amino acid association in combined cohort; $P_{SNP\text{-}Combined}$=SNP association in combined cohort;
Tol=Tolerated, Dmg=Damaging; Del=Deleterious; Prob Dmg= Probably Damaging; Pos Dmg= Possibly damaging

Table 4.5: HaploReg prediction of 5'-UTR *HLA-A* SNP variants.

CHR	SNP	Pos b37	Ref	Alt	[a]Promoter histone marks	[a]Enhancer histone marks	[b]DNAse	[c]Proteins bound	[d]Motifs changed	RefSeq genes
6	rs9260067	29908649	T	A,C,G	None	State 5 Strong enhancer	None	None	None	1.6kb 5' of *HLA-A*
6	rs9260072	29908838	G	C	None	State 4 Strong enhancer	None	None	None	1.4kb 5' of *HLA-A*
6	rs9260077	29908954	G	A	None	State 4 Strong enhancer	Present	None	HDAC2,Pax-5	1.3kb 5' of *HLA-A*
6	**rs41545520 G>T**	**29910265**	**G**	**T**	**State 1 Active promoter**	**None**	**Present**	**NFKB,OCT2,POL2,POL24H8,POU2F2,RFX5,SP1,TBP,YY1**	**ATF3,Duxl,Gfi1, HNF1,ATF3 Ref_{LOD} 11.6; Alt_{LOD} 8.6**	**5' of *HLA-A***
6	rs9260120	29910277	G	C,T	State 1 Active promoter	None	Present	NFKB,OCT2,POL2,POL24H8,POU2F2,RFX5,SP1,TBP,YY1	None	5' of *HLA-A*
6	rs114945359	29910324	G	C	State 1 Active promoter	None	Present	NFKB,OCT2,POL2,POL24H8,POU2F2,RFX5,SP1,TBP,YY1	LF-A1	5' of *HLA-A*

[a]Regulatory chromatin states follows the 15-state definition of Ernst *et al*., 2011
[b]Region where chromatin is hypersensitive to Dnase I enzyme cleavage (ENCODE data)
[c]Protein binding regions assayed by ChIP-seq (ENCODE data)
[d]Motif change as predicted by TRANSFAC, JASPAR and protein-binding microarray experiments

4.2 Results for meta-analysis of NPC GWAS

The initial meta-analysis across GWAS included a total of 2,152 cases and 3,740 controls. Results from the meta-analysis are summarized in Appendix M. As described in the Materials and Methods section, 43 SNPs were identified for replication based on the GWAS meta-analysis. For the Malaysian study, the results from the GWAS and replication efforts were combined. No SNP was associated at the genome-wide level ($P<5.0x10^{-08}$) (Table 4.6). The more prominent associations reported were SNPs near *ITGA9* (OR=2; $P=1.22x10^{-04}$) and *MECOM* (OR=1.37; $P=1.34x10^{-04}$) (Table 4.6). When merged with GWAS and replication data from other NPC study groups, *ITGA9* association was not replicated (OR=1.02; $P=8.30x10^{-01}$) while *MECOM* showed consistent association (OR=0.84; $P=1.50x10^{-12}$) (Table 4.7).

In replication efforts performed across four studies, the strongest association with NPC was observed for rs31489 ($OR_{rep}=0.79$; $P_{rep}=4.3x10^{-11}$), an intronic SNP within *CLPTM1L* in the *CLPTM1L/TERT* region (chr.5p15.33). This represents a locus not reported in previously published NPC GWAS. Findings for this SNP were consistent in the mainland Chinese and two Taiwanese replication studies and absent from the Malaysian replication study, the smallest of the replication efforts (Figure 4.8). A second SNP within the *CLPTM1L/TERT* locus (rs2853668; $r^2 = 0.108$ and D' = 0.917 with rs31489 among controls in our replication studies) was also associated with NPC in the replication phase (OR=1.11; $P=5.2x10^{-4}$), but the association was no longer statistically significant in analyses that conditioned on rs31489 (OR=1.05; $P=0.15$).

In analyses that combined the GWAS and replication studies, findings for rs31489 were strengthened (OR across GWA+replication studies = 0.81; *P*-value = $6.3x10^{-13}$; Table 4.7). Some evidence for heterogeneity across studies was observed (*P*-heterogeneity = 0.035). Additional associations ($P\leq 1x10^{-7}$) were observed in our combined GWA plus replication studies meta-analysis for rs6774494 ($P=1.5x10^{-12}$;

MECOM gene region), rs9510787 (P=5.0x10^{-10}; *TNFRSF19* gene region), rs1412829, rs4977756, and rs1063192 (P=2.8x10^{-8}, P=7.0x10^{-7}, and P=8.4x10^{-7}, respectively; *CDKN2A/2B* gene region; Table 4.7 and Figure 4.8).

Table 4.6: Results for 43 SNPs in Malaysian Chinese combining GWAS and replication samples.

								GWAS		Replication study		Combined	
SNP	**Nearest gene**	**CHR**	**Pos hg19**	**Selection criteria[a]**	**MAF (Ctrls)**	**Major allele**	**Minor allele**	**OR**	***P***	**OR**	***P***	**OR**	***P***
rs31489	*CLPTM1L/TERT*	5	1342714	2	0.22	C	A	1.04	$7.55x10^{-01}$	1.11	$5.09x10^{-01}$	1.07	$4.96x10^{-01}$
rs6774494	*MECOM*	3	169082633	2	0.36	A	G	0.76	$1.29x10^{-02}$	0.69	$4.11x10^{-03}$	1.37	$1.34x10^{-04}$
rs9510787	*TNFRSF19*	13	24205195	2	0.35	A	G	1.23	$5.33x10^{-02}$	1.04	$7.35x10^{-01}$	1.14	$1.01x10^{-01}$
rs1412829	*CDKN2A/2B*	9	22043926	1	0.11	T	C	0.8	$1.78x10^{-01}$	1.15	$4.79x10^{-01}$	1.07	$5.94x10^{-01}$
rs4977756	*CDKN2A/2B*	9	22068652	1	0.22	A	G	0.85	$2.02x10^{-01}$	1.31	$6.76x10^{-02}$	1.02	$8.13x10^{-01}$
rs1063192	*CDKN2A/2B*	9	22003367	1	0.17	T	C	0.89	$3.97x10^{-01}$	1.1	$5.73x10^{-01}$	1.03	$7.98x10^{-01}$
rs2853668	*CLPTM1L/TERT*	5	1300025	2	0.31	C	A	1.09	$5.13x10^{-01}$	1.02	$8.98x10^{-01}$	1.05	$5.70x10^{-01}$
rs3731239	*C9orf53,CDKN2A*	9	21974218	1	0.13	T	C	0.92	$5.88x10^{-01}$	1.03	$8.62x10^{-01}$	1.03	$7.70x10^{-01}$
rs1572072	*TNFRSF19*	13	24127210	2	0.26	G	T	1.02	$8.49x10^{-01}$	1.15	$2.94x10^{-01}$	1.08	$4.10x10^{-01}$
rs3109384	*LOC646388*	11	40118598	1	0.26	C	T	0.9	$3.95x10^{-01}$	0.84	$1.77x10^{-01}$	1.15	$1.10x10^{-01}$
rs9928448	*ALDOA,PPP4C*	16	30072530	2	0.41	T	C	1.05	$6.26x10^{-01}$	1.14	$2.62x10^{-01}$	1.09	$2.57x10^{-01}$
rs10120688	*RP11-145E5.4*	9	22056499	2	0.28	A	G	0.9	$3.51x10^{-01}$	0.8	$7.78x10^{-02}$	1.17	$6.63x10^{-01}$
rs2877822	*MUC13*	3	124645034	2	0.04	C	T	0.72	$1.80x10^{-01}$	1.58	$9.22x10^{-02}$	1.03	$8.79x10^{-01}$
rs6671127	*LOC100133029, GPR177*	1	68571220	1	0.37	A	C	1.07	$5.20x10^{-01}$	1.06	$6.40x10^{-01}$	1.07	$4.29x10^{-01}$
rs10796139	*FRMD4A*	10	13892298	1	0.36	A	G	0.82	$8.72x10^{-02}$	0.95	$6.65x10^{-01}$	1.14	$1.26x10^{-01}$
rs1331627	*NTNG2*	9	135091879	1	0.42	C	T	0.84	$9.95x10^{-02}$	0.97	$7.56x10^{-01}$	1.12	$1.63x10^{-01}$
rs11672613	*C3*	19	6705246	1	0.42	T	C	0.87	$1.91x10^{-01}$	0.9	$3.58x10^{-01}$	1.13	$1.12x10^{-01}$
rs6468749	*YWHAZ*	8	102008828	1	0.37	T	C	1.04	$7.20x10^{-01}$	1.17	$1.97x10^{-01}$	1.1	$2.61x10^{-01}$
rs12577139	*BARX2*	11	129301284	2	0.15	C	T	0.91	$5.51x10^{-01}$	0.82	$2.44x10^{-01}$	1.15	$1.98x10^{-01}$
rs7119879	*BARX2*	11	129305687	2	0.16	G	A	0.84	$2.21x10^{-01}$	0.88	$4.24x10^{-01}$	1.17	$1.50x10^{-01}$
rs1991007	N/A	5	55968018	1	0.08	C	A	1.63	$1.16x10^{-02}$	0.59	$2.10x10^{-02}$	1.09	$5.76x10^{-01}$
rs12570170	*HK1*	10	70801833	1	0.37	G	A	1.13	$2.67x10^{-01}$	0.94	$5.68x10^{-01}$	1.04	$6.23x10^{-01}$
rs2886189	*ZBTB16*	11	113501655	1	0.3	T	C	0.95	$6.67x10^{-01}$	1.02	$8.56x10^{-01}$	1.02	$8.34x10^{-01}$
rs9820110	N/A	3	70469958	1	0.29	G	T	1.24	$6.61x10^{-02}$	1.09	$4.74x10^{-01}$	1.17	$7.04x10^{-02}$
rs17801001	*EPHA3*	3	89414555	1	0.12	A	C	1.56	$5.69x10^{-03}$	0.88	$4.75x10^{-01}$	1.21	$1.15x10^{-01}$
rs11209216	*LOC100133029, GPR177*	1	68571431	1	0.44	C	T	1.05	$6.67x10^{-01}$	1.06	$5.98x10^{-01}$	1.05	$5.03x10^{-01}$
rs6795074	*EPHA3*	3	89516652	1	0.1	T	C	1.36	$8.06x10^{-02}$	0.7	$8.19x10^{-02}$	1.02	$8.56x10^{-01}$
rs9538032	N/A	13	58985847	1	0.25	T	C	1.18	$1.99x10^{-01}$	1.1	$4.99x10^{-01}$	1.14	$1.57x10^{-01}$
rs3181088	*VCAM1*	1	101198708	2	0.11	C	T	1.1	$5.91x10^{-01}$	1.08	$6.63x10^{-01}$	1.09	$4.87x10^{-01}$

Table 4.6, continued.

SNP	Nearest gene	CHR	Pos hg19	Selection criteria[a]	MAF (Ctrls)	Major allele	Minor allele	GWAS		Replication study		Combined	
								OR	*P*	OR	*P*	OR	*P*
rs6800118	*MIRN138-1, hsa-mir-138-1*	3	44141157	2	0.28	A	G	0.99	$9.19x10^{-01}$	0.72	$1.08x10^{-02}$	1.16	$7.71x10^{-02}$
rs7702277	N/A	5	14020756	1	0.12	G	T	1.58	$3.87x10^{-03}$	0.99	$9.49x10^{-01}$	1.28	$3.44x10^{-02}$
rs1296284	N/A	5	55934938	1	0.33	G	A	1.29	$6.84x10^{-02}$	1.13	$3.14x10^{-01}$	1.2	$5.12x10^{-02}$
rs2802402	*ITM2B*	13	47685360	1	0.16	C	T	0.85	$2.30x10^{-01}$	1.04	$8.01x10^{-01}$	1.08	$4.64x10^{-01}$
rs695207	*MIR3134,ROD1*	9	114056169	1	0.27	T	G	1.14	$2.29x10^{-01}$	0.89	$3.66x10^{-01}$	1.02	$8.07x10^{-01}$
rs189897	*ITGA9*	3	37518545	2	0.04	A	T	2.52	$4.13x10^{-06}$	1.23	$5.15x10^{-01}$	2	$1.22x10^{-04}$
rs2158250	*ITGB8*	7	20425446	2	0.41	A	G	0.97	$7.72x10^{-01}$	0.92	$4.65x10^{-01}$	1.06	$4.93x10^{-01}$
rs1286041	N/A	6	6839192	1	0.17	A	G	1.41	$1.36x10^{-02}$	0.7	$2.84x10^{-02}$	1.05	$6.63x10^{-01}$
rs4714505	*LOC100130606, TFEB*	6	41648147	1	0.11	C	T	0.7	$3.79x10^{-02}$	1.13	$4.99x10^{-01}$	1.13	$3.16x10^{-01}$
rs7014115	*ASPH*	8	62649567	1	0.12	T	G	1.63	$3.84x10^{-03}$	1.01	$9.55x10^{-01}$	1.3	$3.15x10^{-02}$
rs4936612	N/A	11	121203120	1	0.4	A	G	0.92	$4.30x10^{-01}$	0.97	$8.01x10^{-01}$	1.06	$4.50x10^{-01}$
rs11637457	*AGBL1*	15	87572506	1	0.16	C	T	0.88	$4.21x10^{-01}$	1.34	$5.17x10^{-02}$	1.1	$3.97x10^{-01}$
rs1324103b	N/A	6	93901016	1	0.42	A	G	0.84	$1.10x10^{-01}$	0.75	$1.57x10^{-02}$	1.25	$3.84x10^{-03}$
rs9924017	*HS3ST4*	16	25849321	1	0.36	A	G	1.1	$4.17x10^{-05}$	1.09	$4.63x10^{-01}$	1.09	$2.70x10^{-01}$

[a]1=Selected based on GWAS meta-analysis results; 2= Selected as an additional candidate based on *a-priori* literature.
[b]Replaced rs6931820 with $P=3.47x10^{-06}$ in GWAS meta.

Table 4.7: Results from GWAS meta-analysis and replication study for 43 SNPs selected for replication (Bei *et al*., 2016).

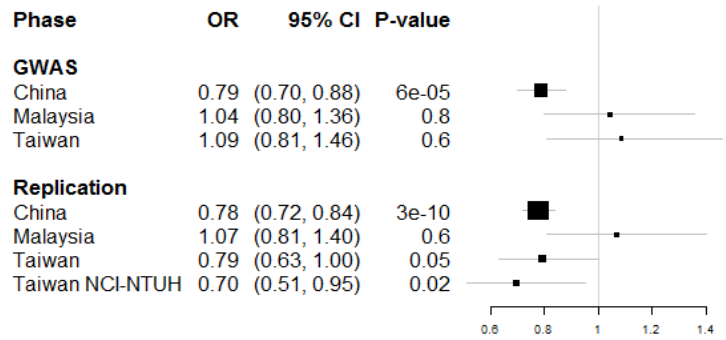
								GWAS meta-analysis		Replication study		Combined	
SNP	**Nearest gene**	**CHR**	**Pos hg19**	**Selection criteria[a]**	**MAF (Ctrls)**	**Major allele**	**Minor allele**	**OR**	***P***	**OR**	***P***	**OR**	***P***
rs31489	*CLPTM1L/TERT*	5	1342714	2	0.22	C	A	0.85	$1.80x10^{-03}$	0.79	$4.30x10^{-11}$	0.81	$6.30x10^{-13}$
rs6774494	*MECOM*	3	169082633	2	0.36	A	G	0.81	$4.00x10^{-07}$	0.86	$3.40x10^{-07}$	0.84	$1.50x10^{-12}$
rs9510787	*TNFRSF19*	13	24205195	2	0.35	A	G	1.2	$1.90x10^{-05}$	1.14	$4.10x10^{-06}$	1.16	$5.00x10^{-10}$
rs1412829	*CDKN2A/2B*	9	22043926	1	0.11	T	C	0.72	$2.80x10^{-06}$	0.85	$4.20x10^{-04}$	0.8	$2.80x10^{-08}$
rs4977756	*CDKN2A/2B*	9	22068652	1	0.22	A	G	0.8	$9.70x10^{-06}$	0.9	$2.70x10^{-03}$	0.87	$7.00x10^{-07}$
rs1063192	*CDKN2A/2B*	9	22003367	1	0.17	T	C	0.77	$2.40x10^{-06}$	0.9	$6.00x10^{-03}$	0.86	$8.40x10^{-07}$
rs2853668	*CLPTM1L/TERT*	5	1300025	2	0.31	C	A	1.15	$1.70x10^{-03}$	1.11	$5.20x10^{-04}$	1.12	$3.60x10^{-06}$
rs3731239	*C9orf53,CDKN2A*	9	21974218	1	0.13	T	C	0.77	$6.30x10^{-05}$	0.87	$1.60x10^{-03}$	0.84	$1.30x10^{-06}$
rs1572072	*TNFRSF19*	13	24127210	2	0.26	G	T	0.89	$1.3x10^{-02}$	0.92	$1.10x10^{-02}$	0.91	$4.80x10^{-04}$
rs3109384	*LOC646388*	11	40118598	1	0.26	C	T	0.83	$3.30x10^{-05}$	0.93	$1.80x10^{-02}$	0.89	$1.60x10^{-05}$
rs9928448	*ALDOA,PPP4C*	16	30072530	2	0.41	T	C	1.17	$1.50x10^{-04}$	1.07	$2.40x10^{-02}$	1.1	$6.50x10^{-05}$
rs10120688	*RP11-145E5.4*	9	22056499	2	0.28	A	G	0.84	$1.80x10^{-04}$	0.94	$4.30x10^{-02}$	0.91	$1.50x10^{-04}$
rs2877822	*MUC13*	3	124645034	2	0.04	C	T	0.68	$1.10x10^{-04}$	1.14	$5.40x10^{-02}$	0.96	$5.20x10^{-01}$
rs6671127	*LOC100133029, GPR177*	1	68571220	1	0.37	A	C	1.2	$1.60x10^{-05}$	1.05	$8.50x10^{-02}$	1.1	$1.20x10^{-04}$
rs10796139	*FRMD4A*	10	13892298	1	0.36	A	G	0.82	$1.30x10^{-05}$	0.96	$1.20x10^{-01}$	0.91	$2.10x10^{-04}$
rs1331627	*NTNG2*	9	135091879	1	0.42	C	T	0.84	$4.70x10^{-05}$	1.04	$1.30x10^{-01}$	0.98	$2.90x10^{-01}$
rs11672613	*C3*	19	6705246	1	0.42	T	C	0.83	$1.10x10^{-05}$	0.96	$1.50x10^{-01}$	0.92	$2.40x10^{-04}$
rs6468749	*YWHAZ*	8	102008828	1	0.37	T	C	1.21	$1.00x10^{-05}$	1.04	$1.50x10^{-01}$	1.09	$2.20x10^{-04}$
rs12577139	*BARX2*	11	129301284	2	0.15	C	T	0.84	$2.10x10^{-03}$	1.06	$1.50x10^{-01}$	0.98	$5.70x10^{-01}$
rs7119879	*BARX2*	11	129305687	2	0.16	G	A	0.84	$1.60x10^{-03}$	1.05	$1.90x10^{-01}$	0.98	$4.80x10^{-01}$
rs1991007	N/A	5	55968018	1	0.08	C	A	1.38	$2.50x10^{-05}$	1.05	$3.20x10^{-01}$	1.15	$1.30x10^{-03}$
rs12570170	*HK1*	10	70801833	1	0.37	G	A	1.19	$5.30x10^{-05}$	1.03	$3.50x10^{-01}$	1.08	$2.10x10^{-03}$
rs2886189	*ZBTB16*	11	113501655	1	0.3	T	C	0.83	$4.30x10^{-05}$	0.97	$3.80x10^{-01}$	0.93	$2.40x10^{-03}$
rs9820110	N/A	3	70469958	1	0.29	G	T	1.24	$2.60x10^{-06}$	1.03	$3.80x10^{-01}$	1.09	$7.10x10^{-04}$
rs17801001	*EPHA3*	3	89414555	1	0.12	A	C	1.32	$7.70x10^{-06}$	1.03	$4.40x10^{-01}$	1.11	$1.70x10^{-03}$
rs11209216	*LOC100133029, GPR177*	1	68571431	1	0.44	C	T	1.18	$5.50x10^{-05}$	1.02	$4.90x10^{-01}$	1.07	$4.60x10^{-03}$
rs6795074	*EPHA3*	3	89516652	1	0.1	T	C	1.38	$3.30x10^{-06}$	1.03	$5.10x10^{-01}$	1.13	$1.70x10^{-03}$
rs9538032	N/A	13	58985847	1	0.25	T	C	1.21	$5.50x10^{-05}$	0.98	$5.10x10^{-01}$	1.05	$8.20x10^{-02}$
rs3181088	*VCAM1*	1	101198708	2	0.11	C	T	1.28	$2.60x10^{-04}$	1.03	$5.80x10^{-01}$	1.1	$1.20x10^{-02}$

Table 4.7, continued

SNP	Nearest gene	CHR	Pos hg19	Selection criteria[a]	MAF (Ctrls)	Major allele	Minor allele	GWAS meta-analysis		Replication study		Combined	
								OR	*P*	OR	*P*	OR	*P*
rs6800118	*MIRN138-1, hsa-mir-138-1*	3	44141157	2	0.28	A	G	0.84	$1.20x10^{-04}$	1.02	$6.20x10^{-01}$	0.96	$8.00x10^{-02}$
rs7702277	N/A	5	14020756	1	0.12	G	T	1.39	$8.00x10^{-08}$	0.98	$6.60x10^{-01}$	1.1	$6.50x10^{-03}$
rs1296284	N/A	5	55934938	1	0.33	G	A	1.21	$2.90x10^{-05}$	1.01	$7.00x10^{-01}$	1.07	$7.90x10^{-03}$
rs2802402	*ITM2B*	13	47685360	1	0.16	C	T	0.77	$2.90x10^{-06}$	0.99	$8.10x10^{-01}$	0.91	$4.40x10^{-03}$
rs695207	*MIR3134,ROD1*	9	114056169	1	0.27	T	G	1.2	$7.30x10^{-05}$	0.99	$8.20x10^{-01}$	1.06	$3.50x10^{-02}$
rs189897	*ITGA9*	3	37518545	2	0.04	A	T	N/A	N/A	1.02	$8.30x10^{-01}$	1.02	$8.30x10^{-01}$
rs2158250	*ITGB8*	7	20425446	2	0.41	A	G	0.86	$4.80x10^{-04}$	1	$8.70x10^{-01}$	0.95	$3.70x10^{-02}$
rs1286041	N/A	6	6839192	1	0.17	A	G	1.26	$3.10x10^{-05}$	1.01	$8.90x10^{-01}$	1.08	$1.40x10^{-02}$
rs4714505	*LOC100130606, TFEB*	6	41648147	1	0.11	C	T	0.71	$1.70x10^{-07}$	1	$9.30x10^{-01}$	0.9	$4.30x10^{-03}$
rs7014115	*ASPH*	8	62649567	1	0.12	T	G	1.33	$6.10x10^{-06}$	1	$9.50x10^{-01}$	1.09	$1.30x10^{-02}$
rs4936612	N/A	11	121203120	1	0.4	A	G	0.85	$6.30x10^{-05}$	1	$9.50x10^{-01}$	0.95	$2.70x10^{-02}$
rs11637457	*AGBL1*	15	87572506	1	0.16	C	T	0.8	$7.70x10^{-05}$	1	$9.50x10^{-01}$	0.93	$3.10x10^{-02}$
rs1324103b	N/A	6	93901016	1	0.42	A	G	0.84	$1.40x10^{-05}$	1	$9.60x10^{-01}$	0.94	$1.20x10^{-02}$
rs9924017	*HS3ST4*	16	25849321	1	0.36	A	G	1.19	$4.70x10^{-05}$	1	$9.70x10^{-01}$	1.06	$2.20x10^{-02}$

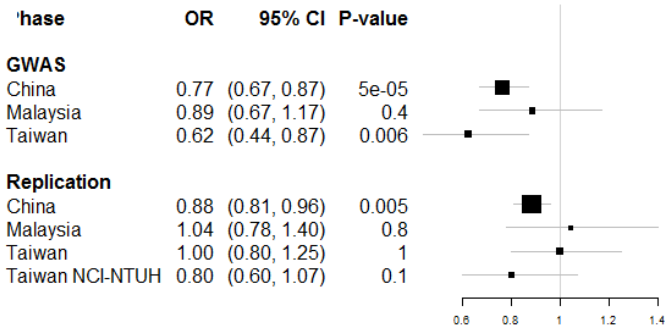
[a]1=Selected based on GWAS meta-analysis results; 2= Selected as an additional candidate based on *a-priori* literature.
[b]Replaced rs6931820 with $P=3.47x10^{-06}$ in GWAS meta.

A. rs31489



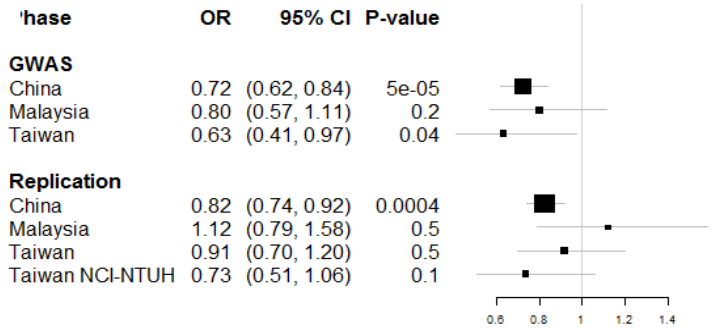
P-heterogeneity across studies: 0.035

B. rs1063192



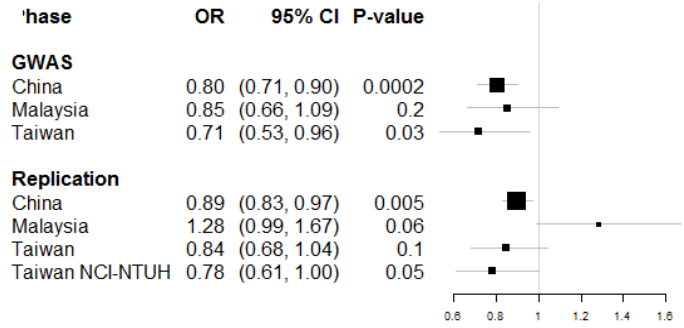
P-heterogeneity across studies: 0.097

C. rs1412829



P-heterogeneity across studies: 0.25

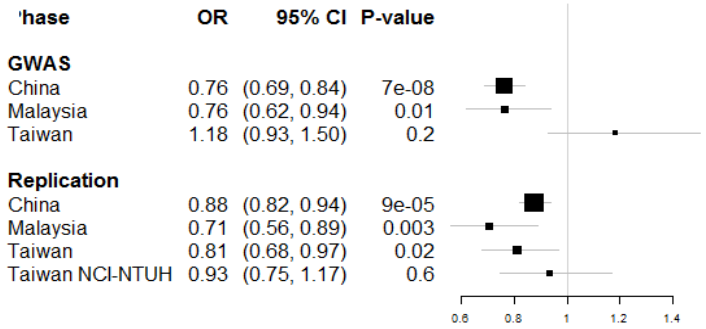
D. rs4977756



P-heterogeneity across studies: 0.039

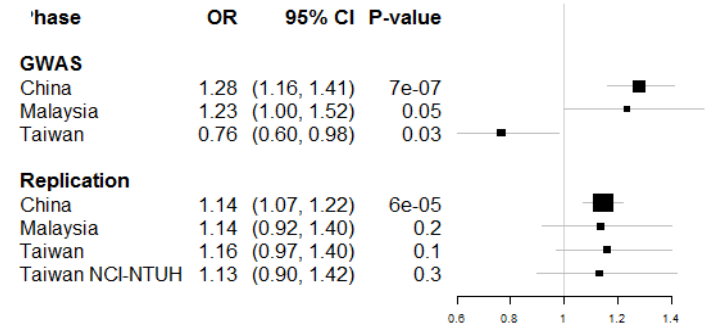
Figure 4.8: Individual Study Results from GWAS Meta-Analysis and Replication Studies for Selected SNPs (Bei *et al*., 2016).

E. rs6774494



P-heterogeneity across studies: 0.0093

F. rs9510787



P-heterogeneity across studies: 0.016

Figure 4.8, continued. Individual Study Results from GWAS Meta-Analysis and Replication Studies for Selected SNPs (Bei *et al*., 2016).

4.3 Results for integrated pathway analysis of NPC

4.3.1 GWAS and gene expression data

The PCA indicated that NPC patients and controls were genetically matched, with minimal evidence of population stratification (Figure 4.9; Figure 4.10). Total of 6,702,151 autosomal SNPs overlapping Illumina HumanHap 550K and Illumina Human OmniExpress_12 v1.1 were retained post-imputation after quality control measures were applied (Figure 4.11). SNP association P-values have been adjusted for age, gender and population structure. Quantile–quantile plot analysis detected a small inflation factor (λ_{gc}) of 1.03, indicating minimal inflation of the genome-wide association due to population structure (Figure 4.12). Post-imputation SNPs were mapped to 20,273 genes. Total of 18,905 gene scores were calculated out of 20,273 genes and were used for GSEA. The remaining 1,368 genes were removed either due to lack of a gene score or were situated in the MHC region. Gene scores calculation have been adjusted for confounding factors.

Differential gene expression analysis was performed on GSE12452 data (31 nasopharyngeal carcinomas; 10 non-NPC nasopharynx tissues) from a Taiwanese case control cohort using GEO2R employing a Student's t-test, adjusted for $\log_2$ fold-change. Total of 21,586 genes were evaluated, of which 5,680 genes showed differential expression, $P_{GEO2R}<0.05$. All 21,586 *P*-values from GEO2R were used as gene level *P*-values for GSEA analysis, screening 2,757 pathways across BioCarta, GO, Ingenuity, KEGG, Panther and Reactome databases.

Pathways identified through joint GWAS and published gene expression data GSE12452 were replicated in a separate cohort of 10 NPC and 7 non-NPC nasopharynx tissues (Table 4.8; Table 4.9). Sample details are listed in Table 4.10. Whole genome gene expression analysis was performed using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit. Total of 3,218 genes showing normalized read counts <10

were removed from analysis. PCA plot (Figure 4.13), heatmap of sample-to-sample distance (Figure 4.14) all showed consistent clustering and demarcation of sample phenotypes. Differential expression analysis for remaining 17,595 genes was performed in DESeq2, and *P*-values for genes that form the target replication pathways were used for GSEA in GSA-SNP.

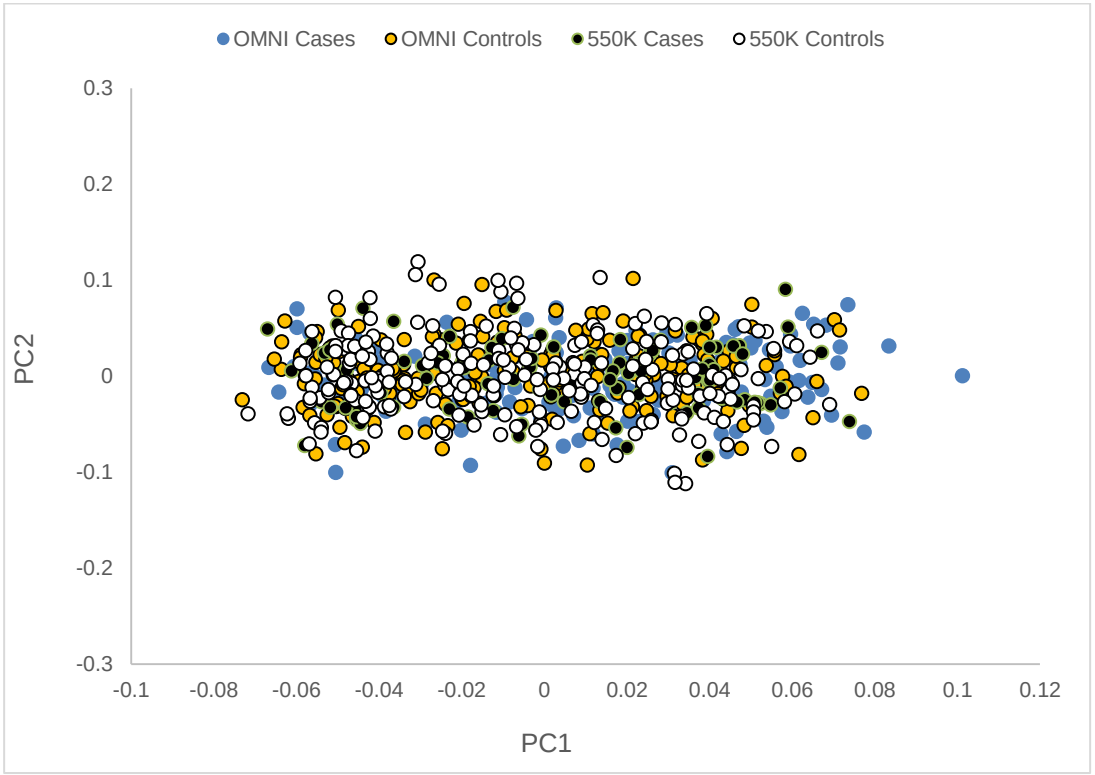


Figure 4.9: PCA plot of HumanOmniExpress_12 v1.1 and HumanHap550K samples after outlier removal. Overlapping SNPs were pruned (r^2<0.2) and first 2 principal components (PC1 vs PC2) plotted. Blue markers signify OMNI chip cases (NPC patients), orange markers signify OMNI chip controls (healthy controls), black markers signify 550K chip cases (NPC patients) and white markers signify 550K chip controls (healthy controls).

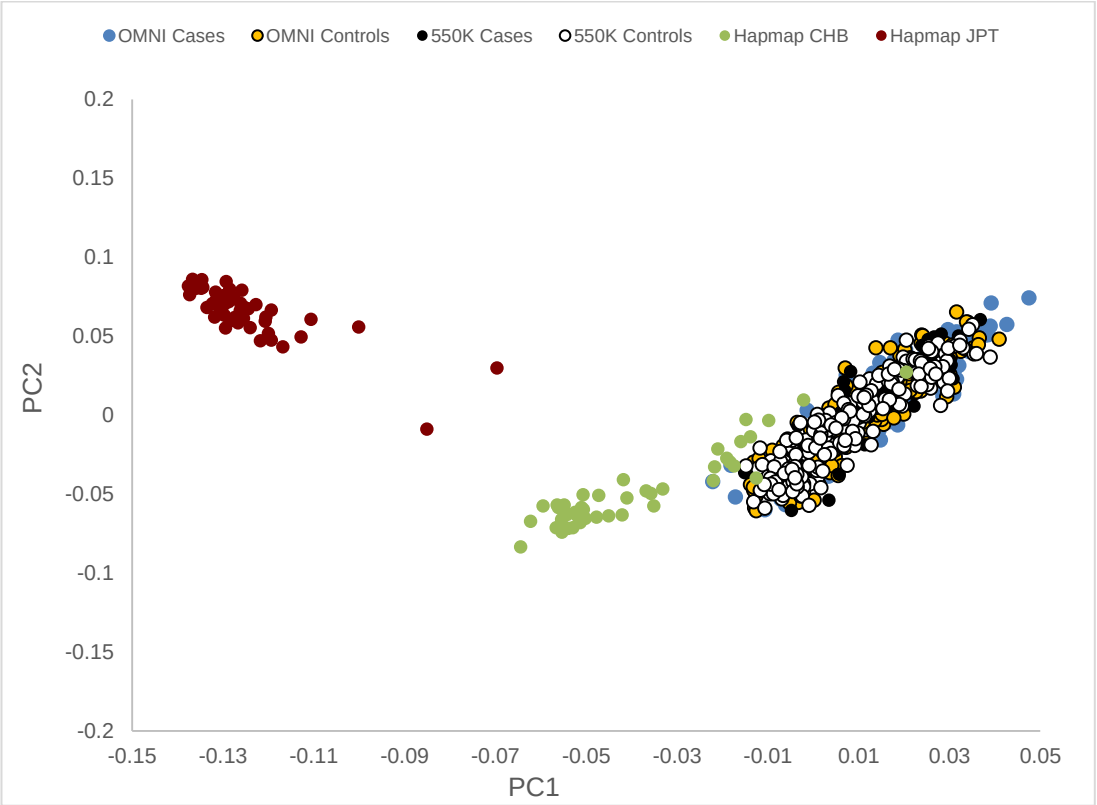


Figure 4.10: PCA plot of HumanOmniExpress_12 v1.1 and HumanHap550K against Hapmap CHB and JPN samples after outlier removal. Overlapping SNPs were pruned (r^2<0.2) and first 2 principal components plotted. Blue markers signify OMNI chip cases (NPC patients), orange markers signify OMNI chip controls (healthy controls), black markers signify 550K chip cases (NPC patients), white markers signify 550K chip controls (healthy controls), green markers signify Hapmap Chinese Han Beijing samples and red markers signify Hapmap Japanese samples.

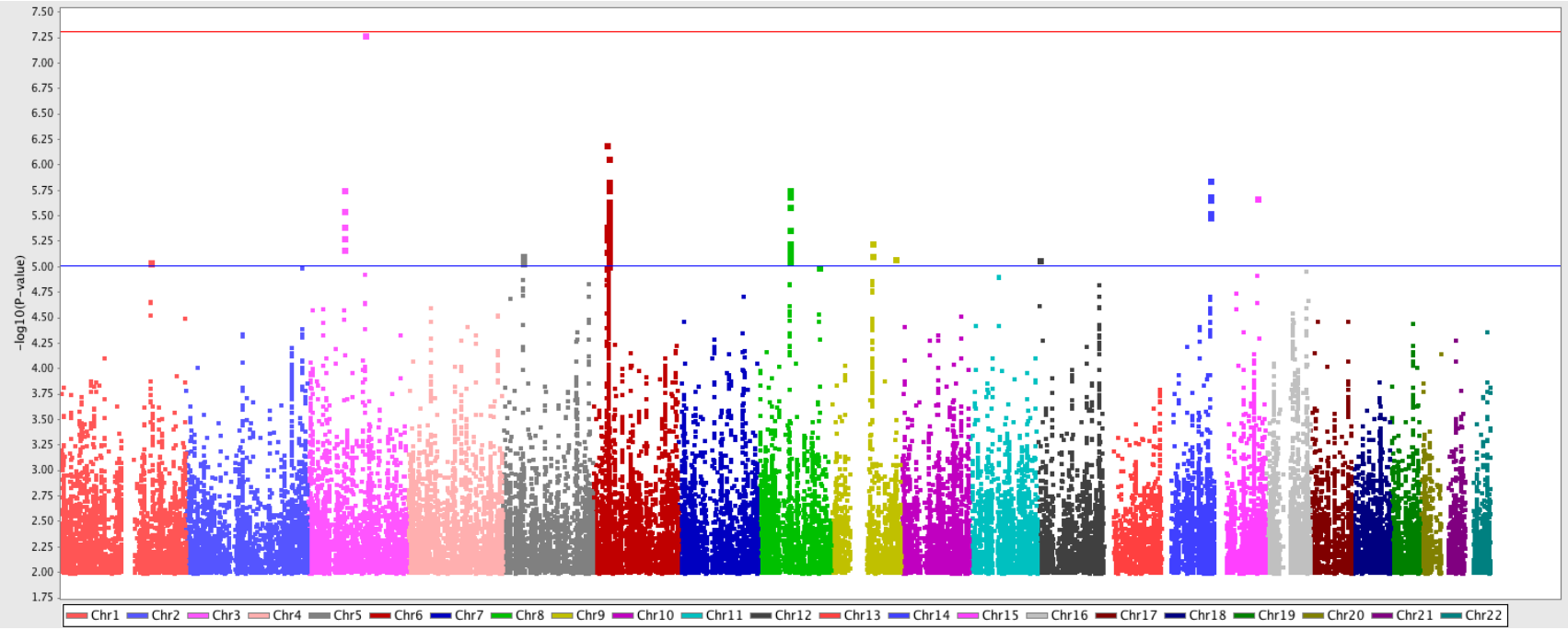


Figure 4.11: Manhattan plot of HumanOmniExpress_12 v1.1, HumanHap550K and all imputated SNPs. Only SNPs showing $P<0.01$ are shown. The blue line marks suggestive association ($P<1.0\text{x}10^{-5}$) while the red line marks the genome-wide association ($P<5.0\text{x}10^{-8}$).

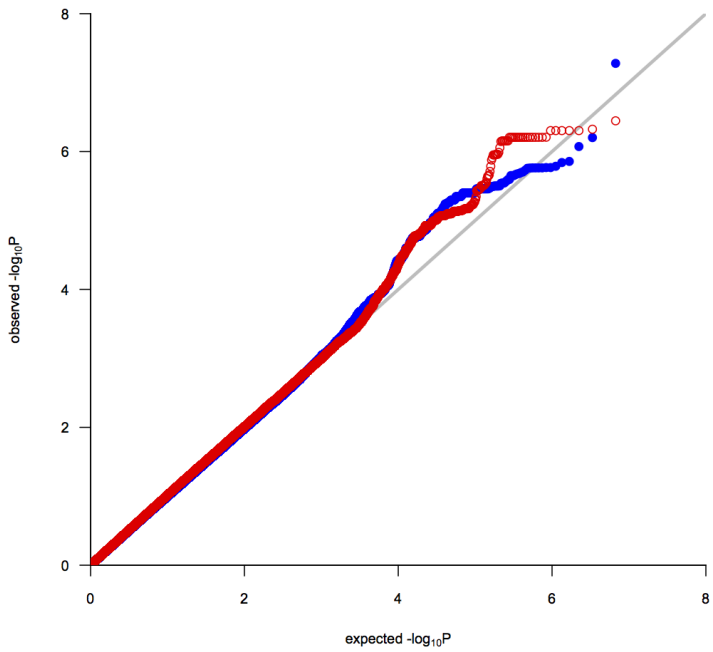


Figure 4.12: QQ plot of merged HumanOmniExpress_12 v1.1 and HumanHap550K post imputation. Red markers signify unadjusted *P*-values and blue markers signify adjusted *P*-values. Genomic inflation factor λ_{gc}=1.03

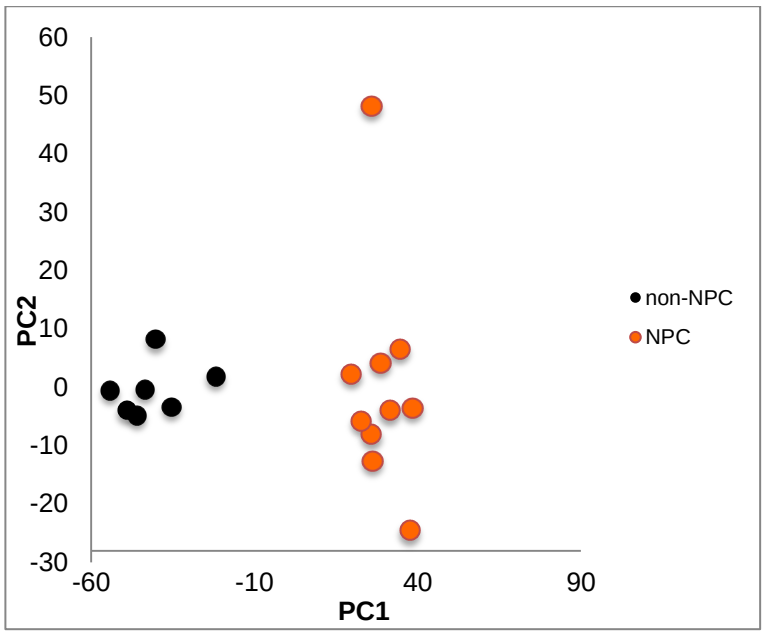


Figure 4.13: PCA plot. The 10 NPC and 7 non-NPC tissues clustered across their first two principal components based on regularized log (rlog) of expression data from Ion AmpliSeq™ Transcriptome Human Gene Expression Kit.

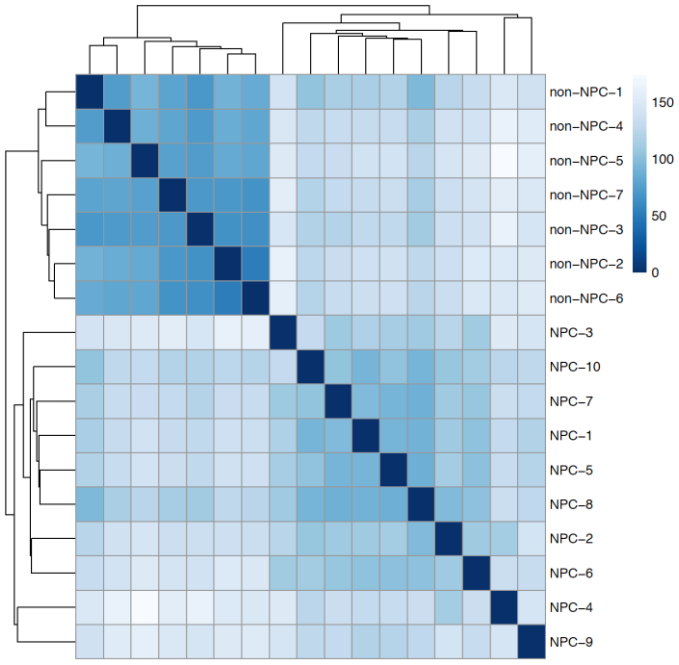


Figure 4.14: Sample-to-sample distances. Heatmap showing the Euclidean distances between the samples as calculated from the regularized log transformation count data from Ion AmpliSeq™ Transcriptome Human Gene Expression Kit.

Table 4.8: Pathways associated with NPC in a Malaysian Chinese cohort.

DATABASE	GENE SET	# GENE	[a]MAGENTA GSEA	[b]GSA-SNP GSEA	[c]$P_{Combined}$	[d]$P_{Bonf\text{-}Combined}$	[e]AmpliSeq GSEA
Gene Ontology	Axonemal dynein complex	12	0.0198	$1.27x10^{-24}$	$1.50x10^{-24}$	$4.15x10^{-21}$	$6.56x10^{-4}$
Gene Ontology	Chromosome, centromeric region	46	0.0235	$3.54x10^{-8}$	$1.82x10^{-8}$	$5.02x10^{-5}$	0.95

[a]Nominal GSEA calculated by MAGENTA
[b]Nominal GSEA calculated by GSA-SNP
[c]Combination *P*-values of nominal MAGENTA and GSA-SNP *P*-values using Fisher's method
[d]Bonferroni corrected combination *P*-values
[e]GSEA of DESeq2 FDR-corrected Wald test *P*-values from AmpliSeq data

Table 4.9: List of genes in pathways associated with NPC.

GENE SET	# GENE	ENTREZ GENE ID	GENE	[a]MAGENTA PVAL	[b]GEO2R	[c]Log_2FC GEO2R	[d]AmpliSeq PVAL	[e]Log_2FC AmpliSeq
Axonemal dynein complex	12	1767	*DNAH5*	$1.30x10^{-2}$	$3.07x10^{-7}$	-2.64	$1.28x10^{-6}$	-2.82
		1769	*DNAH8*	$8.22x10^{-1}$	$7.4X10^{-1}$	-0.05	$2.08x10^{-2}$	-2.14
		1770	*DNAH9*	$3.80x10^{-2}$	$6.79X10^{-8}$	-2.16	$5.60x10^{-6}$	-3.59
		7802	*DNALI1*	$4.23x10^{-1}$	$4.24X10^{-9}$	-3.05	$4.31x10^{-24}$	-5.60
		8632	*DNAH17*	$3.74x10^{-1}$	$3.34X10^{-1}$	-0.11	$3.81x10^{-4}$	2.51
		8701	*DNAH11*	$4.37x10^{-1}$	$5.37X10^{-6}$	-0.64	$2.52x10^{-4}$	-2.31
		10126	*DNAL4*	$6.03X10^{-1}$	$7.65X10^{-3}$	-0.45	$3.05x10^{-1}$	0.35
		25981	*DNAH1*	$9.07X10^{-1}$	$3.14X10^{-7}$	-0.59	$3.66x10^{-5}$	-1.83
		55567	*DNAH3*	$4.85X10^{-2}$	$2.82X10^{-8}$	-0.99	$2.84x10^{-6}$	-4.40
		56171	*DNAH7*	$1.62X10^{-1}$	$1.83X10^{-7}$	-1.72	$1.19x10^{-5}$	-3.33
		64446	*DNAI2*	$4.64X10^{-1}$	$3.04X10^{-7}$	-2.48	$1.96x10^{-16}$	-6.81
		146754	*DNAH2*	$2.65X10^{-1}$	$1.11X10^{-7}$	-1.88	$4.81x10^{-5}$	-3.93

Table 4.9, continued.

GENE SET	# GENE	ENTREZ GENE ID	GENE	[a]MAGENTA PVAL	[b]GEO2R	[c]Log_2FC GEO2R	[d]AmpliSeq PVAL	[e]Log_2FC AmpliSeq
Chromosomal, centromeric region	46	332	*BIRC5*	$1.38X10^{-2}$	$1.05x10^{-5}$	1.56	$1.39x10^{-4}$	1.58
		1058	*CENPA*	$8.96X10^{-1}$	$1.68x10^{-4}$	1.27	$5.78x10^{-4}$	1.46
		1059	*CENPB*	$5.82X10^{-1}$	$3.96x10^{-2}$	0.22	$3.70x10^{-1}$	0.27
		1062	*CENPE*	$7.34X10^{-1}$	$4.25x10^{-5}$	1.18	$3.21x10^{-2}$	1.17
		1063	*CENPF*	$6.65X10^{-1}$	$3.73x10^{-6}$	1.51	$1.14x10^{-4}$	1.66
		2491	*CENPI*	N/A	$3.97x10^{-4}$	0.83	$1.06x10^{-3}$	1.28
		3070	*HELLS*	$3.17X10^{-1}$	$7.11x10^{-8}$	2	$6.98x10^{-4}$	1.13
		3619	*INCENP*	$8.51X10^{-1}$	$2.76x10^{-1}$	-0.1	$4.63x10^{-1}$	0.26
		4288	*MKI67*	$1.27X10^{-1}$	$2.79x10^{-5}$	0.94	$3.29x10^{-2}$	1.06
		5515	*PPP2CA*	$6.48X10^{-1}$	$4.14x10^{-1}$	0.20	$4.40x10^{-1}$	0.31
		5516	*PPP2CB*	$8.28X10^{-1}$	$1.22x10^{-1}$	-0.22	$1.35x10^{-1}$	0.45
		5518	*PPP2R1A*	$4.54X10^{-1}$	$7.12x10^{-1}$	-0.08	$5.43x10^{-1}$	0.15
		5525	*PPP2R5A*	$7.31X10^{-1}$	$2.41x10^{-3}$	-0.55	$6.88x10^{-5}$	-1.12
		5527	*PPP2R5C*	$1.38X10^{-1}$	$7.45x10^{-2}$	-0.54	$1.26x10^{-6}$	-1.49
		5663	*PSEN1*	$7.42X10^{-2}$	$1.00x10^{-1}$	-0.13	$2.53x10^{-1}$	-0.27
		6839	*SUV39H1*	N/A	$5.37x10^{-1}$	0.08	$5.09x10^{-2}$	0.45
		8690	*JRKL*	$7.10X10^{-1}$	$1.16x10^{-1}$	-0.14	$8.26x10^{-1}$	-0.09
		10403	*NDC80*	$5.16X10^{-1}$	$4.00x10^{-6}$	1.97	$3.09x10^{-2}$	0.97
		10664	*CTCF*	$2.33x10^{-1}$	$4.59x10^{-2}$	-0.31	$8.95x10^{-2}$	-0.51
		10951	*CBX1*	$1.40X10^{-1}$	$6.32x10^{-3}$	0.82	$1.06x10^{-7}$	2.28
		11004	*KIF2C*	$1.81X10^{-1}$	$1.19x10^{-4}$	0.90	$1.29x10^{-4}$	1.57
		11339	*OIP5*	$5.11X10^{-1}$	$1.87x10^{-4}$	1.48	$3.72x10^{-5}$	1.82
		23421	*ITGB3BP*	$6.04X10^{-1}$	$1.89x10^{-4}$	0.77	$3.89x10^{-2}$	0.78
		27309	*ZNF330*	$6.76X10^{-1}$	$4.27x10^{-1}$	-0.13	$3.56x10^{-1}$	0.25
		54069	*MIS18A*	$6.65x10^{-1}$	$6.51x10^{-4}$	1.16	$2.30x10^{-4}$	0.83
		55143	*CDCA8*	$5.31X10^{-1}$	$2.32x10^{-2}$	0.38	$1.79x10^{-3}$	1.19
		55166	*CENPQ*	$7.77X10^{-1}$	$1.40x10^{-3}$	0.79	$4.77x10^{-2}$	0.91

Table 4.9, continued.

GENE SET	# GENE	ENTREZ GENE ID	GENE	[a]MAGENTA PVAL	[b]GEO2R	[c]Log_2FC GEO2R	[d]AmpliSeq PVAL	[e]Log_2FC AmpliSeq
		55320	*MIS18BP1*	$7.29x10^{-1}$	$1.23x10^{-2}$	0.55	$1.66x10^{-1}$	-0.40
		55355	*HJURP*	$3.85X10^{-1}$	$1.52x10^{-3}$	0.63	$6.63x10^{-4}$	1.33
		55920	*RCC2*	$6.02X10^{-1}$	$6.13x10^{-3}$	0.47	$1.74x10^{-3}$	1.05
		79075	*DSCC1*	$4.31X10^{-1}$	$4.95x10^{-6}$	1.49	$1.33x10^{-3}$	1.57
		79723	*SUV39H2*	$2.83X10^{-2}$	$8.58x10^{-4}$	1.27	$1.73x10^{-6}$	1.80
		81789	*TIGD6*	$4.09X10^{-3}$	$9.65x10^{-1}$	0.01	$8.56x10^{-1}$	-0.11
		83540	*NUF2*	$1.67X10^{-2}$	$2.75x10^{-5}$	2.06	$1.94x10^{-4}$	1.47
		84948	*TIGD5*	$5.23X10^{-1}$	$1.65x10^{-1}$	0.21	$4.03x10^{-3}$	0.78
		91151	*TIGD7*	$3.95X10^{-1}$	$5.48x10^{-2}$	-0.56	$4.33x10^{-1}$	-0.67
		91687	*CENPL*	$6.66X10^{-1}$	$1.06x10^{-3}$	0.65	$2.21x10^{-3}$	1.13
		151246	*SGOL2*	$4.34X10^{-2}$	$3.59x10^{-3}$	0.88	$1.45x10^{-2}$	0.82
		151648	*SGOL1*	$1.89X10^{-1}$	$1.02x10^{-2}$	0.22	$5.26x10^{-4}$	1.30
		166815	*TIGD2*	$5.61X10^{-1}$	$1.21x10^{-2}$	0.64	$3.56x10^{-2}$	0.56
		200765	*TIGD1*	$9.12X10^{-1}$	$1.29x10^{-1}$	0.61	$2.72x10^{-2}$	-0.59
		201254	*STRA13*	$4.17X10^{-2}$	$3.00x10^{-1}$	0.27	$2.84x10^{-1}$	0.37
		201798	*TIGD4*	$6.68X10^{-1}$	$1.31x10^{-1}$	-0.19	N/A	N/A
		220359	*TIGD3*	$3.69X10^{-1}$	$1.94x10^{-1}$	-0.14	$4.06x10^{-1}$	-0.81
		378708	*APITD1*	$5.39X10^{-1}$	$6.94x10^{-1}$	0.12	$2.97x10^{-1}$	0.48
		387103	*CENPW*	$8.99X10^{-1}$	$1.04x10^{-2}$	0.97	$1.85x10^{-5}$	1.71

[a]Gene P-val assigned using best SNP association; 10kb UTR 2kb DTR
[b]Gene differential expression calculated by GEO2R (limma package) adjusting for log_2 fold change
[c]Log_2 fold change calculated in the direction of NPC cases vs non-NPC nasopharynx tissue
[d]Gene differential expression calculated by DESeq2 from 10 NPC and 7 non-NPC nasopharynx tissues from a Malaysian cohort
[e]Log_2 fold change calculated in the direction of NPC vs non-NPC nasopharynx tissue from a Malaysian cohort

Table 4.10: Sample details of NPC and non-NPC nasopharynx tissues from a Malaysian cohort used in gene expression analysis.

Sample ID	Phenotype	Gender	Ethnic group	Histology
non-NPC1	non-NPC	Male	Bajau	lymphoid tissue with 40% normal epithelial
non-NPC2	non-NPC	Female	Other Malaysian	lymphoid tissue with 30% epithelial
non-NPC3	non-NPC	Female	Bajau	lymphoid tissue with 20% normal epithelial
non-NPC4	non-NPC	Female	Kadazan Dusun	lymphoid tissue with 30% epithelial
non-NPC5	non-NPC	Male	Indian	lymphoid tissue with 20% epithelial
non-NPC6	non-NPC	Female	Malay	lymphoid tissue with 20% epithelial
non-NPC7	non-NPC	Male	Malay	lymphoid tissue with 40% epithelial
NPC1	NPC	Male	Other Malaysian	80% cancer cells
NPC2	NPC	Male	Bajau	80% cancer cells
NPC3	NPC	Female	Kadazan Dusun	80% cancer cells
NPC4	NPC	Male	Kadazan Dusun	80-90% cancer cells
NPC5	NPC	Female	Other Malaysian	80% cancer cells
NPC6	NPC	Male	Other Malaysian	80-90% cancer cells
NPC7	NPC	Female	Kadazan Dusun	80% cancer cells
NPC8	NPC	Male	Kadazan Dusun	90% cancer cells
NPC9	NPC	Male	Malay	90% cancer cells
NPC10	NPC	Male	Chinese	laser-captured microdissection

4.3.2 GWAS and Gene Expression Pathway analysis

For GSEA of both GWAS and gene expression data, 2,757 pathways was screened with gene sizes of 10-100 genes across BioCarta, GO, Ingenuity, KEGG, Panther and Reactome databases. GSEA of GWAS data in MAGENTA identified 92 pathways associated with NPC, showing nominal $P_{\text{GWAS-GSEA}}<0.05$ (Appendix N). GSEA of gene expression data in GSA-SNP identified 312 pathways associated with NPC, with a similar threshold of nominal $P_{\text{EXPR-GSEA}}<0.05$ (Appendix N). A total of 8 overlapping pathways were identified across the two platforms, assuming the nominal threshold of $P<0.05$ (Appendix O). We used Fisher's method to integrate the GSEA *P*-values from GWAS and gene expression data.

Two pathways were identified to be associated with NPC, namely the GO axonemal dynein complex ($P_{\text{GWAS-GSEA}}=1.98\text{x}10^{-2}$; $P_{\text{exp-GSEA}}=1.27\text{x}10^{-24}$; $P_{\text{Combined-FDR}}=1.38\text{x}10^{-22}$) and the chromosomal centromic region ($P_{\text{GWAS-GSEA}}=2.35\text{x}10^{-2}$; $P_{\text{exp-GSEA}}=6.03\text{x}10^{-9}$; $P_{\text{Combined-FDR}}=1.03\text{x}10^{-5}$) pathway (Table 4.8; Appendix P). Upon replication using NPC and non-NPC nasopharynx tissues, only the Gene Ontology (GO) axonemal dynein complex association was replicated ($P_{\text{AmpliSeq-GSEA}}=6.56\text{x}10^{-4}$) while the chromosomal centromic region association was not replicated ($P_{\text{AmpliSeq-GSEA}}=0.95$) (Table 4.8). Of the 12 genes that form this axonemal dynein complex pathway, 2 genes showed nominal significance in both GWAS and gene expression data with $\log_2$ fold-change $\leq$ -2, namely *DNAH5* ($P_{DNAH5\text{-GWAS}}=1.30\text{x}10^{-2}$; $P_{DNAH5\text{-exp}}=3.07\text{x}10^{-7}$; $\log_2\text{FC}=-2.64$) and *DNAH9* ($P_{DNAH9\text{-GWAS}}=3.8\text{x}10^{-2}$; $P_{DNAH9\text{-exp}}=6.79\text{x}10^{-8}$; $\log_2\text{FC}=-2.16$) (Table 4.9). The difference in gene expression of *DNAH5* and *DNAH9* between NPC and non-NPC nasopharynx tissues was replicated (DESeq2 FDR-corrected Wald test $P<0.05$, $\log_2\text{FC}\leq-2$) in our replication cohort ($P_{DNAH5\text{-AmpliSeq}}=7.75\text{x}10^{-5}$, $\log_2\text{FC}=-2.44$; $P_{DNAH9\text{-AmpliSeq}}=4.50\text{x}10^{-6}$, $\log_2\text{FC}=-3.67$) (Table 4.9). These results implicate key genes in the axonemal dynein complex pathway driving NPC development.

CHAPTER 5: DISCUSSION

5.1 Discussion for NPC GWAS study

This study describes a NPC GWAS of the Malaysian Chinese population, with emphasis on the *HLA-A* region. NPC GWAS studies to date have consistently implicated the *HLA-A* as a strongly associated locus towards NPC (Bei *et al*., 2010; Tang *et al*., 2012; Tse *et al*., 2009). In this study, strong SNP and amino acid association signals were detected, driven by both susceptible *HLA-A* allele *HLA-A*02:07* and protective *HLA-A*11:01*. Controlling for effects of *HLA-A*02:07* reduced the associations of both damaging variants, HLA-A-99Tyr/Cys-rs1136697-A/G and HLA-A-145Arg/His-rs1059520 while controlling for effects of *HLA-A*11:01* reduced the associations of *HLA-A* 5'-UTR SNPs and adjacent *GABBR1*, *HLA-F* and *HCG9* loci (Appendix E-F, Appendix J-K). Thus, *HLA-A* association is largely driven by effects of *HLA-A*02:07* and *HLA-A*11:01*, with internal and adjacent SNPs and amino acid variants acting as proxies.

HLA-A alleles display diverse occurrence across different populations. The *HLA-A*02:07* allele shows a frequency ranging from 6% to 13% among Southern Han Chinese individuals, including those of Hong Kong, Taiwan, Singapore and Malaysia (González-Galarza *et al*., 2015). In stark contrast, *HLA-A*02:07* is rare in Japan, with only frequency of less than 1% (González-Galarza *et al*., 2015). The *HLA-A*11:01* allele shows an even more striking contrast between Southern Han Chinese individuals and Japanese individuals with frequencies of approximately 30% in Southern Han Chinese while it is reported to be only 5% in the Japanese population (Nakaoka & Inoue, 2015). As *HLA-A* alleles are linked to NPC occurrence, its diverse distribution could be a factor in the distinct distribution of NPC incidence.

In our present study, *in silico* methods of PROVEAN (Choi *et al*., 2012), SIFT (Kumar *et al*., 2009) and Polyphen-2 (Adzhubei *et al*., 2010) predicted damaging variants for HLA-A-99Tyr (pocket groove binding) and HLA-A-145Arg (T-cell receptor binding site). HLA-A-99Tyr is a residue for pocket A, B, C and D binding. The stability of pocket B is critical as it is the binding groove for anchor position 2 of HLA-A associated peptides (Matsui *et al.*, 1993). From our study, multi-allelic variants are observed for HLA-A-aa-site-99, namely HLA-A-99Phe and HLA-A-99Cys. However, HLA-A-99Phe is predicted to be a benign or tolerated variant (Table 3.4). Thus, the damaging variant HLA-A-99Cys could possibly be influencing the peptide loading ability of HLA-A, and subsequently altering the cytotoxic T lymphocyte (CTL) recognition of the MHC-peptide complex. The stability of cell-mediated immune responses is also modulated by the T-cell receptors. In HLA-A molecules, the T-cell receptor acts to bind and stabilize the cytotoxic T-cells-MHC-peptide complex, while the CTL elicits an apoptotic response to destroy the infected cell (Salter *et al.*, 1990). A damaging variant on HLA-A-145Arg was detected. It is a T-cell receptor binding site of the HLA-A molecule. This variant is located in the α3 domain encoded by exon 3 of the *HLA-A* gene, the binding site of the CD8 co-receptor (Salter *et al*., 1990). Despite not being the main contact point for the CD8 receptor (residue 223-229), it is possible that mutation in HLA-A-145Arg could de-stabilize the T-cell receptor binding to the MHC-peptide complex, disrupting the antigen-specific activation. Although *HLA-A* variants have been identified to be associated with NPC in this study, the results fail to exclude the influence of EBV in NPC development. This limitation is because EBV data such as serology or DNA viral load is not available for our samples.

EBV genotypes have been found to be unique for specific tissue types (Chen *et al*., 1996) as well as ethnicity (Zhou *et al*., 2008). EBV genotypes show distinct *BZLF* gene variation in blood and tissue samples (Chen *et al*., 1996). In Southern China, the

type C EBV variant is more prevalent. This variant shows a loss of a *Bam*HI site between the *Bam*Hl Wl* and I1* regions. In addition, an "f" variant harboring an extra *Bam*HI site in the *Bam*HI F region is also detected (Lung *et al*., 1990). The type C variant is less prevalent in non-endemic regions, as observed in NPC cases in Japan whereby EBV type A is the more prevalent genotype (Zhou *et al*., 2008). The different EBV genotypes are speculated to influence NPC development differently. However, most reports merely document association and correlation. No studies thus far have reported on how the EBV genotype influences NPC occurrence.

The high SNP exclusion rate from our GWAS could possibly result in lower power of detection. The excluded SNPs were cross-checked against the National Human Genome Research Institute (NHGRI) Catalog of Published Genome-Wide Association Studies (GWAS) Catalog (Welter *et al.*, 2014), the Catalogue of Somatic Mutations (COSMIC) (Forbes *et al.*, 2011) and the Genetic Association Database (GAD) (Becker *et al.*, 2004). No previous GWAS and COSMIC loci were found among our excluded SNPs. As for the GAD database, there were potential NPC associated genes that were implicated such as *ABCB1*, *GSTM1*, *MDM2*, *MMP2*, *NAT2*, *NQO1*, *TLR10 and TLR4* and these loci were excluded due to low genotyping rate (call rates < 99%) or rare minor alleles (MAF < 1%). In addition, removal of sample outliers showing abnormal population structure or cryptic relatedness is important to avoid false positives. Samples of different ethnicity would introduce a bias and skew the SNP frequencies, affecting the association analysis. Similarly, presence of relatives would inflate the frequencies of SNP alleles and affect the association analysis as well. In GWAS studies, the common practice would be to remove all unexpected relatedness, including 4^{th} and 5^{th} degree relatives. This would minimize the effect of cryptic relatedness on the association analysis.

5.2 Discussion for meta-analysis of NPC GWAS

This study presents results from a meta-analysis of NPC GWAS followed by replication studies across three regions in Asia. A novel association was observed within the *CLPTM1L/TERT* locus. This finding is of note given that SNPs in this region were reported from GWAS conducted for numerous other cancers, including lung, bladder, pancreas, testis, and central nervous system (Mocellin *et al.*, 2012). A recent meta-analysis of 85 studies including over 490,000 subjects that evaluated 67 *TERT/CLPTM1L* locus polymorphisms and 24 tumor types identified 11 SNPs with strong cumulative evidence for an association with at least one cancer type. rs31489 was one of these SNPs and was found to have strong cumulative evidence for association with testicular cancer among Caucasians and moderate cumulative evidence for association with Asian lung cancer (Mocellin *et al*., 2012). Furthermore, a review of the literature identified candidate gene studies (two that evaluated SNPs and a third that evaluated a microsatellite marker) that reported an association between polymorphisms within the *CLPTM1L/TERT* locus and NPC (Fachiroh *et al.*, 2012; Yee Ko *et al.*, 2014; Zhang *et al.*, 2011). Two of the three SNPs evaluated in these studies are in LD with rs31489 (rs401681 r^2=0.427 in 1 kG ASN and 0.512 in 1 kG CHB; rs402710 r^2=0.433 in 1 kG ASN and 0.569 in 1 kG CHB). The third SNP is not in LD with rs31489, suggesting the possibility for the existence of greater than one independent susceptibility variant within the *CLPTM1L/TERT* locus (rs2736098 r^2=0.016 in 1 kG ASN and 0.049 in 1 kG CHB). Our findings in the *CLPTM1L/TERT* locus gain added significance given the role of *TERT* in telomere length regulation (Bellon & Nicot, 2008), the finding that telomerase overexpression is observed in NPC (Cheng *et al.*, 1998), and that the EBV protein LMP1, a protein frequently expressed in NPC, activates TERT expression and enhances telomerase activity (Mei *et al.*, 2006; Terrin *et al.*, 2008). There was evidence for possible heterogeneity in effect observed for rs31489

across study populations (P_{het}=0.035). The evidence for heterogeneity was of marginal statistical significance and was driven primarily by the two Malaysian studies included in this study. It is unclear at this time whether our findings reflect true heterogeneity. This observation deserves further consideration in future studies.

Additional associations ($P \leq 1x10^{-7}$) were observed in our combined GWA plus replication studies meta-analysis for rs6774494 (P=1.5x10^{-12}; *MECOM* gene region), rs9510787 (P=5.0x10^{-10}; *TNFRSF19* gene region), rs1412829, rs4977756, and rs1063192 (P=2.8x10^{-8}, P=7.0x10^{-7}, and P=8.4x10^{-7}, respectively; *CDKN2A/2B* gene region; Table 4.3 and Figure 4.1). These associations were previously reported from the Mainland China NPC GWAS and their potential biological implications discussed (Bei *et al*., 2010); the study data provide support for these associations.

Strengths of the study include the fact that it evaluated associations with NPC across multiple GWAS and the large size of its replication effort. Limitations include the inability to further investigate potential heterogeneity of effects by exposure status or geographic/ethnic groups. This study also did not identify functional variants of the *CLPTM1L/TERT* locus. This aspect is important considering cells with mutated *TERT* promoters have elongated telomeres, with the possibility of promoting immortalization and tumorigenesis of cancer cells (Chiba *et al*., 2015). Future studies should explore the associations reported herein in additional populations with differing ethnic makeup.

5.3 Discussion for integrated pathway analysis of NPC

This work reports an integrated approach of investigating pathway associations towards NPC. Using this study design that combines GWAS and gene expression data, the joint contributions of genomic variants and differential gene expression towards NPC development can be identified. Also, focusing on pathway-based associations removes the need to adhere to strict multiple testing thresholds commonly practiced in GWAS studies, enabling inclusion of moderate to small effect loci. GSEA was chosen for pathway analysis of both GWAS and gene expression data to standardize the pathway analysis methodology and avoid discrepancies brought upon by other pathway analysis methods. The choice is strongly influenced by MAGENTA and its ability to account for confounding factors on the association scores of genes for GWAS data, a feature not available for other GSEA methods (Segre *et al*., 2010).

GWAS and gene expression data used in this study are from independent sources. Also, the epigenetic effect is not addressed in this study. These are possible reasons leading to low concordance of gene association strength and differential gene expression. Nonetheless, the results identified a link between the axonemal dynein complex and NPC. This association was also replicated in a separate cohort of NPC and non-NPC tissues, further strengthening our findings.

Axonemal dynein complex are critical components in the ciliated cells. The axonemal dynein complex functions in mucociliary clearance, failure of which would lead to infection in the upper and lower respiratory tract (Palmblad *et al.,* 1984). Ciliated cells line the upper and lower respiratory tract, the nasopharynx included. Coordinated movement of the cilia sweeps mucus out of the upper and lower respiratory tract, acting as a primary defense mechanism of the airways (Knowles & Boucher, 2002). Seeing that NPC is a viral cancer caused by EBV infection as well as nasopharynx exposure to carcinogens, the implications of impaired mucociliary

clearance, in particular in the upper respiratory tract, are highly relevant to NPC.

Two genes grouped under the axonemal dynein complex, namely *DNAH5* and *DNAH9* showed consistent association to NPC across both genomic and gene expression data. *DNAH5* codes for a heavy chain of the dynein complex. Mutations in *DNAH5* have been linked to cilia immotility (Olbrich *et al.*, 2002). DNAH9 co-exists with DNAH5, but is localized in a distal position of the cilia. It also codes for a heavy chain of the dynein complex and functions to drive beating of the cilia. Both *DNAH5* (GEO2R $\log_2$FC=-2.64; AmpliSeq $\log_2$FC= $\log_2$FC=-2.44) and *DNAH9* (GEO2R $\log_2$FC=-2.16; AmpliSeq $\log_2$FC=-3.67) are lowly expressed in NPC tissues, suggesting a loss of function. This study was unable to investigate any links of EBV genes to host gene expression due to lack of EBV genes expression data. In addition, there has been no previous reports documenting the influence of EBV genes in modulating expression of axonemal dynein genes.

This study is not without its limitations. The association of the axonemal dyenein gene set is largely driven by the expression data. In addition, although *DNAH5* and *DNAH9* are lowly expressed in NPC tissues, there is no data available indicating direct impairment of cilia function. The results are also unable to identify the main reason behind the dysregulation of the axonemal dyenein gene set. Other probable causes include epigenetic signatures, in particular methylation. There is no paired epigenetic data to corroborate this hypothesis. Likewise, the absence of paired genomic data also hinders identifying genomic variants that directly correlate with gene expression, thus implying possible regulatory function.

CHAPTER 6: CONCLUSION

The advent of high throughput GWAS studies have accelerated the discovery of potential susceptible variants in our genome. The amount of data generated is unprecedented, and have led to better understanding of disease etiology. Despite the wealth of data available, GWAS studies remains at best, an epidemiological study. Despite our best efforts, we have yet to translate our findings to the clinic. Correlation does not imply causation. The challenge remains to identify disease causing genomic variants with direct consequence to disease. Current limitations include the high cost of next generation sequencing technologies to enable large scale sequencing of samples, generating even more detailed maps of our genome. This can be corroborated with other data, for example transcriptomics, proteomics and epigenomics to construct a more wholistic representation of the dynamics of disease onset.

In the case of NPC, there is no doubt it displays a multi-factorial etiology. NPC onset can range from environmental factors such as diet, lifestyle habits, viral infection or even genetics. The challenge moving forward would be to identify functional variants of NPC. To unravel the complex etiology of NPC, high throughput platforms will play a crucial role in accelerating the search for prospective therapeutic candidates. This coupled with the emergence of machine learning or artificial intelligence will shape the future direction of cancer research in our never ending quest to understand and combat cancer.

The antigen presentation mechanism of the *HLA-A* is a complex process, instrumented by the peptide loading complex (PLC) that consists of a myriad of assembly molecules (Purcell & Elliott, 2008). Polymorphisms in the *HLA-A* gene only constitute a small portion of the processes involved in *HLA-A*-peptide presentation. Any defect in the cascade of processes, starting from the formation of the nascent class I heavy chain until the final stage of transporting the MHC-peptide complex to the cell

surface, would result in a defective MHC-peptide complex. Not forgetting, the EBV virus through the EBNA-1 is capable of eluding the cells immune-surveillance by expressing a Gly-Ala repeat sequence, thus preventing its proteosomal breakdown (Young & Rickinson, 2004). Solving the genetic predisposition of NPC remains a challenging task. Nevertheless, the plethora of genomic data available would greatly facilitate exploring the functional basis of NPC development, in particular, the *HLA-A* region.

The GWAS meta-analysis and replication effort has identified an additional susceptibility locus for NPC within the *CLPTM1L/TERT* region of chromosome 5p15.33 and provides support for several previously reported NPC susceptibility loci. However, heterogeneity does exists across studies, as evidenced by the lack of association of the Malaysian NPC loci, *ITGA9* in other NPC studies.

The integrated approach of investigating pathway associations towards NPC, utilizing joint contributions of genomic variants and differential gene expression data. The results uncovered a pathway with possible influence on NPC development. The limited concordance between GWAS association data and differential gene expression levels put forth the challenges of adopting a multi-platform approach in identifying aberrant or dysregulated pathways in NPC. An eQTL approach analyzing correlation of genomic variants to gene expression of a common cohort could enhance the resolution of pathway-based GWAS approaches and enable us to drill down to the key pathways or genes affecting NPC.

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List of Publications and Papers Presented

Pubilcations

1. Chin, Y. M., Mushiroda, T., Takahashi, A., Kubo, M., Krishnan, G., Yap, L. F., . . . Ng, C. C. (2015). HLA-A SNPs and amino acid variants are associated with nasopharyngeal carcinoma in Malaysian Chinese. *International Journal of Cancer, 136*(3), 678-687.

2. Bei, J. X., Su, W. H., Ng, C. C., Yu, K., Chin, Y. M., Lou, P. J., . . . International Nasopharyngeal Carcinoma Genetics Working Group. (2016). A GWAS meta-analysis and replication study identifies a novel locus within CLPTM1L/TERT associated with nasopharyngeal carcinoma in individuals of Chinese ancestry. *Cancer Epidemiology Biomarkers & Prevention, 25*(1), 188-192.

3. Chin, Y. M., Tan, L. P., Norazlin Abdul Aziz., Mushiroda, T., Kubo, M., Nor Kaslina Mohd Kornain, . . . Ng, C. C. (2016). Integrated pathway analysis of nasopharyngeal carcinoma implicates the axonemal dynein complex in the Malaysian cohort. *International Journal of Cancer, 139*(8), 1731-1739.

Presentations

1. Chin, Y.M., Mushiroda, T., Ng, C.C. and The Malaysian NPC Study Group. (2013). High resolution genotyping of *HLA-A* reveals stronger association of HLA-A single SNPs compared to *HLA-A* allele subtypes to nasopharyngeal carcinoma susceptibility in Malaysian Chinese. Poster presented at the Joint Conference of HGM 2013 and 21st International Congress of Genetics, The Sands Expo and Convention Centre, Marina Bay Sands, Singapore.

2. Chin, Y.M. (2014). Imputation of NPC genome-wide SNP data reveals single gene and gene-set pathway associations. Paper presented at the 4th NPC Research Day, University Malaya, Kuala Lumpur.

3. Chin, Y.M. (2016). Genome-wide association studies (GWAS) of nasopharyngeal carcinoma in the Malaysian cohort using single genes, meta-analysis and pathway analysis approaches. Paper presented at the 2nd International Symposium on Molecular Medicine, Tokushima University, Japan.

4. Chin, Y.M., Tan, L.P., Tan, G.W., Norazlin Abdul Aziz., Nor Kaslina Mohd Kornain, Pua, K.C., . . . The Malaysian NPC Study Group. (2016). Identification of candidate loci for nasopharyngeal carcinoma through GWAS and eQTL. Poster presented at NIH Research Week 2016, Institute for Health Management (IHM), Bangsar Kuala Lumpur.