SCREENING OF MALAYSIAN PATIENTS WITH NASOPHARYNGEAL CARCINOMA (NPC) USING COMPARATIVE GENOMIC HYBRIDIZATION (CGH) TECHNIQUE FOR IDENTIFICATION OF SPECIFIC GENETIC ALTERATION

by

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PENYARINGAN PESAKIT NASOPHARYNGEAL CARCINOMA DI MALAYSIA MENGGUNAKAN TEKNIK PERBANDINGAN HIBRIDASI GENOMIK UNTUK MENGENALPASTI PERUBAHAN GENETIK YANG SPESIFIK

oleh

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Tesis yang diserahkan untuk
memenuhi keperluan bagi
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LIST OF ABBREVIATIONS

A260/A280	: ratio of 260 absorbance over 280 absorbance
A	: adenine
aCGH	: array Comparative Genomic Hybridization
AJCC/UICC	: American Joint Committee on Cancer/ Union Internationale Contre le Cancer
ADM2	: Aberration Detection Method 2
Alu 1	: restriction endonucleases
ANOVA	: analysis of variance
ASXL3	: additional sex combs like 3
BAC	: Bacterial Artificial Chromosome
BAX	: BCL2-associated X protein
BCAT1	: branched chain aminotransferase 1, cytosolic
BCL2	: B-cell lymphoma 2
BLU	: zinc finger, MYND domain containing 10
Bp	: base pair
BSA	: bovine serum albumin
B-raf	: v-raf murine sarcoma viral oncogene homolog B1
BRCA1	: breast cancer 1
BRG1	: Brahma-related gene 1
BRD7	: bromodomain containing 7
Buffer AE	: elution buffer
Buffer AL	: lyses buffer
Buffer ATL	: lyses buffer
Buffer AW	: wash buffer
Buffer BL	: lyses buffer
Buffer BW	: wash buffer
Buffer TW	: wash buffer
C	: cyanine
CACNA2D2	: calcium channel, voltage-dependent, alpha 2/delta subunit 2
CaCX	: Carcinoma of Uterine Cervix
CCD	: charge-couple device

CCND	: cyclin D1
Cdk4	: cyclin dependent kinase 4
cDNA	: complementary deoxyribonucleic acid
CGH	: Comparative Genomic Hybridization
CI	: confidence interval
CNV	: Copy number variation
CLL	: Chronic Lymphoid Leukaemia
CMA	: Chromosomal Microarray
c-myb	: cellular-myb
c-myc	: cellular-myc
CNV	: Copy number variation
CpG	: genomic regions that contain a high frequency of GC contents
CT	: computerized tomography
Cy-3	: cyanine 3
Cy-5	: cyanine 5
DAG	: diacylglycerol
DAPI II	: 4,6'-diamidino-2-phenylindole dihydrochloride
dATP	: deoxyadenine triphosphate
dCTP	: deoxycytosine triphosphate
ddH2O	: deionized distilled water
dGTP	: deoxyguanine triphosphate
dH2O	: distilled water
DNA	: deoxyribonucleic acid
dNTP	: dinucleotide triphosphate
dTTP	: deoxythymine triphosphate
dUTP	: deoxyuridine triphosphate
DNA	: deoxyribonucleic acid
EA	: early antigen
EBNA	: Epstein-Barr nuclear antigen
EBV	: Epstein- Barr Virus
EDTA	: Ethylenediaminetetraacetic acid
eIF5A2	: eukaryotic translation initiation factor 5A2

FGF3	: fibroblast growth factor 3
FISH	: fluorescent in-situ hybridization
FITC	: Fluorescein isothiocyanate
FUS1	: fused in sarcoma
FVT1	: follicular lymphoma variant translocation 1
G	: guanine
GLI	: glioblastoma
GNAT1	: guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1
GSTM1	: glutathione S-transferase M1
GSTT1	: glutathione S-transferase theta 1
HCl	: hydrogen chloride
HCP5	: Human leukocyte antigen complex P5
HLA	: human leukocyte antigen
Hi-RPM	: hybridization buffer
HOXD13	: homeobox D13
HPV	: Human papillomavirus
H & E	: Haematoxylin & Eosin
HRPZ II	: Hospital Raja Perempuan Zainab II
HSI	: Hospital Sultan Ismail
HSNZ	: Hospital Sultanah Nur Zahirah
HTAA	: Hospital Tengku Ampuan Afzan
HUSM	: Hospital Universiti Sains Malaysia
HYAL2	: hyaluronoglucosaminidase 2
IMR	: Institute Medical Research
ISH	: In Situ Hybridization
ISCN	**:** International System for Human Cytogenetic Nomenclature
Kbp	: kilo base pair
KCNJ8	: potassium inwardly-rectifying channel, subfamily J, member 8
KRAS2	: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
L	: Liter

LKB1	: serine/threonine kinase 11
LOH	: loss of heterozygosity
MALT1	: mucosa associated lymphoid tissue lymphoma translocation gene 1
MCL	: myeloid cell leukemia sequence
MDM2	: murine double minute 2
MEN1	: multiple endocrine neoplasia I
MICA	: MHC class I polypeptide-related sequence A
Mm	: milimeter
M	: meter
M	: Molar
Ml	: mililiter
Mg	: milligram
MRI	: magnetic resonance imaging
mRNA	: messenger ribonucleic acid
MSH2	: mutS homolog 2, colon cancer, nonpolyposis type 1
MSO	: Methylation-Specific Oligonucleotide
MYCN	: v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
MYO18B	: myosin XVIIIB
NaCl	: Natrium Chloride
NaOH	: Natrium Hydroxide
NCBI	: *National Center for Biotechnology Information*
NP-40	: nonyl phenoxylpolyethoxylethanol
NPRL2	: tumor suppressor candidate 4
Nm	: nanometer
OD	: Optical Density
ORL	: Otorhinolaryngolgy
PCR	: polymerase chain reaction
PEW	: Polyester wax
PHA	: phytohemaglutinin
PIKC3A	: phosphoinositide-3-kinase, catalytic, alpha polypeptide
PL6	: placental protein 6

PTCH	: patched homolog
PTHLH	: parathyroid hormone-like hormone
PTX1	: prostate nuclear gene
QCMT	: QC metrics
RAD51	: homolog (RecA homolog, E. coli) (S. cerevisiae)
RASSF1	: Ras association domain family member 1
Rb1	: retinoblastoma susceptibility gene
RBSP3.18	: chromosome 3 open reading frame 8
REL	: v-rel reticuloendotheliosis viral oncogene homolog
RNA	: ribonucleic acid
Rpm	: rotator per minute
Rsa 1	: Ribosome Assembly
RT-PCR	: reverse transcriptase polymerase chain reaction
SETBP1	: SET binding protein 1
SD	: standard deviation
Skp2	: S-phase kinase-associated protein 2
SKOR2	: SKI family transcriptional corepressor 2
SNP	: single nucleotide polymorphism
SSC	: saline-sodium citrate
T	: thiamine
TE	: tris-EDTA
TBE	: tris-borate-EDTA
TIM	: Rho guanine nucleotide exchange factor (GEF) 5
TNM	: tumor node metastasize
TP53	: tumor protein p53
Tris-HCl	: tris- hydrogen chloride
TSC2	: tuberous sclerosis 2
UV	: ultra-violet
Vca	: viral caspid antigen
WHO	: World Health Organization
WNT4	: wingless-type MMTV integration site family, member 4
101F6	: cytochrome b-561 domain containing 2
µg/µL	: microgram per microliter

µm	: micro
x g	: times gravity

LIST OF SYMBOLS

< : less than

> : more than

^{o}C : degree celcius

μ : micro

% : percentage

β : beta, power of study (1- β)

PENYARINGAN PESAKIT NASOPHARYNGEAL CARCINOMA DI MALAYSIA MENGGUNAKAN TEKNIK PERBANDINGAN HIBRIDASI GENOMIK UNTUK MENGENALPASTI PERUBAHAN GENETIK YANG SPESIFIK

ABSTRAK

Kanser nasofaring (NPC) adalah kanser yang berasal daripada lapisan epitelium lekuk-rosen Muller pada nasofarink. Kanser ini banyak terdapat di China Selatan dan Asia Tenggara termasuk Malaysia. Di dalam kajian ini, perubahan genetik pada empat puluh empat pesakit NPC daripada 5 buah hospital di Malaysia telah disaring. Teknik hibridasi perbandingan genomik (CGH) telah digunakan untuk pengenalpastian sebarang perubahan genetik. CGH adalah salah satu teknik sitogenetik molekul yang digunakan untuk mengenalpasti ketidakstabilan perubahan genetik yang terdapat pada penyakit kanser ini. Tisu biopsi nasofarink yang dipilih telah dipastikan mengandungi sekurang-kurangnya 75% tumor terlebih dahulu dengan menggunakan teknik pewarnaan Haematoxylin & Eosin (H&E) sebelum penggunaan teknik CGH. DNA tumor telah diekstrak daripada empat puluh empat biopsi NPC, sementara empat puluh empat DNA rujukan telah diekstrak daripada empat puluh empat darah periferi dari subjek normal. DNA tumor dan DNA normal telah dilabel dengan kaedah translasi nick (nick translation) menggunakan pewarnaan berpendarfluor hijau dan merah. DNA itu telah dihibridasi pada metafasa kromosom dan diwarnakan dengan DAPI II. Akhirnya, gambar kromosom telah diambil dan dianalisa.Kebanyakan sampel adalah lelaki berbanding perempuan dengan kadar 2.4:1. Kumpulan etnik Melayu mempunyai peratus yang lebih tinggi

daripada Cina iaitu masing-masing 57% dan 43%. Kes NPC dari kalangan Cina menunjukkan insiden yang tinggi pada dua buah hospital di negeri yang mempunyai penduduk Cina yang tinggi iaitu Johor dan Pahang. Purata umur pesakit pada diagnosis pesakit NPC ialah 49.45 tahun (14.74) [purata (SD)]. Tiada asosiasi yang signifikan ditemui di antara umur dengan jantina dan kumpulan etnik. Kebanyakan pesakit (38 pesakit) adalah daripada klasifikasi WHO Jenis III kecuali 5 pesakit adalah WHO jenis II dan seorang pesakit adalah WHO jenis I. Tiada asosiasi yang signifikan di antara jenis WHO dengan kumpulan etnik dan dengan jantina pesakit NPC. Analisa juga menunjukkan tiada asosiasi yang signifikan di antara jenis WHO dengan purata umur pesakit NPC. Di dalam kajian ini, perubahan kromosom pada 41 kes telah dijumpai manakala 3 kes tiada perubahan kromosom. Lima belas perubahan penambahan kromosom yang lazim telah dikenalpasti iaitu kromosom 2p, 2q, 3p, 3q, 4p, 4q, 5p, 5q, 6q, 7q, 8q, dan 12q manakala 10 perubahan pengurangan kromosom yang lazim telah dijumpai iaitu 1p, 9q, 15q, 16p, 16q, 17p, 19p, 19q, dan 12q. Tiada asosiasi yang signifikan ditemui di antara jantina dan penambahan genetik kecuali kromosom 3q manakala dalam perubahan pengurangan kromosom, terdapat asosiasi yang signifikan pada 4 daripada 10 kromosom iaitu pada kromosom 1p ($p=0.013$), 15q ($p=0.012$), 16q ($p=0.040$) dan 19p ($p=0.033$). Asosiasi yang signifikan juga tidak ditemui di antara 3 jenis NPC dengan perubahan genetik yang lazim. Pengesahan menggunakan teknik jujukan hibridasi perbandingan genomik (aCGH) menunjukkan hasil yang sepadan dengan teknik CGH secara konvensional. Berdasarkan artikel yang sedia ada, kajian ini adalah yang pertama dijalankan terhadap pesakit NPC di Malaysia dengan menggunakan teknik CGH. Dari kajian ini, beberapa onkogen dan gen penindas tumor telah dipetakan pada perubahan kromosom yang lazim seperti c-myc onkogen pada 8q24, MDM2

onkogen pada 12q13-14 dan TSC2 gen penindas tumor pada 16p. Kajian ini telah menyediakan landasan kepada kajian seterusnya untuk menempatkan sebarang gen penindas tumor dan onkogen pada pesakit NPC di Malaysia. Untuk mengenalpasti sebarang gen penindas tumor dan onkogen pada pesakit NPC di Malaysia, saiz sampel haruslah ditingkatkan lagi pada kajian masa hadapan.

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ABSTRACT

Nasopharyngeal Carcinoma (NPC) is a malignancy that originates from the epithelial lining of Fossa of Rosen Muller in the nasopharynx. It is a highly prevalent disease in Southern China and Southeast Asia including Malaysia. In this study, the genetic alterations from forty-four NPC patients from five hospitals in Malaysia were screened. Comparative Genomic Hybridization (CGH) technique was used for screening the genetic alterations. CGH is a molecular cytogenetic technique which is used to identify imbalanced genetic alterations that is present in this malignant disease. NPC tissues that were biopsied were first confirmed to have at least 75% tumour cells using Haematoxylin & Eosin (H & E) staining before proceeding to CGH. Tumor cells were extracted from forty-four NPC biopsies while forty-four references DNA were extracted from peripheral blood of forty-four normal controls. Tumour DNA and normal DNA was labelled by nick translation method with green and red fluorescent dyes. They were hybridized at metaphase chromosomes DNA and counterstained with DAPI II. Finally, the image was captured and analyzed. Most of the samples were male compared to female with a ratio of 2.4 : 1. Malay ethnic group was higher than Chinese which is 57% and 43% respectively. Chinese

cases showed higher incidence in the two hospitals from the states with majority Chinese ethnic group. The mean age of diagnosis of the NPC patients is 49.45 years old (14.74) [mean (SD)]. There were no significant difference between age with gender and ethnicity. Most of the patients (38 patients) were NPC WHO Type III except 5 patients who were Type II and one Type I. There is no significant difference between WHO type with ethnic group and with gender of NPC patients. There is also no correlation between types of NPC with mean age of diagnosis. In this study, chromosomal alterations were found in 41 cases but none in 3 other cases. Fifteen common chromosomal alterations gains which were chromosome arms 2p, 2q, 3p, 3q, 4p, 4q, 5p, 5q, 6q, 7q, 8q and 12p while ten common chromosomal alterations loss were 1p, 9q, 15q, 16p, 16q, 17p, 19p, 19q and 22q. There were no genetic differences between the gender and genetic gain except for chromosome 3q while in chromosomal aberration loss there were genetic differences in 4 out of 10 chromosome arm which were chromosome arms 1p ($p=0.013$), 15q ($p=0.012$), 16q ($p=0.040$) and 19p ($p=0.033$). In addition, there were no genetic differences between the three types of NPC with common genetic alterations findings except for chromosome 9q loss. Loss of 9q was detected in 80% of Type II and 32% of Type III tumours. Validation using Array Comparative Genomic Hybridization (aCGH) showed compatible results with conventional CGH. Based on the available literature, this is the first study conducted on Malaysian NPC patients utilizing CGH technique. From this study, several oncogenes and tumour suppressor genes were mapped at our common genetic alterations such as c-myc oncogene at 8q24, MDM2 oncogene at 12q13-14 and TSC2 tumour suppressor gene at 16p. This study has provided the platform for further investigations to locate any tumour-suppressor genes and oncogenes in Malaysian NPC patients. In order to determine any tumour-suppressor

genes and oncogenes in Malaysian NPC patients, it is suggested that a larger sample size of NPC patients should be conducted in future research.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Genetic factors have been reported as one of the most important etiological factors of Nasopharyngeal Carcinoma, but the molecular basis for tumorigenesis is still unclear. Therefore, screening of genetic imbalances in patients with NPC would be useful for early diagnosis of NPC. In this study, Comparative Genomic Hybridisation (CGH) was used to detect genetic alterations that include gain or loss in the chromosomes of NPC tissues and validation by array-based Comparative Genomic Hybridization (aCGH) was utilized. Prior to make further explanation of this study, clear information about NPC and CGH are needed.

1.2 General description of the nasopharynx

The nasopharynx is a portion of the pharynx that lies behind the nasal cavity and above the soft palate. According to medical dictionary, the word 'nasopharynx' is derived from a combination of Latin and Greek. 'Naso-' is derived from the Latin word nasus, which means nose while 'Pharynx' is from a Greek word for throat and the Greco-Roman term 'nasopharynx' was used in 1877 (Shiel and Stoppler, 2008a). Nasopharynx is the uppermost division of the pharynx while oropharynx is the middle part, followed by hypopharynx which is the third part of the pharynx (**Figure 1.1**). The air inhaled from atmosphere through the nose crosses the nasopharynx and enters the trachea for respiration.

The size of the nasopharynx in adults is approximately 4cm in length, 4 cm wide and 3 cm in an anteroposterior dimension (Chew, 1997). The nasopharynx consists of an anterior wall, floor, lateral wall, roof and posterior wall. The anterior wall of the nasopharynx is formed by the choana and posterior margin of the nasal septum. The floor is formed by the upper surface of the soft palate and is continued into the oropharynx. The lateral wall on either side in bony and presents two important features, which the pharyngeal orifice of the Eustachian tubes and the fossae of Rosenmuller. The roof continues into posterior wall separate smoothly and lies on the body of sphenoid, basiocciput and basisphenoid (Chew, 1997).

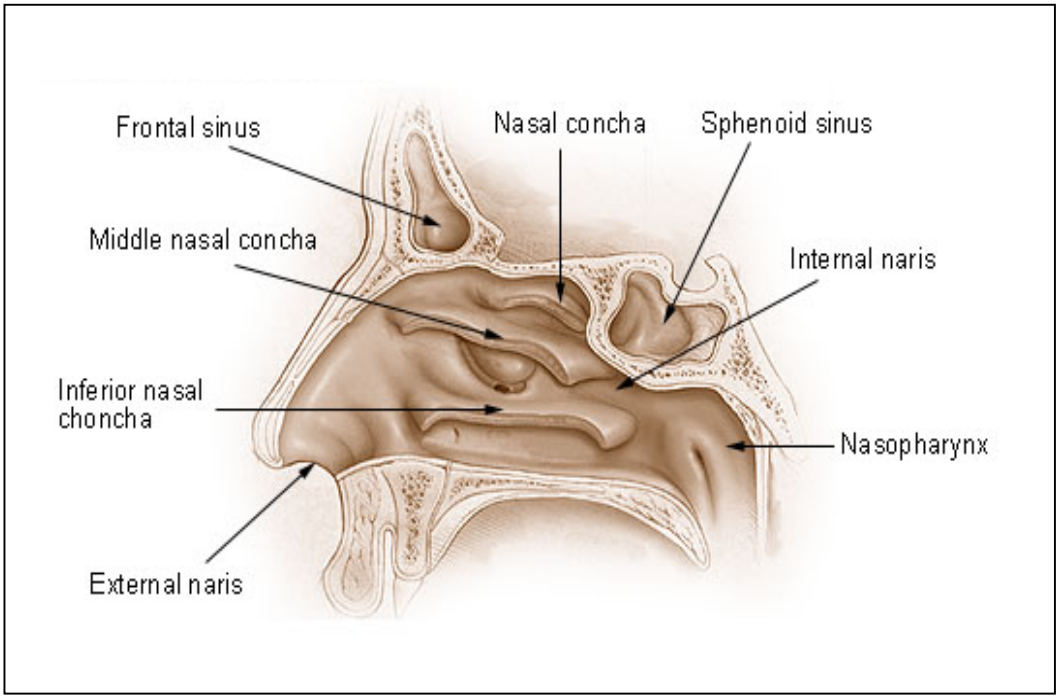

Figure 1.1: Schematic diagram of nasopharynx. Nasopharynx is the uppermost division of the pharynx (www.aboutcancer.com/nasopharynx).

1.3 Neoplasm

Neoplasm or tumor is abnormal cell growth which may be benign, pre-malignant (carcinoma in-situ) or malignant (cancer). According to the medical dictionary (Shiel and Stoppler, 2008a) the word ‘neoplasm’ is adapted from Greek which is ‘neo’ means new and plasma means growth. Thus, it is translated as a new growth.

Neoplasm is classified as benign, premalignant and malignant. Benign neoplasm consists of non-cancerous cell that are unable to metastasize or invade to surrounding tissue. Pre-malignant neoplasm or carcinoma in-situ is tissue that has potential to be transformed into malignant cells after certain duration of time (Chabner, 2007). These forms of tissue are not invasive unless they are transformed into malignant tissues. Malignant cells have the quality to invade the surrounding tissues and destroy them. Moreover, they have the property to metastasize locally, regionally or to distant organs of the body.

1.4 Cancer

Cancer is a disease where the growth of the cells are beyond control (Crosta, 2010). When they grow, they do not push the surrounding structures but rather they infiltrate and invade into them, causing the disruption of their normal walls, surfaces, borders and boundaries. This does not limits to the soft tissues only, but most of them even erode the surrounding bony contours and destroy the anatomical land marks. Another hallmark is spread to other locations via lymphatics or blood vessels and metastazing into the distant organs of the body. There are many types of cancers depending upon where the cells are initially affected such as cervical cancer, colorectal cancer, lung cancer and nasopharyngeal cancer.

Cancer cells are formed when these two processes which are mitosis and protein synthesis, are disturbed. The character of cancer cells are anaplastic which make the cells failed to carry on the function of mature cells when their DNA stops making codes (Chabner, 2007). In normal cell cycle regulation, normal cells will become mature and die after certain time via a process called apoptosis. In cancer cells, the mechanism of apoptosis also breaks down making the cells reproduce continuously. Apoptosis or programmed cell death makes the uncontrolled cell growth that leads to a mass formation(Crosta, 2010). Besides, altered DNA materials and cellular programs make new signals that enables cell proliferation, movement of cells, invasion of adjacent tissue and metastases (Chabner, 2007).

The cause of the cancer development can be divided into two major groups which are genetic hereditary and environmental factors (Chabner, 2007). Preetha Anand et. al. reported that, environmental factor plays an important role of causing cancer (Anand *et al.*, 2008). About 90-95% of all cancer cases can be attributed to environment and lifestyle, while only 5-10% are attributed to genetic hereditary factors (Anand *et al.*, 2008). Therefore, major lifestyle changes such as smoking cessation, increased ingestion of fruits and vegetables, moderate use of alcohol, caloric restriction, exercise, avoidance of direct exposure to sunlight, minimal meat consumption, use of whole grains, use of vaccinations, and regular check-ups are needed to prevent cancer development and progression (Anand *et al.*, 2008).

1.4.1 Classification of Cancerous Tumour

Cancer is classified into three major groups which are carcinoma, sarcoma and mixed-tissue tumours. They are divided according to histogenesis which is by identifying from where the particular tumours cell arise (Chabner, 2007). Carcinomas are the largest group that contribute approximately 90% of all malignancies. It is a solid tumour that is derived from epithelial tissue that covers internal and external body organs such as skin, urinary, colon, and reproductive organs. Benign tumours that are derived from epithelial cells are usually designated with the term adenoma, while malignant tumours that are derived from epithelial tissue are designated the term carcinoma (Chabner, 2007).

Sarcomas are derived from connective tissue and are less common than carcinomas. Examples of connective tissue are bone, fat, muscle and bone marrow. While mixed-tissue tumours are uncommon tumours which are derived from tissue that composed of epithelial and connective tissue such as kidney, ovaries and testes.

1.4.2 Cancer in Malaysia

According to the Malaysia Cancer Statistic 2006, 131.3 per 100,000 Malaysians were diagnosed as having cancer and NPC is one of the most common cancers in Peninsular Malaysia after breast, colorectal, lung and cervical cancer (Zainal *et al.*, 2006). The variation of cancer gives the difference in cancer occurrence of age, ethnicity, and sex. Certain types of cancer mainly occur in certain age group, ethnicity and sex. The most common cancer in children (from age 0-14 years old) is leukaemia. The most common cancer in men are nasopharynx, colorectal, lung, brain

and leukaemia while the most common cancer in women are breast, cervix, thyroid gland, ovary and colorectal (Zainal *et al.*, 2006).

1.5 Nasopharyngeal Carcinoma (NPC)

Nasopharyngeal carcinoma (NPC) is a malignancy originating in the epithelial lining of the nasopharynx, commonly arising from the fossa of Rosenmuller, the recess located medial to the medial cruca of the Eustachian tube (Wei and Sham, 2005). **Figure 1.2** shows the Computerized Tomography (CT) image of nasopharyngeal carcinoma. It is one of the most common cancers in Malaysia, a country in Southeast Asia with a multiracial population (Prasad and Rampal, 1992). In Malaysia, the National Cancer Registry stated that NPC was the second most common cancer in men: Chinese men had the highest age-standardised incidence, with 18.1 cases per 100,000, Malay men, with 4.8 cases per 100,000, and Indian men, with 2.6 cases per 100,000 (Lim *et al.*, 2004). According to Watkinson et. al., the incidence of nasopharyngeal cancer in Singapore was 87% among the Chinese, 10% among Malays, 3% among Europeans, and negligible in the Indian population (Watkinson *et al.*, 2000). Whereas in Hong Kong in the southern part of the Guangdong, the incidence rate of NPC among men and women are 20-30 per 100, 000 and 15 to 20 per 100,000 respectively (Parkin *et al.*, 1997). The high incidence of NPC among the Chinese ethnic group still remains unknown when they immigrated to Southeast Asia and North American countries but conversely when they were born in North America (Dickson and Flores, 1985). Wei and Sham suggested that this incidence may be related to genetic, ethnic and environmental factor (Wei and Sham, 2005).

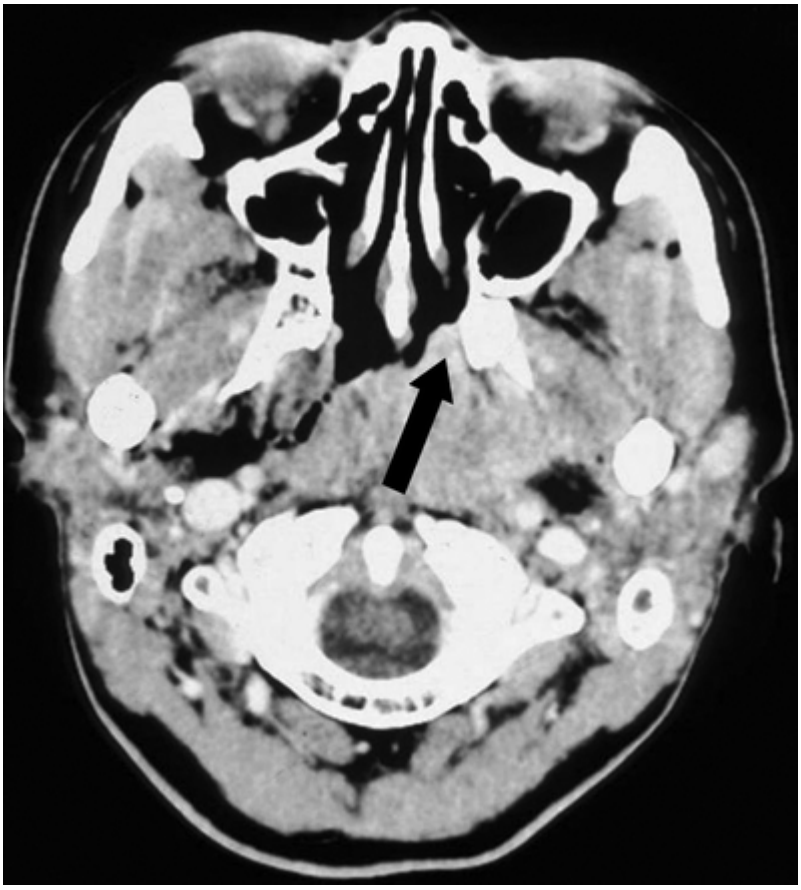

Figure 1.2: CT image of NPC. The arrow shows the nodular tumour abutting the left pterygoid process (Shatzkes *et al.*, 2006).

1.5.1 Clinical features of NPC

NPC patients usually presents with multiple symptoms. However early tumour may remain asymptomatic due to the anatomy of the nasopharynx which has the space that allows air passages (Woo and Van Hasselt, 2008). This may cause difficulty to examine the tumour at an early stage. According to a study in Hong Kong, the commonest clinical presentation of NPC is cervical lymphadenopathy where 76% of NPC patients complaints of neck swelling, followed by nasal symptoms 73%, aural symptoms 62% and cranial nerve palsy in 20% of the patients (Lee *et al.*, 1997). This is similar with a study in Malaysia where the commonest symptoms reported which are neck swelling (57%), followed by nasal bleeding (45%) and headache (41%) (Suzina and Hamzah, 2003).

Patients with NPC present one or more symptoms which depend on the location of the primary tumour, their infiltration of the structures and the metastases to the cervical lymph nodes (Wei and Sham, 2005). At the beginning, a tumour mass can lead to unilateral nasal obstruction and discharge. The symptoms become bilateral and presents with epistaxis when the tumour grows and ulcerates (Wei and Sham, 2005). The Eustachian tube becomes dysfunctional and presents with conductive deafness when the tumour bulk in the nasopharynx extends with or without posterolateral extension into the paranasopharyngeal space (Wei and Sham, 2005).

The patient become the symptomatic when the primary tumour grows superiorly to infiltrate the skull base while diplopia is present when the tumour extends upwardly which affects the cavernous sinus, the lateral wall, the third, fourth and sixth cranial nerves (Wei and Sham, 2005). The patient will complaint of having facial pain and

numbness when the tumour extends to the foramen ovale which affects the fifth cranial nerve (Wei and Sham, 2005). Whilst the commonest complain arise is neck mass formation due to metastasizes of the tumour to the regional cervical lymph nodes (Wei and Sham, 2005).

1.5.2 Incidence of NPC

In Malaysia, NPC was the most common cancer among men (Zainal *et al.*, 2006). The Age Standardized Rate (ASR) of 8.5 and 2.6 per 100,000 populations for males and females, respectively were registered as NPC. The incidence rate among Chinese males was the highest with the ASR of 15.9 per 100,000 populations (Zainal *et al.*, 2006). In Southern China, NPC was the serious health problem with the incidence rate of approximately 25-30 per 100 000 population which was the highest rate worldwide (Lo *et al.*, 2004). While in Taiwan, NPC was estimated to be in the top ten ranking of incidence and cause of death from cancer (Chen *et al.*, 1999).

1.5.3 Histopathology of NPC

NPC is composed of malignant epithelial cells in which their nuclei are round or oval with scanty chromatin and distinct nucleoli (Wei and Sham, 2005). NPC is also known as malignant lymphoepithelioma because the tumour cells frequently infiltrate with lymphoid cells in the nasopharynx (Woo and Van Hasselt, 2008). Besides, there are many term have been used to describe these tumour cells such as undifferentiated carcinoma, transitional cell carcinoma and anaplastic carcinoma (Woo and Van Hasselt, 2008). Therefore, different histological classifications of NPC were used to classify this tumour (McGuire and Lee, 1990).

Since 1978 till now, NPC classification was based on the WHO (Shanmugaratnam, 1978) classification which is divided into three histological subtypes type I, II and III on the basis of the light microscopic appearance (Woo and Van Hasselt, 2008). Type I is either squamous cell carcinoma or keratinizing carcinoma with well differentiated, moderately differentiated and poorly differentiated while type II is nonkeratinizing carcinoma and type III is undifferentiated carcinoma. The WHO (Shanmugaratnam and Sobin, 1991) classification was revised then in the same year into two grades. Grade 1 consist of type I, keratinizing squamous cell carcinoma and grade 2 consist of type II and type III which is nonkeratinizing squamous cell carcinoma and undifferentiated carcinoma (Woo and Van Hasselt, 2008).

WHO type I NPC (**Figure 1.3**) showed the squamous differentiation with intercellular bridges and keratin over most of its extent while WHO type II (**Figure 1.4**) showed the differentiation with a maturation sequence in the cells (Shanmugaratnam *et al.*, 1979). There was no squamous differentiation, mucin production and granular differentiation shown in type II NPC (Shanmugaratnam *et al.*, 1979). Shanmugaratnam *et. al.*, also described that type II NPC cells consist of fairly well defined cells and show an arrangement that is stratified or pavemented and not syncytial (Shanmugaratnam *et al.*, 1979). Shanmugaratnam et. al. also described the histology of Type III NPC (**Figure 1.5**) as oval or round vesicular nuclei and prominent nucleoli which were arranged in irregular and moderately well defined masses. Moreover, it was formed in strands of loosely connected cells in lymphoid stroma (Shanmugaratnam *et al.*, 1979). Contrary to type II tumour, type III cell margins are indistinct, shows mucin production and the tumour shows a syncytial and not pavemented appearance (Shanmugaratnam *et al.*, 1979). It also

shows the spindle-shaped tumour cells with some of them showing hyperchromatin nuclei (Shanmugaratnam *et al.*, 1979).

Previous study showed that NPC grade 2 are higher than grade 1 in high incidence area such as Taiwan and Southeast Asia which was about 90% of all cases (Chen *et al.*, 1999, Jayasurya *et al.*, 2000). While NPC grade 1 was more common in low incidence area such as North America (Nicholls, 1997). Grade 2 is more related to EBV virus which can be detected within the tumour cells and in the peripheral circulation of patients (Woo and Van Hasselt, 2008). Contrary to grade 1 tumour which s not related to the EBV virus, they are less aggressive than grade 2 tumours and less sensitive to radiotherapy treatment (Woo and Van Hasselt, 2008).

1.5.4 Staging of NPC

Many stages of NPC have been designed for better correlation with prognosis. The staging is based on the combined assessment of patient's tumour in nasopharynx (T), regional lymph nodes (N) and presence of any distant metastases (M) (Woo and Van Hasselt, 2008). The American Joint Committee on Cancer/ Union Internationale Contre le Cancer (AJCC/ UICC) system 1997 was preferred as the latest system should be used replacing the Ho's staging system (Ho, 1978) even though Ho's system is more effective in stratifying patients evenly into different prognostic group (Woo and Van Hasselt, 2008). Moreover, the new staging system allows the disease to be staged more precisely according to the extensiveness of the stage that separate patient into cohorts of more equal size and shown to predict survival (Cooper *et al.*, 1998, Chiu *et al.*, 2003). The differential systems between AJCC/UICC stage and Ho stage is that the nodal classification of Ho stage indicates better prognostic

significance (Wei and Sham, 2005). The AJCC/UICC stage is shown in **Table 1.1**. The staging procedure is decided by the physician based on physical examination, endoscopy of the upper airway and radiological imaging by CT and/or MRI. These are different from other head and neck cancer which need evaluation under general anaesthesia (Woo and Van Hasselt, 2008).

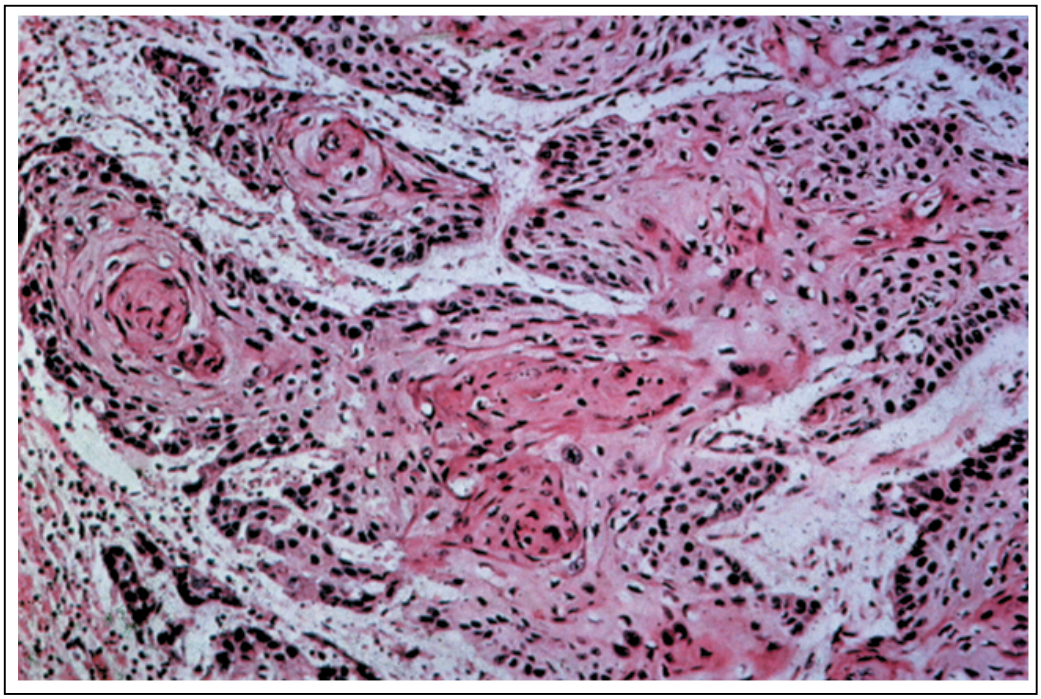

Figure 1.3: Schematic diagram of keratinizing squamous cell carcinoma of nasopharyngeal carcinoma, WHO classification type I. This type is characterized by the presence of keratinization and intercellular bridge (Woo and Van Hasselt, 2008).

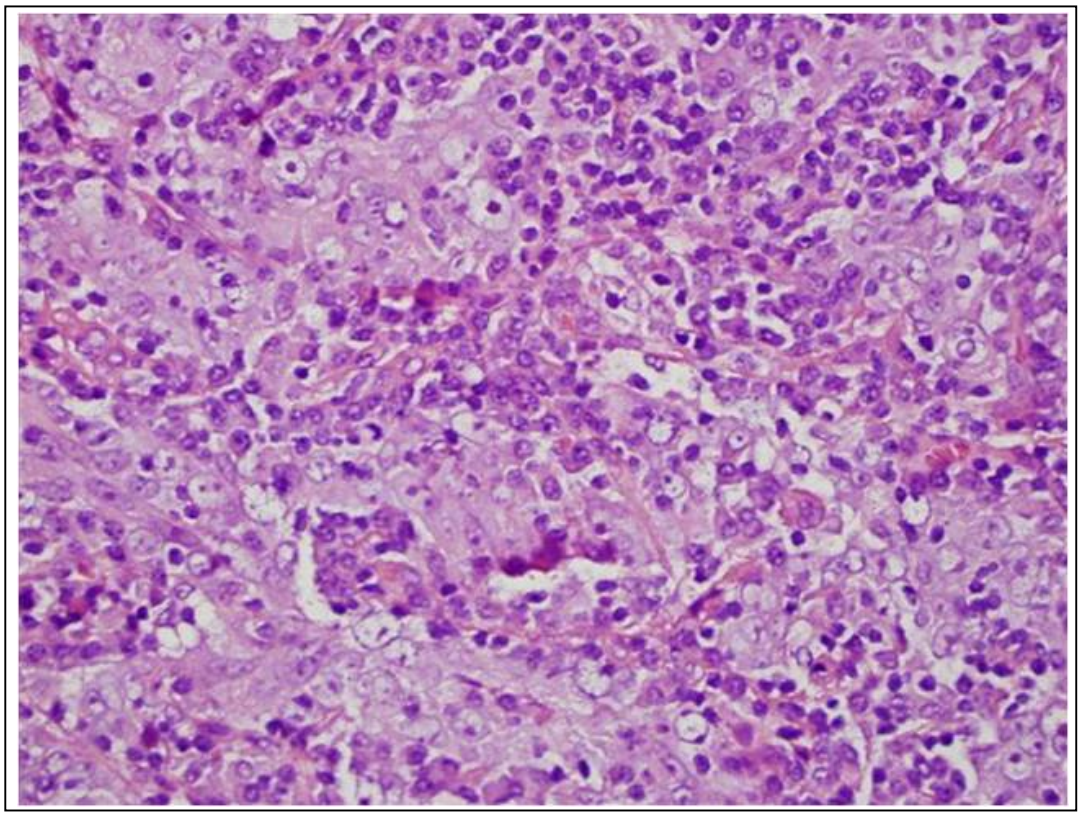

Figure 1.4: Schematic diagram of non-keratinizing carcinoma of NPC tissue, WHO classification type II. This type II is characterized by the presence of interconnecting cord of neoplastic cells and the absence of keratinisation (http://www.orthopaedia.com/display/PORT/Nasopharyngeal+Carcinoma)

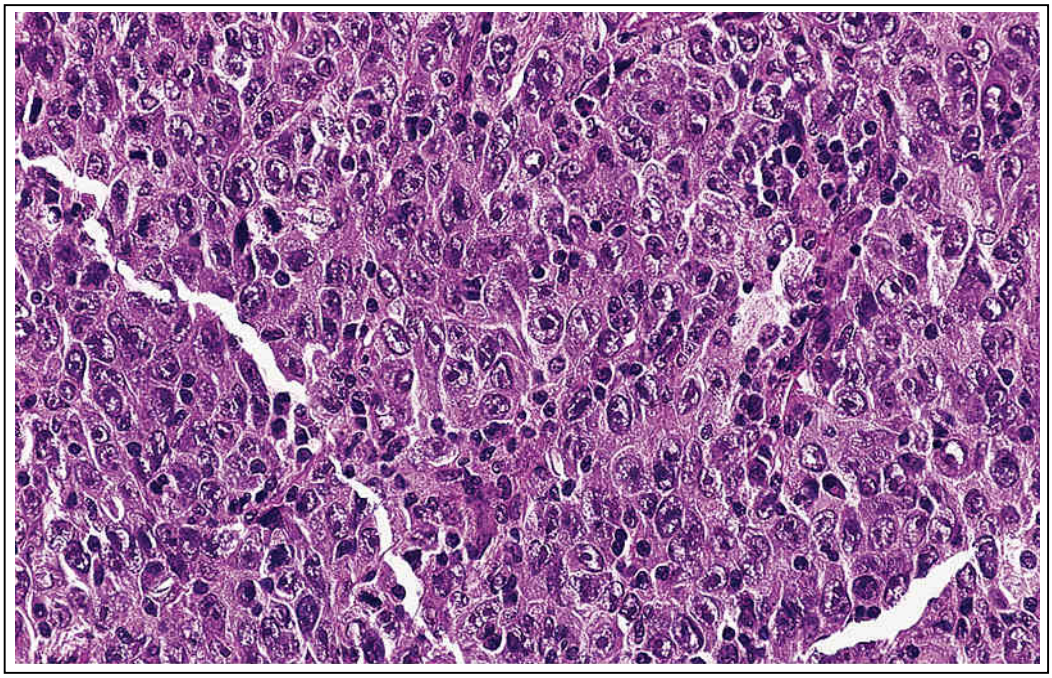

Figure 1.5: Schematic diagram of undifferentiated carcinoma of nasopharyngeal carcinoma tissue, WHO classification type III. This type III is characterized by the cohesive growth of neoplastic cells, enlarged nuclei with vesicular chromatin, poorly defined cell margins and prominent nuleolei (http://www.pathconsultddx.com/pathCon/diagnosis).

CLASSIFICATION			
PRIMARY TUMOUR (T)			
TX	PRIMARY TUMOUR CANNOT BE ASSESSED		
T0	NO EVIDENCE OF PRIMARY TUMOUR		
TIS	CARCINOMA *IN SITU*		
T1	TUMOUR CONFINED TO THE NASOPHARYNX		
T2	TUMOUR EXTENDS TO SOFT TISSUES		
T2A	TUMOUR EXTENDS TO THE OROPHARYNX AND/OR NASAL CAVITY WITHOUT PARAPHARYNGEAL EXTENSION[A]		
T2B	ANY TUMOUR WITH PARAPHARYNGEAL EXTENSION		
T3	TUMOUR INVOLVES BONY STRUCTURES AND/OR PARANASAL SINUSES		
T4	TUMOUR WITH INTRACRANIAL EXTENSION AND/OR INVOLVEMENT OF CRANIAL NERVES, INFRATEMPORAL FOSSA, HYPOPHARYNX, ORBIT OR MASTICATOR SPACE		
REGIONAL LYMPH NODE (N)			
NX	REGIONAL LYMPH NODES CANNOT BE ASSESSED		
N0	NO REGIONAL LYMPH NODE METASTASIS		
N1	UNILATERAL[B] METASTASIS IN LYMPH NODE(S), 6 CM OR LESS IN THE GREATEST DIMENSION, ABOVE THE SUPRACLAVICULAR FOSSA[B]		
N2	BILATERAL[B] METASTASIS IN LYMPH NODE(S), 6 CM OR LESS IN THE GREATEST DIMENSION, ABOVE THE SUPRACLAVICULAR FOSSA		
N3	METASTASIS IN LYMPH NODE(S),>6 CM AND/OR INVOLVE THE SUPRACLAVICULAR FOSSA		
N3A	GREATER THAN 6 CM IN DIMENSION		
N3B	EXTENSION TO THE SUPRACLAVICULAR FOSSA		
METASTASIS (M)			
MX	DISTANT METASTASIS CANNOT BE ASSESSED		
M0	NO DISTANT METASTASIS		
M1	DISTANT METASTASIS		
STAGE GROUPING			
STAGE 0	TIS	N0	M0
STAGE I	T1	N0	M0
STAGE IIA	T2A	N0	M0
STAGE IIB	T1-2A	N1	M0
	T2B	N0-1	M0
STAGEIII	T1-2B	N2	M0
	T3	N0-2	M0
STAGE IVA	T4	N0-2	M0
STAGE IVB	ANY T	N3	M0
STAGE IVC	ANY T	ANY N	M1

APARAPHARYNGEAL EXTENSION DENOTES POSTEROLATERAL INFILTRATION OF TUMOUR BEYOND THE PHARYNGOBASILAR FASCIA.

BSUPRACLAVICULAR FOSSA IS A TRIANGULAR AREA ORIGINALLY DESCRIBED BY HO. IT IS DEFINED BY THREE POINTS: (1) THE SUPERIOR MARGIN OF THE STERNAL END OF THE CLAVICLE; (2) THE SUPERIOR MARGIN OF THE LATERAL END OF THE CLAVICLE; (3) THE POINT WHERE THE NECK MEETS THE SHOULDER. UNILATERAL OR BILATERAL LYMPH NODES RATHER THAN IPSILATERAL OR CONTRALATERAL LYMPH NODES ARE NOTED, AS THE NASOPHARYNX IS CONSIDERED A MIDLINE STRUCTURE.

Table 1.1: Table shows the AJCC stage classification for NPC (Woo and Van Hasselt, 2008)

1.5.5 Aetiology and risk factor of NPC

The development of NPC is contributed by several factors such as genetic factors, Epstein - Barr virus (EBV) infection and exposure to environmental carcinogens. The historical finding of EBV genome was first discovered by Laing in 1969 (Laing, 1969) and nowadays researchers have discovered that the EBV genome is frequently detected in the biopsy of NPC suggesting the EBV infection plays a pivotal role in tumorigenesis (zur Hausen *et al.*, 1970). NPC appears to be related to the viral infection as patients develop an antibody response to the viral caspid antigen (VCA), the early antigen (EA) and the nuclear antigen (EBNA) (Watkinson *et al.*, 2000). Chien *et. al.* found that two specific serologic markers of EBV infection such as IgA antibodies and anti-EBV DNase antibodies can appear long before the development of NPC which allows for early detection of NPC in high-risk population (Chien *et al.*, 2001b).

Previous studies on NPC have shown that NPC tumorigenesis is closely related to environmental factor such as consumption of smoked and preserved foods, exposure to soot and dust and occupational exposure to formaldehyde and various herbal oils containing Epstein-Barr Virus (EBV) activating compounds (Yu and Yuan, 2002, Chen *et al.*, 1999, Feng *et al.*, 2007). In Hong Kong and Southern China, NPC was common among fisher folk whose diet is high in salted fish, preserved food which lacks fresh vegetables (Watkinson *et al.*, 2000), fruit and deficient of vitamin C such as dried fish, salted duck eggs, salted mustard green and fermented soya bean paste (Watkinson *et al.*, 2000). Salted fish contains volatile nitrosamines (Watkinson *et al.*, 2000, Woo and Van Hasselt, 2008) which are alkylating agents and known to induce malignant nasal tumors (Yu *et al.*, 1986). Continuous consumption of salted fish

among emigrant Chinese showed the high incidence of NPC cases (Woo and Van Hasselt, 2008).

Genetic factor plays an important role for developing NPC. Yu *et. al.* described that the incidence of developing NPC in the first degree relatives of patients is six times higher than in normal individuals (Yu *et al.*, 1990). Two areas of genetic that have been examined in the study of NPC include human leucocyte antigen (HLA) typing and chromosomal level which is related to oncogenes and tumour suppressor genes. In this study, we were interested to study the genetic changes at the chromosomal level. Previous study in other countries have reported consistent chromosomal abnormalities such as deletion in chromosomes 3 and 9 and gain in chromosomes 1 and 12 (Chien *et al.*, 2001a, Hui *et al.*, 1999) observed in NPC biopsies suggesting the NPC tumour suppressor gene and oncogenes occur at these gene loci (Woo and Van Hasselt, 2008).

1.6 Identification of Genetic Alterations

Many techniques can be utilized to identify genetic alterations such as Comparative Genomic Hybridization (CGH), Fluorescence *In Situ* Hybridization (FISH), Single Nucleotide Polymorphism array (SNP array), allele-specific Polymerase Chain Reactions (allele-specific PCR), Loss of Heterozygosity (LOH) analysis and sequencing (Weiss *et al.*, 1999). Compared with other techniques, CGH is a technique that provides a global overview of chromosomal gain and losses throughout the whole genome from a tumour sample (Weiss *et al.*, 1999).

1.7 Comparative Genomic Hybridization (CGH) Technique

Comparative Genomic Hybridization (CGH) is a molecular cytogenetic technique that allows comprehensive analysis throughout the entire tumour genome for regions of DNA sequences to detect gain and losses without the need of cell culture (Kallioniemi *et al.*, 1992). The technique provides the important benefit of application for basic research and clinical practice especially in the area of tumour genetics. This is because DNA copy number aberrations are an important pathogen in cancer research (Tachdjian *et al.*, 2000) where it can detect amplified genes and locate their location on normal chromosomes (Joos *et al.*, 1995). Moreover, in clinical genetics, CGH can diagnose unbalanced chromosomal rearrangement (Levy and Hirschhorn, 2002) . CGH was first reported in 1992 by Kallioniemi *et. al.* as a new chromosomal analysis technique. This is followed by another report by Manoir et al in 1993 (Weiss *et al.*, 1999, Kallioniemi *et al.*, 1992, du Manoir *et al.*, 1993).

According to Weiss *et. al.*, from 1992 to 1999, approximately 300 articles on CGH that been published around the world including reviews and technical papers (**Figure 1.6**) (Weiss *et al.*, 1999). The CGH method become famous among researchers around the world because the many reasons: modified technique in CGH which does not need cell culturing, the delay in diagnosis of conventional cytogenetics (Zudaire, 2002), poor quality of metaphases obtained from solid tumour compared to obtained from blood and bone marrow (James, 1999), reduced demands on the material used for CGH which made it possible to use archival formalin fixed and paraffin embedded materials, smaller lesion, (Weiss *et al.*, 1999) and solid tumours (Zudaire, 2002).

1.7.1 Principles of CGH

CGH is a modified method from *in situ* hybridization (Kallioniemi *et al.*, 1994). DNA which is used in CGH method, is independent of tumour cell growth, will give invaluable information on genetic alteration in many types of solid tumours. According to Kallioniemi *et. al.*, who is the developer for this technique, approximately equal amounts of labelled DNA was used. Green fluorochrome is labelled to the test DNA and red fluorochrome is labelled to the normal DNA. There were then cut into small fragments before hybridized to normal metaphase spread with the presence of Human Cot-1 DNA to block the repetitive sequences. The metaphase chromosomes are first fixed conventionally to the slide which is used as a targeted DNA (Tachdjian *et al.*, 2000). Both green and red DNA fragments will compete during hybridization to their locus of origin on the chromosomes on the metaphase slide (Kallioniemi *et al.*, 1994). After hybridization for 2 to 3 days, the images of the metaphases were captured by fluorescence microscope and analyzed using CGH software (Kallioniemi *et al.*, 1994). The green to red ratio represents the aberration in the chromosome where underrepresentations (ratio< 1) indicates loss in the chromosomes while over-representation (ratio > 1) indicates gain in the chromosomes (**Figure 1.7**) (Weiss *et al.*, 1999). According to Lucille Voullaire and Leeanda Wilton the basic assumption in CGH is that the hybridization kinetics of the test and reference DNA are independent. Therefore, the ratio of binding of the DNA is proportional to the ratio of the copy numbers of the sequences in the DNA samples at a specific locus (Voullaire and Wilton, 2007). The relative amounts of test and reference DNA hybridizing to the target DNA are estimated by measuring the green and red fluorescence intensities at each point on the target metaphase chromosomes using fluorescence microscopy and digital image analysis through green and red

filters, respectively (Tachdjian *et al.*, 2000).The regions of the copy number change are determined by the relative fluorescence intensities of the test DNA to the references DNA hybridized to metaphase chromosomes (Voullaire and Wilton, 2007). Comparison of the test fluorescence signals and the references fluorescence signals allow the detection of chromosomal gains and losses (Zudaire, 2002).

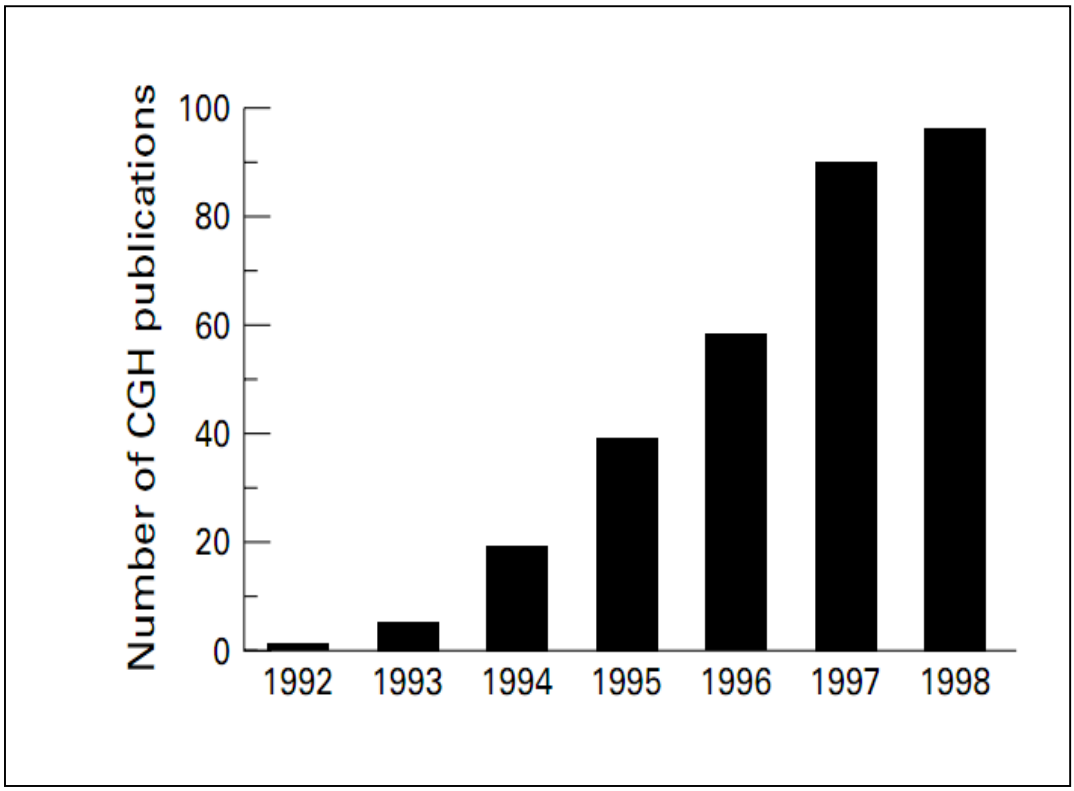

Figure 1.6: Graph shows the number of publications using CGH from 1992 to 1998 (Weiss *et al.*, 1999).

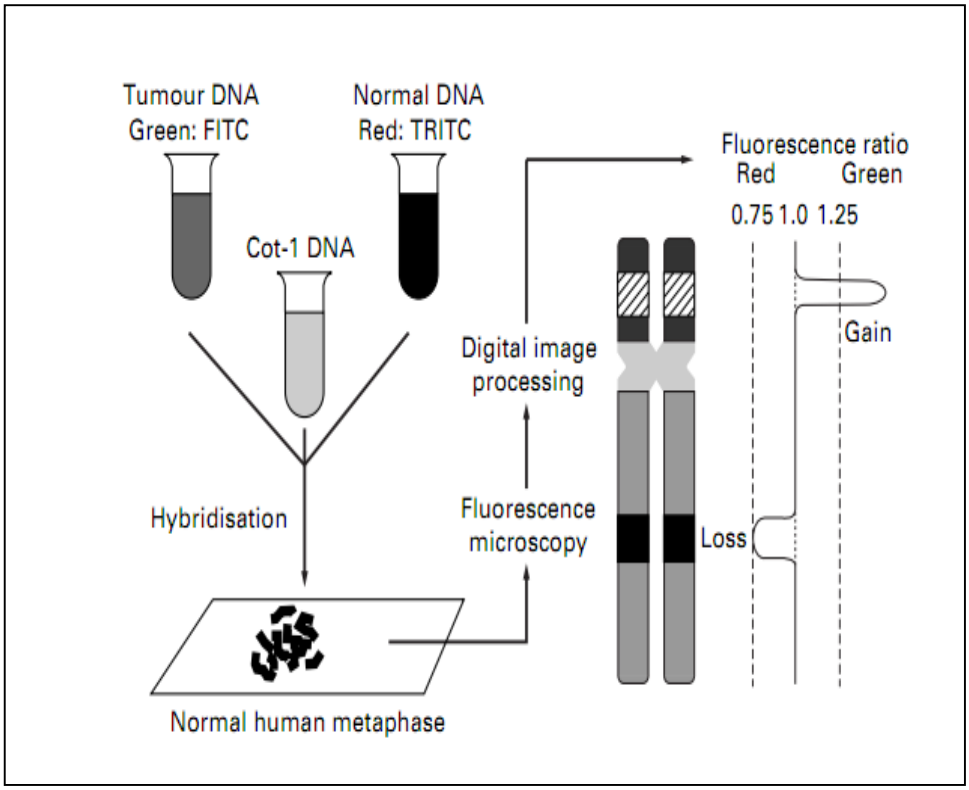

Figure 1.7: Schematic diagram shows an overview of principle of CGH (Weiss *et al.*, 1999).

1.7.2 Application of CGH in cancer research

CGH is a useful technique that is beneficial in cancer research. There are many application of CGH including screening of tumours for genetic aberrations (Chien *et al.*, 2001a, Fang *et al.*, 2001, Chen *et al.*, 1999, Hui *et al.*, 1999, Yan *et al.*, 2001), searching for genes involved in the carcinogenesis of cancers (Simon *et al.*, 1998) and analyzing tumours in experimental models to obtain an insight into tumour progression (Tienari *et al.*, 1998). Moreover CGH can also be applied in diagnostic classification (Simon *et al.*, 1998) and prognostic assessment (Isola *et al.*, 1995). CGH has also been used to study chromosomal alterations in neonatal genomes (Daniely *et al.*, 1998, Yu *et al.*, 1997, Wang *et al.*, 1995).

1.7.3 CGH findings in NPC

There are many studies of NPC using CGH technique around the world especially in Asia such as China and Taiwan (Hui *et al.*, 1999, Chien *et al.*, 2001a, Chen *et al.*, 1999, Fang *et al.*, 2001, Yan *et al.*, 2001) . Research has shown many chromosomal alterations gains and losses in NPC patients in their respective countries (**Table 1.2**). Since many studies showed evidence of chromosomal loss at chromosome arm 3p21 and chromosomal gain at chromosome arm 12p12, therefore, this study will focus on chromosome arm 3p21 and 12p12.