CHAPTER II

Background

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is unique among epithelial malignancies because of its epidemiologic characteristics (Chin et al.,1992). It occurs infrequently in Europeans and North American Caucasians; but the incidence of NPC in Chinese living in South China, Hong Kong, Taiwan, and Singapore is about 50-100 times higher than that in Caucasians living in the European and American continents(Chin et al.,1992).The difficulty in early diagnosis and failure to achieve complete cure are the possible major contributing factors. More understanding about the cause, etiologic factor and newer technology have contibuted to better diagnosis and management but the complete cure is still insufficient.

1. Classification of nasopharyngeal carcinoma

1.1 Histopathological type : According to the World Health Organization (WHO), the NPC has been classified into three types : squamous cell carcinoma (SCC, WHO type 1), nonkeratinizing carcinoma (NKC, WHO type 2), and undifferentiated carcinoma (UC,WHO type 3). The classification is relied entirely on the basis of light microscopic appearances of the tumour cells.

1.1.1 SCCs are tumours in which evidence of keratinization is unequivocally present as evidenced by the presence of intercellular bridges and the production of keratin, either intracellular or extracellular. SCC of nasopharyngeal origin may be graded as well, moderately, or poorly differentiated cancers. A desmoplastic reaction to invading tumour cell is common, and the intense infiltrating lymphocytes, so conspicuous in the other histological types of NPC, are often lacking in SCC. (Rajadurai et al., 1995)

1.1.2 NKC and UC are distinguished from SCC by the failure to demonstrate evidence of squamous differentiation on light microscopy. Tumour cells in NKC generally exhibit a pavemented or stratified arrangement, and individual tumour cells are separated by clearly defined cell margins. UC, cell borders are indistinct and tumour masses appear as syncytia sheet-like masses. A characteristic and striking is vesicularity of the nuclei and the presence of prominent nucleoli in UC cells. Additionally,in NKC and UC, a substantial lymphoid infiltrate is frequently present. (Rajadurai et al.,1995)

2. Epidemiology & Incidence

The age-adjusted incidence rate of NPC is less than 1/100,000 persons per year in American Caucasian which is contrasted to the people in the developing countries especially in Southern China, Hong Kong, Thailand, Singapore, Malaysia, and the Philippines (Rajadurai et al.,1995). NPC may represent 25% of all cancers in southern China, the age-standardised annual incidence rate is approximately 30-80/100,000 persons per year (Abderrahim and

Esteban,1995) for male and 13/100,000 persons per year for female (Muir et al., 1987). An increased incidence has been reported from the Caribbean nations, Saudi Arabia and North Africa,which showed an intermediate incidence 8-12/100,000 persons per year (Abderrahim and Esteban,1995). From annual report of cancer at Chulalongkorn hospital in 1993, NPC was ranked fourth among all cancers in male (Figure 2) and ranked seventh in the annual report of National Cancer Institute (1993), of the Ministry of Public Health Thailand (Figure 3).

3. Etiology

Epsteins-Barr virus infection, genetic, environmental and dietary factors have all been implicated as etiological agents (Zheng et al.,1994;Abderrahim and Esteban,1995).

3.1 Epstein-Barr virus

The Epstein-Barr virus is a member of human herpes virus family, with over 90% carrier state in adult population worldwide (Freeman et al.,1994). The association of Epstein-Barr virus (EBV) with serveral lymphoid malignancies such as Burkitt's lymphoma (BL) and Hodgkin's disease (HD) is well established (Zur Hausen et al.,1970; Herbst et al.,1990). EBV is belived to be closely associated with nasopharyngeal carcinoma due to the regularly occurance of EBV genome in tumour cells and specific anti-EBV antibody profiles presented in patients with NPC compared to normal control population (Zheng et al.,1994; Rajadurai et al.,1994). The analysis of EBV latent protein expression in NPC has

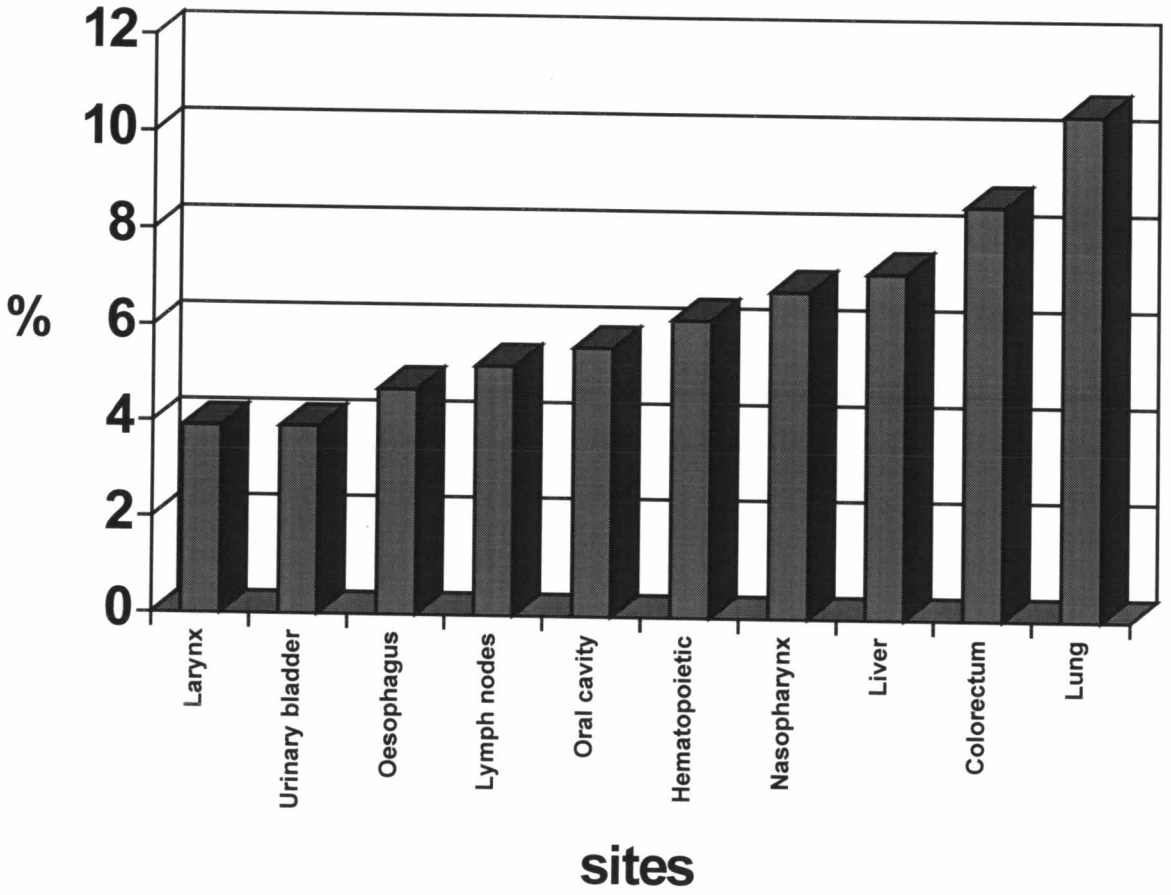

Figure 2 : Leading cancers in male at Chulalongkorn hospital 1993)

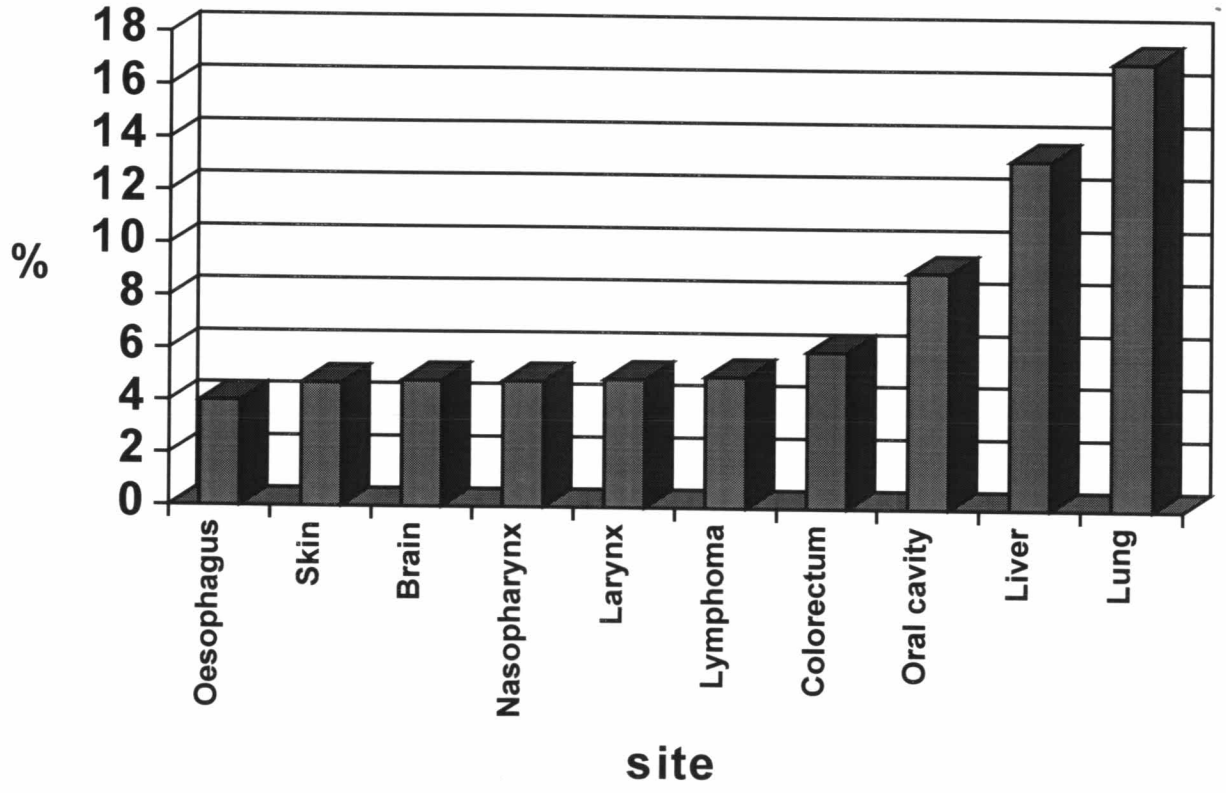

Figure 3 : The 10 leading site of cancer in male in Bangkok (1993)

been studied, allowing the identification of nuclear protein (Epstein-Barr virus nuclear antigen [EBNA], membrane proteins (Latent membrane protein [LMP]), and small nuclear EBV-encoded RNAs (EBER-1, EBER-2) (Abderrahim and Esteban, 1995; Rajadurai et al.,1995). Analysis of the subtypes by using anti-complement immunofluorescence for the detection of the EBV-encoded nuclear antigen complex (EBNAs) suggested an association of the virus with undifferentiated NPC but not with squamous cell NPC (Niedobitex et al.,1993). The expression of EBNA 2 was related to histologic differentiation of the disease. All cases of SCC (WHO type 1) failed to show LMP or EBNA 2 and about half of differentiated nonkeratinizing carcinomas were positive, whereas all undifferentiated NPCs had positive reactions with both LMP and EBNA 2. This results has been confirmed by recent studies using DNA-DNA *in situ* hybridization for the demonstration of the viral genome (Weiss et al.,1989; Niedobitex et al.,1993). In 1993, Hording and collegue examined the presence of the EBV genome by polymerase chain reaction in tumour samples from 37 NPC patients. There was EBV DNA presented in all nonkeratinizing NPC tumours but only two of the 14 keratinizing tumours. In 1995, Rajadurai and collegue analyzed EBV infection and gene expression, using various techniques for the detection of the EBV genome in tumour cell. The EBV-encoded RNAs (EBER) were readily detected by EBER *in situ* hybridization (EBER-ISH) in most tumour cells of all three WHO types of NPC, while the latent membrane protein (LMP-1) expression was detected by immunohistochemical staining in 72% (36/50) of the nonkeratinizing carcinoma and undifferentiated samples (WHO type 2 and 3) and using polymerase chain reaction amplification of EBNA-1,EBNA-2 in all samples, indicating that all forms of NPC express the same EBV genes. The expression of

specific EBV genes suggest that EBV infection in nasopharyngeal epithelium may be a prerequisite for the development of NPC.

3.2 Genetic factors

Genetic factors have also been implicated in the etiology of NPC, a disease that occurs relatively more often in the Chinese population which affected by undifferentiated carcinoma of the nasopharyngeal type (UNCT) in multiethnic prevalence areas suggested a genetic predisposition for this disease (Zheng et al.,1994). Several studies, use several genetic systems to investigate of UNCT patients in China and other parts of Asia. Early studies reported HLA (Human leukocyte antigen) allele association with NPC in Chinese people (Simons et al.,1974). These included three alleles at the HLA-B locus (BW 46 and B17/BW58), the HLA-A locus (A2) and HLA-BS-in-2. Subsequently, HLA haplotypes cotaining both A2 and BW46 were shown to confer an approximately two fold increased risk (Levine et al.,1992). The HLA types AW19, BW46, and B17 are also reported to be associated with an increase risk,whereas HLA-A11 is associated with a decreased risk (Lu et al.,1990). Interestingly, HLA-A2 has been recently reported to be linked with lower incidence in whites, more markedly when the HLA-A2 locus was homozygote (Burt et al.,1994). These studies showed evidence of a possible linkage between the HLA and NPC susceptibility.

3.3 Environment and dietary factors

To identify the factors which may be linked to the development of NPC, several studies on the lifestyle and food habits in Cantonese Chinese were

conducted (Yu et al.,1990;Zheng et al.,1994). After a comparative analysis the conclusion was that traditional preserved food preparations could represent the common factors(Herbert et al.,1993). Several case-control studies in Malaysia, Hong Kong and the Guangxi province of China, stated that the consumption of salted fish was found to have a significant association with the increased risk of NPC (Armstrong et al., 1983; Yu et al., 1986, 1988; Zheng et al., 1994). Many studies reported on the detection of nitrosamines in salted fish, which were strongly suspected to play a role in the development of NPC. The consumption of herbal tea, and the early exposure to domestic wood fires were significantly associated with high risk of UCNT, whereas the consumption of leafly vegetables was associated with a reduce risk (Zheng et al., 1994). Some studies reported an increased risk of NPC in adulthood with the frequent consumption of salted soybeans, canned salted or pickled vegetables, and salted mustard greens (Lee et al., 1994). In contrast, the role of tobacco and alcohol consumption are not related with NPC type2 and 3 but the role of tobacco and alcohol are related with NPC type1. Chow et al., 1993 reported a positive correlation between cigarette smoking and the development of NPC among white US veterans.

Molecular study

Cancer is essentially a genetic disease at the cellular level. Studying the mechanism of carcinogenesis is developed rapidly, owing to the discovery of genes and gene products involved in the regulation of cell proliferation and differentiation and the identification of genetic events implicated in tumour formation (Varmus, 1984). Many of these genes that involved in proliferation and

differentiation have been classified as proto-oncogenes and tumour suppressor genes. The development of tumours involves the loss or inactivation of multiple tumour suppressor genes and/or the activation of proto-oncogenes and the development of tumours is a multistep process (Fearon and Vogelstein; 1990).

1. Carcinogenesis is a multistep process

Carcinogenesis is a multistep process driven by carcinogen-induced genetic and epigenetic damage in susceptible cells that gain a selective growth advantage and undergo clonal expansion as the result of activation of proto-oncogenes and/or inactivation of tumour suppressor genes (Harris, 1992) (Figure 4). The first stage of the carcinogenesis process, tumour initiation, involves exposure of normal cells to chemical, physical or microbial carcinogens that cause a genetic change (s), both providing the initiated cells with an altered responsiveness to their microenvironment and exerting normal cells (Weinstein et al, 1991). The initiated cells may have decreased responsiveness to the intercellular signals that maintain normal tissue growth and regulate the homeostatic growth and mutation of cells (Weinstein et al, 1991; Harris, 1992). Tumour progression results in greater proliferation and survival of the initiated cells than the normal cells and enhances the probability of additional genetic damage, including endogenous mutations that accumalate in the expanding population of these cells (Harris,1992). The initiated cell that converted to malignancy can increase substantially by their furture exposure to DNA damaging agents (Henning et al, 1983, 1989). This may further activate proto-oncogenes (Yuspa et al., 1990) and /or inactivate tumour suppressor genes.

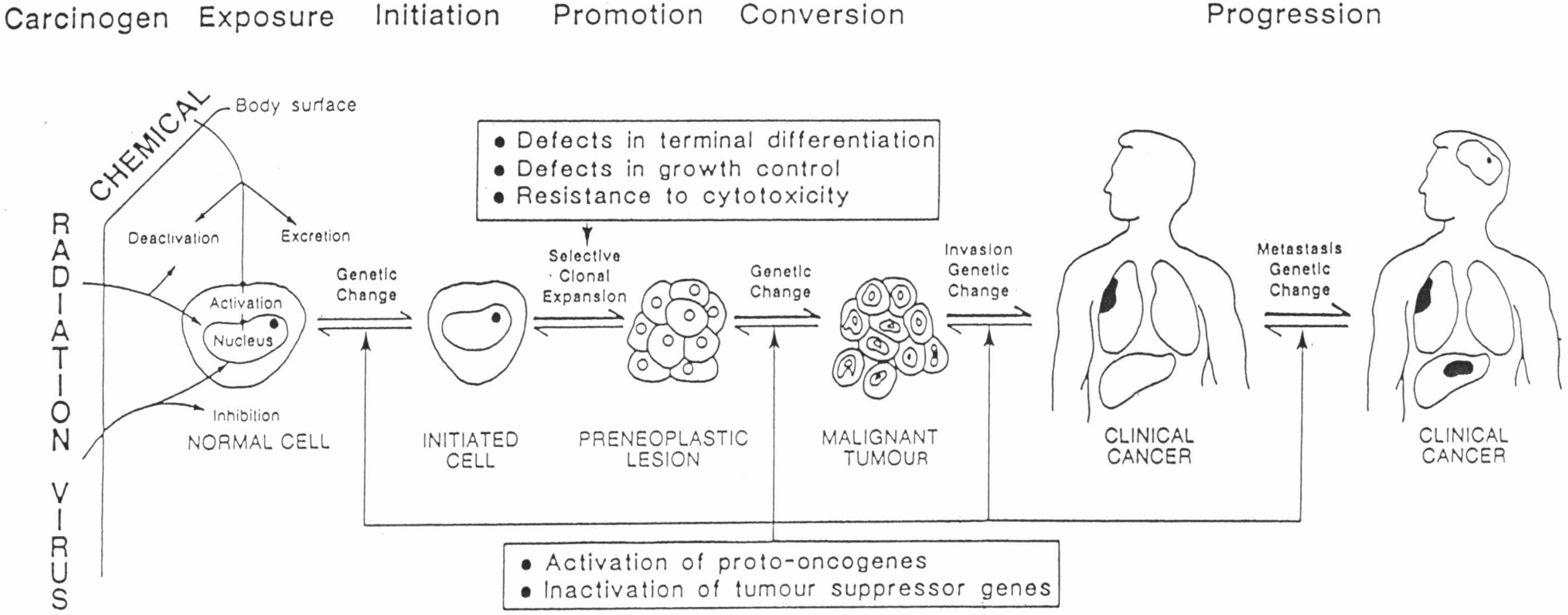

Figure 4 : Carcinogenesis is a multistage process involving multiple genetic and epigenetic events in proto-oncogene, tumour suppressor genes. (Modified from Harris, 1992)

The tumour cells continue to exhibit abnormal number and structure of chromosomes, gene amplification and altered gene expression (Rowly, 1990). In most common human tumours (e.g., of the lung, colon and breast), multiple tumour suppressor genes are frequently affected, indicating that malignant growth is subjected to several levels of negative control (Callahan and Campbell, 1989; Weston et al,1989). An example, the model of multistep pathway in colorectal cancer demonstrated that the accumulation of multiple mutations in several tumour suppressor genes and oncogenes over a lifetime results in cancer (Fearon and Vogelstein, 1990) (Figure 5).

2. Proto-oncogene and tumour suppressor gene

Proto-oncogenes and tumour suppressor genes encode a diverse range of proteins whose physiological role are the maintenance of normal growth. Alterations or mutations in these genes, have been shown to occur in each tumour. These changes affect in cell growth proliferation, differentiations (Fearon and Vogelstein, 1990). Table1 showing the two classes of genes, proto-oncogenes and tumour suppressor genes, are involved in the evolution of most, if not all cancers (Barrett, 1992).

2.1 Proto-Oncogenes

Proto-oncogenes are normal cellular genes that, play an essential physiological role in normal cell growth, normal cellular proliferation and differentiation (Bishop,1987). When Proto-oncogenes were inappropriately activated as "oncogene", they caused dysregulation of growth and differentiation

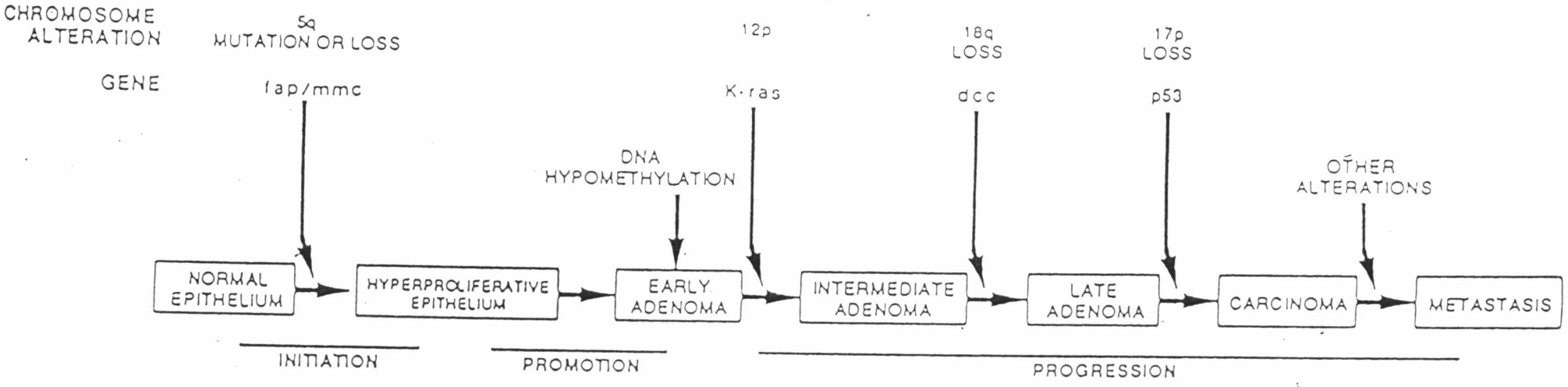

Figure 5 : Multistep model of colorectal carcinogenesis. (Modified from Harris, 1992)

Table 1 : Two classses of genes involved in carcinogenesis

Proto-oncogenes	Tumour-suppressor genes
-Involved in cellular growth and differentiation	- Involved in cellular growth and differentiation (negative regulator of cell growth)
-Family of genes exists	- Family of genes exists
-Activated (quantitatively or qualitatively) in cancers	-Inactivated or lost in cancers
-Activation of only one allele required to contribute to cancer. Activation by point mutation,Chromomal translocation or gene amplification.	-Inactivation of both alleles : The first allele inactivated by point mutation or deletion, whereas the second allele inactivated by chromosomal loss, nondisjunction, somatic recombination or gene conversion.
-Dominant in cellular level	-Recessive in cellular level

Modified from Harris, 1992

pathways and enhanced the probability of neoplastic transformation (Harris,1992). The sites and modes of action of many proto-oncogenes have been defined (Table 2), which included growth factors, growth factor receptors, signal transducers and nuclear binding proteins

2.1.1. Growth factors

Growth factors are molecules which act via cell surface receptors to induce cellular division. Some growth factors will lead to transformation of normal cells in vitro. Alternatively, the process of transformation itself can produce growth factors (Tronick and Aaronson, 1995).

2.1.2 Growth factor receptors

Several proto-oncogenes can produce proteins from components of growth factor receptors at the cell surface. Binding of growth factors to their respective membrane receptors is the first step in the delivery of mitogenic signals to the initiate cell division. An example of this is the *fms* proto-oncogene which forms the CSF-1 (Colony stimulating factor) receptor in differentiating macrophages (Tronick and Aaronson, 1995).

2.1.3. Signal transduction

In the normal stage, an extracellular growth factor has bound to its cell surface receptor, where it is able to transfer (transduce) its signal to the nucleus. The process of action may involve with a second messengers, an example of this is the ras family:H-*ras*, K-*ras*, N-*ras*. Its function is in modulation

of cellular proliferation via the transduction of signal from the cell surface to the nucleus (Tronick and Aaronson, 1995)

2.1.4. Nuclear binding protein

Nuclear binding proteins are molecules which are produced from nucleus. The functions of these genes are transcription factors, that regulate other genes action or function (Hollywood and Barton, 1994).

2.2 Tumour suppressor gene

In contrast to proto-oncogenes, tumour suppressor genes are normal cellular genes that play negative regulatory roles in cell proliferation, differentiation, and other cellular and systemic processes. It is their loss or inactivation that is oncogenic (Sager, 1989; Weinberg, 1991). Oncogenes are identified by their positive role in the transformation of appropriate host cells (Sager,1989). Tumour suppressors, on the other hand, have an essentially negative effect, blocking transformation and driving cells toward normality (Sager,1989). Evidence of cancer pathogenesis has come from three observations: cell hybrids, inherited cancer syndromes and loss of heterozygosity in tumours.

2.2.1. Somatic cell hybrids

The murine cell hybrids formed by fusion between normal and tumour cells were found to revert to the normal phenotype (Harris,1988). These experiments showed that the normal cells were donating genetic information capable of suppressing the neoplastic phenotype of their tumour cell partners

Table 2 : Some proto-oncogenes and their modes of action.

Proto-oncogenes are normal genes involved in cellular proliferation and differentiation.

Proto-oncogenes	**example**	**function**
Growth factors	*sis*	B chain of PDGF
	int-2, hst	Fibroblast growth factor related
Growth factor receptors	*erb*B	Epidermal growth factor receptor
	fms	CSF-1 receptor
Signal tranducer	H, K, and N-*ras*	GTP-binding/GTPase
	abl,src,fes,	Protein tyrosine kinases
	mos,raf	Prptein serine kinases
Nuclear trancription factors	*myc,* N-*myc*	DNA- binding protein
	myb	DNA -binding protein
	jun, fos	DNA-binding protein
	*erb*A	DNA-binding protein (thyroid hormone receptor)

Modified from H. Earl Ruley, 1993; J.L. Bos and C.F. van Kreijl, 1992.

(Weinberg,1991). When the chromosomes from the normal parent were lost, the hybrid cells would often revert back to a tumorigenic state (Weinberg, 1991). Correlation of this reversion to tumourigenicity with the loss of specific normal chromosomes led to the conclusion that these chromosomes carried genes that were missing from the genomes of tumour cells and could act to normalize the growth program of the cancer cells (Weinberg,1991).

2.2.2 Inherited cancer syndromes

The second clue that suggested the existance of tumour suppressor genes was came from the inherited cancer syndromes study. Since 1970, Knudson had proposed the two-hit model of tumourigenesis to demonstrate the two mutational events that involved in the development of 2 forms of retinoblastoma cancer: In sporadic retinoblastoma, seen in children without a family history of the disease, he suggested that both lesions have been sustained in the retinal cell lineage as somatic mutations occurred long after conception. For familial retinoblastoma, he proposed that one of the two mutations has been acquired from a genetically afflicted parent or originates during gametogenesis; the second required mutation then occured as a somatic event (Knudson,1971). Until 1983, Benedict and collegue were proven Knudson's hypothesis and suggested that the two mutational events proposed involved the inactivation of both functional copies of the 13q14-associated RB gene. The demonstrated loss of RB gene function caused this gene to ally with the "tumour suppressor genes" (Sager,1989). Moreover, it became clear that single inactive RB allele acts within cells throughout the body in a recessive manner, as children who are effectivly hemizygous for RB undergo essentially normal

development. Only the rare cell that loses its remaining wild-type allele shows evidence of growth deregulation (Weinberg,1991).

2.2.3. Loss of heterozygosity

A third clue for the discovering of tumour suppressor gene was suggested by the genetic mechanisms used by evolving tumour cells to eliminate both copies of genes like RB (Sager, 1989; Weinberg, 1991; Mulligan and Mole, 1993). The first copy of a tumour suppressor gene is inactivated by a somatic (or a germ line) mutation. The chromosome region carrying the remaining normal allele may then be eliminated by machanisms like base-substitution mutations, deletions, chromosome nondisjunction, mitotic recombination and gene conversions (Figure 6) (Cavenee et al., 1983; Sager, 1989; Weinberg, 1991). Most tumours that lack functional copies of a suppressor gene (like RB) display two identically mutated alleles. These steps that lead to homozygosity of a mutant suppressor allele usually involve the flanking chromosomal regions as well. DNA markers mapping to nearby tumour suppressor allele, which may have shown heterozygosity prior to tumour progression, will suffer a parallel reduction to homozygosity or loss of heterozygosity (LOH).

In order to recognize these events, DNA samples from tumour and normal tissue from the same individual are compared using DNA markers closely linked to the disease gene. If one of these mechanisms of somatic loss has occured, the alleles associated with the missing "normal" gene copy will be present in the normal tissue and absent in the tumour DNA samples (Mulligan

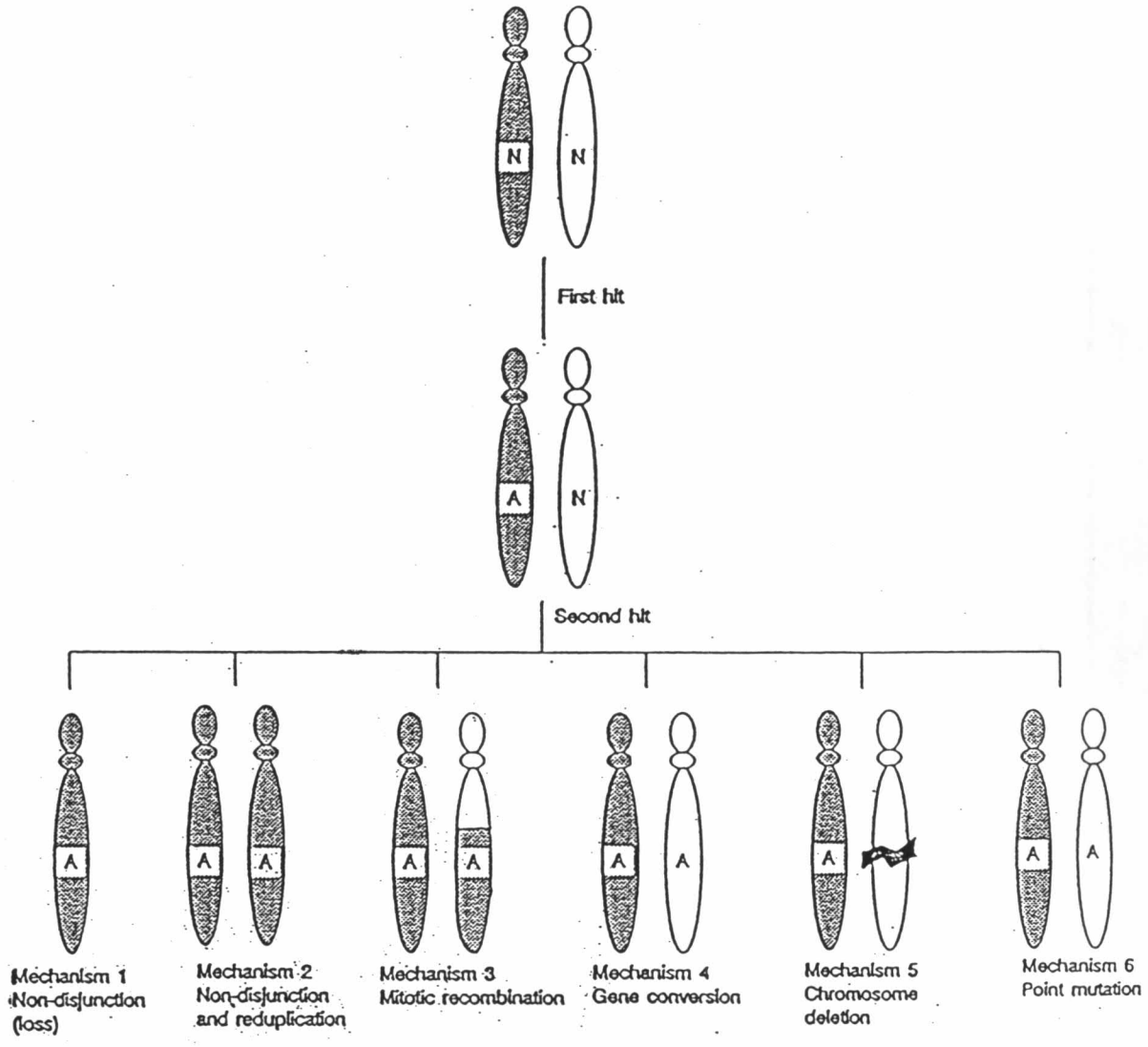

Figure 6 : Knudson ' s two-hit hypothesis : a schematic representation of the development of loss of heterozygosity. The first allele of tumour suppressor gene is inactivated by point mutation (or germ line). The second allele carrying the remaining normal is inactivated by mechanisms involving larger regions, including nondisjunction, and subsequent loss of the normal chromosome, or mitotic recombination, gene conversion, chromosome deletion. These latter events are frequently recognized as a somatic loss of constitutional heterozygosity (LOH) in tumour.

(Modified from Apiwat mutirangura)

and Mole, 1993). Examples of candidate tumour suppressor genes involved in human carcinogenesis are shown in Table 3-4.

3. Dection of Loss of heterozygosity or Allelic loss (LOH)

The analysis of LOH pattern in tumour specimens is based on detecting the differences between normal maternal alleles and tumour DNA. If a chromosome locus has been lost in a tumour one of the two normal alleles will be absent in the tumour DNA when compared with the constitutional DNA. A common standard method used to detect LOH is restriction fragment length polymorphism (RFLP), an original method using polymorphisms in DNA as a linkage markers (Botstein et al., 1980). The variations (polymorphism) of DNA sequence can be detected by using restriction enzymes which cut DNA at specific recognition sites. The disadvantage of this analysis in detecting LOH are due to several reasons : (1) RFLP is depended on the extraction of large amounts of high molecular weight DNA ; (2) This technique is a time-consuming process ; (3) Many of the RFLP probes are limited by decreased degrees of polymorphism ; (4) RFLP studies of tumour can be hampered by the presence of normal tissue, which may obscure the alleles loss. More recently, short tandem repeat polymorphic markers (STRPs) or microsatellites, a type of newly described DNA markers, have been used as an alternative for LOH analysis (Louis et al.,1992 ; Huang et al.,1993). The STRP is a short tandem repeat DNA sequence distribute throughout the human genome and is hypervariable with heterozygosities of 60% to 90% (Weber and May, 1989; Litt and Luty, 1989;

Table 3 : Cloned familial cancer genes

Entity	Gene symbol	Chromosome location
Retinoblastoma	RB1	13q14
Wilms'tumour	WT1	11p13
Li-Fraumeni syndrome	TP53	17p13
Familial adenomatous polyposis	APC	5q21
Neurofibromatosis type1	NF1	17q11
Neurofibromatosis type2	NF2	22q12
Von Hippel-Lindau syndrome	VHL	3p25
Multiple endocrine neoplasia type 2A	MEN2A	10q11
Familial breast cancer	BRCA1,BRCA2	17q21,13q12-q13

Table 4 : Uncloned but mapped familial cancer genes

Entity	Gene symbol	Chromosomal location
Multiple endocrine neoplasis type 1	MEN1	11q13
Familial melanoma	MLM	9p21
Neuroblastoma	NB	1p36
Basal cell nevus syndrome	BCNS	9q31
Beckwith-Wiedemann syndrome	BWS	11p15
Renal cell carcinoma	RCC	3p14
Tuberrous sclerosis 1	TSC1	9q34
Tuberous sclerosis 2	TSC2	16p13

from Knudson, 1993

Weber, 1990). STRPs typically occur in 6-30 units with those longer repeat, (12 units of dinucleotides) to be polymorphic (Weber, 1990). The polymorphism at these loci is the results of the variation in length of the number of repeat blocks (Hearne et al.,1992; Weber, 1990). The allele fragments can be generated by the polymerase chain reaction (PCR) and assayed by using specific primer sequences closely flanking the repeat-containing units (Litt and Luty, 1989). Additionally, this PCR based typing of STRP is more efficient and requires smaller amounts of genomic DNA than the standard RFLP analysis. The STRP markers can be analysed simultaneously by using multiplex PCR methods (Huang et al., 1993). The PCR-generated DNA fragment are further resolved by polyacrylamide gel electrophoresis (Litt and Luty, 1989; Weber and May, 1989) (Figure 7-8).

Because of their hypervariability and abundance, STRPs are ideal markers for LOH analysis. Several studies have used STRP for detecting the LOH in several carcinoma and have shown that LOH at the specific chromosome sites is frequently associated with development of various cancers (Marshall, 1991 ; Goddard and Solomon, 1993) (Table 5).

The allelic loss studies which involved every arm of every chromosome are called *"allelotyping study"*. By analysing the pattern of allelic loss with STRP, it is possible to define regions of loss that are common to multiple tumours of the same type. The localization of allelic loss to specific chromosomal regions has led to the identification of tumour suppressor genes important in the tumourigenesis.

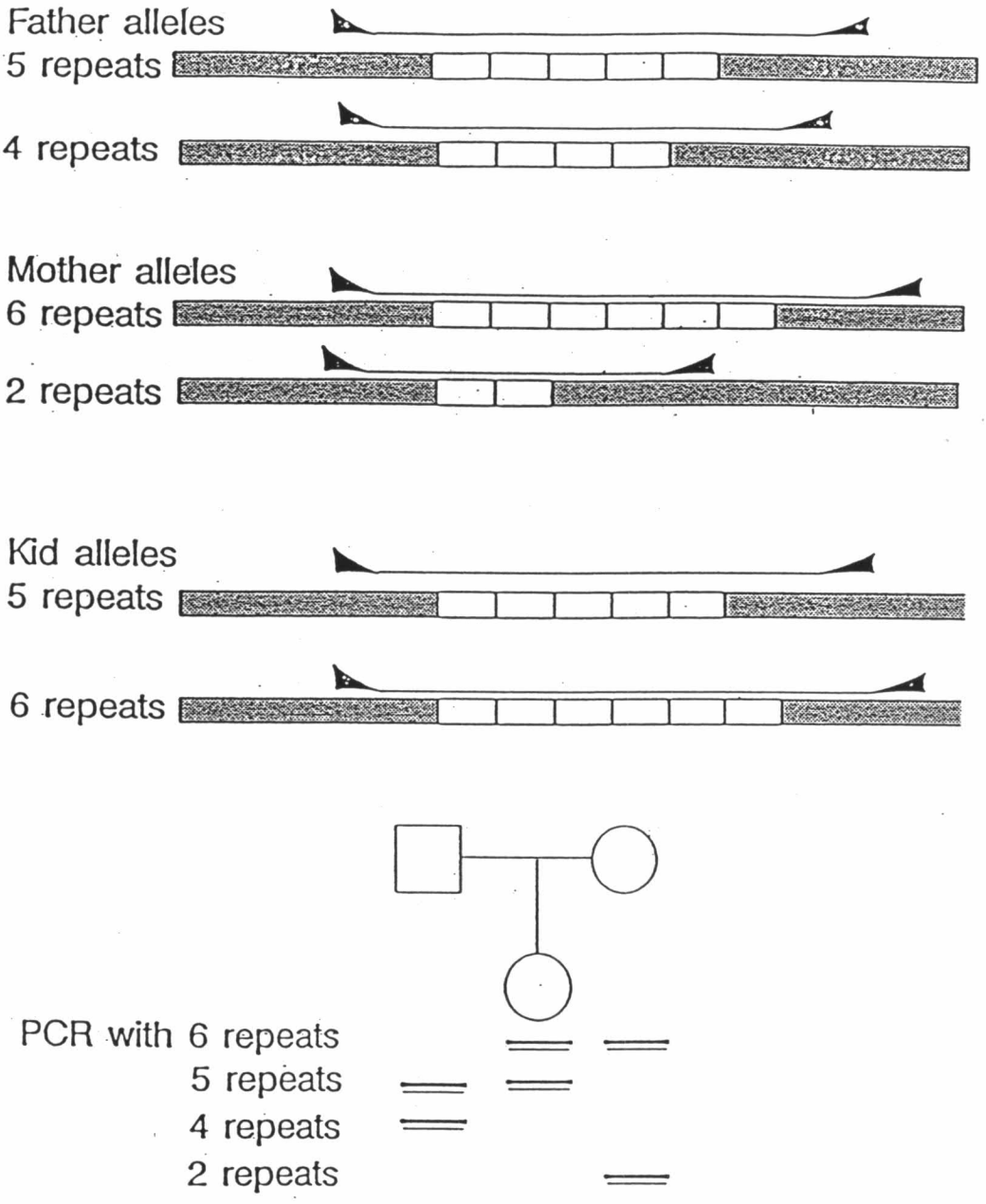

Figure 7 : The polymorphisms in length of STRP allele can be generated by the Polymerase Chain Reaction (PCR) assay and resolved by polyacryl - amide gel electrophoresis (Modified from Apiwat Mutirangura).

○ = female □ = male □□□ = STRP repeat block unit

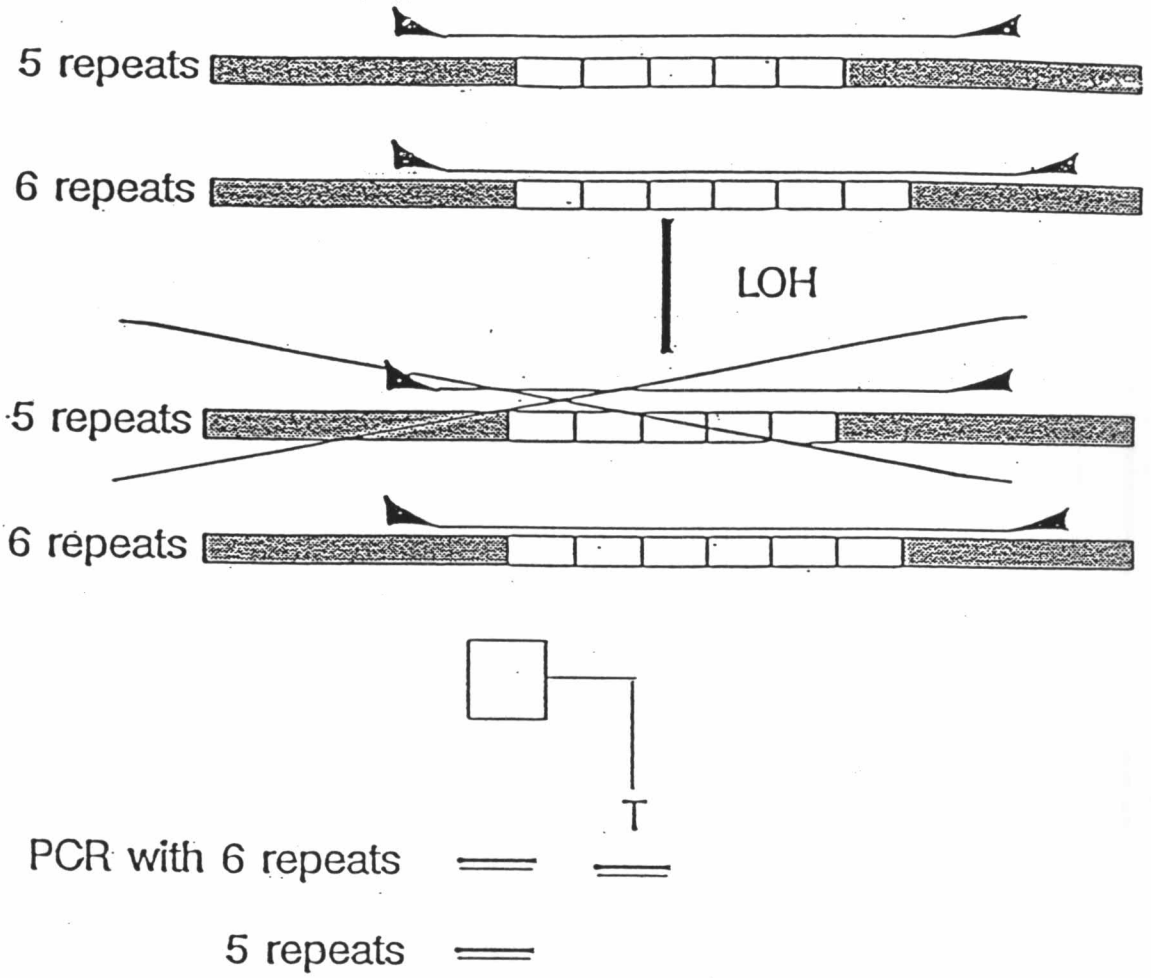

Figure 8 : The analysis of LOH pattern in tumour specimen by using STRP closely linked to the tumour suppressor gene. If a chromosome locus has been lost in a tumour one of the two normal alleles will be absent in the tumour DNA when compared with the constitutional DNA. N, DNA from normal tissue; T, DNA from tumour tissue (Modified from Apiwat Mutirangura).

□ = male ▭▭▭ = STRP repeat block unit

Table 5 : Loss of heterozygosity in some human tumours.

A second somatic event will result in loss of the remaining normal gene activity

Tumour	Implicated chromosome(s)
Breast carcinoma	1p, 1q, 3p, 11p, 13p, 17p, 17q, 18q
Lung carcinoma	3p, 13q, 17p
Colorectal carcinoma	5q, 17p, 18q, 22q
Cervical carcinoma	3p, 4q, 5p, 5q, 11p, 18p
Multiple endocrine neoplasia type 1, 2	11q,1p respectively
Astrocytoma	10p, 10q, 17p
Bladder carcinoma	9p, 9q, 11p, 17p
Renal carcinoma	3p
Head and Neck carcinoma	3p, 5q, 9p, 11q, 17p
Endometrial carcinoma	10q, 17p, 18q
Retinoblastoma	13q
Osteosarcoma	13q, 17p
Neuroblastoma	1p, 14q
Wilms'tumour	11p13, 11p15.5
Melanoma	1p, 9p, 17p
Ovarian carcinoma	3p, 6p, 6q, 11p, 11q, 13q, 17p, 17q, Xp
Hepatocellular carcinoma	11p, 13q

Modified from Arnold J. Levine,1993