CHAPTER IV

Method

1. Sampling of specimens

The specimens used in this study were consisted of both fresh tumour tissues and peripheral blood leukocytes obtained from twenty three previously histologically confirmed nasopharyngeal carcinoma patients, whom had been diagnosed at Chulalongkorn hospital during 1994-1995.

1.1 Tumour specimens: Tumours were obtained by either surgically resection or fine-needle aspiration at palpable nodes. A sample was taken for histopathological assessment. Tumours were graded according to WHO classification and staged according to the tumour-nodes-metastasis classification by American Joint Committee on Cancer-1988. The general information on cancer staging and grading are shown in Appendix A. The tumour specimens were evaluated histologically by cryostat sectioning to select tissue that contained > 70% of tumour cells and the remainder of the tissue were frozen in liquid nitrogen for DNA extraction.

1.2 Peripheral blood samples : 5-10 ml of peripheral blood sample was collected from each patient in an EDTA coated tube, the DNA extracted from this blood was used as self normal control.

2. DNA extraction

2.1 Tumour tissue : Tissue sized approximately 0.5x0.5 cm, was rapidly frozen and crushed to produce readily digestible pieces, then followed by:

2.1.1 Resuspended the tissue in 1 vol digestion buffer (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS, 0.1 mg/ml Proteinase K).

2.1.2 The sample was incubated with shaking at 50°C overnight in capped tubes. After incubation the tissue should be almost indiscernible.

2.1.3 The sample was extracted with equal volume of Phenol-Chloroform-Isoamyl alcohol.

2.1.4 Centrifuge 10 min at 1700xg in a microcentrifuge. If the phases do not separate well, add another volume of digestion buffer, omitting proteinase K, and repeat the centrifugation. If there is a thick layer of white material at the interface between the organic phase and inorganic phase, repeat the organic extraction.

2.1.5 Tranfer the aqueous (top) layer to a new tube, add 1/2 vol.of 7.5 M CH_3COONH_4 and 2 vol of 100% ethanol and gently mix. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 1700 xg for 2 min.

2.1.6 Rinse the pellet with 70% ethanol. Decant the ethanol and air dried the pellet. (It is important to rinse well to remove any residual salt and phenol.)

2.1.7 Resuspend the DNA in double distilled water at 37 °C until dissolved. The DNA samples can be shaken gently at room temperature or at 65°C for several hours to facilitate solubilization.

2.1.8 To calculate the DNA concentration, measure the density of the sample at 260 nm and 280 nm. If OD ratio of A_{260} to A_{280} is >1.6, the DNA sample is pure enough for further study.

2.1.9 Store the DNA samples at 4°C until used. The DNA samples should be kept at -70°C for longer period.

2.2 Peripheral blood leukocyte : The extraction of DNA from peripheral blood leukocyte was performed as followed :

2.2.1 5-10 ml of whole blood is freezed and quick thawed at 37°C several times until dissolved. Add an equal volume of $T_{10}E_{10}$(10mM Tris pH 7.0 and 10mM EDTA pH 8.0).

2.2.2 Vortex vigorously for 10 sec. and centrifuge for 10 mins at 1,000-2,000 rpm.

2.2.3 Resuspend the pellet in $T_{10}E_{10}$mix then spin down. Repeat step 2.2.1-2 until the pellet appears white.

2.2.4 Resuspend the pellet in 3 ml of $T_{20}E_5$ (20mM Tris and 5mM EDTA), 0.2 ml of 10% ultrapure SDS, and 0.5 ml Protienase K solution (2 mg proteinase K in 1.0 ml of 1% SDS-2mM EDTA, should be prepared 30 min before use.) Mix vigorously for 15 sec.

2.2.5 Incubate the tube(s) in 55°C shaking waterbath overnight for complete digestion.

2.2.6 Add 1 ml saturated 6M NaCl, shake vigorously for 15 sec. and centrifuge at 2,500-3,000 rpm for 15 min.

2.2.7 Tranfer the supernatant from each tube (contains DNA) to a new tube. (You should have an obvious protien pellet before you pour off supernatant. If you don't get a protien pellet add another ml of NaCl solution and shake-spin again.)

2.2.8 Add 2 volumes of 95% EtOH (room temperature) to each DNA tube.

2.2.9 Agitate tubes to precipitate DNA.(Not vigorously vortex). You should be able to see the DNA.

2.2.10 Spin tubes at 3,000 rpm for 3-4 min to pellet DNA, rinse the DNA twice with cold (-20°C) 70% EtOH.

2.2.11 Air dry the pellet briefly or use Speed-vac evaporator.

2.2.12 Resuspend the dry DNA pellet in approximately 100 μl distilled water.

2.2.13 Incubate the DNA samples at 37°C overnight to completely dissolve.

2.2.14 If the DNA does not dissolve, repeat the extraction by adding equal volume of Phenol-Chloroform-Isoamyl alcohol.

2.2.15 Mix and centrifuge for 5 mins at 2,000-3,000 rpm.

2.2.16 Transfer the supernatant to a new tube. Add 1/10 volumes of 3M sodium acetate (NaOAc) and 2 volumes of 95% EtOH

2.2.17 Recover the DNA by centrifugation at 2,000-3,000 rpm for 3 min.

2.2.18 Repeat step 10-13.

2.2.19 Measure the optical density of sample at 260 nm and 280 nm.

2.2.20 Calculate 260/280 ratio to show deproteination. The 260/280 ratio should be grater than 1.6.

3. Calculation of DNA concentration

The reading at 260 nm is used for calculating the concentration of nucleic acid of the samples. An OD of 1 corresponds to approximately 50 μg /ml for double-straned DNA.Therefore DNA concentration is calculated from the following:

DNA concentration = OD x 50 x dilution ratio (μg /ml)

4. PCR analysis

DNA from tumour and peripheral leukocyte blood was analyzed for LOH after PCR amplification at the short tandem repeat polymorphic markers (STRPs). There are 89 STRPs markers used in this study. These STRPs are distribute through all 39 chromosomal arms as shown in Table 6.

4.1 End-labeling primer

One strand of the forward of each primer pair was end-labeled for 1-2 hrs at 37°C in a volume of 10 μl containing 10 μM primer, 0.025 mCi (γ-^{32}P)

ATP at 3,000 Ci/mmol, 10mM MgCl2, 70mM Tris HCl pH 7.6, 5mM DTT and 10 units of T4 polynucleotide kinase. Without further separation of the unincorporated nucleotides, the kinase reaction mixture was directly added to the PCR buffer mix.

4.2 PCR amplification

The PCR reaction was performed in a total volume of 10 μl using 50 ng of genomic DNA in 200 μM each of deoxynucleotidetriphosphates (dNTPs), 10mM tris HCl pH 8.4, 50mM KCl, 1.5 mM $MgCl_2$ (for all reactions; excepted NFI, D20S470 added 2.5 mM and 2.0 mM $MgCl_2$ respectively).Each of the primer pair was performed in optimal concentration ranged in 0.05-0.5 μM (Table 6). The marker set of (D3S1600, D3S966, D9S169), (D11S534, GABRB3, D9S51, D10S169), (GLUT2, D2S102, TCRD), (D13S119, D5S82), (IL2Rβ, D8S88), (D16S287, D4S174, D17S520), (D15S131, D15S123), (D21S258, MFD133, D20S17, D1S103), (D7S517, IGF2R), (D12S341,D19S221), and (D2S131, D10S249) were analysed for LOH by multiplex PCR (more than one locus amplified simultaneously in one reaction tube).The other markers were amplified as single PCR reaction. The list of markers are shown in Table 6. In the mutiplex PCR reaction, the initial denaturation step was 95°C for 4 min then followed by 25 cycles of denaturation at 94 °C for 1 min,annealing at 55°C for 2 min,extension at 72°C for 2 min and a final extension at 72°C for 7 min. For those primers with an asterik symbol(*) the amplification was performed as followed :

* ; initial denaturation step was 95°C for 4 min,then followed by 5 cycles of step down PCR denaturation at 94°C for 1 min,annealing at 60°C, 59°C, 58°C, 57°C, and 56°C for 1 min respectivly,extension at 72°C for 2 min and 25 cycles f denaturation at 94°C for 1 min,annealing at 55°C for 1min,extension at 72°C for 2 min and a final extension at 72°C for 7 min.

** ; initial denaturation was at 95°C for 4 min followed by 28 cycles of denaturation at 94°C for 1 min,annealing at 52°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min.

*** ; initial denaturation was at 95°C for 4 min followed by 25 cycles of denaturation at 95°C for 1 min,annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7min.

4.3 Polyacrylamide Gel Electrophoresis and Autoradiography

Electrophoresis is the standard method used to separate and identify DNA fragments. Polyacrylamide gels are most effiective for separating small fragments of DNA (5-500 bp). Their resolving power is extremely high, and fragments of DNA that differ in size by as little as 1 bp can be separated from one another. It is optimize to separate and identify STRPs. PCR products were analyzed on denatured 6% polyacrylamide / 7M urea sequencing gel in 1xTBE buffer. Final products (3μl) of each reaction were mixed with 1.5 μl formamide-loading buffer and then loaded onto the gel. The DNA fragments were size-fractionated on a 35x43 cm gel for 3 hr at 80 W until the tracking dye (bromphenol-blue) reached the bottom of the gel. After electrophoresis,

the wet gel was wrapped with clean plastic wrap and exposed to Kodak T-mat X-ray film for 12-24 hr at -70°C with and intensifying screens.

5. Analysing the DNA band patterns

LOH was scored by direct visual comparison of allelic intensity between normal and tumour samples. No allele loss was scored, when the intensity of the 2 allelic bands from tumour sample was equal to those from leukocyte. Allele loss was scored when the reduce intensity of one allele was observed. The DNA band patterns was considered to be *"informative"*, when observed heterozygous allele (2 band) in normal DNA sample at a given locus and considered to be *"uninformative"* when observed homozygous (1 band) in normal DNA sample.

Table 6 : List of STRP loci for allelotyping study

Chromosome arm		Loci	Location	Size	Primer conc(μM)
1P	***	D1S243	1p36.1-36.2	142-170	0.3
1Q		D1S103	1q31-q32	82-102	0.1
2P	**	D2S131	2p	229-247	0.4
	*	D2S405	2p	275	0.2
2Q		D2S102	2q33-q37	138-162	0.3
3P	**	D3S1038	3p25VHL	115	0.2
	**	D3S192	3p25VHL	96-118	0.3
	***	D3S1600	3p14	182-198	0.1
	***	D3S966	3p21.3	147	0.3
	***	D3S1480	3p	87-125	0.2
	*	D3S1255	3p25VHL	150	0.3
	*	D3S1217	3p13-14	170	0.3
	*	D3S1481	3p14	120	0.3
	*	D3S1214	3p21.3	170	0.3
3Q		GLUT2	3q26.1-q26.3	184-222	0.05
	***	D3S1744	3q23-q24	170	0.1
4P		D4S174	4p11-p15	175-195	0.5
	***	D4S1599	4q	142-156	0.3
4Q	*	D4S1554	4q11-q35	184-208	0.3
	***	D4S1625	4q	194	0.1
5P	***	D5S392	5p	83-117	0.2
	*	D5S819	5p	281	0.2
5Q		D5S82	5q14-21	169-179	0.3
	***	D5S346	5q21-q22	96-122	0.3
6P	*	D6S309	6p	254-272	0.2
	*	D6S477	6p	238-280	0.4
6Q		IGF2R	6q27	158-166	0.2
	**	D6S503	6q	270	0.1
	*	D6S292	6q	141-161	0.2
7P		D7S517	7p	239-257	0.1
	*	D7S460	7p	234	0.3
7Q	*	D7S486	7q31	133-146	0.2
	*	D7S821	7q	281	0.1
8P	***	NEFL	8p	137-147	0.3
	***	D8S87	8p21.3-p22	170	0.1
	***	D8S110	8p	281	0.1
8Q		D8S88	8q22	76-100	0.1

Table 6 : STRP loci for alleltyping study (continue)

Chromosome arm		Loci	Location	Size	Primer conc(μM)
8Q		MCC	8q24	87-125	0.3
9P	***	D9S169	9p21	259-275	0.1
	*	D9S165	9p	250	0.2
	*	IFNA	9p22	130	0.1
9Q		D9S51	9q	135-159	0.05
	**	D9S290	9q34	148-160	0.2
	***	ABLI	9q34	130	0.1
10P	***	D10S89	10p11.2-pter	140	0.3
	**	D10S249	10p	118-134	0.2
10Q		D10S169	10q11.2-qter	99-117	0.1
	***	D10S677	10q	271	0.1
11P	***	WTI	11p13	150	0.2
	*	D11S554	11p	234	0.2
11Q		D11S534	11q13	228-244	0.2
	***	D11S956	11q13	247-308	0.3
	***	D11S976	11q23	130	0.2
	*	D11S897	11q23	98-120	0.3
	***	INT2	11q13.3	161-177	0.3
12P	*	D12S341	12p	114-130	0.1
	*	D12S62	12p	194	0.4
12Q		MFD133	12q	161-175	0.2
	*	D12S86	12q	124-160	0.2
13Q	*	D13S284	13q13RBI	197-227	0.2
		D13S119	13q	197-213	0.2
14P		TCRD	14q11.2	118-128	0.1
	*	D14S118	14q	230	0.2
15Q		GABRB3	15q11-13	181-201	0.1
	***	D15S131	15q	238-274	0.2
	***	D15S123	15q	191-207	0.2
16P		D16S287	16p13.11	201-225	0.2
	*	D16S748	16p13.2-13.13	230	0.4
16Q	*	D16S511	16q22-24	182-222	0.2
	***	D16S539	16q24.2-q24.3	194	0.1
17P		D17S520	17p12	130-144	0.15
	***	D17S945	17p13	186-208	0.3
	*	D17S1176	17p	95-109	0.3
17Q	*	KRT9	17q21NMEI	182-198	0.2
	***	NFI	17q11.2	234	0.1
18P	***	D18S59	18pter-p11.22	148-164	0.2
18Q		D18S35	18q21.2-q21.3	104-124	0.05

Table 6 : STRP loci for allelotyping study(continue)

Chromosome arm	Loci	Location	Size	Primer conc(μM)
18Q	DCC	18q21.1	106-160	0.3
***	D18S535	18q	150	0.3
19P *	D19S221	19p	191-211	0.1
19Q *	D19S412	19q	89-113	0.3
***	D19S246	19q	270	0.1
20P **	D20S470	20p	280	0.2
***	D20S27	20p12	128-138	0.2
20Q	D20S17	20q12-q13.1	130-140	0.2
21Q	D21S258	21q	184-206	0.2
***	D21S11	21q21	271	0.1
22Q	IL2RB	22q	149-163	0.2
**	D22S446	22q11NF2	260	0.2