Table 1. Clinical data and telomerase activity in oral leukoplakia and HNSCC.

Patient code	age	sex	location	onset (mth)	R	diagnosis	histo	K	telomerase +/-	telomerase dosage
CU1	37	F	buccal and tongue	6	-	erythroplakia	GI	-	+	<100
CU2	74	F	lat border tongue	2	-	leukoplakia	G0	+	-	
CU2R	74	F	lat border tongue	1	+	leukoplakia	G0	+	+	<100
CU6	57	M	lower dental ridge	12	-	leukoplakia	G0/GI	+	+	<100
CU11	45	M	lat molar trigone	8	-	leukoplakia	ND	ND	-	
CU13	40	F	lat border tongue	5	-	leukoplakia	G0/GI	+	-	
CU14	64	M	retro molar trigone	2	-	leukoplakia	G0	-	-	
CU15	70	M	hard palate	48	-	leukoplakia	G0	+	-	
CU16	60	F	buccal mucosa	12	-	leukoplakia	G0	+	-	
CU17	59	F	buccal mucosa	3	-	leukoplakia	G0	+	-	
CU18	40	M	buccal mucosa	0.5	-	leukoplakia	G0	+	-	
CU18R	40	M	buccal mucosa	1	+	leukoplakia	G0	+	-	
CU20	58	M	buccal mucosa	2	+	leukoplakia	G0	+	+	<100
CU21	42	M	buccal mucosa	40	-	leukoplakia	G0	+	-	
CU23	40	F	buccal mucosa	12	-	leukoplakia	G0	+	-	
CU24	70	F	buccal mucosa	72	+	leukoplakia	G0	+	+	>1000
CU25	64	F	buccal mucosa	2	-	leukoplakia	G0	+	+	100-1000
CU26	51	M	lat tongue	1.5	-	leukoplakia	ND	ND	-	
CU34	61	M	lat tongue	2	-	leukoplakia	GII	-	+	>1000
CU37	58	F	buccal mucosa	12	+	leukoplakia	G0	+	-	
CU39	39	F	buccal mucosa	40	-	leukoplakia	G0	+	-	
CU44	46	F	buccal mucosa	1/4	-	leukoplakia	G0	-	-	
CU42	78	M	buccalmucosa	2	-	erythroplakia	G0	+	+	100-1000
CU41	42	F	tongue	6	-	leukoplakia	G0/GI	+	+	<100
RAMA5	73	F	upper first molar	2	-	leukoplakia	G0	+	-	
RAMA13	62	F	lower lip	40	-	erythroplakia	GI	-	+	<100
CU3	63	F	retromolar trigone			verucous CA	V		+	<100
CU5	80	F	lat tongue			verucous CA	V		+	100-1000
CU10	78	M	lat tongue			SCC	G1		+	100-1000
CU22	40	F	lat tongue			SCC	G1		-	
CU9	78	F	buccal mucosa			SCC	G1		+	<100
CU19	55	M	tongue			SCC	G1		+	100-1000
CU29	80	M	floor of mouth			SCC	G1		-	
CU31	60	M	tongue			SCC	G1		+	>1000
CU35	65	F	lower lip			SCC	G1		+	>1000
CU40	62	F	lower gum			SCC	G2		+	<100
CU28	63	M	floor of mouth			SCC	G2		+	100-1000
CU30	58	F	tongue			SCC	G2		+	>1000
CU32	72	M	glottic			SCC	G2		+	<100
CU33	83	M	hard palate			SCC	G2		+	>1000
CU38	81	F	aryepiglottic fold			SCC	G2		+	>1000
CU27	50	M	nasopharynx			NPC	G3		+	<100

R, recurrence from previous laser surgery; K, keratosis; F, female; M, male; +, positive; -, negative; ND, not done; G0, hyperplastic lesion; GI, mild dysplasia; GII, moderate dysplasia; GIII, severe dysplasia; V, verrucous carcinoma; SCC, squamous cell cancer; NPC, nasopharyngeal cancer; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

Table 2. Telomerase activity in HNSCC and oral leukoplakia

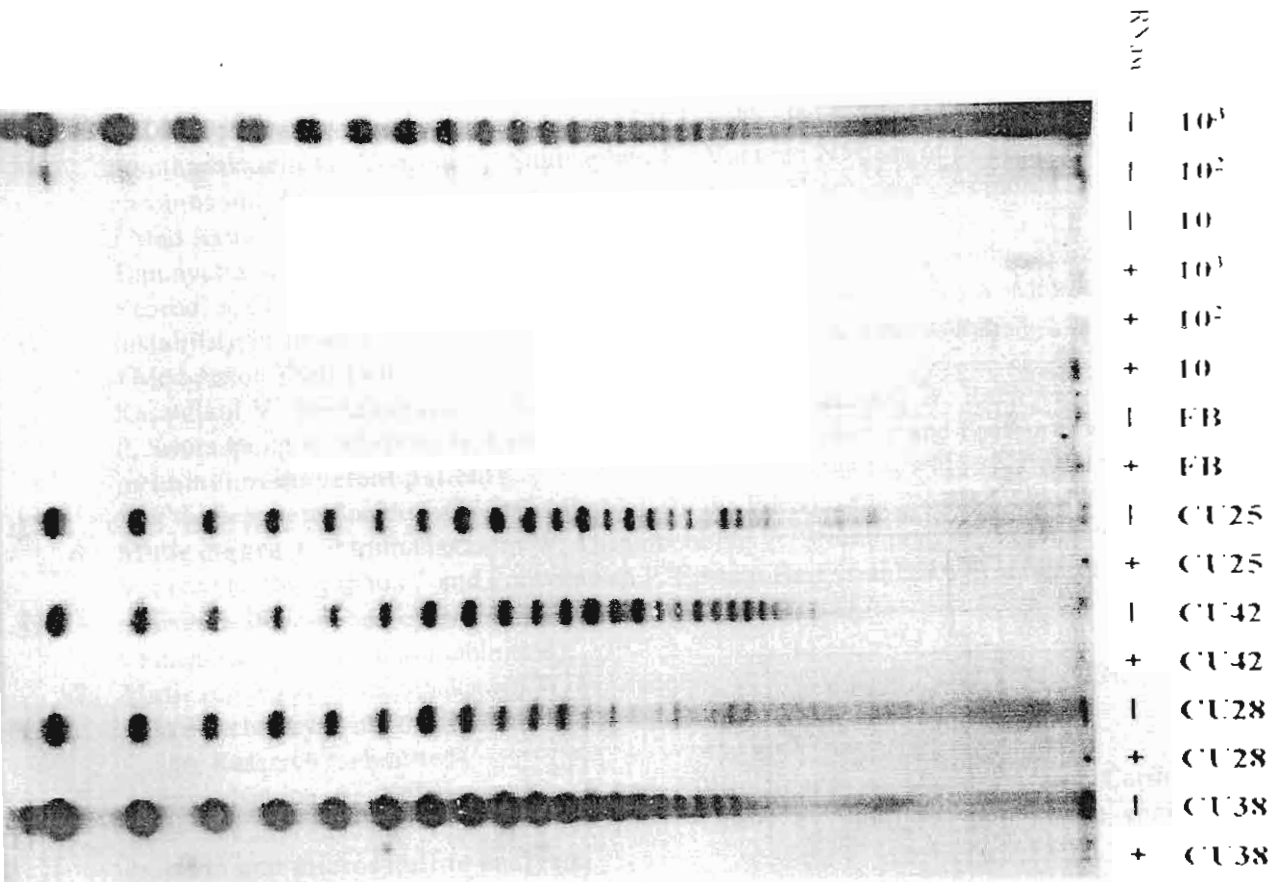
	telomerase activity positive	telomerase activity negative	Total
Cancer			
Verucous carcinoma	2	0	2
Squamous cell cancer	11	2	13
Nasopharyngeal cancer	1	0	1
Total HNSCC	**14**	**2**	**16**
Oral premalignant lesion			
Gross appearrance			
Leukoplakia	7	16	23
Erythroplakia	3	0	3
Total oral leukoplakia and erythroplakia	**10**	**16**	**26**
Histological finding			
G0 without history of recurrence after treatment	2	11	13
G0 with history of recurrence after treatment	3	2	5
Total cases with G0, hyperplasia	**5**	**13**	**18**
G0/GI	2	1	3
GI	2	0	2
GII	1	0	1
Total cases with dysplasia	**5**	**1**	**6**

G0, hyperplasia; G0/GI, mixed between hyperplasia and mild dysplasia; GI, mild dysplasia; GII, moderate dysplasia.

Figure legend

Fig 1. Telomerase activity in leukoplakia and HNSCC. +/-, with/without RNase pretreatment. Extracts of Ebstein-Barr virus immortalized human lymphocyte cell line B958 of 10^3, 10^2 and 10 cells were used as the positive controls. Normal cultured fibroblast, FB, was the negative control. Leukoplakia (CU25 and CU42), and HNSCC (CU28 and CU38) were assayed in aliquot containing 6 μg of protein. All cancer and leukoplakia samples showed detectable telomerase activity.

Figure 1


RNase
- 10³
- 10²
- 10
+ 10³
+ 10²
+ 10
- FB
+ FB
- CU25
+ CU25
- CU42
+ CU42
- CU28
+ CU28
- CU38
+ CU38

Output

วารสารระดับนานาชาติ

1. **Mutirangura A**, Supiyaphun P, Trirekapan S, Sriuranpong V, Sakuntabhai A, Yenrudi S and Voravud N. Telomerase activity in oral leukoplakia and head and neck squamous cell carcinoma.
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* The last two publications were not proposed but TRF was acknowledged.
*Appendix included reprint #1, 2, 3, 4, 8, and 9.
*Reprint #5,6 and 7 will provide when available.

Appendix:

Reprints from output number 1, 2, 3, 4, 8 and 9

of corresponding histopathological or clinical indications to this effect.

The adenoma karyotypes had several similarities to those of carcinomas, as expected for lesions that are accepted as forming a neoplastic continuum. First, all tumours in both groups (adenomas and carcinomas) had clonal chromosome aberrations. Second, structural chromosome rearrangements and more than one abnormality were identified in five out of the six adenomatous polyps examined. In three of the adenomas (case IV/lesion 1, case V/lesion 2 and case VII/lesion 2), the karyotype was as complex as in the carcinoma of the same case. In contrast, only two (case I/lesion 2 and case VI/lesion 5) of the six cytogenetically abnormal hyperplastic or inflammatory polyps showed clones with structural aberrations and only one (case VI/lesion 5) had a clone with multiple changes, all of them numerical.

The recurrent chromosome aberrations detected in the present series, +X, +Y, –Y, –1, del(1)(p36), –4, +5, +7, der(8;17)(q10;q10), +13, –14, –15, –17, –18, +20, –21, and –22, have been previously detected in colorectal tumours (Mitelman, 1994; Heim and Mitelman, 1995). It is of interest, however, that two of these aberrations, der(8;17)(q10;q10) and –14, were found only in carcinomas. This indicates that although most of the chromosomal changes that occur non-randomly during colorectal tumorigenesis may be found already at the benign stage, as has also been suggested previously (Bardi et al, 1997), some could be carcinoma specific. Data from previous studies (Mitelman, 1994) support this interpretation, at least as far as der(8;17)(q10;q10) is concerned. This rearrangement has never been seen in adenomas, whereas whole-arm translocations between the long arms of chromosomes 8 and 17, as well as several other chromosomes are common in colorectal carcinomas. Monosomy 14 has been detected in adenomas (Mitelman, 1994), albeit rarely. As it is so much more common in carcinomas (Bardi et al, 1997), it is tempting to suggest that it is usually acquired during malignant transformation and that it indeed may play a causal role on the process. The finding that loss of heterozygosity on the long arm of chromosome 14 is found only in advanced colorectal carcinomas (Ookawa et al, 1993) seems to be consonant with this view.

Some of the chromosome aberrations that occur non-randomly in both adenomas and carcinomas of the large bowel (this report; Bardi et al, 1997) are found at clearly different rates. Trisomy for chromosomes 7 and 13 is more common in adenomas, whereas the frequency of –17 and –18 has been at least two times higher in carcinomas than in adenomas. In the present study, loss of one chromosome 18 was found in all cases with adenomatous polyps. In case II, monosomy 18 was detected only in the largest of the three adenomas, in agreement with earlier observations (Fearon, 1994) that this change occurs late in adenoma development.

The only carcinoma carrying clones with simple numerical aberrations was the one of case VI. We have previously suggested that colorectal adenocarcinomas with simple numerical aberrations arise through pathogentic mechanisms different from those operative in karyotypically complex carcinomas (Bardi et al, 1995). The carcinoma of case VI was located in the rectosigmoid region, as are most tumours with simple numerical changes (Bardi et al, 1995). The patient also had a flat adenoma, a type of tumour that has not previously been cytogenetically characterized but which might constitute a precursor lesion for some infiltrating carcinomas (Wolber and Qwen, 1991). It is obviously too early on the basis of the aberrations detected in this tumour (–Y, +20 and –22 in different clones) to draw any conclusions about the general karyotypic profile of these colorectal lesions.

With the exception of case III, cytogenetically related clones were detected in the carcinoma and in at least one benign lesion from the same patient. This is evidence that these macroscopically distinct tumours arose as part of the same neoplastic process, in spite of the fact that the distance between them was at least 3 cm. The only alternative explanation would be that the same oncogenetic enviromental factor induced identical chromosomal rearrangements in more than one cell. In the absence of any positive evidence in favour of the latter scenario, however, we deem it less likely.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish, Danish and Norwegian Cancer Societies.

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British Journal of Cancer (1997) 76(6), 770–776

Genomic alterations in nasopharyngeal carcinoma: loss of heterozygosity and Epstein–Barr virus infection

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Summary Nasopharyngeal carcinoma is a subset of head and neck squamous cell cancers with unique endemic distribution and aetiological co-factors. Epstein–Barr virus has been revealed to be an important aetiological factor for most nasopharyngeal carcinomas. Nevertheless, additional genetic alterations may be involved in their development and progression. The aim of this study was to determine the likely chromosomal locations of tumour-suppressor genes related to Epstein–Barr virus-associated nasopharyngeal carcinoma. Fifty-six microsatellite polymorphic markers located on every autosomal arm were used to estimate the incidence of loss of heterozygosity in 27 Epstein–Barr virus-associated nasopharyngeal carcinomas. High frequencies of allelic loss were observed on chromosome 3p (75.0%) and 9p (87.0%). Chromosome 9q, 11q, 13q and 14q displayed loss in over 50%, while chromosome 3q, 6p, 16q, 19q and 22q exhibited loss in 35–50%. Furthermore, several other chromosomal arms demonstrated allelic loss in 20–35%. Additionally, 1 of the 27 cases showed microsatellite instability at multiple loci. These findings provide evidence of multiple genetic alterations during cancer development and clues for further studies of tumour-suppressor genes in Epstein–Barr virus-associated nasopharyngeal carcinoma.

Keywords: nasopharyngeal carcinoma; loss of heterozygosity; Epstein–Barr virus; allelotype; tumour-suppressor gene

Nasopharyngeal carcinoma (NPC) is a subset of head and neck squamous cell cancers (HNSCC) with unique endemic distribution and aetiological co-factors (Fandi et al, 1994). Although NPC is rare among Caucasians in Europe and North America, it is one of the most common cancers in southern China and among Eskimos in Arctic regions, where it has an incidence of 20–50 per 100 000 men. An intermediate incidence is noted in South-East Asia (Voravud, 1990). While HNSCC is closely associated with exposure to tobacco and alcohol, Epstein–Barr virus (EBV) appears to be an important aetiological factor for most NPC (Liebowitz, 1994).

Loss of function of tumour-suppressor genes has been implicated as being essential for solid tumour development and related to chromosomal rearrangement regarding the loss of normal chromosomes or segments (Knudson, 1971; Zhu et al, 1992). Various studies in NPC reported frequent allelic loss on chromosome 3p, 9p and 11q and homozygous deletion or hypermethylation of the *p16* gene (Huang et al, 1991; Choi et al, 1993; Lo et al, 1995–1996, Hui et al, 1996). The aim of this study was to investigate whether other tumour-suppressor genes are also involved in NPC development by analysing the loss of heterozygosity (LOH) on every autosomal arm. Interestingly, allelotyping of HNSCC have been well characterized (Nawroz et al, 1994; El-Naggar et al, 1995; Field et al, 1995). Allelic loss on chromosome 3p, 9p and 11q are also frequent events in HNSCC. In addition, frequent LOH was observed on other chromosomes, e.g. 6p, 8, 13q, 14q, 17p, 18q and 19q (Nawroz et al, 1994; El-Naggar et al, 1995; Field et al, 1995). It would be of great interest and importance to elucidate whether the genetic events in Epstein–Barr virus-associated NPC are similar or distinct from HNSCC.

Received 6 December 1996
Revised 14 February 1997
Accepted 20 February 1997

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MATERIALS AND METHODS

Tissues and DNA extraction

Primary NPC tissues were collected from 27 patients before treatment at Chulalongkorn University Hospital. The tissues were divided into two pieces. The first part was sent for routine histological examination. The second part was immediately stored in liquid nitrogen until further use. All the tumours were histologically ascertained to be undifferentiated NPC, according to the WHO classification. The 27 tumours included stages ranging from II to IV. Blood samples obtained by venipuncture from the same patients were used as constitutional controls. DNA was extracted from the tumour tissues and blood leucocytes by methods previously described (Maniatis et al, 1989).

EBV detection and typing by PCR

For the detection and typing of EBV DNA in the tumour tissues, three previously described polymerase chain reaction (PCR) protocols were used with some modifications (Sample et al, 1990; Feinmesser et al, 1992; Lin et al, 1993). DNA from cell line B95-8, EBV-transformed human lymphocytes (American Type Culture Collection), was used as positive control and double-distilled water as negative control.

Duplex PCR was performed to detect EBV using two sets of primers. The first amplified the non-polymorphic EBV nuclear antigen 1 (EBNA-1), generating an approximately 610-bp DNA

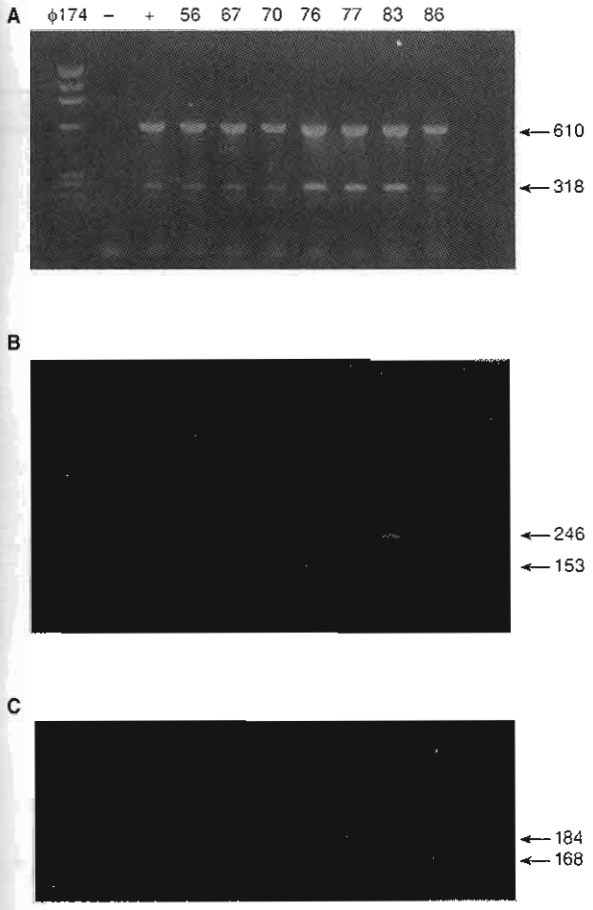

Figure 1 Autoradiographs showing PCR genotyping of EBV-infected NPC on a 2% agarose gel stained with ethidium bromide. The first lane from the left is φ174 *Hae* III standard DNA size marker. + and – are PCR products from positive controls, B958 cell line, and negative controls, double distilled water, respectively. Numbers indicate corresponding PCR products from NPC patients. (**A**) Duplex PCR generating 610-bp and 318-bp DNA fragments for EBNA-1 and human β-actin genomic sequence respectively. (**B**) PCR generating 246-bp and 153-bp DNA fragments for EBNA-3C of EBV type B and type A respectively. (**C**) PCR generating 184 bp and 168 bp DNA fragments for EBNA-2 of EBV type B and type A respectively

fragment. The second amplified a human β-actin genomic sequence, generating an approximately 318-bp DNA fragment. The oligonucleotide sequences for both sets of PCR primers were identical to the ones previously reported (Feinmesser et al, 1992).

Two sets of PCR primers were used for EBV typing. The first primer amplified the EBV nuclear antigen 2 (EBNA-2), generating a DNA fragment of 168 bp for EBV type A and of 184 bp for EBV type B. The second one amplified the EBV nuclear antigen 3C (EBNA-3C), generating a DNA fragment of 153 bp for EBV type A and of 246 bp for EBV type B. The oligonucleotide sequences for both sets of PCR primers were identical to the ones previously reported (Sample et al, 1990; Lin et al, 1993).

The PCR reactions were performed in a total volume of 20 µl using 50 ng of the corresponding tumour DNA in 200 µM dNTP each, 1.5 mM magnesium chloride, 50 mM potassium chloride, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.5 units of *Thermus aquaticus* DNA polymerase (Promega) and 0.5 µM of each primer. The PCR amplifications were performed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, with an extension at 72°C for 1 min and a final extension at 72°C for 7 min. The PCR products were then analysed using 2% agarose gel electrophoresis.

Allelotyping

Fifty-six microsatellite markers for PCR analysis are listed in Table 1. For each chromosomal arm one to five markers were tested.

One strand of each primer pair was end labelled at 37°C for 1–2 h in a total volume of 10 µl containing 10 µM primer, 0.025 mCi [γ-^{32}P]ATP (Amersham) at 3000 Ci mmol^{-1}, 10 mM magnesium chloride, 5 mM DTT, 70 mM Tris-HCl (pH 7.6) and 10 units of T4 polynucleotide kinase (New England Laboratories). Without further separating of the unincorporated nucleotides, the kinase reaction was added to the PCR buffer mix.

The PCR reactions were performed in a total volume of 10 µl using 50 ng of genomic DNA in 200 µM dNTP each, 10 mM Tris-HCl (pH 8.4), 50 mM potassium chloride, 1.5 mM magnesium chloride (for all reactions, except NF1 and D20S470, 2.5 and 2.0 mM magnesium chloride, respectively, was added), 0.5 units of *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus) and the concentration was calculated from 0.05–0.5 µM of each primer.

The marker sets of (D3S1600, D3S966, D9S169), (D11S534, GABRB3, D9S51, D10S169), (GLUT2, D2S102, TCRD), (IL2RB, D8S88), (D16S287, D4S174), (D21S258, MFD133, D20S17, D1S103), (D12S341, D19S221) and (D2S131, D10S249) were analysed for LOH using multiplex PCR. The others were amplified as simplex PCR (Mutirangura et al, 1993).

Several PCR reactions, indicated in parentheses in Table 1, have been optimized for each primer set as follows: for reaction 1 and 3, the initial denaturation step at 95°C for 4 min, then followed by 25 cycles of denaturation at 94°C for 1 min, with 1 min of annealing at 55°C for reaction 1, or 52°C for reaction 3, extension at 72°C for 2 min and a final extension at 72°C for 7 min; for reaction 2, the initial denaturation step was 95°C for 4 min, then followed by

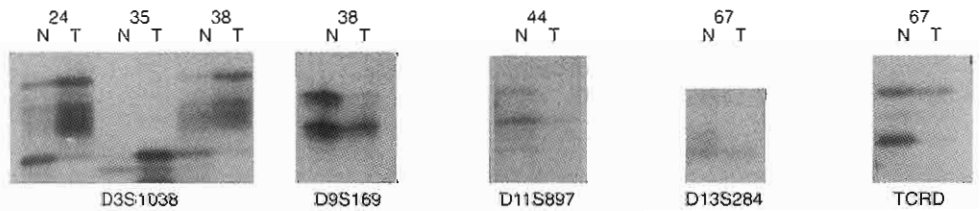

Figure 2 Autoradiographs showing LOH analysis using microsatellite markers. Representative NPC tumours (T) and corresponding normal leucocytes (N) are shown with microsatellite markers indicated on the bottom. Markers D9S169, D11S897 and D13S284 reveal loss of upper alleles, and markers D3S1038 and TCRD reveal loss of lower alleles

Table 1 LOH and MSI data for each locus of 27 NPCs

	Locus(C)[a]	Location	L/I(%)	11	18	19	24	31	35	38	44	45	5
Stage				III	IV	IV	IV	R	IV	II	III	IV	III
T				3	3	2	4		4	2	2	1	4
N				0	2[b]	2[c]	3		3	0	1	2[b]	2
M				0	0	0	0		1	0	0	0	0
WHO				II	II	II	III	II	III	II	II	II	II
EBV				A	A	A	A	A	A	A	A	A	A
	D1S243 (1)	1p36.1–2	7/24 (29.2)	–	–	U	–	+	–	+	–	U	–
	D1S103 (1)	1q31q32	5/25 (20.0)	+	+	–	–	–	–	i	–	–	–
	D2S131 (3)	2p	2/20 (10.0)	–	U	–	–	–	–	i	–	–	–
	D2S102 (1)	2q33q37	5/22 (22.7)	–	+	–	–	+	–	i	+	–	U
	D3S1038 (3)	3p25	18/24 (75.0)	U	–	U	+	+	+	+	+	U	–
	D3S192 (3)	3p25		U	–	U	+	N	N	+	+	–	–
	D3S966 (1)	3p21.3	12/18 (66.6)	–	U	+	+	+	–	i	+	U	U
	D3S1600 (1)	3p14	15/20 (75.0)	U	–	U	+	+	+	i	+	–	–
	GLUT2 (1)	3q26.1–2	11/23 (47.8)	U	+	+	–	–	–	i	+	+	U
	D3S1744 (1)	3q23q24		–	+	U	N	N	–	i	+	+	N
	D4S174 (1)	4p11p15	6/23 (26.1)	–	–	–	–	–	–	i	+	U	–
	D4S1554 (2)	4q11q35	4/23 (17.4)	–	U	+	N	–	+	i	–	–	–
	D5S392 (1)	5p	6/25 (24.0)	+	–	N	N	–	N	+	–	U	N
	D5S819 (2)	5p		U	U	–	N	U	–	–	U	U	–
	D5S82 (1)	5q14q21	7/21 (33.3)	–	–	+	+	–	U	i	+	U	U
	D6S309 (2)	6p	10/21 (47.6)	+	–	+	+	–	U	i	+	–	+
	D6S503 (3)	6q	7/22 (31.8)	–	–	–	U	U	+	–	–	–	U
	D7S517 (1)	7p	4/24 (16.7)	–	+	–	–	–	–	+	+	–	–
	D7S486 (2)	7q31	6/26 (23.1)	–	–	–	–	–	–	+	+	–	–
	NEFL (1)	8p	3/23 (13.0)	–	–	–	U	–	U	+	–	–	–
	D8S88 (1)	8q22	3/22 (13.6)	–	–	–	U	–	–	i	–	–	U
	D9S169 (1)	9p21	20/23 (87.0)	+	–	+	+	+	–	+	+	+	+
	IFNA (2)	9p22		N	–	U	N	N	–	U	+	+	+
	D9S51 (1)	9q	11/22 (50.0)	–	+	+	–	+	U	+	–	–	+
	ABL1 (1)	9q34		N	+	+	U	U	U	U	U	U	N
	D10S249 (3)	10p	4/21 (21.1)	–	–	–	N	–	–	–	–	–	–
	D10S169 (1)	10q11.2	3/22 (13.6)	U	–	–	N	U	U	i	–	U	–
	D10S677 (1)	10q		–	N	N	N	–	–	–	–	–	N
	WT1 (1)	11p13	7/25 (28.0)	U	U	+	N	–	–	+	U	+	–
	D11S554 (2)	11p		–	–	–	N	N	–	U	+	–	–
	D11S534 (1)	11q13	7/27 (25.9)	U	–	–	N	–	U	i	–	–	U
	D11S956 (1)	11q13		U	–	U	U	N	–	+	–	–	–
	INT2 (1)	11q13.3		–	–	–	–	–	U	i	–	U	–
	D11S976 (1)	11q23	14/26 (53.8)	U	–	U	N	N	+	+	+	+	–
	D11S897 (2)	11q23		–	–	–	–	–	U	+	+	+	U
	D12S341 (2)	12p	7/23 (30.4)	–	–	–	+	+	–	i	U	–	+
	MFD133 (1)	12q	4/20 (20.0)	U	–	–	+	–	–	i	+	–	–
	D13S284 (2)	13q14.2	14/22 (63.6)	N	+	+	+	+	–	i	+	–	+
	D13S119 (1)	13q14.3-q22	6/20 (30.0)	–	U	+	N	–	U	–	+	U	U
	TCRD (1)	14q11.2	10/19 (52.6)	–	U	U	U	+	U	U	U	+	–
	D14S118 (1)	14q	6/14 (42.9)	U	+	U	N	–	+	U	+	U	U
	GABRB3 (1)	15q11q13	4/22 (18.2)	–	–	U	N	–	+	i	–	–	U
	D16S287 (1)	16p13.11	4/22 (18.2)	–	U	–	+	+	–	–	–	–	+
	D16S511 (2)	16q22q24	11/23 (47.8)	–	–	–	–	+	+	i	+	+	U
	D17S520 (1)	17p12	8/27 (29.6)	–	–	–	–	–	–	i	–	–	–
	D17S1176 (2)	17p		–	–	–	–	–	–	+	–	–	–
	KRT9 (2)	17q21	6/19 (31.6)	+	U	–	–	–	–	i	–	–	+
	D18S59 (1)	18p11.2	0/20 (0)	U	–	–	–	–	–	–	–	–	–
	D18S35 (1)	18q21.2	6/24 (25.0)	U	U	–	+	–	U	+	–	+	U
	DCC (1)	18q21.1		U	–	U	+	–	+	i	U	–	–
	D19S221 (2)	19p	9/25 (36)	–	+	–	U	–	–	–	+	+	–
	D19S412 (2)	19q	2/19 (10.5)	U	–	–	U	U	–	i	–	U	U
	D20S470 (3)	20p	4/20 (20.0)	–	+	–	N	–	U	i	–	+	U
	D20S17 (1)	20q12	3/20 (15.0)	–	–	U	+	–	–	U	+	–	U
	D21S258 (1)	21q	3/20 (15.0)	–	U	U	U	–	–	–	+	U	–
	IL2RB (1)	22q	10/22 (45.5)	U	–	+	N	+	–	+	–	+	–

[a]C, PCR condition; L, number of positive LOH cases; I, number of informative cases; R, recurrence; A, EBV type A; B, EBV type B; +, positive LOH result; –, negative LOH result; i, microsatellite instability; U, uninformative result; N, not done.

51	53	56	67	70	76	77	83	86	93	102	103	105	112	123	138
IV	III	IV	IV	IV	IV	IV	IV	III	IV	IV	IV	IV	IV	IV	III
3	3	3	2	4	2	2	3	3	4	4	3	2	3	4	3
3	1	3	2[a]	2[c]	2[b]	3	3	0	1	2[c]	2[c]	2[c]	2[b]	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
III	II	II	II	III	III	III	II	II	III	III	III	III	II	III	II
A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
+	–	+	–	–	–	–	–	–	U	+	+	+	–	–	–
+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	+	U	–	–	–	–	U	–	+	U	U	–	–	–
+	U	–	–	–	–	–	U	–	+	–	–	U	–	–	–
U	+	+	U	+	+	+	+	+	+	U	+	+	U	U	U
+	+	+	U	+	U	U	+	+	+	+	U	U	+	–	–
+	+	+	–	–	–	U	+	U	–	+	+	U	+	U	N
–	+	+	+	–	–	+	+	+	+	+	+	U	U	U	N
U	U	U	U	+	–	U	–	+	+	+	–	–	+	–	U
+	N	–	N	N	N	+	–	U	+	+	–	–	+	–	–
+	+	+	–	–	U	–	+	–	–	U	–	–	–	–	–
+	U	–	–	–	–	–	–	–	–	–	+	–	–	–	–
–	N	–	–	N	+	–	+	–	U	–	+	–	–	–	–
–	–	–	U	–	U	U	N	U	–	+	+	–	–	–	N
+	–	–	+	–	–	–	–	–	–	+	+	–	U	–	–
+	–	–	U	+	–	+	U	U	+	+	–	U	–	–	–
–	+	U	–	–	–	+	+	–	U	–	+	–	+	+	–
–	+	–	–	U	–	–	–	U	–	–	U	–	–	–	–
–	+	U	–	+	–	–	+	–	+	–	–	–	–	–	–
–	+	–	U	–	–	–	–	U	–	+	–	–	–	–	–
–	–	–	+	–	–	–	U	+	–	–	–	+	–	U	–
–	+	U	+	U	U	U	N	U	+	+	+	U	U	U	+
–	+	+	+	U	+	+	+	U	U	+	+	U	+	U	+
–	–	+	+	–	U	U	–	–	U	U	U	U	U	U	U
–	+	U	N	U	U	U	U	–	U	–	U	U	U	+	+
+	+	–	–	–	U	–	N	–	U	U	+	U	–	–	–
U	U	U	–	U	U	–	+	+	–	U	U	U	U	U	U
–	N	–	N	–	N	–	+	+	–	U	–	–	N	–	–
+	+	–	–	–	U	U	U	–	U	U	–	U	–	U	U
–	N	–	N	–	–	–	–	–	–	+	–	–	–	–	–
–	U	+	U	U	–	–	–	U	–	–	–	U	U	U	+
i	+	+	+	–	–	N	N	–	i	–	U	+	U	–	+
–	U	+	+	–	–	–	–	–	–	–	–	U	+	–	+
U	U	+	N	+	N	+	N	–	–	+	U	+	+	–	+
–	+	U	+	+	–	+	U	–	U	+	–	+	+	–	+
–	+	–	–	+	–	–	N	–	–	N	+	+	–	–	–
–	–	U	–	–	–	U	–	–	U	+	U	–	–	–	U
+	+	+	+	–	–	U	–	–	U	+	+	+	–	+	U
–	–	–	–	U	–	–	–	–	–	+	+	+	–	–	+
–	+	+	+	+	–	–	+	+	U	+	U	–	–	–	–
–	+	+	U	–	+	–	U	–	U	U	U	U	U	U	–
–	–	–	–	–	–	–	–	U	+	+	+	–	–	–	–
+	N	–	U	–	U	–	–	–	–	–	U	–	–	–	–
+	+	–	–	–	–	–	–	+	+	+	+	–	–	U	+
–	+	+	U	–	–	+	U	U	–	+	+	+	–	–	–
–	+	+	–	–	–	+	–	+	–	+	+	U	U	–	–
+	+	+	–	–	–	+	N	U	–	U	U	U	–	U	–
–	U	–	–	–	–	U	–		U	–	–	–	U	–	U
–	N	+	+	–	U	–	U	–	i	–	U	U	U	–	–
U	–	+	U	–	–	–	N	U	–	U	–	U	U	–	–
–	+	–	+	–	+	–	–	+	+	+	U	–	–	U	–
+	–	–	–	+	–	–	–	–	–	–	–	U	–	–	U
–	+	–	–	–	U	–	–	–	–	–	+	U	–	–	–
–	+	–	U	–	–	U	–	–	U	–	–	–	–	U	–
–	+	–	U	+	–	–	U	–	–	–	–	U	–	–	–
+	+	+	+	+	–	–	U	U	+	–	–	–	–	U	–

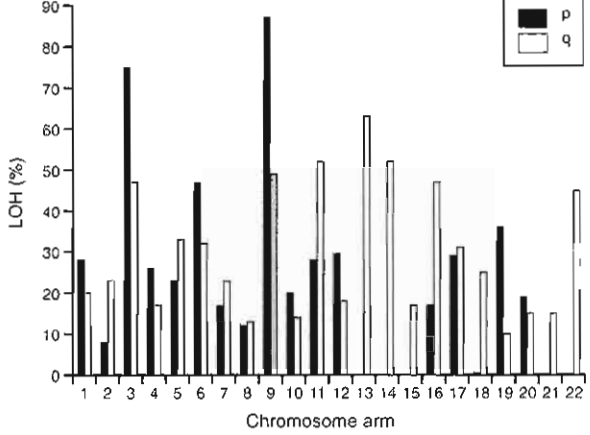

Figure 3 Frequency of allelic loss for autosome in NPC. Allelotyping was accomplished using polymorphic microsatellite analysis. The probes used are listed in Table 1

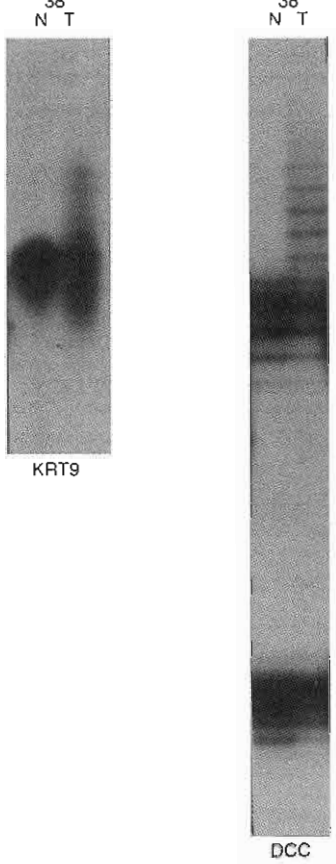

Figure 4 Autoradiograph showing the microsatellite instability at KRT9 and DCC loci. N, normal DNA; T, tumour DNA

five cycles of step-down PCR denaturation at 94°C for 1 min, with 1 min of annealing at 60°C, 59°C, 58°C, 57°C and 56°C, extension at 72°C for 2 min and 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, with extension at 72°C for 2 min and a final extension at 72°C for 7 min.

Two microlitres of each reaction were mixed with 1 µl of formamide-loading buffer, heated at 95°C for 2 min, put on ice for 30 s and then loaded onto 6% polyacrylamide/7 M urea gel. DNA fragments were size fractionated at 70 W until the tracking dye reached the appropriate point on the gel. After electrophoresis, the wet gel was transferred to filter paper (Watman), wrapped with Saran wrap and exposed to Kodak T-mat radiographic film for 6–24 h at –70°C with an intensifying screen.

RESULTS

Twenty-seven EBV-associated NPC samples were selected for LOH studies. All biopsied specimens were histologically confirmed. Among these 27 cases, 15 were WHO type II and the others were WHO type III. Twenty-six cases were infected with EBV subtype A and one case with type B (Figure 1 and Table 1).

LOH in NPC

A panel of 56 microsatellite polymorphic markers representing every chromosomal arm was used to screen for LOH frequency. Table 1 shows the polymorphic loci used to test each chromosomal arm, patient staging, EBV typing and LOH, as well as microsatellite instability (MSI) results. Results representative for LOH are shown in Figure 2. Frequencies of LOH for each autosomal arm are represented in Figure 3.

The frequencies of LOH from each chromosomal arm varied from 0% to 87%. Chromosome 3p and 9p with 78% and 87%, respectively, revealed higher incidence than other chromosomal arms. For chromosome 3p, further analysis displayed that there were at least two LOH loci, which were 3p14, D3S1600 and 3p25, D3S192 and D3S1038. Two cases, 51 and 70, showed LOH from 3p25 but not 3p14. In contrast, two cases, 47 and 50, revealed loss from 3p14 but not 3p25. Two cases, 35 and 93, presented loss from both 3p14 and 3p25 but not 3p21. Thus, 3p14 and 3p25 were two separate LOH loci. Other regions with allelic loss over 50% were on chromosome 9q (50.0%), 11q (53.8%), 13q (63.6%) and 14q (52.6%). Further analyses of chromosome 11q revealed higher frequency of LOH on 11q23 (53.8%), while only 7 out of 27 (25.9%) had LOH on 11q13. Additional analyses on chromosome 13 displayed that the incidence of LOH for D13S284, located on 13q14, was higher than D13S119, located on 13q14.3-q22. LOH between 35% and 50% was noted on chromosome 3q (47.8%), 16q (47.8%), 19p (36.0%) and 22q (45.5%). Finally, several other chromosomes demonstrated allelic loss in 20–35%.

MSI in NPC

Out of 27 samples tested, MSI for multiple loci was presented in only one case, i.e. 38. MSI was revealed in 25 of 56 loci. In addition, sample 93 demonstrated MSI on two loci, D11S956 and D18S35, and sample 53 showed MSI on one locus, D11S956. Representative results for MSI are shown in Figure 4. No significant clinical difference was noted regarding these cases.

DISCUSSION

Several areas of chromosomal loss during cancer development and progression are associated with inactivation of both tumour-suppressor gene alleles (Huang et al, 1991; Zhu et al, 1992; Nawroz et al, 1994). In addition, they are correlated with the histopathology, staging and clinical outcome of cancer (Broder et al, 1995). Here we demonstrated several chromosomes with significant LOH in

NPC. While several LOH loci are common to HNSCC, some appear to be unique to NPC progression.

Previously Choi et al (1993) showed LOH on chromosome 3p for all of the informative 35 NPC cases and Huang et al (1994) studied chromosome 9p and found allelic loss on 11 samples from 18 NPC. Consistently, in this study, two of the highest incidences of allelic loss have been shown on chromosomes 3p (78%) and 9p (87%). Three LOH loci have been reported on chromosomes 3p, 3p14, 3p21 and 3p25 (Maestro et al, 1993). This study revealed that, at least, the 3p14 and 3p25 loci are associated with NPC development. It should also be noted that a putative tumour-suppressor gene, *VHL*, is the candidate gene on chromosome 3p25 (Latif et al, 1993). For 9p, the LOH locus has been defined on chromosome band 9p21; and gene *p16*, which controls cell cycles, has previously been shown to have homozygous deletion or hypermethylation (Lo et al, 1995, 1996). Interestingly, 9p LOH has now been well documented in precancerous lesions of HNSCC (El-Naggar et al, 1995). It is interesting to investigate whether genetic alterations of chromosome 9p might also be an early event of NPC carcinogenesis.

Allelic loss of chromosome 11q has been observed in several other tumour types, such as HNSCC, breast, ovary and lung (Nawroz et al, 1994; Gudmundsson et al, 1995*a*; Iizuka et al, 1995). Poor prognosis of breast and ovarian cancer has been associated with 11qLOH (Gudmundsson et al, 1995*a*). At least three LOH loci have been delineated on chromosome 11q, 11q13, 11q15 and 11q23 (Iizuka et al, 1995). In addition, 11q13 is a chromosome region with a high frequency of amplification in HNSCC (Meredith et al, 1995). This results in allelic imbalance and may be difficult to distinguish from LOH. This study described a higher frequency of allelic loss in NPC on 11q23 than on 11q13. It should also be noted that the LOH on 11q23 may be related to the ataxia-telangiectasia locus (Savitsky et al, 1995).

Chromosome 13qLOH is also frequently detected in several cancers, such as retinoblastoma, breast cancer and HNSCC (Zhu et al, 1992; Nawroz et al, 1994; Gudmundsson et al, 1995*b*). This study has shown that the common LOH locus in NPC may be located proximal to 13q14.3. At least two tumour-suppressor genes are located proximal to this region. The first is *Rb*, retinoblastoma gene, and the other is *BRCA2*, the candidate gene for the second locus of the familial breast cancer syndrome (Zhu et al, 1992; Gudmundsson et al, 1995*b*). Previous mutation analysis of *Rb* on NPC demonstrated negative results (Sun et al, 1993). Thus, it is tempting to hypothesize that the *BRCA2* tumour-suppressor gene may be responsible for NPC development.

The allelic loss of chromosome 14q was also frequently found in other types of cancer, e.g. bladder cancer, neuroblastoma, colorectal cancer and HNSCC (Fong et al, 1992; Young et al, 1993; Nawroz et al, 1994; Chang et al, 1995). A recent study has delineated two tumour-suppressor gene loci on chromosome 14, i.e. 14q12 and 14q32 (Chang et al, 1995). Interestingly, LOH of 14q is associated with an advanced phenotype of neuroblastoma and frequently found in advanced colorectal cancer (Fong et al, 1992; Young et al, 1993).

Chromosome 17p is one of the most common regions with genetic alterations reported in cancer. *p53*, the best known tumour-suppressor gene, is located on this chromosome (Carson et al, 1995). *p53* alterations, including protein expression and mutations, are common in HNSCC while mutation of *p53* in NPC is infrequent (Field et al, 1991; Boyle et al, 1993; Shin et al, 1994; Brennan et al, 1995; Chakrani et al, 1995). The LOH study of this chromosome supported the mutation data in which allelic loss of chromosome 17p was noticed in only 30% of the NPC, while previous reports revealed 50% LOH of the HNSCC (Nawroz et al, 1994; Field et al, 1995).

Among 27 cases tested, 15 were WHO type II and 12 were WHO type III. The average frequencies of LOH for each chromosome region were 0.34 and 0.31 for WHO type II and III respectively. Interestingly, LOH was found to be more frequent on chromosome 4p, 7p, 9q, 11q and 22q for WHO type II, while a higher frequency of LOH for WHO type III was reported on chromosome 6p and 15q. However, because of the limited number of tumours, these comparative data are not statistically significant.

MSI is presented as variations in the length of microsatellite repeats in tumour DNA when compared with matched normal DNA. The abnormality in the size of the microsatellite loci has been observed in various types of cancer as well as in hereditary non-polyposis colorectal cancer (HNPCC) (Thibodeau et al, 1993). In HNPCC, mutations in a number of DNA mismatch repair genes (*hMSH2*, *hMLH1*, *hPMS1* and *hPMS2*) have been reported. Thus, MSI may be the consequence of decreased accuracy of the DNA mismatch repair system during DNA replication, which might facilitate the accumulation of mutations (Rhyu, 1996). This study has presented MSI of multiple loci in only one out of 27 NPC samples tested. This suggests that the phenomenon of MSI is a relatively rare event during NPC development.

NPC is a unique subclassification of HNSCC as a result of its endemic distribution and aetiological cofactors. It would be of great interest and importance to elucidate whether the genetic events in Epstein–Barr virus-associated NPC are similar or distinct from HNSCC. Previous HNSCC studies have demonstrated a high frequency of LOH on chromosomes 3p, 3q, 6p, 8p, 8q, 9p, 11q, 13q, 14q, 17p, 18q and 19q (Nawroz et al, 1994; El-Naggar et al, 1995; Field et al, 1995). Nawroz et al (1994) studied 29 HNSCCs and showed 67, 50, 38, 40, 38, 72, 61, 54, 39, 52, 23 and 40% allelic losses respectively. Additionally, Field et al (1995) tested 80 specimens and found LOH more frequently on chromosome 3p, 8p, 9p, 13q, 17p, 18q and 19q for 52, 35, 62, 27, 50, 49 and 29% respectively. Finally, El-Naggar et al (1995) studied 20 patients for LOH on chromosome 3p, 5q, 8p, 9p, 9q, 11q and 17p, and a high incidence of LOH in invasive carcinoma was observed at 9p (72%), 8p (53%), 3p (47%), 9q (35%) and 11q (33%). Similar incidences on chromosomes 3p (78%), 3q (48%), 6p (48%), 9p (87%), 11q (54%), 13q (64%) and 14q (43%) have also been detected regarding NPC. However, NPC revealed lower incidences of LOH on chromosomes 8p (13%), 17p (30%) and 19q (11%). In contrast, this study has shown frequent allelic loss regarding NPC on chromosomes 16q (48%) and 22q (46%). As several genetic alterations of NPC and HNSCC are similar, the multistep processes for the development and progression of both cancers overlap. However, some genetic changes seem to be unique in the biology of NPC development.

It would also be of interest to compare these allelotyping data with the allelic loss pattern of other EBV-associated neoplasias, for example post-immunosuppression/transplant, AIDS-related and Burkitt's lymphomas. However, there is only limited knowledge of LOH for comparison at present.

ACKNOWLEDGEMENTS

The authors are deeply indebted to the staff of the Department of Otolaryngology and the Radiotherapy Section, Department of

Radiology, Chulalongkorn University Hospital, for the recruitment of patients and collection of materials. We also thank Dr David H Ledbetter and Dr Lisa G Shaeffer for providing some STR primers, Mrs Bungon Changchup and Mr Anucha Karnthaworn for technical assistance and Dr Henry Wilde, Ms Petra Hirsch, Dr Dong M Shin and Dr Karol Sikora for their critical review of the manuscript. This work was supported by the Rachadapisek Sompoj China Medical Board research fund, Molecular Biology Project, Faculty of Medicine, Chulalongkorn University and the Thailand Research Fund.

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[CANCER RESEARCH 56, 3530–3533, August 1, 1996]

Telomerase Activity in Oral Leukoplakia and Head and Neck Squamous Cell Carcinoma[1]

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ABSTRACT

The expression of telomerase, a ribonucleoprotein complex, is necessary to overcome cellular senescence, and it is associated with immortal cells and cancer. However, its role in precancerous lesions such as oral leukoplakias is less known. The purpose of this study is to investigate the presence of telomerase activity in oral leukoplakia and the relationship between the enzyme and multistep tumorigenesis. Telomerase activity was detectable in 14 of 16 human head and neck squamous cell carcinomas and 10 of 26 oral leukoplakia tissues. We also showed that the expression of telomerase in the premalignant lesions was associated with phenotypic progression, the degree of dysplasia. These results indicate that telomerase is activated frequently during the late stage of oral premalignancy and may play a crucial role in head and neck squamous cell carcinogenesis.

INTRODUCTION

Head and neck carcinogenesis is a process of "field cancerization," the repeated exposure of an entire field of tissue to carcinogenic insults (*e.g.*, tobacco and alcohol), which increases the risk for development of multiple independent premalignant and malignant foci (1). The evolution of HNSCC[3] requires multiple steps in which the gradual accumulation of somatic mutations alters cellular growth, proliferation, and differentiation and is expressed histologically as a progression from normal epithelium to precancerous lesions and invasive carcinoma (2–4). The genetic alterations in the formation of HNSCC, especially mutational activation or overexpression of oncogenes and loss of normal function of tumor suppressor genes, have been identified (5). However, most cells with cumulative mutations do not progress toward malignant transformation. Only few cells with genetic aberrations will succeed in carcinogenesis (6). The crucial genetic events in the development of HNSCC are not well understood.

Oral leukoplakia and erythroplakia are seen grossly as white and red patches or plaques that cannot be classified with other entities (7, 8). Both show ranges of histological changes including epithelial thickening due to increased cell layers and varying degrees of accumulation of surface keratin. Some show evidence of dysplasia or early invasive carcinoma. Abnormal vascular patterns and inflammation in the submucosa are often noted and may contribute to the red appearance in erythroplakia. Both leukoplakia and erythroplakia are precancerous lesions and provide unique models to study multistep tumorigenesis (7, 8). The malignant transformation rate of oral leukoplakia varies from 0 to 20% over 20 years and up to 40% for erythroplakia (4, 9). Previous investigators have been searching for biomarkers for risk assessment in these premalignant lesions. Histological evaluation for the presence or absence of dysplasia is the most reliable indicator of carcinomatous development (10).

Telomerase is a ribonucleoprotein enzyme that synthesizes telomeres, the specialized structures containing unique simple repetitive sequences (TTAGGG in vertebrate) at the end of chromosomes (11, 12). The enzyme compensates for the end replication problem and allows cells to proliferate indefinitely (13). Recent studies, using the TRAP, have shown that telomerase is activated in most human cancer tissues but not in most normal tissues and tissues adjacent to malignant or benign tumors (14). In addition, previous studies have shown that the lack of telomerase activity correlates with critically shortened telomeres and frequent spontaneous cancer remission (15). Thus, the expression of telomerase is important and may be a rate-limiting step for tumor progression (14).

The expression of telomerase occurs in different stages of cancer progression depending on types of malignancy. In certain tumor types, such as non-small cell lung cancer and gastric cancer, telomerase is activated in higher frequency in the late stages of tumor progression (16, 17). In tumors such as hepatocellular carcinoma and mouse skin cancer, telomerase activity has, however, been detected in premalignant stages (18, 19). To identify telomerase activity in the multistep carcinogenesis of HNSCC, we performed a TRAP assay in oral leukoplakia and HNSCC. In addition, we studied the clinicopathological correlation of oral leukoplakia tissues and their telomerase activities.

MATERIALS AND METHODS

Tissues. Samples were obtained by punch biopsy or surgical resection from 26 oral leukoplakias, 16 HNSCCs, and 18 normal oral tissues adjacent to leukoplakias (14 cases) or cancer (4 cases). These samples were each divided into two pieces. The first part was sent for routine histological examination. The second part was immediately stored in −80°C until used. All H&E-stained slides were reviewed by one pathologist (S. Y.) to determine histological differentiation of tissues according to the criteria described previously (8, 20, 21). The hyperplastic lesions, G0, were classified according to the increased number of cells in the epithelium. For the classification of dysplasia, the nomenclature of the CIN classification was used (GI-GIII as compared with CIN I, CIN II, and CIN III, *i.e.*, mild, moderate, and severe dysplasia, respectively). The squamous cell carcinomas were also classified according to the loss of differentiation that the lesions exhibited, using the Union International Contre Cancer classification (G1 for well differentiated, G2 for moderately differentiated, and G3 for poorly differentiated squamous cell cancer).

Telomerase Assay. TRAP was performed as described previously (18). Each sample of 10–100 mg frozen tissue was first washed in 500 μl ice-cold PBS (calcium and magnesium free), then homogenized in 20–200 μl of ice-cold CHAPS lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol, diethylpyrocarbonate H_2O] depending on the size of sample, using a manual homogenizer. After 30 min of incubation on ice, the lysate was centrifuged at 14,000 × *g* for 30 min at 4°C. The supernatant was aliquoted, flash frozen in liquid nitrogen, and stored at −80°C. The protein concentration was determined by Micro TP assay kit (Wako Pure Chemicals,

Received 3/15/96; accepted 5/24/96.

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[1] This work was supported by the Rachadapisek Sompoj China Medical Board Research Fund; the Thailand Research Fund; and the Molecular Biology Project, Faculty of Medicine, Chulalongkorn University.

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[3] The abbreviations used are: HNSCC, head and neck squamous cell cancer; TRAP, telomeric repeat amplification protocol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CIN, cervical intraepithelial neoplasia.

Industries, Ltd.). The typical protein concentration of the extract was between 1 and 10 μg/μl, and an aliquot of the extract containing 6 μg of protein (standard condition) was used for each TRAP assay.

Telomerase activity was measured using Epstein-Barr viral-transformed human lymphocytes (American Type Culture Collection cell line B958). Cells were harvested and washed twice with ice-cold PBS, then resuspended in 100 μl of ice-cold CHAPS lysis buffer per 10_6 cells and processed as for tissue extracts. The supernatant, which had a cell concentration of 10_4 cells/μl, was diluted serially at 1:10 and 1:100 to obtain the standard 10_3 and 10_2 cells/μl, respectively. The same protocol without serial dilution was used for normal cultured fibroblasts as negative control.

The TRAP assay was performed as follows. Appropriately diluted extracts (6 μg protein) were assayed in 50 μl of reaction mixture containing 20 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 63 mM KCl; 0.005% Tween-20; 1 mM EGTA; 50 μM dGTP, dATP, and dTTP; 5 μM dCTP; 0.1 μg of the deoxyoligonucleotide primer TS (5′-AATCCGTCGAGCAGAGTT-3′); 1 μg of T4g32 protein (Boehringer Mannheim); 4 μl of α-$_{32}$p dCTP (10 μci/μl, 3000 ci/mmol); and 2 U of *Taq* polymerase (Perkin-Elmer Corp.) and diethylpyrocarbonate H_2O in 0.5 ml tube. The tube contained 0.1 μg of the deoxyoligonucleotide CX (5′-CCCTTACCCTTACCCTTACCCTAA-3′) sequestered at the bottom by a wax barrier (Ampliwax; Perkin-Elmer Cetus). After 10 min of incubation at 23°C to allow telomerase-mediated extension of the TS primer, the reaction tube was then subjected to 30 PCR cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Aliquots (5 μl) of the PCR mixture were analyzed on 8% nondenaturing polyacrylamide gel in 0.6× Tris-borate EDTA buffer until the xylene cyanol had migrated 17 cm from the origin (gel size 20 × 40 cm). The gels were then exposed to Kodak XAR-5 X-ray film at −70°C with an intensifying screen. The positive results were compared with the telomerase activity of 100 and 1000 cells of B958. Duplicate assays were performed on all samples with and without RNase pretreatment to a final concentration of 0.05 mg/ml for 10 min at room temperature. Data were collected blindly and decoded later.

RESULTS

Telomerase Activity in Oral Premalignancy and HNSCC. In the present study, we analyzed 16 samples of HNSCC, 26 of oral leukoplakias, and 18 of normal oral tissues adjacent to 14 leukoplakias and 4 cancers (Tables 1 and 2). B958 cells were used as positive controls and cultured normal fibroblasts as negative controls (Fig. 1). Telomerase activity was detectable in most of the cancer tissues, 14 of 16 cases (87.5%). Only 10 of 26 (38.5%) oral leukoplakia and erythroplakia samples were positive. All but one, CU34, adjacent to normal oral epithelium, lacked detectable TRAP activity (Fig. 1). Pretreatment of all extracts with RNase abolished telomerase activity.

Because both leukoplakia and cancer showed variation in the intensity of the TRAP signals, we compared the intensity of the ladder signals with serial dilution of cell line B958, 100 and 1000 cells, respectively. A gradual increment of TRAP intensity was observed and varied directly with the number of the immortalize cells tested. We then semiquantitatively compared the density of each positive TRAP result with the serially diluted B958 cell line. The dosage of

Table 1 *Clinical data and telomerase activity in oral leukoplakia and HNSCC*

Patient code	Age	Sex	Location	Onset (month)	R[a]	Diagnosis	Histological classification	K	Telomerase	
									+/−	Dosage
CU1	37	F	Bucca and tongue	6	−	Erythroplakia	GI	−	+	<100
CU2	74	F	Lateral border tongue	2	−	Leukoplakia	G0	+	−	
CU2R	74	F	Lateral border tongue	1	+	Leukoplakia	G0	+	+	<100
CU6	57	M	Lower dental ridge	12	−	Leukoplakia	G0/GI	+	+	<100
CU11	45	M	Lateral molar trigone	8	−	Leukoplakia	ND	ND	−	
CU13	40	F	Lateral border tongue	5	−	Leukoplakia	G0/GI	+	−	
CU14	64	M	Retromolar trigone	2	−	Leukoplakia	G0	−	−	
CU15	70	M	Hard palate	48	−	Leukoplakia	G0	+	−	
CU16	60	F	Buccal mucosa	12	−	Leukoplakia	G0	+	−	
CU17	59	F	Buccal mucosa	3	−	Leukoplakia	G0	+	−	
CU18	40	M	Buccal mucosa	0.5	−	Leukoplakia	G0	+	−	
CU18R	40	M	Buccal mucosa	1	+	Leukoplakia	G0	+	−	
CU20	58	M	Buccal mucosa	2	+	Leukoplakia	G0	+	+	<100
CU21	42	M	Buccal mucosa	40	−	Leukoplakia	G0		+	−
CU23	40	F	Buccal mucosa	12	−	Leukoplakia	G0	+	−	
CU24	70	F	Buccal mucosa	72	+	Leukoplakia	G0	+	+	>1000
CU25	64	F	Buccal mucosa	2	−	Leukoplakia	G0	+	+	100–1000
CU26	51	M	Lateral tongue	1.5	−	Leukoplakia	ND	ND	−	
CU34	61	M	Lateral tongue	2	−	Leukoplakia	GII	−	+	>1000
CU37	58	F	Buccal mucosa	12	+	Leukoplakia	G0	+	−	
CU39	39	F	Buccal mucosa	40	−	Leukoplakia	G0	+	−	
CU44	46	F	Buccal mucosa	¼	−	Leukoplakia	G0	−	−	
CU42	78	M	Buccal mucosa	2	−	Erythroplakia	G0	+	+	100–1000
CU41	42	F	Tongue	6	−	Leukoplakia	G0/GI	+	+	<100
RAMA5	73	F	Upper first molar	2	−	Leukoplakia	G0	+	−	
RAMA13	62	F	Lower lip	40	−	Erythroplakia	GI	−	+	<100
CU3	63	F	Retromolar trigone			V carcinoma	V		+	<100
CU5	80	F	Lateral tongue			V carcinoma	V		+	100–1000
CU10	78	M	Lateral tongue			SCC	GI		+	100–1000
CU22	40	F	Lateral tongue			SCC	GI		−	
CU9	78	F	Buccal mucosa			SCC	GI		+	<100
CU19	55	M	Tongue			SCC	GI		+	100–1000
CU29	80	M	Floor of mouth			SCC	GI		−	
CU31	60	M	Tongue			SCC	GI		+	>1000
CU35	65	F	Lower lip			SCC	GI		+	>1000
CU40	62	F	Lower gum			SCC	G2		+	<100
CU28	63	M	Floor of mouth			SCC	G2		+	100–1000
CU30	58	F	Tongue			SCC	G2		+	>1000
CU32	72	M	Glottis			SCC	G2		+	<100
CU33	83	M	Hard palate			SCC	G2		+	>1000
CU38	81	F	Aryepigolottic fold			SCC	G2		+	>1000
CU27	50	M	Nasopharynx			NPC	G3		+	<100

[a] R, recurrence from previous laser surgery; K, keratosis; F, female; M, male; +, positive; −, negative; ND, not done; G0, hyperplastic lesion; GI, mild dysplasia; GII, moderate dysplasia; GIII, severe dysplasia; V, verrucous carcinoma; SCC, squamous cell cancer; NPC, nasopharyngeal cancer; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

Table 2 *Telomerase activity in HNSCC and oral leukoplakia*

Conditions	Telomerase activity		
	Positive	Negative	Total
Cancer			
Verucous carcinoma	2	0	2
Squamous cell cancer	11	2	13
Nasopharyngeal cancer	1	0	1
Total HNSCC	14	2	16
Oral premalignant lesion			
Gross appearance			
Leukoplakia	7	16	23
Erythroplakia	3	0	3
Total oral leukoplakia and erythroplakia	10	16	26
Histological finding			
G0[a] without history of recurrence after treatment	2	11	13
G0 with history of recurrence after treatment	3	2	5
Total G0 cases	5	13	18
G0/GI	2	1	3
GI	2	0	2
GII	1	0	1
Total cases with dysplasia	5	1	6

[a] G0, hyperplasia; G0/GI, mixed between hyperplasia and mild dysplasia; GI, mild dysplasia; GII, moderate dysplasia.

telomerase activity was then recorded as <100, 100-1000, or >1000, indicating whether the intensity of those bands was less than 100, or between 100 and 1000, or more than 1000 B958 cells, respectively (Fig. 1). The variation in expression of telomerase has been detected in both oral leukoplakias and HNSCCs. Nonetheless, in general the leukoplakias expressed less activity than the cancers. From 10 leukoplakias with positive TRAP assays, six (60%) showed activity less than 100 B958 cells. Only four (40%) exhibited activity more than 100 B958 cells; of these four leukoplakias, two (20%) were between 100 and 1000 cells, and the other two (20%) were more than 1000 B958 cells. Although the cancer samples also showed variation in intensity of TRAP assay, the activity tended to be higher than in leukoplakia. From 14 positive samples, only five (35.7%) demonstrated activity less than 100 B958 cells. Nine (64%) had activity higher than 100 cells, four (28%) with 100-1000, and the other five (35.7%) with more than 1000 B958 cells (Table 1).

Telomerase Activity and Clinical Data in Oral Leukoplakia and HNSCC. The clinical data, histological findings, and TRAP assay of the oral leukoplakia and HNSCC patients are summarized in Tables 1 and 2. Telomerase activity was examined in 16 HNSCC tissues, which included 13 squamous cell cancer tissues, two verrucous cancer tissues, and one nasopharyngeal cancer WHO type III with positive Epstein-Barr viral infestation shown by PCR assay (data not shown). The enzyme activities were detected in all but two cases. Both negative cases were from well-differentiated squamous cell cancers.

Ten samples from the 26 premalignant tissues (38.5%) exhibited positive TRAP results. The presence of telomerase activity was associated with the more progressive (Table 2). Eighteen hyperplastic lesions were tested. Five of them had a history of recurrence after previous laser surgery in which two also had leukoplakia samples collected prior to surgery (CU2 and CU18). Five of the 18 (27.3%) hyperplastic lesions were positive. Three of the five (60%) recurrent hyperplasias showed positive TRAP results. Interestingly, one of the three cases (CU2), the sample prior laser surgery, showed negative telomerase activity. The hyperplastic oral epithelia without history of previous surgery demonstrated fewer positive TRAP results in only 2 of 13 samples (15.8%). The dysplastic lesions demonstrated a higher frequency of detectable telomerase activity. Six samples were tested, one with moderate dysplasia (GII), two with mild (GI), and three with a mix between mild dysplasia and hyperplasia (GI/G0). Five of these (83.3%) demonstrated telomerase activity. In only one sample with GI/GI, the TRAP assay was negative. Telomerase activity was more frequently detectable in the erythroplakia samples than in leukoplakia. All three erythroplakia tissues demonstrated positive TRAP assay (100%). On the contrary, only 7 of 23 (30.4%) leukoplakias showed positive results. No obvious correlation was demonstrated with age, sex, history of risk factor exposure (data not shown), location of leukoplakia, and onset of the diseases.

DISCUSSION

This study has demonstrated that telomerase activity was detectable not only in almost all (87.5%) of the HNSCCs but also in 38.5% of premalignant lesions, such as oral leukoplakias. In leukoplakia, positive activities were associated more frequently with dysplasia (83.3%) than with hyperplasia (27.3%). This result may indicate that telomerase mainly expresses in late head and neck squamous cell carcinogenesis but prior to a fully developed cancer phenotype. Telomerase activity also had positive correlation with erythroplakia samples. Interestingly, erythroplakia has been shown to have a malignant transformation rate higher than that of leukoplakia (4, 7).

Nevertheless, these data also indicate that telomerase is not always activated during the dysplastic stage. Not all malignant and dysplastic lesions exhibited telomerase activity. This suggests that telomerase

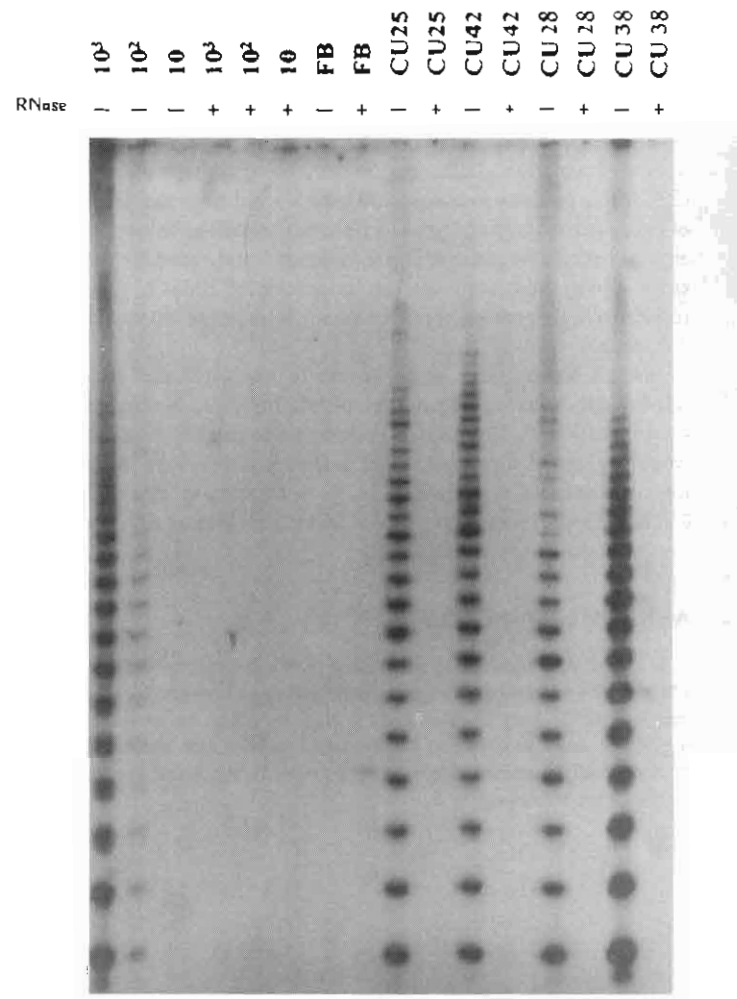

Fig. 1. Telomerase activity in leukoplakia and HNSCC. + and –, with and without RNase pretreatment, respectively. Extracts of Epstein-Barr virus-immortalized human lymphocyte cell line B958 of 10^3, 10^2, and 10 cells were used as the positive controls. Normal cultured fibroblast (*FB*) was the negative control. Leukoplakia (*CU25* and *CU42*), and HNSCC (*CU28* and *CU38*) were assayed in an aliquot containing 6 μg of protein. All cancer and leukoplakia samples showed detectable telomerase activity.

have demonstrated similar results (14, 16, 17).

Some hyperplastic lesions demonstrated telomerase activity. This might be due to the presence of contamination with dysplastic cells, which were not visualized under a light microscope but were detectable by TRAP assay. However, it is possible that telomerase activity is present in some premalignant cells without dysplastic change. It is interesting to note that the detection of telomerase activity was more frequent in cases with a history of recurrence after laser surgery, although one previously showed negative activity. This might be the result of the selective advantage of previously unidentified immortalize cells. However, it is also possible that the surgery indirectly induced a number of cell divisions and consequently an increased chance of telomerase activation.

Because leukoplakia is often associated with inflammation in the submucosa, it is possible that telomerase activity of normal lymphocytes associated with these lesions may confound the interpretation of the results. However, this should be unlikely because normal lymphoid organs, *e.g.,* lymph node and spleen, had no detectable TRAP assay, and these should represent telomerase activity of normal lymphocytes (22, 23).

This study suggests that telomerase is frequently activated at late-stage carcinogenesis of HNSCC. Nevertheless, the onset of activation can vary considerably starting from hyperplasia to late-stage cancer. Moreover, some cancers may not require immortalization during multistep carcinogenesis. How this enzyme is activated during tumorigenesis is not yet elucidated. A previous complementation study suggested that immortalization was recessive and of genetic heterogeneity (24). Immortalization during tumorigenesis may be a consequence of mutation(s) occurring in the telomerase repression pathway (23). If this pathway is unique and independent from the development of invasive cancer phenotypes, cellular immortalization can develop at any step of carcinogenesis. The significant correlation between telomerase activity and late-stage carcinogenesis of HNSCC may be the result of a higher mutation rate involving telomerase repression during this stage.

Not all leukoplakias will convert to cancer. Some lesions are self-limited, whereas others may persist for years before malignant transformation (9, 10). Thus, it is desirable to identify biomarkers that would be useful for detection of precancerous lesions. Because telomerase activity is detectable in the intermediate step of HNSCC carcinogenesis, it may serve as a biomarker for cancer risk assessment in patients with oral leukoplakia.

ACKNOWLEDGMENTS

We are deeply indebted to the staff of the Department of Otolaryngology, Chulalongkorn University Hospital, for recruiting patients and for collection of materials; to Dr. Jerry W. Shay and Dr. Nam Woo Kim for providing us with the TRAP assay protocol; to Dr. Piyarat Tosukhovongs and Dr. Parvapan Bhattarakosol for technical advice; and to Dr. Henry Wilde for his critical review of the manuscript.

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Loss of Heterozygosity for Chromosome 11 in Epstein-Barr-Virus Associated Nasopharyngeal Carcinoma

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Nasopharyngeal cancer (NPC) is a subset of head and neck squamous cell cancers (HNSCC) with unique endemic distribution and etiologic co-factors[1-3]. Although NPC is rare among European and North American Caucasians, it is one of the most common cancers in Southern China and among Eskimos in Arctic regions where it has an incidence of 20 to 50 per 100,000 males. An intermediate incidence is noted in Southeast Asia. Several etiologic factors have been identified for NPC. These include the Epstein-Barr virus (EBV), environmental factors, genetic susceptibility, and human leukocyte antigens[1-4]. EBV has been considered a crucial factor for NPC clonal evolution especially for NPC type II and III, WHO classification[5]. Several genetic changes in NPC were also reported[3,6,7]. Loss of heterozygosity (LOH) of chromosome 3p and 9p were common events[6,7]. In addition, homozygous deletion on chromosome 9p21-22 was detected in some cases[6]. Certain oncogenes and tumor suppressor genes have been studied in NPC. Bcl-2, c-myc, ras and p53 has been displayed[7-10]. Thus it is tempting to hypothesis that during NPC development, EBV acts during initiator as well as promotion to introduce multiple genetic changes leading to cancer. Study roles of EBV and genetic alterations in NPC development are a necessary step towards understanding it.

The goal of this project was to describe which loci of chromosome 11 exhibit LOH in NPC. Allele imbalance on chromosome 11 loci is a frequent event in several cancers, for example, lung cancer, head and neck squamous carcinoma, breast cancer and bladder[11-14]. LOH studies on chromosome 11 have revealed at least three genomic regions, 11p13 and 11q23 that commonly undergo loss in solid tumors[15]. However, thus far there have not been any reports regarding the possible involvement of this region in NPC.

In this study we used a panel of short tandem repeats polymorphic markers (STRPs) to investigate region showing LOH from NPC on chromosome 11.

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MATERIAL AND METHOD

Tissues and DNA Extraction

Primary NPC tissues and blood were collected from 25 patients prior to treatment at Chulalongkorn University Hospital. Tissues were divided into two parts. The first part was sent for routine histological examination. The second part was immediately stored in liquid nitrogen until used. The biopsied samples were selected for further analysis if they were histologically diagnosed as NPC according to the WHO classification and if they contained neoplastic cells at more than 70 per cent of the total tissue. DNA was extracted from tumor tissue and blood by the method previously described[16].

EBV detection by PCR

For the detection of EBV DNA in tumor tissue, a previously described PCR protocol was used with some modifications[4]. Cell line B958, EBV transformed human lymphocytes (American Type Culture Collection) were used as positive controls and the patients' leukocytes as negative controls. Two sets of primers were used. The first was 5'GTAGAAGGCCATTTTTCCAC3' and 5'CTCCATCGTCAAAGCTGCA3', amplifying the non-polymorphic EBV nuclear antigen 1 (EBNA-1) and generating a product of approximately 610 bp. The second was a human b-actin genomic sequence, 5'ATCATGTTTGAGACCTTCAA3' and 5'CATCTCTTGCTCGAAGTCCA3' which generated a product of approximately 318 bp.

The duplex PCR reaction was performed in a total volume of 20 µl using 50 ng of DNA with the following final concentrations: 200 µM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (*p*H 9.0), 0.1% Triton X-100 and 0.5 units of Thermus aquaticus DNA polymerase (Promega). The final concentration for each primer was 0.5 µM. For the PCR reaction, initial denaturation was at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, with an extension at 72°C for 1 min and a final extension at 72°C for 7 min. The PCR products were then analyzed on a 2 per cent agarose gel. The DNA from NPC tissues which presented EBV genomes was used for allelotyping.

Allelotyping

Seven STRPs for PCR analysis were tested, two STRPs on 11p13, WT1 and D11S554, three STRPs on 11q13, D11S534, D11S956 and INT2 and two STRPs on 11q23, D11S976 and D11S897.

One strand of each primer pair was end-labeled for 1-2 hours at 37°C in a volume of 10 µl containing 10 µM of primer, 0.025 mCi [32p] ATP (Amersham) at 3,000 Ci/mmol, 10 mM $MgCl_2$, 5 mM DTT, 70 mM Tris-HCl (*p*H 7.6), and 10 units of T4 polynucleotide kinase (New Englands). Without further separation of the unincorporated nucleotides, the kinased reaction was added to the PCR buffer mix.

The PCR reaction was performed in a total volume of 10 µl using 50 ng of genomic DNA in 200 µM each of dNTPs, 10 mM TrisHCl (*p*H 8.4), 50 mM Kcl, 1.5 mM $MgCl_2$ and 0.5 units of Thermus Aquaticus DNA polymerase (Perkin Elmer Cetus). Each of the primer pairs was performed in optimal concentration, 0.2 µM for WT1, D11S554, D11S534 and D11S976, and 0.3 µM for D11S956, D11S897 and INT2.

Two PCR reactions have been optimized for each primer set. For WT1, D11S534, D11S956, INT2 and D11S976, the initial denaturation step was 95°C for 4 min then followed by 25 cycles of denaturation at 94°C for 1 min, with 1 min annealing at 55°C for reaction 1 extension at 72°C for 2 min and a final extension at 72°C for 7 min.

For D11S554 and D11S897 the 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, with 1 min annealing at 60°C, 59°C, 58°C, 57°C, and 56°C respectively, extension at 72°C for 2 min and 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, with 2 min extension at 72°C and a final extension at 72°C for 7 min.

Two µl of each reaction was mixed with 1 µl formamide-loading buffer, heated at 95°C for 2 min, put on ice for 30 sec and then loaded onto 6 per cent polyacrylamide/7 M urea gel. DNA fragments were size-fractionated at 70 watts until the tracking dye reached the appropriate point of the gel. After electrophoresis, the wet gel was wrapped with plastic wrapping films and exposed to Kodak T-mat X-ray film for 6-24 hours at -70°C with an intensifying screen.

RESULTS

Twenty five EBV-associated-NPC samples were selected for allelotyping. All biopsied tissues

Table 1. STR results on 25 NPC patients.

Locus	Location	Tumours number																									
		11	18	19	24	31	35	38	44	45	46	47	50	51	53	56	57	67	70	72	76	83	86	102	103	105	LOH %
WT1	11p12	U	U	+	N	-	-	+	U	+	U	-	U	+	+	-	U	-	-	-	U	U	-	U	-	U	30.4
D11S554	11p	-	-	-	N	N	-	U	+	-	-	-	U	-	N	-	-	N	-	N	-	-	-	+	-	-	
D11S534	11q13	U	-	-	N	-	U	N	-	-	N	U	U	-	U	+	-	U	U	-	-	-	U	-	-	-	
D11S956	11q13	U	-	U	U	N	-	+	N	-	-	-	-	N	+	+	N	+	-	-	-	N	-	-	U	+	20
INT2	11q13	-	-	-	-	-	U	N	-	U	U	-	U	-	U	+	U	+	-	-	-	-	-	-	-	-	
D11S976	**11q23**	U	-	U	N	N	+	+	+	N	N	N	+	U	N	N	-	N	N	N	N	N	-	+	U	+	45.5
D11S897	**11q23**	-	-	-	-	-	U	+	N	+	U	-	U	-	+	U	U	+	+	-	-	U	-	+	-	+	

U: uninformative, N: not done, +: positive for LOH, -: negative for LOH

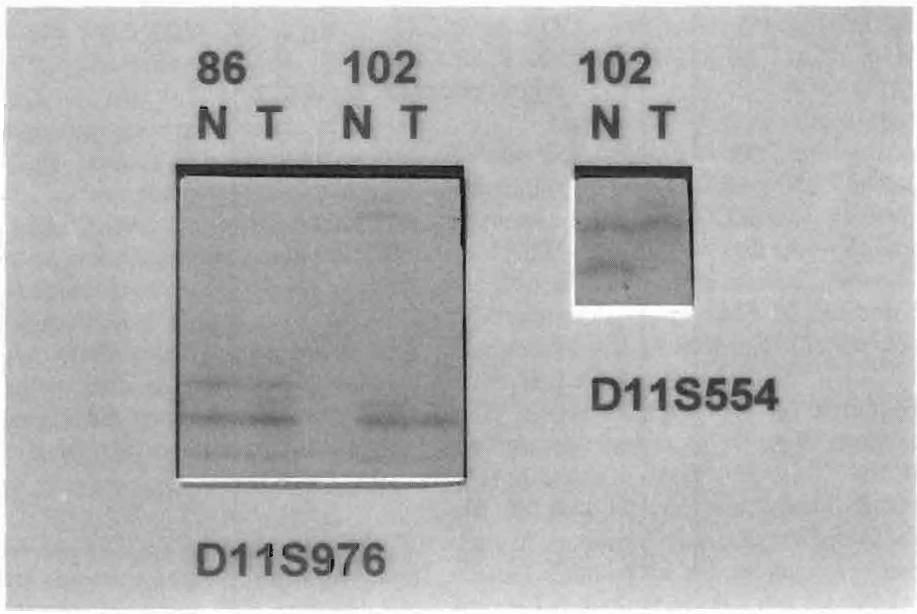

Fig. 1. Autoradiograph showing loss of heterozygosity on analysis with STRs. Representative NPC tumours (T) and corresponding normal leukocytes (N) are shown with STR indicated on the bottom. Marker D11S976 reveals losss of upper band on patient 102 and the allele was retained on case 86. Marker D11S554 reveals LOH on patient 102.

were histologically confirmed as tumors. Among these 25 cases, 16 were WHO type II and the others were WHO type III.

A panel of 7 STRPs representing three loci on chromosomal 11 were used to screen for LOH frequency. Table 1 shows the polymorphic loci used includes the number of positive LOH and informative cases for each marker. Representative results are shown in Fig. 1. Twelve from 25 informative cases (46%) showed LOH for at least 1 locus on chromosome 11. Further analysis of chromosome 11q revealed that LOH loci might be linked to 11q23. Ten from 22 NPC patients (45.5%) manifested LOH on 11q23, using D11S976 and D11S897 microsatellites, while 5 from 25 (20%) had LOH on 11q13, from D11S534 and D11S956.

DISCUSSION

Inactivation of both tumor suppressor gene alleles results in abnormal cellular growth, proliferation, and differentiation which are necessary for multistep cancer progression. This process requires two steps, called, the two hits hypothesis. The first hit is to inactivate one allele. The second hit mostly occurs through chromosomal alterations leading to the loss of the other normal allele as well as the nearby chromosomal genome. Using linked polymorphic markers, restriction fragment length polymorphism (RFLP) or microsatellite, will recognize the chromosome changes as a loss of one allele in tumor tissue, LOH[17,18].

Several areas of chromosomal losses are frequent events for cancer progression and/or correlated with the histopathology, staging and clinical outcome of cancers[19]. There are at least three loci on chromosome 11 that are crucial for cancer development. Chromosome 11p posses the Wilms' tumour 1 (WTI) gene. The MEN-1 locus has been mapped to the 11q13 region[20] and the ataxia telangiectasia (AT) genes have been mapped to the 11q22-23 region[21]. In addition, 11q13 is a chromosome region with high frequency of amplification in HNSCC[22]. This results in allelic imbalance and may be difficult to distinguish from LOH. Here we demonstrated that significant LOH on chromosome 11 in NPC. While LOH on 11q13 is less significant, more frequent LOH was detected on 11p and 11q23. Thus it is possible that the AT

gene and WT1 may be significant for NPC development.

SUMMARY

In order to demonstrate and define possible tumor suppressor gene loci on chromosome 11 associated with NPC, we used 7 STR to test for LOH on 25 NPC samples. LOH was detected in 46 per cent of cases. Most LOH loci were clustered on the long arm. Further study demonstrated 22 per cent and 45.5 per cent of cases with LOH on 11q13 and 11q23 respectively.

ACKNOWLEDGEMENT

This work was supported by the Rachadapisek Sompoj China Medical Board research fund, the Thailand Research Fund, and Molecular Biology Project, Faculty of Medicine, Chulalongkorn University.

The authors are deeply indebted to the staff of the Department of Otolaryngology, and the Radiotherapy Section, Department of Radiology, Chulalongkorn University Hospital for the recruitment of patients, and collection of materials, to Mrs Bungon Changchup and Mr. Anucha Karnthaworn for technical assistant.

(Received for publication on September 2, 1996)

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