Discussion

We have established that chromosome 14q11, 14q12-13, and 14q32-qter represent three minimal LOH regions in nasopharyngeal carcinoma. Two regions, chromosome 14q12-13 and 14q32, have also been demonstrated to be lost in ovarian and bladder carcinomas (14,15). Thus our finding confirmed the presence of tumor suppressor genes in these two regions.

In addition to LOH on 14q12-13 and 14q32, we demonstrated a third region of allelic loss to be the TCRD locus. This could indicate another tumor suppressor gene linked to TCRD. Alternatively, our data suggested that there might be a deletion hot spot between TCRD and D14S283. Consequently, LOH in this region might be secondary to chromosomal rearrangement.

Chromosomal rearrangement of 14q11 was commonly found in hematologic malignancies especially in acute lymphoblastic leukemia (16-18). These translocations had breakpoints occurring within the TCRD locus which were probably mediated by an illegitimate V(D)J recombination (16-18). Our data suggested a specific chromosomal breakage within or near the TCRD locus in NPC. It would be interesting to investigate whether this rearrangement in NPC involves the V(D)J recombinase or if genetic alterations in NPC bear any similarity to hematologic malignancies on the molecular level.

Chromosome 14q LOH has also been shown to relate to the development of several forms of cancer, including meningioma, renal cell carcinoma, neuroblastoma and gastric carcinoma (10-13). In addition, 14qLOH was demonstrated to correlate with colorectal carcinoma metastases (21) and with adverse prognosis for renal cell carcinoma (11). This study demonstrated a high frequency of 14qLOH in NPC. Thus, putative tumor supressor gene loci on chromosome 14 might play a relatively significant role in NPC development. Although initially there was no significant correlation of 14qLOH with a clinicopathological phenotype, its significance as an independent prognostic factor remains to be determined. Finally, characterization of these tumor suppressor genes and of the mechanism of chromosomal rearrangement on chromosome 14q would be of major importance for the understanding of molecular progression of human neoplasms.

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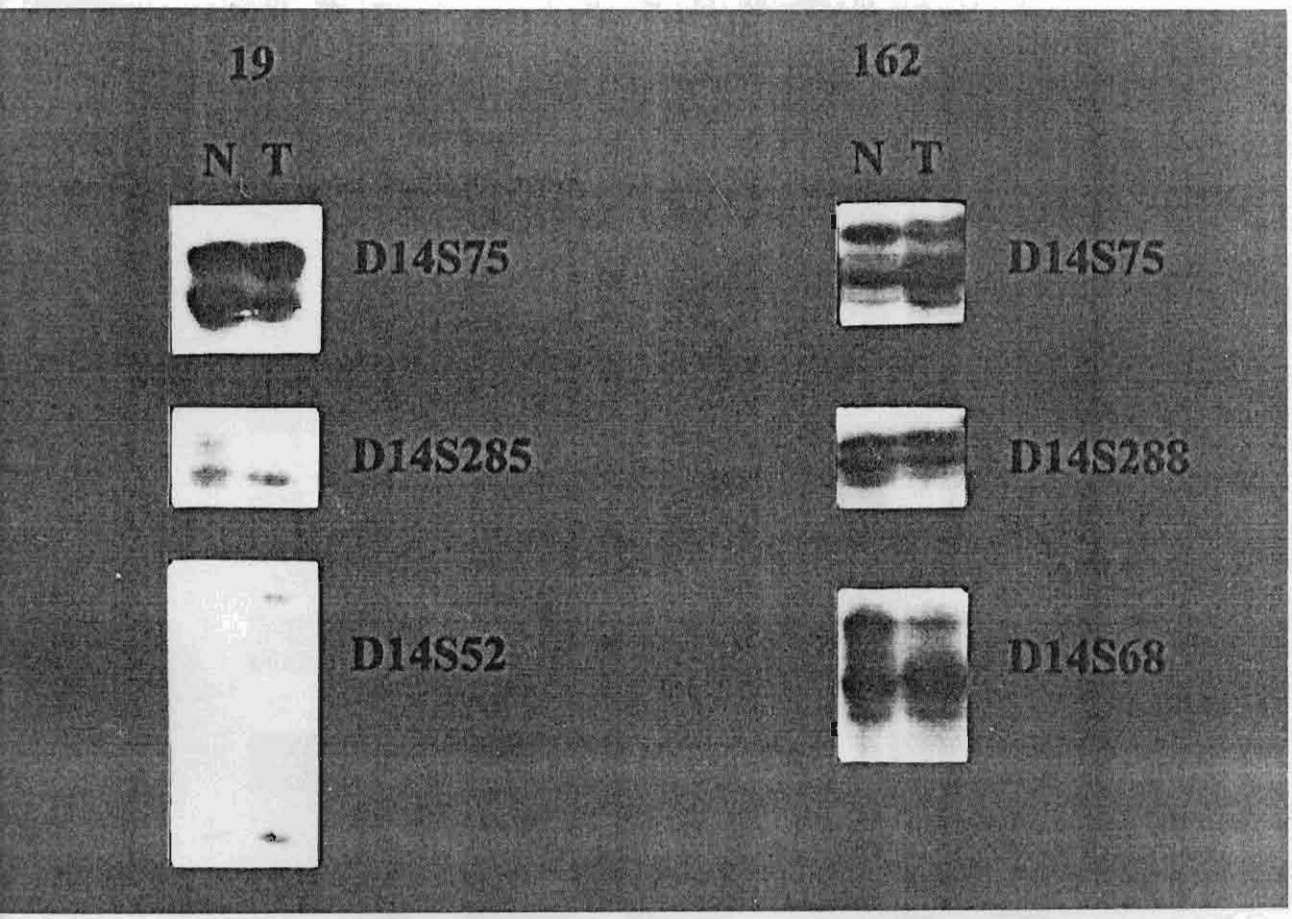
Figure Legends

Fig 1. Microsatellite analysis in selected nasopharyngeal carcinomas showing partial loss of chromosome 14q. Tumor 19 demonstrates retention of both alleles at D14S52 and loss at D14S75 and D14S285, respectively. Tumor 162 demonstrates retention of both alleles at D14S288 and loss at D14S75 and D14S68.

Fig 2. Deletion map of chromosome 14q in nasopharyngeal carcinoma. Cytogenetic location and microsatellite markers (STR) are illustrated on the left. Solid bars on the right indicate the minimal area of loss at 14q11, 14q12-13 and 14q32.

Fig 3. Microsatellite analysis in selected nasopharyngeal carcinoma showing deletion breakage between TCRD and D14S283. Tumor 78, 113 and 183 demonstrate loss at TCRD but retention of both alleles at D14S283.

Figure 1



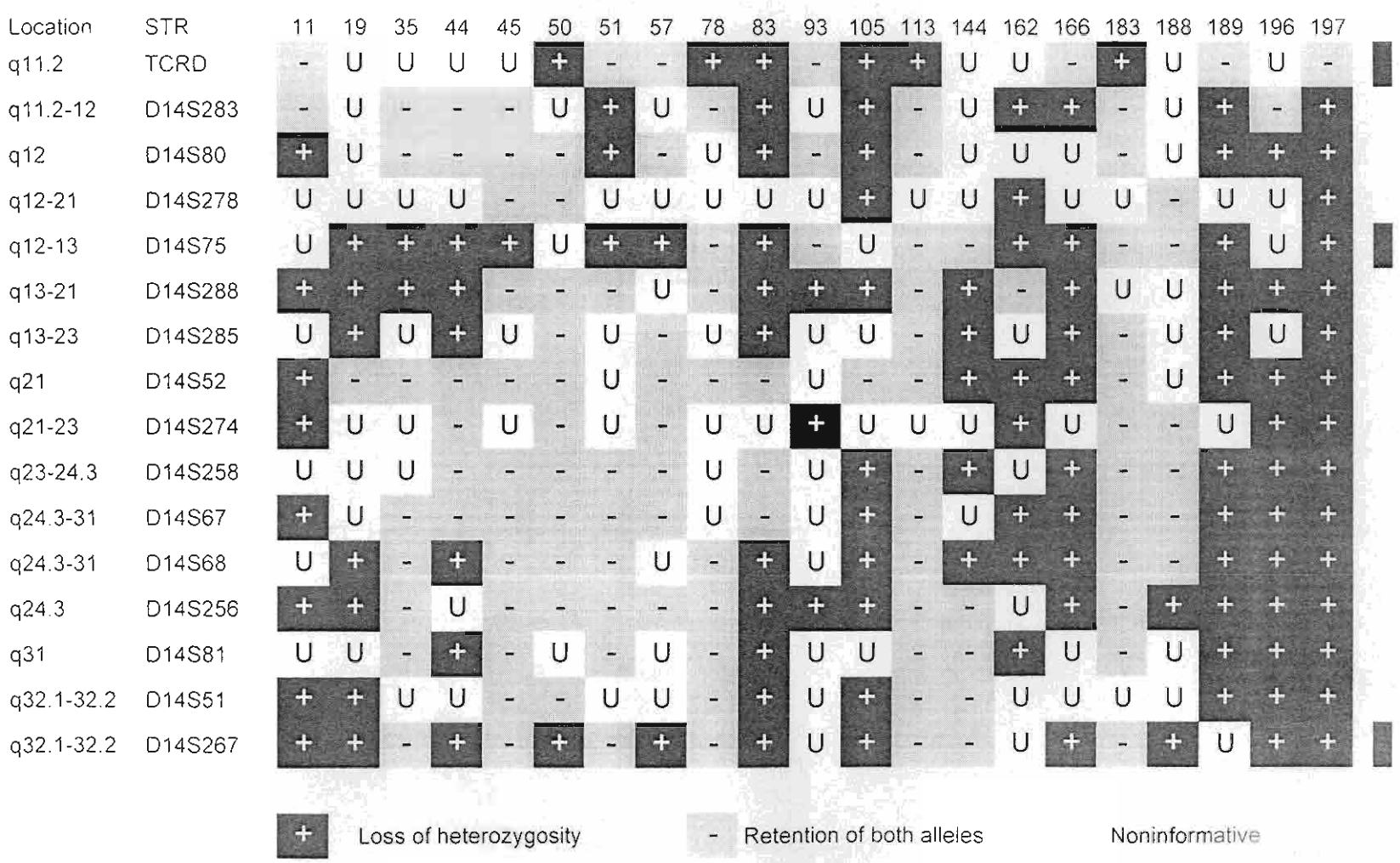
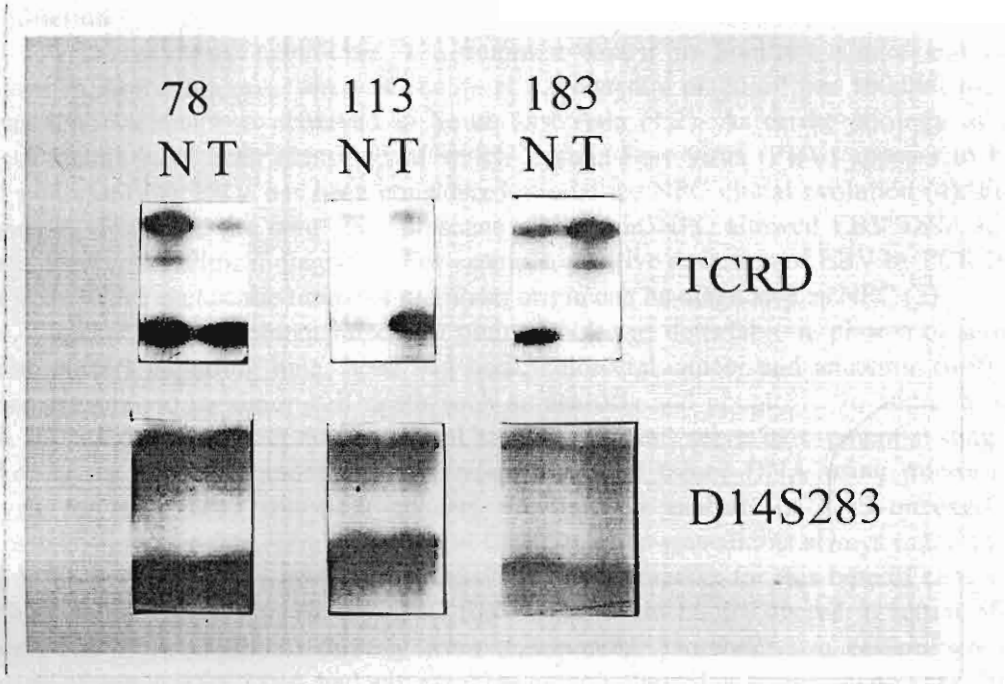

Location
STR
11 19 35 44 45 50 51 57 78 83 93 105 113 144 162 166 183 188 189 196 197
q11.2 TCRD
q11.2-12 D14S283
q12 D14S80
q12-21 D14S278
q12-13 D14S75
q13-21 D14S288
q13-23 D14S285
q21 D14S52
q21-23 D14S274
q23-24.3 D14S258
q24.3-31 D14S67
q24.3-31 D14S68
q24.3 D14S256
q31 D14S81
q32.1-32.2 D14S51
q32.1-32.2 D14S267
+ Loss of heterozygosity
- Retention of both alleles
Noninformative

Figure 3



Chapter 3

EBV DNA in Serum of Patients with NPC

This results presented in this chapter have been taken, in part, from a published manuscript

- **Mutirangura A**, Pornthanakasem W, Theamboonlers A, Sriuranpong V, Yenrudi S, Voravud N, Supiyaphun P and Poovorawan P. Epstein-Barr viral DNA in serum of patients with nasopharyngeal carcinoma.
  Clinical Cancer Research (Submitted).

Introduction

Nasopharyngeal carcinoma is a common cancer in Southern China and among Eskimos in Arctic regions, where it occurs at a frequency of 20-50 per 100,000 men. An intermediate incidence is observed in South-East Asia (1,2). As to the etiology of NPC, several factors have been identified of which Epstein-Barr virus (EBV) appears to be the most important (3). EBV has been considered crucial for NPC clonal evolution (4). From a diagnostic viewpoint, the consistent presence of EBV in NPC allowed EBV DNA to be a genetic marker for clinical diagnosis. For example, positive detection of EBV by PCR from a neck node with a metastatic tumor of unknown origin can be diagnosed as NPC (5).

Recent studies demonstrated that tumor DNA was detectable in plasma or serum of several cancers including lung, head and neck, colorectal cancer and leukemia (6-9). This finding may relate to tumor cell death such as necrosis and apoptosis. In addition, tumor DNA in serum may reflect some clinical significance and serve as a potential diagnostic marker in the future. These previous studies identified tumor DNA using microsatellite analysis for lung, head and neck cancers, or mutation analysis of RAS oncogenes for colorectal cancer and leukemia. Since latent EBV infection was almost always found in NPC, EBV DNA may serve as a specific and sensitive genetic marker for this type of cancer (10). In the course of this study, we tested if EBV DNA could be discovered in serum of NPC patients and if it originated directly from tumor cells. Moreover, we determined if the presence of serum EBV DNA had any association with clinical or tumor phenotypes, such as staging, invasion, metastasis and tumor cell death, especially apoptosis.

Materials and Methods

Sample Collection

Primary NPC tissues were collected from 46 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 tumors were histologically ascertained to be undifferentiated NPC according to the WHO classification. The 46 tumors included stages ranging from I to IV.

Blood samples were obtained by venipuncture from the same patients and 84 healthy blood donors. To obtain serum, clotted blood specimens were centrifuged at low speed for 5 min, and the serum was stored at -20°C before use.

DNA Isolation

NPC tissue was treated with SDS and proteinase K at 50 °C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA (11). Serum DNA was purified on Qiagen columns (Qiamp Blood Kit, Qiagen, Basel, Switzerland) according to the "blood and body fluid protocol". Serum (2-3 ml) was passed on the same column. 1/10 of DNA extracted was then used for PCR analysis.

ELISA Test

Serum from healthy blood donors was examined for previous EBV infection. For detection of antibody of the IgG class to EBV viral capsid antigen (anti-EBV-VCA IgG) a commercially available ELISA kit (Human, Gesellschaft fur Biochemica und Diagnostica mbH) was used.

EBV Detection and Typing by PCR

For the detection and typing of EBV DNA in the tumor tissues, three previously described polymerase chain reaction (PCR) protocols were used (5,12,13). DNA from cell line B958, 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 fragment. The second amplified a human β-actin genomic sequence, generating an approximately 310 bp DNA fragment (5).

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 antigen 3C (EBNA-3c) generating a DNA fragment of 153 bp for EBV type A and of 246 bp for EBV type B. (12,13). Only EBNA-2 was used for detection and typing of EBV DNA in serum due to the quality of the PCR product. Primers GH20 and PCO4 were used to amplify β-globin in order to test the presence of amplifiable human DNA in all sera (14), generating a DNA fragment of 260 bp. The oligonucleotide sequences for all sets of PCR primers were identical to the ones previously reported.

The PCR reactions were performed in a total volume of 20 μl using 50 ng of the corresponding tumor DNA or 1/10 total DNA extracted from 3 ml serum 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 and 0.5 μM of each primer. The PCR amplification was performed as follows: initial denaturation at 94 °C for 5 min, followed by 40 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 analyzed using 2% agarose gel electrophoresis.

To confirm the presence of EBNA-2 PCR product in the serum DNA tested, the gels were subsequently transferred to a Hybond N^+ membrane (Amersham Corp.) applying a routine Southern blot protocol, and the membrane was hybridized to EBNA-2 common probe that had been end-labeled with ^{32}P using T4 polynucleotide kinase (13).

DNA Fragmentation

10 μg of tumor DNA were treated with 50 ng of RNaseA for 30 min. The DNA was then ethanol precipitated, resuspended in 10 μl of TE and analyzed on a 1.8% agarose gel run in 1xTBE at 50V for 3 hrs.

Correlation between Clinical Data, Apoptosis and Serum EBV DNA

Data regarding histology, tumor staging, EBV detection and typing, and apoptosis were collected in a double-blind fashion until analyzed. Fisher's exact test was used to compare the results from serum analysis with clinical and tumor parameters.

Results

EBV DNA in Serum

In order to determine whether EBV DNA can be detected in sera of NPC patients and whether it represents tumor DNA, DNA from sera of two groups was studied. The first one

comprised 46 NPC patients and the other one 84 healthy blood donors (Table 1 and 2). The average age of NPC patients was 43.2 years, ranging from 16 to 81 years. Twenty six of them were male and 20 were female. All the primary tumors and sera of these patients were tested for EBV infection (Figure 1, 2 and 3). All the tumors showed positive results with 44 cases infected with type A and the other two cases with type B, respectively (Table 1, Fig 1). Nevertheless, only 13 serum samples were positive for EBV. Interestingly, these 13 cases exhibited an EBV type identical to the one encountered in the primary tumors, 11 of which were type A and 2 type B, respectively (Table 1, Fig 1 and 2). This observation suggests that serum EBV DNA originates from NPC and thus can be used as a marker for the tumor DNA.

In order to exclude the possibility of having obtained serum EBV DNA from other latently infected cells, sera of 84 blood donors were tested (Table 2). The average age was 31.8 years, ranging from 19 to 59 years. Thirty-two individuals were female and the others were male. Almost all of them, 82 out of 84 cases, tested positive for anti-EBV-VCA IgG suggesting previous infection. However, none of them tested positive for serum EBV DNA.

Serum EBV DNA, Apoptosis and Clinical Correlation.

In order to elucidate importance and meaning of NPC DNA present in serum, the correlation between the presence of serum EBV DNA, clinical data and programmed cell death was established (Table 1). In 4 of the 46 cases, β-globin could not be amplified and they were therefore excluded from the correlation study. Clinical data including age, sex, staging, invasion and metastasis are included in table 1. None of them seemed to have a statistically significant correlation with the presence of EBV DNA in serum.

Since serum or plasma DNA has been hypothesized to be due to the leaking of DNA from dead cells (15), we explored whether apoptosis relates to the presence of tumor DNA in serum of NPC patients. All 42 tumor DNAs were tested for DNA fragmentation and 10 of them were positive (Table 1, Fig 4). Like serum EBV DNA, the apoptosis could not be correlated with any clinical data in a statistically significant manner. However, apoptosis and the serum EBV DNA were correlated with a p-value of 0.046. Thus, the presence of NPC DNA in serum is significantly associated with apoptosis of the tumor.

Discussion

Though the presence of EBV in tumor tissue is unique to NPC, especially in Southern China and South East Asia, EBV infection is very common worldwide (16). In addition, a recent study showed that EBV infection is very common in the Thai population and that it happens early in life (17). Primary infection with EBV usually leads to clinical manifestations ranging from mild self-limited illness to infectious mononucleosis. Most EBV infection in human originates in the oropharyngeal epithelium. These cells are permissive for virus replication. A persistent active lytic infection can continue at some level for many years. After the primary infection, EBV can diffuse across the basal membrane and cause latent infection in B lymphocytes. This infection is important regarding the dissemination of infection to distal epithelial surfaces such as the nasopharynx (3). This study identified EBV DNA in the sera of 13 out of 46 NPC patients. In addition, EBV typing from sera and primary tumor DNA yielded identical results suggesting the EBV DNA in sera originating from tumors. To prove that serum EBV DNA did not originate from other latently infected cells, such as the oral epithelium or B lymphocytes, we tested a number of healthy subjects previously infected with EBV. None of their sera was positive for EBV DNA and consequently, the EBV DNA found in serum must have originated from NPC cells.

How tumor DNA comes to be present in serum is not yet known. Nevertheless, plasma or serum DNA could originate from dead cells (15). In order to investigate whether NPC DNA in serum is associated with tumor cell death, we studied the correlation between apoptosis and serum EBV DNA and founded it statistically significant. Among 13 cases positive for EBV DNA, 6 cases showed apoptosis while there were 4 other cases with

apoptosis but without serum EBV DNA. Thus it is possible that the cases positive for serum EBV DNA but not undergoing apoptosis might experience some degree of necrosis and therefore have tumor DNA leaking into serum. In an apoptotic event, the cytoplasm of dead cells would be phagocytosed by macrophages (18). In any case, PCR for serum EBV-DNA might be a very sensitive method capable to detect some cases of tumor DNA leaking from the phagocytic process.

This study showed no correlation between serum EBV DNA and/or apoptosis and other clinical phenotypes including sex, age, staging, invasion and metastasis. Prospective trials will be necessary to determine whether serum EBV DNA is an independent prognostic factor. Nevertheless, since the serum EBV DNA may be associated with tumor cell death, this PCR method could be used for follow up regarding the rate of tumor regression or as a marker to determine the response to radiation and chemotherapy.

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Table 1. Serum EBVDNA, Apoptosis and Clinical Staging of 42 NPC Patients

	Total	Serum EBVDNA		p value
	[x]	+	-	
NPC patient	42 [4]	13	29	
EBV type (A:B)	(40:2)	(11:2)	(29:0)	0.09
Age <40:40-60: >60	13:18:11	2:6:5	11:12:6	
Sex (M:F)	23:19	9:4	14:15	0.32
Stage (I:II:III:IV)	2:5:9:26	0:2:1:10	2:3:8:16	
WHO typing(II:III)	34:8	10:3	24:5	0.69
Local Invasion (+:-)	17:25	7:6	10:19	0.31
Skull or Nerve Involvement(+:-)	8:34	5:8	3:26	0.08
LN metastasis (+:-)	28:14	10:3	18:11	0.48
Distance Metastasis	0			
Serum EBVDNA (+:-)	13:29			
Apoptosis (+:-)	10:32	6:7	4:25	0.046

[x], number excluded because of negative PCR for β globin;(+), positive; (-), negative; A:B, EBV type A and type B, respectively; <40:40-60: >60, age less than 40, between 40 and 60, and above 60 years, respectively; M:F, male and female, respectively; I:II:III:IV, stage I, II, III and IV, respectively; II:III, WHO type II and III, respectively.

Table 2 Prevalence of anti-EBV-VCA IgG and Serum EBVDNA among healthy Thai controls

Age (years)	No.	No. Positive Anti-EBV	% Positive	No. Positive Serum EBVDNA	% Positive
19-29	41	39	95.1	0	0
30-39	23	23	100	0	0
40-49	13	13	100	0	0
50-59	7	7	100	0	0
Total	84	82	97.6	0	0

Figure legends

Figure 1: PCR genotyping of EBV-infected NPC on a 2% agarose gel stained with ethidium bromide. From left to right: ϕx174 *Hae* III standard DNA size marker. (+) and (-) are PCR products of positive controls, B958 cell line, and negative controls, double distilled water, respectively. Numbers indicate corresponding PCR products of NPC patients. (A) Duplex PCR generating 610-bp and 318-bp DNA fragments of EBNA-1 and human β-actin genomic sequence, respectively. (B) PCR generating 246-bp and 153-bp DNA fragments of EBNA-3C of EBV type B and type A, respectively. (C) PCR generating 184 bp and 168 bp DNA fragments of EBNA-2 of EBV type B and type A, respectively.

Figure 2: PCR genotyping of sera of EBV-infected NPC patients on a 2% agarose gel stained with ethidium bromide. From left to right: ϕx174 *Hae* III standard DNA size marker, (+) and (-) are PCR products of positive controls, B958 cell line, and negative controls, double distilled water, respectively. Numbers indicate corresponding PCR products of NPC patients. (A) PCR generating 184 bp and 168 bp DNA fragments of EBNA-2 of EBV type B and type A, respectively. (B) PCR generating 260 bp DNA fragments of β-globin.

Figure 3: Hybridization after PCR genotyping sera of EBV-infected NPC patients using EBNA-2 PCR products as template and its internal primer as probe. Samples are loaded in the same order as shown in figure 2.

Figure 4: DNA fragmentation of EBV-infected NPC on a 1.8% agarose gel stained with ethidium bromide. From left to right: ϕx174 *Hae* III standard DNA size marker followed by numbers indicating corresponding tumor DNA of NPC patients.

Figure 1

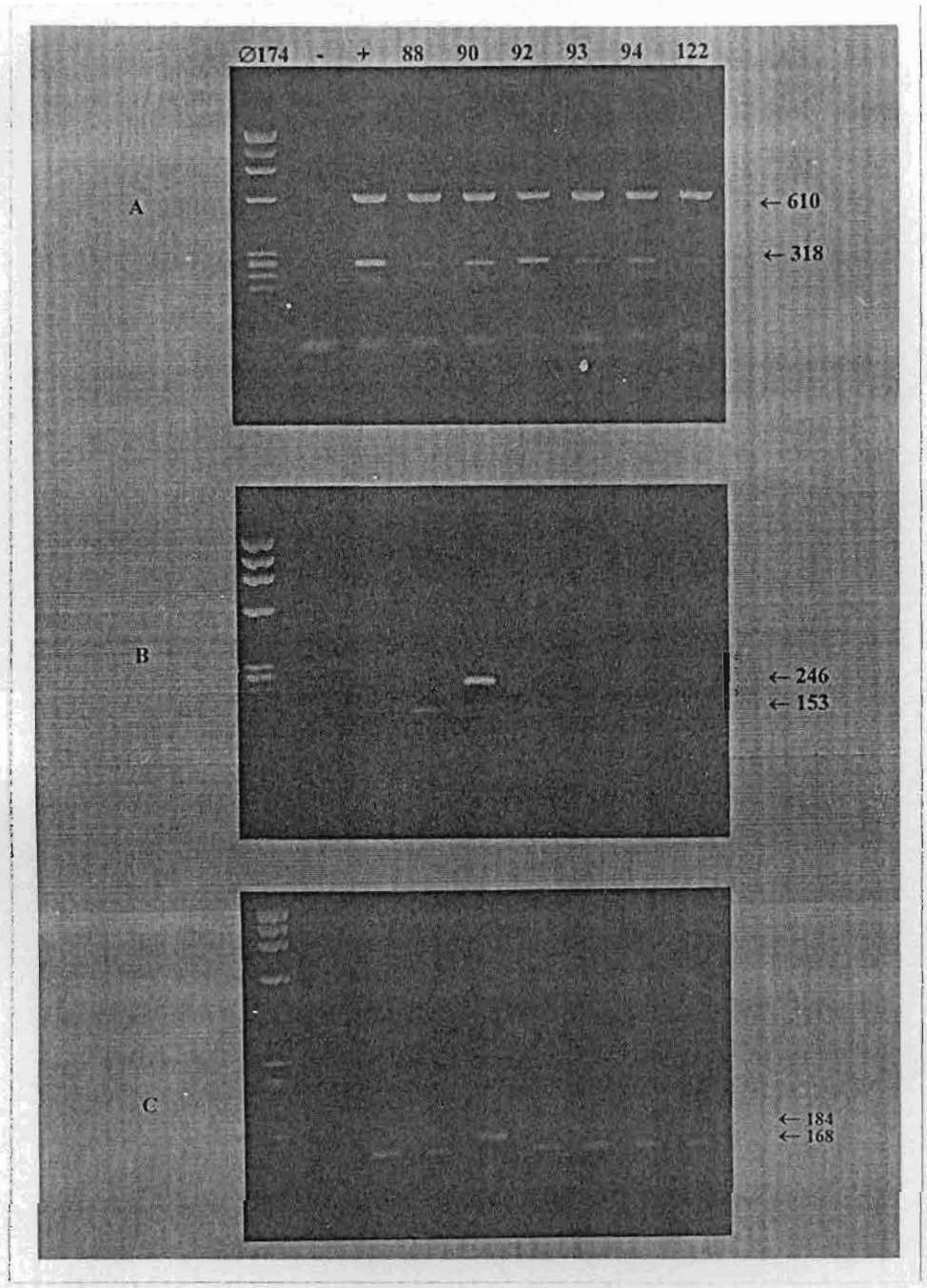


Figure 2

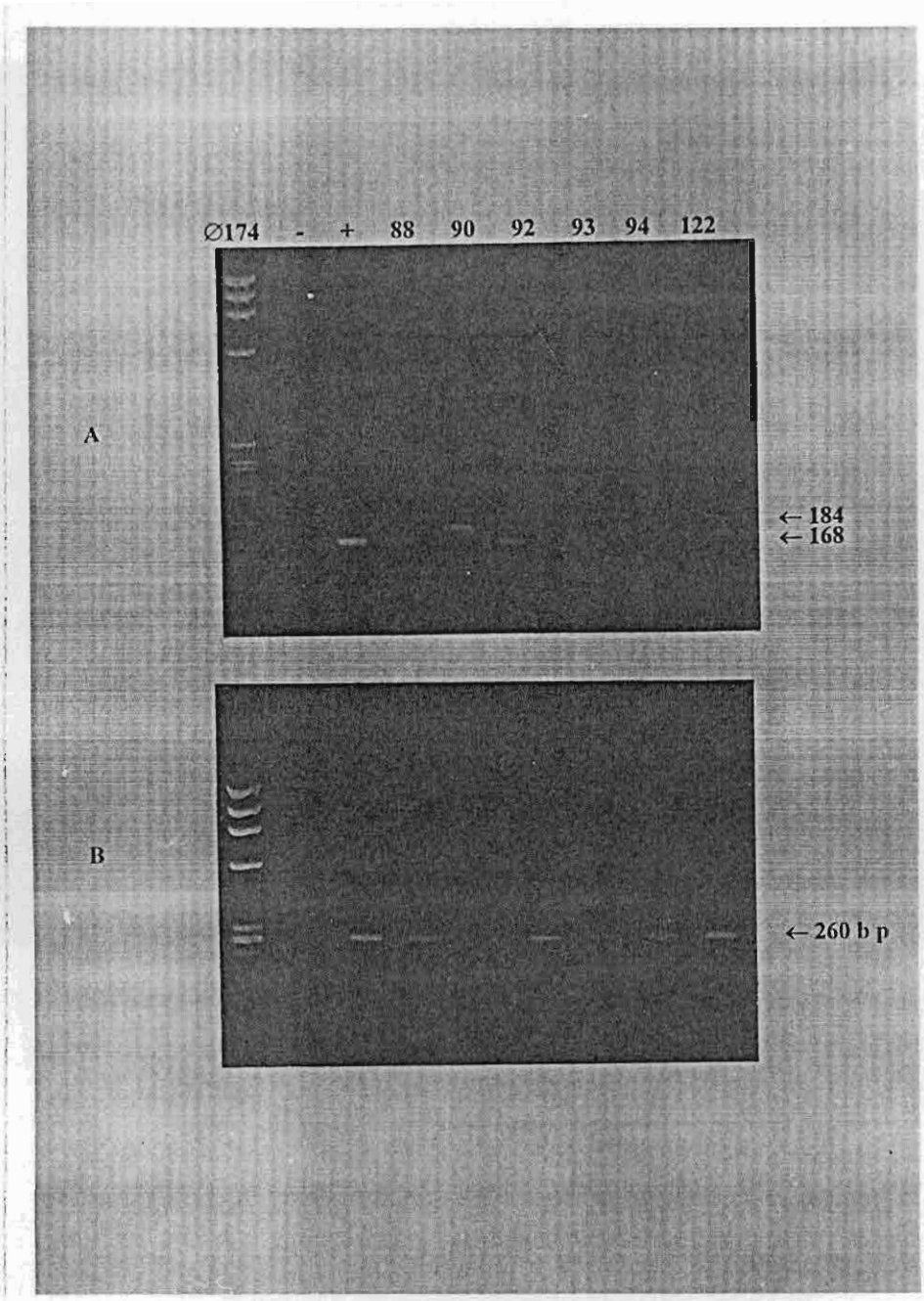


Figure 3

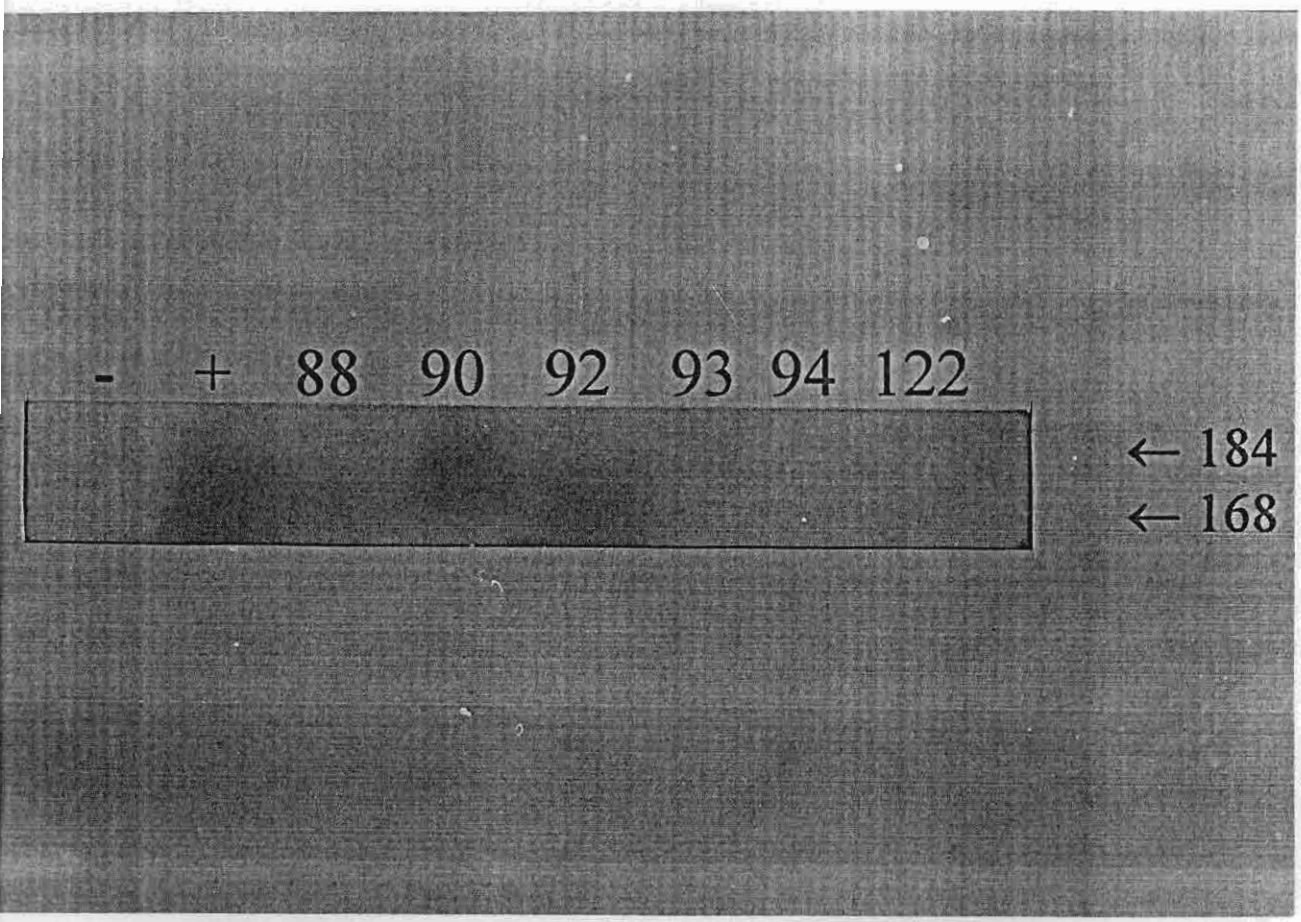
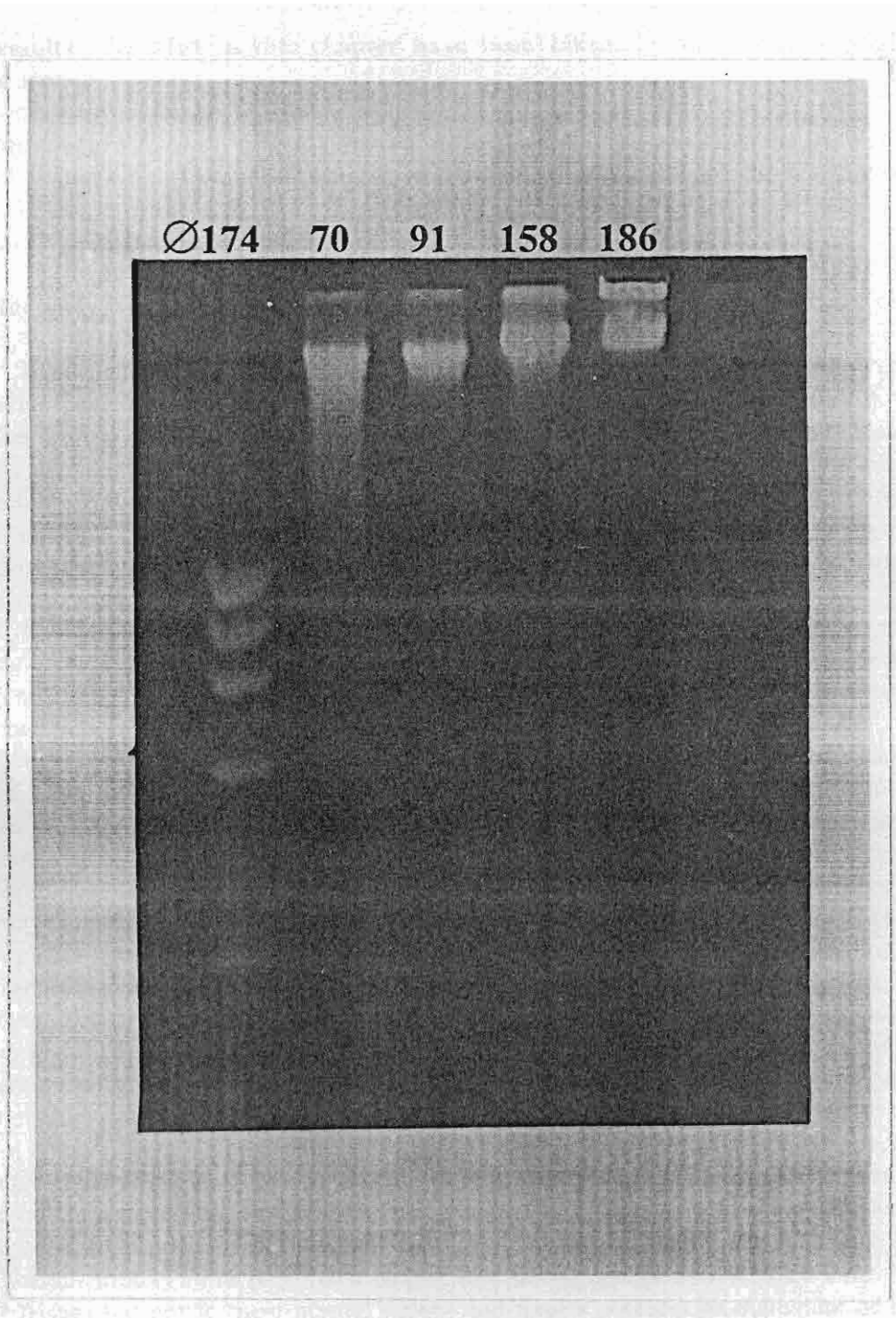


Figure 4



Chapter 4

Telomerase Activity in Oral Leukoplakia and HNSCC.

This results presented in this chapter have been taken, in part, from a published manuscript

- **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.
  Cancer Research; 1996; 56, 3530-3533.

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 head and neck squamous cell cancer (HNSCC) requires multiple steps in which the gradual accumulation of somatic mutations alters cellular growth, proliferation and differentiation and is histologically expressed as a progression from normal epithelium to precancerous lesions and invasive carcinoma (2,3,4). 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 grossly seen as white and red patches or plaques which cannot be classified with other entities (7,8). Both show ranges 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 telomeric repeat amplification protocol (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 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 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 leukoplakia, 16 HNSCC and 18 normal oral tissues adjacent to leukoplakia (14 cases) or cancer (4 cases). These samples were 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 hematoxylin-eosin-stained slides were reviewed by one pathologist (S.Y.) to determine histological differentiation of tissues according to the criteria decribed 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 (cervical intraepithelial neoplasia) classification was used (GI-GIII as compared to CIN I-CIN III that is, mild, moderate, and severe dysplasia). The squamous cell carcinomas were also classified according to the loss of differentiation which the lesions exhibited (UICC classification; G1 for well differentiated, G2 moderately differentiated, and G3 poorly differentiated squamous cell cancer).

Telomerase Assay TRAP was performed as previously described (18). Each sample of 10-100 mg frozen tissue was first washed in 500 μl ice-cold PBS (Ca, Mg free), then homogenized in 20-200 μl of ice-cold CHAPS lysis buffer [10mM Tris-Hcl (pH 7.5), 1mM $MgCl_2$, 1mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% Glycerol, DEPC H_2O] depending on the size of sample, using manual homogenizer. After 30 minutes of incubation on ice, the lysate was centrifuged at 14,000 g for 30 minutes 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). The typical protein concentration of the extract was between 1-10 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 tranformed 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 cell and processed as for tissue extracts. The supernatant, which had a cell concentration of 10^4 cell/μl, was serially diluted 1:10, and 1:100 to obtain standard 10^3 and 10^2 cell/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 (pH8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 μM dGTP, dATP, dTTP and 5 μM dCTP, 0.1 μg of the deoxyoligonucleotide primer TS (5'-AATCCGTCGAGCAGAGTT-3'), 1μg of T4g32 protein (Boehringer Mannheim), 4 μl of α-^{32}p dCTP (10μci/μl, 3,000ci/mmol), 2 U of Taq polymerase (Perkin Elmer) and DEPC 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 barries

(Ampliwax; Perkin Elmer). After 10 minutes 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 minute. Aliquots (5 µl) of the PCR mixture were analyzed on 8% non-denaturing polyacrylamide gel in 0.6 x TBE buffer until the xylene cyanol had migrated 17 cm. from the origin (gel size 20X40 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 1,000 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 leukoplakia and 18 of normal oral tissues adjacent to 14 leukoplakias and 4 cancers (Table 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%) of 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.

Since 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 1,000 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 serial diluted B958 cell line. The dosage of telomerase activity was then recorded as <100, 100-1,000 or >1,000 whether the intensity of those bands were less than 100 or between 100 and 1,000 or more than 1,000 B958 cells, respectively (Fig. 1). The variation in expression of telomerase has been detected in both oral leukoplakia and HNSCC. Nonetheless, in general the leukoplakia expressed less activity than the cancers. From 10 leukoplakia with positive TRAP assays, six (60%) showed activity less than 100 B958 cells. Only four (40%) exhibited activity more than 100 B958 cells, in which two (20%) were between 100 and 1,000 cells and the other two (20%) were more than 1,000 B958 cells. Though 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 100-1,000 and the other five (35.7%) with more than 1,000 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 table 1 and 2. Telomerase activity was examined in 16 HNSCC tissues, which included 13 squamous cell cancer tissues, two verucous cancer tissues and one nasopharyngeal cancer (NPC) 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 5 (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 epitheliums 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. Only in one sample with G0/GI, the TRAP assay was negative. Telomerase activity was more frequently detectable in the erythroplakia samples than in leukoplakia. All three erythroplakia tissues demontrated 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 head and neck squamous cell cancers but also in 38.5% of premalignant lesions, such as oral leukoplakia. In leukoplakia, positive activities were more frequently associated 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 leukoplakia (4,7).

Nevertheless, these data also indicates that telomerase is not always activated during the dysplastic stage. Not all malignant and dysplastic lesions exhibited telomerase activity. This suggests that telomerase activity may be required for most but not all cancers. Previous studies 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 light microscope but 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 even though 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.

Since 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, for example 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) that occurs 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 leukoplakia 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. Since 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.

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