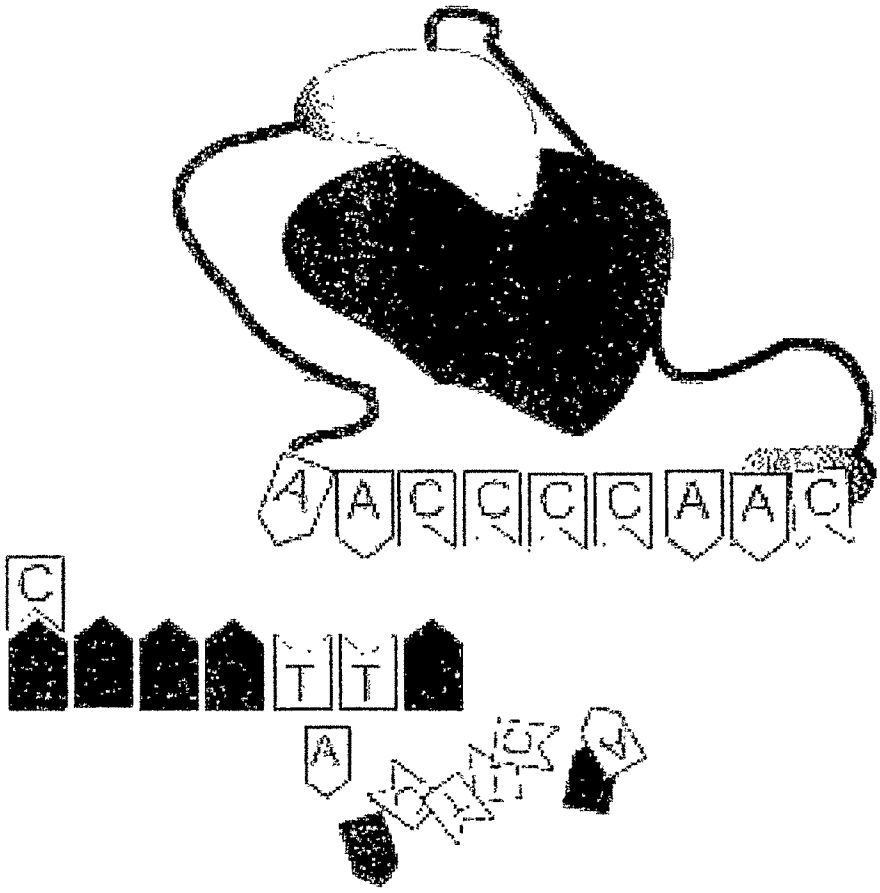


If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

Materials & Methods

One hundred and ten head and neck cancer patients referred to The Gujarat Cancer & Research Institute were enrolled in the study. These included patients with cancer of lip [International Classification of Disease, Oncology section (ICD)-O C00], tongue (ICD-O C02), buccal mucosa (ICD-O C00.3,4, C06.0,1,2), gums (ICD-O C03), hard palate (ICD-O C05.0), floor of mouth (ICD-O C04), pharynx (ICD-O C01, C05.1,2, C09.0,1,9, C10.0,2,3) and larynx (ICD-O C32.0,1,2). The study was approved by the hospital committee. Diagnosis of the disease was established on the basis of clinical, radiological and histopathological examinations by the specialists of the Institute. The patients with any other major illness of any other vital organ were excluded. TNM classification and staging of the disease was done according to the standard criteria of AJCC (1997). The clinical and pathological details of these patients are shown in **Table-1**. The male: female ratio of the cancer patients was 2.5:1. The median age of cancer patients was 48 years. The habit of tobacco consumption was observed in 81.8% of the patients. Majority of the patients i.e. 79.1% had cancer of oral cavity. Histological findings revealed that all of the patients showed squamous cell carcinoma. The patients were followed-up regularly and their clinical findings as well as treatment details were recorded. Information regarding treatment outcome were also collected during follow-ups of the patients.

Table-1

Clinicohistopathological details of the head and neck cancer patients

Site	Lip	3	Floor of Mouth	6
	Buccal Mucosa	38	Tongue	24
	Gums	15	Pharynx	4
	Hard Palate	1	Larynx	19
Age	Range	27-75 years		
	Median	48 years		
Gender	Male	79		
	Female	31		
Tobacco consumption	With habit	90		
	Without habit	20		
Stage	I	4	III	14
	II	14	IV	78
Histopathology	Squamous Cell Carcinoma			
Nodal Involvement	Present	65		
	Absent	45		
Histological Grade	Well differentiated	48		
	Moderately differentiated	45		
	Poorly differentiated	17		
Nuclear Grade	I	44		
	II	52		
	III	9		
	NA	5		

NA: Not available

Fourty patients referred to the hospital as suspected cases of head and neck cancer or precancer and diagnosed as having precancerous or benign conditions were also enrolled for the study as the pathological controls. The precancerous or benign conditions included submucous fibrosis, leukoplakia, erythroplakia and hemangioma. Clinical and pathological details of these patients are shown in Table-2.

Table-2

Clinical details of the patients with precancerous conditions

Age	Range	16 - 70 years
	Mean	43 years
Gender	Male	36
	Female	4
Tobacco consumption	With habit	37
	Without habit	3
Histopathology	Leukoplakia	18
	Submucous fibrosis	17
	Erythroplakia	1
	Hemangioma	4

Tumor tissues were collected on ice at the time of biopsy or surgery. Tissue specimens were selected by concerned surgeon and/or pathologist, immediately after surgical resection. Normal tissues adjacent to the tumors were also collected whenever possible. The adjacent normal tissue was at least 1 cm away from the tumor. Tissue specimens were snap frozen in liquid nitrogen and stored at -80°C until analysis. Both normal and tumor tissues from the same patient were processed simultaneously to avoid any experimental variations. At the time of analysis, tissue specimens were thawed, washed with tris-buffered saline (pH 8.0), crushed under liquid nitrogen by mortar and pestle and divided into two parts. These parts were proceeded for telomerase assay and telomere length.

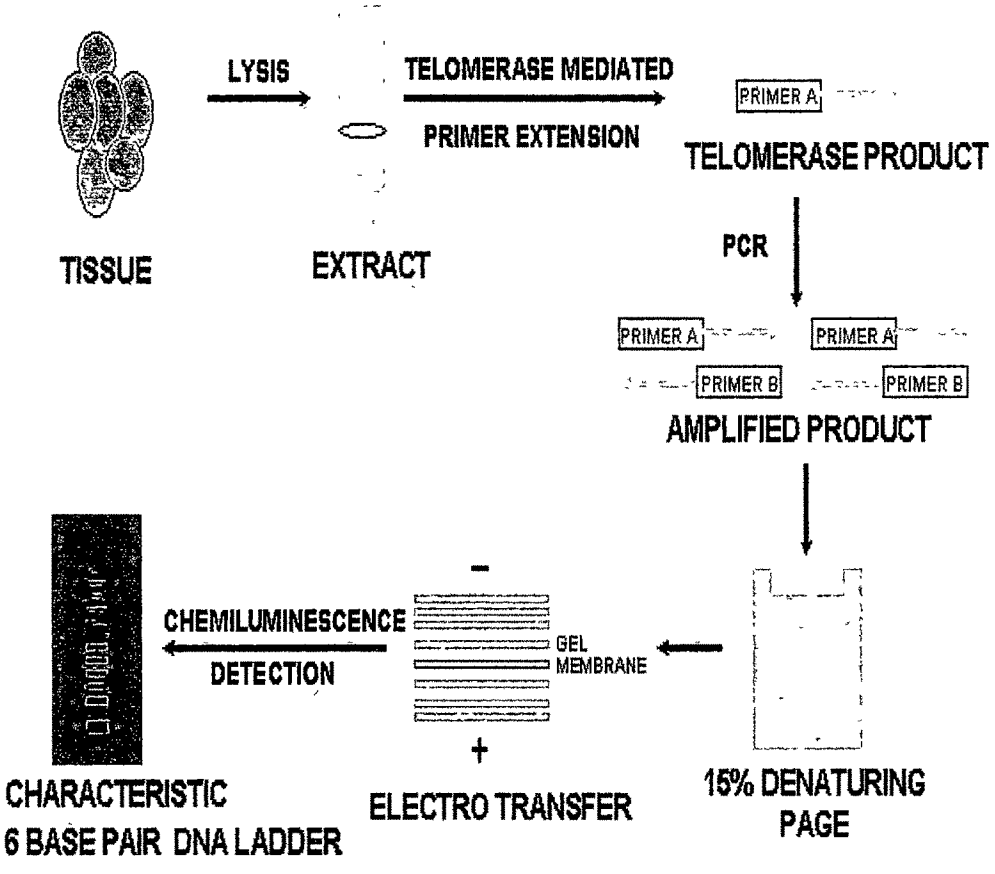
The experiments were performed using fine chemicals from Sigma (USA) and analytical grade reagents from Qualigens (India). Molecular biology grade and electrophoresis grade reagents were used wherever required and were procured from either Sigma (USA), Amresco (USA), or Bangalore Genei (India). Experiments kits, nylon membrane and chemiluminescence films were procured from Boehringer Mannheim (Germany). The experiments were carried out using DNase and RNase free plasticwares.

TELOMERASE ACTIVATION

Telomerase activation was determined using Telomeric Repeat Amplification Protocol (TRAP) as described by Kim et al. (1994) and Piatyszek et al. (1995), with necessary minor modifications. Telomerase PCR-ELISA Kit (Boehringer Mannheim, Germany) was used for the TRAP assay. Manufacturer's instructions were followed. As visualization of the characteristic telomerase 6 basepair ladder is the desirable method of determination of telomerase activation, the ladder was visualized by separating the PCR amplified telomerase product on denaturing polyacrylamide gel and subsequent chemiluminescent detection. Biotin Luminescence Detection Kit (Boehringer Mannheim, Germany) was used for chemiluminescence detection of the telomerase product. A brief protocol for determination of telomerase activation from the tissue specimens is shown in **Figure-9**.

Figure-9

Brief protocol for determination of telomerase activation by TRAP assay



TELOMERIC REPEAT AMPLIFICATION PROTOCOL

The Telomeric Repeat Amplification Protocol was performed using Telomerase PCR-ELISA Kit (Boehringer Mannheim, Germany), which follows the original method for determination of telomerase activation described by Kim et al. (1994). It allows highly specific amplification of telomerase-mediated elongation products combined with non-radioactive detection following an ELISA protocol.

In the first step, telomerase added telomeric repeats (TTAGGG) to the 3' end of the biotin-labeled synthetic P1-TS-primer. In the second step, these elongation products were amplified by polymerase chain reaction using the primers P1-TS and P2. Telomerase is a processive enzyme. It pauses after synthesis of each set of six nucleotide telomeric unit. Therefore, a PCR product with the telomerase specific 6 nucleotide increments was generated. This product, when electrophoresed on a polyacrylamide gel, created a "ladder" pattern of bands. The products can be detected by either ELISA method following the kit procedure, or by visualization of the telomerase specific 6 nucleotide ladder.

Reagents

1. Lysis reagent
   Ready to use solution provided with the kit
2. Reaction mixture
   2X solution provided with the kit
   Tris buffer containing Telomerase substrate, biotin-labeled P1-TS primer, P2 primer, nucleotides, and Taq polymerase.

Procedure

- Tissue specimens were crushed using mortar and pestle, under liquid nitrogen.
- After crushing the tissue specimens, 200 µl lysis reagent was added to lyse the cells and incubated on ice for 30 minutes.
- The lysates were centrifuged at 16000X g for 20 minutes at 4°C. The supernatant was transferred into fresh tube, aliquoted, shock frozen in liquid nitrogen and stored at -80°C until analysis.
- Protein concentration was measured by Lowry's method (Lowry et al., 1951).
- Cell extract equivalent to 50 µg total protein was proceeded for TRAP. Cell extract prepared from immortalized telomerase expressing cells (293 cells) provided in the kit was used as positive control. Telomerase positive cell extract heated at 80°C for 30 minutes was used as negative control.

- For TRAP, cell extracts were mixed with 25 µl reaction mixture in a 0.2 ml PCR tube. The volume was made up to 50 µl by sterile D.D.W. All these steps were carried out on ice.
- Combined primer elongation and amplification reactions were carried out in the thermal cycler (Perkin Elmer, USA). Telomerase mediated primer elongation was done by incubating the tubes at 25°C for 30 minutes. The reaction was terminated by inactivating telomerase at 94°C for 5 minutes. Telomerase product was amplified by 30 cycles of polymerase chain reaction (denaturation: 94°C for 30s; annealing: 50°C for 30s; polymerisation: 72°C for 90s; final incubation: 72°C for 10 minutes) followed by cooling at 4°C for 10 minutes. The PCR products were stored at -20°C until electrophoresed.

ELECTROPHORESIS

The kit protocol detects PCR amplified telomerase products by ELISA. However, it is desirable to visualize the telomerase-specific 6 nucleotide ladder. Therefore, in this study, the PCR-amplified telomerase products were electrophoresed on 15% denaturing polyacrylamide gel, which separates 30-150 base nucleotides.

Reagents

1. *Acrylamide Stock (40%)*

   38.0 gm Acrylamide

   2.0 gm N, N' Methelene bis-acrylamide

   Autoclaved double distilled water (D.D.W.) to make 100 ml

2. *10X TBE Buffer*

   108 gm Tris buffer

   55 gm Boric acid

   40 ml 0.5 M Ethylene diamine tetra acetic acid (EDTA); pH 8.0

   pH adjusted to 8.1-8.2 with solid boric acid

   D.D.W. to make 1000 ml

3. *10% Ammonium persulfate (APS)*

   10% APS w/v in autoclaved D.D.W.

4. *15% Denaturating Polyacrlamide Gel Mixture (Separating Gel)*

   37.5 ml 40% Gel

   10.0 ml 10X TBE Buffer

   40.0 ml Formamide

   42.04 gm Urea

   80 µl N,N,N',N'-Tetra methylene diamine (TEMED)

   800 µl 10% APS

   Volume was made up to 100 ml by autoclaved D.D.W.

   (The mixture was degassed prior to addition of APS and TEMED)

5. *8% Denaturing Polyacrylamide Gel Mixture (Stacking Gel)*

   20.0 ml 40% Gel

   10.0 ml 10X TBE Buffer

   40.0 ml Formamide

42.04 gm	Urea
300 µl	TEMED
3.0 ml	10% APS

Volume was made up to 100 ml by autoclaved D.D.W.

(The mixture was degassed prior to addition of APS and TEMED)

6. *Loading buffer*

4.0 ml	Formamide
5.0 µl	10 M NaOH
1.0 µl	0.5 M EDTA
4.0 gm	Sucrose
25.0 mg	Bromophenol blue

Total volume was made up to 5 ml.

7. *Electrode buffer*

10X TBE diluted with autoclaved D.D.W. to make 1X TBE

Procedure

- Fifteen percent denaturing polyacrylamide slab gel of 10 cm X 14 cm X 1.5 mm was polymerized over a period of 45 minutes, with a stacking of 8% denaturing polyacrylamide gel.
- 20 µl of PCR product was mixed with 5 µl loading buffer and electrophoresed at a constant voltage of 250 volts until the tracking dye, bromophenol blue ran off the gel.

ELECTROTRANSFER AND FIXING OF THE DNA

The method of horizontal electrophoretic transfer of protein or DNA from a gel to an immobilizing membrane was used for southern transfer of oligonucleotides. In this study, Multiphor II Nova Blot electrophoretic transfer unit (Pharmacia LKB Biotechnology, Sweden) was used for transfer of nucleotides from gel to positively charged nylon membrane (Boehringer Mannheim, Germany). The protocol is based on a semi-dry electrophoretic transfer technique. It used fiter papers soaked in buffer as the only buffer reservoir.

Reagents

1. *1X TBE*
   10X TBE diluted 1:10 in D.D.W. (v/v)

Procedure

- After electrophoresis, the PCR products were transferred to positively charged nylon membrane (Boehringer Mannheim, Germany) by electroblotting using Nova Blot unit following the instructions. Continuous buffer system of 1X TBE was used in the filter paper layers as buffer reservoir.
- DNA was fixed on the membrane by baking at 120°C for 60 minutes.

DETECTION

Telomerase product was immunodetected on the nylon membrane using Biotin Luminescence Detection Kit from Boehringer Mannheim, Germany. The nylon membrane was subjected to Streptavidin-Alkaline Phosphatase conjugate which binds to the biotin labeled primers onto which the telomerase product was synthesized during TRAP assay. Membrane was subsequently subjected to CSPD, a chemiluminescence substrate for Alkaline Phosphatase. Enzymatic dephosphorylation of CSPD by alkaline phophatase led to a light emission at a maximum wavelength of 477 nm which was finally captured on chemiluminescence films.

Reagents

1. *Maleic acid buffer*

   0.1 M Maleic acid

   0.15 M NaCl

   pH adjusted to 7.5 with solid NaOH.

   Buffer was autoclaved.

2. *Washing buffer*

   Autoclaved Maleic acid buffer plus 0.3% Tween 20(v/v)

3. *Blocking reagent stock solution (10X)*

   10% Blocking reagent (w/v) in maleic acid buffer

Blocking reagent, provided in the kit, was dissolved in Maleic acid buffer by constantly stirring on a heating block (60°C) and autoclaved.

4. *5X Blocking solution*
   Stock solution was diluted 1:1 in maleic acid buffer.

5. *Detection buffer*
   0.1M Tris-HCl
   0.1M NaCl
   pH was adjusted to 9.5
   Buffer was autoclaved

6. *Streptavidin-Alkaline Phosphatase (AP) conjugate*
   Provided with the kit
   Diluted 1:5000 in 5X blocking solution; final concentration 200 mU/ml before use.

7. *CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'- (5'-chloro)* tricyclo[3.3.1.13,7]decan}4-yl)phenyl phosphate)
   Ready to use solution provided with the kit

Procedure

- The membrane was initially rinsed in washing buffer for 5 minutes.
- To prevent non-specific binding, the membrane was then incubated twice in 5X blocking solution for 30 minutes at room temperature.

- The membrane was then incubated in working Streptavidin-AP conjugate solution for 30 minutes at room temperature. The Streptavidin –AP conjugate binds to biotin tagged to the primer.
- The membrane was rinsed twice in washing buffer for 15 minutes at room temperature to remove excess antibody.
- Equilibration was carried out by incubating the membrane in detection buffer for 5 minutes.
- Membrane was placed on a transparent acetate sheet (DNA side up) and 2 ml diluted CSPD solution was spread over it. CSPD was dephosphorylated by alkaline phosphatase. This lead to light emission at a maximum wavelength of 477 nm.
- After 5 minutes, another acetate sheet was placed over the membrane and excess liquid was squeezed out. The membrane was then incubated at 37°C for 15 minutes to enhance the luminescent reaction.
- Luminescensce was captured on chemiluminescence film by exposing the membrane to the film for 10-30 minutes.
- The samples producing characteristic 6 basepair DNA ladder were considered to be telomerase positive.

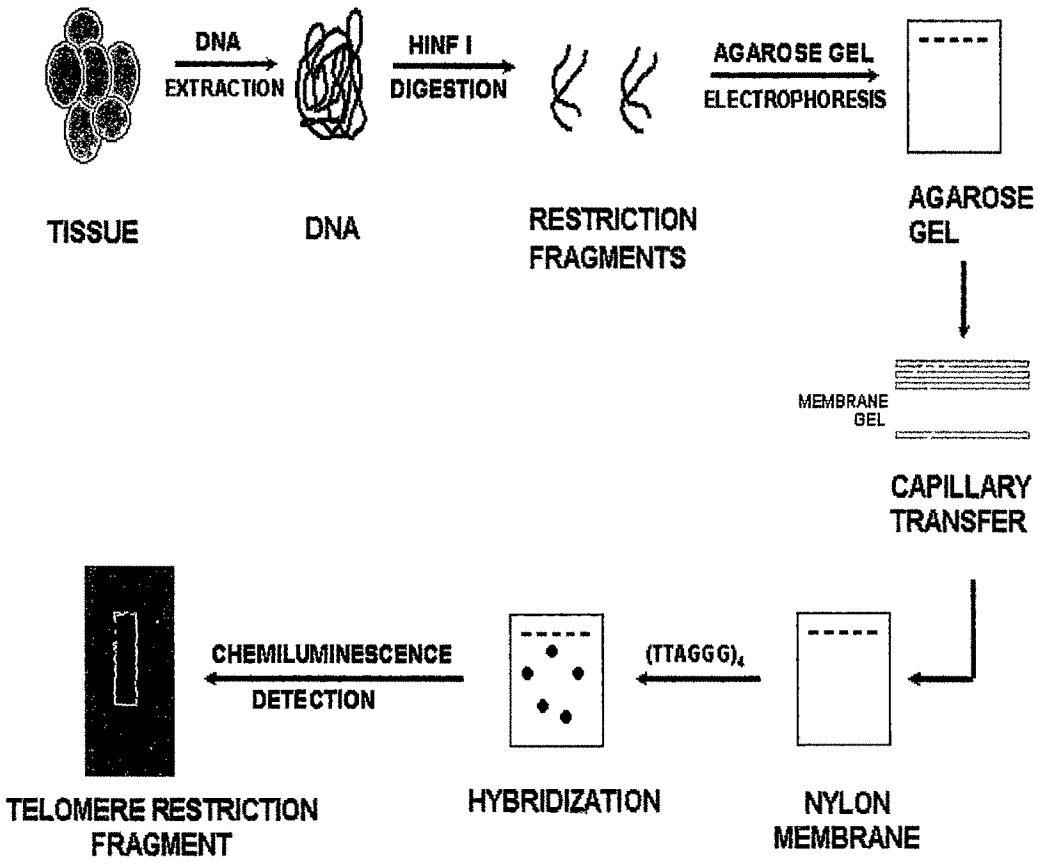
TELOMERE LENGTH

Telomere length was quantitatively assessed by measuring the terminal restriction fragment (TRF) size following standard assays, which used southern analysis (Allshire et al., 1989; de Lange et al., 1990; Harley et al., 1990). Briefly, the tissue was crushed, DNA was extracted from the tissue and digested by restriction enzyme. The restriction fragments were run on agarose gel and subsequently transferred to the nylon membrane. Restriction fragments on the nylon membrane were hybridized to digoxigenin labeled telomeric probe. Telomeric probes were labeled with digoxigenin using DIG 3' end labeling kit (Boehringer Mannheim, Germany). Terminal Restriction Fragments (TRFs) were visualized by chemiluminescence detection using DIG luminescence detection kit (Boehringer Mannheim, Germany). **Figure-10** shows a brief protocol for determination of TRF length by southern hybridization.

Figure-10

Brief protocol for determination of telomere length

by Southern Hybridization



Reagents

1. *Lysis buffer* (autoclaved)

   50 mM Tris, pH 8.0

   10 mM EDTA, pH 8.0

   10 mM NaCl

   2% SDS

   Proteinase K 150 µg/ml (added at the time of use)

2. *TE buffer* (autoclaved)

   100 mM Tris, pH 8.0

   1 mM EDTA, pH 8.0

3. *Tris saturated Phenol*

   Commercially available phenol was distilled and mixed with 1M Tris buffer (pH 8.0) until the phenol phase showed pH of 7.9-8.0. The Tris phase was removed and two washes of 100 mM Tris (pH 8.0) plus 100 µl 2-mercaptoethanol per 100 ml of buffer was given to phenol.

4. *Chloroform: Isoamyl alcohol (24:1, v/v)*

5. *3M Sodium acetate, pH 5.5* (autoclaved)

6. *Ethanol*

7. *70% Ethanol*

   Ethanol diluted (v/v) to 70% in autoclaved D.D.W.

Procedure

- After crushing in liquid nitrogen, the tissue specimens were mixed with 3ml lysis buffer and incubated at 37°C overnight.

- Equal volume of Tris saturated phenol was added, mixed for 10 minutes and centrifuged at 8000 rpm for 20 minutes.
- The supernatant was taken into a fresh tube. Half volume of Tris saturated phenol and half volume of chloroform: isoamyl alcohol (24:1) was added, mixed for 10 minutes and centrifuged at 8000 rpm for 20 minutes.
- The supernatant was transferred to a fresh tube. 1/30th volume of sodium acetate and equal volume of ice-cold ethanol was added and swirled slowly to precipitate DNA.
- DNA precipitate was washed with 70% ethanol and subsequently with absolute ethanol, air-dried and dissolved in TE buffer.
- Purity of the DNA samples was checked by calculating $O.D._{260}/O.D._{280}$. DNA concentration was determined by taking $O.D._{260}$.

DNA DIGESTION

DNA was digested by the restriction enzyme *Hinf I*, which is synthesized from Haemophilus influenzae Rf. The restriction site for this enzyme is as follows:

G'ANTC
CTNA,G

This enzyme does not have restriction site in the telomeric region; therefore full length telomeres are obtained after digestion.

Reagents

1. *Hinf I* (Bangalore Genei)
   From Haemophilus influenzae Rf

2. *Buffer 10X* (Provided with the enzyme); (Bangalore Genei)

10 mM	Tris HCl (pH 7.8)
50 mM	NaCl
10 mM	$MgCl_2$
10 mM	2-Mercaptoethanol
100 µg/ml	Nuclease free BSA

Procedure

- Following mixture was prepared in a microfuge tube

10.0 µg	DNA
5.0 µl	10X Buffer
50.0 units	*Hinf I*

  Total volume made up to 50 µl by autoclaved D.D.W.
- This mixture was incubated overnight at 37°C for DNA digestion.
- The reaction was stopped by heating mixture at 75°C for 15 minutes.

DNA ELECTROPHORESIS

After digestion of DNA, the restricted DNA fragments obtained were electrophoresed on 0.7% agarose gel (13 cm x13 cm) in 1X TBE.

Reagents

1. *Agarose Gel mixture*
   0.7% Agarose in 1X TBE (w/v)
2. *Loading buffer*
   0.25% Bromophenol blue
   40% Glycerol
   1X TBE
3. *Electrode buffer*
   1X TBE
4. *DNA Molecular Weight Marker II, DIG-labeled* (Boehringer Mannheim, Germany): 0.12-23.1 kbp (8 fragments of 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp)

Procedure

1. Agarose gel mixture was heated until agarose dissolved completely. It was allowed to cool to 45°C, poured into 13 cm X 13 cm gel tray and allowed to polymerize for 45 minutes.
2. 25 µl DNA digest was mixed with 5µl loading buffer and proceeded for submarine gel electrophoresis using agarose gel. Constant voltage of 30 volts was maintained. 5 µl DIG-labeled DNA molecular weight markers were also run simultaneously.
3. DNA digest was allowed to run till dye reached the end.

SOUTHERN TRANSFER OF DNA

The DNA in the gel was depurinated and denatured to facilitate the transfer on to nylon membrane. The gel was neutralized and subsequently DNA was transferred to nylon membrane by capillary transfer.

Reagents

1. *250 mM* *HCl*
2. *Denaturation solution*
   0.5 N NaOH
   1.5 M NaCl
3. *Neutralization solution*
   0.5 M Tris-HCl, pH 7.5
   3 M NaCl
4. *20X Saline Sodium Citrate (SSC)*
   3 M NaCl
   300 mM Sodium citrate, pH 7.0

Procedure

- Gel was depurinated by gently shaking in 250 mM HCl for 5 minutes at room temperature.
- Gel was rinsed in D.D.W.

- Denaturation was carried out by shaking the gel gently in denaturation solution at room temperature twice for 15 minutes. This step was carried out to convert double stranded DNA is converted into single stranded DNA. These two steps of depurination and denaturation were carried out to facilitate the subsequent transfer of DNA from gel to nylon membrane.
- Gel was rinsed with D.D.W.
- Gel was treated with neutralization solution for 15 minutes at room temperature.
- DNA was transferred onto positively charged nylon membrane by capillary action overnight. 20X SSC buffer was used for the transfer.
- After the transfer, membrane was rinsed in 2X SSC and air-dried. DNA was fixed to the membrane by baking at 120°C for 30 minutes.

LABELING OF PROBES

Telomeric probe $(TTAGGG)_4$ was used to detect telomeric DNA on the membrane. After stripping, minisatellite probe $(CAC)_5$ was hybridised to the membrane to check DNA digestion. Both probes were labeled with digoxigenin. For this, DIG Oligonucleotide 3'-End Labeling Kit procured from Boehringer Mannheim, Germany was used. The probes

were non-radioactively labeled by incorporation of a digoxigenin-labeled nucleotide. The steroid hapten digoxigenin (DIG) was linked via a spacer arm to the dideoxy uridine-triphosphate. The probes were enzymatically labeled at the 3'-end with terminal transferase by incorporation of the digoxigenin-labeled dideoxyuridine-triphosphate (DIG-ddUTP).

Reagents

1. *5X Reaction buffer*, pH 6.6 (Provided with the kit)

1 M	Potassium cacodylate
0.125 M	Tris-HCl
1.25 mg/ml	Bovine serum albumin

2. *$CoCl_2$ solution* (Provided with the kit)

25 mM	$CoCl_2$

3. *DIG-ddUTP solution* (Provided with the kit)

1 mM DIG-ddUTP in D.D.W.

4. *Terminal Transferase* (Provided with the kit)

50 units/μl in:

0.2 M	Potassium cacodylate
1 mM	EDTA
200 mM	KCl
0.2 mg/ml	Bovine serum albumin, pH 6.5
50%	Glycerol, (v/v).

5. *Glycogen solution* (Provided with the kit)

20 mg/ml Glycogen solution

6. *Oligonucleotide probes* (custom synthesized)
   Procured from Bangalore Genei. Dissolved in D.D.W.

Procedure

- Following mixture was prepared in a microfuge tube on ice:
  4 µl Reaction buffer
  4 µl $CoCl_2$ solution
  100 pmol Oligonucleotide
  1 µl DIG-ddUTP solution
  1 µl (50 units) Terminal transferase
  Volume was made up to 20 µl with D.W.
- The reaction mixture was incubated at 37°C for 15 minutes. DIG-ddUTP is added at the 3' end of the oligonucleotide by the enzyme Terminal transferase.
- After the incubation, reaction mixture was cooled on ice. 1 µl Glycogen solution was mixed in 200 µl 0.2 mM EDTA solution, pH 8.0 and 2 µl of this dilution was added to the reaction mixture to stop the reaction.
- This mixture was directly used for hybridization.

SOUTHERN HYBRIDIZATION

The membrane was first subjected to prehybridization solution, containing blocking reagent, to block non-specific binding. TRF were then hybridised to the DIG labeled telomeric probe overnight.

Reagents

1. *Prehybridization solution*
   5X SSC
   1% Blocking Reagent
   0.1% N-lauryl sarcosine
   0.02% SDS

2. *Hybridization solution*
   20 ml Prehybridization + 20 µl Digoxigenin labeled probe

3. *2X Wash solution*
   2X SSC
   0.1% SDS

4. *0.1X Wash solution*
   0.1X SSC
   0.1% SDS

Procedure

- After southern transfer and fixing of DNA onto the membrane, the membrane was treated to prehybridization solution for 90

minutes at 55°C in a hybridization oven. This helped in blocking the binding of probe at non-specific sites in subsequent step.

- Prehybridization solution was discarded and the membrane was shaken at 55°C in hybridization solution containing Dig-labeled telomeric probe overnight.
- The membrane was then given stringency washes in 2X wash solution twice for 5 minutes at room temperature and 0.5X wash solution twice for 15 minutes at 42°C. This removed extra probe and thus helped in reducing background signals.
- The membrane was then proceeded for detection.

DETECTION

Immunodetection was carried out using DIG luminescence detection kit from Boehringer Mannheim. Manufacturer's instructions were followed. The membrane was subjected to anti digoxigenin, Fab fragment conjugated to alkaline phosphatase, which binds to digoxigenin labeled probes. Membrane was subsequently subjected to CSPD, a chemiluminescence substrate for Alkaline Phosphatase. Enzymatic dephoshorylation led to a light emission at a maximum wavelength of 477 nm. Chemiluminescence was finally captured on chemiluminescence films.

Reagents

1. *Maleic acid buffer*
   As described before
2. *Washing buffer*
   As described before
3. *Blocking stock solution*
   As described before
4. *1X Blocking solution*
   Stock solution was diluted 1:10 in maleic acid buffer.
5. *Detection buffer*
   As described before
6. *Anti-dig- AP (Anti-digoxigenin, Fab fragments conjugated to alkaline phosphatase)*
   Provided with the kit.
   Diluted 1:10000 in 1X blocking solution before use.
7. *CSPD*
   Provided with the kit. Diluted 1:100 in detection buffer.

Procedure

- The membrane was rinsed briefly (5 minutes) in washing buffer.
- The membrane was incubated in 1X blocking solution twice for 30 minutes to prevent non-specific binding of antibody in the subsequent step.

- It was then incubated in diluted anti-dig-AP for 30 minutes at room temperture.
- Extra antibody was removed by shaking the membrane twice in washing buffer.
- The membrane was equilibrated in detection buffer for 5 minutes.
- Membrane was placed on a transparent acetate sheet (DNA side up) and 2 ml diluted CSPD solution was spread over it. CSPD, the chemiluminescence substrate, was dephosphorylated by Alkaline phosphatase. This lead to light emission at a maximum wavelength of 477 nm.
- After 5 minutes, another acetate sheet was placed over the membrane and excess liquid was squeezed out. The membrane was then incubated at 37°C for 15 minutes to enhance the luminescent reaction.
- Luminescensce was captured on chemiluminescence film by exposing the membrane to the film for 30 minutes.
- The films were developed and scanned densitometrically on a Gel Documentation System and analyzed using Molecular Analyst software(BioRad, USA).
- Mean telomere restriction fragment length was measured as the length corresponding to the densitometric peak.

STRIPPING AND REHYBRIDIZATION

The membrane was stripped and rehybridized with minisatellite probe. This rehybridization step was carried out to ensure complete DNA digestion by the restriction enzyme and proper run.

Reagents

1. *Stripping solution*
   0.2M NaOH
   0.1% SDS
2. *2X SSC*
3. *4X Wash solution*
   4X SSC
   0.1% SDS

Procedure

- The membrane was briefly rinsed in D.D.W. and washed twice in stripping solution at 37°C to remove the Dig-labeled telomeric probe.
- The membrane was then rinsed thoroughly in 2X SSC.
- The membrane was hybridized to minisatellite probe $(CAC)_5$ following the same procedure except that prehybridization and hybridisation were done for 90 minutes and overnight respectively at 42°C. The stringency washes were given as follows: 2 X 5

minutes at room temperature and 2 X 15 minutes at 42°C in 4X wash solution.

STATISTICAL METHODS

Molecular Analyst software was used to calculate the peak TRF length (BioRad, USA). SPSS statistical software (Version 10; Inc, Chicago, IL) was used to perform statistical analysis. Comparison between peak TRF length in different groups was performed using the student 't' test analysis. Paired 't' test analysis was performed to compare results between the adjacent normal tissue and malignant tissue specimens in individual patients. Correlation of telomerase activity and TRF lengthwith clinicopathological characteristics was evaluated using multivariate analysis. Correlation of telomerase activity and TRF length with disease free survival was established by Kaplan-Meier analysis. Spearman's correlation test was performed to analyze the correlation between telomerase activation and peak TRF length.