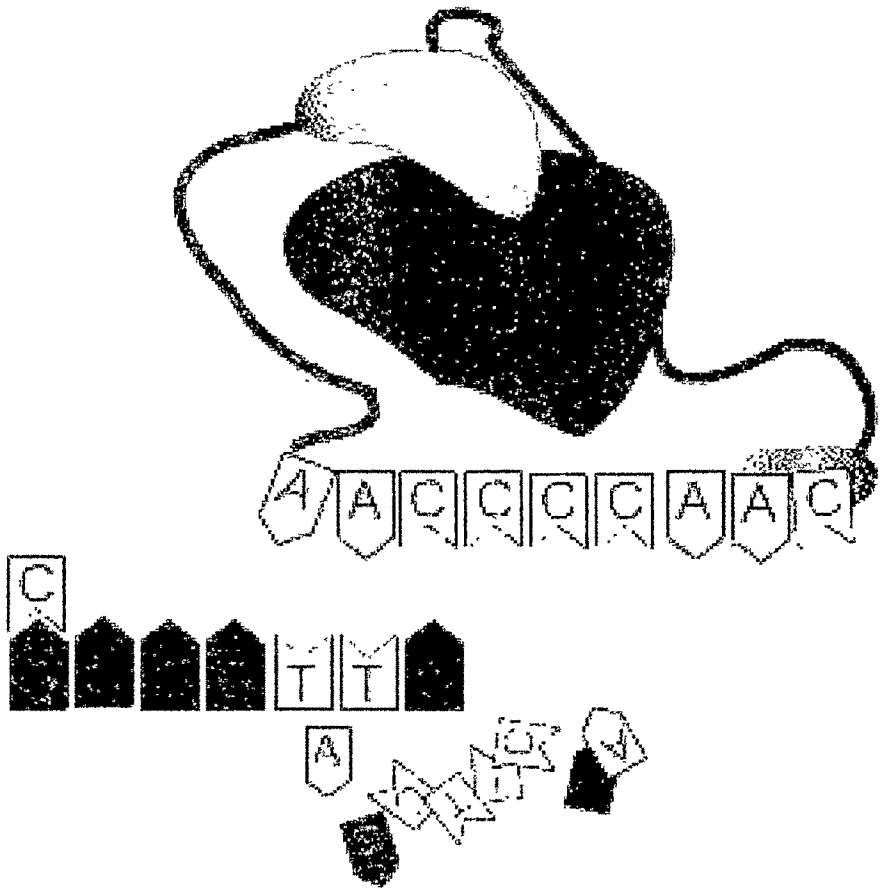


There is no more difficult art to acquire than the art of observation...

William Osler

Results

ESTIMATION OF PROTEIN CONTENT IN TISSUE SPECIMENS

Tissue specimens were homogenised in 200µl lysis buffer. Protein content was estimated spectrophotometrically in each cell extract using 5µl aliquot, on the same day, by Lowry's method (Lowry et al., 1951). Protein content in different specimens varied from 10µg/5µl to 150µg/5µl depending on the amount of initial tissue. The cell extract was aliquoted, snap frozen and stored at -80°C immediately after extraction. Cell extract equivalent to 50µg protein was processed for telomerase assay.

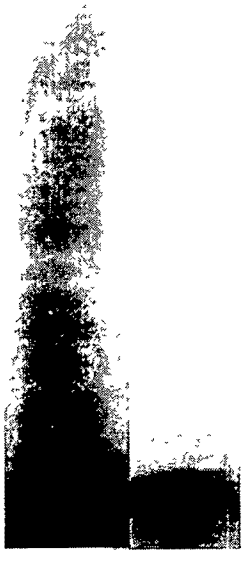
TELOMERASE ACTIVATION

Telomerase activation was determined by Telomeric Repeat Amplification Protocol in tissue specimens from patients with head and neck cancer and patients with related precancerous diseases. The Telomerase PCR ELISA Kit (Boehringer Mannheim, Germany) allows detection of telomerase activity by means of ELISA protocol. However, as visualisation of the typical telomerase mediated 6-nucleotide-ladder is desirable, and more dependable, we followed PCR amplification of telomerase product by its electrophoretic separation and detection using Biotin Luminescence Detection Kit (Boehringer Mannheim, Germany). **Figure-11** shows the characteristic 6 basepair DNA ladder observed in

specimens showing telomerase activation. Lane 1 shows telomerase activation in the positive control, the cell extract prepared from immortalized telomerase expressing kidney cells (293 cells) provided along with Telomerase PCR-ELISA Kit. Lane 2 shows the absence of telomerase activation in the negative control. Positive control heated to 80°C for 30 minutes served as negative control.

Figure-11

Characteristic telomerase mediated 6 base pair DNA ladder



Lane 1: Positive control

Lane 2: Negative control

Telomerase Activation in Precancerous Tissues

Head and neck carcinogenesis is a multistep process, consisting of tranformation of normal cells to initiated cells, to precancerous cells, and finally to malignant cells, during which there are multiple changes taking place in the cell. It is very important to study these molecular and biological events that take place in the cell. Therefore telomerase activation was analysed in tissue specimens obtained from patients with precancerous diseases. **Figure-12** depicts the representative patterns of telomerase activation observed in tissue specimens from patients with precancerous diseases. Lane 1 represents a tissue specimen of oral leukoplakia. Lane 2 represents a tissue specimen of submucous fibrosis. Lane 3 also represents a tissue specimen of submucous fibrosis. Lane 4 represents a tissue specimen of hemangioma. Lanes 5 and 6 represent positive and negative controls, respectively. **Table-3** describes the frequency of telomerase activation in tissue specimens of precancerous conditions/diseases. Thirty-five out of 40 (87.5%) of the precancerous tissue specimens showed telomerase activation.

Figure-12

Representative patterns of telomerase activation

in tissue specimens of precancerous conditions

Lane 1: Leukoplakia
Lane 2: Submucous fibrosis
Lane 3: Submucous fibrosis
Lane 4. Hemangioma
Lane 5 Positive control
Lane 6: Negative control

Table-3

Frequency of telomerase activation in precancerous tissues

Tissue (N)	Telomerase Activation	
	Present N (%)	*Absent* N (%)
Precancerous tissues (40)	35 (87.5%)	5 (12.5%)

Telomerase Activation in Tissue Specimens from Head and Neck Cancer Patients

The representative patterns obtained from telomerase assay in tissue specimens from head and neck cancer patients are illustrated in **Figure-13**. Lanes 1 and 2 represent telomerase negative adjacent normal tissue specimen and telomerase positive malignant tissue specimen, respectively from a patient with carcinoma of tongue. Lanes 3 and 4 represent telomerase positive specimens of adjacent normal and malignant tissues respectively, from a patient with carcinoma of buccal mucosa. Lanes 5 and 6 represent positive and negative controls, respectively.

Figure-13

Telomerase activation in different tissue specimens from head and neck cancer patients

Lanes 1 and 2: Adjacent normal and malignant tissue specimens respectively from a patient with carcinoma of tongue

Lanes 3 and 4: Adjacent normal and malignant tissue specimens respectively from a patient with carcinoma of buccal mucosa

Lane 5 and 6: Positive control and negative control respectively

Table-4 shows the frequency of telomerase activation in different tissue specimens from head and neck cancer patients. Majority of the malignant tissue specimens i.e. 75 out of 110 (68.2%) showed telomerase activation. On the other hand, a surprisingly high number of adjacent normal tissues i.e. 52 out of 103 (50.5%) were also found to show telomerase activation.

Table-4

Frequency of telomerase activation in head and neck cancer

Tissue (N)		Telomerase activation	
		Present N (%)	*Absent* N (%)
Adjacent normal tissue	(103)	52 (50.5%)	51 (49.5%)
Malignant tissue	(110)	75 (68.2%)	35 (31.8%)

Thus, it is evident that telomerase activation was observed in most of the malignant tissue specimens. On the other hand a surprisingly high number of adjacent normal and precancerous tissue specimens showed telomerase activation. To rule out the possibility of false positive results due to cross contamination, the experiments were repeated by

processing the cell lysates from adjacent normal, precancerous and malignant tissues specimens in different batches. However, similar results were obtained. The presence of telomerase activation in the adjacent normal tissues may suggest the presence of tumor cells in the apparently adjacent normal tissues. The presence of telomerase activation may aid in predicting occult malignant disease.

Telomerase Activation in Oral Cavity Cancer

Oral cavity cancer formed the major bulk (79.1%) of the head and neck cancer in the present study. This group of cancer included the anatomical sites, which are generally, chronically and directly, exposed to the carcinogenic insults mainly in the form of tobacco. Therefore, it may be fruitful to elucidate the picture of molecular changes in this major subsite. **Table-5** shows the frequency of telomerase activation in tissue specimens obtained from patients with oral cavity cancer. A similar trend of telomerase activation to that observed in the head and neck cancer was also seen in this group. Telomerase activation was observed in 70.1% of malignant tissue specimens. Once again, as high as 53.8% of adjacent normal tissue specimens, showed telomerase activation.

TABLE-5

Frequency of telomerase activation in oral cavity cancer

Tissue (N)	Telomerase activation	
	Present N (%)	*Absent* N (%)
Adjacent normal tissue (80)	43 (53.2%)	37 (46.2%)
Malignant tissue (87)	61 (70.1%)	26 (29.9%)

It is clear that, as oral cavity is the major group of subsites in head and neck region, the picture of telomerase activation observed in oral cavity cancer patients is similar to that in all head and neck cancer patients.

Telomerase Activation and Stage wise Disease Activity

The most important factor for determining the prognosis and management of cancer is the tumor stage, which is based primarily on anatomical considerations and histopathological evaluations. **Table-6** shows the stage wise classification of telomerase activation in tissue specimens from head and neck cancer patients. The frequency of telomerase activation in malignant tissues did not vary much in the

tumors of different stages; it ranged from 50% to 71.4%. Interestingly, adjacent normal tissues in the stage IV patients showed highest frequency of telomerase activation, i.e. 57.7% of the adjacent normal tissues from stage IV patients were found to be positive for telomerase activation.

Table-6

Stage wise distribution of frequency of telomerase activation in head and neck cancer patients

Stage	Telomerase activation	
	Adjacent normal tissues	*Malignant tissues*
	Present/Total (%)	Present/Total (%)
Stage I	01/04 (25.0%)	02/04 (50.0%)
Stage II	03/14 (21.4%)	09/14 (64.3%)
Stage III	07/14 (50.0%)	10/14(71.4%)
Stage IV	41/71 (57.7%)	54/78 (69.2%)

Comparison of Telomerase Activation in Early and Advanced Stage of Disease

Generally, the malignant tumors in stage I and stage II are clubbed together as early stage of disease, because they represent local disease with no tumor spread. Malignant diseases of early stage are mostly treated by curative therapy. On the other hand, the tumors in stage III and IV, which may involve regional or systemic tumor spread, are clubbed together as advanced stage of disease. These advanced stage diseases are treated by palliative therapy. The percentages of telomerase activation were similar in malignant tissues in both the groups. However, more strikingly, telomerase activation in adjacent normal tissues was more frequent in advanced stage of the disease **(Table-7)**. Telomerase activation was observed in 56.5% of the adjacent normal tissues in advanced stage as against 21.4% of the adjacent normal tissues in early stage of the disease.

The analysis of telomerase activation in patients with different stages of disease, showed that telomerase activation was observed in adjacent normal tissue specimens from high number of patients with advanced disease i.e. stage III and stage IV disease. This result could possibly suggest the spread of malignant cells in the adjacent normal tissue in advanced stage disease.

Table-7

Frequency of telomerase activation in early and advanced stage head and neck cancer patients

Stage	Telomerase activation	
	Adjacent normal tissues	*Malignant tissues*
	Present/Total (%)	Present/Total (%)
Early	03/14 (21.4%)	11/18 (61.1%)
Advanced	48/85 (56.5%)	64/92 (69.6%)

Telomerase Activation in Patients With and Without Nodal Involvement

Tumor spread is an important criterion for determination of severity of the disease. Involvement of the local nodes indicates the spread of malignant cells. The presence or absence of lymph node metastases is a powerful predictor of survival in cancer patients. **Table-8** indicates the frequency of telomerase activation in tumor tissues from patients with and without nodal involvement. Similar frequency of telomerase activation was observed in malignant tissue specimens from node

negative and node positive patients. Telomerase activation was observed in 67.7% of node negative and 68.9% of node positive head and neck cancer patients.

Table-8

Frequency of telomerase activation in node negative and node positive head and neck cancer patients

Nodal Involvement	Telomerase activation	
	Adjacent normal tissues	*Malignant tissues*
	Present/Total (%)	Present/Total (%)
Negative	25/41 (61.0%)	31/45 (68.9%)
Positive	27/62 (43.5%)	44/65 (67.7%)

Telomerase activation was found in a similar number of patients in both node negative and node positive patients.

Telomerase Activation in Tumors of Different Histologic Grades

Histologic grade of the tumor indicates the progression of malignant changes in the cells. Differentiation of a cell refers to the extent to which they resemble their normal cells of origin and includes the extent to which they achieve their fully mature morphologic and functional characteristics. As malignancy progress, a cell passes from well to moderately, to poorly differentiated states. **Table-9** illustrates the frequency of telomerase activation depending on the different histologic grades of the tumor Poorly differentiated tumors showed the highest frequency of telomerase activation i.e. 58.8% and 76.5% in both adjacent normal and malignant tissues, respectively.

Table-9

Telomerase activation in tissue specimens from head and neck cancer patients with tumors of different histologic grades

Histologic Grade	Telomerase activation	
	Adjacent normal tissues	*Malignant tissues*
	Present/Total (%)	Present/Total (%)
Well Differentiated	22/46 (47.8%)	32/48 (66.7%)
Moderately Differentiated	20/40 (50.0%)	30/45 (66.7%)
Poorly Differentiated	10/17 (58.8%)	13/17 (76.5%)

Telomerase Activation in Tumors of Different Nuclear Grade

Nuclear grade of the tumor is determined on the basis of the nuclear pleomorphism, mitotic index, nuclear cytoplasmic ratio, etc. **Table-10** provides the frequency of telomerase activation in tumors of different nuclear grades of the tumor. The highest frequency of telomerase activation was observed in the grade III tumors in both adjacent normal and malignant tissues. Telomerase activation was evident in 66.7% of adjacent normal tissues and 88.9% of malignant tissues in head and neck cancer patients with grade III tumors.

Table-10

Telomerase activation in tissue specimens from head and neck cancer patients: correlation between different nuclear grades

Nuclear Grade	Telomerase activation	
	Adjacent normal tissues	*Malignant tissues*
	Present/Total (%)	Present/Total (%)
Grade I	23/39 (59.0%)	29/44 (65.9%)
Grade II	20/46 (43.5%)	36/52 (69.2%)
Grade III	06/09 (66.7%)	08/09 (88.9%)

Thus, it was observed that, telomerase activation was present in higher number of patients with poorly differentiated tumors and in patients with nuclear grade III tumors.

Correlation of Telomerase Activation with Clinicopathological Characteristics

Multivariate analysis was performed to evaluate any possible correlation between telomerase activation and different clinicopathological parameters including age, gender, site of tumor, stage of tumor, nodal involvement, histologic grade of tumor and nuclear grade of tumor. As clear from **Table-11**, telomerase activation was found to be an independent parameter in head and neck cancer patients. No significant correlation was observed between these clinicopathological parameters and telomerase activation. *It is clear from the data that telomerase activation showed no correlation with other clinical and histopathological characteristics and that it was an independent feature.*

Table-11

Correlation of telomerase activation with clinicopathological characteristics in head and neck cancer patients

Parameter	Adjacent Normal Tissue		Malignant Tissue	
	F value	*Significance*	*F value*	*Significance*
Age	1.307	0.284	1.050	0.405
Gender	0.033	0.858	1.434	0.239
Habit of tobacco	2.521	0.122	0.002	0.969
Site	0.797	0.621	0.528	0.844
Stage	3 467	0.068	0.088	0.768
Nodal status	0.078	0.781	0.002	0.968
Histologic Grade	0.002	0.998	0.078	0.926
Nuclear Grade	1.062	0.353	1.47	0.238

Correlation of Telomerase Activation with Disease Free Survival of the Patients

Over the past several decades, clinicopathologic investigations have noted that recurrence and overall survival of cancer patients are linked to tumor size, histologic grade, clinical stage of disease, perineural and vascular involvement, and lymph node and distant metastasis. We investigated whether telomerase activation and telomere length had the capability to reflect the proliferative nature of the tumor as well as the correlation of these parameters with the clinical course of the disease.

All the patients were followed-up at regular intervals and details regarding anticancer treatment and disease status throughout the follow-up period were recorded. However, it was observed that many patients were lost to follow-up at various stages of the study. The correlation between telomerase activation status and the two year disease free survival was analysed by plotting Kaplan and Meier survival plots (**Figure-14A**). The two year disease free survival analysis showed that telomerase activation in the malignant tissue had no effect on the disease free survival of the patients. However, clinical course of the disease significantly correlated with the status of telomerase activation in the adjacent normal tissues. A significantly better two year disease free survival was observed in the patients not showing

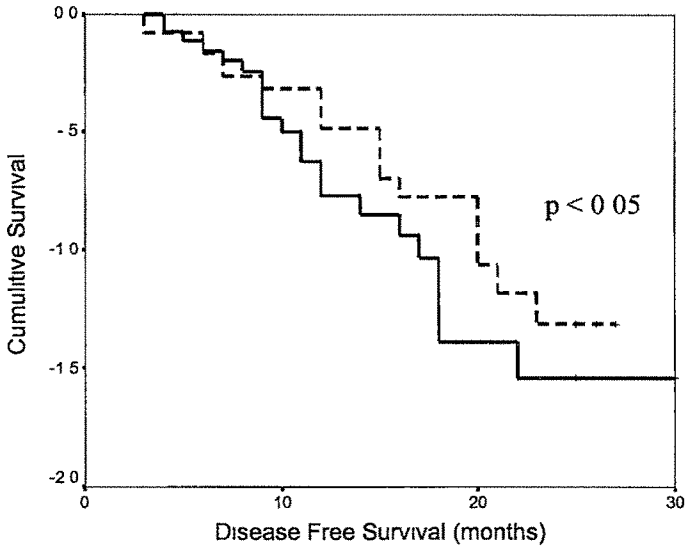
telomerase activation in adjacent normal tissues as compared to the patients showing telomerase activation (**Figure-14B**).

Thus, it can be observed that the status of telomerase activation in the adjacent normal tissues may predict prognosis of the disease. The presence of telomerase activation in adjacent normal tissues may be suggestive of spread of the tumor to the adjacent normal tissues or molecular changes in the apparently normal cells. Analysis of telomerase activation in head and neck cancer patients may help in discerning the patients with a poor prognosis, who may be in need of a close follow-up and an aggressive anticancer treatment.

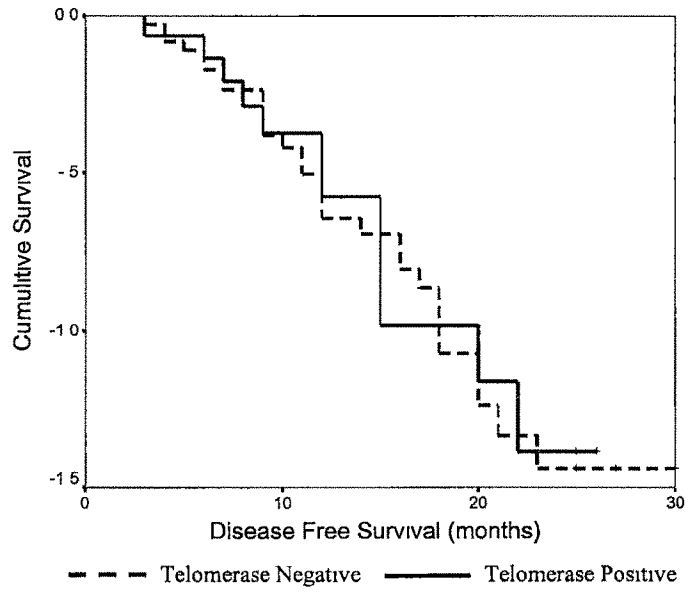
Figure-14

Correlation of telomerase activation with disease free survival

A: Adjacent normal tissues



B: Malignant tissues



A. Correlation of telomerase activation in adjacent normal tissues with disease free survival

B: Correlation of telomerase activation in malignant tissues with disease free survival

DNA ANALYSIS

DNA was extracted from tissue specimens obtained from head and neck cancer patients. Integrity of each DNA sample was assessed by checking the $O.D._{260}$/ $O.D._{280}$ ratio, which should be approximately 1.8. The ratio deviates if the sample is contaminated with protein or RNA. All DNA samples were also checked for degradation by agarose gel electrophoresis. Then the samples were processed for telomere length analysis by restriction digestion followed by southern hybridization.

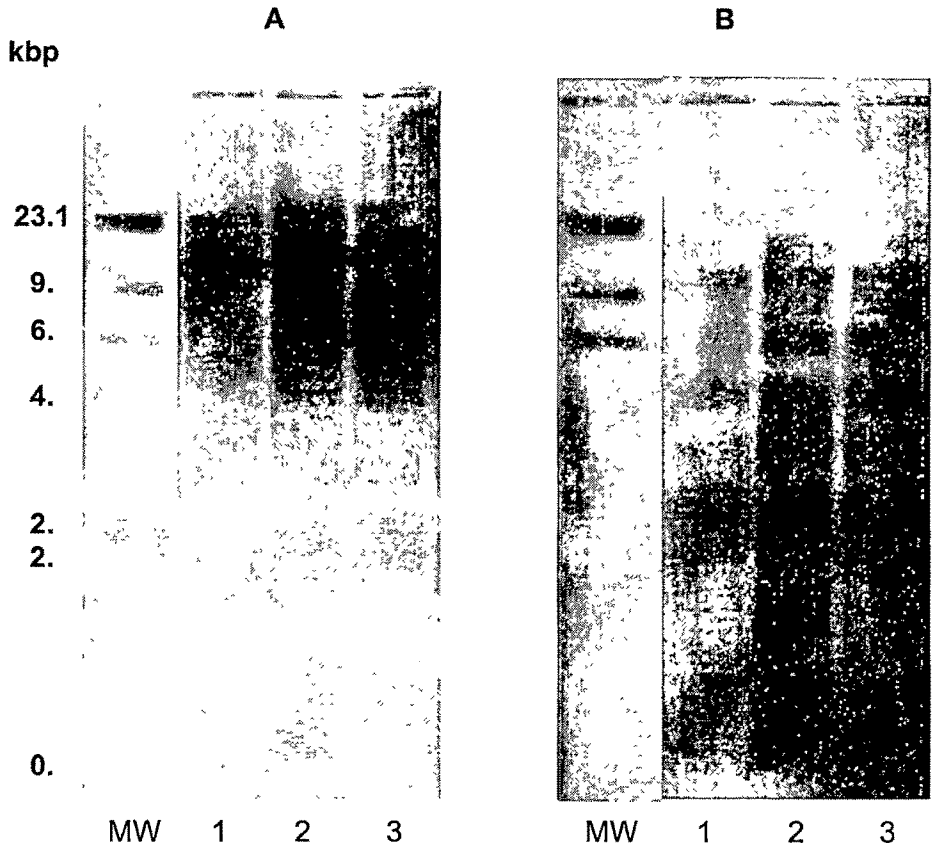
TERMINAL RESTRICTION FRAGMENT LENGTH ANALYSIS

The telomere length in tissue specimens from head and neck cancer patients was analyzed by southern blot analysis. Representative results are illustrated in **Figure-15** and **Figure-16**. Lanes 2, 3 and 4 in **Figure-15A** represent adjacent normal tissue, submucous fibrosis and malignant tissue specimens respectively from a patient with carcinoma of buccal mucosa. **Figure-15B** shows the results of rehybridization of the same membrane with minisatellite probe $(CAC)_5$. This step was performed to check proper digestion of each DNA sample. The films were scanned on a gel documentation system and analysed densitometrically using Molecular Analyst software (Bio-Rad, USA). This software is a powerful tool for analysing images and data from the

image analysis system. The Molecular Analyst/PC graphical interface operates to simplify image manipulation and to determine relative mobility and molecular weight of the samples. Densitometric scans obtained for the three specimens are shown in **Figure-15C**. It is clearly evident from this figure that TRF length in malignant tissue specimens was shorter as compared to the adjacent normal and precancerous tissue specimens. The difference in TRF length can be noted both in the southern blots a well as the densitometric scans.

Figure-15

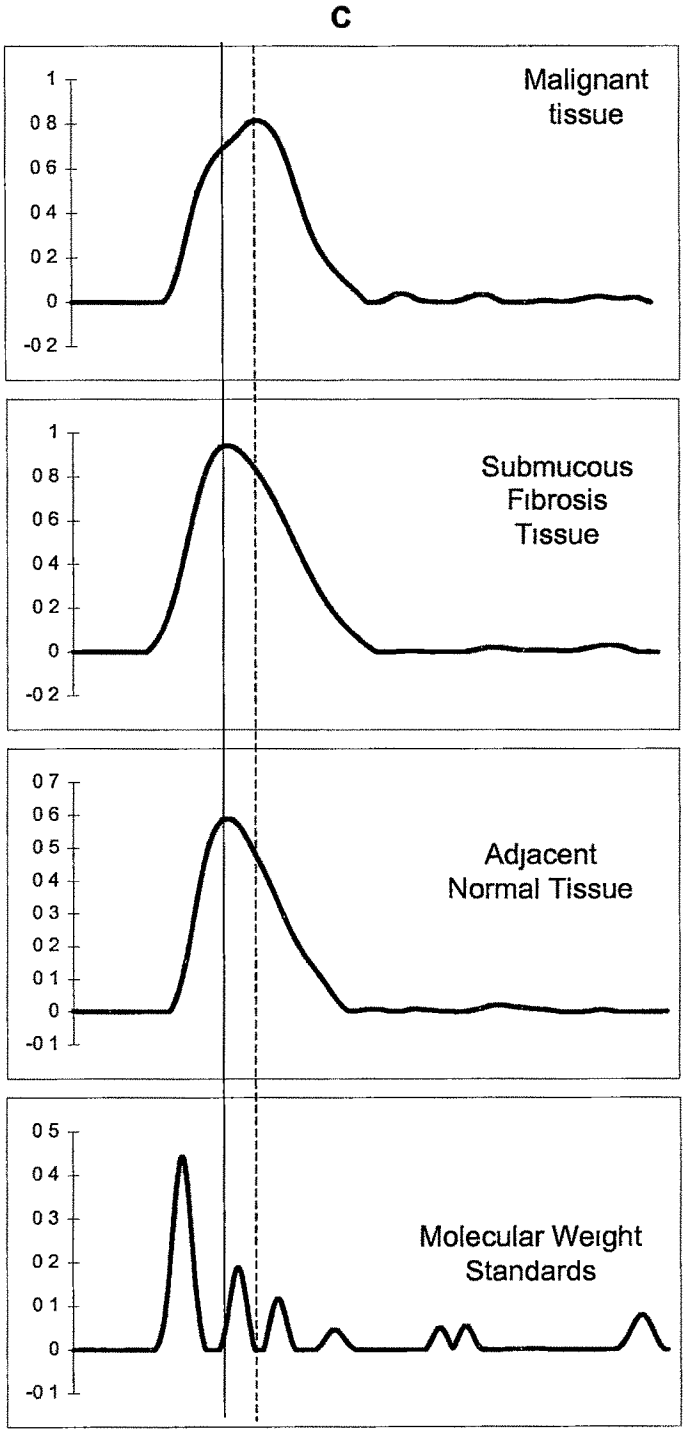
TRF length analysis in different tissue specimens from head and neck cancer patients



A: TRF length analysis.

Lane 1 molecular weight markers, lane 2. adjacent normal tissue,
Lane 3 submucous fibrosis; lane 4 malignant tissue

B: Rehybridization of the same membrane with minisatellite probe $(CAC)_5$



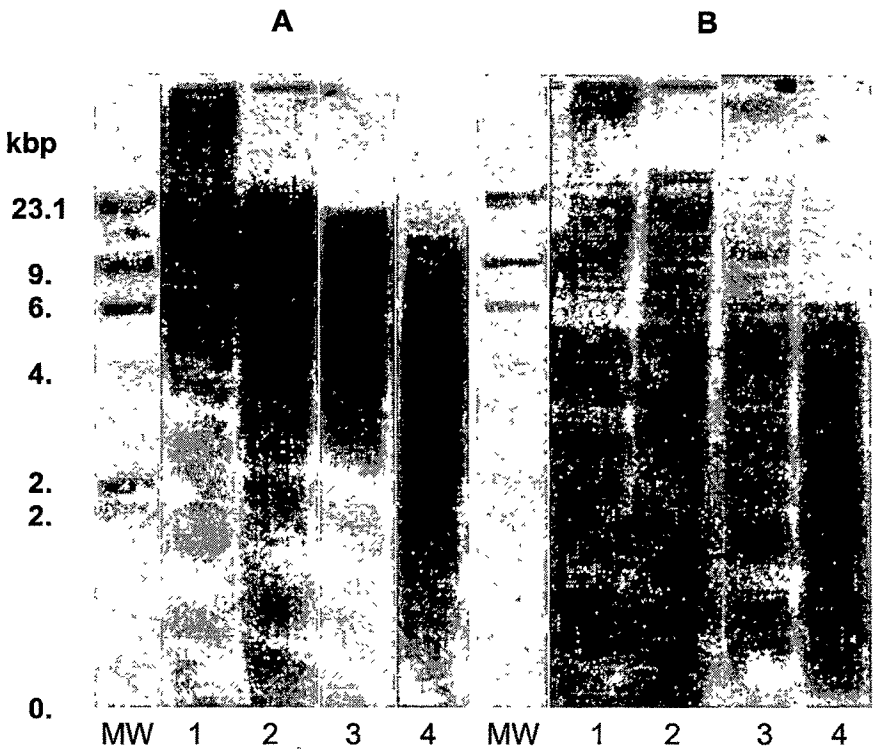
C: Densitometric scans of the blots

Figure-16 depicts the TRF pattern, the minisatellite pattern and densitometric scans of DNA samples from patients with head and neck cancer. Lanes 2 and 3 in **Figure-16A** show TRF pattern in adjacent normal and malignant tissue specimens respectively from a patient with carcinoma of tongue. While lanes 4 and 5 represent adjacent normal and malignant tissue specimens respectively from a patient with carcinoma of hard palate. **Figure-16B** shows the results of rehybridization of the same membrane with minisatellite probe $(CAC)_5$. Densitometric scans obtained for these three specimens are shown in **Figure-16C**. Decrease in TRF length in malignant tissue specimens as compared to the adjacent normal tissue specimens can be noted in the blots as well as the densitometric scans.

All the samples were analysed densitometrically. Density peak of each sample was considered to be its peak TRF length. Peak TRF length of each sample was calculated by comparison with the molecular weight standards, which were always run on every gel processed for TRF analysis. The calculations were carried out with the help of Molecular Analyst PC image analysis software provided with the Gel Documentation System (Bio-Rad, USA).

Figure-16

TRF length analysis in adjacent normal and malignant tissues from patients with head and neck cancer



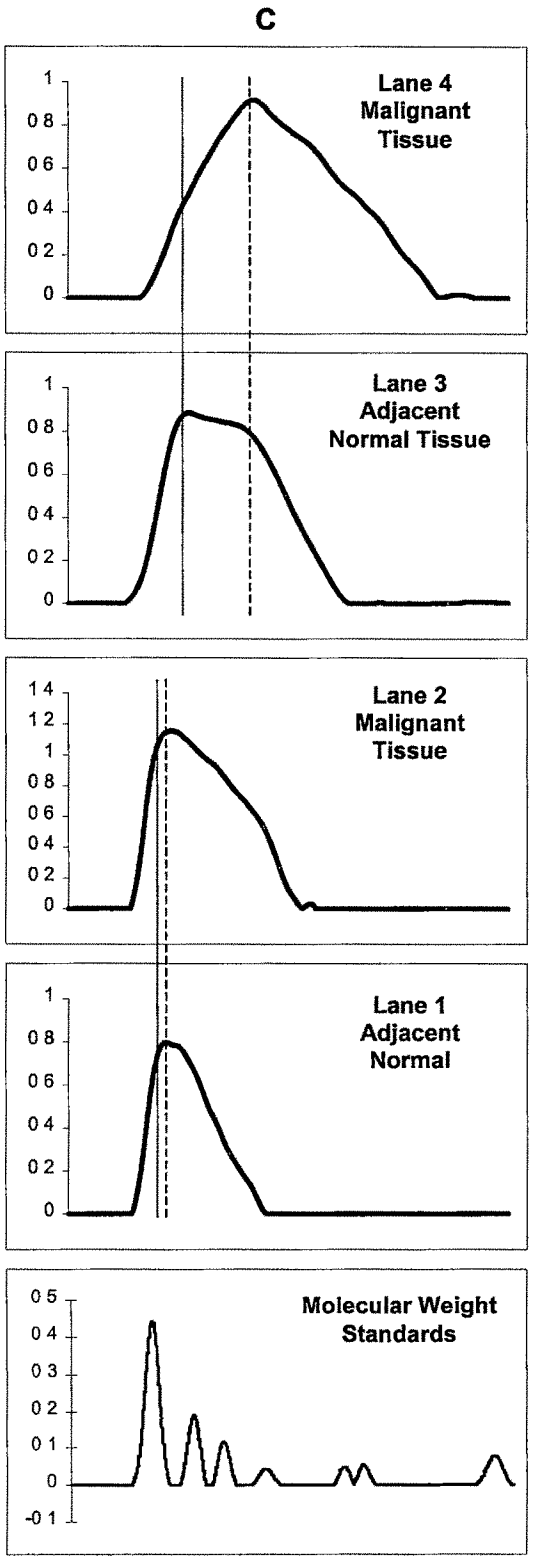
A. TRF length analysis.

Lane MW: molecular weight markers

Lanes 1 and 2: adjacent normal and malignant tissue from same patient

Lanes 3 and 4. adjacent normal and malignant tissue from same patient

B: Rehybridization of the same membrane with minisatellite probe $(CAC)_5$



C: Densitometric scans of the blots

Table-12 shows the range and mean of the peak TRF length in tissue specimens from cancer patients. It was observed that different tissue specimens revealed wide variation in the peak TRF length. The peak TRF length ranged from 4.59 kb to 18.96 kb in adjacent normal tissues and 3.89 kb to 15.58 kb in malignant tissues. However, the noteworthy finding was that the mean peak TRF length in malignant tissues was significantly lower as compared to that in adjacent normal tissues ($p<0.001$). The mean peak TRF length in malignant tissue was 8.23 kb as against 10.14 kb in adjacent normal tissues.

Table-12

TRF length in tissue specimens from patients with head and neck cancer

Tissues (N)	Peak TRF length	
	Range (kb)	*Mean ± S.E. (kb)*
Adjacent normal tissues (84)	4.59 - 18.96	10.14 ± 0.2795
Malignant tissues (91)	3.89 - 15.58	8.23 ± 0.3166
"t" value: 4.504; significance: $p<0.001$		
paired "t" value: 5.133; significance: $p<0.0001$		

Paired "t" test analysis, which compared the peak TRF length beteween adjacent normal and malignant tissue specimens in individual patients, also revealed the similar results. The peak TRF length in malignant tissues was significantly lower as compared to that in adjacent normal tissues ($p<0.0001$).

TRF Length in Oral Cavity Cancer

As oral cavity represents the major subsite of head and neck region and is very important to elucidate the molecular characteristics of tumors of these sites, peak TRF length was calculated in patients with oral cavity cancer. **Table-13** provides the range and mean of peak TRF length observed in patients with oral cavity cancer, which represented the major part of head and neck cancer in this study. The results were similar to that observed in head and neck cancer patients, i.e. peak TRF length in malignant tissues was significantly lower as compared to the adjacent normal tissues in patients with oral cavity cancer.

Thus, the results obtained for telomere length analysis in oral cavity cancer patients reflect the overall picture of head and neck cancer patients, which shows declined peak TRF length in malignant tissues.

Table-13

TRF length in tissue specimens from oral cavity cancer patients

Tissues (N)	Peak TRF length	
	Range (kb)	*Mean ± S.E. (kb)*
Adjacent normal tissues (70)	4.59 - 18.96	9.89 ± 0.4143
Malignant tissues (67)	3.89 - 15.58	8.38 ± 0.3076
"t" value: 2.936; significance: p<0.01		
paired "t" value : 4.145; significance: p<0.0001		

Correlation between TRF Length Stage wise Disease Activity

Table-14 shows the mean peak TRF length in patients with different stages of disease. As detailed in the table, the mean peak TRF length in malignant tissues was significantly lower than that in adjacent normal tissues only in stage IV patients. In comparison between early disease and advanved disease, the group of patients with advanced stage of disease showed significantly lower peak TRF length. Moreover, there was no significant difference in the mean TRF length in malignant

tissues in patients with early stage of disease and patients with advanced stage of the disease.

Table-14

Comparison between mean peak TRF length in different tissue specimens and stage of the disease

Stage	TRF length Mean±S.E. (N)		"t" value	Significance
	Adjacent Normal	*Malignant*		
I	7.44 ± 0.760 (04)	6.61 ± 0.624 (04)	0.845	NS
II	10.84 ± 1.398 (09)	8.28 ± 0.624 (13)	1.669	NS
III	10.97 ± 0.689 (15)	9.63 ± 0.660 (13)	1.410	NS
IV	10.02 ± 0.369 (56)	8.03 ± 0.361 (61)	4.115	p<0.0001
Early	9.71 ± 1.05 (13)	7.87 ± 0.615 (17)	1.509	NS
Advance	10.22 ± 0.325 (71)	8.32 ± 0.325 (74)	4.119	p<0.0001
Early Vs. Advanced (Malignant): "t" value 0.6611; Significance: NS				

NS Not significant

Paired "t" test analysis revealed that the patients with stage I and stage IV disease showed significantly shorter peak TRF length in malignant tissue specimens as compared to adjacent normal tissue specimens.

Further, when grouped together, patients with both early and advanced stage of disease showed a significant decrease in peak TRF length in malignant tissues as compared to adjacent normal tissues (**Table-15**).

Table-15

Paired "t" test analysis for peak TRF length in patients with different stages of disease

Stage (N)	"t" value	Significance
I (4)	6.180	p<0.01
II (8)	1.972	NS
III (13)	1.927	NS
IV (48)	4.334	p<0.001
Early (12)	2.686	p<0.02
Advanced (61)	4.751	p<0.0.001

NS- Not significant

TRF Length Analysis Based on Nodal Status

As evident from **Table-16**, even when the patients were grouped according to nodal status, the peak TRF length was significantly shorter in malignant tissues (8.21 kb and 8.28 kb, respectively) as compared to

the adjacent normal tissues (9.77 kb and 10.55 kb, respectively) in both node negative and node positive patients with head and neck cancer. The peak TRF length in node nagative and node positive patients with head and neck cancer was also compared using paired "t" test to evaluate case-wise significance (**Table-17**). It was again found that the peak TRF length was significantly shorter in malignant tissues as compared to the adjacent normal tissues in both node negative and node positive patients with head and neck cancer.

Table-16

Comparison of mean peak TRF length in tissue specimens in node negative and node positive head and neck cancer patients

Nodal status	Mean Peak TRF Mean±S.E. (N)		"t" value	Significance
	Adjacent Normal	*Malignant*		
Negative	9.77±0.4043(45)	8.21±0.4391(57)	2.898	p<0.01
Positive	10.55±0.4975(39)	8.28±0.4558(34)	3.3697	p<0.01
"t" value	1.2182	0.1259		
Significance	NS	NS		

NS Not significant

Table-17

Paired "t" test analysis of peak TRF length

in node positive and node negative head and neck cancer patients

Nodal status	"t" value	Significance
Node negative	3.3768	$p<0.02$
Node positive	4.0021	$p<0.01$

Peak TRF Length in Tumors of Different Histologic Grades

Table-18 documents comparison of mean peak TRF length in patients with different histologic grades, which are considered to be important histologic features for prognostication of the disease. Mean peak TRF length was found to be significantly shorter in well differentiated tumors (8.13 kb) as compared to adjacent normal tissues (10.37 kb). However, no significant difference was observed between mean peak TRF length in tumors with well, moderate or poor differentiation.

Paired "t" test analysis in the results from head and neck cancer patients with different histologic grades of the tumor showed that peak

TRF length was shorter in malignant tissues as compared to adjacent normal tissues in all the groups, which can be seen in **Table 19**.

Table-18

Comparison of mean peak TRF length in tumors with different histologic grades

Histologic grade (Differentiation)	**Peak TRF length** Mean±S.E. (N)		**"t" value**	**Significance**
	Adjacent Normal	*Malignant*		
Well (a)	10.37 ± 0.512 (42)	8.13 ± 0.386 (44)	3.488	p<0.001
Moderate (b)	9.77 ± 0.440 (27)	8.54 ± 0.504 (30)	1.834	NS
Poor (c)	10.16 ± 0.899 (10)	7.80 ± 0.734 (17)	2.033	NS
"t" value	aVs.b: 0.901	aVs.b: 0.6479		
	bVs.c: 0.3886	bVs.c: 0.835		
	cVs.a: 0.1730	cVs.a: 0.4006		

NS: Not significant, Vs versus

Table-19

Paired "t" test analysis of peak TRF length in head and neck cancer patients showing different tumor hoistologic grades

Differentiation	"t" value	Significance
Well	3.7716	$p<0.001$
Moderate	2.1375	$p<0.05$
Poor	3.3566	$p<0.01$

Peak TRF Length in Tumors of Different Nuclear Grades

Table-20 represents the comparison of mean peak TRF length in head and neck cancer patients with different nuclear grades. The nuclear grade represents the nuclear characteristics of the tumor which are important for elucidation of the disease status. As detailed in the table, mean peak TRF length was found to be significantly shorter in malignant tissues as compared to adjacent normal tissues in patients with nuclear grade I and III tumors. However, no significant difference was observed between mean peak TRF length in tumors with nuclear grade I, II or III.

Table-20

Comparison of mean peak TRF length

in tumors of different nuclear grades

Nuclear grade	Peak TRF length Mean±S.E. (N)		"t" value	Significance
	Adjacent Normal	*Malignant*		
I (a)	10.37±0.511 (40)	8.02±0.402 (43)	3.488	p<0.001
II (b)	9.74±0.512 (32)	8.58±0.485 (30)	1.834	NS
III (c)	10.16±0.899 (10)	8.50±0.949 (17)	2.033	p<0.01
"t" value	aVs.b: 0.876	aVs.b: 0.892		
	bVs.c: 0.710	bVs.c: 0.076		
	cVs.a: 0.417	cVs.a: 0.467		

NS Not significant, Vs Versus

Paired "t" test analysis of the results obtained for TRF length analysis in head and neck cancer patients based on the nuclear grade of the tumor revealed that peak TRF length was significantly shorter in malignant tissues as compared to adjacent normal tissues in grade I and II patients. This can be observed in **Table-21**.

Table-21

Paired "t" test analysis of peak TRF length in head and neck cancer patients showing different tumor nuclear grades

Nuclear Grade	"t" value	Significance
I (36)	3.3368	$p<0.02$
II (27)	3.9410	$p<0.01$
III (7)	1.0839	NS

NS Not significant

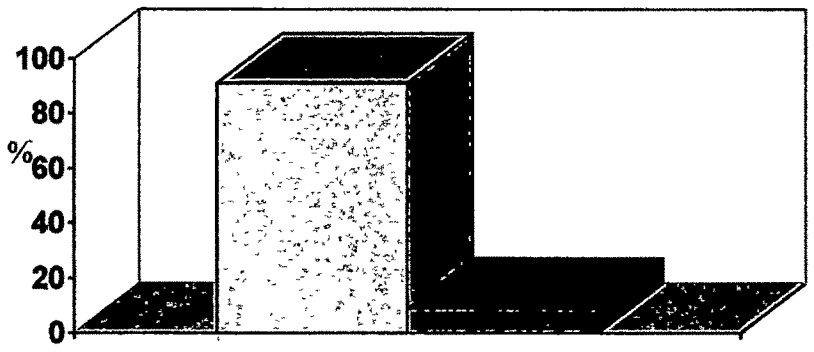
Thus, it is clear that there was no significant difference in mean TRF length in either malignant or adjacent normal tissues in tumors with different stages of disease, different nodal status or different histologic and nuclear grades. It is evident that telomere length did not change significantly with severity of the disease.

Patients with shorter TRF length in malignant tissues

It is important to evaluate the telomere length in each head and neck cancer patient; to check the difference in telomere length in individual patient. The histogram in **Figure-17** documents that more than 90% of the patients showed shorter peak TRF length in malignant tissues as compared to adjacent normal tissues.

Figure-17

Patients showing shorter peak TRF length in malignant tissues as compared to adjacent normal tissues



Correlation of peak TRF length with Clinicopathological Characteristics

Multivariate analysis was performed to establish any possible correlation between peak TRF length and different clinicopathological parameters like age, gender, site of tumor, stage of the disease, nodal involvement, histologic and nuclear grades of tumor (**Table-22**). However, no correlation could be observed between these parameters indicating peak TRF length was an independent feature of the tumors. *It is clear from the data that telomere length was independent of the clinical and histopathological features.*

Table-22

Correlation of peak TRF length with clinicopathological characteristics of the head and neck cancer patients

Parameter	Adjacent Normal Tissue		Malignant Tissue	
	F value	*Significance*	*F value*	*Significance*
Age	0.113	0.976	0.466	0.760
Gender	0.011	0.919	1.087	0.314
Habit	0.625	0.441	0.022	0.833
Site	0.427	0.871	1.575	0.217
Stage	1.331	0.258	1.520	0.227
Nodal status	0.041	0.841	0.345	0.561
Histologic Grade	1 521	0.235	0.184	0.833
Nuclear Grade	0.103	0.902	0.128	0.880

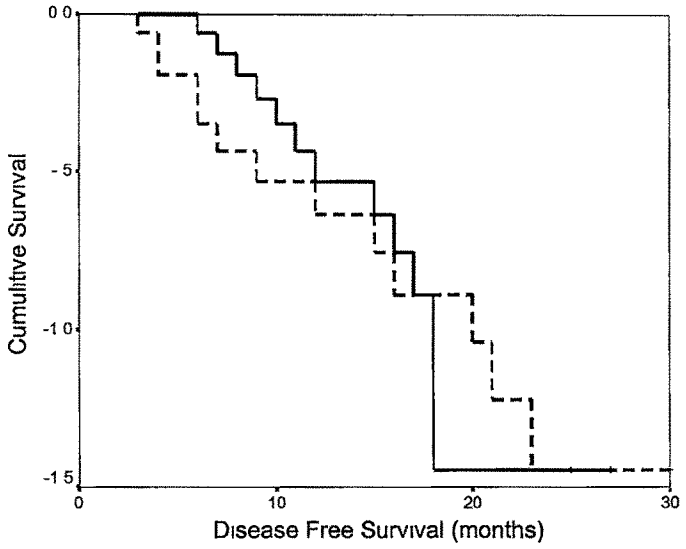
Correlation of Peak TRF Length with Disease Free Survival of the Patients

It is essential to evaluate the prognostic value of any cellular or molecular marker while studying its clinical utility. Therefore, we analysed whether peak TRF length showed any correlation with the disease status of the patient after anticancer therapy. Median value of peak TRF length in both the groups i.e. adjacent normal and malignant tissues was calculated. Patients were divided into two groups: (i) patients with peak TRF length less than the median value and (ii) patients with peak TRF value greater than the madian value. The correlation between peak TRF length and the two year disease free survival was analysed by plotting Kaplan and Meier survival plots **(Figure-18A).** The two year disease free survival analysis showed that peak TRF length in the adjacent normal tissue had no effect on the disease free survival of the patients. However, clinical course of the disease significantly correlated with the peak TRF length in the malignant tissues. A significantly better two year disease free survival was observed in the patients with shorter TRF length in malignant tissues as compared to the patients showing longer TRF length (**Figure-18B**).

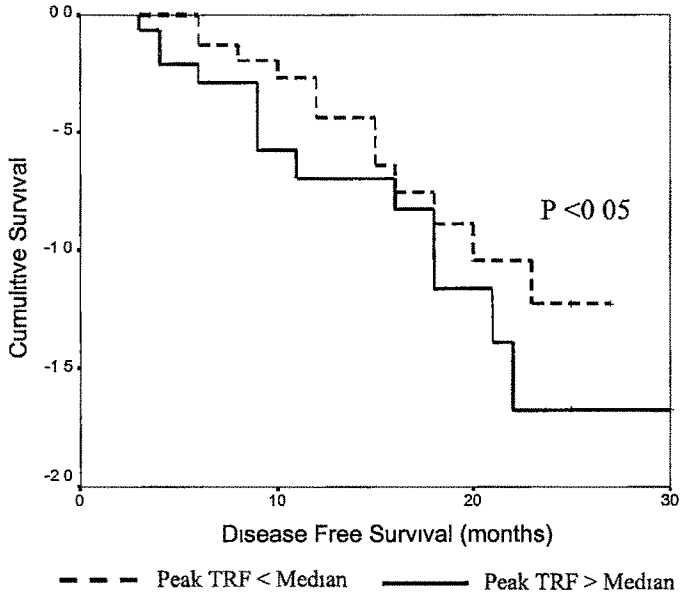
Figure-18

Correlation of peak TRF length with disease free survival

A: Adjacent normal tissues



B: Malignant tissues



A: Correlation of peak TRF length in adjacent normal tissues with disease free survival.

B: Correlation of peak TRF length in malignant tissues with disease free survival.

The Kaplan and Meier plots revealed that the analysis of TRF length in malignant tissue may be helpful in predicting the prognosis of the disease. TRF length analysis may serve as an aid in discerning the patients who are going to have poor prognosis and therefore are in need of a closer follow-up and aggressive anticancer therapy.

Correlation Between Telomerase Activation and TRF length

Spearman's correlation test was performed to evaluate the correlation between telomerase activation and peak TRF length, the results of which are shown in **Table-23**.

Table-23

Correlation between telomerase activation and peak TRF length

Telomere activation Vs. TRF length	Spearman's Rho	Significance
Malignant tissues	-0.093	0.486
Adjacent Normal tissues	-0.253	0.065

As clear from the table, no correlation was observed between both the parameters, in either malignant or adjacent normal tissues. Telomerase activation and peak TRF length was independent of each other.