

Assaying Telomerase Activity in Three Different Malignant Conditions in Egyptian Patients Employing the Polymerase Chain Reaction Technique

EMAD IBRAHIM ABDEL-FATTAH, M.D.*; SAMIR MOHAMMAD ABDEL-MONEIM, M.D.*;
EMAD ELDIN FOUAD ISMAIL, M.D.*; MOHAMAD TAREK MANSOUR, Ph.D.** and
TAHER IBRAHIM EI-SERAFI, M.D.*

*Biochemistry Dept., Faculty of Medicine, Suez Canal University and **Virology and Immunology Dept,
National Cancer Institute, Cairo University.

ABSTRACT

Background: Telomerase enzyme adds hexameric repeats of 5'-TTAGGG-3' to the end of mammalian chromosomal DNA (telomeres) to compensate for the progressive loss that occurs with successive rounds of DNA replication. Significant telomerase activity has been identified in human germline cells in neoplastic immortal somatic cells, in normal human lymphocytes and in stem cells, but not in most normal somatic cells, which senesce after a certain number of cell divisions.

Objective: The present study was conducted to examine telomerase activity in three different common malignant conditions in Egypt: 1) treated and untreated chronic myeloid leukemia (CML), 2) hepatocellular carcinoma (HCC) with and without type B or C viral hepatitis and 3) urinary bladder carcinoma (UBC) with and without bilharziasis.

Material and Methods: In CML, twenty subjects were included in each group and results were compared to a control group of age-matched apparently normal individuals. In HCC and UBC specimens were included in each group. In CML cases, peripheral blood mononuclear cells were analyzed, while in HCC and UBC cases, tumor biopsy specimens were used. Telomerase activity was estimated by polymerase chain reaction (PCR) followed by ELISA technique, which is a modification of the original telomerase repeat amplification protocol (TRAP). This technique allows highly specific amplification of telomerase-mediated elongation products combined with non-radioactive detection following an ELISA protocol.

Results: In CML, 85% of untreated patients were telomerase positive which was significantly higher than treated patients (65%) or control group (30%). In HCC, 57% of cases were telomerase positive. A significantly higher percentage of telomerase positive specimens were found among HCC cases associated with either HCV (75%) or HBV (71.4%) infection than in HCC cases alone (22.2%). In UBC, 69% of patients were telomerase positive without differences between bilharzial and non-bilharzial cases.

Conclusion: Elevated telomerase enzyme activity is associated with untreated or viral- complicated malignancies and can be used as an adjuvant to parameters used for monitoring tumor progression or the effect of therapy.

Key Words: *Telomerase activity - Telomerase repeated amplification - Hepatocellular carcinoma - Urinary bladder cancer - Chronic myeloid leukemia - Hepatitis C virus - Hepatitis B virus.*

INTRODUCTION

Ends of linear chromosomes are capped by specialized nucleoprotein structures termed telomeres. Telomeres comprise tracts of noncoding hexanucleotide repeat sequences that, in combination with specific proteins, protect against degradation, rearrangement, and chromosomal fusion events. Due to the polarity of conventional DNA synthesis, a net loss of telomeric sequences occurs at each cell division. It has been proposed that this cumulative telomeric erosion is a limiting factor in replicative capacity and elicits a signal for the onset of cellular senescence. To proliferate beyond the senescent checkpoint, cells must restore telomere length. This can be achieved by telomerase, an enzyme with reverse-transcriptase activity. This enzyme is absent in differentiated somatic tissues, but telomerase reactivation has been detected in most tumors [19].

Telomerase has been detected in the majority of human malignant tumors, making telomerase activity one key difference between mortal and immortal cells [18]. Therefore, telomerase offers the potential opportunity to control cell

proliferation by interfering with a totally new and unique biological process which is cell senescence [12].

In leukemia, telomerase is specifically activated during the progression stages of the disease [5]. In chronic myeloid leukemia (CML), there is detectable telomerase activity above background, and high frequency of additional cytogenetic changes [16].

Telomerase activity was reported in liver samples from patients with hepatitis and liver cirrhosis but no tumor pathology. Tahara et al., [20] studied telomerase activity in 105 frozen samples from human normal liver tissues, chronic liver disease, and hepatocellular carcinoma (HCC). They found that telomerase activity was positive in 28 of 33 HCC tissues regardless of tumor stage or size. Telomerase activity was expressed in 15 of 18 differentiated HCC nodules smaller than 3 cm. HCC tissues from all eight hepatitis B virus-positive patients were telomerase positive, while telomerase activity was not detected in normal liver tissues (0 of 4). Weak telomerase activity was only detected in 1 of 22 non-tumor liver tissues from HCC patients. Interestingly, in 19 of 38 hepatitis tissues and 6 of 8 cirrhotic liver tissues from apparently cancer-free patients, very weak telomerase activity was detected.

Yang et al. [22], reported the telomerase in 95.7% of both cancer tissues and bladder wash specimens. Telomerase activity was undetected in all normal tissues except one, which was obtained from a patient with carcinoma in situ.

Tumor specimens from a cohort of patients with bladder cancer showed telomerase activity in exfoliated cells in 23 (55%) of the 42 spontaneously voided urine specimens and in 36 (84%) of the 43 bladder-washing fluids examined. Considering voided urine specimens and bladder-washing fluids together, telomerase was detected in exfoliated cells from 40 (89%) of the 45 patients [8].

Yoshida et al., [23] suggested that telomerase could be a good diagnostic marker for the early noninvasive identification of patients with bladder carcinoma by facilitating the detection of exfoliated immortal cancer cells in urine.

This work was directed to study telomerase activity in peripheral blood mononuclear cells of treated and untreated chronic myeloid leu-

emia patients, in hepatocellular carcinoma, in liver diseases and in urinary bladder carcinoma with and without bilharziasis.

PATIENTS AND METHODS

MATERIAL:

CML Patients:

Patients with Philadelphia translocation-positive CML were seen at the Suez Canal University Hospital and the National Cancer Institute, Cairo University. All cases were diagnosed by competent hematologists in the above institutions. The subjects were divided into 3 groups, each containing 20 individuals: 1) Newly diagnosed CML patients who have not taken any treatment, 2) Established cases of CML patients under regular treatment of Hydroxyurea and 3) a control group of apparently healthy individuals from blood bank donors.

HCC Tissue Samples:

This study was carried out on 64 liver tissue samples including 18 specimens with hepatocellular carcinoma, 18 specimens with hepatocellular carcinoma with HBV, 18 specimens with hepatocellular carcinoma with HCV and 10 apparently normal liver tissue samples that served as controls. The tissue samples were obtained from the National Cancer Institute, Cairo University. Samples were frozen in liquid nitrogen and stored at (-80°C) until used. The samples were taken by ultrasound guided biopsy or during operation and were already diagnosed pathologically.

UBC Tissue Samples:

This study was carried out on 42 urinary bladder cancer tissue specimens: 16 with bilharziasis and 16 without bilharziasis, and 10 apparently normal bladder tissue samples that served as controls. Samples were obtained from the National Cancer Institute, Cairo University.

METHODS:

I- Sample Collection:

* *CML Patients:*

Five millilitres (5ml) of blood were collected in sterile sodium heparin vacutainer tubes. Mononuclear cells were obtained by the ficoll-Hypaque 1077 (cell separating media) density gradient method, using sodium diatrizoate-poly sucrose gradient, resulting in blast-enriched

fractions consisting of >80% blast cells in the blastic phase [3].

** HCC Tissue Samples:*

Thin slices (around 10-15 mm thick) of frozen tissue specimens were prepared on sterile disposable Petri dishes with surgical disposable knife blades to obtain around 50 thin flakes, which were then immediately transferred to sterile Eppendorf tubes containing 200uI ice-cold lysis buffer and incubated on ice for 30 minutes. Then the lysate was centrifuged at 16000xg for 20 minutes at 4°C in a refrigerated centrifuge. Only 175uI of the supernatant were carefully removed and transferred to 1 fresh Eppendorf tube and then protein concentrations were determined by the Pasteur Lab. protein assay kit Lot No. 222. Protein content was fixed at 20 ug for all samples. The tissue extracts were shock frozen in liquid nitrogen and stored at -8 0°C until used in the second step.

** UBC Tissue Samples:*

This study was carried out on 32 urinary bladder tissue samples including 16 specimens with bilharzial bladder cancer and 16 with non-bilharzial bladder cancer. The tissue samples were snap frozen in liquid nitrogen and stored at -80°C until used. The samples were already diagnosed pathologically and their clinical data were retrieved from the patients' files.

II- Telomerase Activity Assay By PCR:

Telomerase activity was assessed according to the telomeric repeat amplification protocol (TRAP method) as described by Kim et al. [7] with minor modifications. In this protocol, telomerase adds telomeric repeats (TTAGGG) to the 3' end of the biotin-labeled synthetic P1-TS-primer. These elongation products are amplified by PCR using the primers P1-TS and P2 reverse primer, generating a PCR product with the telomerase-specific 6 nucleotide increments (Telomerase PCR ELISA KIT Cat. No. 1 854 666. By Boehringer Mannheim, Germany).

III- Hybridization and ELISA detection:

An aliquot of the PCR product was denatured and hybridized to a digoxigenin-labeled telomeric repeat-specific detection probe. The resulting product was immobilized via the biotin-labeled primer to a streptoavidin-coated microtiter plate. The immobilized PCR product is then detected with an antibody against digoxigenin that is conjugated to peroxidase. The probe

is visualized by virtue of peroxidase-metabolizing TMB to form a yellow colored reaction product. (Telomerase PCR ELISA KIT Cat. No. 1 854 666 (Boehringer Mannheim, Germany)).

RESULTS

CML Patients:

The study subjects were classified into telomerase positive and telomerase negative based on a cutoff optical absorbance value (0.127) that was calculated according to the assay kit manufacturer's recommendations as follows:

$$\text{Cutoff} = \text{mean} \pm 2 \text{ S.D} \quad 0.127 = 0.57 \pm 2 \times 0.35$$

As shown in table (1) and fig. (1), 6 out of 20 subjects in the control group (30%) were telomerase positive which was statistically significantly less than telomerase negative ones [14 out of 20 (70%)] ($p < 0.001$). Conversely, in untreated CML patients, 16 out of 20 patients (80%) were telomerase positive which was statistically significantly higher than telomerase negative ones [4 out of 20 (20%)] ($p < 0.001$). Similarly, in treated CML patients, 13 out of 20 patients (65%) were telomerase positive which was statistically significantly higher than telomerase negative ones [7 out of 22 (35%)] ($p < 0.001$). On comparing untreated CML and control groups, the percentage of telomerase positive subjects in untreated CML patients (80%) was statistically significantly higher than that in control group (30%) ($p < 0.05$). Also, on comparing treated CML and control groups, the percentage of telomerase positive subject and in treated CML patients [13 out of 20 (65%)] was statistically significantly higher than that in control group [6 out of 20 (30%)] ($p < 0.05$). However, on comparing untreated and treated CML patient groups, there was no statistically significant difference in telomerase activity.

Table (1): Comparison between CML patients and the control group as regards the distribution of telomerase activity.

Telomerase activity	Control		Untreated CML		Treated CML	
	No.	%	No.	%	No.	%
Positive	6	30	16	80	13	65
Negative	14	70	4	20	7	35
Total	20	100	20	100	20	100

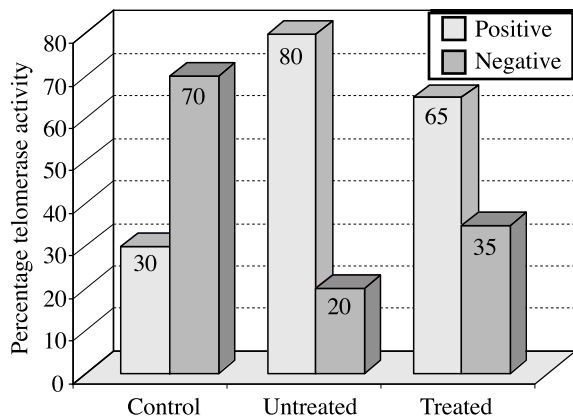


Fig. (1): Comparison between CML patients and control group as regards the distribution of telomerase activity.

On employing simple linear regression analysis to detect any correlation between absorbance of telomerase activity and hematological parameters, a statistically significant direct correlation between mean absorbance of telomerase activity and blast cell percentage in telomerase positive subjects in untreated CML patients was found ($r = 0.7855, p < 0.001$). Fig. (2) shows the correlation between mean absorbance of telomerase activity and blast cell percentage in telomerase positive untreated CML patients.

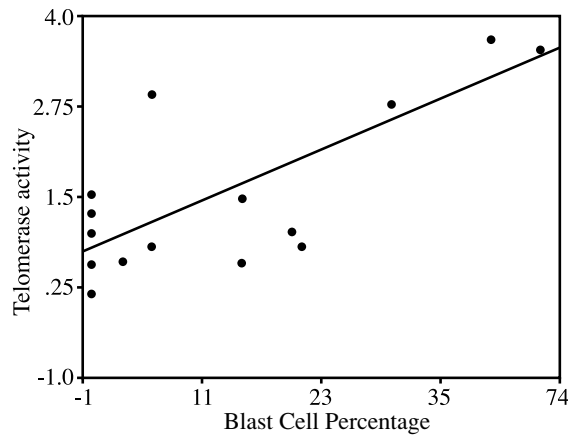


Fig. (2): Correlation between mean absorbance of telomerase activity and blast cell percentage in telomerase positive untreated CML patients.

Table (2): Telomerase activity among all tissue samples of HCC.

Telomerase activity	No.	%
Positive	16	57.14
Negative	12	42.86
Total	28	100

In HCC:

Assay of telomerase activity in all tissue samples of HCC showed positive results in 16 tissue samples out of 28 (57.14%) as shown in table (2) and fig. (3). All control specimens were telomerase negative.

Table (3) and fig. (3) show the distribution of telomerase activity among HCC alone, HCC with HBV and HCC with HCV. In HCC alone, telomerase activity was positive in 2 samples out of 9 (22.2%). In HCC with HBV, 5 samples out of 7 (71.4%) were positive and in HCC with HCV, 9 samples out of 12 (75%) were positive.

Table (3): Telomerase activity among HCC alone, HCC with HBV and HCC with HCV.

Telomerase activity	HCC alone		HCC with HBV		HCC with HCV	
	No.	%	No.	%	No.	%
Positive	2	22.22	5	71.43	9	75.0
Negative	7	77.78	2	28.57	3	25.0
Total	9	100	7	100	12	100

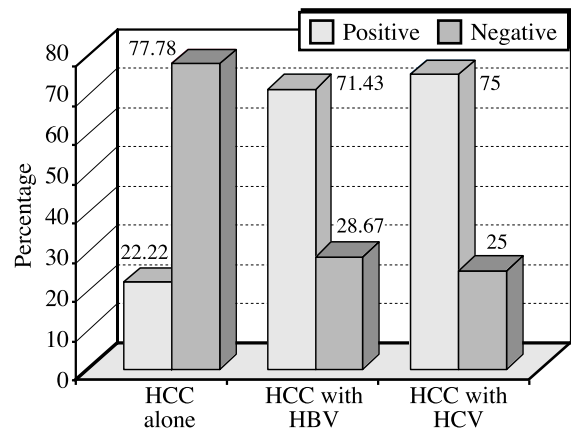


Fig. (3): Telomerase activity among HCC alone, HCC with HBV and HCC with HCV.

In UBC:

Assay of telomerase activity in all bilharzial and non-bilharzial bladder cancer tissue samples showed positive results in 22 tissue samples out of 32 (69%). The distribution of telomerase activity in bilharzial and non-bilharzial bladder cancer was equal in each group: 11 samples out of 16 (69%) as shown in table (4) and fig. (4). All control specimens were telomerase negative.

Table (4): Telomerase activity among bilharzial and non-bilharzial bladder cancer samples.

Telomerase activity	HCC alone		HCC with HBV		HCC with HCV	
	No.	%	No.	%	No.	%
Positive	11	69	11	69	22	69
Negative	5	31	5	31	10	31
Total	16	100	16	100	32	100

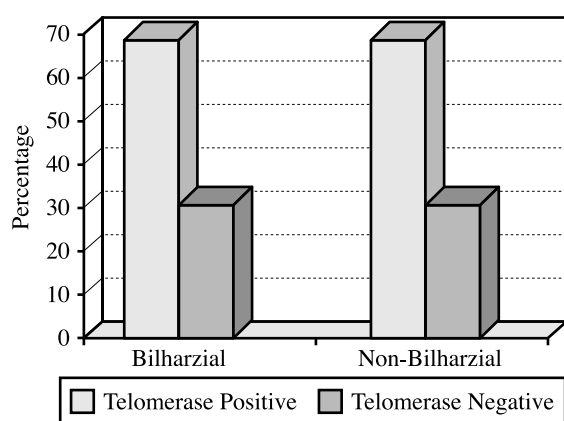


Fig. (4): Telomerase activity among bilharzial and non-bilharzial bladder cancer samples.

DISCUSSION

Mammalian cells have evolved complex mechanisms for regulating cellular life span. Normal cells demonstrate a strictly limited growth potential and senescence after a defined number of cell divisions. Cellular senescence is one of the bases of organismal aging. In contrast, tumor cells often exhibit an apparently unlimited proliferation potential and are called (immortalized). Some investigators have proposed that the progressive shortening of the tips of the eukaryotic chromosomes-the telomeres-is an important component of the senescence and is involved in control of the cell cycle. The enzyme telomerase adds TTAGGG repeats onto mammalian telomeres, which prevents their shortening. Telomerase is ordinarily inactive in most somatic cells but can be detected in tumor cells. The activation of telomerase in malignant cancers seems to be an important step in tumorigenesis, whereby the cell gains the ability of indefinite proliferation to become immortal. As detailed information accumulates about how telomeres and telomerase dynamics are involved in the regulation of the cell cycle events, one

can expect new opportunities for application to gerontology and cancer therapy [2].

CML Patients:

In the control group, telomerase activity was detected in the peripheral blood mononuclear cells in 6 out of 20 (30%) subjects. This finding is in agreement with Broccoli et al., [1] who found telomerase activity in peripheral blood leukocytes in normal volunteers.

Similarly, Tatematsu et al. [21] and Iwama et al. [6] found detectable telomerase activity in the peripheral blood of normal individuals. Also, Iwama et al. [6] found that the level of telomerase activity in hematopoietic cells was insufficient to prevent progressive shortening of the terminal restriction fragments (TRF) since reduction of TRFs in the peripheral blood cells correlates with increased age.

In this study, the frequency of telomerase positive patients in untreated CML group was 16 out of 20 (80%). This is in agreement with Broccoli et al. [1], Iwama et al. [6] and Ohyashiki et al. [16] who found that 100% of CML patients studied had telomerase positivity in their peripheral blood cells. On the other hand, our findings differ from those of Tatematsu et al., [21], who showed that telomerase activity was undetectable in peripheral blood of CML patients. This discrepancy of telomerase activity in CML patients may be due to difference in methodology used to measure telomerase activity because some of those investigators used fluorescent-labeled telomeric repeat quantitative amplification protocol assay, while others used a novel quantitative stretch PCR assay to detect increase in telomerase activity. We used telomere repeat amplification protocol (TRAP) which is a qualitative telomerase PCR ELISA method.

More recently, Li et al. [13] demonstrated that telomerase activity progressively increases as the bone marrow cells acquire increasing proliferative potential as in CML.

In our study, the frequency of telomerase positive patients in the treated CML group was 13 out of 20 (65%) and this frequency was less than that in the study by Iwama et al. [6], in which 100% of treated CML patient had telomerase activity in their peripheral blood. This difference also could be due to different methodology used to measure telomerase activity.

The frequency of telomerase-positive patients in the untreated CML group [16 out of 20 (80%)] was significantly higher than that in control group (30%). This agrees with Ohyashiki et al., [16] who found that 79% of CML patients in the chronic phase had elevated telomerase activity compared to the normal peripheral blood cells. Also, the frequency of telomerase-positive patients in the treated CML group [13 out of 20 (65%)] was significantly higher than that in the control group.

In the current study, there was no significant difference between the frequency of telomerase-positive untreated CML patients (80%) and those in treated CML group (65%). This agrees with the results of Ohyashiki et al., [16] and Iwama et al. [6], where the frequency of telomerase-positive untreated CML patients and telomerase-positive treated CML patients was 100% in both.

However, there was a statistically significant direct correlation between mean absorbance of telomerase activity and blast cell percentage in telomerase positive subjects in this group. On the other hand, the mean blast percentage in telomerase-positive untreated CML patients was significantly higher than that in telomerase-positive treated CML patients. Also, none of telomerase-negative patients in treated CML patients had any blast cells in their peripheral blood, while the mean blast percentage in telomerase positive patients in the same group was (2.54 ± 0.81) blasts.

These findings agree with the results of Ohyashiki et al., [16] who reported that blast cells had short TRFs and 50% had elevated telomerase activity compared to that in the chronic phase. One possibility is that progressive telomere shortening leads to a critical point and only those cells that upregulate telomerase activity in the blast phase survive. The same authors also added that, in CML, a clone of blast crisis may also exist in the chronic phase as a minor clone. Thus, if the high telomerase activity reflects blast crisis phase, they may also exist in the chronic phase. However, such cells might be quiescent in the chronic phase and eventually when they increase proliferation will be detected at the clinical level [16].

More recently, Li et al., [13] reported that telomerase activity progressively increases as the bone marrow cells acquire increasing prolifera-

tive potential. These data demonstrate that the increasing proliferative potential of the marrow which occurs during the development of AML is associated with a simultaneous increase in telomerase activity.

Could CML patients benefit from telomerase inhibitors? Until 2000, it was a fantastic topic for answering the key question asked by experimental and medical oncologists. Will telomerase be a therapeutic target for the third millennium? The most convincing argument for going ahead with this target is obviously the strong correlation existing between the level and frequency of telomerase expression and the malignant properties of tumors [12].

In HCC:

In this our study, telomerase activity was not detected in all tissue samples of hepatocellular carcinoma. This may agree with the other studies where telomerase activity could not be detected in 100% of their cases [11]. We found telomerase activity in 57.14% of our samples (16 out of 28). Nouse et al., [15] reported that 11 out of 29 (38%) HCC samples were weakly telomerase positive and there was no telomerase activity in normal liver samples. Huang et al., [4] demonstrated that telomerase activity was positive in 24 out of 39 HCC tissues (61.5%) and in 6 out of 39 non-tumor liver tissues (15%).

Park et al., [17] found that telomerase was strongly activated in 79% (19/24) of the hepatocellular carcinomas. All of 3 normal control livers showed no telomerase activation. No relationship could be observed between the enhancement of telomerase activity and tumor nature. Nagao et al., [14] found that telomerase activity was detected in 17 out of 19 hepatocellular carcinoma cases (89.47%).

In the current study, telomerase activity was detected in 75% of cases of HCC with HCV, and in 71% of cases of HCC with HBV, while in HCC alone, telomerase was positive in only 22% of cases, but this difference was not statistically significant which may be due to the low number of samples in each group.

However, this difference indicates that viral hepatitis infection may be a factor affecting telomerase activity in hepatocellular carcinoma, where viral DNA of HBV is integrated in DNA of hepatocytes and may affect expression of telomerase. HCV RNA may cause activation or

induction of telomerase activity. This agrees with the findings of Nouse et al., [15] who found that telomerase was positive in 50% (3/6) of patients with HCC and HBsAg positive, 91.7% (11/12) of HCC patients with positive HCV-Ab, and in 85.7% (6/7) of non B-non C patients with no significant difference between the groups.

In another study, telomerase activity was detected in 100% of HCC samples, and the activity correlated with the degree of differentiation. These results suggest that telomerase activity may be required as a critical step in the multi-genetic process of hepato-carcinogenesis and also may be useful in the characterization or prognostication of HCC [9].

In UBC:

In this study, telomerase activity was detected in 22 tissue samples of urinary bladder carcinoma out of 32, representing 69% of cases. Yoshida et al., [23] found that telomerase activity was detected in 48 (86%) of 56 urinary bladder carcinoma cases regardless of tumor stage or differentiation, whereas it was not found in any normal bladder tissue specimen.

Similarly, Kyo et al., [10] reported that different levels of telomerase activity were detected in all 22 urothelial tumors, including 13 bladder cancers, 8 ureteric cancers and one renal pelvic cancer but not detected in any adjacent normal tissue samples. Also, Kinoshita et al., [8] demonstrated that telomerase activity was found in 41 (98%) of the 42 urinary bladder carcinoma samples. In contrast, it was not detected in normal bladder tissue. Telomerase activity was detected in 22 (95.7%) of 23 tissue samples with transitional cell carcinoma of the urinary bladder, but not detected in any of the normal tissue samples [22].

On comparing the results of the present study with those listed above, one can find that our figure is lower than theirs. This can be attributed to the different pattern and characters of bladder cancer in Egypt from western countries with the supposition that it might possess inhibitors of telomerase or Taq polymerase, hindering detection of telomerase activity. Another possibility is that some cases may have a very low level of expression that can-not be detected by the current assays.

Will telomerase be a therapeutic target for

the third millenium? The most convincing argument (which is a scientifically documented one) for going ahead with this target is obviously the strong correlation existing between the level and frequency of telomerase expression and the malignant properties of tumors. This has been now largely documented in established tumor cell lines and fresh tumor samples obtained from patients. Noteworthy is the very important difference of telomerase expression between malignant and normal tissues. This difference is much higher than those observed for classical enzymatic targets of chemotherapy such as thymidylate synthetase, dihydrofolate reductase and topoisomerases. If this translates to the clinical situation, telomerase inhibitors might display a good selectivity for tumor cells with a minimal toxicity for normal tissues [12].

It is concluded that elevated telomerase enzyme activity is associated with untreated or viral-complicated malignancies and can be used as an adjuvant to parameters used for monitoring tumor progression or the effect of therapy.

REFERENCES

- 1- Broccoli D., Young J.W. and de Lang T.: Telomerase activity in normal and malignant hematopoietic cells. *Proc. Natl. Acad. Sci., USA* 90: 9082-9086, 1995.
- 2- Dahse R., Fudler W. and Ernst G.: Telomeres and telomerase: biological and clinical importance. *Clin. Chem.*, 43 (5): 708-714, 1997.
- 3- Ferrante A. and Thong Y.H.: Optimal condition of simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood by the Hypaque-Ficoll method. *J. Immunol. Methods.*, 36: 109-117, 1980.
- 4- Huang G.T., Lee H.S., Chen C.H., Chirn L.L. and Lin Y.W.: Telomerase activity and telomere length in human hepatocellular carcinoma. *Eur. J. Cancer*, 34 (12): 1946-9, 1998.
- 5- Ishikawa F.: Telomeres and telomerase. Department of life Science, Tokyo Institute of technology, Japan. *Human-cell*, 9 (4): 287-94, 1996.
- 6- Iwama H., Ohyashiki K., Ohyashiki J.K., Hayashi S., Kawakubo K., Shay J.W. and Toyama K.: Telomerase activity and cytogenetic changes in chronic myeloid leukemia with disease progression. *Leukemia*, 11 (2): 190-4 Feb., 1997.
- 7- Kim N.W., Piatyszek M.A., Weinrich S.I. and West M.D.: Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cells Sci.*, 17: 1-15, 1996.
- 8- Kinoshita H., Ogawa O., Kakehi Y., Mishina M., Mitsumori K., Itoh N., Yamada H., Terachi T. and Yoshida O.: Detection of telomerase activity in exfoliated

- cells in urine from patients with bladder cancer. *J. NCI*, 89 (10): 724-730, 1997.
- 9- Kishimoto K., Fujimoto J., Takeuchi M., Yamamoto H. and Okamoto E.: Telomerase activity in hepatocellular carcinoma and adjacent liver tissues. *J. Surg. Oncol.*, 69: 119-124, 1998.
 - 10- Kyo S., Kunini K., Uchibayashi T., Namiki M. and Hove M.: Telomerase activity in human urothelial tumors. *Am. J. Clin. Pathol.*, 107 (5): 555-560, 1997.
 - 11- Lanford R.E., Chavez D., Chisari F.V. and Sureau C.: Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood nonnuclear cells and other hepatic tissues by RT PCR. *J. Virol.*, 69: 8079-83, 1995
 - 12- Lavelle F., Riou J., Laoui A. and Milliet P.: Telomerase: a therapeutic target for the third millennium? *Crit. Rev. Oncol. Hematol.*, 34 (2): 111-126, 2000.
 - 13- Li B., Young J., Andrews C., Chen Y X., Toofanfared P., Huang R.W., Horvath E., Chopra H., Raza A. and Preisler H.D.: Telomerase activity in preleukemia and acute myelogenous leukemia. *Leuk. Lymphoma.*, 36 (5-6): 579-587, 2000.
 - 14- Nagao K., Tomimatsu M., Endo H. and Hisatomi H.: Telomerase reverse transcriptase mRNA expression and telomerase activity in hepatocellular carcinoma. *J. Gastroenterol.*, 34 (1): 83-7, 1999.
 - 15- Nouse K., Urabe Y., Higashi T., Nakatsukasa H., Iimura N., Ashida K., Kinugasa N., Yoshida K., Vematsu S. and Tsuji T.: Telomerase as a tool for the differential diagnosis of human hepatocellular carcinoma. *Cancer.*, 78 (2): 232-236, 1996.
 - 16- Ohyashiki K., Ohyashiki J.K., Iwama H., Hayashi S., Shay J.W. and Toyama K.: Telomerase activity and cytogenetic changes in chronic myeloid leukemia with disease progression. *Leukemia*, 11 (2): 190-4, 1997.
 - 17- Park Y.M., Choi J.Y., Byun B.H., Cho C.H., Kim H.S. and Kim B.S.: Telomerase is strongly activated in hepatocellular carcinoma but not in chronic hepatitis and cirrhosis. *Exp. Mo. Med.*, 30 (1): 35-40, 1998.
 - 18- Poremba C., Shroyer K.R., Frost M., Diallo R., Fogt F., Schafer K.L., Burger H., Shroyer A.L., Dockhorn B., Boecker W.: Telomerase is a highly sensitive and specific molecular marker in fine needle aspiration of breast lesion. *Clin. Oncol.* 17 (7): 2020, 1999.
 - 19- Rha S.Y., Izbicka E., Lawrence R., Davidson K., Sun D.: effect of telomere and telomerase interactive agents on human tumor and normal cell. *Clin. Cancer Res.*, 6 (3): 987-993, 2000.
 - 20- Tahara H., Nakanishi T., Kitamoto M., Nakashio R., Shay J.W., Tahara E., Kajiyama G. and Ide T.: Telomerase activity in human liver tissues: Comparison between chronic liver disease and hepatocellular carcinomas. *Cancer Res.*, 55 (13): 2734-2736, 1995.
 - 21- Tatematsu K., Nakayama J., Danbara M., Shionoya S., Sato H., Onine M. and Shikawa: A novel quantitative stretch PCR assay that detects dramatic increase in telomerase activity during the progression of myeloid leukemia. *Oncogen*, 13 (10): 2265-2274, 1996.
 - 22- Yang S.C., Hee D.H., Hong S.J., Chung B.H. and Kim I.Y.: Telomerase activity: a potential marker of bladder transitional cell carcinoma in bladder washes. *Yonsei medical Journal*, 38 (3): 155- 159, 1997.
 - 23- Yoshida K., Sugino T., Tahara H., Woodman A., Bolodeoku J., Nargund V., Fellows G., Goodison S., Tahara E. and Tarin D.: Telomerase activity in bladder carcinoma and its implication for noninvasive diagnosis by detection of exfoliated cancer cells in urine. *Cancer*, 79 (2): 362-369, 1997.