Comparative Expression of Human Telomerase Catalytic Subunit in Normal and Tumor Breast Cell Lines

Denise Collado
Biology Department, Fordham University, Bronx, NY 10458
dcollado@fordham.edu

Abstract

Telomerase is a ribonucleoprotein responsible for maintaining the length of telomeres by adding DNA sequences to the ends of chromosomes. Without the activity of telomerase, the length of telomeres would decrease with each cell division, causing cells to undergo cell cycle arrest. Through the use of RT-PCR expression of human telomerase reverse transcriptase mRNA (the catalytic protein subunit of telomerase) was detected in MCF10A, a normal immortalized breast cell line, MCF7 a noninvasive tumor breast cell line, and HTB26 an invasive tumor breast cell line. The results reveal that all three cell lines express detectable amounts of telomerase reverse transcriptase. The levels of telomerase reverse transcriptase mRNA was much higher in the two tumor breast cell lines than in the immortalized normal breast cell line. These finding support the reports that link telomerase activity with the proliferating capacity of cells and suggest that telomerase activity may serve as a marker for the detection of cancerous cells.

Introduction

Telomerase is a reverse transcriptase ribonucleoprotein that is able to generate and maintain the length of telomeres (DNA sequences that make up the ends of chromosomes). The RNA component of telomerase (hTR) serves as the template for elongation of telomeric DNA. The catalytic protein subunit of telomerase (hTERT, 127kDa in humans) contains the activity of a DNA polymerase and has a conserved amino acid motif also found and highly conserved in other reverse transcriptases(2,7). Telomerase elongates the 3' ends of preexisting telomeres by reverse transcribing the template region of its RNA component, generating DNA sequences that consist of tandem repeats of (TTAGGG)(1,4,7,9). By doing so, it addresses the "end-replication

problem" (1,11) of cells in that results in a loss or shortening of terminal telomeric sequences during normal replication of chromosomes. The shortening of the telomeric sequences is due to DNA polymerase's inability to fully replicate the 3' end of the lagging strand of linear DNA. In the absence of telomerase, telomeres progressively shorten every cell cycle causing a cell to eventually undergo senescence. Thus the length of telomeres acts as a molecular clock(8) that dictates what will be the Hayflick limit or the maximum number of times a cell can replicate (1,3).

Telomerase, which were first identified in Tetrahymena Thermophilia, have since been detected in mammals, amphibians, and plants (10,2). In humans, telomerase activity is strictly regulated during development and is found to be present in a variety of tissues. During early development, it is expressed at low levels and in later stages of development it becomes undetectable (1,3,5,4,7). Telomerase activity is significantly expressed, in highly proliferating cells, such as male germ cells and stem cells (1,6). It is up-regulated in the majority of known cancer cell lines and immortalized cells (5), allowing these cell types to maintain the lengths of their telomeres and overcome the senescence phase.

Inhibiting telomerase activity in immortal cells causes them to undergo apoptosis whereas transfecting telomerase negative cells with hTERT cDNA causes cells to replicate for longer periods of time (1, 7). These findings link telomerase with the proliferation potential of cells. Telomerase has been viewed by the cancer research field as a potential marker for identifying cancerous cells and as a target for anticancer therapeutics. It is found in over 90% of breast cancer cells, while not expressed in most normal human cells (12). But an assay for telomerase activity alone will not be enough

to determine the existence of cancerous cells since it has also been reported that not all immortalized or actively proliferating cells express telomerase (5). These cells maintain the lengths of their telomeres through alternative mechanisms that are currently under investigation (5,9).

We report here the detection of hTERT mRNA in different cell lines through the use of RT-PCR. We show that the level of hTERT mRNA is a good indication of the level of telomerase activity occurring in different cell lines. These findings show that measurement of telomerase activity can be used to distinguish most cancerous cells from normal cells.

Materials and Methods

Cell Cultures.

MCF-10A (normal breast cell line), MCF-7 (tumorgenic non-invasive breast cell line) and HTB-26 (tumorgenic invasive breast cell line) were cultured at 37º C with 7% CO2. MCF-10A cells were grown in 1:1 DMEM:F12 media with 5% Horse Serum (both from Gibco), 20mM Hepes, 10ng/ml EGF (Invitrogen), 10,000 U/ml penicillin, 10,000ug/ml streptomycin, 29.2 mg/ml L-glutamine (Gibco), 10g/ml insulin (Invitrogen), 0.1 ug/ml Cholera Toxin (Sigma), and 500ng/ml Hydrocortisone (Sigma). MCF-7 cells were grown in DMEM (Gibco) supplemented with 2mM L-glutamine (Gibco),1mM sodium pyruvate (Gibco), 5000U/ml penicillin, and 5000 g/ml streptomycin (Gibco), and 10% fetal bovine serum (Hyclone). These cells were kindly provided by Dr. Raj Kandpal.

RNA isolation.

RNA was purified using the Tri Reagent kit (Molecular Research Center Inc.) according to the manufacturer's protocol.

RT-PCR amplification.

The primers used for RT-PCR were designed to amplify a 158bp product from the cDNA or a 2.6kb product from genomic DNA corresponding to variant 1 of human telomerase reverse transcriptase or hTERT (Accession # NM_003219). The forward primer (5'-TCTTCGACGTCTTCCTAC-3') spanned exon 8 from nucleotide # 2469-2486 and the reverse primer (5'-AATCCCCGCAAACAGCTTG-3') spanned exon 9 from nucleotide #2608-2626. Exons 8 and 9 are separated by a 2,480 bp intron in genomic DNA. The Quiagen One-Step RT-PCR kit was used for all reactions. RT-PCR amplification of B-actin was used to control for the amount of RNA present in each of the samples. The B-actin forward primer (5'-CTGACTGACTACCTCATGAAG-3') and reverse primer (5'-GAGGAGCAATGATCTTGATCT-3') spanned nucleotide 553-573 and 1161-1141 respectively, of B-actin cDNA (Accession # NG_002724). The primer set for B-actin was designed to amplify a 444 bp product. Each RT-PCR reaction mix had a final volume of 10 ul and contained the following:

100 ng of RNA, 0.6ul of each primer (primers were diluted to 10pmol/ul), 0.4ul dNTPs, 0.4 ul enzyme mix, 2ul 5x reaction buffer, and 1ul dH_2O. The conditions for RT-PCR were as follows: one cycle of 50ºC for 30 min. and 95ºC for 15 min., followed by 50 amplification cycles of 94ºC for 30s, 60ºC for 30s, 72ºC 30s, and a final extension of 72ºC for 5 min. For the amplification of actin products, only 18 cycles were used. As a

negative control, reactions were also carried out in the absence of an RNA template. PCR products were separated using a 1% agarose gel (run at 130 volts) and purified using the Rapid PCR purification kit (Marligen Bioscience) according to the manufacturer's protocol. A total of 4ul of a 100 bp marker was loaded on the agarose gel for size comparison.

DNA Sequencing.

The dideoxy chain termination method was used along with the AmpliCycle sequencing kit to determine the nucleotide sequences of the PCR products. The sequencing reaction mix contained the following: 4ul of 10x sequencing buffer, 0.25ul of -^{33} P dATP, 2ul of forward or reverse primer, 1ul of purified DNA (36 ng/ul), 12.8ul of dH_2O. The conditions for the sequencing reaction were: 94ºC for 3 min., followed by 35 PCR cycles of 94ºC for 30 sec., 60ºC for 30 sec., and 72ºC for 1 min. The resulting nucleotide sequence was blasted against NCBI's database to verify that it corresponded to variant 1 mRNA of telomerase reverse transcriptase (Accession #: NM_03219).

Results

Expression of hTERT in normal and cancerous breast cell lines

The expression of telomerase reverse transcriptase was studied in a normal human mammary epithelial cell line (MCF10A) and two human tumor breast cell lines (MCF7 and HTB26) using RT-PCR. The set of primers used for RTPCR, recognized variant 1 mRNA of telomerase reverse transcriptase (hTERT Accession #NM_003219). The forward primer spanned a region in exon 8 and the reverse primer spanned a region in

exon 9 of telomerase reverse transcriptase. In genomic DNA, these two exons are separated by an intron that is 2,480 bp in size (fig.1).

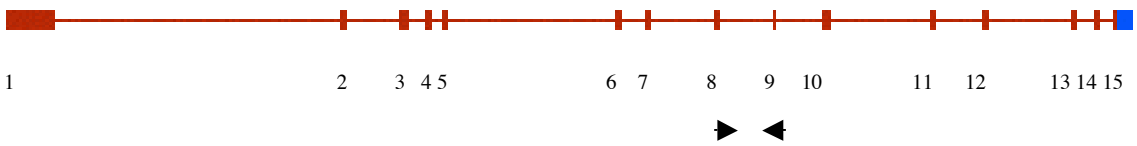

Fig.1: The primer set used for RT-PCR spanned regions of exon 8 and 9 of the cDNA for telomerase reverse transcriptase (Accession #NM_003219). In between these two exons there is a 2, 480bp intron in genomic DNA. The forward primer spanned exon 8 from base pair #2469-2486 of variant 1 mRNA. The reverse primer spanned exon 9 from base pair #2608 –2626. The primer set amplifies a 158bp PCR product.

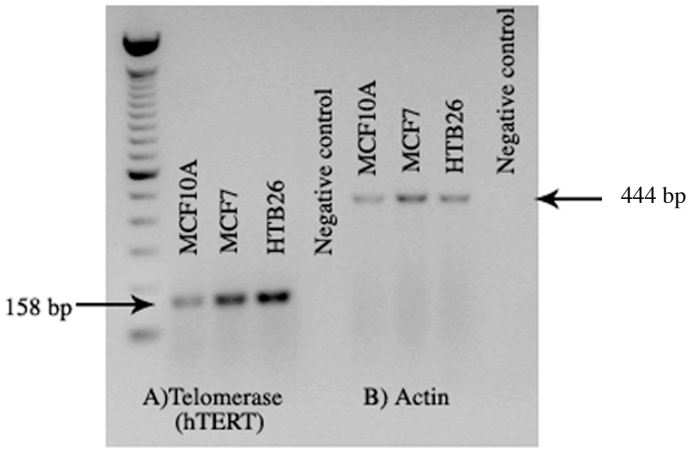

Fig. 2: Detection of telomerase reverse transcriptase expression in cells using RT-PCR. A) A total of 100 ng of RNA isolated from the indicated cell lines was subjected to RT-PCR using a primer set that spanned regions of exons 8 and 9 of telomerase reverse transcriptase cDNA (Accession # NM_003219). This primer set amplified a158bp product from telomerase reverse transcriptase cDNA, indicated by the left arrow. B) To control for the amount of RNA used in each sample, RTPCR for B-actin (Accession # NG_002724) was performed using a primer set that amplified a 444 bp product, indicated by the right arrow.

Equal amounts of the PCR products were loaded and visually analyzed on a 1% agarose gel (fig.2). RT-PCR was performed three times using the same conditions (see materials and methods) for each of the three cell lines. In all three cell lines, the primer set amplified a predicted 158bp product from telomerase reverse transcriptase cDNA. Equal amounts of RNA from all three cell lines was also amplified using primers designed to amplify a 444bp product from B-actin cDNA. This was done to control for the amount of RNA present in each of the samples analyzed on the 1% agarose gel.

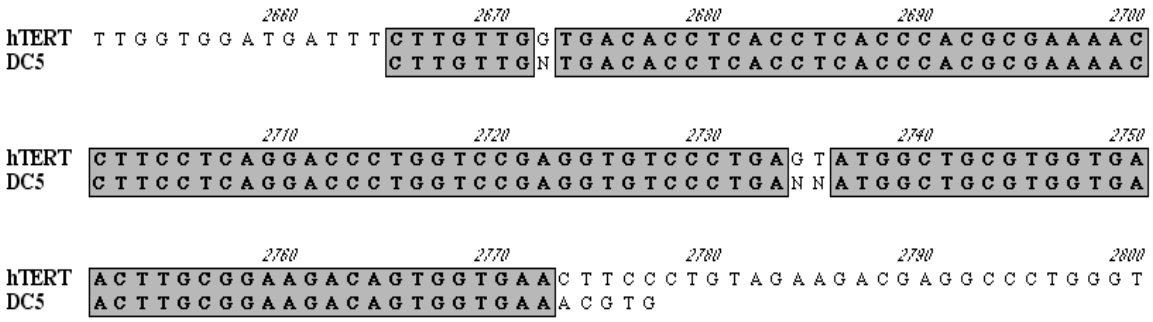

Fig.3: Sequencing results for the amplified product of Telomerase Reverse transcriptase (hTERT). The nucleotide sequence of the amplified products (shown in fig.2A) was blasted against NCBI's nucleotide databases. The results of the alignment confirm that the amplified 158 bp product corresponds to sequences in the variant 1 mRNA of hTERT (Accession Number NM_003219). The N's in the above sequence alignment indicate regions on the sequencing gel that were unreadable.

To confirm that the observed bands represented sequences amplified from the cDNA of telomerase reverse transcriptase (hTERT), the bands were purified from the gel and sequenced using the dideoxy chain termination method. The resulting nucleotide sequence was blasted against NCBI's databases. The results of the blast confirmed that the amplified PCR products (labeled DC5) corresponded to nucleotide # 2665-2772 in the hTERT mRNA(Accession # NM_003219). For aesthetic purposes the MacVector

software was used to create a diagram showing the results of the nucleotide sequence alignment (fig. 3). The letter N in the sequence of the amplified PCR product (referred to as DC5) is used to indicate positions on the sequencing gel that were not readable.

Discussion

Telomerase maintains the lengths of telomeres and is only expressed in a small number of normal adult proliferating cell types, like germ and somatic stem cell lines. Other somatic cells lack telomerase activity, causing their telomeres to shorten with each cell division until they enter senescence. Cancer cells are able to continuously divide by expressing higher levels of telomerase activity than normal cells. This allows most cancer cells to maintain their telomeres and overcome senescence. Studies on different cancer breast cell lines revealed that 95% of all breast cancer cells express detectable levels of telomerase activity (12). It has also been reported that the amount of telomerase activity expressed by a cancer cell, can be used to determine its level of malignancy(12).

In this study, the expression of telomerase reverse transcriptase or hTERT was compared between a normal immortalized breast cell line (MCF10A), a noninvasive tumor breast cell line (MCF7) and an invasive tumor breast cell line (HTB26) through the use of RT-PCR. The detection of telomerase reverse transcriptase (hTERT) mRNA and not hTR mRNA (the RNA component of telomerase) is used to identify cells with telomerase activity. It has been shown that all human cells express hTR (1) but only telomerase activity has been detected in cells that expressed hTR and hTERT. Cells that

lack expression of hTERT (the catalytic subunit of telomerase) also lack telomerase activity.

The results of the RT-PCR for this study revealed that all three cell lines contained detectable levels of telomerase reverse transcriptase mRNA. Other reports have also shown that immortalized and cancerous cell lines contain a higher level of telomerase activity than normal cell lines (which do not have the ability to continuously divide). Compared to the MCF10A band resulting from RT-PCR, the expression of hTERT mRNA seems to be more prominent in the two cancer breast cell lines(MCF7 and HTB26), indicating that there is a higher amount of telomerase activity in those cell lines than in the normal immortalized breast cell line. Like previous reports mentioned above (12), these results reveal that the amount of telomerase activity is an indication of the level of malignancy of a cancerous cell.

From the RT-PCR results, one can predict by looking at the intensity of the bands that MCF7 and HTB26 cell lines are more malignant than the MCF10A cell line, since they express higher amounts of hTERT mRNA. The MCF10A cell line contained detectable levels of hTERT mRNA was because it was immortalized. This does not mean that MCF10A is cancerous (because it is still contact inhibited and will not form tumors if injected in an organism) but just that it can now continuously divide, maintaining its telomeres through telomerase activity. If it were not immortalized, no detectable levels of telomerase mRNA would have been expressed. MCF7 and HTB26 cell lines, on the other had are not contact inhibited and will form tumors in an organism. These results show that detection of telomerase activity can be a useful method for

identifying cancerous cells and even their degree of malignancy when compared to normal cells.

Currently a more sensitive assay is being used for the detection of telomerase activity called the TRAP assay (or Telomerase repeat amplification protocol assay), which can detect as few a one to ten telomerase positive cells (12). But an assay for telomerase alone will not be an efficient method for detecting all cancerous cells since it has been reported that some cancer cell lines do not express telomerase activity. These cancer cells maintain their telomeres through an alternative method that is currently under investigation. It is recommended that an assay for the detection of telomerase activity should be used in addition to other methods that can identify other possible markers of cancerous cells. This will ensure the efficient detection of all cancerous cells, including the ones that do not express telomerase activity.

Acknowledgements

I would like to thank Mr. Jingson Qui, Mr. Brian Fox and my fellow classmates for their encouragement, support and helpful discussions. I am thankful to Dr. Raj Kandpal for providing me with the cells used in this project. Dr. Rubin, thank you for providing us with the opportunity to use the knowledge we have learned in class and for pushing us to do our best.

References

1) Cong, Yu-Sheng, Wright, Woodring E., Shay, Jerry W. 2002. Human Telomerase and Its Regulation. *Microbiol. Mol. Biol. Rev*. 66:407-425.

2) Martin-Rivera, L., E. Herrera, J.P. Albar, and M. A. Blasco. 1998. Expression of mouse telomerase catalytic subunit in embryos and adult tissues. *Proc. Natl. Acad. Sci.* USA, 95:10471-10476.

3) Prescott, JC & Blackburn, EH.1999. Telomerase: Dr. Jekyll or Mr. Hyde? *Curr. Opin. Genet. Dev*. 9:368-373.

4) Mattson, M.P.,Fu,W., Zhang, P. 2001.Emerging roles for telomerase in regulating cell differentiation and survival: a neuroscientist's perspective. *Mech. Ageing Dev*. 122: 659-671.

5) Greider, C. W. 1998.Telomerase activity, cell proliferation, and cancer. *Proc. Natl. Acad. Sci*. USA, 95:90-92.

6) Finnon, P., Silver,A.R., Bouffler, S.D.2000. Upregulation of telomerase activity by X-irradiation in mouse leukaemia cells is independent of Tert, Terc, Tnks and Myc transcription. *Carcinogenesis* 21: 573-578.

7) Guenes,C., Lichtsteiner, S., Vasserot, A.P., Englert,C.2000. Expression of the hTERT Gene Is Regulated at the Level of Transcriptional Initiation and Repressed by Mad1. *Cancer Res.* 60: 2116-2121.

8) Collins,K., Gandhi,L.1998. The reverse transcriptase component of the Tetrahymena telomerase ribonucleoprotein complex. *Proc. Natl. Acad. Sci.* USA, 95: 8485-8490

9) Kilian, A., Bowtell,D.D., Abud, H.E., Hime, G.R., Venter, D.J. 1997. Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum. Mol. Genet*, 6:2011-2019.

10) Fitzgerald, M.S., McKnight, T.D, Shippen, D.E.1996. Characterization and developmental patterns of telomerase expression in plants. *Proc. Natl. Acad. Sci.* USA, 93:14422-14427.

11) Mattson, M.P., Klapper,W. 2001.Emerging Roles for Telomerase in Neuronal Development and Apoptosis. *Journal of Neuroscience Research* 63: 1-9.

12) Herbert, Brittney-Shea, Wright, W.E., Shay, J.W. 2001. Telomerase and Breast Cancer. *Breast Cancer Research* 3: 146-149.