

## Profile of POT1 as telomerase shelterin component discriminates between cervical cancer and normal cervical cells

Andreas Budi WIJAYA<sup>1\*</sup>, Furqan HIDAYATULLAH<sup>1</sup>, Verina Veronica SETYABUDHI<sup>1</sup>,  
Faizal Reza PAHLEVI<sup>1</sup>, Rasyad INDRA<sup>2</sup>, Tatit NURSETA<sup>3</sup>

<sup>1</sup>Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia

<sup>2</sup>Department of Physiology, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia

<sup>3</sup>Department of Gynecologic and Oncologic of Saiful Anwar General Hospital, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia

Received: 22.12.2015 • Accepted/Published Online: 07.07.2016 • Final Version: 18.04.2017

**Background/aim:** Telomerase activity is influenced by hTERT transcriptional regulation, shelterin, and posttranscriptional alternative splicing. Telomerase shelterin such as POT1 is highly correlated with various cancers. However, the profile of POT1 in cervical cancer has not been clearly identified. Therefore, it is important to identify its profile in cervical cancer biopsy tissue and normal cervical smears.

**Materials and methods:** Biopsy tissue of cervical cancer patients and normal cervical smears were characterized using SDS-PAGE and western blot. Sixteen biopsy tissues of cervical cancer patients and 15 normal cervical smears were measured for POT1 level using ELISA.

**Results:** The inline band at 70 kDa indicated that all samples had protein that was identified as POT1. Western blot showed that telomerase antibody only recognized POT1 in biopsy tissue of cervical cancer patients. There was a significant difference ( $P = 0.01$ ) in POT1 level between biopsy tissue of cervical cancer patients and normal cervical smears.

**Conclusion:** POT1 was identified at 70 kDa in biopsy tissue of cervical cancer patients and its level was higher than that in normal cervical smears. The high level of POT1 in the biopsy tissue of cervical cancer patients showed the influence of this shelterin component in cervical carcinogenesis and also cell immortalization.

**Key words:** Cervical cancer, POT1, shelterin, telomerase

### 1. Introduction

Cervical cancer is the leading cause of death from cancer in Indonesia, about 25.91% of all cancer cases (1). The incidence of cervical cancer has increased by 0.6% every year (2). Chronic infection by oncogenic human papilloma virus (HPV) is related to 99.7% of cervical cancer cases. In fact, 67% of Indonesian woman with normal cytological smears are HPV infected. HPV infection is involved in telomerase reactivation and also responsible for cancer's cell immortality. Cervical cancer's malignancy was associated with 95% telomerase activity (3).

Telomerase, a reverse transcriptase enzyme that adds DNA sequence repeats to the 3' end of the telomeric DNA strand in the telomere region, is responsible for preventing constant DNA loss each time the chromosome is doubled. Overexpression of telomerase is associated with malignant

cell transformation. Most somatic tissues do not show telomerase activity, except proliferating stem cells and activated lymphocyte (4). Telomerase activity is very important for carcinogenesis and cell immortalization. In human cells, telomerase activity is influenced by transcriptional regulation of its catalytic subunit, hTERT; the shelterin complex (TRF1, TRF2, RAP1, TIN2, POT1, and TPP1); and also posttranscriptional alternative splicing (5).

POT1 (protector of telomeres 1) plays a major role in the regulation of telomere length through multiple binding properties with TRF1, TPP1, and TIN2. It regulates telomerase-mediated telomere elongation and protects telomeres from chromosomal fusion. Many studies have consistently shown that the profile of POT1 is highly correlated with gastric cancer, thyroid cancer,

\* Correspondence: andreasbudiwijaya@gmail.com

breast cancer, and leukemia. However, POT1 and shelterin profiles in cervical cancer tissue have not been sufficiently identified (4). Therefore, it is important to identify POT1 profiles of biopsy tissues of cervical cancer patients and normal cervical smears. The present study addressed some important basics for a cervical cancer screening approach based on POT1 level.

## 2. Materials and methods

### 2.1. Cervical smears and biopsy tissues collection

Patients were recruited from the outpatient clinic of the Department of Gynecology and Oncology, Saiful Anwar Public Hospital, Malang, Indonesia. All cancer patients were mostly referred by their general practitioner in the period of March to June 2013. They were asked to participate in this study during their initial visit to the outpatient clinic. All patients with moderately dyskaryotic smears were eligible for participation. The mean age of the 16 cervical cancer patients was 51.76 years old (ranging from 37 to 72). All subjects with radiotherapy history and history of previous chemotherapy were excluded.

All specimens were collected during the initial visit, before staging/diagnostic procedures. All cancer patients underwent biopsies. The collected tissues were suspended in 5 mL of ice cold phosphate buffer saline (6.4 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , 0.14 M NaCl, and 2.7 mM KCl (pH 7.2)) and kept in ice until further processing ( $-40^\circ\text{C}$ ). The study protocol was approved by the medical ethical committee of the Saiful Anwar Public Hospital.

The cervixes of normal and eligible women were scraped with the blunt or pointed end of an Ayre's spatula and with an endocervical brush. All normal patients mostly came for routine screening (pap smear). The mean age of the 15 patients with normal cervical smears was 39.33 years old (ranging from 26 to 51). All subjects with previous cancer and history of cervical intraepithelial neoplasia were excluded. The scraped cells were suspended in 5 mL of ice cold phosphate buffer saline (6.4 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , 0.14 M NaCl, and 2.7 mM KCl (pH 7.2)) and kept in ice until further processing ( $-40^\circ\text{C}$ ). Ethical clearance was obtained from Regional Medical Sciences Research Ethics Committee of Faculty of Medicine, Brawijaya University.

### 2.2. Ethical approval

The use of human tissues in this research was approved by local ethical committees, and all patients gave written consent prior to their participation. This research was conducted according to the Declaration of Helsinki Principles.

### 2.3. Sample extraction

For ectocervical and endocervical samples (normal cervical smear), 4 mL of cell suspension was centrifuged and washed with wash buffer (10 mM HEPES/KOH (pH 7.5), 1.5 mM  $\text{MgCl}_2$ , 10 mM KCl, and 1 mM dithiothreitol). Supernatant was removed and lysis buffer (100 mM NaCl; 10 mM Tris-HCl, pH 7.6; 1 mM EDTA, pH 8.0; 1  $\mu\text{g}/\text{mL}$  aprotinin; and 100  $\mu\text{g}/\text{mL}$  PMSF) was added. Biopsy tissues were homogenized before the lysis buffer was added. The suspension was incubated on ice for 30 min and then centrifuged again on a Hettich centrifuge ( $4^\circ\text{C}$ ) (6000 rpm for 15 min). The supernatant was collected and placed in a new Eppendorf for further analysis.

### 2.4. Assessment of protein profile

Normal cervical smears, human telomerase peptide, and biopsy tissues of cervical cancer patient were resolved by using SDS-PAGE 12.5%. For sample preparation, 2.5  $\mu\text{L}$  of human telomerase peptide was combined with 7.5  $\mu\text{L}$  of Tris Cl, and 10  $\mu\text{L}$  of each was taken from biopsy tissue of the cervical cancer patients and normal cervical smear samples. Electrophoresis was performed for 100 min (100 V).

### 2.5. Telomerase antibody preparation

This experimental study used a 3-month male albino rabbit to produce polyclonal telomerase antibody. Human telomerase peptide (Abcam) was used for antibody production, composed with Freund's adjuvant. The initial injection was proposed in the first week, followed by the first booster in the second week and the second booster in the seventh week. As a control, blood was taken from the rabbit before immunization. In the third week after the initial injection, a blood sample was collected continuously every week. The blood sample was taken from the auricular vein and placed in a heparin vacutainer. After blood was collected five times, the second booster was injected to provide a higher titer of antibody. In order to get purified polyclonal IgG concentrate, the SAS 50 method was applied to the collected serum (6). ELISA was used to confirm the highest titer of telomerase antibody, which was collected 10 times (5 times after the first booster and 5 times after the second booster).

### 2.6. Immunoblotting

Western blot was used to analyze the binding site between telomerase antibody and all samples, which were assumed to contain telomerase components. For immunoblotting, 40  $\mu\text{L}$  of polyclonal telomerase antibodies was prepared as primary antibody (1:50) in the case of western blot for telomerase component analysis. Normal cervical smears (ectocervix and endocervix), human telomerase peptide, and biopsy tissue of cervical cancer patient samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane. The membranes were probed with 40  $\mu\text{L}$  of polyclonal antibodies, followed by 1  $\mu\text{L}$  of phosphatase

labelled anti-rabbit IgG (KPL). TBS-BSA 0.2% was used as working dilution. BCIP/NBT phosphatase substrate (KPL) was prepared as substrate for western blot. The highest titer of telomerase antibody (primary antibody) based on the ELISA result was prepared for this immunoblotting.

### 2.7. Telomerase component level determination

Indirect ELISA was performed to measure the levels of telomerase components in all samples. Supernatant of all samples was diluted using assay buffer (1:10, 10  $\mu$ L of sample and 90  $\mu$ L of assay buffer). After antigen had been coated onto a micro plate, it was incubated at 4 °C overnight. Then the coating solution was removed and the plate was washed using PBS-Tween (3 times). The remaining protein-binding site in the coated wells was blocked by adding 50  $\mu$ L of blocking buffer (BSA 1% in PBS, 45 min). The plate was washed again three times using PBS-Tween. Diluted primary antibody was added to each well (100  $\mu$ L of telomerase antibody) in PBS-BSA 1% (1:250, for 2 h). The plate was washed twice using PBS-Tween. Then, 50  $\mu$ L of conjugated secondary antibody (Alkaline Phosphatase Labelling) was diluted in tris

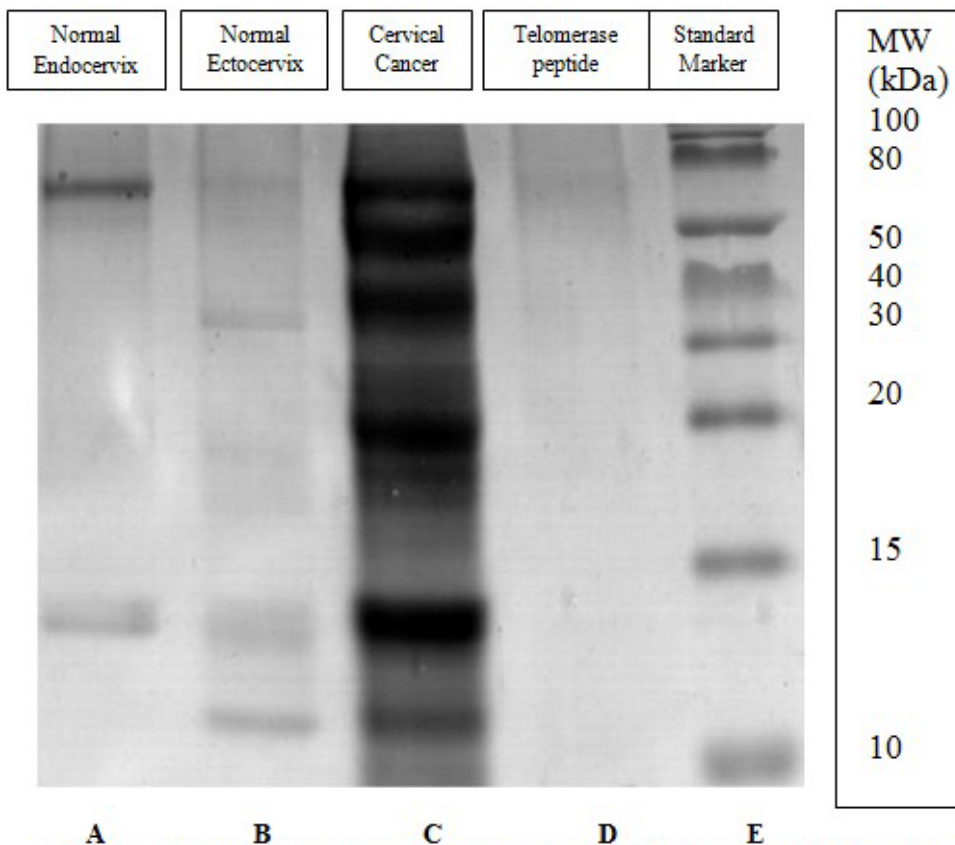
buffer saline (1:1000, 90 min). After washing twice, BCIP/NBT substrate was added to each well and incubated for 30 min. Finally, an equal volume of stopping solution (NAOH 3 M) was added. The result was read by observing the optical density at 450 nm using a plate reader. The independent t-test was performed to find out whether the levels of telomerase component between two subjects were significantly different.

## 3. Results

### 3.1. Telomerase shelterin profile

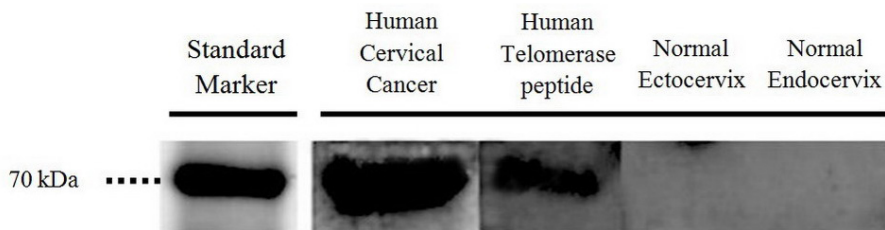
Biopsy tissue of cervical cancer patients, human telomerase peptide, and normal ectocervical and endocervical smear samples were characterized using SDS-PAGE. There was an inline band at 70 kDa that indicated that all samples had 70 kDa proteins, which were identified as POT1, the human telomerase shelterin component (Figure 1). Human telomerase peptide was expressed at 70 kDa (Figure 2).

Western blot was executed using rabbit serum with the highest level of telomerase antibody. The peak level of antibody was acquired in the second week after the



**Figure 1.** Protein profiling and characterization of cervical cancer biopsy tissue extract, normal cervical smear extract, and human telomerase peptide antigen.

A. Normal endocervical smear extract, B. Normal ectocervical smear extract, C. Cervical cancer biopsy extract, D. Standard marker.



**Figure 2.** Western blotting showed 70 kDa protein in cervical cancer tissue extract and human telomerase peptide, suggested as POT1.

second booster. There was a single band at 70 kDa in the biopsy tissue of cervical cancer patients identical to the human telomerase peptide band. On the other hand, there was no such identical band in normal ectocervical and endocervical smear samples (Figure 2). It showed that telomerase antibody only recognized POT1 in the biopsy tissue of cervical cancer patients.

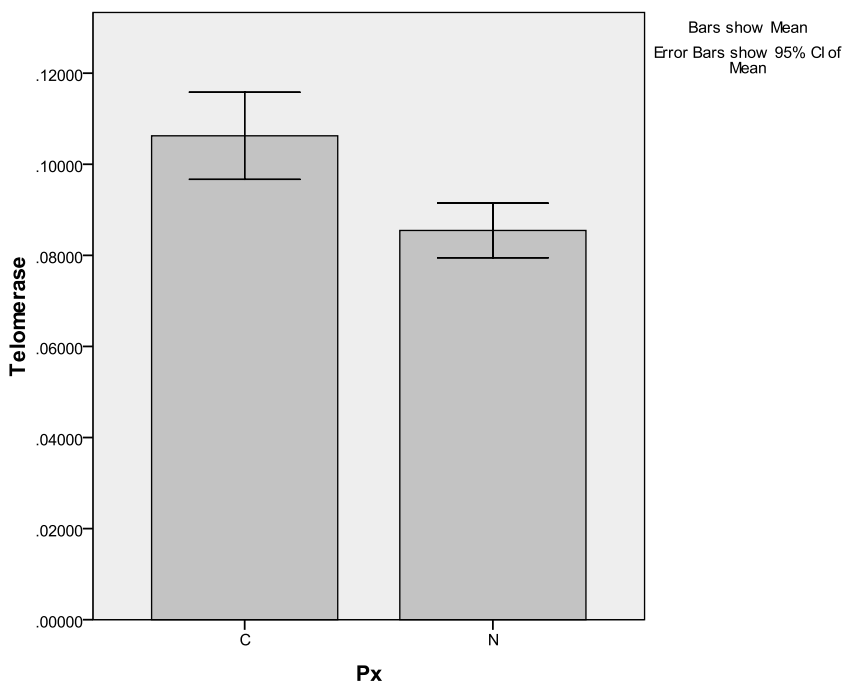
**3.2. POT1 level determination**

Since telomerase antibody only recognized POT1 in the biopsy tissue of cervical cancer patients, we can discriminate between cervical cancer and normal cervical cells based on POT1 level. All biopsy samples were collected from 16 cervical cancer patients with different stages (IB–IIIB). Fifteen normal cervical smear samples were collected from patients with normal cytological features (no dysplasia or metaplasia). The independent

t-test was performed to observe whether both groups of subjects were significantly different in terms of POT1 level. Based on the normality test, the collected data were normally distributed ( $P = 0.127$  ( $P > 0.05$ )). It was also found that POT1 level measurement between the biopsy tissue of cervical cancer patients (C) and normal cervical smears (N) was significantly different ( $P = 0.01$  ( $P < 0.05$ )) (Figure 3).

**4. Discussion**

Human telomerase complex is influenced by conformational binding between hTERT, RNA template (hTR), and shelterin complex (7). An inline band at 70 kDa showed that biopsy tissue of cervical cancer patients, normal cervical smears, and human telomerase peptide had the same protein, which was identified as POT1. As a shelterin



**Figure 3.** POT1 level difference between cervical cancer patients (C) and patients with normal cytological features (N).

component-telomerase ribonucleoprotein complex, POT1 is important for telomeric regulation, prevention of DNA damage, and chromosomal degradation. Its expected molecular weight is between 65 and 70 kDa (8). Western blot showed that there was a single band at 70 kDa in the biopsy tissue of cervical cancer patients, identical to the human telomerase peptide band, which was identified as POT1. Since there was no such identical band in normal ectocervical and endocervical smear samples, it is possible that POT1 was expressed inadequately or had a slightly different structure compared to cancer cells' POT1. This finding indicates that the profile of POT1 in normal cervical smears was different from that in the biopsy tissue of cervical cancer patients.

POT1 plays major roles in the regulation of telomere length through multiple binding properties with TRF1, TPP1, and TIN2. It may indicate that T-loop stabilization provided by POT1 serves to prevent excessive telomere elongation by telomerase. G-quadruplex disruption will inhibit telomerase access to the telomere end (9). POT1 may block the action of telomerase (8); on the other hand, telomerase recruitment and elongation of telomeres were preceded by POT1 action (10,11).

Telomerase recruitment initiation occurs when full-length POT1 binds to TPP1 through its C-terminal domain. Binding between full-length POT1 and TPP1 must take place on the inner side of telomeric DNA, which has an overhang telomeric end (more than 30 terminal or 2 oligonucleotide binding). On the other hand, telomerase activity inhibition occurs when POT1-N (POT1 isoform without C-terminal domain) binds to TRF1-TIN2-PTOP/PIP1/TINT1 complex. When this complex is formed on the outer side of telomeric DNA (less than 30 terminal or 2 oligonucleotide binding), there will be no telomerase that can bind to the telomeric end (10,11).

POT1 expression was higher in the biopsy tissue of cervical cancer patients than that in normal cervical smears. In previous studies, POT1 had high and strong expression in HeLa cells (12). Consistent with this, POT1 also had high expression in the biopsy tissue of cervical cancer patients, as well the HeLa cell line. Expression of POT1 has been identified in some cancer cell lines such as gastric cell lines SGC-7901 and MKN28 and larynx squamous carcinoma cell lines Hep-2 and Hep-2R (12).

The role of POT1 was associated with cell invasion, proliferation, and apoptosis (13–15). Kondo et al. found that POT1 level was highly associated with tumor stage. Upregulation of POT1 occurred mainly in late stage gastric cancer. POT1 inhibition will cause telomere shortening and telomerase recruitment disturbance (16). Tumor serosal invasion and metastasis nodules show a significant correlation with high level of POT1. Its inhibition will decrease cell proliferation, colony formation, and

invasiveness and increase cell apoptosis (14). Therefore, POT1 level might be related to cervical cancer invasiveness, proliferation, and also progressiveness.

There was a significant difference ( $P = 0.01$ ) in POT1 level between the biopsy tissue of cervical cancer patients and normal cervical smears. This finding showed that POT1 level will increase significantly in the biopsy tissue of cervical cancer patients, rather than normal cervical smears. POT1 was conserved in the biopsy tissue of both cervical cancer patients and normal cervical smears. The high level of POT1 in the biopsy tissue of cervical cancer patients showed the influence of this shelterin component in cervical carcinogenesis and also cell immortalization.

The regulation of telomerase activity is complex, owing to its relation to transcriptional control, posttranslational modification, and alternative splicing (17). Shelterin and positively–negatively acting factors also play a major role in telomerase. hTERT regulation is the most rate-limiting factor of telomerase activity. Alternative splice variants of hTERT,  $\alpha$ - and  $\beta$ -deletion isoforms, have been studied in a few cell lines. These isoforms generally have negative effects on telomerase activity. Overexpression of this variant will result in telomerase activity abolishment, which leads to significant reduction and telomere shortening (18).

Telomerase activity in human cells is correlated significantly with hTERT splice variant. All hTERT alternative spliced forms show no reversed transcriptase activity. They cause frame shift and premature termination of open reading frame (ORF). Only full-length hTERT with 16 exons has full reversed transcriptase activity. In addition to normal full-length isoform,  $\beta$ -deletion isoform hTERT was found in lung and colon tumors, and also in adjacent tissue. Similarly,  $\alpha$ -deletion isoform was also found in lung and colon tumors. On the other hand,  $\gamma$ -deletion hTERT was not found in lung and colon tumors or in adjacent tissue (17). However,  $\alpha\beta\gamma$ -deleted variant of telomerase reverse transcriptase was found in gastrointestinal cell lines (19,20).

Due to the fact that  $\gamma$ -deletion hTERT was not found in some tumor cells but was detected in gastrointestinal cell lines, it is assumed that  $\alpha\beta\gamma$ -deleted hTERT might be dominant in normal cells rather than in cancer cells. This alternative spliced variant is supposed to show no reversed transcriptase activity in normal cells. Therefore, no telomerase activity was detected in most somatic tissue. Full length and  $\alpha\beta$ -deleted hTERT showed a conserved region at position 1 to 710 aa (Figure 4). The  $\alpha\beta\gamma$  deleted variant of hTERT has a totally different structure in comparison with full length hTERT (Figure 5).

Characterization of  $\alpha\beta\gamma$ -deleted variant of hTERT in somatic cell should be explored to know which possible explanation can support telomerase activity downregulation in normal somatic cells. Due to different profiles of POT1



Figure 4. Sequence alignment and conserved region between full length hTERT and its splice variant, αβγ-deleted hTERT.

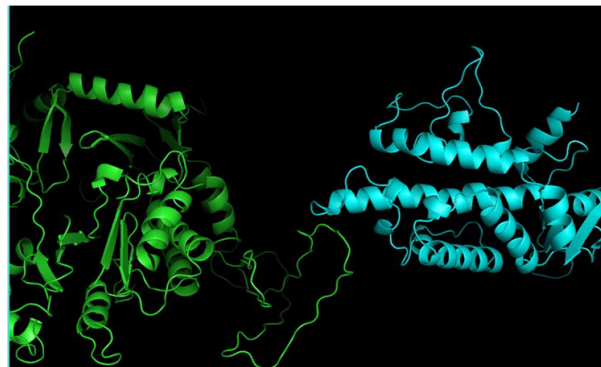


Figure 5. Protein structure between full length hTERT (green) and its splice variant, αβγ-deleted hTERT (blue).

in the biopsy tissue of cervical cancer patients and normal cervical smears, it is important to reveal the amino acid sequence of POT1 between both samples. The amino acid sequence of POT1 will provide structural differences between both samples. A similar protein structure will help researchers to focus on determining how POT1 may affect and promote telomerase activity in normal and cancer cells. Better understanding of POT1's role and mechanism of alternative splicing in tumorigenesis may lead to novel directions for future cancer studies.

As mentioned earlier, the regulation of telomerase activity is complex as it is related to transcriptional control, shelterin regulation, and alternative splicing. Shelterin

such as POT1 itself, which was identified at 70 kDa in the biopsy tissue of cervical cancer patients, plays various major role in telomerase-mediated telomere elongation. This research has demonstrated that POT1 level in the biopsy tissue of cervical cancer patients was higher than that in normal cervical smears. Thus, POT1 cut-off level determination will discriminate between cervical cancer patients and patients with normal cervical smears. Since a high level of POT1 was also associated with tumor stage, measurement of POT1 level in human cervical tissue samples might determine the prognosis of cervical cancer patients. POT1 level might also be used to support cervical cancer staging and posttreatment monitoring.

In terms of alternative splicing, telomerase activity in human cells is correlated significantly with hTERT splice variant. It is assumed that  $\alpha\beta\gamma$ -deleted hTERT might be dominant in normal cells rather than in cancer cells. The

conserved region of full length and  $\alpha\beta\gamma$ -deleted hTERT started from position 1–710 aa.  $\alpha\beta\gamma$ -deleted hTERT has a totally different structure in comparison with full length hTERT.

## References

1. National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention. Fact Sheet: Genital HPV; 2010.
2. Forouzanfar MH, Lozano R, Lopez AD, Delossantos AM, Foreman KJ, Murray CJ. Breast and cervical cancer in 187 countries between 1980 and 2010: a systematic analysis. *Lancet* 2011; 378: 1461-1484.
3. Catarino R, Araújo A, Coelho A, Gomes M, Nogueira A, Lopes C, Medeiros RM. Prognostic significance of telomerase polymorphism in non-small cell lung cancer. *Clinical Cancer Research* 2010; 16: 3706-3712.
4. Artandi SE, De Pinho RA. Telomeres and telomerase in cancer. *Carcinogenesis* 2010; 31: 9-18.
5. Zhen WS, Heng SJ, Wei Z, Qian JS, Ping WH, Sheng JY, Ping Q, Yi L, Mo L. Telomerase activity in cervical intraepithelial neoplasia. *Chinese Medical Journal* 2004; 117: 202-206.
6. Liddell, JE. *Antibody Purification by Ammonium Sulfate Precipitation*. University of Cardiff; 2003.
7. Wojtyła A, Gladych M, Rubis B. Human telomerase activity regulation. *Mol Biol Rep* 2011; 38: 3339-3349.
8. Ponsot E, Laguna AE, Delis AM, Kadi F. Telomere length and regulatory proteins in human skeletal muscle with and without ongoing regenerative cycles. *Exp Physiol* 2012; 55: 21-28.
9. Wanga F, Tanga M, Zeng Z, Wua R, Xue Y, Hao Y, Pang D, Zhao Y, Tana Z. Telomere- and telomerase-interacting protein that unfolds telomere G-quadruplex and promotes telomere extension in mammalian cells. *Biochemistry* 2012, PNAS Early Edition: 1-6.
10. Xin H, Liu D, Wan M, Safari A, Kim H, Sun W, O'Connor MS, Songyang Z. TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. *Nature* 2007; 445: 559-562.
11. Wang F, Podell ER, Zaug AJ, Yang Y, Baciú P, Cech TR, Lei M. The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature* 2007; 445: 506-510.
12. Liu F, Pu XY, Xiang GM, Jiang DN, Hou G, Huang DN. Expression of hPOT1 in HeLa cells and the probability of gene variation of *hpot1* Exon14 in endometrial cancer are much higher than in other cancers. *Asian Pacific J Cancer Prev* 2012, 13: 5659-5663.
13. Fujii K, Sasahira T, Moriwaka Y. Protection of telomeres 1 protein levels are associated with telomere length in gastric cancer. *Int J Mol Med* 2008; 5: 599-604.
14. Wan SM, Tie J, Zhang YF. Silencing of the hPOT1 gene by RNA interference promotes apoptosis and inhibits proliferation and aggressive phenotype of gastric cancer cells, likely through up-regulating PinX1 expression. *J Clin Pathol* 2011; 12: 1051-1057.
15. Tang T, Zhou FX, Lei H. Increased expression of telomere-related proteins correlates with resistance to radiation in human laryngeal cancer cell lines. *Oncol Rep* 2009; 6: 1505-1509.
16. Kondo T, Oue N, Yoshida K, Mitani Y, Naka K, Nakayama H, Yasui W. Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. *Cancer Research* 2004; 64: 523-529.
17. Larssen SS, Fossberg E, Gaudernack G. Characterization of novel alternative splicing sites in human telomerase reverse transcriptase (hTERT): analysis of expression and mutual correlation in mRNA isoforms from normal and tumour tissues. *BMC Molecular Biology* 2006; 7: 26.
18. Bollmann FM. Physiological and pathological significance of human telomerase reverse transcriptase splice variants. *Biochimie* 2013; 95: 1965-1970.
19. Wong MS, Chen L, Foster C, Kainthla R, Shay JW, Wright WE. Regulation of telomerase alternative splicing: a target for chemotherapy. *Cell Reports* 2003; 3: 1028-1035.
20. Nagao K, Katsumata K, Aizawa Y, Saito N, Hirata H, Sasaki H, Yamamoto S, Hikiji K, Koiwa T, Hisatomi H. Differential alternative splicing expressions of telomerase reverse transcriptase in gastrointestinal cell lines. *Oncol Rep* 2004; 11: 127-131.