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นิพนธ์ต้นฉบับ
ORIGINAL ARTICLE

การตรวจวัดความยาวของเทโลเมียร์จาก Pap smear ในผู้ป่วยมะเร็งปากมดลูกด้วยเทคนิค SYBR-green quantitative real time PCR

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บทคัดย่อ

มีรายงานค่าความยาวของเทโลเมียร์แตกต่างกันในมะเร็งแต่ละชนิด ผู้วิจัยจึงทำการวัดค่าสัมบูรณ์ของความยาวของเทโลเมียร์ (absolute telomere length; aTL) ด้วยวิธี SYBR-green quantitative real time PCR (SYBR-green qRT-PCR) ในผู้ป่วยมะเร็งปากมดลูก โดยใช้ตัวอย่างเซลล์ปากมดลูกจาก Pap smear ของผู้ป่วยมะเร็งปากมดลูกชนิด squamous cell carcinoma และชนิด adenocarcinoma รวม 71 ราย เทียบกับกลุ่มควบคุมซึ่งมีอายุเท่ากันเป็นคู่ ผลการศึกษาพบว่า ค่ามัธยฐานของ aTL (median of absolute telomere length) ในผู้ป่วยมะเร็งปากมดลูกยาวกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ (1.50 kb/diploid genome, 95% CI = 1.11-1.81 และ 0.73 kb/diploid genome, 95%CI : 0.46-1.01 ตามลำดับ, $p < 0.001$) อย่างไรก็ดีแม้ว่าค่ามัธยฐาน aTL ของผู้ป่วยมะเร็งปากมดลูกชนิด squamous cell carcinoma จำนวน 59 ราย จะยาวกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ (1.63 kb/diploid genome, 95% CI = 1.23-1.98 และ 0.57 kb/diploid genome, 95% CI = 0.41-0.93 ตามลำดับ, $p < 0.001$) แต่ไม่พบความแตกต่างของความยาวเทโลเมียร์ในผู้ป่วยมะเร็งปากมดลูกชนิด adenocarcinoma จำนวน 12 ราย เมื่อเปรียบเทียบกับกลุ่มควบคุม (0.86 kb/diploid genome, 95% CI = 0.35-1.78 และ 1.18 kb/diploid genome, 95% CI = 0.74-2.12 ตามลำดับ, $p = 0.62$) ผลการศึกษาครั้งนี้ชี้ให้เห็นว่า สามารถนำเทคนิค SYBR-green qRT-PCR มาประยุกต์ใช้ในการตรวจหาความยาวของเทโลเมียร์จากเซลล์เนื้อเยื่อปากมดลูกจาก Pap smear ได้ การวัดค่า aTL น่าจะมีประโยชน์ต่อการรักษาผู้ป่วยมะเร็งปากมดลูกชนิด squamous cell carcinoma จึงควรมีการศึกษาจำนวนตัวอย่างเพิ่มมากขึ้นต่อไป นอกจากนี้ยังพบว่า ผู้ป่วยมะเร็งปากมดลูกชนิด squamous cell carcinoma มีแนวโน้ม aTL ยาวเมื่อระยะโรคเลวลง ขณะที่ adenocarcinoma จะมี aTL สั้นลง

คำสำคัญ : Cervical cancer, Cervical tissue, Absolute telomere length, SYBR-green qRT-PCR

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ORIGINAL ARTICLE

Detection of telomere length from Pap smear in cervical cancer patients by SYBR-green quantitative real time PCR

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Abstract

There are several studies about the difference of telomere length in each type of cancers. Therefore, we aim to measure absolute telomere length (aTL) from cervical tissues by using SYBR-green Quantitative Real-time PCR (SYBR-green qRT-PCR) in cervical cancer patients. The cervical tissues of Pap smear samples were obtained from 71 cases of squamous cell carcinoma and adenocarcinoma patients compare with age-match controls. The results showed that the median of aTL in cervical cancer patients was significantly longer compared to the controls (1.50 kb/diploid genome, 95% CI = 1.18.1-11 and 0.37 kb/diploid genome, 95% CI = 0.1.01-64, respectively, p<0.001). However, the aTL median of 59 squamous cell carcinoma patients was significantly longer compared to the controls (1.63 kb/diploid genome, 95% CI = 1.23-1.98 and 0.57 kb/diploid genome, 95% CI = 0.41-0.93, respectively, p<0.001) but there was no significant difference in 12 adenocarcinoma patients compared to the controls (0.86 kb/diploid genome, 95% CI = 0.35-1.78 and 1.18 kb/diploid genome 95% CI = 0.74-2.1, respectively, p = 0.62). The results showed that SYBR-green qRT-PCR technique can be applied to measure aTL of cervical tissues from Pap smears. The aTL value might be a potential useful prognostic treatment for squamous cell carcinoma. The further study should be conducted in larger sample size. Moreover, longer aTL of squamous cell carcinoma and shorter adenocarcinoma patients tends to have poor prognosis.

Keywords: Cervical cancer, Cervical tissue, Absolute telomere length, SYBR-green qRT-PCR

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Introduction

Situating at the end of the chromosome, telomere is noncoding DNA which is composed of six base-pair repetitive DNA sequences 5′-TTAG-GG-3′ and nucleoprotein.[1]The main function of telomeres is to protect the end of chromosome from enzyme and abnormal fusion reaction. [2] In somatic cells, telomere length is shortened by the time since there is some telomere lost from each cell division because of end replication problem from lagging strand synthesis.[3] At the critical point that the length of telomere is dramatically short, chromosome is unstable leading to p53 activation, cell senescence and apoptosis. Cancer cell is a cell that can escape from the senescence and apoptosis after cell reaches at the critical point. It has a mechanism that maintains the stability of telomere length. Some somatic gene mutation causes abnormal cell division and development of cancer cells eventually.[4]

Cervical cancer is the second most common cancer found (the first one is breast cancer) and is also the third causes of cancer deaths in women worldwide (the first and second causes of cancer deaths are breast and lung cancers, respectively). [5] The major cause of cervical cancer is human papillomavirus (HPV) infection, especially HPV type 16 and 18.[6] There are three types of cervical cancer based on pathological classification and frequency including, squamous cell carcinoma, adenocarcinoma and other epithelial tumors, respectively.[7] There are several studies about the changes of telomere length in various types of cancers. However, the results are unclear. Some researchers reported that the longer telomere length was associated with the risk of cancers, while the others revealed that the shorter telomere length was related to cancers. Moreover, some studies reported that telomere length was not involved in some cancers. [8] It should be noted that investigation of telomere length with different samples and methods leads to different results. [8] However, there is no report about telomere length of cervical cancer patients. This is the first study using SYBR-green qRT-PCR technique to determine the telomere length from extracted DNA from Pap smear of cervical cancer patients compared to telomere length of normal control. Our findings provided the basic knowledge about the molecular changes in telomere length of cervical cancer patients to further cancer prognosis.

Materials and methods

Study populations

A retrospective case-control study of 142 females who were screened with Pap smear were derived from Kalasin Hospital, Thailand. There were 71 cervical cancer patients with Pap smear positive and all the cases were pathologically confirmed by Institute of Pathology, Department of medical Service of Ministry public Health, Bangkok, Thailand. In addition, the 71 women with Pap smear negative and no cancer were randomly selected. Controls were matched with cases according to age and were selected from the querying medical records.

DNA extraction from pap smear

Cervical cells preparation was modified from deRoda Husman et al.[9] Pap smear were placed in xylene and left overnight until the coverslips could easily be removed. Then the cells were collected from Pap smear with a new sterile razor blade and transferred into a micro centrifuge

tube. One milliliter of absolute ethanol was added. Then the tube was mixed and centrifuged at 14,000 rpm, for 10 min. After that the supernatant was discarded as much as possible. The cells were washed twice and brought to extract the DNA. Genomic DNA was extracted from Pap smear using Genomic DNA Mini Kit, modified from the manufacturer's instruction (Geneaid Biotech Ltd, Taiwan). DNA was quantified by an Eppendorf BioSpectrometer® fluorescence. Extracted DNA specimens were stored at 4°C or<-20°C until used.

Telomere length measurement

Telomere length measurement for aTL determination was measured by SYBR-green qRT-PCR method as modified from O'Callaghan et al.[10] The standard curve was established using serial dilution 10^{1} through to 10^{-5} of known quantities of synthesized 84 mer oligonucleotide with a MW of 26667.3 containing only TTAGGG repeats 14 times. All oligonucleotides were synthesized from 600 pg/ul to 0.06 pg/ul. The linear standard curve of 60.0-0.6 pg/ul (6.00E1 to 6.00E-1) **(Figure 1)** was selected for aTL calculation. DDW was added to each standard to maintain a constant 20 ng of DNA per reaction. Each dilution was run triplicate. Each 20 ul of the PCR mixture for standard amplification was consisted of 20 ng DNA, 2x SYBR green master mix, 20 uM long oligomer for standard telomere (5'-TTAGGGTTAGGGTTAGG-GTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGG-GTTAGGGTTAGGGTTAGGGTTAGGG-3') PCR reaction was run in separate 96 well plates on Roche Lightcycler®480 platform (Roche Molecular Diagnostics, USA). The thermal cycling conditions for std. 84 bp oligomers was: 10 min at 95°C, followed by 32 cycles of 95°C for 15 sec, 60°C for 1 min and 72°C for 20 sec. Specificity of the amplified product was assessed by performing a melting curve analysis were 1 cycle of 95°C for 10 sec, 60°C for 1 min and continuous at 98°C then cooling at 40 °C for 10 sec. Each 20 ul of the PCR mixture for the telomere amplification was consisted of 30 ng DNA, 2x SYBR green master mix, 20 uM telomere forward primer (CGGTTTGTTTG-GGTTTGGGTTTGGGTTTGGGTTTGGGT), 20 µM telo-mere reverse primer (GGCTTGCCTTACCCTTACCCT-TACCCTTACCCTTACCCT). In every single 96 well plate, negative control and positive control were included. Each sample, negative control and positive control were run duplicate. The PCR cycle of sample was the same as standard curve performing. After amplification was complete, the Lightcy-cler®480 software produced a value for each reaction that was equivalent to kb/reaction based on the telomere standard curve value. The final calculations were done to determine aTL as kilobases per diploidgenome.

Statistical analysis

Wilcoxon rank-sum (Mann-Whitney) test was used to compare the mean rank differ of absolute telomere length between cases and controls. *p*-values below 0.05 were considered statistically significant. The STATA version 10 software was used to perform all of the analyses.

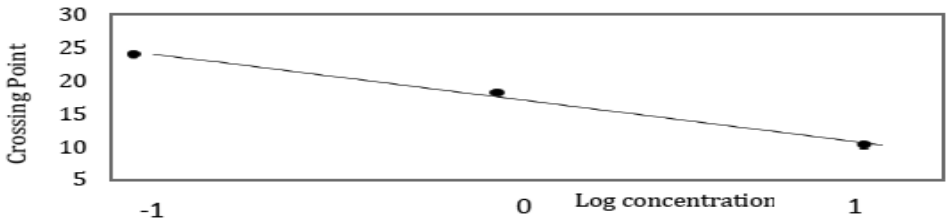
Ethical Clearance

This study was approved by the ethic committee of Khon Kaen University and the ethic committee of Kalasin Hospital.

Results

Demographic characteristics of 71 cervical cancer patients

Demographic characteristics of 71 cases were shown in **Table 1.** From the 71 cervical cancer patients, the median age was 52 years with a range of 31 to 68 years. 59 (83.10%) and 12 (16.90%) patients were squamous cell carcinoma and adeno-carcinoma, respectively.



Crossing Point	Standard
13.28	6.00E1
18.57	6.00E0
24.06	6.00E-1

Figure 1. Standard curve of SYBR Green qRT-PCR, generated by LightCycler® 480 real time PCR system (Roche Diagnostics GmbH, Mannheim, Germany), for telomere length determination by using known quantities of synthesized 84 mer oligonucleotide with a MW of 26667.3 consisted of TTAGGG repeats 14 times.

Table 1. Demographics and clinical characteristics of cervical cancer patients

Demographics	Patients	
	N (71)	%
Age (years)		
Median	52	
Range	31-68	
<30	-	-
30-34	3	4.23
35-39	4	5.63
40-44	3	4.23
45-49	11	15.50
50-54	27	38.03
55-59	11	15.50
60-64	10	14.08
65-69	2	2.82
>69	-	-
Histology		
Squamous cell carcinoma	59	83.10
Adenocarcinoma	12	16.90

The absolute telomere length

The absolute telomere length (aTL) of cervical cancer patients and controls expressed total telomere length (kb per diploid genome). The median of aTL in cervical cancer patients was significantly longer compared to the controls (1.50 kb/ diploid genome, 95% CI = 1.11–1.81 and 0.37 kb/diploid genome, 95% CI = 0.46–1.01 in cases and controls, respectively, $p < 0.001$). The median of aTL of 59 squamous cell carcinoma patients was also determined. The results showed that the median of aTL of squamous cell carcinoma patients was significantly longer compared to the controls (1.63 kb/diploid genome, 95% CI = 1.23-1.98 and 0.57 kb/diploid genome, 95% CI = 0.41-0.93, in cases and control, respectively, $p < 0.001$). However, there was no significant difference in adenocarcinoma patients compared to the controls (0.86 kb/diploid genome, 95% CI = 0.35-1.78 and 1.18 kb/diploid genome 95% CI = 0.74-2.12 in cases and controls, respectively, $p = 0.62$) **(Table 2)**. The aTL was also determined based on clinical characteristics of cervical cancer patients as shown in Table 3. The results showed that in cervical cancer patients (squamous cell carcinoma), the aTL of moderate differentiated type (5.31 kb/diploid genome) was longer compared to the aTL of poorly differentiated (1.5 kb/diploid genome). The aTL of 2 cervical cancer patients stage IIB squamous cell carcinoma (2.15, 1.06 kb/diploid genome) were longer compared to the aTL of stage II adenocarcinoma (0.35 kb/ diploid genome). In addition, the aTL of stage II adenocarcinoma patients (0.35 kb/diploid genome) was shorter compared to stage I patients (0.88 kb/diploid genome).

Table 2. The median of absolute telomere length (aTL) of cervical cancer.

	All case / control (n = 71//71)	SCC[1] / control (n = 59/59)	Adeno[2] / control (n = 12/12)
Median of aTL (kb/diploid genome)	1.50 (1.11-1.81) / 0.73 (0.46-1.01)[3]	1.63 (1.23-1.98) / 0.57 (0.41-0.93)[3]	0.86 (0.35-1.78) / 1.18 (0.74-2.12)[3]
p-value	< 0.001	< 0.001	0.62

[1] SCC = Squamous cell carcinoma

[2] Adeno = Adenocarcinoma

[3] median of aTL /diploid genome (95%CI)

Table 3. Absolute telomere length (aTL) distribution by clinical characteristics of cervical cancer patients.

Cervical cancer type	n	aTL (kb /diploid genome)
Squamous cell carcinoma		
- poorly differentiated carcinoma non keratinizing type	1	1.5
- moderate differentiated (non-keratinized)	1	5.31
- Stage IIB	2	2.15, 1.06
Adenocarcinoma		
- Stage I	1	0.88
- Stage II	1	0.35

Discussion

From the previous study, they reported that changes in the length of telomere, both longer and shorter, compared to the control were benefit for prognosis. The short telomere was a risk factor in bladder, esophageal, gastric, head and neck, ovarian, renal, and overall incident cancer patients.[8] Moreover, the long telomere was a risk factor and poorer outcome in prostate cancer[11], breast cancer[12]non-Hodgkin lymphoma[13] and hepatocellular carcinoma patients.[14] This is the first study investigating the aTL in cervical tissues. The results showed that cervical cancer patients had median of aTL longer compared to the control. Abnormal cervical cells which transform to cervical cancer cells has not been described clearly. However, it is well-known that E6 protein found in HPV type 16 and 18 and E7 protein found in HPV type 16 only are the cause of cervical cancer. HPV protein, E6 protein, binds with normal

cell p53 protein leading to *p53* gene mutation whereas E7 protein binds with pRb protein leading to retinoblastoma (*Rb*) gene mutation. The mutations of *p53* and *R*b genes cause alteration of cell-cycle checkpoint. These abnormal cells have proliferation and growth continuously and develop to cervical cancer cells eventually.[15] According to types of cervical cells, we found that the aTL of 59 (83.10%) squamous cell carcinoma cases was significantly longer compared to the controls whereas the aTL of 12 (16.90%) adenocarcinoma was not seen significantly differences compared to the controls; however, the trend of aTL of adenocarcinoma was shorter compared to the controls. The difference of telomere length in these two types of cervical cells may be caused by different pathogenesis. There was a report revealed that telomere length was changed depending on types of cancers and body response for each cancer disease.[16] Squamous cell carcinomas begin from the squmocolumnar junction of extocervix. The lesions may be keratinizing or nonkeratinizing, while adenocarcinomas developed from the mucus-producing gland cells of the endocervix.[17] Moreover, the causative viruses of these two types of cervical cancer are also different. Bulk et al[18] reported that HPV-16 was a causative virus for squamous cell carcinoma and adenocarcinoma whereas HPV-18 was a causative virus for adenocarcinoma only. In addition, Yang et al [19] investigated the effect of the HPV genotype as a prognostic factor, the study showed that HPV-18 was a poor prognostic factor for in cervical cancer patients as stage I-IIA following primary surgical treatment. Lombrad et al[20]31 revealed that cervical cancer patients infected with HPV-18 had 2.4 greater relative risk of death compared to cervical cancer patients infected with HPV-16. Patient data derived from Kalasin Hospital revealed that an adenocarcinoma stage II of cervical cancer patient had telomere length shorter compared to an adenocarcinoma stage I. This indicated that shorter telomere in adenocarcinoma was a poor prognosis. In contrast, longer telomere in squamous cell carcinoma was a poor prognosis. Moreover, a moderate differentiated of cervical cancer patient had telomere length longer compared to a poorly differentiated one. In addition, 2 cervical cancer patients stage II B had telomere length longer compared to an adenocarcinoma stage II. However, we need to collect more samples to increase reliable results. This is a retrospective study which has limitations about gathering patient data and controlling all factors that are involved in changes of telomere length.[21] In this study, we selected the same age of participle in case and control groups. Moreover, participants who had some underlying diseases were excluded. Moreover, the adenocarcinoma samples are not many. The further study should be conducted with more sample size to gather reliable results. For DNA extraction, cells from Pap smear samples should be placed on ¼ of slide to assure that the cells are adequate for investigation by PCR.

Conclusion

We applied the SYBR Green qRT-PCR for measurement the aTL of Pap smear derived from cervical tissue samples for the first time which might be useful for cervical cancer prognosis.

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