Development of Lateral Flow Assay for Detecting HPV-16 E6 Oncoprotein

การพัฒนาวิธี Lateral Flow เพื่อตรวจหาโปรตีนก่อมะเร็ง HPV-16 E6

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ABSTRACT

Cervical cancer, the fourth leading cause of cancer death in women worldwide, is associated with persistent of HPV infection especially type 16 which covered more than 50% of the cases. Nowadays, the screening methods for cervical cancer are based on cytology and nucleic acid detection which are time consuming, require pathologist and special equipments. During cancer development, high expression of E6 oncoprotein is demonstrated thus detection of HPV-16 E6 protein might be helpful for early detection of cancer development. Since the lateral flow assay is simple, rapid, cost-effective and no instrument requirement, in this study, a rapid lateral flow assay combining nanogold-antibody conjugates was developed to detect HPV-16 E6 oncoprotein. The developed test has been established and further evaluation of the test is investigated.

บทคัดย่อ

ในปัจจุบันพบว่ามะเร็งปากมดลูกเป็นโรคมะเร็งที่คร่าชีวิตของผู้หญิงเป็นอันดับที่ 4 ของผู้หญิงทั่วโลก โดยสัมพันธ์กับการคงอยู่ของการติดเชื้อไวรัสแปปิโลมาโดยเฉพาะชนิด 16 (HPV-16) ซึ่งพบได้มากกว่าร้อยละ 50 ของผู้ป่วย ปัจจุบันการตรวจคัดกรองหามะเร็งปากมดลูกใช้วิธีตรวจทางเซลล์วิทยาและการตรวจหาสารพันธุกรรมของเชื้อ แต่ต้องใช้เวลานาน ต้องมีแพทย์ผู้เชี่ยวชาญ และเครื่องมือพิเศษ ระหว่างการพัฒนาเป็นมะเร็งนั้น มีการแสดงออกของโปรตีนก่อมะเร็ง E6 ในปริมาณสูง การตรวจหาโปรตีนก่อมะเร็ง E6 ของ HPV-16 น่าจะช่วยในการตรวจพบการเป็นมะเร็งในระยะแรกได้ เนื่องจากวิธี Lateral flow assay เป็นวิธีที่ทำได้ง่าย รวดเร็ว ราคาถูก และไม่ต้องการเครื่องมือใดๆ ในการศึกษาครั้งนี้จึงทำการพัฒนาวิธีรวดเร็ว Lateral flow assay ร่วมกับอนุภาคนาโนทองคำ การพัฒนาวิธีประสบความสำเร็จและอยู่ระหว่างการประเมินวิธีการทดสอบต่อไป

Key Words: HPV-16 E6, Lateral flow, Gold nanoparticles

คำสำคัญ: เอชพีวี 16 อี 6 การไหลแบบเลเทอรอล อนุภาคนาโนทองคำ

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Introduction

Cervical cancer is the third most commonly diagnosed cancer and is the fourth leading cause of cancer death in women worldwide. There are more than 525,000 new cases of cervical cancer and approximately 275,000 deaths due to cervical cancer each year. More than 85% of the global burden occurs in developing countries, including Thailand (Ferlay et al.,2010). Human Papillomavirus (HPV) is a major causative agent. At least 70-80% of cervical cancer patients are associated with the infection of HPV type 16 (HPV-16) and 18 (HPV-18) (de Sanjose et al.,2010). Nowadays, the diagnostic method of cervical cancer is based on the detection of cervical cell dysplasia and HPV DNA. The clinical appearance of cervical cancer is observed for changing in cells of transform zone of cervix by cytology and histology. However, this method has some limitations. The results take time and false negative rate as high as 20-30% has been reported due to clumping cells. In contrast to HPV DNA detection, the method has high sensitivity and specificity but requires special instruments, experience technician and very expensive (Burd, 2003; Schwaiger 2012). Moreover, the presence of HPV-DNA does not always correlate to the cancer development. Previous studies strongly indicated that HPV E6 oncoprotein is overexpressed in cervical cancer cells (Ganguly N, 2009); therefore a test to detect HPV E6 mRNA has been developed for early detection of cancer development stage. Besides E6 mRNA, E6 protein is also another target for cancer screening test. According to Kamonwan (2009), the immunogoldagglutination assay was developed for detecting HPV-16 E6 and L1 protein directly from clinical specimen and the result was visibly detected by an agglutinate of the reaction. The assay used AuNP conjugated with either HPV-16 E6 or HPV-16 L1 polyclonal antibodies. However, this assay has low sensitivity and takes time (overnight). Thus, this study aims to improve the sensitivity and time of the detection by using the lateral flow assay instead of agglutination assay. In addition, the lateral flow assay is simple, rapid, cost-effective and possibly future to be point-of-care test.

Objectives of the study

To develop a gold nanoparticles lateral flow assay for detecting of HPV-16 E6 protein.

Methodology

Materials

Gold nanoparticles (AuNP) in diameter 10- and 40-nm were purchased from Sigma Aidrich. The gold particles were either suspended in phosphate buffered saline or 0.01% tannin acid with 0.04% trisodium citrate, 0.26 mM potassium carbonate, and 0.02% sodium azide as preservative. The antibodies used in this study are listed in Table 1.

Preparation of HPV-16 E6 recombinant proteins

The procedure is followed to Kamonwan (2009). In short, the HPV-16 E6 protein was expressed in *E. coli* BL-21 (DE3) containing pGEX-3X-MT-E6 using IPTG induction at 25 °C for 6 hours. Recombinant protein was purified using GST fusion protein affinity column purification kit (GE healthcare CO., USA). The eluate was quantitated by Quant-iT protein assay kit (Qubit fluorometer; Invitrogen Co., USA) and analysed the purity by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Recombinant proteins were used as a positive sample which amount was 20 ug per reaction.

Table 1 List of antibodies used in this study

Type of antibody	Company	Concentration
Polyclonal goat anti HPV-16 E6 (sc-1584)	Santacruz Biotechnology,	1-10 ug/ml goldnanoparticle or 1-2 ug/ line
Monoclonal donkey anti-goat (sc-2020)	Santacruz Biotechnology,	0.5-2 ug/ line
Monoclonal mouse anti HPV-16 E6 (sc-460)	Santacruz Biotechnology,	1-10 ug/ml goldnanoparticle or 1-2 ug/ line
Monoclonal goat anti-rabbit (sc-2004)	Santacruz Biotechnology,	0.5-2 ug/ line
Monoclonal goat anti-mouse (Pierce 31160)	Pierce, USA	0.5-2 ug/ line
Monoclonal mouse anti HPV-16 E6 (ab30716)	Abcam, UK	1-10 ug/ml goldnanoparticle or 1-2 ug/ line
Monoclonal mouse anti HPV-16 E6 (ab70)	Abcam, UK	1-10 ug/ml goldnanoparticle or 1-2 ug/ line
Polyclonal rabbit anti HPV-16 E6 (ABIN733448)	Antibodies-online.com, Germany	1-10 ug/ml goldnanoparticle or 1-2 ug/ line

Preparation of AuNPs-antibody (Ab) conjugate

The gold nanoparticle labeled antibody was prepared according to the method of Yokota et al (1992). First, the condition of AuNP and antibody was optimized. Standard curves were determined for each antibody in order to determine the minimum amount of antibody that stabilizes gold nanoparticle. Each AuNP solution (10-nm and 40-nm) was adjusted to pH 8 with 0.2 M Na_2CO_3 and checked by pH paper. A range of antibody amounts (final concentration 0 to 10 µg/ml gold) was added to 100 µl of AuNP. After that, the mixtures were incubated for 10 min at room temperature, 10 µl of 10% NaCl were added to each tube. The solution was mixed rapidly and allowed to stand for 5 min before determining the optical density at 580 nm. The color of samples changes from brilliant red to blue as the concentration of antibody decreases. The optimum concentration of antibody for AuNP labeling was the lowest concentration of antibody that did not change the color.

One ml of AuNP solution was adjusted to pH 8 with 0.2 M Na_2CO_3 and checked by pH paper. For preparing an AuNP conjugate, the anti-HPV16 E6 antibody (Table 1) was added to the 1 ml of the 10-nm or 40-nm AuNP solution at an optimal final concentration. After gently mixing for 60 min at room temperature, 0.1 ml of blocking solution (either 10% BSA in or 3% PEG, both was in Milli Q water) was added to block the AuNP surface. After gently mixing for 30 min at room temperature, the mixture was centrifuged at 25,000 rcf for 10-nm AuNP and 6,000 rcf for 40-nm AuNP at 4 °C for 30 min. The supernatant was discarded and the AuNP conjugate was resuspened in storage buffer (20 mmol/L sodium borate, pH 8, 1% BSA และ 0.1% sodium azide). The centrifugation and resuspension steps were repeated and the AuNP conjugate was finally suspended in 0.1 ml storage buffer and then stored at 4 °C.

Development of the lateral flow device

The lateral flow device consists of four components: a sample pad (1.5 x 0.4 cm^2 cellulose fiber, Millipore), a conjugation pad (0.8 x 0.4 cm^2 glass fiber, Millipore), a nitrocellulose membrane (2.2 x 0.4 cm^2 NCM, Millipore), and an absorbent pad (1.5 x 0.4 cm^2). Most components were fixed on a backing pad (6 x 0.4 cm^2). A schematic diagram of the lateral flow device is shown in fig. 1. Primary antibody, anti HPV-16 E6 polyclonal or monoclonal antibody, and the secondary antibody (against the Ab on the AuNP surface) were applied in a test line and a control line, respectively on a piece of NCM. The distance between the test line and control line was 0.5 cm. Various types of antibody, sizes of AuNP, and blocking solution were optimized. After drying for 1 h in desiccator at RT, the membrane was blocked with 1% BSA and then dried, washed with milli Q water twice, dried and stored under dry conditions.

The conjugate pad was prepared by pouring the 10 ul of AuNPs-antibody conjugate and then drying for at least 1 h in desiccator at RT.

Finally, the sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad were assembled sequentially on backing pad. Each segment overlapped 0.2 cm to facilitate the migration of solution during the assay.

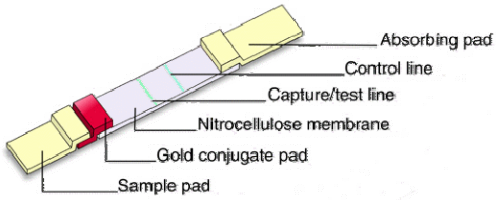

Figure 1 A schematic diagram of the lateral flow device

Results

HPV-16 E6 recombinant proteins

The expression of *E.coli* BL-21 (DE3) containing pGEX-3X-MT-E6 induced by IPTG was at 25°C for 6 h after that the proteins were purified by GST affinity chromatography and further analysed by SDS-PAGE and Western blot as shown in fig. 2. The E6 fusion protein band was about 43 kDa and the GST band was about 26 kDa.

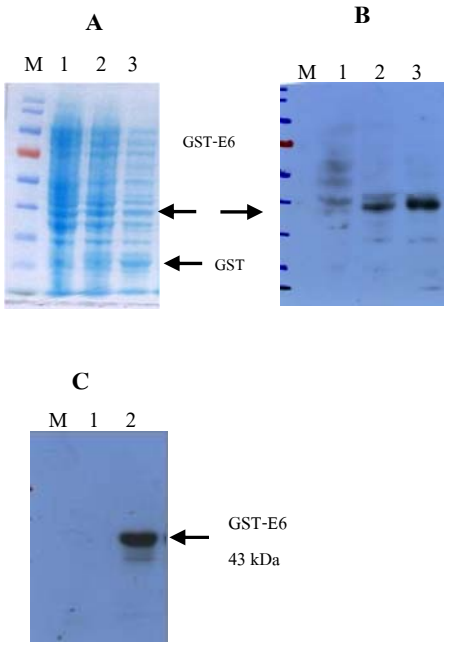

Figure 2 SDS-PAGE and Western blot (WB) analysis of GST-HPV-16 E6 A:SDS-PAGE stained with coomasie blue, B:WB, lane 1; whole cell lysate (WCL) from *E. coli* BL(DE3) non-induced by IPTG, lane2 and 3;WCL induced by IPTG lot 1 and 2, respectively., C: WB of purification of GST-HPV-16 E6, lane 1; WCL from *E. coli* BL(DE3) non-induced by IPTG, lane 2; purified of GST-HPV-16 E6

Optimizations of the lateral flow assay

10-nm and 40-nm AuNP were recruited. To optimize concentration of Ab used to conjugate with AuNP, Ab titration was performed. The results indicated that the minimum quantity of each antibody needed to stabilize either 10- or 40-nm AuNP was 7-10 µg/ml gold. Finally, a concentration of 10 µg/ml gold was selected.

The assay consists of a test line and control line. The sandwich reaction between antigen-AuNP conjugated Ab complex and capture antibody occurred in the test line, while no reaction AuNP conjugate was bound to antibody against Ab on the AuNP surface at control line. In the presence of HPV-16 E6 antigen, the signal appears at the test and control lines, whereas without that, the signal showed only at the control line.

In the assay, DW, Milli Q water or 0.1x PBS were used as a negative sample while HPV-16 E6 recombinant protein as a positive sample. Several types of Ab shown in Table 2 (both monoclonal and polyclonal from Santa Cruz) were applied since some Ab gave non-specific binding to the test line indicating false positive results (Fig 3). To reduce non-specific binding, blocking solution was also optimized between 10% BSA and 3% PEG, both were in Milli Q water.

Finally, the conditions for detecting HPV-16 E6 protein by the lateral flow assay were as followed,

(i) Monoclonal mouse anti-HPV-16 E6 (ab30716) as the AuNP-conjugated Ab.

(ii) Polyclonal rabbit anti- HPV-16 E6 antibody (1 mg/ml) at the test line

(iii) Size of 10-nm AuNP in 0.01% tannin acid with 0.04% trisodium citrate, 0.26 mM potassium carbonate, and 0.02% sodium azide solution

(iv) 3% PEG in Milli Q water as blocking solution

Under the optimized condition, 20 µg of recombinant HPV-16 E6 proteins were appeared within 5 min after reaction (Fig. 4)

Discussion and Conclusions

Cervical cancer worldwide is infected with specific types of HPV DNA mainly HPV-16 and HPV-18 (de Sanjose et al., 2010). In Thailand, the incidence of cervical cancer ranks top among cancers in women and was reported that HPV-16 causes 55.9% of cervical cancer (WHO/ICO, 2009). To early diagnosis of the cancer development, HPV-16 E6 is suggested to be a candidate marker. In this study, a rapid lateral flow assay was developed using antibody combining nanogold technology. The factors which may affect the lateral flow reaction were examined. First, the size of AuNP, Several publications report on a comparison of the size of the nanoparticles. Better sensitivity using larger particles was observed; however, the instability of AuNP decreases as the size increases (Posthuma-Trumpie et al., 2009). Second, the stability of AuNP depends on the suspending solution. The 10-nm AuNP suspended in 0.01% tannin acid with 0.04% trisodium citrate, 0.26 mM potassium carbonate, and 0.02% sodium azide as preservative was found to be more stable than in PBS or water. Third is the type of antibody (polyclonal or monoclonal) as shown in Table 1. Using different Ab types as indicated in Table 2 showed mostly false-positive results. One reason is that polyclonal antibody is produced by the immunization of whole antigen resulted in low specificity (Varma et al., 2002 and Lipman et al., 2005). After optimization, the Ab used to conjugate with AuNP is mouse monoclonal Ab whereas the capture Ab on the test line is rabbit polyclonal Ab

(Table 2). Interestingly, species of the animal induced Ab might affect the reaction as well.

Our study demonstrated a successfully developed Lateral flow assay using nanogold particles. However, this has just begun for the future evaluation of the assay. The sensitivity and the specificity of the test will be further investigated. Moreover, this assay will be directly tested in clinical specimens with various stages of clinical pathology to evaluate the benefit of the test in early cancer diagnosis.

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Table 2 The results of optimization of the lateral flow assay conditions

Size of AuNPs (nm)	Anti-E6 conjugated gold		Capture Ab Test line (anti-E6), T	Capture Ab Control line, C	Results		
	Ab Conc. (ug/mL gold)	Type			Sample	T	C
10 40	2, 10 4, 6, 10	Goat polyclonal (sc-1584)	Goat polyclonal (sc-1584)	Donkey anti-goat (sc-2020)	DW	+	+
					Milli Q	+	+
					0.1xPBS	+	+
10, 40	10	Goat polyclonal (sc-1584)	Mouse monoclonal (sc-460)	Donkey anti-goat (sc-2020)	Milli Q	+	+
					0.1xPBS	+	+
40	10	Mouse monoclonal (sc-460)	Goat polyclonal (sc-1584)	Goat anti-mouse (Pierce 31160)	Milli Q	+	+
					0.1xPBS	+	+
40	10	Mouse monoclonal (sc-460)	Mouse monoclonal (sc-460)	Goat anti-mouse (Pierce 31160)	Milli Q	+	+
					0.1xPBS	+	+
10, 40	10	Goat polyclonal (sc-1584)	Normal Goat IgG	Donkey anti-goat (sc-2020)	Milli Q	+	+
			Milli Q		Milli Q	-	+
40	10	Mouse monoclonal (sc-460)	Normal Goat IgG	Goat anti-mouse (Pierce 31160)	Milli Q	+	+
			Milli Q		Milli Q	-	+
10	10	Mouse monoclonal (ab30716)	Rabbit polyclonal (ABIN733448)	Goat anti-mouse (Pierce 31160)	Milli Q	-	+
					20 ug of E6 protein	+	+

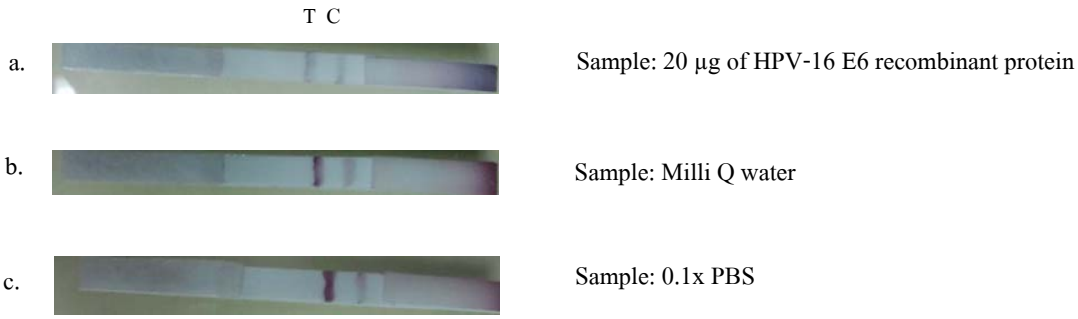

Figure 3 The false-positive results on lateral flow strip

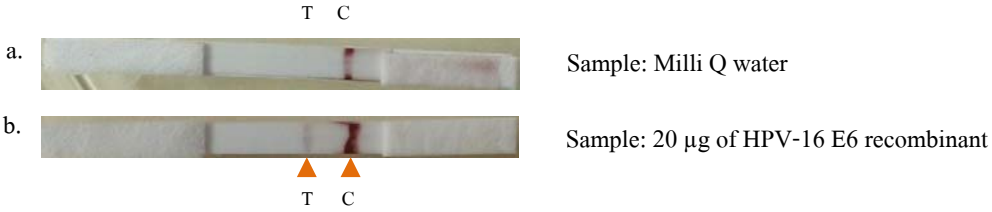

Figure 4 The result of lateral flow assay