

Development of In Situ Hybridization with Tyramide Signal Amplification (ISH with TSA) for Human Papillomavirus (HPV) Detection Using Biotin Labeled Universal Oligonucleotide Probe (BLOP)

การพัฒนาวิธี in situ hybridization โดยใช้สาร tyramide เพื่อตรวจเชื้อ
อีวแมนแพบพิวโลมาไวรัสโดยใช้โพรบสายสั้นที่ติดฉลากด้วยไบโอติน

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ABSTRACT

To detect HPV DNA and evaluate the HPV infected cells, In situ Hybridization with Tyramide signal amplification using a universal oligonucleotide probe was developed. The probe was designed by bioinformatics analysis and labeled with biotin by Biotin Universal Linkage System . The biotin labeled oligonucleotide probe was evaluated by dot blot hybridization using plasmid DNA templates extracted from 11 specific HPV clones. In situ Hybridization with Tyramide signal amplification was performed on Caski cell line. 3,3'-Diaminobenzidine tetrahydrochloride and Aminoethyl carbazole were used as chromogenic substrates in detection system. The results showed that the designed universal oligonucleotide probe contained 50 bps of late gene and had ~ 90% specific homology to all high risk HPV. Biotin labeled oligonucleotide probe could specifically identify all HPV DNA extracted from specific HPV clones by dot blot hybridization. For HPV DNA detection and evaluation of HPV infected cells, biotin labeled oligonucleotide probe was used in In situ Hybridization with Tyramide signal amplification that performed in HPV positive cell lines. HPV DNA was detected in the nuclei of Caski cells which demonstrated single dots or punctuate signal representing integration at multiple sites. The background decreased when Aminoethyl carbazole was used as a substrate. These results suggest that biotin labeled oligonucleotide probe can be used to identify HPV DNA and evaluate the physical status of HPV in the infected cells by In situ Hybridization with Tyramide signal amplification. This biotin labeled oligonucleotide probe will be a universal probe that further applied to identify several HPV physical status in infected cells.

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บทคัดย่อ

การศึกษานี้ทำการพัฒนาวิธีการตรวจหา HPV DNA ในเซลล์ติดเชื้อ HPV โดยสร้างโพรบหรือสายนิวคลีโอไทด์ขนาด 50 นิวคลีโอไทด์เพื่อใช้ในการตรวจหาเชื้อฮิวแมนแพปพิโลมาไวรัสกว่า 40 ชนิดที่ก่อให้เกิดการติดเชื้อที่บริเวณอวัยวะสืบพันธุ์โดยวิธี In situ Hybridization with Tyramide signal amplification โพรบจะถูกติดฉลากด้วยสาร Biotin โดยวิธี Universal Linkage System หรือเรียก biotin labeled oligonucleotide probe และเพิ่มความไวในการตรวจโดยใช้สาร tyramide ในการทดลองครั้งนี้ได้ทำการเปรียบเทียบ substrate 2 ชนิดเพื่อใช้เป็นสารก่อเกิดสัญญาณซึ่งทำให้เกิดตะกอนสีที่ละลายน้ำเพื่อให้ตรวจพบได้ คือ substrate 3,3'-Diaminobenzidine tetrahydrochloride ซึ่งให้ตะกอนสีน้ำตาลและ substrate Aminoethyl carbazole ซึ่งให้ตะกอนสีแดง และสามารถอ่านผลได้โดยใช้กล้องจุลทรรศน์แบบ standard bright field microscope จากการทดสอบความจำเพาะของโพรบกับพลาสมิดของเชื้อ HPV 11 ชนิดพบว่าโพรบสามารถทำปฏิกิริยากับพลาสมิดทั้ง 11 ชนิดโดยวิธี dot blot hybridization และเมื่อนำโพรบมาทำการตรวจหา HPV DNA โดยวิธี In situ Hybridization with Tyramide signal amplification บนเซลล์เพาะเลี้ยงมะเร็งปากมดลูก Caski ที่มีการติดเชื้อ HPV type 16 พบว่าเกิดจุดขึ้นในนิวเคลียสโดยพบจุดเดี่ยวๆหลายจุด โดยจะเกิดเป็นตะกอนสีแดงเมื่อใช้ Aminoethyl carbazole และน้ำตาลเมื่อใช้ 3,3'-Diaminobenzidine tetrahydrochloride ให้เห็นบนเซลล์ที่ศึกษาซึ่งจุดที่เกิดขึ้นเป็นลักษณะที่แสดงว่าเชื้อมีการแทรกจีโนมของเชื้อเข้าสู่จีโนมของคนคือมี physical status แบบ integrated form การใช้ Aminoethyl carbazole เป็น substrate จะมี background น้อยกว่าการใช้ 3,3'-Diaminobenzidine tetrahydrochloride จากผลการทดลองนี้แสดงว่าโพรบขนาด 50 นิวคลีโอไทด์ที่ถูกสร้างขึ้นมาสามารถตรวจพบ HPV DNA ในเซลล์ติดเชื้อและวินิจฉัยภาวะหรือ physical status ของเชื้อได้โดยใช้วิธี In situ Hybridization with Tyramide signal amplification ได้ ในการศึกษาครั้งนี้สรุปว่า biotin labeled oligonucleotide probe น่าจะเป็น universal probe ที่สามารถนำไปตรวจหา HPV DNA อย่างไม่จำเพาะชนิดรวมทั้งภาวะการติดเชื้อในเซลล์ติดเชื้อได้

Key Words: In situ hybridization with tyramide signal amplification, Biotin labeled universal oligonucleotide probe (BLOP), Human papillomavirus (HPV)

คำสำคัญ : In situ hybridization ใช้สาร Tyramide โพรบติดฉลากด้วยไบโอติน ฮิวแมนแพปพิโลมาไวรัส

Introduction

The human papillomavirus (HPV) is a DNA virus that belongs to the family of *Papovaviridae*. It can infect cutaneous and mucosa tissue and causes warts or benign tumor. More than 130 types of HPV have been identified so far. Approximately, 40 genotypes have been isolated from anogenital lesions. According to their biological oncogenic potential, HPV could be divided into two groups: low risk HPV types and high risk HPV types.

The low risk group that more frequently associated with low grade squamous intraepithelial lesions (SIL) or so-called cervical intraepithelial neoplasia I (CIN I) lesions and condyloma acuminatum, comprises HPV types 6, 11, 40, 42, 43, 44, 54, 61, 72 and 81. The high risk HPV types include types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 73, and 82 (Munoz et al., 2003). They frequently associated with high-grade SIL or CIN II, CIN III

and invasive carcinoma of uterine cervix. The rate of HPV infection in women with all ages is variable. Infected patients younger than 35 year old have about 70–80% spontaneous regression within 2–3 years. The remaining (20%) have persistent infection and may develop cervical cancer (Steenbergen et al., 2005).

The Pap test (Papanicolaou test or Pap smear) has been used to screen women for cancerous and precancerous cells of the cervix for more than 50 years and is an important contribution leading to a decrease in the incidence of cervical cancer as decreased rate is 75%. However, Pap test has been shown to have low sensitivity and the rate of false negative and false positive is high. The recommendation for cervical cancer screening in the developed countries is the addition of HPV testing with pap test (Bristow RE, 1998). Several tests have been used such as PCR, RT-PCR and Hybrid capture II (HC II), etc. However, they need specific tools and are not suitable for routine laboratory in developing countries. The spontaneous regression is one factor that affects HPV DNA detection in specimen.

It has been well established that the persistence of HPV infection and HPV integration into host genome is the mechanism of cervical carcinogenesis. HPV integration can be determined by detection of physical state of HPV DNA in host cells, demonstrated as integrated form. In this consequence, the disruption of E2 gene occurs and subsequently E6 and E7 genes are overexpressed. Genetic instability is a consequence of HPV infection and integration. The overexpressed E6 and E7 viral oncoproteins of HR HPV inactivate pRB and p53, respectively and resulting in cervical cancer progression. Integrated form of HPV was mostly

found in cervical cancer cells. Increasing levels of HPV integration are also associated with the severity of lesion. The HPV DNA integration was detected of 0, 5–87 and 80–100 % in CIN I, CIN II–III and cervical cancer, respectively, according to the testing methods (Evans and Cooper, 2004). The detection of HPV integration can be used as a marker of cervical cancer progression by oncogenic HPV infection.

Various methods have been used to detect the HPV physical status such as southern blot analysis of restriction enzyme digest patterns (Cullen et al., 1991; Shirasawa, 1986), southern blot analysis and 2D gel electrophoresis (Cullen et al., 1991; Hudelist et al., 2004), PCR analysis of E2 region (Gallo et al., 2003; Park et al., 1997; Tonon et al., 2001), RT-PCR analysis of E6/E7 transcript (Klaes et al., 1999; Park et al., 1997), real time PCR–E2:E6 amplification (Nagao et al., 2002). All of these methods are highly sensitive and reliable, but each of them has certain specifications and high cost that is not suitable for routine laboratory. Some methods cannot differentiate the physical status of HPV infection in the cell.

Recently, the method of in situ hybridization is very popular, because it enables correlation between the detection of HPV DNA and morphological changes in the tissue. The sensitivity of ISH has been greatly improved by tyramide amplification reagent and fluorescent substrate that promise the potential detection of low/single copy HPV as PCR technique (Zehbe et al., 1997). GenPoint™ technique has been developed and using chromogenic substrate that can be examined by light microscope (Lizard G, 2001). Two forms of HPV physical status can be identified by this

technique, punctuate signal pattern representing integration and diffuse representing episomal HPV. However, this technique needs specific probe to detect specific type of HPV.

Because of low sensitivity of pap test in cervical cancer screening, those with negative results or undetermined results are managed by cytology follow-up or confirmed by specific methods such as colposcopy. One of disadvantage of Pap test is unable to identify the HPV infection that is at risk to develop cervical cancer. For effective and rapid detection of abnormal cell with HPV infection, this study aims to develop the tyramide based ISH for the detection of HPV physical status using universal oligonucleotide probe. Tyramide amplification reagent and chromogenic substrates will be used to improve the sensitivity of the technique and can be examined by light microscope. It enables correlation between the detection of HPV DNA and morphological changes in the abnormal cervical cells that will improve the cervical cancer screening program and prevent cancer progression.

Materials and Methods

Cell lines.

The cervical carcinoma cell line Caski which contains 600 copies of HPV type 16 (HPV-16) DNA per cell and HPV negative cell line Vero (green monkey kidney) were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum and antibiotics.

Plasmids.

Plasmids containing HPV genomic DNA that were used in this study were HPV-11, -16, -18, -31, -33, -35, -39, -45, -51, -52, and -58.

Design of universal Oligonucleotide probe

Complete HPV sequences were obtained from GenBank with the following accession numbers: HPV-6, NC001355; HPV-11, M14119; HPV-16, NC001526; HPV-18, NC001357; HPV-26, NC001583; HPV-45, X74479 ; HPV-53, NC001593; HPV-54, NC001676; HPV-51, M2877; HPV-52, X74481; HPV-56, X74483; HPV-58, D90400; HPV-31, M14119; HPV-33, M12732; HPV-35, M74117; HPV-39, M62849; HPV-59, X77858; HPV-66, U31794; HPV-73, X94165; HPV-82, AB027021; HPV-40, X74478; HPV-42, M73236; HPV-43, AJ620205; HPV-44, U31788; HPV-61, U31793; HPV-72, X94164; HPV-81, AJ620209; HPV-57, X55965; HPV-30, X74474; HPV-34, NC001587; HPV-32, NC001586; HPV-62, AY395706; HPV-67, D21208. L1 region sequences were aligned. A multiple sequence alignment was done using Multalin and ClustalW. Universal HPV oligonucleotide probe for genital HPVs was designed and biotin was labeled by universal linkage system

Biotin Universal Linkage System (ULS®)

Designed oligonucleotide probe was chemically labeled with biotin-universal linkage system (ULS®) labeling kit (Fermentas) which uses a special platinum compound. The Pt compound has two free binding sites, one of which is used to bind a marker group that is biotin, thus forming a biotin/platinum complex. The other binding site is used to link the complex to the purines of designed oligonucleotide probe. 3 Units of biotin ULS® reagent to 1 ug of oligonucleotide probe was mixed and incubated at 85°C for 30 min.

Dot blot hybridization

Biotin labeled oligonucleotide probe was evaluated by dot blot hybridization. 50 ng of each HPV plasmid (11, 16, 18, 31, 33, 35, 39, 45, 51, 52 and 58) and extracted DNA from Caski, SiHa, HeLa and Vero was denatured and applied to nylon membranes (Biodyne C) with dot blot apparatuses (Bio-Rad, Hercules, Calif.). The membrane was hybridized at 42°C overnight with biotin labeled oligonucleotide probe. Following hybridization, membrane was washed at 50°C to remove nonspecifically bound probe. This ensures efficient removal of the nonspecifically bound probe and optimal specific hybridization. The bound probe was detected with streptavidin-horseradish peroxidase (Zymed) and chemiluminescence detection kit (LumiGLO, KPL). Membrane was exposed to Kodak Medical X-ray film. HPV positivity by dot blot was determined by establishment of a negative cutoff and signals above the cutoff were positive.

In situ hybridization with tyramide signal amplification (ISH with TSA)

Human cervical cancer cell line containing HPV-16 (Caski 600 copies) was washed in phosphate buffered saline and applied to glass slides, air-dried at room temperature, fixed in 1% buffered formalin for 1 hour, absolute ethanol at -20°C for 10 minutes and stored at -20°C. Target DNA was unmasked by placing the slides in 10 mM citrate buffer (pH 6.0) at 95°C for 40 minutes. After cooling at room temperature in citrate buffer, the slides were digested with 50 µg/ml proteinase K in TE buffer at 37°C for 20 minutes and proteinase K was inactivated by washing in distilled water, following by quenching

of the endogenous peroxidase with 0.6% hydrogen peroxide in methanol for 30 minutes at room temperature and rinsed in two washed of distilled water. After the oligonucleotide probe was added, the slides were covered with coverslips, and target DNA and the probe were denatured by heating at 96°C for 12 minutes by using water bath. The slides were hybridized at 42°C overnight in a humid chamber. After hybridization, the coverslips were removed by soaking in 2XSSC/0.05% tween20. Stringent wash was performed by incubating slides in 1XSSC/0.1%SDS at 48°C twice 10 minutes each. The slides were blocked in blocking solution for 30 minutes. The amplification cycle followed, which consisted of the application of primary streptavidin horseradish peroxidase (Zymed) 1:200 dilution in PBS for 30 minutes, five minutes wash three times in PBS/0.05% tween20, followed by the application of biotinyl-tyramide solution (PerkinElmer Life Sciences) 1:50 in the buffer for 15 minutes, followed by five minutes wash three times in PBS/0.05% tween20, followed by secondary streptavidin horseradish peroxidase (Zymed) 1:200 dilution in PBS for 30 minutes, followed by five minutes wash three times in PBS/0.05% tween20. The signal was developed by adding either diaminobenzidine (DAB) (Sigma) or amino ethyl carbazole (AEC) (Sigma) for 10 minutes and counterstained in Mayer's hematoxylin before mounting with mounting solution and visualized by bright field microscopy.

Results***A universal probe design and evaluation***

Complete HPV sequences were obtained from GenBank and used for alignment of L1 region

sequences. The L1 region is relatively well conserved. A multiple sequence alignment was done using Multalin and ClustalW. The sequences of the oligonucleotide which was finally used in this study is 50 bp of 5'CAGGGCCATAACAATGGTATTTGTTGGGGTAATCAATTATTTGTTACTGT 3'. This sequence stretched showing 90% identities among HPV types (Figure 1). The specificities of the oligonucleotide probe were checked using BLAST within the NCBI website. This probe was labeled with biotin by universal linkage system.

Evaluation of the biotin labeled oligonucleotide probe (BLOP)

BLOP was evaluated using dot blot hybridization with the plasmid DNA template extracted from 11 HPV clones. BLOP could specifically identify all extracted HPV DNA of specific HPV clones (Figure 2). When hybridization was performed under stringent conditions, the probe did not detect human genomic DNA in normal cells or HPV negative cells. This result demonstrated that BLOP is specific and universal for HPV DNA detection of several HPV types.

Development of ISH with TSA on Caski cell line

To detect HPV DNA as well as evaluate HPV physical status in HPV infected cells, ISH with TSA on Caski cell line was performed and BLOP was used as a probe. In line with previous reports, Caski cells were harbored with integrated form of HPV16. This technique showed the positive signal of HPV DNA in nucleus of Caski cells that demonstrated dots or punctuate signal at multiple sites that was likely to be responsible for HPV integration form in the Caski cell (Figure 3). More than 80 % of Caski cells showed positive signal. For the detection system, AEC substrate presented lower background with more contrast color than DAB. No signal was observed in HPV negative cells. This result suggested that BLOP should be a universal oligonucleotide probe for HPV DNA detection and identification of HPV physical status by ISH with TSA technique.

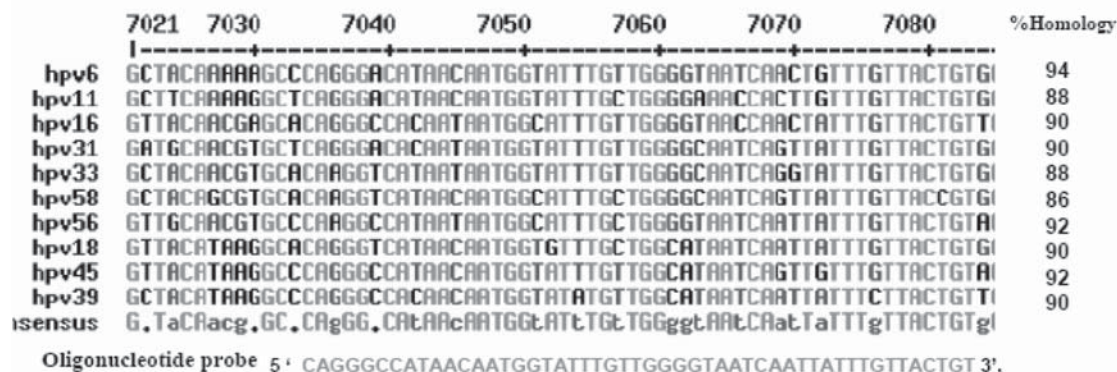


Figure 1 A multiple sequence alignment using Multalin designed oligonucleotide probe at position from 7034-7083.

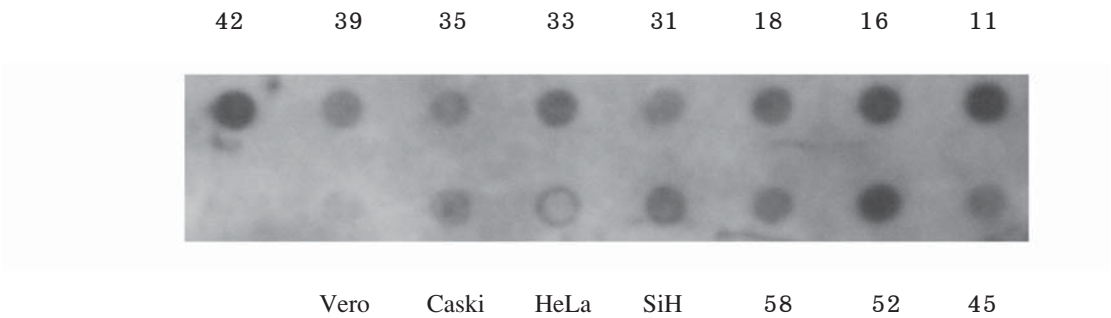


Figure 2 Biotin labeled oligonucleotide probe (BLOP) was tested with HPV DNA extracted from 11 HPV clones, HPV infected cell lines (Caski, HeLa and SiHa) and HPV negative DNA extracted from HPV negative cells (Vero)

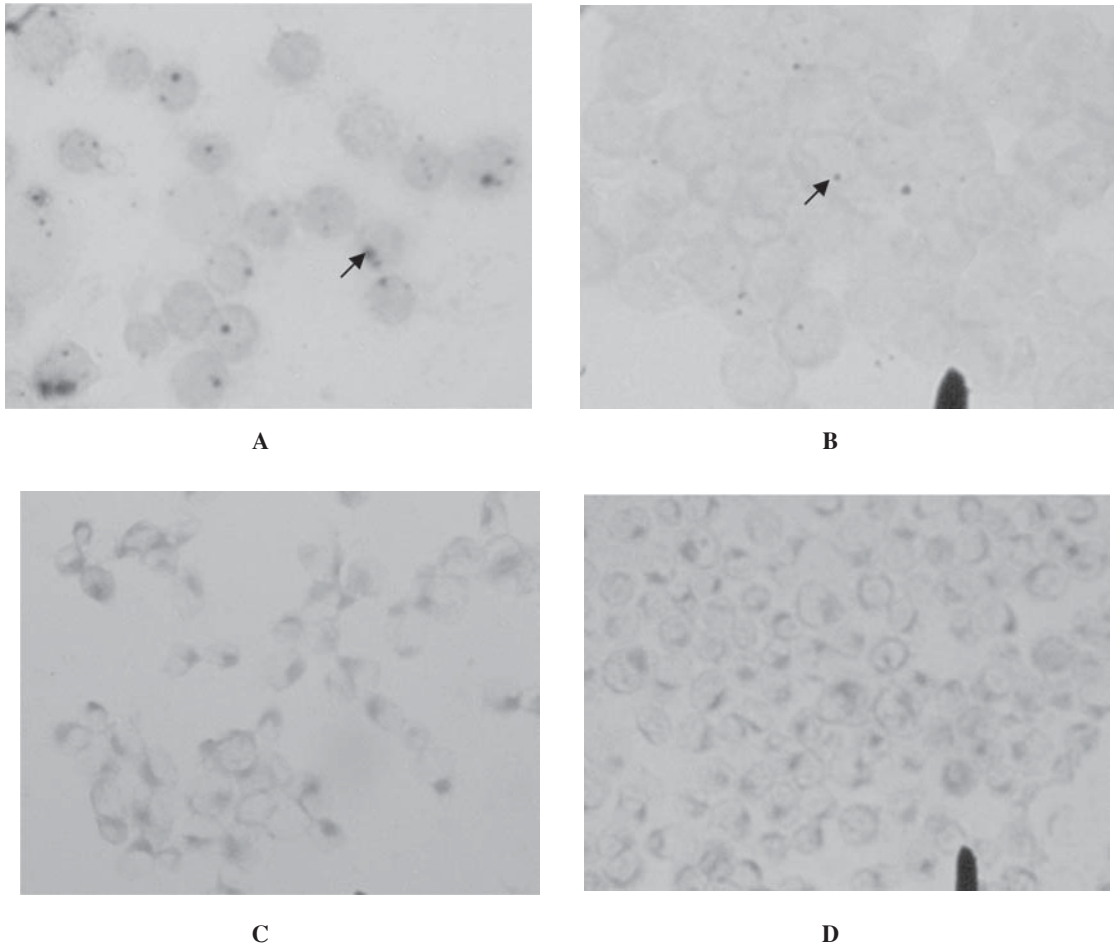


Figure 3 A. Caski cell line demonstrating HPV-16 integration, hybridization signals demonstrated with DAB (brown dot). B. Caski cell line demonstrating HPV-16 integration, hybridization signals demonstrated with AEC (red dot).C and D. HPV negative cells (Vero), no hybridization signals demonstrated with DAB and AEC respectively.

Discussion and Conclusion

The main goal of this work was the development of a novel universal oligonucleotide probe targeting the L1 region to detect genital Human papillomavirus (HPV) types by in situ hybridization (ISH) technique. The sensitivity of biotin labeled oligonucleotide probe (BLOP) for ISH technique was increased by using tyramide signal amplification and with high proportion of biotin molecules incorporated into the probe. Biotin universal linkage system (ULS) technique was chosen for biotinylation of oligonucleotide probe. Advantages of oligonucleotide probe are as follows. (i) It was designed by BLAST search to match specifically highly conserved HPV L1 region and avoiding cross-reactivity with other bacterial genomes and viral genomes. (ii) Its small size allows for easy penetration into the cells. (iii) It is stable making it less prone to degradation during hybridization procedures. (iv) It is easy to produce by an automated chemical synthesis. (v) No self-hybridization. (vi) The detection by bright field microscope makes adaptable to routine clinical laboratories (McNicol and Farquharson, 1997). The 50-bp universal oligonucleotide probe that was designed had >90 % identity to about 40 high risk HPV type. This probe should universal to detect several HPV DNA by ISH technique.

We evaluated this probe and investigated whether the 50-bp universal oligonucleotide probe region permitted identification of HPV types using dot blot hybridization. To increase the specificity of the BLOP, moderate stringency washing condition was carried out with high washing temperature (50 °C). This ensures efficient removal of the nonspecifically bound probe and optimal specific

hybridization. By this condition, all 11 HPV genotypes and extracted DNA from Caski, SiHa, HeLa cells were correctly identified, indicating the high specificity of the BLOP. In contrast to [32P]-end - labeled oligonucleotide probe that described by Burner GC *et al.* (Burner *et al.*, 1990) for the detection of HPV-18 was incapable of detecting extracted HPV-18 DNA from HeLa cells by dot blot hybridization. Is it specific probe?.

BLOP was evaluated on cervical cell lines (Caski cells) by ISH with TSA. Hybridization in the cells has a lower stringency than hybridization on the membrane in terms of temperature. Hybridization temperature was similar to dot blot hybridization, however, washing temperature was carried out with temperature at 48 °C. HPV DNA was detected in the nuclei of Caski cells as single dots or punctuate signal at multiple site representing HPV integration. The positive signal demonstrated by DAB and AEC were dots or punctuates that were smaller than using the specific HPV plasmid probes (Evans *et al.*, 2003) and DNA probes (Huang *et al.*, 1998; Lizard *et al.*, 2001; Samama *et al.*, 2002). However, the dots that detected in this study were similar to the detection using 120 bp oligonucleotide probe (Day *et al.*, 1990). The single dots can be enlarged by repeated cycles of tyramide signal amplification (Huang *et al.*, 1998). Evan *et al* (Evans and Cooper, 2004) described the different of detection system between DAB and AEC and suggested that AEC substrate presented with clear background. This study, however, showed that DAB and AEC gave the same background, whereas AEC could provide excellent color contrast with Mayer's hematoxylin counterstained. In this study, the result demonstrated that BLOP

could be applied to detection HPV DNA by ISH with TSA and identified HPV physical status. The BLOP be the universal probe and provide a useful tool to detect physical status of human papillomavirus in clinical specimens.

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