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In Situ Detection of the p53-E6 and pRb-E7 Complex Formation in EC109 Cell by Proximity Ligation Assay

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Abstract

Human papillomavirus is an important cancer factor in many cancers. E6E7 is the important viral oncogene and plays an important role. It is known that its function is to regulate p53 and pRb. In this paper, *in situ* detection was performed to determine the interaction between them; the research provides visual evidence of their interaction. **Research methods:** *In situ* PLA reaction was used to detect the relationship between two different protein. **Results:** The quantity of the HPV18E6 expression is much higher than the expression of HPV18E7 protein in HeLa and EC109 cells; the interaction between p53-E6 and pRb-E7 was clearly observed and this effect could be visualized by this method. **Conclusion:** Interaction of the HPV18E6 protein combined with p53 protein and HPV18E7 protein combined with pRb protein could be visualized in cell.

Keywords

HPV E6, HPV E7, p53, pRb, PLA

1. Introduction

The carcinogenic mechanism of high-risk HPV involved in human cancers has been well established by early evidences in the 1980s. The cloned DNAs of high-risk HPV which are associated with lesions that have a high risk for malignant progression encode cellular transformation properties in established rodent fibroblasts [1], in primary rodent cells [2] [3] [4], and in primary human cells [5] [6] [7]. HPV 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7 [8]. Interaction of the prod-

ucts of oncogenes with tumor is suppressed or gene is believed to be an important step of cellular transformation as a consequence of disruption of the normal functions of the specific tumor suppressors [9]. A possible mechanism by which the high-risk HPV E6 and E7 proteins abrogate the tumor suppressor function of p53 and pRb, respectively has been demonstrated by the findings that the E6 protein promote the degradation of p53 and E7 protein form complexes with pRb *in vitro* [10] [11]. The interaction was shown by the coimmunoprecipation of E6 with p53 and E7 with p-Rb in rabbit reticulocyte mixed lysates using the p53-specific monoclonal antibody PAb 421 and pRb-specific monoclonal antibody C36, followed by visualization of radioactively labeled proteins by autofluorography.

Coimmunoprecipation and other protein pull-down assays allow stable protein interactions to be investigated *in vitro* translation system, but they fail to easily reveal transient interactions and the location of interacting proteins within cell. Additionally, there may be considerable variation in synthetic proteins with respect to structure and expression level between *in vitro* and *in vivo* condition. Therefore, the methods with high specificity and sensitivity need be required to directly observe interacting endogenous proteins in cells and subcellular compartments. Few assay techniques are applied to investigate and verify the interaction between oncoproteins encoded by HPV and cell cycle regulatory proteins in cell level other than coimmunoprecipation in recent years. For this purpose, we applied the *in situ* proximity ligation assay technology (*in situ* PLA) for highly sensitive detection of the complex formation of E6-p53 and E7-pRb. This method was early reported by Fredrikssonin 2002 [12] and subsequently was generally applied in a majority of researches. The proximity ligation allows proteins to be represented as information carrying DNA strands through rolling-circle amplification (RCA). A pair of oligonucleotide probes-labeled secondary antibodies generates a signal that serves as surrogate markers for the detected protein molecules only when the two PLA probes have bound in close proximity, either to the same primary antibody or two primary antibodies that have bound to the sample in close proximity. The signal from each detected pair of PLA probes is visualized as an individual spot. These PLA signals can be quantified and assigned to a specific subcellular location. In a word, PLA is characterized by both specificities of antigen-antibody binding and sensitivity of PCR.

2. Material and Method

2.1. Cell Culture and Pre-Treatment

HeLa cell, EC109 cell, and HEK293 cell were cultured in DMEM medium containing 10% FBS supplemented with 1% penicillin and streptomycin at 37°C and harvested before reaching confluency. We made cytospin preparations that were air-dried for 30 min, fixed with 4% paraformaldehyde, and washed with PBS once.

2.2. *In Situ* PLA Reaction

Duolink II Fluorescence reagents were purchased from Olink Bioscience for *in situ* PLA detection. The detailed user manual can be available online at https://www.sigmaaldrich.com/china-mainland/zh/technical-documents/protocols/biology/duolink-fluorescence-user-manual.html#fluorescence.

Duolink® PLA is based on the proximity ligation assay (PLA) principle, and combines the specificity of secondary antibodies with the sensitivity afforded by rolling circle amplification to detect endogenous proteins in fixed cells and tissues. A pair of oligonucleotide labeled antibodies (PLA probes) generates an amplified signal only when the probes are in close proximity (<40 nm).

Primary antibodies for detection of protein interactions—Duolink IQ

Duolink provides an excellent way to detect protein interactions and/or heterodimeric targets. This is done using two primary antibodies, each directed against one of the targets of interest. The two primary antibodies must either have been raised in different species or been modified with different haptens. Also, both primary antibodies must bind to the target under the same conditions.

Primary antibodies for detection of single protein expression—Duolink Q

When single protein targets are to be detected you can use either one or two primary antibodies against your target (Figure 1).

Usage of two primary antibodies will give you an assay with superior specificity. When using two primary antibodies, they must be directed against different, noncompeting epitopes, on the same target molecule. The two primary antibodies must either have been raised in different species or been modified with different haptens. Also, both primary antibodies must bind to the target under the same conditions (Figure 2).

The cytospin preparations on glass slide were blocked with 4% BSA in PBS for 1 h at 37˚C in a humidity chamber before incubation at 4˚C with rabbit anti-HPV18E6 antibody, rabbit anti-HPV18E7 antibody, mouse anti-p53 antibody, and mouse anti-pRb antibody respectively for detecting these individual proteins. To investigate the complex formation of E6 with p53 andE7 with pRb, we added the mixtures of anti-HPV18E7 antibody and anti-pRb antibody as well as of anti-HPV18E6 antibody and anti-p53 antibody into the sample, respectively for incubation. The slides were then incubated with the secondary antibodies conjugated with PLA oligonucleotide probes (MINUS and PLUS) for 1 h at 37˚C
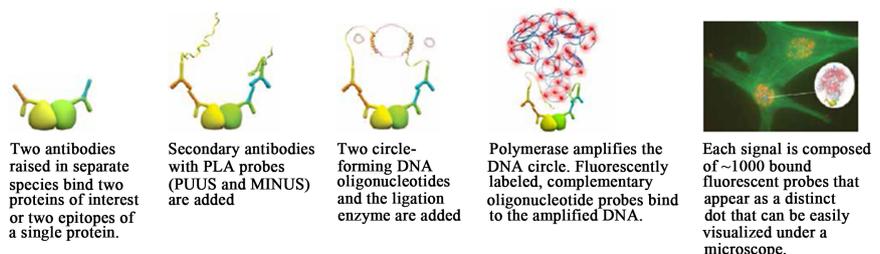

Two antibodies raised in separate species bind two proteins of interest or two epitopes of a single protein.

Secondary antibodies with PLA probes (PUUS and MINUS) are added

Two circle-forming DNA oligonucleotides and the ligation enzyme are added

Polymerase amplifies the DNA circle. Fluorescently labeled, complementary oligonucleotide probes bind to the amplified DNA.

Each signal is composed of ~1000 bound fluorescent probes that appear as a distinct dot that can be easily visualized under a microscope.

Figure 1. Step-by-step to show how to perform a Duolink® Proximity Ligation Assay experiment.
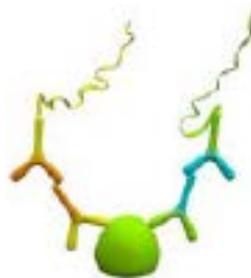
Figure 2. To detect and quantify one single protein with high specificity, use two different primary antibodies directed against two different epitopes on the same protein.

after washes. The ligation solution containing ligase and nucleotides was added to the sample for forming circles using as templates the two oligonucleotides if the PLA probes are in close proximity. We performed ligations for 30 min at 37˚C. After washes, we performed the RCA using one of PLA probes as a primer and the ligated circle as template by hybridization with fluorescence-labeled probe for 100 min at 37˚C. We used the Laser Scan Confocal Microscope (LSM700, Zeiss) for imaging.

3. Results

Visualizing the Protein-Protein Interactions in Cells

E6-p53 protein complexes and E7-pRb protein complexes were detected by double recognition, and the distribution of red fluorescent dots was seen in both EC109 and HeLa cells, which represent the target protein complexes we wanted to detect. It was found that the signal density of E6-p53 protein complexes was lower in EC109 cells, but the signal density was higher in HeLa cells. Similarly, the signal density of E7-pRb protein complexes is high in both EC109 and HeLa cells. In addition, no fluorescent dots were produced in EC109 and HeLa cells, when an anti-GAPDH alternative was used for pRb against E7. The reason is that E7 does not form complexes with pRb proteins (Figure 3). We also used single antibody for the detection of fluorescence signals of HPV18E6, E7pRb and p53 proteins in EC109 cell and HeLa cell which are also shown (Figure 4 and Figure 5).

4. Discussion

In recent decades, rare studies reported transient interactions between onco-proteins encoded by HPV and cell cycle regulatory proteins *in situ* level. To our knowledge, this study firstly detected the complex formations of E6 with p53 and E7 with pRb in HPV-positive tumor cell lines using PLA technique. The signals that represent the corresponding single molecule or complex formations have been easily observed in cytospin preparation materials.

It is well known that the p53 encoded by wide-type p53 gene is generally quite low due to a short half-life [13] [14]. However, the p53 encoded by abnormality p53 gene were detected in tumor cells or tissues by using western blot or IHC,
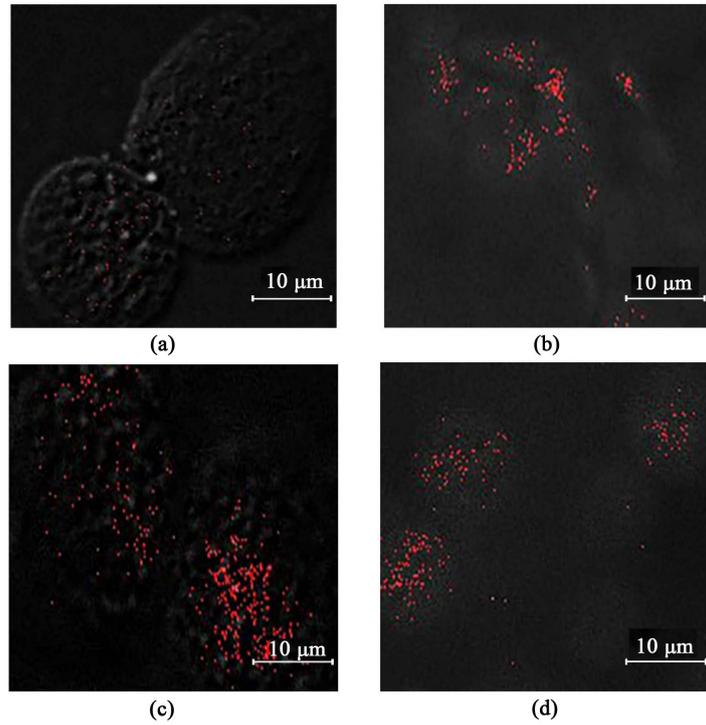
Figure 3. (a) Detection of interaction between pRb and HPV18E7 protein in EC109 cell; (b) the detection of interaction between pRb and HPV18E7 protein in HeLa cell; (c) the detection of interaction between p53 and HPV18E6 protein in EC109 cell; (d) the detection of interaction between p53 and HPV18E6 protein in HeLa cell.
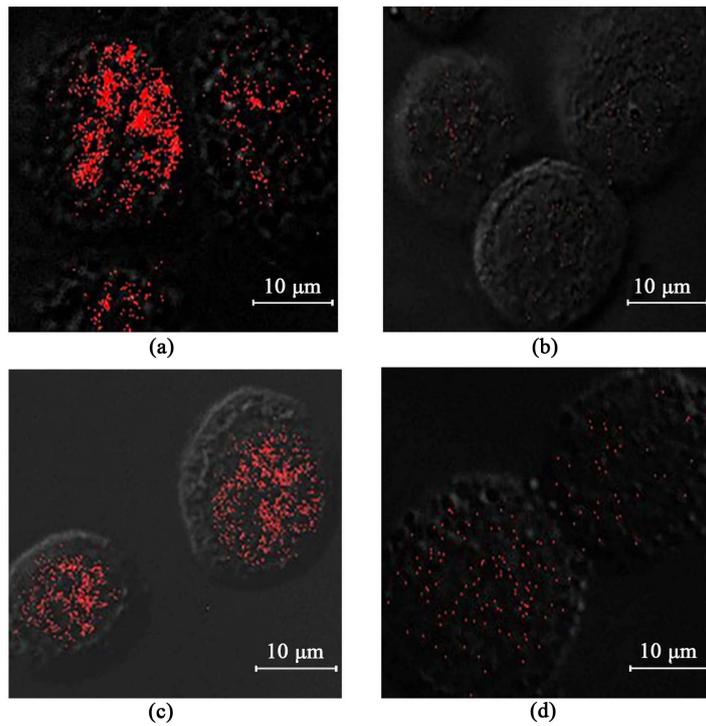

Figure 4. (a) the detection of single HPV18E6 protein in EC109 cell; (b) the detection of single HPV18E7 protein in EC109 cell; (c) the detection of single HPV18E6 protein in HeLa cell; (d) the detection of single HPV18E7 protein in HeLa cell.
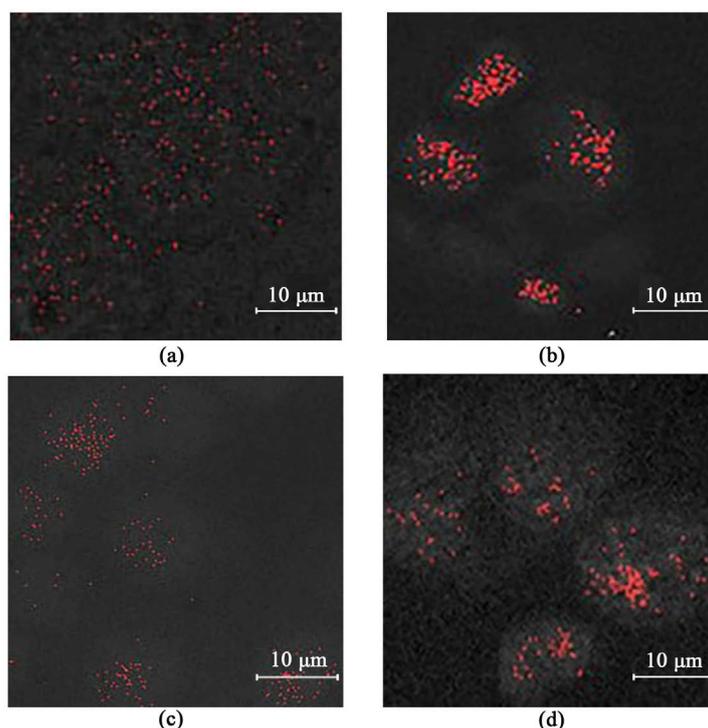
Figure 5. (a) the detection of single pRb protein in HeLa cell; (b) the detection of single pRb protein in EC109 cell; (c) the detection of singlep53 protein in HeLa cell; (d) the detection of single p53 protein in EC109 cell.

because of the accumulation of p53 that has a prolonged half-life [14] [15] [16] [17]. Researchers have demonstrated that no mutation of p53 gene was observed in HPV-positive cervical cancers and derived cell lines [18] [19]. Therefore, the p53 protein was reported to be undetectable in HeLa cell with conventional methods as a consequence of both innate instability and E6-mediated degradation. And likewise, the p53 protein was not detected in EC109 cell positive for HPV18. In this study, our western blot results of HeLa and EC109 lysate were corresponding to the previous findings. Fortunately, the normal p53 was intuitively found in HeLa and EC109 cell by *in situ* PLA technique, which presented in the form of red fluorescent spots. E6 and E7 oncoproteins were not detected in HeLa and EC109 lysate using western blot, but were detected in cytospin preparation applying *in situ* PLA, although a low intensity of signals. The previous studies hardly detected the E6/E7 protein in cell lysate with mono- or polyclonal antibody. These findings may be partially attributed to low expression levels of oncoproteins in HeLa and EC109 cell. Another possible explanation of this phenomenon is that the complex formation partially masked the epitope of oncoprotein recognized by the antibody resulting in low affinity of primary antibody to antigen. Due to the high sensitivity of PLA, low level of target molecular can be easily detected and the signals even be quantified by using specific software enabling signal cell statistical analysis of expression levels in tissue or cell populations. Theoretically, the greater the affinity of the primary antibody is, the higher the assay sensitivity is, because the assays are performed below tar-

get-saturating conditions. However, it is also noted that only a fraction of target molecules are detected as fluorescent spots. This probably reflects less than 100% efficiency in the multiple steps of the procedure, including binding of antibodies, oligonucleotide ligation, amplification, and imaging. On the other hand the background of false positive signals generated by nonspecific adsorption of antibodies will be a great challenge at any time. Therefore, an optimized assay approach should be acquired in high sensitivity and low background. *In situ* PLA is a further development and improvement of early DNA-based protein detection strategy such as immune-PCR [20] and protein-binding DNA aptamers [21] [22] [23]. This assay has generally been applied to monitor other molecular events including protein phosphorylation [24] [25], mRNA-cytoskeleton interaction [26] [27], DNA methylation [28], and location of molecule in subcellular organelles [29]. Additionally, *in situ* PLA can theoretically be used as a molecular ruler that measures the distances between epitopes by varying the protein binders and the lengths of the oligonucleotides on the proximity probes. In conclusion, *in situ* PLA will have a promising prospect in basic and clinical researches.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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