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Original Research

The correlation between infections by human papillomavirus types 6/11 and 16/18 and mammary gland hyperplasia with glandular thickening

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Abstract: The aim of the present study was to investigate the correlation between human papillomavirus (HPV) type 6/11 and 16/18 infections and glandular thickening mammary gland hyperplasia in order to explore methods for preventing glandular thickening mammary gland hyperplasia. A total of 240 patients with glandular thickening mammary gland hyperplasia who were treated by surgery in our hospital from January 2012 to June 2017 were enrolled in the present study. The hyperplastic breast tissue and adjacent normal breast tissue were taken to test HPV type 6/11 and 16/18 infections using conventional PCR and *in situ* hybridization techniques. The correlations between HPV type 6/11 and 16/18 infections and glandular thickening mammary gland hyperplasia were analyzed using statistical methods of chi-square test. The infection rates of HPV type 6/11 and 16/18 in the hyperplastic breast tissue were 31.95% and 34.91%, respectively and 11.83% and 14.79% in the normal breast tissue, respectively. The differences were statistically significant (all $p<0.05$). HPV type 6/11 and 16/18 infections may be closely related to the development of glandular thickening mammary gland hyperplasia, and may be one of the causes of glandular thickening mammary gland hyperplasia.

Key words: Human papillomavirus; HPV 6/11; HPV 16/18; Glandular thickening mammary gland hyperplasia.

Introduction

Mammary gland hyperplasia is a benign breast disease (BBD) caused by abnormal development and degeneration of the breast (ANDI) (1, 2), which is essentially due to the hyperplasia of the mammary gland and interstitial resulting in structural disorders. Glandular thickening mammary gland hyperplasia is one of common types of mammary gland hyperplasia, in which breast acinar and small ducts are focally hyperplasia, and there are varying degrees of connective tissue hyperplasia. In addition, the lobular structure essentially loses its normal form (3).

Human papillomavirus (HPV) is a non-enveloped virus composed of double-stranded loop of approximately 8000 base pairs DNA and an icosahedral stereosymmetric protein, which belongs to Capsid Polyomavirus subfamily (4). HPV infects human epidermal and epithelial cells and can cause a variety of benign and malignant tumors (5). At present, more than 150 types of HPV viruses have been isolated and identified (6). It is currently believed that HPV virions infect epithelial tissue through tiny breaks and are likely to bind to receptors including integrin, laminin, *etc*. According to its carcinogenesis, HPV is usually divided into low-risk type causing various sputum and high-risk type causing carcinoma (7). HPV 16/18 is the most common oncogenic HPV, with more than 70% of cervical cancer and more than 50% of CIN3 cases worldwide caused by it (8). In contrast, HPV 6/11 is the most common low-risk HPV, with 90% of genital warts cases caused by it (9).

Glandular thickening mammary gland hyperplasia is generally considered benign, but there is a certain risk of canceration. Therefore, we hypothesized that high-risk HPV (HPV16/18) may be associated with breast cancer, while low-risk HPV (HPV6/1 1) may be associated with papilloma. The aim of the present study was to investigate the correlation between human papillomavirus (HPV) type 6/11 and 16/18 infections and glandular thickening mammary gland hyperplasia in order to explore methods for preventing glandular thickening mammary gland hyperplasia.

Materials and Methods

Samples and patients

One hundred and sixty-nine (169) cases of histopathologically confirmed glandular thickening mammary gland hyperplasia who were treated by surgery in our hospital from January 2012 to June 2017. The average ages of the patients were 53 years. All the specimens were formalin-fixed, paraffin-embedded, and diagnosed by two pathologists.

DNA was extracted from formalin-fixed and paraffin embedded tissue blocks by preparing 20 mm thick sections collected in sterile microtubes. All precautionary measures were taken to avoid cross-contamination of the samples. DNA was extracted by the TIANamp Genomic Kit (Tiangen Biotech, Beijing, China) according to manufacturer's protocol. The integrity of DNA extracted from tissues was assessed by PCR with b-globin primers. Positive control DNA of HPV type 58 was

extracted from Escherichia coli (E. coli) harboring HPV 58 whole genomic DNA available in our laboratory. All reagents were purchased from Applied Bio-systems, USA.

PCR Amplification for HPV 6/11 and 16/18 DNA

The HPV 6/11 and 16/18 specific primers were designed by the Primer 6 software based on the gene sequences. The forward primer: 5′-CGAGGATGAAATAGGCTTGG-3′and reverse primer: 5′-ACACAAACGAACCGTGGTGC-3′generate 109 bp fragment of E7 region of HPV 6/11. The forward primer: 5′-CGAGTATGCGATAGCGTTCC-3′and reverse primer: 5′-ACACCGACTTACATTGGTCG-3′generate 109 bp fragment of E7 region of HPV 16/18. β-globin forward primer: 5′-ACACAACTGTGTTCACTAGC-3′and reverse primer: 5′-CAACTTCATCCACGTTCACC-3′generate 110 bp fragment.

Before analyzing the extracted DNA for HPV, the integrity of the extracted DNA was confirmed by using the b-globin. The standard PCR protocol was carried out with an initial 5 min denaturation step at 95°C coupled to a repeating cycle of 30 sec at 94°C (denaturation), 30 sec at annealing temperature, and 30 sec at 72°C (extension) for 35 cycles, followed by a 5 min completion step at 72°C, and final cooling to 4°C. Each PCR run included reactions with DNA templates from positive and negative controls as well as a no template control containing all PCR components but without the template DNA to ensure that the reagent mix was free of contaminants. Amplification products were visualized by ethidium bromide staining under ultraviolet light, after agarose (2.0%) gel electrophoresis.

In situ Hybridization for HPV 6/11 and 16/18 DNA

From the 169 of glandular thickening mammary gland hyperplasia specimens, 5-mm thick sections were prepared from formalin-fixed-paraffin-embedded blocks and stored at -20°C until required for use. For use in *in situ* hybridization (ISH), the oligonucleotide probes 5′-GCCAGATGGACAAGCACAACGGCCACAGCTAA TTACTACATTGTAACTTG-3′ and 5′-GTTAGTAGC ACAAGCACTTCGGGCACAGCTCGTACTACATT GTCGCATT-3′targeting HPV 6/11 and 16/18 respectively were designed with primer 6 software. The specificities of the oligonucleotide probes were analyzed by BLAST software (http://www.ncbi.nih.gov). The probes were labeled by tailing the oligonucleotides with digoxigenin-11-dUTP kit (Roche, Mannheim, Germany). ISH was performed according to a standard protocol. Briefly, the paraffin was removed and the sections were hydrated. All slides were incubated with 0.4% pepsin at 37°C for 30 min, refixed and then treated with 0.2N HCl followed by dehydration. After pre-hybridization for 2 hr at 42°C, the hybridization reaction was carried out at 42°C for 22 hr in hybridization buffer. After stringent posthybridization wash, detection was accomplished with anti-digoxigenin-AP conjugate. The color reaction was developed in NBT-BCIP and counterstained with 1% nuclear fast red. Purple blue precipitation in nucleus under light microscope was considered as positive hybridization signal. For negative controls, probes and antibodies were replaced with PBS.

Statistics

The positive rates of HPV 6/11 and 16/18 DNA between hyperplastic breast tissue and adjacent normal breast tissue were analyzed using the χ^2 test. Cohen's kappa measure was used for the evaluation of the agreement between PCR and ISH. Fisher's exact test was used to test the frequency of HPV positive and negative cases by clinicopathological characteristics. All the *P*-values presented are two-sided. Statistical calculations were performed using SPSS version 17.0 for Windows.

Results

HPV 6/11 and 16/18 DNA Detected by PCR

All specimens of 169 cases were positive for the β-globin gene (110 bp). HPV 6/11 DNA was detected in 54 out of 169 hyperplastic breast tissues (31.95%) by PCR. In the normal breast tissues, only 20 out of 169 specimens showed HPV 6/11 DNA positivity (11.83%). HPV 16/18 DNA was detected in 59 out of 169 hyperplastic breast tissues (34.91%) by PCR. In the normal breast tissues, only 25 out of 169 specimens showed HPV 16/18 DNA positivity (14.79%).

The size of the PCR products was consistent with the expected band size for HPV 6/11 and 16/18 primers (109 bp) (Figure 1). The positive rates of HPV 6/11 and 16/18 DNA detected by PCR between hyperplas-

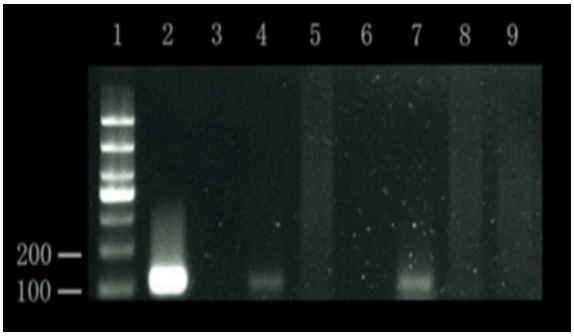

Figure 1. PCR products of representative samples of hyperplastic breast tissues detected with HPV 6/11 and 16/18 primers. 1: DL1000 DNA Marker, 2: positive control, 3: negative control, 4-9: the genomic DNA samples extracted from hyperplastic breast tissues.

Table 1. The positivity of HPV 6/11 and 16/18 DNA between the hyperplastic breast and normal breast tissues.

	HPV 6/11		HPV 16/18	
	PCR	ISH	PCR	ISH
Hyperplastic breast tissue [n (%)]	54 (31.95)	57 (33.73)	59 (34.91)	51 (30.18)
Normal breast tissue [n (%)]	20 (11.83)	11 (6.51)	25 (14.79)	27 (15.98)
χ^2	11.107	17.125	10.344	7.656
p	0.001*	0.000*	0.003*	0.008*

* means significant difference.

Table 2. The results of detection HPV 6/11 in 338 samples by PCR and ISH.

PCR	ISH		Total
	+	-	
+	66	8	74
-	2	262	264
Total	68	270	338
χ^2		17.125	
□		0.000*	

* means significant difference.

Table 3. The results of detection HPV 16/18 in 338 samples by PCR and ISH.

PCR	ISH		Total
	+	-	
+	59	25	84
-	19	235	254
Total	78	260	338
χ^2		7.656	
p		0.008*	

* means significant difference.

tic breast tissue and adjacent normal breast tissue were significantly different (χ^2=11.107, 10.344, respectively; P=0.001, 0.003, respectively) (Table 1).

HPV 6/11 and 16/18 DNA Detected by in situ Hybridization

The results of *in situ* hybridization showed that 57 out of 169 (33.73%) and 51 out of 169 (30.18%) hyperplastic breast tissues were positive for HPV 6/11 and 16/18, respectively, and only 11 out of 169 (6.51%) and 27 out of 169 (15.98%) normal breast tissues were positive, respectively.

The positive rates of HPV 6/11 and 16/18 DNA detected by ISH between hyperplastic breast and normal breast tissues were also significantly different (χ^2=17.125, 7.656, respectively, P=0.000, 0.008, respectively). (Table 1). In addition, substantial agreement was observed between PCR and ISH (kappa=0.781, 0.765, respectively, P=0.000, 0.000, respectively). (Table 2, 3).

HPV 6/11 and 16/18 DNA was detected in the nucleus of cells. Interestingly, HPV 6/11 DNA positive cells were observed in terminal duct epithelial cells (Figure 2A). The positive cells were seen in the lesion of ductal hyperplasic tissues (Figure 2B).

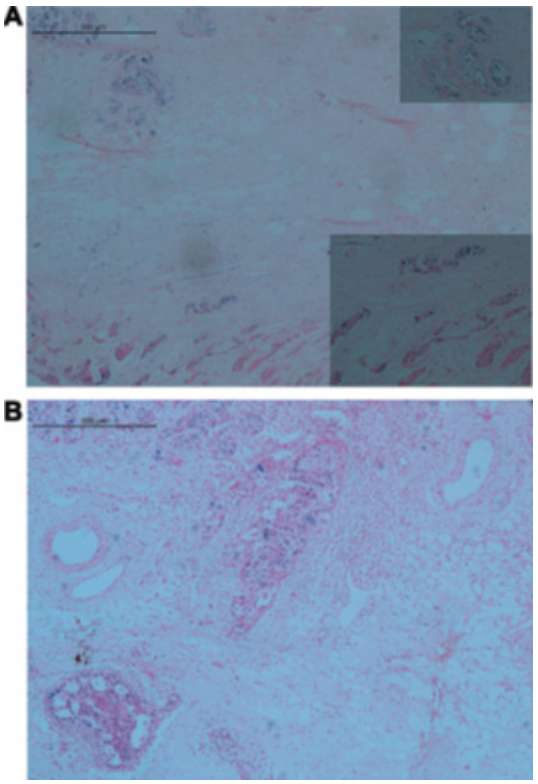

Figure 2. In situ hybridization of hyperplastic breast tissue with HPV 6/11 and 16/18 oligonucleotides probe. The probe was labeled with digoxigenin-11-dUTP, immunostained with anti-digoxigenin AP, and color developed with NBTBCIP substrate.

Discussion

The pathophysiological mechanisms of glandular thickening mammary gland hyperplasia have not been fully elucidated. Most researchers believed that it is related to low level of progesterone and high level of estrogen (2). Huang *et al* (10) compared the plasma estrogen and progesterone concentrations in 1669 patients with benign breast diseases including mastopathy, fibroadenosis, sclerosing adenosis, *etc* and 569 healthy women. The results showed that the plasma estrogen concentration during follicular phase was much higher in the benign breast disease group than that in the control group. This result suggested that patients with benign breast disease have hypothalamic-pituitary-ovary axis dysfunction, resulting in the premature and excess secretion of estrogen. The estrogen has sustained stimulation of target organs, which is likely to be the cause of benign breast disease. However, the results from different researchers on this aspect are not consistent. Brooks *et al* (11) observed that plasma progesterone concentration during luteal phase was lower in the patients with benign breast disease than that in healthy women. In addition, plasma estrogen concentrations were similar between the two groups.

At present, the detection methods of HPV mainly include morphological observation, electron microscopy, immunological hybridization capture (HC2), and viral DNA detection (12). Viral DNA detection includes PCR

and ISH. PCR is a standard test method for HPV, which has the advantage of detecting specific HPV types and detecting viral load using PCR technology. Its sensitivity is high and can detect less than 10 copies of HPV genomic DNA (13). ISH has high sensitivity and specificity, usually used for tissue sectioning or cell smear examination, which can provide not only the precise positioning of the target sequence but also the details of tissue or cell morphology (14). Therefore, application of ISH can not only observe histological changes but also detect the status of HPV expression. PCR was more sensitive than ISH in detecting HPV infections.

In the present study, HPV 6/11 and 16/18 DNA was detected in considerably high number of the hyperplastic breast tissues as compared to the normal breast tissues both by PCR and ISH. Both PCR and ISH demonstrated that the positive rates of HPV 6/11 and 16/18 were significantly higher in the hyperplastic breast tissue than those in the normal breast tissue. Interestingly, substantial agreement was observed between PCR and ISH in the present study.

The HPVs are accepted as carcinogen in human cervical and anogenital cancer. The relationship between HPV infection and breast cancer has also been reported. Although HPV transmission route was not yet determined, some types of HPV were found in both tumors (cervical and breast) (15). The detection of HPV in breast cancer in this study is consistent with majority of the previous publications reporting the presence of HPV in breast cancer world-wide with a prevalence of ranging from 4–86% (16, 17), although a few studies revealed that HPVs could not be detected in breast cancer and normal tissues (18-21). The differences in published reports may be attributed to the numbers of samples tested, methodological differences, and the sensitivity of methods used, such as use of different primer sets.

In conclusion, the present study demonstrated that HPV 6/11 and 16/18 may have a possible causal role in glandular thickening mammary gland hyperplasia pathogenesis. This work provides a solid morphological evidence of the involvement of HPV 6/11 and 16/18 in glandular thickening mammary gland hyperplasia development. On the other hand, more evidence is still needed about the route of HPV infection, the biological behavior of infecting HPV in the duct epithelial cells of breast and the steps of HPV induced glandular thickening mammary gland hyperplasia.

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Conflict of Interest
There are no conflict of interest in this study.

Author's contribution
All work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its content. The study was conceived and designed by Yang Xiaojuan; Deng xiaoqun, Long Xiaoli, Li Jun and Yang Xiaojuan collected and analysed the data; Deng xiaoqun wrote the text and all authors have read and approved the text prior to publication.

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