Genotype Distribution of High Risk Human Papillomavirus in Patients with Breast Cancer

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Abstract
Breast cancer is the most common cancer diagnosed in women, accounting for more than 1 in 10 new cancer each year. It is the second most common cause of death from cancer among women in the world. Viral infections have been accounted for approximately 18–20% of breast cancer. This makes the human papilloma virus (HPV) a strong candidate for initiation and development of breast cancer. High-risk HPV E6 and E7 early oncoproteins were shown to be involved in cell-cycle progression, cellular transformation, and cancer development. In this study, 62 Formalin fixed paraffin-embedded tissue (FFPE) and 40 fresh biopsies were collected from women at different stages of breast cancer. The prevalence of HPV specific DNA in women with breast cancer was detected in 16% of FFPE samples and in 20% of fresh biopsies using nested PCR. When molecular genotyping was carried out using a multiplex PCR, it appeared that HPV-16 was the predominant genotype in 94% of positive cases of breast cancerin both sample types(FFPE and fresh tissues).
Keywords: Breast cancer, HPV, high risk, Iraq

1. Introduction

Breast cancer (BC) is the most commonly diagnosed malignancy in women worldwide as well as the leading cause of cancer-related death in this gender. It was estimated that 2,261,419 new cases of breast cancer and 684,996 deaths worldwide were attributable to this malignant tumor in 2020 (1).There were several risk factors associated with breast cancer such as aging, female gender, smoking, alcohol, obesity, hormonal-replacement therapy and family history. The development of evidence indicates that a handful of oncogenic viruses may have a role in breast cancer. These viruses are mouse mammary tumor virus (MMTV), bovine leukemia virus (BLV), human papillomaviruses (HPVs), and Epstein–Barr virus (2). HPV genotypes have been identified to include 16 HPV high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 68, 73, 82), 12 HPV low risk (6, 11, 34, 40, 42, 43, 44, 61, 70, 72, 81, 89) (3). The prevalence of HPV in BC varied widely from 1.6–86.2% among the different continents of the world. Nine HPV types (HPV- 6, 11, 16, 18, 31, 33, 35, 45 and 52) are evident in BC across different population around the world. The prevalence of these HPV types showed variation among different population. The HPV-16 was prevalent in American BC patients, whereas HPV18 and HPV33 were frequent in Australian and Chinese BC patients (4). HPV E6 and E7 early proteins are oncoproteins that stimulate cell-cycle progression, cellular transformation, and cancerdevelopment. The E6 protein binds to the p53 tumor suppressor protein resulting in accelerated ubiquitin-mediated degradation. E7 protein interacts with the so-called "pRb-associated pocket proteins," including the retinoblastoma protein pRb, which are negative cell-cycle regulators resulting in enhanced phosphorylation anddegradation(5).

2. Materials and Methods

Sample collection

Breast cancer tissues were obtained from a hundred and two patients (62 Formalin fixed paraffin-embedded (FFPE) and 40 fresh tissues) with breast abnormalities and cancer which referred to the Gynecological Oncology Department of Baghdad teaching hospital, In addition 40 healthy women volunteers were used as a control group. The age of patients ranged between 25-75 years old.

Genomic DNA extraction

For the extraction of DNA from FFPE and fresh samples, a commercial kit was used (G-spinTM Total DNA Extraction Kit, iNtRON). Briefly, the first step for DNA extraction from FFPE using Xylene first to get rid of the paraffin, followed by addition of absolute ethanol to wash the sample pellet. Tissue lysis buffer (200µl CL) and 20µl proteinase K (11mg/ml) were added, then incubated at 60°Cfor 1 hour. After that BL (200µl) was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 70°Cfor 15 minutes. While the same steps were used for fresh tissues, except no xylene used. Then for both sample types (FFPE and fresh), absolute ethanol was added for DNA precipitation using spin column and washed (WA buffer), air dried and pellets resuspended in CE buffer.

Detection of HPV Using PCR and Nested PCR Techniques

Initially, detection of HPV was carried out on both sample types by using a conventional PCR and specific primers primers (MY09/MY11) (Forward:

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CGTCCMARRGGAWACTGATC; Reverse: GCMCAGGGWCATAAYAATGG). The amplification conditions used were: denaturation, 95°C for 30 sec; annealing, 55°C for 1 min; and extension, 72°C for 1 min for 35 cycle.

A nested PCR technique and two sets of primers were used to amplify HPV-DNA in both samples types (MY09/MY11) and (GP5+/GP6+). The sequences of primers (GP5+/Gp6+) were (GP5+: TTGTTACTGTGGTAGATACTAC) and (GP6+:AAAAATAAACTGTAAATCATATTC). Amplification conditions used were as follows: Denaturation, 95°C for 30 sec; Annealing, at 54.6°C for 30 sec; and Extension at 72°C for 1 min for 35 cycles. The detail of nested PCR primers for HPV-DNA were done according to (6).

Genotyping of HPV by using real-time

In his study genotyping of HPV-DNA, primers and probes specific for HPV16andHPV18were (forward: GTGGTAGATACACGCAGTAC; reverse:ATATTCCTCCCCATGTCGTAGG and probe:TGTGCTGCCATATCTACTTCAGAACCTand forward: TGTGCTTCTACACAGTCT reverse: CCTCACATGTCTGCTATACTGC and probe: ACCTGGGCAATATGATGCTACCAAATT. The Real-Time PCR primers and probes used for genotyping of HPV-16 and HPV-18 were done according to (7), which were synthesized and supplied by Macrogen (Korea).

3. Results

Detection of HPV DNA in Formalin fixed paraffin-embedded (FFPE) samples

In this study, 62 samples were obtained as a formalin-Fixed Paraffin-Embedded (FFPE)tissues from patients with breast cancer and DNA subjectedto a nested PCR resulted in a successful amplification of ten positive samples out of 62 samples (16%) of total samples. The nested PCR was carried out according to (8).

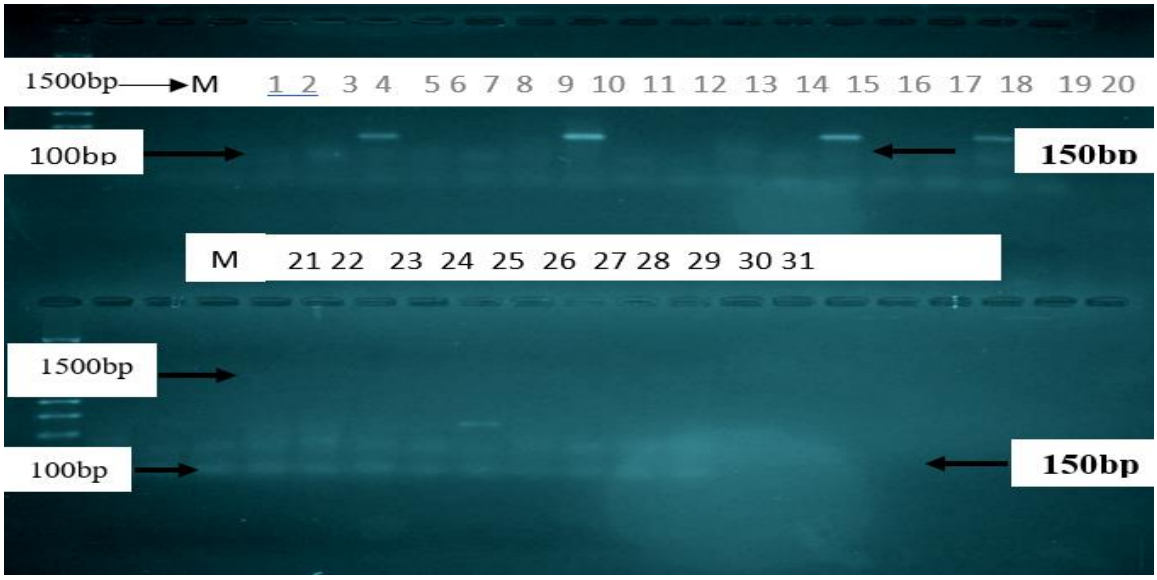

Figure (1). Detection of human papillomavirus (HPV) DNA extracted from formalin fixed paraffin-embedded (FFPE) tissue by using a nested PCR amplification technique. Amplicons run on a 1.5% agarose gel, electrophoresed and other conditions were similar to those mentioned in (Figure 3-1). M: DNA ladder marker (1500-100bp); lanes 1-31, shows amplification results of HPV DNA of representative samples (out of 26 total samples) with a molecular size of 150 bp in length.

Detection of HPV DNA in Fresh breast cancer biopsies

When the some DNA extracted from fresh breast tissues, subjected to the nested PCR, eight samples had positive amplifications of 150bp, specific to the anticipated amplicons of HPV region (Figure 2).

Detection of HPV DNA in Fresh breast cancer biopsies

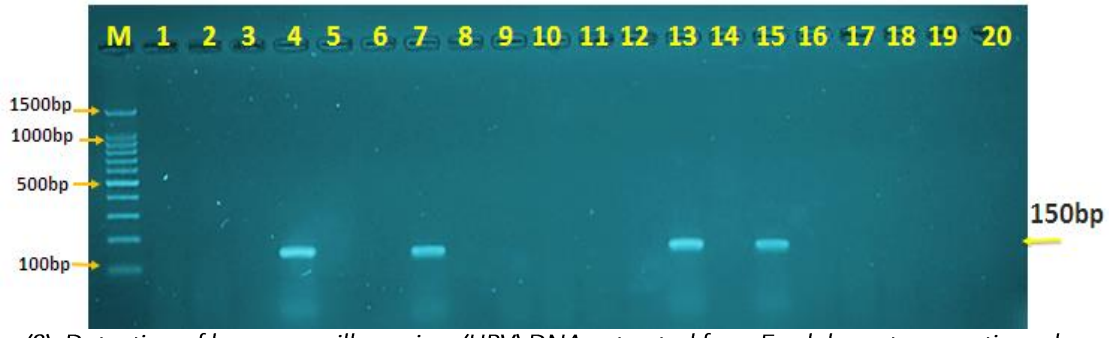

Figure (2). Detection of human papillomavirus (HPV) DNA extracted from Fresh breast cancer tissue by using a nested PCR amplification technique. Amplification and electrophoresis conditions were similar to those described in Figure (1). M: DNA ladder marker (1500-100bp); lanes 1-20, show negative and positive HPV samples. The specific HPV amplicons of representative samples (out of 40 total samples) appeared with size of 150 bp in length.

Genotyping by Real-Time PCR

The eighteen positive samples for HPV DNA were genotyped for the high risk HPV using real-time PCR and primers and probes for HPV-16 and HPV-18. The genotyping result revealed seventeen (94.4%) samples were from a genotype 16 of HPV and one sample was identified as non HPV-16, non HPV-18, Obviously the HPV-16 genotype was the most common in this study (Table 1).

Table (1). The results of genotyping for 18 positive HPV samples by using real-time PCR

HPV-18 genotypes	Percentage (%)	HPV-16 genotypes	No. of positive HPV	No. of samples	Type of sample
----	14.51%	9	10	62	Formalin fixed paraffin embedded (FFPE)
----	20%	8	8	40	Fresh tissue
---	---	94.4%	17.64%	----	Percentage (%)

4. Discussion

To the best of our knowledge,this is the first study of applying newly developed nested PCR for detecting on the presence of high risk Human papillomavirus (HPV) in women with breast cancer in two types of total 102 samples (Formalin fixed paraffin-embedded (FFPE) tissue and fresh biopsies) and assessing the role of HPV E6 and E7 oncogenes in breast cancer progression in Iraqi women. Results of nested PCR showed 16% and 20% of FFPE tissue and fresh biopsies, respectively, cancers were infected of HPV. This revealed a relatively high prevalence of high-risk HPV-DNA in breast cancer samples. Several studies reported that the presence of HPVs in breast cancer women around the world including Qatar where HPV present in only 10% among women with breast cancer (9), but this ratio increased to 65% in 2021 (10). High ratio appeared in in Vanzuela the ratio was 41.67% (11). In Lebanese women, it was 65% (12) and in Iran it was 48.6% (13). This study involved developing a nested PCR technique for the detection of HPV-DNA in women with breast cancer in Iraq, for the first time.The key findings in this study showed the predominance of HPV-16 genotype in most breast cancer samples (94%). In Congo,the HPV-16 was found to be as high as 83.7%(14), While in Italy, it appeared in 29.4% of breast cancer samples (15) and in Rwandese patients, HPV16 prevaled as the main subtype in 77.27% of patients (16). In Iraq, HPV-16 was the dominant (94.6%) high risk genotype in malignant and benign tumors (17). In Spain, HPV-16 prevalence was (13.7%) (18).The transformation of HPV infected cells depends on expression of E6/E7 oncoproteins which contributes to the process of carcinogenesis by increasing cellular proliferation leading to more genomic instability and inhibition of apoptosis (19). The expression of both oncoproteins is controlled by viral protein E2, which often gets abrogated due to viral integration in the host genome through the E2 region (20). It has been previously demonstrated that E6/E7 of HPV converts non-invasive and non-metastatic breast cancer cells into invasive and metastatic ones(10). HPV oncogenes in cervical cancer appeared to be a mechanism could alsobe involved in breast cancer. HPV infection can upregulate and lead to mutations in APOBEC3B, which in tumors may increase the risk of breast cancer. APOBEC3B assists to protect against the harmful effect of viruses (21,22,23). After viral DNA integration, mutations in APOBEC3B can lead to host genome instability and then to breast cancer progression (22). HPV infections can adversely influence additional members of the APOBEC family (24,25). Moreover, HPV can interact with BRCA (BReast CAncer) genes and leading to mutations which may cause breast cancer (26).In breast and other tissues, the BRCA1 and BRCA2 are expressed and involved in repairing process of damaged DNA which can lead to cancer (27). It has been shown that E6 and E7 proteins are able to interact with the BRCA1 (as the antagonists) and alter its activity. Moreover, BRCA1 interacts with RB and p53. This interaction is required for the RB functioning in G1 checkpoint of cell cycle. Also, BRCA1 acts as a co-activator of p53-mediated transcription (26). Thus, HPV proteins (E6 and E7), may influence RB and p53 functions with indirect interference through BRCA1 pathway(13).

5. Conclusion

HPV-DNA was detected in (16-20%) of FFPEand fresh biopsies taken from women with breast cancer and the HPV-16 was the most common HPV genotype in 94.4% of HPV positive samples.

Authors' contribution

Laboratory work, data collection, analysis, and writing were performedby Zahraa A. Mohammed. Study design and supervision, reviewing andediting of manuscript were done by Hassan M Naif.

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Financial and competing interest disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

The study was ethically approved by the Ministry of Health of Iraq(decree order 1650 dated 2/11/2020)

and by the Scientific Committeeof the College of Biotechnology, Al-Nahrain University, Baghdad, Iraq..

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