Detection of high and low-risk HPV DNA in archived breast carcinoma tissues from Ethiopian women

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Abstract

Background: HPV have been implicated in the development of cancer of the cervix, mouth and throat, anus, penis, vulva, or vagina, but it has not been much considered as a cause of breast cancer. However, a growing number of investigations have linked breast cancer to viral infections. High-risk HPV types, predominantly (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, and -59) are established as carcinogens in humans, while HPV-68 is probably carcinogenic. In this study we aimed to detect 19 high risk and 9 low risk HPVs from archived breast tumor tissue among Ethiopian women.

Methods: In this study, 75 breast cancer patients from Tikur Anbassa Specialized Hospital in Addis Ababa (Ethiopia) were included. HPV detection and genotyping were done using the novel Anyplex™ II HPV-28 Detection Assay at the Orebro University Hospital, Sweden. The Anyplex^{TM}II PCR System detects 19 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types(6, 11, 40, 42, 43, 44, 54, 61, 70). IHC for p16 was done in automated system using the Dako Autostainer Link.

Results: Out of the 75 valid tests 2 were found to be positive (2.7%) for HPV. One of the cases were positive for high risk HPV16 genotype while the other were positive both for high risk HPV39 and low risk HPV6. The cell cycle protein p16 was highly expressed in the case positive for the high risk HPV16 but it was not expressed in the case positive for HPV39.

Conclusion: With limited number of cases positive for HPV in this study, it is our conclusion that cervical cancer prevention strategies may help protection of breast cancer only in small groups of patients. Due to limitation of the number of participants in the study as well as possible other mechanisms of carcinogenesis, our observation should be reconfirmed using a larger set of patients and in case-control design.

Background

Human Papilloma Virus (HPV) have been implicated in the development of cancer of the cervix, mouth and throat, anus, penis, vulva, or vagina, but it has not been much considered as a cause of breast cancer. However, a growing number of investigations have linked breast cancer to viral infections, including Human Papillomavirus (HPV), Epstein–Barr virus (EBV), Mouse Mammary Tumor Virus (MMTV), and Human Cytomegalovirus (HCMV)(1).

Human papilloma viruses (HPVs) are non-enveloped DNA virus belonging to the Papillomaviridae family (2, 3). Over 170 types of HPV have been identified (3), the majority of which affect the genital tract epithelia, the mucosa of the upper respiratory tract and the skin (2, 3). HPVs are categorized as high risk or low risk, depending on their carcinogenic potential. High-risk HPV types causes cancer, however Low-risk types are not carcinogenic but cause benign anogenital warts and recurrent respiratory papillomatosis(3, 4).

High-risk HPV types, predominantly (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, and – 59) are established as carcinogens in humans, while HPV-68 is probably carcinogenic (5). HPV types 16 and 18 are the most common high-risk types and are responsible for > 70% of all cervical cancer cases (5). HPV are characterized by the presence of three functional code regions in their genome: The E region that codes the early viral function, the L region which is responsible for the late viral function and the long control region (LCR)(2). Even though HPVs are known to be responsible for the development of cervical cancers, HPV infections are often asymptomatic, and most sexually active individuals become infected with HPV at least once in their lifetime (3). A recent systematic review and metanalysis by Ren et al examined 37 Case–control studies containing 3,607 Breast cancer cases and 1,728 controls in a wide range of countries that compare the prevalence of high-risk HPVs in breast cancer as compared to benign breast or normal breast (6). In their study Ren et al showed an increase in breast risk with human papillomavirus (HPV) positive [summary odds ratio (SOR) = 6.22, 95% confidence interval 4.25 to 9.12; P = 0.0002]. Ren et al. also showed three high risk HPV types (HPV16, 18 and 33) to be positively correlated to breast cancer(6). Similarly, a metanalysis conducted by Lawson et al in 2015 showed prevalence of HPV is fourfold higher in breast cancer (21.5%) than controls (5.1%)(7). In their study Lawson et al has shown that HPVs are detected from 2 to 74% of all case (7).

In another study, it has been shown that women with HPV- associated cervical pathology are at increased risk from the same HPV type positive breast cancer which implies a possible link between HPV and breast carcinogenesis(8). There is only one published study so far done to assess the prevalence of HPV in the African continent. This study from Rwanda (9)was conducted in 47 archived formalin-fixed paraffin-embedded tissues to detect and genotype HPV DNA. They reported prevalence of HPV at 46.81% of cases. The most common genotype in this study were HPV16 (77%) followed by HPV33 (14%) and HPV31 (9%)(9).

According to the 2019 IARC report, about 6,294 new cervical cancer cases are diagnosed annually in Ethiopia and HPV is the cause of almost all cases (10). The IARC report also indicates cervical cancer as the 2nd leading cause of female cancer following breast cancer in Ethiopia (10). A study done in 2014 found the most common genotype among cervical cancer patients in Ethiopia was HPV 16, followed by HPV 52, HPV 56 and HPV 31 (11). Another study done in 2013 also found HPV 16 as the most common genotype followed by HPV 52, 58, and 18 (12). There is no published study so far done to assess the role of HPV among breast cancer cases in Ethiopia. Therefore, in this study we aimed to detect 19 high risk and 9 low risk HPVs from archived breast tumor tissue to look at the prevalence among Ethiopian women.

Methods

Patient and Sample Characteristics

These cohorts were previously investigated by our groups in our published work to characterize the molecular distribution and clinicopathological features of breast cancer among Ethiopian women and in

an Androgen receptor study (13, 22). The patient and sample characteristics are summarized as follows: The study initially recruited 114 cases with a pathology confirmed invasive breast carcinoma who visited Tikur Anbessa Specialized Hospital (TASH) in Addis Ababa (Ethiopia) for treatment. After obtaining a written informed consent, participants age, tumor grade, stage of disease, and type of pathology were collected from their medical records. Formalin-fixed paraffin embedded (FFPE) tissue samples were obtained from TASH and St Paul's Hospital Millennium Medical College (SPHMMC) pathology laboratories because some of the biopsies were tested at this hospital. All FFPE blocks were sectioned and H&E stained and evaluated at the Orebro University Hospital, Sweden. TMA were constructed for immunohistochemistry evaluation and tumor cores for molecular testing were taken from the primary donor blocks and transferred to clean eppendorf microcentrifuge tubes.

Tissue core samples for PCR

TMA grand master automated system (3DHISTECH Ltd., Budapest, Hungary) was used to sample cores for PCR analysis. A pathologist marked representative parts of the individual invasive tumor with percentage of tumor cells for sampling of tissue for PCR. A 0.6-millimeter punch biopsies corresponding to the marked area were taken from paraffin blocks. For each patient two biopsy cores were taken from the same tumor.

DNA Extraction

DNA was isolated from tissue core samples of each tumor specimens. The presence of malignant cells was assessed in all samples by evaluation of slides stained with Hematoxylin and eosin (H&E). Genomic DNA (gDNA) was isolated from the tumor cores using the QIAamp DNA Mini Kit (Qiagen), including proteinase K treatment (Qiagen) followed by purification using the QIAcube automated system (Qiagen). The DNA concentration was measured using a Nanodrop ND-1000 (Nanodrop Technologies). The gDNA samples were stored at 4 °C.

Multiplex real time PCR

The multiplex quantitave real time PCR was done at the Orebro University Hospital. Real-time PCR amplification was performed using the Anyplex™ II HPV-28 Detection Assay (Seegene, Seoul, Korea), in accordance with the manufacturer's protocol, in a CFX96 real- time thermocycler (Bio-Rad, Hercules, CA, USA). The Anyplex™ II HPV-28 Detection is a novel multiplex real-time PCR assay that permits the simultaneous amplification, detection and differentiation of target nucleic acids of 28 HPV types and Internal Control (IC). The Anyplex^{TM}II PCR System detects 19 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types(6, 11, 40, 42, 43, 44, 54, 61, 70).

Immunohistochemistry for p16

Immunohistochemistry (IHC) for p16 was done in automated system using the Dako Autostainer Link. Formalin fixed, paraffin sections were cut at 4 microns and rehydrated to water. Heat induced epitope retrieval was performed with FLEX TRS High pH Retrieval buffer for 20 minutes. After peroxidase

blocking, the specific monoclonal antibody (Source and dilution: p16 clone G175-405 from BD, USA and diluted 1/25) were applied at room temperature for 20 minutes. The FLEX + Rabbit EnVision System was used for detection. DAB chromogen was then applied for 10 minutes. Slides were counterstained with Mayers hematoxylin for 5 seconds and then dehydrated and coverslipped. Slides were scanned on a Pannoramic 250 digital scanner (3D HISTECH Ltd., Budapest, Hungary) and images scored using the software program ‘Case viewer’ (3D HISTECH Ltd., Budapest, Hungary). Negative controls were included in the run.

Statistical Analysis

Statistical analysis was done using SPSS for windows version 21. Continuous data are reported as mean ± SD or Number (proportions). Skew distributions are reported as the median value with minimum and maximum.

Ethics

The research proposal was reviewed and approved by the Department of Biochemistry and Institutional Review Board (IRB) of Faculty of Medicine, Addis Ababa University. It is also approved by the National Research Ethics Review Committee at the ministry of Science and Technology.

Results

Patients

There were 114 participants with FFPE available for this study. Only 75 cases had valid result in the genotyping experiment for HPV. Mean age at diagnosis of the 75 cases was 42 years (SD12) and median age was 40 (range 22–75). Most of the participants (40%) were < 40 years old. About 31% of the participants were ≥ 50 years and 28% were 40–49 years old.

Histopathological characteristics

Of the 75 tumors, 7% were grade 1, 29% grade 2 and 33% grade 3. The stages were as follows: Stage 1: 20%; Stage 2: 31%; Stage 3: 35% and Stage 4:1%. The most common type of histology which is presented in “Table 1” was infiltrating ductal (60%) and the lobular type was only 5%.

Table 1
Baseline Pathological Characteristics of the study participants (n = 75)

Variables	N(%)
Histological Grade	
I	5(7)
II	22(29)
III	25(33)
Missing	23(31)
Histological Type	
Infiltrating Ductal	45(60)
Lobular	4(5)
Others/Not classified	17(23)
Missing	9(12)
Stage	
I	15(20)
II	23(31)
III	26(35)
IV	1(1)
Missing	10(13)

HPV Detection

Of the 114 tumors, only 75 had detectable internal control therefore the rest were rejected. Out of the 75 valid tests 2 were found to be positive (2.7%). One of the cases were positive for high risk HPV16 genotype while the other were positive both for high risk HPV39 and low risk HPV6.

p16 Immunostaining

Strong expression p16 was detected in the case positive for HPV 16 but not detected in the case dual positive for HPV39 and HPV6. The expression of p16 across our cohorts is summarized in “Table 2”. Immunohistochemical staining of p16 for the two HPV positive cases is presented in “figure 1”.

Table 2
p16 protein expression among the study participants.

p16 expression	Number of cases	Percentage (%)
Neg	54	72
Pos (Weak/Moderate)	9	12
Pos (Strong)	12	15
Total	75	100

Discussion

In this study, we explored the presence of 19 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types(6, 11, 40, 42, 43, 44, 54, 61, 70). The Anyplex Multiplex qPCR technology for detection and genotyping of HPVs were utilized. We detected the high-risk HPV DNA 2/84 (2.4%) of the study participants from Ethiopian breast cancer patients. Researchers from different countries reported an overall prevalence of 2–74% HPV in breast cancer as reported by Lawson et al(14). Our observation is consistent with two different studies from China by Peng et al. (2%) and Li et al. (2%)(15, 16). The variation in prevalence of HPV in breast cancer in different countries may relate to the existence of geographic differences in HPV infection(14, 17). Lawson et al. showed that countries with low rates of HPV associated cervical cancer appear to have high rates of breast cancer and vice versa. Hence, they argue HPV may not play a major role in breast carcinogenesis however suggest the possibility that HPVs may be involved in some but not all breast cancers(14). Similarly, Lawson et al refer the work done by Grulich and Vajdic(18) which showed that the prevalence of breast cancer is not increased in immunocompromised patients (patients with HIV infections or organ transplant recipients) as supportive evidence for indirect and minor role of HPV in breast cancer(14, 18). The prevalence of HPV in our study (2.7%) is significantly different than the Rwandan study (47%) and this may be due to true epidemiological difference in the distribution of HPV in the two countries or it may be attributed to variation in methodologies applied to each of the studies(9).

Various types of HPV have been identified from different countries with HPV-16 reported as the most common HPV followed by the HPV 18 and 33(6, 14). In our study, the HPV type detected in breast cancer samples positive for HPV DNA was high risk HPV 16 in 1 case (1.2%) and high-risk HPV 39 in another case (1.2%). One of the subject positive for high risk HPV 39 were also positive for low risk HPV type 6 though the low-risk HPV6 have not been implicated in cancers but is major cause of genital warts(2, 19).

Additionally, the High risk HPV39 which is detected in our study was not found in any of the 24 studies reviewed by Lawson et al. However, it was the major high-risk HPV detected in the recent UK study by Salman et al. (14, 20).

The p16 (cell cycle protein) expression is detected both in HPV positive and negative cases in our cohorts. There is evidence that indicates strong-expression as opposed to low or medium expression of p16 is

associated with HPV biological activity and high expression of p16 can be used as a surrogate for indication of transcriptional activity of HPV(21). In our study, the tumor positive for the high risk HPV16 was strongly positive for p16 and this may support previous observations made by other researchers that HPV16 may play an active role in the carcinogenesis of a small proportion of breast cancers (8, 14, 15).

Conclusion

Our study found low prevalence of high-risk HPV in breast carcinoma. These adds to other findings which detected high-risk HPV in breast carcinoma and strengthen the possible association of high-risk HPV viruses with breast cancer in small proportion of patients. Due to limitation of the number of participants in the study as well as possible other mechanisms of oncogenesis as suggested by other researchers, our observation should be reconfirmed using a larger set of patients and in case-control design. Therefore, it is our conclusion that cervical cancer prevention strategies may could help protection of breast cancer in small proportion of patients.

Abbreviations

AHRI	Armauer Nansen Research Institute
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr Virus
HCMV	Human Cytomegalo Virus
HPV	Human Papilloma Virus
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR
SOR	Summary Odds Ratio
SPHMMC	St. Paul’s Hospital Millennium Medical College
TASH	Tikur Anbessa Specialized Hospital
TOCE	Tagging Oligonucleotide Cleavage and Extension

Declarations

Ethics approval and consent to participate

The study was first approved by the ethical review committee of the department of Biochemistry and then approved by Institutional Review Board (IRB) of College of Health Science, Addis Ababa University. Ethical approval was also obtained from St. Paul's Hospital Millennium Medical College to collect archived FFPE tissue samples from enrolled patients. The study is also approved by the National Research Ethics Review Committee at the Ethiopian ministry of Science and Technology. Written and informed consent was obtained from every patient.

Consent to publish

Consent to publish is granted by the ethical review committees mentioned under the "Ethics approval and consent to participate" part. All authors read the manuscript and agreed to publish.

Availability of data and materials

All materials used in the study are available and can be provided as necessary.

Competing interests

The authors declare that they have no competing interests.

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Authors Contribution

EH participated in the study design, data collection and coordinated data management, Laboratory work and statistical analysis. EH also wrote the initial manuscript. DS participated in study design, data collection and data management. MK and CK participated in study design, Laboratory work and data management. GLL participated in Laboratory work. The rest of investigators participated in data analysis and manuscript preparation. DS contributed as senior supervisor of the research work in the Ethiopian side. MK and CK contributed in supervising the work as a senior scientist in the Sweden side. All authors have read and approved the manuscript.

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Figures

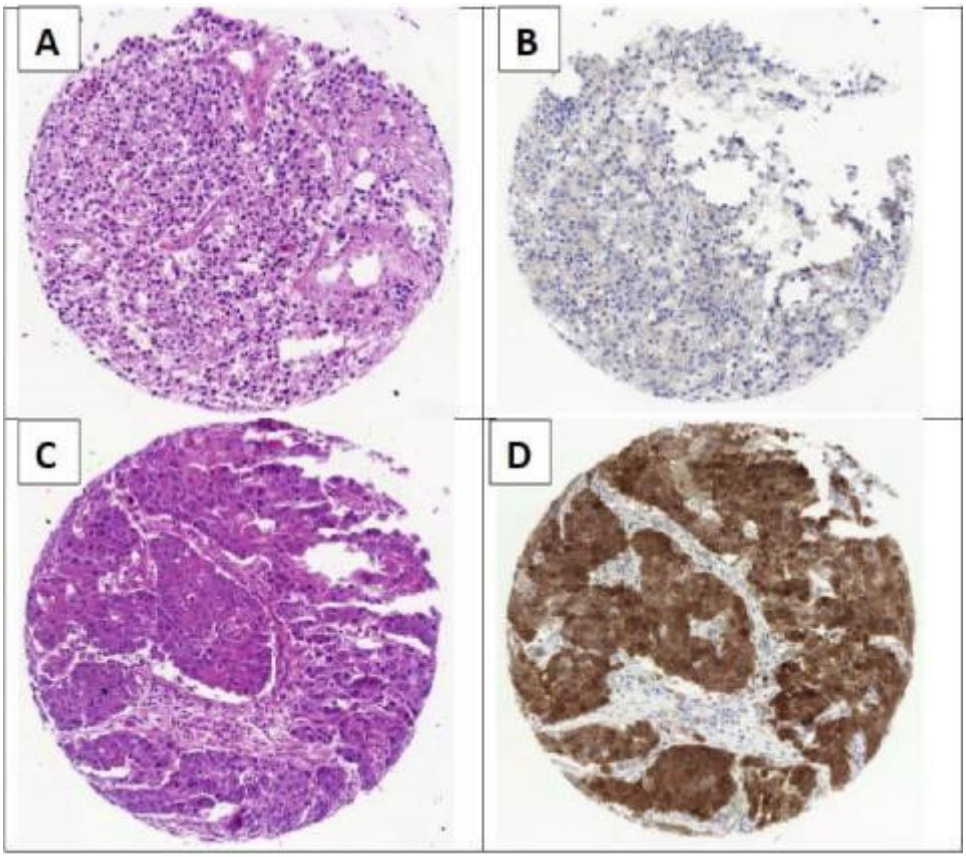

Figure 1

p16 Immunostaining of HPV+ breast tumors. A, H&E staining of the HPV39 & HPV6 positive case. B, Negative p16 immunostaining of the HPV39 & HPV6 dual positive case. C, H&E staining of the HPV16 positive case. D, Strong; diffuse nuclear and cytoplasmic p16 immunostaining of the HPV16 positive case.