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Prevalence of mucosal and cutaneous human papillomavirus in Moroccan breast cancer

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ABSTRACT

Background: Due to recent technical improvements and some encouraging new results, there has been a resurgence of interest in the possibility that a substantial proportion of breast cancers (BCs) may be caused by viral infections, including Human papillomavirus (HPV). The aim of this study was to determine the prevalence of mucosal and cutaneous HPV in tumours from Moroccan BC patients.

Materials and methods: Frozen tumours from 76 BC cases and 12 controls were evaluated for the presence of 62 HPV-types using highly sensitive assays that combine multiplex polymerase chain reaction and bead-based Luminex technology.

Results: HPV DNA was found in 25.0% of BC tumours and only 8.3% of controls. Beta and gamma HPV types were found in 10.5% and 6.6% of BC tumours, respectively. High-risk mucosal types HPV16 and 18 were not detected in the subjects, but other probable/possible high-risk or high-risk -HPV types (HPV51, 52, 58, 59, and 66) were found in 5.3% of BC tumours. Statistical analysis showed no significant difference between, controls, BC cases and the inflammatory status ($p > 0.05$).

Conclusion: HPV DNA was found 3 times as frequently in the BC tumours as in the controls. However, this difference requires confirmation in a larger sample.

1. Background

Breast cancer (BC) is the most common cancer and the leading cause of cancer death among women worldwide. Nearly 1.67 million women are diagnosed with BC each year, and this incidence is increasing, notably in developing countries. The International Agency for Research on Cancer (IARC) estimates that in 2012, 62% of all BC deaths occurred in low- and middle-income countries [1]. In Morocco, BC is the most common cancer in women and the leading cause of female mortality from cancer. Each year, 6650 Moroccan women develop BC and 2880 die of it, with an age-standardized incidence of 36.5 cases per 100,000 population [1].

IARC also reports that about 16% of all cancers worldwide are attributable to infectious agents, with the proportion reaching 33% in Africa [2]; such agents are a plausible risk factor for BC. Since the 1944 discovery that a virus causes mammary cancer in mice, scientists have searched for similar evidence in human BC [3]. However, decades of studies provided only inconclusive results, generating considerable controversy [4]. Due to recent technical improvements and some encouraging new results, there has been a resurgence of interest in the possibility that a substantial proportion of human BCs may be caused by viral infections [5]. Most studies conducted to date have focused on

Abbreviations: BC, Breast cancer; HPV, Human papillomavirus; HR, High-risk;; IARC, International Agency for Research on Cancer; IBC, Inflammatory breast cancer; MFI, Median Fluorescence Intensity;; pHR, Probable/possible high-risk; TS-MPG, Type-specific polymerase chain reaction bead-based multiplex genotyping

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three viruses: mouse mammary tumour virus-like sequences [6,7], Epstein–Barr virus [8–10], and human papillomavirus (HPV) [11–13]. These studies have provided substantial, but not conclusive, evidence of a viral role in breast carcinogenesis. A recent systematic review focusing on these three viruses concluded that the evidence available to date remains preliminary, and advocated for key improvements in methodological approaches, notably the use of an appropriate epidemiological design to determine whether the presence of viruses is significantly associated with BC (comparison of cases to free-of-BC controls, or comparison across well-defined subgroups of cases) [14]. The present study builds on this last recommendation and explores whether the prevalence of HPV infection varies across BC cases and controls in an African population. Furthermore, it explores in a preliminary manner the prevalence of HPV infection in inflammatory BC (IBC), a well-defined subgroup of BC and one of the most aggressive forms of BC, which has a relatively higher incidence in North Africa than in developed countries [15,16]. Moreover, risk factors for IBC seem to be different from those for BC in general (i.e. non IBC) [17].

In the present study, 76 frozen BC tumours and 12 controls were screened for the presence of 62 HPV types belonging to three genera (alpha, beta, and gamma), using highly sensitive HPV genotyping assays [18]. We also compared the prevalence of HPV infection in IBC (n = 13) and non-IBC (n = 63) tumours. Our aim was to better understand the association between HPV infection and BC development as part of the international effort investigating the viral aetiology of BC.

2. Materials and methods

2.1. Samples

Subjects for inclusion in this study were randomly selected from women who had undergone surgical excision for diagnosis or treatment of their breast lumps between 2010 and 2012 at Ibn Rochd hospital (Casablanca, Morocco). The use of this tumour collection for research purposes was approved by the ethics committee of the Pasteur Institute of Morocco.

A total of 103 women have been approached to participate to this study, 88 (85%) of them consented to give their tissues samples. Seventy six of these subjects had BC, while 12 had no BC. Both groups had similar risk factors related to BC, as well as similar demographics, including, age, sex and location.

Detailed clinical and reproductive histories were obtained from medical records for all subjects. Subjects were informed about the study procedures and provided informed consent. Illiterate participants were read the informed consent forms in a presence of a third person, and provided a mark the way they used to for official documents in lieu of signature. The ethics committee of the Pasteur Institute of Morocco approved this consent procedure. Patients who did not give their consent were not included in the study and not registered. The breast tissues were collected directly from the surgical unit and stored in liquid nitrogen until further processing. To avoid the risk of potential contamination, fresh tissues were carefully collected by surgeons according to a standardized protocol, using disposable instruments. Based on the pathological examination, subjects were classified as cases if invasive BC or carcinoma in situ was present (n = 76) or as controls for fibroadenoma (n = 12). Among the BC cases, 13 were diagnosed as IBC, based on clinical history and/or pathological features.

2.2. DNA extraction

DNA extraction was performed using the Qiagen BioRobot EZ1 with the EZ1 DNA tissue kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, frozen tissues were incubated in proteinase K and a buffer G2 (Qiagen, Hilden, Germany) at 56 °C until the tissue was completely lysed. The DNA was stored at − 20 °C until further use.

2.3. Detection of human papillomavirus DNA

The presence of HPV DNA was detected using highly sensitive type-specific polymerase chain reaction (PCR) bead-based multiplex genotyping (TS-MPG) assays that combine multiplex PCR and bead-based Luminex technology (Luminex Corp., Austin, TX, USA), as described previously [18–23]. The multiplex type-specific PCR method uses specific primers for the detection of 19 probable/possible high-risk (pHR) or high-risk (HR) alpha -HPV types (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a and b, 70, 73, and 82), 2 low-risk alpha HPV types (HPV6 and 11), 25 beta HPV types (HPV5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, and 96), and 16 gamma HPV types (HPV4, 65, 95, 60, 48, 50, 88, 101, 103, 108, 109, 112, 116, 119, 121, and 123). Two primers for the amplification of beta globin were also added to provide a positive control for the quality of the template DNA [24]. The cutoff was calculated as previously reported by Schmitt et al. [18]. Following PCR amplification, 10 µl of each reaction mixture was analysed by multiplex human papillomavirus genotyping using the Luminex technology. For each probe, the Median Fluorescence Intensity (MFI) values obtained when no PCR product was added to the hybridization mixture were considered as background values. The cutoff was computed by adding 5 MFI to 1.1 × the median background value as described by Schmitt et al. [18].

Many negative controls were systematically included in all our assays; i.e. (i) several empty tubes were blindly processed together with the other human specimens for the extraction of DNA and the Luminex assay; (ii) no-template PCR controls with water instead of a template; (iii) Luminex beads were hybridized with water in order to estimate the individual bead background. We have preferred to not use viral DNA as positive control, in order to avoid possible source of contaminations during the analysis.

2.4. Statistical analyses

Statistical analyses were performed using the Fisher exact chi-square test (using the online tool at http://www.langsrud.com/fisher.htm), which facilitates comparison between very small numbers.

3. Results

We tested for the presence of 62 mucosal and cutaneous HPV types belonging to three genera (alpha, beta, and gamma) in tumours from 88 subjects: 76 BC tumours (subsequently referred to as cases) and 12 fibroadenomas (subsequently referred to as controls). Of the 76 BC cases, 13 were IBC. The mean ages of patients in the case and control groups were 46.9 years and 38.9 years, respectively. Invasive ductal carcinoma was the most common tumour type (accounting for 84.2% of all cases), followed by the combination of invasive ductal carcinoma + ductal carcinoma in situ and phyllodes tumour, which each accounted for 3.9% of the cases (Table 1).

The presence of amplifiable DNA was confirmed for all 88 specimens by PCR based-technique using primers for a fragment of *β-globin* gene and therefore all DNA samples were adequate for viral DNA analysis. HPV DNA was found in 25.0% (19/76) of the BC cases and 8.3% (1/12) of the controls, although this difference was not statistically significant ($p = 0.28$). Beta and gamma HPV types were found in 10.5% (n = 8) and 6.6% (n = 5), respectively, of the cases, and low-risk and pHR/HR alpha HPV types were found in 7.9% (n = 6) and 5.3% (n = 4), respectively, of the cases (Table 2). Table 3 further details the prevalence of various HPV types in the BC cases. High-risk alpha types HPV16 and 18 were not detected in the subjects, but other HR or pHR-HPV types (HPV51, 52, 58, 59, and 66) were found. Low-risk alpha types HPV6 and 11 were detected in 2.6% and 5.3%, respectively, of the cases. Of the beta HPV types, HPV47 was the most prevalent (n = 3; 3.9%), followed by HPV5 and 8 (n = 2; 2.6% each). The most prevalent

Table 1
Characteristics of the study population.

Characteristic	Controls (n = 12)	Breast cancer cases (n = 76)
Mean age, years	38.9	46.9
Histology, n (%)		
Fibroadenoma	12 (100)	–
Breast cancer		
IDC	–	64 (84.2)
IDC + DC	–	3 (3.9)
PT	–	3 (3.9)
DC	–	2 (2.6)
ILC	–	2 (2.6)
IDC + PG	–	1 (1.3)
IDC + ILC	–	1 (1.3)
Biopsy location, n (%)		
Right breast	5 (41.6)	40 (52.6)
Left breast	7 (58.3)	43 (47.4)

DC, ductal carcinoma in situ; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; PG, Paget disease; PT, phyllodes tumour.

gamma HPV type was HPV50 (3.9%), followed by HPV4 (2.6%) and HPV121 (1.3%). Overall, the most prevalent HPV type was low-risk mucosal HPV11 (5.3%), followed by HPV47 and 50 (3.9% each). Moreover, as shown in Table 2, the frequency of HPV co-infection with multiple HPV genotypes in cancerous breast tissue samples was 6.5% (5/76), including multiple HPV infections with beta HPV types reported in 4 cases, gamma HPV types in 1 case and alpha HPV types in 1 case. Of particular interest, all HPV DNA were detected in non-IBC cases (Table 2).

All HPV-positive tumours were found to be invasive ductal carcinoma in situ; all other types of tumours (accounting for 9% of the cases) were HPV-negative (data not shown).

4. Discussion

To the best of our knowledge, this is the first study of the prevalence of HPV infection in BC tumours from Moroccan women. In this study, a highly sensitive bead-based HPV typing method, TS-MPG was used, for the detection of a large panel of HPV types of the genera alpha, beta, and gamma [18,21,25]. Although our study did not reveal statistically significant differences in the prevalence of HPV infection between BC tumours and controls, the fact that 25.0% of the BC tumours were found to be HPV-positive, compared with only 8% of the controls, requires further exploration in a larger sample.

Our study has several strengths compared with previous studies. These include the very large number of HPV types tested, the high sensitivity of the laboratory assays and the use of rapidly frozen tumour samples, which ensures that viral DNA is perfectly preserved. In this preliminary study we aimed at evaluating the pertinence of further exploration of the prevalence of HPV infection in BC tumours from Africa, and therefore the sample collection and assays were conducted in a very rigorous way.

The association of HPV infection with cervical cancer and head and neck cancers is well established, but its involvement in BC remains controversial. Studies conducted to date have reported various prevalence rates of HPV infection in human BC tumours, ranging from 0% to 86% [12,14,26–30]. These differences may be due to various factors

Table 3
Prevalence of single and multiple HPV infections in cancerous and normal breast tissue samples.

	Single HPV Infection		Multiple HPVs Co-Infection	
	HPV type	N (%)	HPV types	N (%)
Controls	HPV5	1 (8.3)	–	–
	Total	1 (8.3)	–	–
Cases	HPV5	1 (1.31)	HPV5 + 47	1 (1.31)
	HPV24	1 (1.31)	HPV8 + 47 + 50	1 (1.31)
	HPV38	1 (1.31)	HPV8 + 47	1 (1.31)
	HPV4	2 (2.63)	HPV12 + 15 + 6	1 (1.31)
	HPV6	1 (1.31)	HPV22 + 11	1 (1.31)
	HPV50	1 (1.31)	HPV50 + 121	1 (1.31)
	HPV11	2 (2.63)	HPV11 + 59	1 (1.31)
	HPV66	1 (1.31)	HPV52 + 58	1 (1.31)
	HPV51	1 (1.31)	–	–
	Total	**12 (18.4)**	**Total**	**8 (10.5)**

Table 2
Prevalence of human papillomavirus (HPV) DNA in inflammatory and non-inflammatory breast cancer tumours (IBC and non-IBC) and controls.

HPV type	Controls*	Breast cancer cases						
	n (%)	All cases (N = 76) n (%)	P Value	CI, 95%	IBC (n = 13) n (%)	Non-IBC (n = 63) n (%)	P Value	CI, 95%
Any HPV type								
Negative	11 (91.7)	57 (75.0)			13 (100)	44 (69.8)		
Positive	1 (8.3)	19 (25.0)	0.28	[0.87, 1.21]	0	19 (30.2)	1	[0.47, 1.16]
Multiple infections**	0	5 (6.5)	1	[0, 3.72]	0	5 (6.5)	0.58	[0.55, 1.22]
Alpha HPV								
Low-risk								
Negative	12 (100)	70 (92.1)			13 (100)	57 (90.5)		
Positive	0	6 (7.9)	0.591	[0.62, 1.17]	0	6 (9.5)	0.58	[0.64, 1.22]
Multiple infections	0	0	und	und	0	0	und	und
pHR/HR								
Negative	12 (100)	72 (94.7)			13 (100)	59 (93.7)		
Positive	0	4 (5.3)	1	[0.47, 1.16]	0	4 (6.3)	1	[0.48, 1.22]
Multiple infections	0	1 (1.3)	1	[0.47, 1.16]	0	1 (1.3)	1	[0.06, 1.21]
Beta HPV								
Negative	11 (91.7)	68 (89.5)			13 (100)	55 (87.3)		
Positive	1 (8.3)	8 (10.5)	1	[0.47, 1.16]	0	8 (12.7)	0.33	[0.74, 1.23]
Multiple infections	0	4 (5.3)	1	[0.47, 1.16]	0	4 (5.3)	1	[0.48, 1.22]
Gamma HPV								
Negative	12 (100)	71 (93.4)			13 (100)	58 (92.1)		
Positive	0	5 (6.6)	1	[0.55, 1.16]	0	5 (7.9)	0.58	[0.57, 1.22]
Multiple infections	0	1 (1.3)	1	[0.47, 1.16]	0	1 (1.3)	1	[0.06, 1.21]

* No statistically significant differences in the prevalence of the various HPV types were observed between the control group and any of the three breast cancer case groups (all, IBC and non-IBC), or between IBC and non-IBC.

** The HPV-positive category also includes some cases of multiple infections.

including, the sensitivity of the molecular method used for HPV detection and genotyping, and the tumour conservation conditions that affect the HPV DNA quality, possible HPV DNA contamination, as well as genuine variation across populations and disease subgroups. In the present study, all precautions were taken to avoid potential contamination, to ensure perfect tumour conservation and high sensitivity in HPV DNA detection.

High-risk HPV types, such as HPV16 and 18, which have been proposed as a cofactor of BC based on several reports [11,13,26,31–33], were not detected in this study. In our BC sample, the most frequently detected type was HPV11. Interestingly this type has been also found in BC in Iran [34], in Argentina [35] and in Germany [12].

HPV11 is considered a low risk as it is commonly found associated with genital warts and its presence has been demonstrated only rarely in malignancies [36,37]. Moreover, in contrast to HR HPV types, HPV11 does not display transforming activities in in vitro assays [38,39]. Therefore the role of HPV11 in breast carcinogenesis requires further investigations [40].

Beta HPV types were present in 10% of the BC tumours; HPV47 was the most prevalent (4%), followed by HPV5 and 8 (2.6% each). Our findings are in agreement with a previous study that reported the presence of Beta-HPV types in breast cancer [12]. However, another study showed that cutaneous HPV types are rarely present in breast fluids of women at increased risk for BC suggesting that a direct role of HPV in breast carcinogenesis is unlikely [41]. Beta HPV infection has previously been associated with skin cancer in patients with epidermodysplasia verruciformis; these patients frequently develop cutaneous squamous cell carcinoma [42]. Beta HPV infection may also play a role in cutaneous squamous cell carcinoma in immunocompromised non-epidermodysplasia verruciformis and in immunocompetent individuals [43].

Because the prevalence of HPV infection is high among young women, we stratified the HPV DNA detection rate by patient age. HPV DNA was found in both young and older women with no significant difference, (data not shown). This result is in agreement with studies conducted in Norway [44] and Brazil [13], suggesting that the presence of HPV in breast tissue is age-independent. However, other studies conducted in the Syrian Arab Republic [28], Australia [45], and Greece [46] have shown an association between age and HPV positivity, as young women with active sexual behaviours are more likely to be infected with HPV.

In a previous study, conducted on an Algerian sample and using the same detection method (TS-MPG), we found that 15 (12.2%) out of 123 BC cases were HPV-positive (12 alpha HPV and 3 beta HPV) [47]. This prevalence is significantly different from the prevalence found in the present Moroccan sample (25.0%; $p < 0.04$); this difference is probably attributable to the fact that the Algerian tumours were preserved in paraffin, whereas the Moroccan tumours were frozen. Freezing enables much better preservation of viral DNA. Moreover, the HPV types detected in the two populations were somewhat different: in the Algerian sample, HPV16 was the most common (6.5% of BC tumours), and the other types detected were HPV31 (2.4%), 22 (1.6%), and 5 and 6 (0.8% each). In the present Moroccan sample, HPV16, and 31 were not detected and HPV5 and 22 were identified in 2.6% and 1.3% respectively, of the BC tumours.

These two studies were conducted in Casablanca (Morocco) and Annaba (Algeria) that are 2000 km apart, which may contribute to the discrepancies observed; however, these discrepancies also indicate that if HPV is involved in the aetiology of BC, differences across relatively nearby populations may be expected.

We did not find significant differences in the prevalence of HPV infection when comparing IBC tumours with non-IBC tumours or with controls; however, with only 13 IBC tumours, the statistical power was limited. The fact that none of the 13 IBC tumours was found to be HPV-positive does not strongly encourage further exploration of the role of HPV infection in this subgroup. However, when the IBC and non-IBC tumours ($n = 37$ and $n = 86$, respectively) in the Algerian sample were compared, the prevalence of HPV infection was higher in the IBC tumours (18.9%) than in the non-IBC tumours (8.1%), although this difference was not statistically significant, it illustrates another difference between the two studies.

Considered together, the two studies (the present one and the Algerian one) confirm the presence of HPV DNA belonging to different genera in BC tumours, but they do not provide clear results about the HPV types involved, nor strong evidence of an aetiological role for HPV since no significant differences in the prevalence of HPV infection were observed across groups (controls, IBC, and non-IBC tumours).

The main strength of the present study is the technical approach used for HPV detection and genotyping. Its main limitation is the small sample size of the control and IBC groups, but since the incidence of IBC is low (< 3% of BC cases in Europe and < 10% of cases in North Africa), it takes time to accrue a large sample. Collecting malignant and non-malignant tumours in the same surgical ward (in order to ensure comparable quality and freezing procedures) is also challenging.

5. Conclusions

Overall, HPV DNA was found 3 times as frequently in the BC tumours as in the controls, with the presence of HPV DNA belonging to different genera in BC tumours. Our conclusion is that, given the complexity involved and the relatively low prevalence of HPV infection in BC tumours, studies in large samples are required to better understand the role of HPV infection in human BC aetiology.

5.1. Ethical approval and consent to participate

Ethical approval to conduct this study was obtained from the Pasteur Institute of Morocco Research Ethics Committee. Subjects were informed about the study procedures and provided informed consent. Illiterate participants were read the informed consent forms in a presence of a third person, and provided a mark the way they used to for official documents in lieu of signature. The ethics committee of the Pasteur Institute of Morocco approved this consent procedure.

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Consent for publication

Informed consent was obtained from the patient for the publication of this manuscript.

Availability of data and materials

All data are fully available without restriction.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EA: draft the manuscript, carried out the molecular studies and contributed to tumour collection

TG: participated in the design and coordination of the project and reviewed the final manuscript

MB: contributed to tumour collection

SM: contributed to laboratory work.

MA: performed the statistical analyses and contributed to the revision of the manuscript

SS: participated in sample collection, clinical data acquisition and anatomy pathology analyses

MEM: contributed to the revision of the manuscript

MC: contributed to the study design and coordination of the project and provided critical revisions of the manuscript

MT: participated in the conception and design of the study

MK: participated in the design and coordination of the project and contributed to the revision of the manuscript

All authors read and approved the final manuscript.

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