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Short report

No evidence of human papillomavirus DNA in breast carcinoma in Tunisian patients

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ABSTRACT

The aim of this study was to evaluate the prevalence of broad range of anogenital HPVs in a series of 123 Tunisian breast carcinoma cases. PCR assays were performed to amplify regions within the L1, E1, E6 and E7 open reading frames of a broad range of anogenital HPVs and specific types HPV16, 18, 31 and 33. In addition, we performed an in situ hybridization analysis using HPV biotinylated DNA probes for the detection of broad spectrum of anogenital HPV types, high-risk HPV types (16 and 18), intermediate-risk HPV types (31 and 33) and low-risk HPV types (6 and 11). None of the 123 breast carcinoma samples showed PCR amplification of HPV DNA using the broad spectrum consensus primer-pairs E1-350L/E1-547R and GP5+/GP6+ primers. Furthermore, neither high risk nor low-risk HPV types were detected in any of these cases. Moreover, using in situ hybridization for the detection of HPVs, we failed to detect a positive signal in neoplastic cells in any case. Our results suggest that anogenital papillomaviruses are unlikely to play a role in the development of breast carcinomas in Tunisian patients.

Introduction

Breast cancer is one of the most prevalent malignant throughout the world, occupying first place in mortality in women.[1] The etiology of breast cancer is unknown, but there are many risk factors associated with breast cancer development. Multiple genetic, endocrine, environmental and life style factors may contribute to its causation. Also, there is some evidence that breast carcinogenesis may be in some cases associated with viral agents. A possible role of oncogenic HPV in breast cancer has been suspected. Indeed, it has been shown that human breast epithelial cells can be immortalized by HPV types 16 and 18.[2] This raised the possibility that HPV may be etiologically related to some cases of breast cancer.

However, unlike cervical carcinoma, which is almost always associated with HPV, the causal role of HPV infection in the development of breast carcinoma remains a controversial subject. While some studies report the presence of HPV DNA sequences in breast cancer tissue, especially types 16, 18 and 33,[3–17] other studies revealed that HPVs could not be detected in breast cancer.[18–23]

In Tunisia, breast cancer is the leading cause-related death among women, with an annual incidence of 19.6/100,000 women.[1]

The aim of this current study was to evaluate the prevalence of anogenital papillomaviruses in breast cancer in Tunisia. We examined the presence of these viruses in 123 specimens of human breast cancers using polymerase chain reaction (PCR) and in situ hybridization.

Subjects and methods

Patients and tumors samples

Our current study was conducted on 123 Tunisian women with sporadic breast carcinoma. The cases were diagnosed between 1995 and 2006 at the Farhat-Hached Hospital in Sousse, Tunisia.

The age of patients ranged from 31 to 87 years, with mean age of 49.3 years. One hundred and twelve cases were invasive ductal carcinoma, six was medullary carcinoma, and five were invasive lobular carcinoma according to WHO criterias.[24] Invasive ductal carcinomas were graded according to the modified Scarff-Bloom-Richardson (SBR) system as follows: 21.5% grade I, 38.4% grade II, 40.1% grade III.[25] Tumors ranged in size from 1 to 8 cm, with a mean of 2.6 cm. Eighty-one patients had no pathological node involvement, whereas 22 had pathological node involvement. Twenty patients had no surgical axillary dissection. Distant metastases were described in only 11 patients at the time of diagnosis. The estrogen receptor, progesterone receptor and HER2/*neu* status of the tumor was performed by immunohistochemistry and revealed a positive result in 44%, 47% and 26% of cases, respectively.

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Polymerase chain reaction (PCR)

DNA was extracted from tumor frozen tissues according to standard protocol previously described in our earlier studies.[26] Extracted DNA adequacy to PCR analysis was firstly assessed by a PCR assay using a set of primers (GH-20: ACACAACTGTGTTCACTAGC/GH-21: GGAAAATAGACCAATAGGCAG) amplifying a 407-bp fragment in the human β-globin gene as previously described.[27]

The presence of HPV DNA was carried out by polymerase chain reaction (PCR) in three steps. Briefly, a first screening to determine the presence of HPV DNA was performed using two consensus degenerated primers GP5+/GP6+[28] and E1-350L/E1-547R,[29] which targets a conserved 150 bp sequence of the L1 open reading frame (ORF) and a conserved 180 pb sequence spanning the E1 ORF, respectively. These primers permit the detection of a large spectrum of anogenital HPV types (6, 11, 16, 18, 30, 31, 33, 34, 35, 39, 40, 42, 45, 51, 52, 53, 56, 57, and 58). As a second step, two consensus primer pairs, located within the E6 and E7 ORFs, pU-1M/pU-2R and pU-31B/pU-2R were used to amplify HPV DNA from high-risk HPV (types 16, 18, 31, 33, 52b, and 58) and low-risk HPV (types 6 and 11), respectively.[30] Finally, all samples were tested for HPV types 16, 18, 31, and 33 using type-specific primers located within the E6 ORF.[31] Appropriate HPV positive controls were included in all amplification reactions.

In situ hybridization

In situ hybridization was performed on 3 μm thick formalin-fixed paraffin-embedded sections to evaluate the presence of HPV in tumor cells. Formalin-fixed paraffin-embedded sections suitability to in situ hybridization analyze was assessed using biotinylated Human DNA Probe (Ref: X1414, DakoCytomation). All analyzed sections proved adequate for in situ hybridization analysis yielding the presence of dark brown signal in the nuclei of cells.

In brief, for the detection of HPV, paraffin-embedded sections were examined with a broad spectrum HPV biotinylated DNA probe purchased from DakoCytomation, which recognises the anogenital HPV types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51, and 52 (Ref: Y1404, DakoCytomation), and another three specific types of biotinylated DNA probes including: high-risk 16 and 18 (Ref: Y1412, DakoCytomation), intermediate-risk 31 and 33 (Ref: Y1413, DakoCytomation) and low-risk 6 and 11 (Y1411, DakoCytomation) according to the manufacturer's instructions. Positive control formalin-fixed paraffin-embedded tissues infected with HPV were included in every in situ hybridization procedure. Positive signals were seen as dark brown granules in the nuclei of affected neoplastic cells.

Results

The integrity of all the DNA samples was validated by PCR amplification of a 407-bp fragment of the human β-globin gene Fig. 1. Despite the presence of adequate extracted DNA, none of the 123 breast carcinoma samples were HPV positive using consensus E1-350L/E1-547R Fig. 2a. and GP5+/GP6+ Fig. 2b. primers. Neither high risk (HPV 16, 18, 31, 33, 52b, and 58) Fig. 2c. nor low risk (HPV 6 and 11) Fig. 2d. type viruses were detected in any of these cases. In addition using primers specific for HPV 16, 18, 31 and 33 none of cases were positive Fig. 3. Positive controls were always given strong positivity and negative controls were consistently negative. Moreover, in situ hybridization showed no evidence of staining for HPV DNA in any of the cases. All controls tested in the same conditions stained reproducibly.

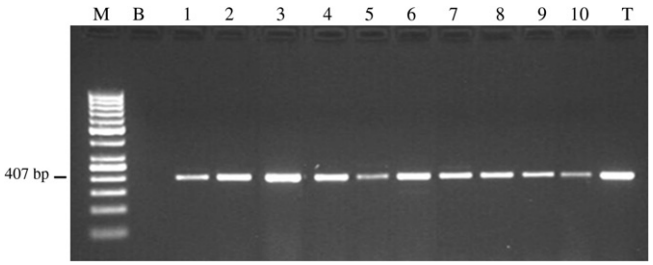

Fig. 1. Representative ethidium bromide-stained agarose gel electrophoresis after specific polymerase chain reaction (PCR) of a 407-bp fragment of the human β-globin gene. Lanes M show 100-bp ladder (Promega); lanes T represent positive controls (tumor DNA); lanes B represent negative control (without DNA template).

Discussion

To date, more than 200 HPV types have been characterized,[32] about 40 of them are associated with anogenital neoplasia.[33] Infection with the low-risk HPV6, 11, 42, 43, 44 are responsible for benign lesions (condylomas). The high-risk viruses such as HPV31, 33, 35, 51, 52 and 58 are detected more frequently in low squamous intraepithelial lesions, whereas HPV16 and 18 are predominantly associated with cancers of anogenitalia especially the uterine cervix.[34]

The anogenital HPV types are found in human infections and cancers at considerably different prevalence and with significant interregional variation.[35] However, available data on the prevalence of HPV within healthy women and women with uterine cervix cancer in Tunisia suffer from severe limitations. Moreover available data are derived from a few cities within this country and conceivably refer to specific populations and social groups. In a previous study, it was reported that HPV16 is the most frequent type in Tunisian cervicovaginal cancer, followed by HPV31 HPV18.[36] In addition the predominant HPV type reported among prostitute's women is HPV16, but in married women is HPV6.[37,38]

In the present study, we evaluated the presence of HPVs in 123 breast carcinoma samples by using DNA PCR amplification of regions within the L1, E1, E6 and E7 open reading frames of wide spectrum of anogenital human HPV genome. The presence of HPVs was also evaluated by in situ hybridization method using specific HPV probes. This is, to the best of our knowledge, the first study investigating the presence of HPVs in breast cancer in Tunisia. Despite the use of high quality of DNA extraction from fresh frozen tissues and the use of high sensitive PCR for the detection of many

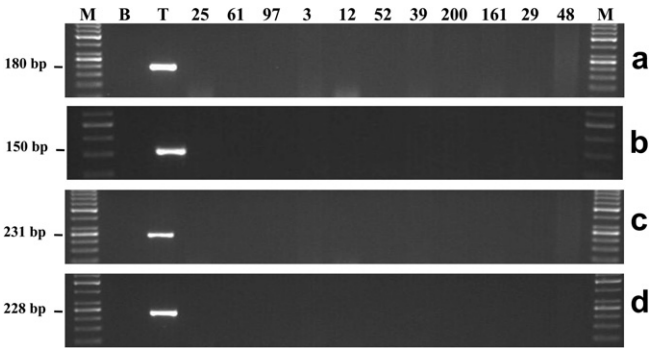

Fig. 2. Representative ethidium bromide-stained agarose gel electrophoresis after specific polymerase chain reaction (PCR) of (**a**) E1 open reading frame of whole spectrum of anogenital HPV types, (**b**) L1 open reading frame of whole spectrum of anogenital HPV types, (**c**) HPV high-risk types, (**d**) HPV low-risk types. Lanes 25, 61, 97, 3, 12, 52, 39, 200, 161, 29 and 48 represent breast carcinoma cases; Lanes M show 50-bp ladder (Promega); lanes T represent positive controls (tumor DNA); lanes B represent negative control (without DNA template).