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Basic Study

Lymphocyte subsets predictive value and possible involvement of human papilloma virus infection on breast cancer molecular subtypes

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Abstract

AIM

To detect human papilloma virus (HPV) presence and to characterize cellular immune response in breast cancer patients.

METHODS

A total of 74 women were included, of which 48 samples were from patients diagnosed with breast cancer and 26 patients with benign pathology of the breast. Molecular subtype classification was performed based on the immunohistochemical reports of the tumor piece. HPV genome detection and genotyping from fresh breast biopsies was performed using the INNO-LIPA HPV Genotyping Extra test (Innogenetics, Ghent, Belgium). CD3+, CD4+, CD8+ and natural killer (NK)+ cells levels from peripheral blood samples from patients with breast cancer and benign pathology were measured by flow cytometry.

RESULTS

Luminal A was the most frequent breast cancer molecular subtype (33.33%). HPV was detected in 25%

of the breast cancer patients, and genotype 18 was the most frequent in the studied population. The mean of CD3+, CD4+ and CD8+ subpopulations were decreased in patients with breast cancer, in relation to those with benign pathology, with a statistically significant difference in CD8+ values ($P = 0.048$). The mean of NK+ cells was increased in the benign pathology group. The average level of CD3+, CD4+, CD8+ and NK+ cells decreased as the disease progressed. HER2+ and Luminal B HER2+ tumors had the lowest counts of cell subsets. HPV breast cancer patients had elevated counts of cellular subsets.

CONCLUSION

Determining level changes in cellular subsets in breast cancer patients is a useful tool to evaluate treatment response.

Key words: Breast cancer; Human papilloma virus; Molecular subtypes; Immune response; T lymphocytes; NK cells

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Core tip: This work detected the presence of the human papilloma virus (HPV) genome in patients with breast cancer and measured the levels of cellular subsets as predictor factors. The viral genome was found in 25% of breast cancer cases, with the high-risk 18 genotype the most frequent. Luminal A tumors represented 33.33% of the sample. The average level of CD3+, CD4+, CD8+ and NK+ cells was decreased in cancer patients compared to the benign pathology group, while the reverse effect was observed in HPV positive patients.

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INTRODUCTION

With more than 408200 new cases and over 92000 deaths, breast cancer is the first cancer in the Americas, in terms of new cases, and the second in terms of women cancer deaths^[1]. In Venezuela, according to the Ministry of Popular Power for Health in 2012, breast cancer ranked first in cancer incidence with 5063 new cases and was the first cause of cancer death in the female population. This was followed by cervix cancer, with 2067 deaths, representing 22.88% of new diagnoses due to cancer and 18.25% of deaths caused by this pathology^[2].

The 13th Conference of St Gallen in 2013 established that it was unnecessary to perform genetic tests on each patient to classify molecular subtypes, which was proposed by Perou *et al.*^[3] in 2000, since histopathological results were comparable. The use of molecular diagnosis was necessary only in atypical behavior cases^[4]. Luminal A breast cancer cases are the most frequent and have good prognosis and response to hormonal treatment. Luminal B HER2- and Luminal B HER2+ breast cancers represent between 15% and 20% of breast tumors, and their response to hormonal therapy is less. HER2+ cancer accounts for 15%-20% of breast cancer subtypes, showing more aggressive biological and clinical behavior, with increased sensitivity to chemotherapy. The triple negative (TN) subtype represents from 8% to 37% of all breast cancers. These tumors are infiltrating ductal types characterized by a solid growth pattern, aggressive clinical behavior and high rate of metastasis to brain and lungs^[4,5]. Molecular subtypes are currently used as predictive factors among breast cancer patients^[6].

Nearly 50% of newly diagnosed breast cancer cases are related to hormonal factors; only 5% to 10% are related to genetic factors, although it is known that these greatly increase the risk of developing the disease. Several studies have determined the physiological, environmental and lifestyle factors related to the incidence of breast cancer, some of which are modifiable through preventive interventions^[1].

The non-genetic factors include: Age of diagnosis after 65 years, which is the most important risk factor, early menarche, menopause after 55-years-old, first live birth after 30-years-old, nulliparity, breast biopsies history, diagnosis of atypical hyperplasia by breast biopsy, obesity, alcohol consumption, hormone replacement therapy and excessive exposure to radiation. Other possible risk factors include high-in-fats and low-in-fiber diet, and little exercise^[7].

Approximately 18% of human cancers are caused by infectious factors, and it is recognized that breast cancer is strongly related to environmental factors, such as viruses, diet, radiation, among others^[8]. Human papilloma virus (HPV) infection distribution reports in breast cancer are controversial. In 2015, an overall prevalence between 0% and 86% was reported, with an average of 30.30%, with the highest frequency reported in Oceania at 44.30%. South America presented 14.60% of HPV infection, exceeding 10.70% of North America^[9].

HPV types are classified as low-risk and high-risk types based on the ability to induce carcinogenesis. HPV 6 and HPV 11 are low-risk subtypes and cause more than 90% of genital warts. High-risk HPV subtypes such as HPV 16, 18, 31, 33, 45 and 52, cause squamous intraepithelial lesions that can progress to invasive squamous cell carcinomas^[10]. This double strand DNA virus expresses *E6* and *E7* oncogenes, which interact with p53 and pRB proteins, respectively, promoting the development of neoplasias due to uncon-

trolled cell cycle activation and inhibition of apoptosis^[11]. The high-risk HPV genome could be integrated into the host genome during the carcinogenesis process, causing the loss of *E6* and *E7* transcription regulation by the interruption of *E2* gene open reading frame^[12].

Besides clinical and treatment parameters, the host immune response might influence the prognosis of cancer patients after standard treatment^[13]. Breast tumor progression is due to a systematic action, affecting the host's physiological processes and triggering responses in peripheral blood cells^[14]. It is known that CD4+ and CD8+ T cells are required for an effective anti-tumor immune response. CD4+ T cells are critical for priming tumor-specific CD8+ T cells and for the secondary expansion and memory of CD8+ T cells as well^[15]. CD8+ T cells have been shown to be mediators of antitumor immunity and act directly over tumor cells. Recent studies have suggested its clinical importance, reporting that an increase of CD8+ T cells correlates with increased survival in large cohorts of various human cancer patients^[13].

The natural killer (NK)+ cells have the ability to produce lysis in tumor cells and cells infected with intracellular viruses or parasites, through cytotoxic mechanisms mediated by preformed molecules, such as perforins and granzymes. They also have the ability to secrete cytokines such as interferon types I and II^[16]. NK cells appears to protect against tumor development and progression^[17].

Evaluation of circulating T lymphocytes, B lymphocytes and NK+ cells may be one of the beneficial ways to understand immune response, assist in clinical diagnosis, provide evidence of pathogenesis, course, and prognosis of disease and determine clinical treatment^[18]. This work aimed to evaluate the possible role of cellular subsets as predictive factors and the association of HPV in patients with breast cancer, according to the molecular subtypes, being the first study of its kind reported in Venezuela.

MATERIALS AND METHODS

Study population

We evaluated prospectively from February 2011 to October 2013 patients attending at the Breast Pathology Unit, in the Gynecology Department, from the University Hospital of Caracas. A total of 74 women were included, of which 48 samples were from patients diagnosed with breast cancer and 26 patients diagnosed with benign pathology of the breast. Patients were invited to participate in the study, with prior information on the design and protocol. Each one signed an informed consent, approved by the hospital Bioethics Committee.

Patients with other tumors or immune system-related disease were excluded. None of the patients had received any form of medical or surgical therapy such as radiotherapy, chemotherapy or treatment with steroids or immunosuppressants prior to the investigation.

Breast cancer molecular subtypes classification

Determination of breast cancer molecular subtype was

performed based on the immunohistochemical reports of the tumor piece, which were obtained from the clinical histories of each patient. According to markers expression, tumors were classified as: (1) Luminal A: RE+; RP+ ($\geq 20\%$); Ki67 ($< 14\%$), HER2-; (2) Luminal B HER2-: RE+; RP ($< 20\%$); Ki67 ($\geq 14\%$), HER2-; (3) Luminal B HER2+: RE+; RP indifferent; Ki67 ($\geq 14\%$); HER2+; (4) HER2+: RE-, RP-, Ki67 ($\geq 14\%$); HER2+; and (5) TN: RE-, RP-, Ki67 ($\geq 14\%$); HER2-^[5].

Tissue samples

Fresh biopsies were obtained from tumors of patients who underwent surgery. Biopsies were frozen at -70°C for molecular analysis.

DNA extraction

To perform DNA extraction from fresh breast biopsies, QIAmp DNA mini kit (250) was used (Qiagen, Hilden, Germany), following the manufacturer's instructions.

HPV detection and genotyping

HPV genome detection and genotyping from fresh breast biopsies was performed using the INNO-LIPA HPV Genotyping Extra test (Innogenetics, Ghent, Belgium), following the manufacturer's instructions. Based on the reverse hybridization principle, 28 different HPV genotypes were identified by detecting specific sequences in the L1 region. The assay uses the proven SPF10 primer set for highly sensitive amplification of the most clinically relevant HPV genotypes.

Blood samples

Before surgery, 5 mL of venous blood was obtained from each patient. The samples were drawn into heparinized tubes and transported to Oncology and Hematology Institute for processing.

Cellular subsets

Cellular subsets quantification was performed by flow cytometry. Fifty microliters of whole blood was taken, and 5 μL of monoclonal antibody was added. The sample was briefly vortexed and incubated for 15 min at room temperature in the dark. Eight hundred microliters of BD 1X lysis solution was added and incubated for 10 min at room temperature in the dark. The sample was centrifuged for 5 min at high speed, and the supernatant was discarded. The pellet was resuspended with 300 μL of FACS Flow and vortexed. Finally, the tube was acquired in the BD FACS Canto II cytometer, of six colors (configuration 4-2), with the BD FACS Diva 6.2.2 application.

Cell surface marker analysis was performed using CD4-FITC/CD8-PE/CD3-PC5 (Beckman Coulter) for CD3+, CD4+ and CD8+ T cells and CD3-APC, CD16-FITC, CD56-PE for NK+ cells (Beckman Coulter, Pasadena, CA, United States). Absolute cell counts were calculated by multiplying the cell subset percentage by the total lymphocyte concentration present in peripheral blood.

Table 1 Demographic characteristics for the breast cancer and benign pathology groups

	Breast cancer (<i>n</i> = 48)	Benign pathology (<i>n</i> = 26)	<i>P</i> value ¹
Mean age (yr)	57.79 ± 14.13 (range: 31-85)	32.54 ± 11.63 (range: 14-60)	0.000
Menarche (yr)	12.42 ± 1.67 (range: 9-16)	12.62 ± 1.69 (range: 9-17)	0.763
Term pregnancy	2.63 ± 2.27 (range: 0-11)	1.50 ± 1.55 (range: 0-5)	0.066
Sexual partners	1.98 ± 1.15 (range: 0-5)	1.76 ± 1.09 (range: 1-3)	0.750
Oral contraceptives	47.92%	65.38%	0.352
Tobacco	50.00%	11.53%	0.002
Alcohol	47.92%	46.15%	0.678
Breast cancer family history	37.50%	19.23%	0.199

¹Breast cancer group *vs* benign pathology group.**Table 2** Mean absolute values for the cellular subsets in the breast cancer and benign pathology groups

	Breast cancer (cel/mm ³)	Benign pathology (cel/mm ³)	<i>P</i> value ¹
TCD3+	1517.95 ± 666.23	1861.68 ± 760.52	0.102
TCD4+	888.73 ± 445.18	974.01 ± 390.17	0.504
TCD8+	551.34 ± 284.35	764.54 ± 431.10	0.048
CD4+/CD8+	1.94 (range: 0.59-6.33)	1.57 (range: 0.64-4.36)	0.271

¹Breast cancer group *vs* benign pathology group.

Statistical analysis

Measures of central tendency and dispersion were used for continuous variables; frequency analysis and contingency tables were used for discrete variables. Analysis of variance between groups, *t*-Student Parametric Test for two independent samples and non-parametric Mann-Whitney *U* test were used to perform hypothesis contrast. Significance level was fixed at *P* < 0.05 (Statistical Software: SPSS in its Version 20 in Spanish; IBM Corp., Armonk, NY, United States).

RESULTS

The study included 74 patients, with menarche age between 9 and 17 years, with 60.81% of menarche age between 12 and 14 years. The average sexual partners number was between 0 and 5, and 74.32% of the patients had 0 to 2 sexual partners. Regarding pregnancy at term, the cases registered were between 0 to 11 per patient, with the highest proportion between 0 and 2 deliveries (67.57%). Overall, 47.29% had an alcoholic habit, 36.49% had a tobacco habit, 54.05% of the patients used oral contraceptives, and 59.46% had a family history of cancer.

Table 1 shows demographic characteristics for the study groups, where 50% of breast cancer patients reported smoking and alcohol habit, and about 40% reported a family history of breast cancer. Mean age and tobacco habit showed a statistically significant difference between breast cancer patients and benign pathology patients.

Of the breast cancer samples, 50.00% corresponded to stage II, followed by stage I (20.83%), stage III (14.58%), stage 0 (8.33%) and stage IV (6.25%). Regarding the histopathological diagnosis, the breast

cancer tumors were infiltrating ductal carcinoma (79.16%), ductal carcinoma *in situ* (8.33%), lobular carcinoma (8.33%) and mucinous carcinoma (4.17%). Luminal A was the most frequent breast cancer molecular subtype (33.33%), followed by Luminal B HER2- (29.17%), Luminal B HER2+ (14.58%), TN (12.50%) and HER2+ (10.42%).

HPV was detected in 25.00% of the breast cancer patients, and genotype 18 was the most frequent in the studied group, followed by types 16, 6 and 31. 41.67% of the patients presented mixed infections, and 75.00% showed infection with high oncogenic risk genotypes in breast fresh tissue biopsies. In the benign pathology group, HPV genome was detected in 7.69%, finding genotypes 18 and 33 of high oncogenic risk, in single infections. Fifty percent of the HPV positive breast tumors were Luminal A, followed by the HER2+ type, with 25%. Luminal B HER2- and TN types represent 16.67% and 8.33%, respectively, among HPV positive breast tumors (*P* = 0.027).

Table 2 shows the mean absolute values for each cellular subset of the breast cancer patients and benign pathology patients. Breast cancer patients showed a decrease in mean values compared to those of benign pathology, with statistically significant difference in CD8+ count between both study groups. The mean of each cellular subset decreased as the stage of the disease increased (*P* > 0.005). Regarding breast cancer molecular subtypes, the HER2+ tumors had the lowest CD4+ and CD8+ values (Table 3).

The NK+ cells counts were elevated in the benign pathology group, with 1217.04 ± 778.69 cel/mm³ (range: 48.30-3193.18), in comparison with breast cancer patients (1053.79 ± 690.56 cel/mm³ (range: 187.55-3675.00)) (*P* = 0.651). A decrease in the mean

Table 3 Correlations between the breast cancer molecular subtypes and the cellular subtypes

	CD4+ (cel/mm ³)	CD8+ (cel/mm ³)	NK+ (cel/mm ³)
Luminal A	916 ± 358.81	645 ± 299.91	1064 ± 455.61
Luminal B HER2-	877 ± 494.16	544 ± 273.04	1120 ± 818.37
Luminal B HER2+ HER2+	914 ± 318.73	491 ± 208.07	926 ± 500.35
HER2+	680 ± 444.86	426 ± 319.13	834 ± 836.75
TN	992 ± 568.02	483 ± 214.90	1183 ± 837.52
P value ¹	0.842	0.527	0.912

¹Between breast cancer molecular subtypes. TN: Triple negative.

Table 4 Correlations between the breast cancer HPV status and the cellular subtypes

	HPV+ (n = 14)	HPV- (n = 34)	P value ¹
CD3+ (cel/mm ³)	1832.12 ± 537.08	1399.56 ± 665.69	0.563
CD4+ (cel/mm ³)	1139.92 ± 416.84	796.56 ± 416.84	0.091
CD8+ (cel/mm ³)	630.66 ± 271.00	518.58 ± 281.53	0.748
CD4+/CD8+	2.11 (range: 0.71-4.17)	1.88 (range: 0.59-6.33)	0.029
NK+ (cel/mm ³)	1350.21 ± 736.79	950.47 ± 634.35	0.082

¹HPV+ vs HPV-. HPV: Human papilloma virus; NK: Natural killer.

of NK+ cells was observed as the stage of the disease increased ($P = 0.0827$). Regarding breast cancer molecular subtypes, HER2+ tumors and Luminal B HER2+ had the lowest values of NK+, while TN tumors had the highest values (Table 3).

As shown in Table 4, HPV+ breast cancer tumors had elevated cellular subtype counts compared to HPV- tumors. A decrease in the mean of NK+ cells was observed as the disease stage increased in the HPV+ tumors.

DISCUSSION

Breast cancer is the most frequently diagnosed neoplastic disease during menopause, leading to a significant reduction of women's quality of life^[19]. In developing countries, the disease emerges as a serious public health problem due to the high economic and social costs associated with its care^[20].

Breast tumors have a very wide phenotypic diversity, which is accompanied by a large variability in gene expression patterns^[3]. Gene signatures are used as predictors of therapy response, and protein gene products have direct roles in the biology and clinical behavior of cancer cells and are potential targets to develop novel therapies^[5].

According to different reports, Luminal A represents 50% to 60% of breast cancer cases, while Luminal B, both HER2- and HER2+, represent 15% to 20% of cases. HER2+ tumors are detected between 15% and 20%, and TN tumors are found between 8% and 37% of breast tumors^[4,5]. In this study, Luminal A was the most frequent group, followed by Luminal B HER2-, Luminal B HER2+, TN and HER2+.

In Venezuela, few studies have characterized breast cancer molecular subtypes. Uribe *et al.*^[21] reported

Luminal A as the more frequent subtype (60.94%), followed by TN (28.75%). López *et al.*^[22] found that of 110 patients evaluated, 40.0% presented with Luminal B HER2- tumors, followed by Luminal A (20.91%).

Identification of genetic and risk factors, such as environmental and hormonal factors, have increasing value and play an important role in breast cancer prevention^[23]. These risk factors increase neoplastic process development probability and depend on the exposure time or individual genetic predisposition. Therefore, they can influence cancer development, even if they do not directly cause the disease^[24].

Regarding non-genetic factors, in our study, 35.42% of patients had a diagnosis age greater than 65 years, 58.33% had menarche before 12 years, 14.58% were nulliparous at the time of the study, and 47.92% indicated alcohol consumption. However, many of the patients who developed breast cancer did not have any identifiable risk factor.

Epidemiological studies gave a first indication of an association between viral agents and specific human cancers. Infectious factors are responsible for approximately 18% of human cancers, and it is well accepted that human breast cancer is highly associated with environmental factors. Among many microorganisms studied, viral infections have been suggested to play a role in cancer development, especially those cancers caused by HPV^[8].

In this study, HPV was detected in 25.00% of the samples. High oncogenic risk genotypes were the most common, with a higher prevalence of type 18 over 16, unlike cases of benign pathology, where the virus detection reached 4.76%. Similar reports indicate frequencies between 15.0% and 29.4% for HPV positivity^[25-29], and Aguayo *et al.*^[30] reported the lowest of this group of studies, with 8.7% in South America. As

for genotypes found, all studies found HPV type 16 was the most frequent in single infections or mixed infections with genotype 18.

However, other studies performed viral detection by polymerase chain reaction and genotyping by sequencing and reported a greater presence of type 18. In 2005, Kan *et al.*^[31] showed 48% positivity for HPV in breast cancer biopsies, with 100% for genotype 18. In addition, Heng *et al.*^[32], Antonsson *et al.*^[33], Glenn *et al.*^[34] and Lawson *et al.*^[35] found genotype 18 in greater proportion in breast cancer tissue. Since adenocarcinoma constitutes the majority of histological breast carcinoma types, it is understandable that HPV 18 was similar or even a little higher than HPV 16 in these studies. Hence, the distribution of high-risk HPV type for breast carcinoma is probably different than that for cervical cancer^[36].

To our knowledge, there are no reports about the correlation between HPV presence and breast cancer molecular subtypes, so our research is the first study to demonstrate this association. Piana *et al.*^[29] correlated HPV presence with TN tumors and showed a viral detection of 15%. However, the authors used paraffin embedded biopsies and did not discriminate between all the molecular subtypes.

More studies are necessary to evaluate the correlations between HPV presence and breast cancer molecular subtypes. With this knowledge, we could determine if the presence of HPV is associated with better prognosis tumors that are more responsive to chemotherapy treatments, as occurs in HPV positive oropharyngeal squamous cell carcinoma (OPSCC). Multiple studies have confirmed that HPV positive OPSCC shows a better response to chemotherapy and radiation, independently of treatment scheme^[37].

Recently, several studies focused on DNA deaminase APOBEC3B (A3B), a source of uracil dependent genomic mutations that is associated with mutagenesis in multiple human cancers, including cancers in breast, head and neck, cervix, bladder, lung, ovary and other tissues. This enzyme belongs to a protein family that has broad and overlapping functions in innate immunity *via* the restriction of viruses, transposons and other foreign DNA elements^[38]. Therefore, some authors have suggested a possible role for viral infections, such as HPV and EBV, in the regulation A3B gene expression in some cases of breast cancer^[38,39].

A3B expression levels are low in most of normal tissues. The E6 HPV oncoprotein offers the first contact in viral infection and A3B-mediated mutagenesis. In one model, high-risk HPV E6 protein inactivates p53, causing the elimination of A3B gene transcription^[38] for cervix cancer, head and neck cancer^[40], and HPV positive breast cancer^[39]. Given the roles of the proteins p53, A3B and E6, when levels of DNA damage and mutations are raised, answers to these damages and apoptosis are prevented.

Regarding the immune response, previous studies have shown a decrease in T lymphocytes proliferation,

low CD3+ and CD4+ count, an increase in CD8+ count and a decrease in CD4+/CD8+ ratio. Other studies have reported a gradual decrease in the CD4+/CD8+ ratio, proportional to the progression of breast cancer^[18]. Currently, data about the cytotoxicity of NK cells and blood levels are contradictory, and there is a lack of information regarding the tumor microenvironment in patients with breast cancer^[41].

An important strength of this work was the inclusion of breast cancer patients samples, which were taken prior to surgical, systemic and radiant treatment. This allowed us to evaluate the differences in the immunological status of the patients without the influence of treatment.

In the breast cancer patients group, there was a decrease in the concentration of TCD4+ and TCD8+ lymphocytes compared to the benign pathology group, while the CD4+/CD8+ ratio was higher in the breast cancer. TCD8+ concentration variation showed a statistically significant difference ($P = 0.048$) in respect to the benign pathology group, representing a possible predictive marker. Regarding the behavior of lymphocytes in relation to breast cancer staging, the CD4+/CD8+ ratio decreased as the stage of the disease increased, which is consistent with Jia *et al.*^[18], who reported a rate decrease in advanced breast cancer.

The average absolute values for NK+ cells in the breast cancer patients group was less than those of the benign pathology group. Verma *et al.*^[42] also reported a lower NK+ cell count in breast cancer patients. They evaluated the variation of these values during and after treatment and found that neoadjuvant therapy increased NK+ cell values above that reported in the healthy group. It has been reported that chemotherapy, by damaging or stressing cells, promotes the release of various signals that activate dendritic and NK+ cells and induces the release of pro-inflammatory cytokines^[43].

It is known that functional capacity of immune cells decline with aging. Diminished phagocytic capacity of dendritic cells leads to impaired antigen presentation and activation of the adaptive immune system. In addition, thymus involution decreases the production of naïve T cells, and memory T cells accumulate, diminishing the T-cell repertoire^[44,45]. Postmenopausal women exhibit a reduced number of total lymphocytes, mainly B and CD4+ cells. Similarly, after surgical menopause, the CD4+/CD8+ ratio and the circulating B cells are decreased, while NK cells are increased^[45]. The breast cancer patients evaluated did not show statistically significant differences with respect to cell subsets and age groups (data not shown).

Regarding breast cancer molecular subtypes, we found that the CD4+ count and the CD4+/CD8+ ratio were decreased in the HER2+ tumor group, and that TN tumors showed an increase in the NK+ cell count. Particularly, the HER2+ group and the Luminal B HER2+ group showed a considerable decrease of NK+ with respect to the rest of the molecular subtypes.

Jia *et al.*^[18] reported a decrease in the CD4+ count

and the CD4+/CD8+ ratio and an increase in CD8+ and NK+ cells in ER-, HER2+, TN tumors, with Ki67 \geq 14%, indicating a greater failure of the immunological response in those tumors with aggressive phenotype. Previous studies have shown that estrogen plays an important role in regulating the activation of T lymphocytes, particularly CD4+ and CD8+; suggesting that the absence of estrogen receptors in HER2+ tumors is related to what was observed in this study population and was affecting the concentration of both CD4+ and NK+ cells.

ER- and TN breast tumors have a worse prognosis than ER positive tumors. These findings indicate a greater degree of immune function suppression and anti-tumor activation, which is reflected in the aggressiveness of HER2+ and TN tumors^[18]. Kim *et al.*^[13] revealed that the decreased number of CD8+ T cells was significantly associated with aggressiveness and malignant features of tumors, including lymph node metastasis, higher stage and higher Ki67. Therefore, immunotherapy is one of the most promising approaches for breast cancer therapy. If new research establishes a role for lymphocyte subsets in the etiology of aggressive phenotypes, such as those characterized by being ER-, HER2+, presenting a Ki67 \geq 14% and TN subtype, new treatment strategies for these breast cancer types should include immunotherapy.

The cellular subset values were increased in the HPV+ tumors compared to those of HPV- patients, and there was a statistically significant difference with respect to the CD4+/CD8+ ratio ($P = 0.029$), due to a considerable increase in the CD4+ count of HPV+ patients. Despite the HPV evasion mechanisms of the immune response, about 90% of genital and skin lesions resolve on average in 2 years. Immunohistochemical studies show that regression of cutaneous, oral and genital warts in animals and humans is characterized by a massive local infiltration with CD4+, CD8+ and macrophages into the lesion, and the expression of Th1 cytokines profile. Despite the intense local response, the systemic antigen-specific T cell responses are weak, transient and difficult to measure^[46].

Compromised adaptive immunity is the basis for high-risk HPV infection to cervical cancer progression. Different immune cell profiles characterize the different stages of the disease progression in cervical intraepithelial neoplasm and invasive cancer. The high-risk HPV infection changes and modifications induced include the adaptation of the immune system to create a suitable microenvironment for persistent infection and lesion progression^[47].

During HPV persistent infection, pro-inflammatory cytokines are not released, and the Langerhans cell and dendritic cell activation and recruitment signals are absent. In fact, cells with viral late gene expression, which may contain high levels of viral proteins, are shed from the surface of the epithelium away from immune surveillance. In general, a failure in developing an effective host immune response correlates with persistent infection and an increased probability of

progression toward invasive cancer^[11].

Although no previous studies have evaluated the profile of cellular subsets in patients with HPV positive breast tumors, we observed that the virus infected group showed higher values of CD4+, CD8+ and NK+ cells in comparison with negative patients. It is possible that these increases are due to an activation of the immune response to viral infection in the mammary tissue.

It has been described in HPV positive cases of OPSCC that patients show a greater proportion of circulating TCD8+ lymphocytes compared with HPV negative cases and that these lymphocyte levels are better predictors of treatment response than HPV status. Other prognostic markers could include CD4+/CD8+ ratio, the circulating levels of the T cell group, the presence of infiltrating lymphocytes in the tumor microenvironment, the expression of MHC class I and the characterization of the immune response by microarray^[48].

It is known that CD8+ T cells play a major role in elimination of viral infection, secreting interferon and displaying cytolytic effects mediated by granzyme and perforin. CD4+ T cells also secrete interferon but instead mediate killing primarily by engagement with ligands for death receptors, such as Fas or TNF-related apoptosis-inducing ligand, resulting in caspase-mediated apoptosis^[49]. The presence of CD8 T cells in cervical lesions is associated with a favorable prognosis, with their numbers inversely correlating with tumor progression.

On the other hand, infection with high-risk HPV genotypes compromises the activation of NK+ cells. In the case of the cervix, NK+ cells predominate in early stages of infection and in low-grade squamous intraepithelial lesions, whereas in cases of cervical cancer, NK+ activation receptors are considerably diminished. These findings imply a low cytotoxic activity by the NK+, thereby facilitating the progression of the lesion and carcinogenesis^[47].

In patients with breast cancer and HPV positive breast tissue, a decrease in the NK+ cell count was observed as the severity of the disease increased, implying a failure in the cytotoxic activity performed by the cells studied. It has been observed that the presence of HPV in mammary tissue modifies the activity of the evaluated cells, at the level of the cellular immune response.

It is well known that the incidence of cervical cancer due to HPV infection is much higher compared to non-genital cancers or oropharyngeal cancers associated with the presence of the virus, so the distribution and application of HPV vaccines for preventing cervical cancer remains as a public health priority. However, by 2008, non-genital and oropharyngeal cancers represented about 80000 new cases of cancer associated with HPV infection worldwide, implying a major health problem^[50].

Evidence obtained from clinical trials indicates that

current HPV vaccines can prevent HPV infections in vulva, vagina, anus and mouth as well as pre-cancerous anogenital lesions in women and oral and anogenital infections, and pre-cancerous lesions in men. However, data comparing the efficacy and effectiveness data of cervical infections and high-grade lesions with those types of injuries are limited^[50,51].

As yet, the implications of HPV vaccination for prevention of non-cervical cancers have not been fully explored. Some countries have recommended HPV vaccination for young males, based on the hypothesis that vaccination will prevent HPV-associated cancers in men as well as theoretical benefits in preventing HPV transmission to women^[50].

Therefore, if HPV vaccines contribute to decrease the rate of non-genital cancers, we could have a group of patients that develop breast cancer due to HPV infection, which could benefit from the use of vaccines available in the international market, which include the bivalent that protects against types 16 and 18, the tetravalent, for types 6, 11, 16 and 18, and the nonavalent, approved in 2014 for types 6, 11, 16, 18, 31, 33, 45, 52 and 58^[52].

ARTICLE HIGHLIGHTS

Research background

Breast cancer is the leading cause of death among women, classified in molecular subtypes according to a genetic profile. Approximately 18% of human cancers are caused by infectious factors, and it is recognized that breast cancer is strongly related to environmental factors, such as viruses, diet, radiation, among others. Human papilloma virus (HPV) has been detected in 0% to 86% of breast cancer tumors, and it represents a possible risk factor. The host immune response might influence the prognosis of cancer patients after standard treatment.

Research motivation

It was recently suggested that HPV presence may act as a risk factor in breast cancer development, but it has not been correlated with molecular subtypes. In addition, it is important to evaluate the immune response of breast cancer patients and to be able to suggest some prognostic values that make it possible to offer better patients treatment.

Research objectives

The main objective is to detect HPV presence and to characterize the cellular immune response in breast cancer patients based on the molecular subtypes.

Research methods

The patients inclusion was done prospectively, and the breast cancer molecular classification was made according to the St. Gallen International Breast Cancer Conference. HPV detection and genotyping were performed using HPV INNO-LIPA Genotyping Extra test, and lymphocyte subsets were measured by flow cytometry.

Research results

Luminal A was the most frequent breast cancer molecular subtype (33.33%), and HPV was detected in 25% of the breast cancer patients, with genotype 18 as the most frequent in the studied population. The means of CD3+, CD4+ and CD8+ subsets were decreased in patients with breast cancer, respective to benign pathology, with a statistically significant difference between CD8+ values ($P = 0.048$). The mean of NK+ cells was increased in the benign pathology group. HER2+ and Luminal B HER2+ tumors had the lowest counts of cell subsets. The HPV breast cancer patients had elevated counts of cellular

subsets.

Research conclusions

It can be observed that HPV positive breast cancer tumors have a better prognosis, correlated with Luminal A subtypes, and show a better cellular immune response, specifically in relation with TCD8+ cells counts, suggesting a better response to chemotherapy and radiant treatment, as in the case of HPV positive oropharynx tumors.

Research perspectives

Future efforts should focus on evaluating patients disease-free survival, based on HPV positivity in the tumor tissue. In addition, viral load and HPV genome integration, the identification of HPV variants by sequencing, and the infiltrating lymphocytes in the tumor bed should also be studied. As a final consideration, the experience of working with fresh samples is complex and involves a process of sample collection in the operating room, in addition to the management of bioethical parameters, and our experience will allow for the possibility of obtaining more accurate results.

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