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

Fernandes A *et al*. Lymphocyte subsets, HPV and breast cancer

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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 INNO-LIPA HPV Genotyping Extra test (Innogenetics, Belgium). CD3+, CD4+, CD8+ and 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 between CD8+ values (*p =* 0.048). The mean of NK+ cells was increased in bening pathology group. The average 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. The HPV breast cancer patients had elevated counts of celular 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:** The present 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 the breast cancer cases, been high-risk 18 genotype the most frequent. Luminal A tumors represent 33.33% of the sample. The average of the CD3+, CD4+, CD8+ and NK+ cells was decreased in the cancer patients, in comparison to the benign pathology group, while the reverse effect was observed in the 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, followed by cervix cancer, with 2067 deaths, representing 22.88% of new diagnoses due to cancer and 18.25% of deaths caussed by this pathology[2].

The 13th Conference of St Gallen, in 2013 stablished that it was unnecessary to perform genetic tests on each patient to classify molecular subtypes, 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 are the most frequent cases of breast cancer, with good prognosis and response to hormonal treatment. Luminal B HER2- and luminal B HER2+, represent between 15% and 20% of breast tumors and their response to hormonal therapy is not so good. HER2+cancer accounts for 15%-20% of breast cancer subtypes, showing more aggressive biological and clinical behavior, with increase 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 research have allowed to determine 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, being the most important risk factor, early menarche, menopause after 55 yeras old, first live birth after 30 years old, nulliparity, breast biopsies history, diagnostic of atypical hyperplasia by breast biopsy, obesity, alcohol consumption, hormone replacement therapy (HRT) 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%, been the highest frequency reported in Oceania, with 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 hability to induce carcinogénesis. HPV 6 and HPV 11 are low-risk subtypes and cause more tan 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 doble strand DNA virus expresses *E6* and *E7* oncogenes, which interact with p53 and pRB proteinas, respectively, promoting the development of neoplasias due to uncontrolled cell cycle activation and inhibition of apoptosis[11]. The high-risk HPV genome could be integrated into the host genome during carcinogenesis process, causing the lost 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 tumors progression is due to a systemically action, affecting the host’s physiological processes and triggering responses in the 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 has been 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 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 interferons 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 was 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 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 investigation.

***Breast cancer molecular subtypes classification***

It 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+ (≥ 20%); Ki67 (< 14%), HER2-; (2) Luminal B HER2-: RE+; RP (< 20%); Ki67 (≥ 14%), HER2-; (3) Luminal B HER2+: RE+; RP inddiferent; Ki67 (≥ 14%); HER2+; (4) HER2+: RE-, RP-, Ki67 (≥ 14%); HER2+; and (5) TN: RE-, RP-, Ki67 (≥ 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 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, Belgium), following the manufacturer’s instructions, based on the reverse hybridization principle, to identify 28 different HPV genotypes, by detecting specific sequences in the L1 region. The assay uses the proven SPF10 primer set for highly sensitive amplification of most clinically relevant HPV genotypes.

***Blood samples***

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

***Cellular subsets***

Cellular subsets quantification was performed by flow cytometry. 50 μl of whole blood was taken and 5 μl of monoclonal antibody was added. It was briefly vortexed and incubated for 15 min, at room temperature, in the dark. 800 μl of BD 1X lysis solution was added and incubated for 10 min, at room temperature in the dark. It 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 6 colors (configuration 4-2), with the BD FACS Diva 6.2.2 application.

Cell surface marker analysis was performed using CD4-FTIC/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). Absolute cell counts were calculated by multiplying the cell subset percentage by the total lymphocyte concentration present in peripheral blood.

***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).

**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%). 47.29% had alcoholic habit, while 36.49% had tobacco habit. 54.05% of the patientd used oral contraceptives and 59.46% had 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 family history of breast cancer. The mean age and tobacco habit showed staystical significant difference between breast cancer patients and beningn 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 finally, stage IV (6.25%). Regarding the histopathological diagnosis, the breast cancer tumors were infiltrating ductal carcinoma (79.16%), ductal carcinoma *in situ* (DCIS) (8.33%), lobullar 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%).

The HPV presence 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 75.0% 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. 50% of the HPV positive breast tumors were Luminal A, followed by the HER2+ type, with 25%. Luminal B HER2- and TN types represents 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, finding statistically significant differences in CD8+ count, between both study groups. The mean of each cellular subset decreased as the stage of the disease increased (*p* > 0.005). According to the breast cancer molecular subtypes, the HER2+ tumors had the lowest CD4+ and CD8+ values (Table 3).

The NK+ cells counts were elevated in the bening pathology group, with 1217.04 ± 778.69 cel/mm3 (range: 48.30–3193.18), in comparition with breast cancer patients (1053.79 ± 690.56 cel/mm3 (range: 187.55–3675.00)) (*p =* 0.651). A decrease in the mean of the NK+ cells was observed as the stage of the disease increased (*p =* 0.0827). According to the 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, the HPV+ breast cancer tumors had elevated cellular subtypes counts, in respect to the HPV- tumors. A decrease in the mean of NK+ cells was observed as the disease stage increased, in the HPV+ tumors.

**DISCUSSION**

In women around menopause, breast cancer is the most frequently diagnosed neoplastic disease, 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 over 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%. HER2+ tumors are detected between 15% and 20%, and TN 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 finally, HER2+.

In Venezuela, there are few studies that 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] indicate that of 110 patients evaluated, 40.0% present Luminal B HER2- tumors, followed by Luminal A (20.91%).

Genetic and risk factors identification, such as environmental and hormonal factors, have an increasing value and play an important role in breast cancer prevention[23]. These risk factors increase neoplastic process development probability, and will depend on the exposure time or individual genetic predisposition. Therefore, these can influence cancer development, even though it don’t directly cause the disease[24].

Among the non-genetic factors described previously, in the study group 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 develop breast cancer did not have any identifiable risk factor.

Epidemiological studies gave a first indication of an association between viral agents with 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 are suggested in cancer development, especially those cancers caused by HPV[8].

In this study, HPV presence was detected in 25.00% of the samples, finding that 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% and 29.4% for HPV positivity[25-29], being the one reported by Aguayo *et al*[30], the lowest on this group of studies and the one corresponding to South America, with 8.7%. As for genotypes found, all indicate the presence of HPV type 16 as the most frequent, in single infections or mixed infections with genotype 18.

However, other studies performed viral detection by PCR and genotyping by sequencing, and reported a greater presence of type 18. Such is the case of Kan *et al*[31], in 2005, who obtained 48% positivity for HPV in breast cancer biopsies, with 100% for genotype 18. As well as Heng *et al*[32], Antonsson *et al*[33], Glenn *et al*[34], and Lawson *et al*[35] who 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 here. Hence, the high-risk HPV types distribution for breast carcinoma are probably different from cervical cancer[36].

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

It is necessary more studies for evaluate the correlations between HPV presence and breast cancer molecular subtypes. With these results we could suggest that the presence of HPV is associated with better prognosis tumors and therefore could present better response to chemotherapy treatments, as in the case of HPV positive oropharyngeal squamous cell carcinoma (OPSCC). Multiple studies have confirmed that HPV positive OPSCC shows better response to chemotherapy and radiation, independently of treatment scheme[37].

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

Due to the low expression levels in most of normal tissues, a mechanism that could be affecting this A3B protein arises. The E6 HPV oncoprotein offers the first contact in viral infection and A3B-mediated mutagenesis. A model in which high-risk HPV E6 protein inactivate p53, causing the elimination of A3B gene transcription was proposed[38], for cervix cancer, head and neck[40], and now, HPV positive breast cancer[39], where the proteins p53, A3B and E6 are involved, raising the levels of DNA damage and mutations, and preventing answers to these damages and apoptosis.

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 decreased 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, besides there is a lack of information referring to 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 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 TCD4+ and TCD8+ lymphocytes concentration, compared to the benign pathology group, while CD4+/CD8+ ratio was higher. 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 lymphocytes behavior in relation to the breast cancer staging, the CD4+/CD8+ ratio decreased as the stage of the disease increased, coinciding with Jia *et al*[18], who reported a rate decrease when breast cancer advances.

The average of absolute values for NK+ cells in the breast cancer patients group was found to diminish compared to the benign pathology group. Verma *et al*[42] reported a lower NK+ cell count in breast cancer patients. They also evaluated the variation of these values during and after treatment, indicating 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 ageing. A diminished phagocytic capacity of dendritic cells leads to impaired antigen presentation and activation of the adaptive immune system. Besides, 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).

According to the breast cancer molecular subtypes, we found that the group of HER2+ tumors presented a decrease in the CD4+ count and in the CD4+/CD8+ ratio, while the TN tumors showed an increase in the NK+ cell count. Particularly the HER2+ group and Luminal B HER2+ 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, in the CD4+/CD8+ ratio, and an increase in CD8+ and NK+ cells, related to RE-, HER2+, TN tumors and with Ki67 ≥ 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+; this could suggest that the absence of estrogen receptors in HER2+ tumors is related to what was observed in this study population, 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, 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 high Ki67. Therefore, immunotherapy is one of the most promising approaches for breast cancer therapy. If new research establishes a role for lymphocytes subsets in the etiology of aggressive phenotypes, such as those characterized by being ER-, HER2+, presenting a Ki67 ≥ 14% and TN subtype, strategies of new treatments for these breast cancer types should include immunotherapy.

The cellular subsets values were increased in the HPV+ tumors, with respect to the HPV- patients, observing 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 in breast tissue. Despite the HPV evasion mechanisms of the immune response, about 90% of genital and skin lesions resolve in an average time of 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 CIN and invasive cancer. The high-risk HPV infection changes and modifications induced, includes 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, and 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 there are no references that evaluate the profile of cellular subsets in patients with HPV positive breast tumors, it was observed how the virus infected group showed higher values of CD4+, CD8+ and NK+ cells, in comparison with negative patients, being able to speculate that it is due to an activation of the immune response to viral infection in the mammary tissue.

It has been described that in HPV positive cases of oropharyngeal squamous cell carcinoma (OPSCC), patients show a greater proportion of circulating TCD8+ lymphocytes, compared with HPV negative cases, and these lymphocyte levels predict treatment response better than the 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 IFN and displaying cytolytic effects mediated by granzyme and perforin. CD4+ T cells also secrete IFN and instead mediate killing primarily by engagement with ligands for death receptors such as Fas or TRAIL, resulting in caspase-mediated apoptosis[49]. So, 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 (LSIL), whereas in cases of cervical cancer, NK+ activation receptors are considerably diminished. That implies a low cytotoxic activity by the NK+, facilitating the progression of the lesion and carcinogenesis[47].

In patients with breast cancer and HPV positive breast tissue, a decrease in the NK+ cells count was observed as the severity of the disease increased, implying a failure in the cytotoxic activity performed by the cells studied. It can be 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, comparing the efficacy and effectiveness data of cervical infections and high-grade lesions with those types of injuries, such data 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) have been detected in breast tumors between 0% to 86%, representing a posible risk factor, beside 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 makes it possible to offer better patients treatment.

***Research objectives***

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

***Research methods***

The patients inclusion was done prospectively and the breast cancer molecular classification were 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%), 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+ subsets were decreased in patients with breast cancer, respect to benign pathology, with a statistically significant difference between CD8+ values (*p =* 0.048). The mean of NK+ cells was increased in bening pathology group. HER2+ and Luminal B HER2+ tumors had the lowest counts of cell subsets. The HPV breast cancer patients had elevated counts of celular subsets.

***Research conclusions***

It can be observed that HPV positive breast cancer tumors have a better prognosis, correlated with Luminal A subtypes and also 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***

We suggest to evaluate the patients disease-free survival, based on HPV positivity in the tumor tissue, besides, to evaluate the viral load and HPV genome integration, the identification of HPV variants by sequencing, and the infiltrating lymphocytes in the tumor bed. As a final consideration, the experience of working with fresh samples is complex, involves a whole process of sample collection in the operating room, in addition to the management of bioethical parameters, generating the possibility of obtaining more accurate results.

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

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Breast cancer (*n* = 48)** | **Benign pathology (*n* = 26)** | ***P* value1** |
| **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 |

**1**Breast cancer group *vs* benign pathology group.

**Table 2 Mean absolute values for the cellular subsets in the breast cancer and bening patholgy groups**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Breast cancer (cel/mm3)** | **Benign pathology (cel/mm3)** | ***P* 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.

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

|  |  |  |  |
| --- | --- | --- | --- |
|  | **CD4+** **(cel/mm3)** | **CD8+** **(cel/mm3)** | **NK+** **(cel/mm3)** |
| 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+ | 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* value1 | 0.842 | 0.527 | 0.912 |

1Between breast cancer molecular subtypes.

**Table 4 Correlations between the breast cancer human papilloma virus status and the cellular subtypes**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **HPV+ (*n* = 14)** | **HPV- (*n* = 34)** | ***P* value**1 |
| **CD3+** **(cel/mm3)** | 1832.12 ± 537.08 | 1399.56 ± 665.69 | 0.563 |
| **CD4+** **(cel/mm3)** | 1139.92 ± 416.84 | 796.56 ± 416.84 | 0.091 |
| **CD8+** **(cel/mm3)** | 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/mm3)** | 1350.21 ± 736.79 | 950.47 ± 634.35 | 0.082 |

1HPV+ *vs* HPV-. HPV: human papilloma virus.