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Triple Marker Immunohistochemestry Analysis in Breast Cancer Mexican Patients

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RESUMEN

El Cáncer de Mama (CM) es un severo problema de salud pública en México. **Objetivo**: determinar los diferentes perfiles de expresión del CM ductal en mujeres mexicanas con base en el análisis de inmunohistoquímica (IHQ) de tres diferentes marcadores (RE, RP y HER2) y comparar dichos perfiles entre mujeres con carcinoma de inicio temprano (≤35 años al diagnóstico) y mujeres de más de 35 años.
Metodología: se estudiaron los casos consecutivos de CM ductal sin patrón específico registrados en el Departamento de Patología. Resultados: Se estudiaron 506 casos de CM; 43 eran de mujeres con CM de inicio temprano (8.5%). Los perfiles de IHQ fueron: triple negativo, 19.1%; HER2, 16.6%; luminal A, 56.3%, y luminal B, 8%. Los perfiles triple negativo, el de sobre-expresión de HER2 y el luminal B, ER+PR- fueron más frecuentes en mujeres jóvenes y se asociaron con mayor grado histológico. La frecuencia del grupo triple negativo (19.1%) fue mayor que lo usualmente reportado en la literatura (15%). La mayoría de estos casos tuvieron grados histológicos altos (II o III). Sin embargo, 2.1% fueron tumores bien diferenciados. *La sobre-expresión de HER2* se observó en el 28% del grupo con carcinoma de inicio temprano mientras que sólo representó el 15.5% en las mujeres de mayor edad.
Conclusión: los perfiles de expresión en mujeres mexicanas presentan características especiales y existe una diferencia significativa entre mujeres mayores y menores de 35 años. Estos hallazgos justifican un estudio prospectivo clínico que relacione los distintos perfiles de expresión por IHQ para determinar su comportamiento biológico. Esta evaluación es importante para la toma de decisiones terapéuticas, en especial, de pacientes de países en desarrollo donde otras técnicas caras no están disponibles para uso generalizado.
Palabras clave: cáncer, mama, inmunohistoquímica, perfiles moleculares.

ABSTRACT

Objective: To assess the different expression profiles of ductal BC in Mexican women based on IHC analysis of three markers (ER, PR, and HER2) and to compare them between women with EOBC (≤35 years of age at diagnosis) and patients over 35 years.
Methodology: All consecutive, new cases of invasive ductal carcinoma not otherwise specified, registered at the Pathology Department files were selected for study. General frequencies of each marker were determined and different profiles in tumor tissue samples were obtained.
Results: 506 tumor samples were studied; 43 of them from women with EOBC (8.5%). The IHC profiles of tumors were: triple negative, 19.1%; HER2, 16.6%; luminal A, 56.3%, and luminal B, 8%. Women with EOBC and older patients have significantly different profiles. The triple negative*, the HER2*-overexpressing, and the luminal B, ER+PR- profiles were more frequent in young women, and they were associated with higher histological grade. The frequency of the triple negative group (19.1%) was higher than usually reported (15%). Majority of triple negative cases had high histological grades (II or III). However, 2.1 % were well differentiated tumors. *HER2*-overexpressing profile predominated in the EOBC (28%) while the other group showed almost half of this frequency (15.5%).
Conclusion: Expression profiles in Mexican women have special features. These findings justify a prospective clinical study in order to determine the biological behavior of the different profiles. This evaluation is important for therapeutic decisions and the quality of life in women from developing countries where expensive diagnostic techniques are not available for all patients.
Key words: Breast, cancer, immunohistochemistry, molecular profiles.

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Breast Cancer (BC) is a worldwide health problem.[1] In Mexico, BC is the second most frequent malignant neoplasm[2] and the first cause of death in women with cancer.[3] The WHO system classifies BC in ductal and lobular carcinomas according to the origin of the malignant cells. Infiltrating ductal cancer is the most common breast cancer type and comprises 70% to 80% of all cases.[4] However, infiltrating ductal cancer is not a homogeneous group. After diagnosis of BC has been histologically confirmed, information concerning molecular markers that influence prognosis and treatment is required. None of the numerous molecular markers available has surpassed hormone estrogen and progesterone receptors and Her2/neu receptor (triple marker profile) regarding their relevance for selection of targeted therapies.[5] The HER2 gene (also known HER2/neu, ErbB-2, ERBB2) codifies for the human epidermal growth factor receptor 2. This gene is amplified from 2- to >20-fold in 30% of tumors. HER2 gene amplification is a significant predictor of both overall survival and time-to-relapse in patients with BC.[6]

During the past years, a new BC classification based on variations in gene expression patterns derived from cDNA microarrays has been developed.[7] BC could be classified into a triple negative group (basal epithelial-like) (ER-, PR-, HER2-), an *ERBB2*-overexpressing group (ER-, PR-, Her2 neu+) and a luminal-like group based on triple marker expression. The luminal epithelial/estrogen receptor-positive group is divided into at least two sub-groups: luminal A (ER+ and/or PR+, HER2–) and luminal B (ER+ and/or PR+, HER2+), each with a distinctive expression profile.[8] Survival analyses have shown significantly different outcomes for patients belonging to the various groups.[8] Different authors have found that both HER2+ and triple negative subtypes are more frequent in younger and premenopausal women, showing a higher percentage of cases of poorly differentiated tumors, higher S-phase fraction, and poor prognosis when compared with those of the luminal subtype.[9]

Some authors have found that inactivation of *BRCA1* gene appears to play a critical role in estrogen receptor (ER)-negative cancer stem cells (CSCs), driving tumor development mainly toward a triple negative or, in some cases, to a luminal B phenotype. The existence of common genomic alterations in triple negative, HER2+, and luminal B breast tumors may suggest a common cell origin arising from an ER-negative CSC. In addition, specific genomic aberrations in ER-positive tumors could provide cellular proliferation advantages when cells are exposed to estrogen. Probably, luminal A tumors arise from an ER-positive progenitor cell.[10]

As mentioned before, molecular classification of BC into distinct sub-groups was based on global mRNA expression profiles.[7] However, immunohistochemistry (IHC) has proved useful in identifying cellular expression status and specific protein location. Studies based on IHC staining of the three previously mentioned markers have corroborated the findings obtained by cDNA microarrays concerning the different prognosis and biological behavior of BC subtypes.[11] With this information in mind, is possible to use the same BC classification based only in IHC analysis.

The genetic background of Latin American population is different from that of Europeans, Asians or Africans. In fact, it is a mixture of them.[12] Therefore, it is necessary to assess the different expression profiles of ductal BC in Mexican women based on IHC analysis of ER, PR, and HER2, and to compare them with those reported in other populations. Knowledge of these profiles is important in the therapeutic management of women with BC. Besides, in Mexico 8%[13] of patients with BC are 35 years or younger at diagnosis (early onset BC/EOBC). This work was designed to determine first the frequency of the different profiles in Mexican women with BC and to study whether these profiles exhibit the same distribution in a group of EOBC patients.

MATERIAL AND METHODS

Protocol was approved by the Scientific and Ethics Committees of the Unidad Médica de Alta Especialización No. 25, Instituto Mexicano del Seguro Social.

Patients sample

All consecutive, new cases of invasive ductal carcinoma not otherwise specified (IDC NOS) registered at the Pathology Department files during the last, complete, three years were selected for study. Exclusion criteria included absence of histological slides and/or IHC stains, or lack of paraffin blocks for obtaining new slides if the original materials were not available. Samples from studies lacking positive control were also excluded.

Proceedings

We formed groups of women with EOBC (≤35 years of age at diagnosis) and patients over 35 years. General frequencies of each marker (ER, PR, and HER2) were determined. The following eight different profiles based on ER, PR, and HER2 staining in tumor tissue samples were obtained: Triple negative group (ER–, PR–, HER2–); *HER2*-overexpressing group (ER–, PR–, HER2+); luminal A group with three variants (ER+, PR+, HER2–), (ER+, PR–, HER2–), and (ER+, PR+, HER2–), and luminal B group with three variants (ER+, PR+, HER2+), (ER+, PR–, HER2+), and (ER–, PR+, HER2+). The IHC profile distribution, the relationship between tumor histological grade and age at diagnosis, the frequency of positive staining for ER, PR, and HER2, and the IHC profile distribution in terms of tumor histological grades were evaluated and compared between the two groups.

IHC analysis

The K1904 ER/PR pharmDxKit (Dako, California USA), a semi-quantitative IHQ assay, was utilized to identify estrogen receptor alpha (ER) and progesterone receptor (PR) expression in neoplastic tissues according to supplier instructions (http://pri.dako.com/28252_er-pr_pharmdx_interpretation_manual.pdf.)

The K5207 Herceptest™ (Dako, California USA), for Dako Autostainer was employed for HER2 assessment (*www.dako.com/prod_productrelatedinformation?url=support_herceptest_elearning_kit_k5207_aut_use_conf.htm)*.

Methodology

All cases were blinded reviewed by a team of two pathologists. If there was a discrepancy between the original report and the new revision, two pathologists reviewed the cases separately and then results were compared and a final decision was emitted. Cases were excluded if they did not satisfy the requirements.

Histological material was reviewed and the tumor was classified according to the modified Scarff-Bloom-Richardson (SBR) histologic grading system.[14] Percentages of tumor cells with positive, clear, and unequivocal nuclear staining for ER and PR and membrane staining for HER2 were determined.[15-17] At least 500 neoplastic cells per sample were analyzed in at least five different areas. The cutoff point for ER- and PR-positive expression was 10%. HER2 expression was considered positive if an intense and uniform (3+) membrane staining was observed in >30% of neoplastic cells.[18]

Statistical analysis

A general description of findings according with age group was made. A Pearson chi-square test was used to know the association between age at diagnosis of women with breast cancer (cutoff point, 35 years of age) and presence or absence of each of the three markers studied (ER, PR, and HER2). The R × C contingency chi-square test was employed to determine heterogeneity between age at diagnosis and the eight IHC profiles studied with its empirical significance level determined by permutations following the algorithm of Roff and Bentzen.[19] The R × C test was used to know the association between age at diagnosis and the three histological- grade tumors. A Pearson chi-square test was utilized to know the association between tumor histological-grade and each marker. The R × C test was employed to determine heterogeneity between the tumor histological grade and the eight IHC profiles. A value of $p<0.05$ was considered significant for these studies.

RESULTS

A total of 1,053 cases of malignant breast tumors were examined and diagnosed. 742 cases were invasive ductal carcinoma not otherwise specified (IDC NOS). Among these, 236 cases were excluded because of unavailable histological material or because the material did not adhere to the complete requirements, including control tissue. After the screening, 506 tumor samples were selected; 43 of them from women with EOBC (8.5%).

In general, ER, PR, and HER2 showed positive staining in 48.4, 52.6, and 24.5% of BC cases, respectively. An univariate analysis showed that IHC markers ER and PR were associated with age at diagnosis. Young women had less percentage of positive ER and PR breast tumors. However, no difference was observed with HER2 (Table 1). There was not association between age at diagnosis and tumor histological grade (Table 2). However, the three markers were associated with tumor histological grade. As showed in Table 3, frequency of nuclear positive staining for ER and PR decreased with higher histological grade. HER 2 membrane staining showed the opposite behavior. When the three markers were combined to obtain the profiles, subtype distribution was as follows: Triple

Table 1. Frequency of three markers (ER, PR and HER2) in women with breast cancer by age at diagnosis

	Age at diagnosis (years)			
Markers	*≤35 (%)*	*>35 (%)*	*Total (%)*	*X^2 (probability)*
ER				
+	30.2	50.1	245 (48.4)	6.22 (0.013)
–	69.8	49.9	261 (51.6)	
PR				
+	34.8	54.2	266 (52.6)	5.89 (0.015)
–	65.2	45.8	240 (47.4)	
HER2				
+	34.8	23.5	124 (24.5)	2.73 (0.098)
–	65.2	76.5	382 (75.5)	
Total	43	463	506	

ER: estrogen receptor; PR: progesterone receptor; HER2: epidermal growth factor receptor-2.

Table 2. Distribution of histological grade tumor by age at diagnosis

*Histological grade**	*Age at diagnosis (years)*				*Total*	*(%)*
	≤35	*(%)*	*>35*	*(%)*		
I	1	(2.3)	14	(3.0)	15	(3.0)
II	22	(51.2)	292	(63.1)	314	(62.0)
III	20	(46.5)	157	(33.9)	177	(35.0)
Total	43		463		506	

*Elston/Nottingham modification of Bloom-Richardson system.
R × C, $p = 0.262$.

negative, 19.1%; HER2, 16.6%; luminal A, 56.3%, and luminal B, 8% (Figures 1 and 2). Women with EOBC and older patients have significantly different profiles. Age at diagnosis and tumor histological grade showed significant association with the different expression profiles (Tables 4 and 5). Triple negative and HER2 overexpression subtypes were more frequent in patients with EOBC and showed higher histological grade. In fact, *HER2*-overexpressing profile was observed in 28% of the EOBC, while the other group had almost the half of this frequency (15.5%).

DISCUSSION

According to the available literature, although molecular classification of BC into distinct sub-groups was based on global mRNA expression profiles,[8] IHC studies paralleled microarrays findings.[20] In this study, 50% of cases expressed ER or PR. These percentages are similar to those described internationally. HER2 expression was nearly 25%, slightly lesser than the 30% reported for other populations.[21,22]

When comparing groups by age at onset, a significant difference in ER and PR expression was observed. Patients with EOBC had lower expression of these markers; nonetheless, HER2 expression was similar in both groups. However, the *HER2*-overexpressing group was more frequent in young women indicating that, in addition to individual expression of each marker, it is important to define the corresponding profile.

Although microarray-based BC classification includes three different profiles in each luminal A and -B type of BC, it appears that these are not similar. A study in patients with single-positive tumors showed that ER+/PR– tumors were found more frequently in elderly postmenopausal women. When compared with the double-negative phenotype, ER+/PR– showed an association with better outcome, but no such survival advantage was detected in the case of ER-/PR+ tumors. According to these authors, ER+/PR– and ER–/PR+ tumors are biologically and clinically distinct groups of BC that may require different treatment strategies, with ER–/PR+ exhibiting more aggressive behavioral characteristics.[23] Therefore, we analyzed the different profiles separately. It was evident that the triple negative and *the HER2*-overexpressing groups were more frequent in young women. Luminal B- type of BC profiles

Table 3. Distribution of positive staining for ER, PR, and HER2 by histological grade tumor

	*Grade**				
Markers	*I*	*II*	*III*	*Total*	*X^2 (probability)*
ER					
+	13	157	75	245	11.69 (0.003)
–	2	157	102	261	
PR					
+	11	183	72	266	16.90 (0.0001)
–	4	131	105	240	
HER2					
+	3	64	57	124	8.72 (0.013)
–	12	250	120	382	
Total	15	314	177	506	

ER: estrogen receptor; PR: progesterone receptor; HER2: epidermal growth factor receptor-2.
*Elston/Nottingham modification of Bloom-Richardson system.

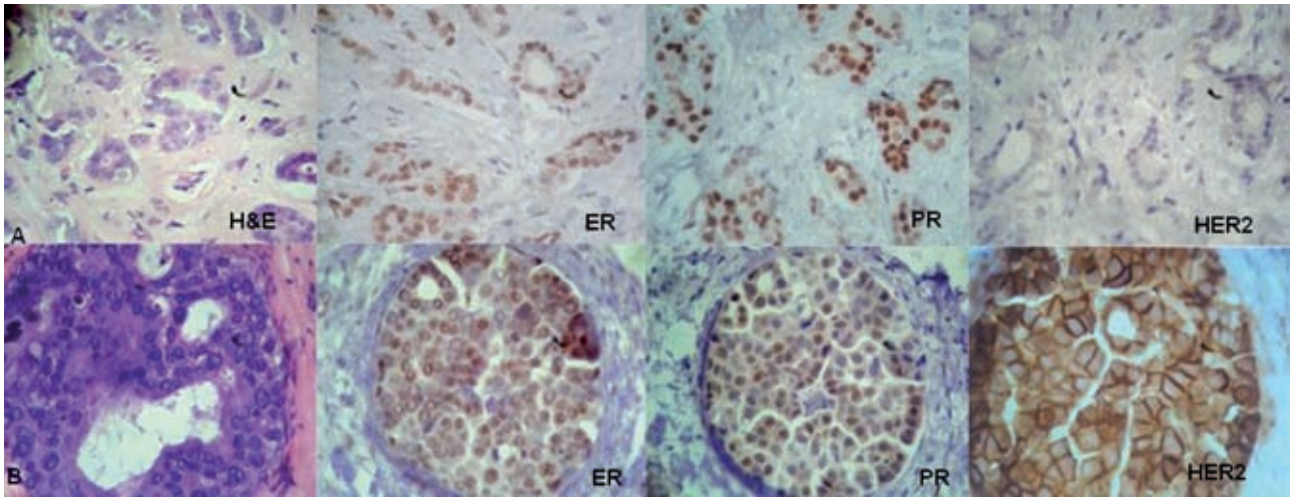

Figure 1. A. Luminal A (ER+, PR+, HER2 -). B. Luminal B (ER+, PR+, HER2+) expression patterns of breast cancer (H&E and IHC study).

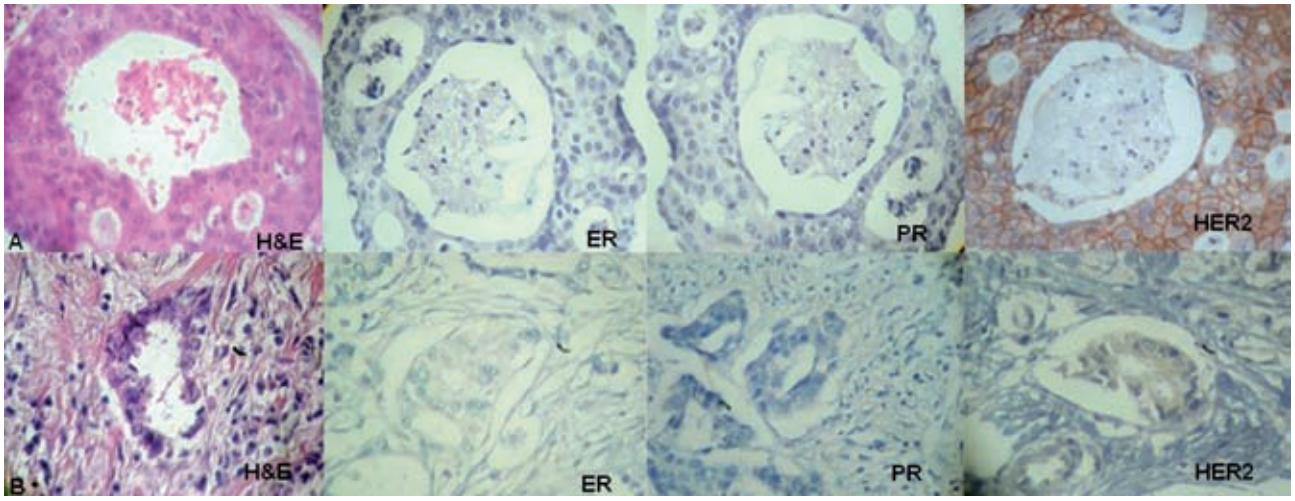

Figure 2. A. *HER2*-overexpressing group (ER–, PR–, HER2+). B. Triple negative- like group (ER–, PR–, HER2–) expression patterns of breast cancer (H&E and IHC study).

exhibited similar frequencies in both groups, except for a slight predominance of type ER+PR– in young women. Luminal-A types with ER+, PR– and ER+, PR+ were observed more frequently in older women.

The frequency of the triple negative group (19.1%), was higher than usually reported (15%).[24] Several reports described higher percentage of triple negative breast cancer in African and African-American premenopausal women. It is important to emphasize that although the majority of triple negative cases have high histological grades (II or III), there are some well differentiated tumors (2.1% of our cases) classified as triple negative, so it is advisable to study these three markers even in low-grade carcinomas.

We did not find association between *HER2*-overexpressing profile and low grade carcinomas. Regarding the prevalence of *HER2*-overexpressing group, it predominated in the EOBC (28%) while the other group had almost half of this frequency (15.5%). We found 8% of luminal-B type BC and the distribution of the variants showed that the triple positive profile was the most common of the three variants (ER+,PR+ HER2 neu+ 3.8%; ER+,PR-, Her2neu+ 1.6%; ER-,PR+, Her2neu+, 2.6%). A study of

Table 4. Distribution of immunohistochemical profiles for ER, PR, and HER2 by age at diagnosis

Profile			*Age at diagnosis (years)*		
ER	*PR*	*HER2*	*≤35 (%)*	*>35 (%)*	*Total (%)*
–	–	–	13 (30.2)	84 (18.1)	97 (19.1)
–	–	+	12 (27.9)	72 (15.6)	84 (16.6)
–	+	–	5 (11.6)	62 (13.4)	67 (13.2)
–	+	+	0 (0.0)	13 (2.8)	13 (2.6)
+	–	–	1 (2.3)	50 (10.8)	51 (10.1)
+	–	+	2 (4.7)	6 (1.3)	8 (1.6)
+	+	–	9 (20.9)	158 (34.1)	167 (33.0)
+	+	+	1 (2.3)	18 (3.9)	19 (3.8)
	Total		43	463	506

ER: estrogen receptor; PR: progesterone receptor; HER2: epidermal growth factor receptor-2.
Basal epithelial-like group: – – –.
HER2-overexpressing group: – –. +.
Luminal A group: – + –, + – –, + + –.
Luminal B group: + + +, + – +, – + +.
R × C, $p = 0.028$.

Table 5. Distribution of the immunohistochemical profile for ER, PR, and HER2 by histological grade tumor

Profile			*Histological grade tumor**						*Total*
ER	*PR*	*HER2*	*I*		*II*		*III*		
			n	*(%)*	*n*	*(%)*	*n*	*(%)*	
–	–	–	2	(2.1)	50	(51.5)	45	(46.4)	97
–	–	+	0	(0.0)	46	(54.8)	38	(45.2)	84
–	+	–	0	(0.0)	52	(77.6)	15	(22.4)	67
–	+	+	0	(0.0)	9	(69.2)	4	(30.8)	13
+	–	–	1	(1.9)	34	(66.7)	16	(31.4)	51
+	–	+	1	(12.5)	1	(12.5)	6	(75.0)	8
+	+	–	9	(5.4)	114	(68.3)	44	(26.3)	167
+	+	+	2	(10.5)	8	(42.1)	9	(47.4)	19
	Total		15		314		177		506

ER: estrogen receptor; PR: progesterone receptor; HER2: epidermal growth factor receptor-2.
*Elston/Nottingham modification of Bloom-Richardson system.
HER2-overexpressing group: – – +.
Luminal A group: – + –, + – –, + + –.
Luminal B group: + + +, + – +, – + +.
R × C, $p = 0.0000001$

2544 cases of invasive breast cancers in American women reported prevalences of 73, 12, 11 and 4% of luminal A, luminal B, triple negative and Her2-overexpressing types respectively.[25] These figures contrast with those found in our patients (luminal A, 56.3%, and luminal B, 8%, Triple negative, 19.1%; HER2, 16.6%). It is understandable that the distribution of the different profiles varies as the population of the Americas consists of a mixture of races in different proportions depending on the geographical location. In Mexico our population is predominantly Mestizo.[12]

In this study, there was no relationship between tumor histological grade and age at diagnosis. Notwithstanding this, it was notorious that ER and PR-positive staining decreased according to higher histological grade. The inverse situation was observed for HER2 expression. Finally, it was evident that the eight different BC profiles had different relationships with histological grades. The triple-negative and HER2-overexpressing groups were

associated with higher histological grade, luminal A subtype cases in general were more frequently grade II, and luminal B ER+, PR-HER2+ was predominantly grade III.[26]

In conclusion, this IHC study based on ER, PR, and HER2 detection demonstrated that expression profiles in Mexican women have special features. To pathologists, this study has demonstrated that there are ductal breast tumors that exhibit all the different combinations of expression of the three IHC markers and that these patterns have relationship with age and histological grades. This study has shown for the first time the overall distribution of the different profiles in Mexican patients and not just the frequency of individual profiles (triple negative or HER2 over-expression profiles rates).[27]

These findings justify a prospective study with a larger sample of different ductal-cell carcinoma subtypes separately, including luminal-A and B-subtype variants, in order to determine their clinical behavior and significance. These evaluations are important for therapeutic decisions in patients from developing countries where expensive diagnostic techniques are not available.

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