ORIGINAL RESEARCH

Breast Cancer Molecular Subtypes in Moroccan Women

Dr Wissal Mahir1, 3*, MD, Dr. Lamiaa Rouas1, 3, MD, Dr. Driss Ferhati2, MD, Dr. Brahim Rhrab2, MD, Dr. Zaitouna Alhamany1, 3, MD, Dr. Nadia Cherradi, MD
1 : UPR d’Anatomie Pathologique. EREPT (équipe de recherche en pathologie tumorale). Faculté de Médecine et de Pharmacie de Rabat, Mohammed V University, Rabat.
2 : Service de Maternité 1 Centre Hospitalier Ibn Sina, Rabat, Maroc

Received 13 July 2016; Revised 11 September 2016; Accepted 15 September 2016.

ABSTRACT

Introduction: Breast cancer remains despite the therapeutic progress, the leading cause of death by cancer among women. It represents a group of very heterogeneous clinical, histopathological and molecular diseases. Molecular heterogeneity has been demonstrated by genomic analysis, even for similar histology cancers. Four subgroups of breast carcinomas are distinguished: Luminal A, Luminal B, HER2 over expression and Basal - like. The Immuno-histo-chemical analysis useip (estrogen receptors) RE, the PR (progesterone receptors), the (Human Epidermal Growth Factor Receptor-2), the Ki67 (proliferation marker) HER2, CK5/6 has shown a subdivision into subgroups similar to those found by genomic analysis. These subgroups are different from the point of view of clinical course and response to adjuvant treatment.

Objectives: The aim of this work is to study the molecular profile of the breast cancers by immunostaining on Moroccan series to a classification with a prognostic value allowing a treatment tailored to each group of patients. Furthermore, the molecular subgroups were correlated to other clinical and histological factors.

Material and methods: It is a prospective study of the laboratory of Anatomy and Pathologic cytology of the children's Hospital, the service I of the maternity hospital in Rabat and in cooperation with the United Nations Centre of pathological anatomy. To do this, 88 cases of breast cancer together were diagnosed between January 1, 2010 and December 31, 2014, taking a period of five years. All tissue samples made subject study of Immuno-histo-chemistry with the following markers: RE, PR, HER2 and Ki67. Only negative triple cases (HR and HER2 negative) benefited from an additional marking with CK5/6 and EGFR to set the basal profile.

Results: Series of 88 cases of mammary carcinomas observed on operating parts, ranged in age between 28 and 84 years old, with an average of 51 ± 12, 8. Carcinoma infiltrating non-specific (DOCTORS) was the most frequent (87.5%). Ranks histo-prognostic Scarff Bloom and Richardson (SBR) 2 and 3 respectively accounted for 45.5 and 51.1% of cases and only 2, 3% of the DOCTORS were grade 1. The Luminal B (53.4%) was under the most common molecular group, followed by Luminal A (23.9%), HER2 + (15.9%) and triple negative (6.8%). The correlation of molecular type of tumors with different prognostic factors showed only one significant connection with the SBR grade.

KEY WORDS: Breast Cancer; Molecular Classification; Immunohistochemistry; Morocco.

Corresponding author:
Dr Wissal Mahir, CEDOC-SVS, Facuté de Médecine et de Pharmacie, Université Mohamed V, Rabat, Morocco.
E-mail : wissal.maher@gmail.com

Copyright © 2016 Mahir Wissal et al.
This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

Breast cancer is a disease characterized by a vast clinical, histo-pathological and molecular heterogeneity, where there is a different prognosis in terms of overall survival and sensitivity to existing therapies. Some patients with similar clinical and morphological characteristics may have an answer and a divergent clinical course evolution. This shows different tumor biology. Although many genes have been studied in cancer of the breast, only two markers, hormone receptors and Her2, are validated in common practice and taken into account to predict response to cancer treatments. The study of
genomic profiles is essential to offer "a la carte" treatment to these patients and to better evaluate the prognosis of their tumors. These studies are done with the new techniques of molecular genetics and high-speed techniques, using DNA or "microarrays" chips his last technique generating "pictures" of the State of the expression of the genes of a cell, allowed to identify new markers and led to new molecular classification of breast cancers. [2] in 2000, Peru and al. were the first to subdivide the breast cancers in molecular subclasses according to their gene expression profile, by applying the technique of analysis by hierarchical clustering not supervised on 65 samples from 42 patients using a list of genes. [3] Initially, they have distinguished four types of carcinomas compared to normal breast tissue: Lminal, HER2, basal-like and normal like.

The luminal group is characterized by the expression of receptors to oestrogens and mainly the genes expressed by the mammary epithelial cells present in the light of the milk ducts: luminales 8, 18 and 19 cytokeratins and the GATA-3 gene. [4] the HER2 group is characterized by the overexpression and amplification of the gene HER2, located on chromosome 17q 12. [5] The Group basal like expressed the same genes as the leaves/myoepithelial cells (Cytokeratine, vimentin...) of normal breast tissue. [6] the Group normal like is characterized by a triple negative profile and the expression of genes in normal breast tissue and adipose tissue.

This group of tumors was waived later saw that it corresponded to a certain contaminated tumors selection bias by normal breast tissue. [7] However, the genetic profile by the method of microarray analysis is not always possible, because of the cost and difficulties of this technique: restricted access requiring hardware frozen tissue material. Thus, several authors substituted it by an Immunohistochemistry study, which may serve as a surrogate for the microarrays in order to define the Sub molecular classes. The technique of Immunohistochemistry (IHC) highlights the protein expression of these breast tumors, by using biomarkers such receptors estrogenic (RE), progesteroniques (RP), factor of growth Her2, Ki-67 and many other markers such as the cytokeratins of high and low molecular weight (CK8 18, CK5/6 etc.). [8,9,10,11] the IHC technique allows to classify correctly at least 75% of breast tumors. These molecular subtypes have been validated by other teams on independent series of breast cancers. Allowing to taking into account in the management of breast cancer. [13,14] However, despite efforts to characterize the protein markers allied to each subclass. He stay 10 to 15 percent of breast tumors that do not belong to any of the groups described above. [15] the purpose of our work is to study the molecular profile of the breast by immunostaining. Molecular subgroups were correlated to other clinical and histological factors. This classification will establish a prognosis and the prescription of a treatment tailored to each group of patients.

MATERIALS AND METHODS

Patients

This work is forward-looking. It was carried on 88 cases of breast cancer, collected and diagnosed at the Hospital of maternity-children in Rabat and at the Centre of United Nations pathological anatomy of Rabat, between January 1, 2010 and December 31, 2014, being a five-year period. The majority of cases have been made to the CHU-maternity Souissi in Rabat. An operating sheet has been established as follows:

- The name and surname of the patient as well as their number of pathological anatomy.
- Their age
- The type of sampling (samples for frozen review, Lumpectomy, mastectomy or quadrantectomy).
- The site of the injury.
- The size of the lesion (in mm) for operating rooms.
- The histological type according to the classification of the 2012 WHO.
- The ganglionic status: total number of metastatic lymph nodes and lymph,
- Grade SBR (grading of Scarff, Bloom and Richardson)
- Presence of vascular emboli
- The expression of hormone receptors (RE and RP).
- The expression of HER2 (Human Epidermal Growth Factor Receptor-2).
- The expression of Ki67 (proliferation marker).
- The expression of Cytokeratine CK5/6 and,
- The expression of the EGFR(Epidermal growth factor receptor).

Methods

Management of the operating parts appealed to samples focused on the tumor or any other macroscopically abnormal area. If the tumor is deep, deep limit is inked and picked. Lumpectomy parts were included in full and their margins have been inked. The axillary glands were taken in full and numbered.

For operating parts after neo-adjuvant chemotherapy, the macroscopic residual tumor size was measured as well as the minimum distance to the riverbanks in millimeter. Parts of Lumpectomy were examined according to a grid. Regarding parts of mastectomy, the tumor nodule was sampled following the ad hoc number of samples for analyzing the remainder in extenso. In the case of remaining unidentifiable macroscopically, the operating room is sampled as exhaustively as possible.

After dehydration and inclusion in paraffin, paraffin blocks were cut in microtome and the obtained ribbons put over blades intended for coloring by the bottle-eosin. Reading the optical microscope stated the following morphological parameters: the histological type, grade SBR, tumor size, and the invasion ganglionic and the presence or not of vascular embolus.

All samples were investigated with the following markers Immuno-histo-chemistry study: RE, PR, HER2, Ki67. Only negative triple cases (HR and HER2 negative) benefited from an additional marking with CK5/6 and EGFR to set the basal profile. Immuno-histo-chemical reactions were performed from tumor cups (5µm) in paraffin previously fixed in buffered 10% formalin. The paraffin block chosen had a non-necrotic tumor fragment and normal mammary structures using internal witness. For the HER2, witnesses’ blades are supplied with the Kit. The cuts have spread over SIL-are blades or pre-treated to gelatin and alum of chrome. After de-waxing at 60 ° for one hour, an antigenic unmasking has been achieved by heat treatment in a microwave oven (800w / during 17 min) in a buffer (citrate or EDTA according to the used Ac). The blades were then placed in an oxygenated solution of H2O2 during 5 min for block endogenous peroxides, and then they have been washed by a PBS (phosphate buffer saline). In order to block nonspecific binding sites, a blocking serum (Ultra- HRP Kit PNI M2391, Immunotech Protein color Agent) has been used. The second rinse in PBS (pH 7.6 during 5 min).

The primary antibodies used as well as the detection Kits are described in the following table 1:
After the revelation, the blades were hand stained to the hematoxylin. The cuts were then dehydrated and mounted between slide and cover slip using a Eukitt® environment. Reading to the optical microscope has defined the slot of each molecular tumor group as shown in table 2.

The correlation has been expressed by Spearman test to calculate the correlation coefficient (p). The research of the factors involved is performed using the χ² test. The threshold of statistical significance is 0.05.

RESULTS

They are given in tables 3 and 4.

A : Histologic section of infiltrating ductal carcinoma. Hematoxylin and eosin stain (Gx25). B: Nuclear immunostaining of ERα in infiltrating ductal carcinoma (Gx40). C: Nuclear immunostaining of PR in infiltrating ductal carcinoma (Gx40). D: Immunostaining of HER2 in infiltrating ductal carcinoma (score 3+) (Gx25). E: Nuclear immunostaining of Ki67F: Immunostaining of CK5/6. G: Membrane staining of HER1

The clinico-pathological study looked at 88 patients with breast cancer. Recruited patients age ranged between 28 and 84 years old, with an average of 51. Sent samples were mainly represented by the mastectomies (56%), followed by a Lumpectomy (35%), frozen review (7% samples) and the quadrantectomies (2%).

Forty six (46) of our patients (52.3%) had a tumor size between 20 and 50mm, and 17% of patients had tumors which size exceeded 50 mm. (Table 3) the infiltrating non-specific carcinoma (DOCTORS) was the most frequent (87.5%), followed by the Intraductal Carcinoma (CIC)(6.8%). Lobular carcinoma infiltrating (CLI) represented 2.3% of cases. Rare histological types met a case of Mucinous Carcinoma, a metaplastic squamous cell carcinoma case and a case of carcinoma infiltrating apocrine.

Table 1: List of antibodies used in this study

<table>
<thead>
<tr>
<th>Ag</th>
<th>Clone</th>
<th>Source</th>
<th>Unmoulding solution</th>
<th>Dilution</th>
<th>Incubation Time</th>
<th>Revelation</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE</td>
<td>Cl Iene ER125, Immunotech</td>
<td>Mouse monoclonal antibody</td>
<td>Citrate buffer Ph 6.0, DiaPath</td>
<td>Ready to use</td>
<td>1H</td>
<td>Biotinylated secondary antibody (Ultratech HRP Kit PNM2391, Immunotech) streptavidine-peroxidase (Ultratech HRP Kit PNM2391, Immunotech) DAB+</td>
</tr>
<tr>
<td>RP</td>
<td>Cl Iene PR10A9, Immunotech</td>
<td>Mouse monoclonal antibody</td>
<td>Citrate buffer Ph 6.0, DiaPath</td>
<td>Ready to use</td>
<td>1H</td>
<td>Biotinylated secondary antibody (Ultratech HRP Kit PNM2391, Immunotech) streptavidine-peroxidase (Ultratech HRP Kit PNM2391, Immunotech) DAB+</td>
</tr>
<tr>
<td>HER2</td>
<td>Ref A0484, Dako</td>
<td>Mouse polyclonal antibody</td>
<td>Citrate buffer Ph 6.0, DiaPath</td>
<td>Diluted to 1/50</td>
<td>30min</td>
<td>Dako REAL™ EnVision™ Detection System, Peroxydase/DAB+</td>
</tr>
<tr>
<td>CK5/6</td>
<td>Cl Iene D5/16 B4, Dako</td>
<td>Mouse monoclonal antibody</td>
<td>EDTA buffer Ph 9, DiaPath</td>
<td>Ready to use</td>
<td>1H</td>
<td>Dako REAL™ EnVision™ Detection System, Peroxydase/DAB+</td>
</tr>
<tr>
<td>Ki67</td>
<td>Cl Iene MIB1, Dako</td>
<td>Mouse monoclonal antibody</td>
<td>EDTA buffer Ph 6.0, DiaPath</td>
<td>Diluted to 1/50</td>
<td>15min</td>
<td>Dako REAL™ EnVision™ Detection System, Peroxydase/DAB+</td>
</tr>
<tr>
<td>EGFR</td>
<td>Cl Iene DAK-H1/1197, Dako</td>
<td>Mouse monoclonal antibody</td>
<td>EDTA buffer Ph 9, DiaPath</td>
<td>Diluted to 1/50</td>
<td>30min</td>
<td>Dako REAL™ EnVision™ Detection System, Peroxydase/DAB+</td>
</tr>
</tbody>
</table>

Table 2: Immuno-histo-chemical Characterization of each sub-group

For hormone receptors, infiltrative carcinomatous component was viewed all over the tumor section. The marking is nuclear and the result is expressed in percentage of cells rounded to the dozen and average intensity of marking (low, medium, high).

The threshold of positivity for RE (estrogen receptors) and PR (the progesterone receptors) is set at 10% of positive cells regardless of the intensity of marking. For the Her2, the results are expressed in percentage of positive infiltrating tumor cells with a circumferential membrane marking. A witness blade provided by Dako is used every time. The score ranges from 0, +1, +2 and +3. A FISH/ICHS (Kit HER2 CISH pharmDx™) complementary has not done for all equivocal cases +2, due to its unavailability in the CHU.

Regarding the Ki67, the result is expressed as a percentage of nuclei stained by the Ki-67 antibody, taking into account all the intensities of marking and not just the areas 'hot spots'. The positivity threshold is 20%. For the CK5/6 and the EGFR, the tumor was noted positive in the presence of a marking membrane without taking into account the percentage.

Statistical analysis: all data are entered using the software SPSS version 20. The intensities of marking and not just the areas 'hot spots' . The positivity threshold is 20%. For the CK5/6 and the EGFR, the tumor was noted positive in the presence of a marking membrane without taking into account the percentage.

Statistical analysis: all data are entered using the software SPSS version 20. The results were expressed in number (and percentage) for qualitative variables and the quantitative variables as average or median.
The histological grading according to Scarff Bloom and Richardson (SBR) helped identify 51.5% of grade III tumors, 45.5% of carcinomas of grade II and 2.3 grade I (table 3) the ganglionic invasion was observed in 44 cases (50%), 20 cases had no ganglionic impairment. Of the 88 cases, 68 (77.3%) were positive for receptors to estrogens (RE) and/or progesterone (PR). A strong over expression of the protein Her2/neu (3 +) was detected in 34 cases (38.6%). Score 0 and 1 + tumors accounted for 35.2% of the cases, while 26.1% of cases showed a low protein expression (score 2 +). For these cases, 69% of them received an amplification of the gene by FISH search, and only 31% had presented the HER2 gene amplification. (Table 3) Ki67 tumoral proliferation index was higher than 20% in 56.1% of the cases. (Table 3) A research of the cytokeratins 5/6 with EGFR to define tumors basal like the number 9 respectively revealed 66.7% and 44.4% positivity. (Table 3) The Sub molecular types are returned in order of frequency: 53.4%, tumors were of type luminal B, 23.9% of tumors are basal like type luminal A, 15.9% of type HER2 + and 6.8% type. (Table 3) The difference in age in different molecular groups was not significant. The correlation of molecular types with different morphological classic prognostic factors showed that the CINS represented the most common histological type for 4 Molecular classes. (Table 4) The SBR grade was significantly associated with the Sub molecular types (p < 0.001); the luminal A group included the highest rate for the histological grade II (18.8%) and the lowest rate for the histological grade I (9.5%), while all of the basal-like tumors were grade III (100%). Vascular embols were more present in HER2 and Luminal B groups (42.9% and 23% respectively) (Table 4) Tumors of size larger than 5 cm were observed especially in Basal like groups (33.3%) and Luminal B groups (42.9% and 23% respectively) (Table 4) Regarding the ganglionic invasion, it was more common in both groups Luminal B and HER2 with 46.4% and 78.8% respectively. (Table 4)
Breast cancer is a very heterogeneous disease clinically, histologically and biologically. The grading is currently based on a number of clinico-pathological prognostic factors and predictive biomarkers limited to hormone receptors and HER2. Immuno-histo-chemical analysis using a RE (the estrogen receptors), PR (progesterone receptors), the HER2 (Human Epidermal Growth Factor Receptor-2), Ki67 (proliferation marker), CK5/6 (cytokeratine 5/6) and EGFR (Epidermal growth factor receptor) showed a subdivision into subgroups similar to those found by genomic analysis. Our work has focused on 88 cases, collected and diagnosed at the Hospital of Maternity-children in Rabat and at the Centre of United Nations pathological anatomy of Rabat. The studied population showed a median age of 51 years (with a standard deviation of 12.8). This result is similar to the Thai and Iranian study where the average age at diagnosis was respectively 52 years and 50 ± 12 years. The Jordanian population showed an average age of around 45 years. Another study at Marshfiled Clinic at St Joseph Hospital of Wisconsin revealed an average age higher than 62.7 years old. The average tumor size is 35 mm (stage 2). The tumors were classified grade II and III of SBR in 45.5 and 51.1% respectively, and grade I in 2.3%. The association between the Sub molecular groups and this factor was significant with a p < 0.001. The SBR rank represents an important and independent prognostic factor for metastatic relapse and survival linked to cancer. The 10-year survival cancer is over 90% for grade I tumors, about 90% of 70% for grades III and II grades. In our series, the dissection study showed a ganglionic invasion in 50% of cases with a number of positive lymph nodes greater than 1 and superior to 10 lymph in 10.2% of the cases. These results are consistent with those found by genomic analysis. The study of Immunohistochemical profile showed that the luminal type prevalence was higher (77.5%) than that of the type not luminal (22.7%). The luminal type represented the most common molecular group in our series. These findings are consistent with the American, Thai, Chinese and Iranian studies. The luminal B was the most common in our study (53.4%). This percentage does not match the result of the team of the CHU of Fez where the Sub group Luminal A was the most frequent (54.3%) [8]. Several studies have found the same result as the Moroccan team (Fez), Iran (63.8%), Thailand (59.3%) and India (34%) [17, 18, 27, 28]. Among non-luminal subtypes in this study, the basal like (6.8%) was less frequent than the slot type HER - 2 + (15.9%). This finding is consistent with the Japanese study, where the percentage of the basal like was of the order of 8% [29] and close to the Brazilian study where the percentage of the Sub group HER2 was 16.4% [30]. On the other hand, Iranian and Thai studies have found that the basal like group is more common than the HER2 group with a percentage of 15.9% and 1.1% [17, 18]. In Iran, the slot type HER - 2 + was the least common among the sub groups. The results of the studies reported in the literature are summarized and compared to our results in table 5. The luminal A group was divided into two subtypes. However, the criteria of definition of the luminal group are still based on different approaches using the Ki67 expression of PR and / or the expression of the Hermitage. It was initially proposed that cancers ER + on expressing the HER2 and being high Ki67 (≥14%) are qualified as luminal B, While those with a negative HER2 and a percentage of low Ki67 (< 14%) as luminal A. Later, during the "St Gallen International Breast Cancer" conference in 2013, the experts recommended a threshold of Ki67 ≥20% as a sign of high status in the definition of cancer type luminal B with a percentage of RP≥20% for the luminal type A. In our study, cancers of the luminal A subgroup, present a grade SBR II (76.2%), with absence of ganglion invasion (33.8%) and vascular embols (61.9%). While tumors of the luminal B subgroup, join the SBR grade III (59.6%), with ganglion invasion (1 to 3 metastatic lymph nodes) in 25.5%. Our results are consistent with the literature; the two subgroups are associated with clinical changes and different therapeutic responses.
Cancers of the luminal A subgroup, correspond to tumors little proliferation of low grade, associated with a better prognosis than other subgroups and respond positively to the hormone treatment so cancers of the luminal B subgroup meet adjuvant chemotherapy. [1] The HER2+ Group brings together the tumors having a strong expression - unequivocally - antibody anti-HER2 (3+), or those who have proven the HER2 gene amplification rated by hybridization techniques in situ (FISH/ISH) in equivocal cases (+2). [5] Thirteen to twenty percent (13-20%) of breast tumors amplify the HER2 gene and about 55% of these cases are ER-negative [16]. In our study, these tumors showed a SBR grade III (57.1) with a ganglion invasion greater than 10 in 35.7% of the cases. This is consistent with the Brazilian study that showed that the HER2 gene amplification is a predictor of poor prognosis [16].

Following the development of a humanized monoclonal antibody directed against HER2, clinical trials have shown the benefit of using a treatment anti-HER2 in patients with HER2-positive breast cancer, such trastuzumab inhibiting the HER2 or lapatinib which inhibits the tyrosines kinases [32]. Following the development of a humanized monoclonal antibody, the HER2 gene was amplified in 35% of cases. This is consistent with the Finnish, Swedish and English studies that have shown that like the basal group is associated with a high tumor aggressiveness and unfavorable prognostic factors. [36, 37, 38] Patients of this subgroup may benefit from therapy that targets the EGFR (HER1). [39]

CONCLUSION
To conclude, the current anatomo-clinical classification remains insufficient to define the adjuvant treatment which is appropriate for each patient, and only the genomic classification of Sørlie and Peru made possible the establishment of distinct molecular groups, highlighting the clinical and morphological heterogeneity. Our study shows that Subtyping based on the immunostaining can be used easily by replacing the genomic techniques to subdivide the breast cancer molecular classes. It is an easy technique, inexpensive and reproducible in ACP Labs, and so would, in our context, be a better support of breast cancers, from the diagnosis step until the therapeutic step and this being as well in the Central devices (rural) structures.

In our series, the study of Immunohistochemical profile showed that the Sub group Luminal B was the most frequent (53.4%), followed with the Luminal A (23.9%) HER2 (15.9%) and Basal Like (6.8%). The correlation between the molecular groups and the histo-forecast factors represented a significant association with the SBR grade only (p < 0.001). These subgroups showed significant differences compared to the size of the tumor, histological grade, ganglionic invasion and the presence of vascular embols. It's the biological entities that consider the implementation of therapeutic trials, particularly for the Group of Basal-like tumors. The latter remains the most aggressive group, biologically and clinically, what drives toward a planification of treatment and the targeted therapy, in addition to justifying the use of markers of base (CK5/6, CK14 or CK17) and/or EGFR. [33, 34] They over express the regulators of the cell cycle, including the tetracycline, the CD44, and the P-cadherin [35] in our series, the tumors Basal like had the highest percentage (83.3%) of tumors of large size (>2.0 cm) and were all classified grade SBR III with a number of metastatic lymph nodes greater than 1 in 39.8% of the cases. Which is consistent with the Finnish, Swedish and English studies that have shown that like the basal group is associated with a high tumor aggressiveness and unfavorable prognostic factors. [36, 37, 38] Patients of this subgroup may benefit from therapy that targets the EGFR (HER1). [39]

PATIENT CONSENT
Written informed consent was obtained from patients for publication of this study.

COMPETING INTERESTS
The authors declare no competing interests.
REFERENCES


