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CONCORDANCE BETWEEN IMMUNOHISTOCHEMISTRY AND RT-qPCR FOR MOLECULAR TYPING OF FEMALE BREAST CANCER IN BOUAKE

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ABSTRACT

Background: Immunohistochemistry is a technique commonly used in the diagnosis of breast cancer. It is performed on histological preparations, following conventional histological examination. This lengthens the time it takes for patients to receive their results. RT-qPCR has been proposed as an alternative for shorter turnaround times compatible with early management. **Objective:** In the present study the aim was to determine the concordance rate between immunohistochemistry and RT-qPCR analyses on fresh biopsy samples from breast cancer patients in Bouaké. **Methods:** Studies on this subject have analyzed frozen paraffin blocks. This prospective descriptive cross-sectional study analyzed the expression of estrogen receptor α (ER1), progesterone receptor (PR) and human epidermal growth factor β (HER2) genes by immunohistochemistry and RT-qPCR. It took place over a 16-month period (September 2023-January 2025). Patients came from the 5 diagnostic and management centers for breast pathologies in the Gbêkê region**. Results:** The mean age was 43.8 ± 11.0 years, with extremes of 24 and 71 years. More than two-thirds of patients (69.8%) had hormone-sensitive cancer, with a preponderance of luminal A (44.2%) followed by luminal B (25.6%). The estrogen receptor gene (ER1) was overexpressed in 44.2% of carcinogenic breast biopsies. PR and HER2 genes expression was elevated in 53.5% and 34.9% of cases respectively. Cohen's κ coefficient of concordance between IHC and RT-qPCR was 0.86, 0.64 and 0.79 for ER1, PR and HER2 respectively. **Conclusion** : Determining gene expression by RT-qPCR on fresh biopsies could prove useful for rapid and efficient patient management.

KEYWORDS: Concordance, immunohistochemistry, RTqPCR, molecular, breast cancer, Bouaké.

INTRODUCTION

Once breast cancer has been diagnosed, it is important for the physician to obtain information about the expression of estrogen and progesterone α hormone receptors and human epidermal growth factor β receptors by the cancer cells.[1] Immunohistochemistry (IHC) is a

commonly used technique for this purpose.[2,3] It is based on the detection of cytoplasmic, nuclear or membrane proteins specific to a cell type and/or function(s), using antibodies directed against the target protein.[2] It has diagnostic and prognostic value.[2] However, it has limitations related to the pre-analytical phase, antibody storage and cross-reactions that lead to false-positive results.[4] IHC presents difficulties in terms of reproducibility of interpretation and scoring of tumor cells. These difficulties sometimes lead to errors in subtype identification, hence the subjective nature of IHC.[5,6,7] In addition, the IHC technique is performed on histological preparations, which lengthens the time required to obtain results.[3] Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) is a molecular biology technique for detecting and quantifying RNA (ribonucleic acid).[8,9] It involves reverse transcription of total RNA or messenger RNA (mRNA) into complementary DNA (deoxyribonucleic acid) (cDNA) using reverse transcriptase, followed by amplification and detection of specific cDNA targets.[10] The amount of DNA is measured in real time at each PCR cycle by measuring the fluorescence emitted.[11] It is useful for quantifying gene expression levels.[12] RT-qPCR has been proposed as an alternative for shorter turnaround times compatible with early management. The study carried out on this subject analyzed frozen paraffin blocks.[5] It demonstrated good concordance of RT-qPCR with IHC, as well as better reproducibility of RT-qPCR compared with IHC.[5] The main drawbacks are the fragility of the RNA and its high cost.[10] In sub-Saharan Africa, and particularly in Côte d'Ivoire, RT-q PCR is not routinely used in the management of breast cancer.

To improve patient care, several groups, including the American Society of Clinical Oncology (ASCO), the National Comprehensive Cancer Network (NCCN) and the St. Gallen Group, have published guidelines and recommendations encouraging the use of molecular analysis for risk stratification and treatment planning in breast cancer.[13,14] The aim of this study was to determine the concordance rate between IHC and RT-qPCR analyses in breast cancer patients in Bouaké.

MATERIAL AND METHODS

Ethical statement

The ethical approval of the protocol and written consent of the participants were secured. All procedures were approved by the National Ethics Committee of Côte d'Ivoire under the authorization number 142-23/MSHPCMU/CNESVS-km. The anonymity and data confidentiality were ensured.

Type, duration and scope of study

This was a cross-sectional, prospective study conducted in the Gbêkê region of Côte d'Ivoire, whose capital is Bouaké. Bouaké is the country's second largest city, located approximately 350 km from Abidjan and 100 km from Yamoussoukro, the political capital. The city has a university hospital (CHU), the only one in the interior of the country. The study period was 16 months (September 2023 to January 2025).

METHODS

Patient flow

The study involved breast cancer patients The patients came from five primary healthcare facilities, which were located in the neighborhoods of Sokoura, Diezoukouamekro, Koko, Dar Es Salam and Belleville in Bouaké. These clinical facilities were part of the regional integrated management program for breast cancer screening and treatment. The CHU also has a central department for the management of cases referred from the periphery, with a gynecology, imaging and pathological anatomy department. The therapeutic itinerary was carried out from the beginning of the study of other peripheries to gynecology, imaging and pathological anatomy. Patients presented with symptoms of breast pathology such as nodules and/or breast discharge. They visited the gynecology department where their clinical examination was carried out. At the end of the consultation, a mammogram was prescribed. This examination was used to classify the patients according to the Breast Imaging Reporting And Data System (BIRADS) classification. BIRADS 4 or BIRADS 5 led to microbiopsy of the affected breast for pathological anatomy. Patients with no carcinoma after pathological examination were not included. Data were collected using a survey form.

Classic Histology

Breast biopsies taken from these patients at Bouaké University Hospital underwent the following steps : dehydration, kerosene embedding, 3µm microtome sectioning, hematoxylin-eosin staining, mounting within 36 to 72 hours to avoid the consequences of cold ischemia. Fixation time was 6 hours.

Immunohistochemistry

Antigenic sites were unmasked by heating at 45◦C in a Binder drying oven (Tuttlingen, Germany) for at least 12 hours. Monoclonal antibodies of murine origin directed against estrogen receptor (antibody SP1), progesterone receptor (antibody 1E2) and human epidermal growth factor receptor β (antibody 4B5) were used (Mannheim, Germany). Immunohistochemistry was performed on the BenchMark GX (Mannheim, Germany). Cancer cells expressing ER, PR and HER2 receptors were stained using the IHC technique and could be counted manually. For hormone receptors, when their proportion exceeded 1% of cancer cells, the result was considered positive. (+).[15] For HER2, immunohistochemical scores were determined according to ASCO recommendations. The Ki67 proliferation index was not performed due to the absence of antibodies. On the basis of IHC results, primary invasive breast carcinomas were classified into four (4) molecular subtypes- Luminal A : ER+ et /ou PR+, HER2 -

- Luminal B: ER+ et /ou PR+, HER2 +

- HER2 enriched: ER- et PR-, HER2+
- Triple negative phenotype: ER- PR -, HER2-

RT-qPCR

RNA was extracted with the RNeasy Plus Universal Tissue extraction kit from (Qiagen, Hilden, Germany) from fresh biopsies collected in cryotubes containing RNA Protect solution (Qiagen, Hilden, Germany) and stored at -80°C. The mRNAs of target genes ER1, PR and HER2 and of housekeeping genes β-actin (BACT), β2-microglobulin (B2M) and calmodulin 2 (CALM2) were amplified. The delta-delta Ct relative quantification method was used[8] Target-specific primers and probes were synthesized in lyophilized form by Integrated DNA Technologies (IDT, Coralville, Netherlands). Their respective sequences are given below.[16]

ER1 Forward primer : 5'- GAGGAGTTTGTGTGCCTCA -3'
ER1 Reverse primer : 5'- GGATATGGTCCTTCTCTTCC-3'
ER1 Probe (ROX) : 5'- ATTCTGGAGTGTACACA-3'
PR Forward primer : 5'- AGATGCTGTATTTTGCACCTGA-3'
PR Reverse primer : 5'- CAAACTCCTGTGGGATCTGC-3'
PR Probe (ROX) : 5'- ATACTAAATGAACAGCGGATG-3 '
HER2 Forward primer : 5'-CTCCACACTGCCAACCG-3'
HER2 Reverse primer : 5'-CTGGACCCCAGCAGTGC-3 '
HER2 Probe (FAM) : 5'- ACGAGTGTGTGGGCGAG-3'
BACT Forward primer : 5'-TGA GCG CGG CTA CAG CTT-3'
BACT Reverse primer : 5'-TCC TTA ATG TCA CGC ACG ATT T-3'
BACT Probe (CY5) : 5'-ACC ACC ACG /TAO/ GCC GAG CGG-3'
B2M Forward primer : 5'- CTGTGCTCGCGCTACTCT-3'
B2M Reverse primer : 5'- CTTTCCATTCTCTGCTGGAT-3'
B2M Probe (CY5) : 5'- CTATCCAGCGTACTCCAA-3'
CALM2 Forward primer : 5'- GCAGAATCCCACAGAAGCA-3'
CALM2 Reverse primer : 5'- TTCTTGCCATCATTGTCAG-3'
CALM2 Probe (CY5) : 5'- GATGCTGATGGTAATGGC-3'

Primers and probes were reconstituted before use. The TaqPath 1-Step Multiplex Master Mix amplification kit (Invitrogen, Manheim, Germany) was used. The 20 µL reaction mixture consisted of 5 µL of 4X Master Mix, 0.5 µL of each specific sense or antisense primer (10 µM), 0.5 µL of probe, 6 µL of RNA extract and RNAse free H2O in sufficient quantity to reach 20 µL. RT-qPCR was performed on the QuantStudio (Invitrogen, Manheim, Germany) in one step. RNA was amplified according to a three-step program. The program included a 10-minute reverse transcription step at 50°C, followed by reverse transcriptase deactivation and Taq polymerase activation. These first two steps are completed by a 50-cycle PCR step consisting of denaturation at 95°C for 5 seconds, hybridization combined with elongation for 1 minute at 60°C. At the end of each PCR cycle, the fluorescence generated by the reaction is measured. Amplification curves with Ct values between 14 and 29 were considered. For expression levels between 0 and 49%, the gene was considered underexpressed.[8] Between 50 and 150%, gene expression was normal.[8] From 151% the gene was overexpressed. The expression status of each molecular target was given by the concordance between at least two normalizations.[8]

Statistical analysis

Data collection and statistical analysis were performed using Excel 2016, SPSS 25 and Stata 12.0 software. Cohen's Kappa concordance test was calculated at the 5% threshold to assess the concordance rate between IHC and RT-qPCR for each target. These tests were applied using 2×2 cross-tabulations. A probability value $p<0.05$ was considered statistically significant. A Kappa coefficient below 0 indicates no concordance; 0 to 20 a slight concordance; 0.21 to 0.40 a moderate concordance; 0.41 to 0.60 a moderate concordance; 0.61 to 0.8 a good concordance and > 0.8 an excellent concordance.[17]

RESULTS

Epidemiological and histopathological data (Table 1)

The mean age was 43.8 ± 11.0 years, with extremes of 24 and 71 years (Table 1). Nearly half the participants were unemployed (46.5%). Pre-menopausal status predominated at 72.1%. Non-specific invasive carcinoma was frequently diagnosed (95.3%) with Nottingham grade II (74.4%). In terms of hormone sensitivity, 37.2% of patients were ER positive and 32.6% PR positive. HER2 oncoprotein was expressed in 27.9% of patients. Overall, the most frequent immunohistochemical type was ER+ and/or PR+ HER2- (37.2%). More than two-thirds of patients (69.8%) had hormone-sensitive cancer, with a preponderance of luminal type A (44.2%).

Table 1: Epidemiological and histopathological data.

Paramètres	Patients (n = 43)	% (100)
Age group		
24-39	17	39,5
40-55	20	46,5
56-71	6	14
Work		
Civil servants	3	7
Non Civil servants	20	46,5
Unemployed	20	46,5
Menopausal status		

Premenopause	31	72,1
Postmenopause	12	27,9
Histopathological type		
Non- specific infiltrating carcinoma	41	95,3
Lobular infiltrating carcinoma	2	4,7
Nottingham Grade		
Grade I	2	4,7
Grade II	32	74,4
Grade III	9	20,9
Immunohistochemical type		
Presence of estrogen receptors	16	37,2
Presence of progesterone receptors	14	32,6
Presence of HER2	12	27,9
ER+ et/ou PR+ HER2-	16	37,2
ER+ et/ou PR+ HER2+	7	16,3
ER- PR-HER2+	5	11,6
ER- PR-HER2-	15	34,9
Molecular Classification		
Luminal A	19	44,2
Luminal B	11	25,6
HER2 enriched	5	11,6
Triple Negative	8	18,6

ER1, PR and HER2 expression levels (Table 2)
The expression of ER1, PR, and HER2 genes explored by RT-qPCR revealed overexpression of ER1 in 44.2% of cancerous breast biopsies.

Table 2: Molecular data.

Parameters			**Patients(43)**	**% (100)**
Presence of mARN spécific (expression)	ER1 mARN	Yes	16	37,2
		No	27	62,8
	PR mARN	Yes	14	32,6
		No	29	67,4
	HER2 mARN	Yes	12	27,9
		No	31	72,1
Expression levels (mRNA)	ER1 mARN	Overexpressed	19	44,2
		Underexpressed	24	55,8
	PR mARN	Overexpressed	23	53,5
		Underexpressed	20	46,5
	HER2 mARN	Overexpressed	15	34,9
		Underexpressed	28	65,1

Immunohistochemistry - RTqPCR concordance (Table3)
There was good concordance between immunohistochemistry and RT-qPCR data. This concordance rate was particularly excellent for estrogen receptors, with a Cohen's κ coefficient of 0.86.

Table 3: Concordance coefficients between immunohistochemistry and RT-qPCR.

Parameters		**ER1 mARN (RT-qPCR)**		**κ of Cohen**
		Positive	**Negative**	
ER Protein (IHC)	Positive	16	0	0,86
	Negative	3	24	
		PR mARN (RT-qPCR)		
		Positive	**Negative**	
PR Protein (IHC)	Positive	15	0	0,64
	Negative	8	20	
		HER2 mARN (RT-qPCR)		

		Positive	Negative	
HER2 Protein (IHC)	Positive	12	0	0,79
	Negative	4	27	

DISCUSSION

The average age of 43.8 years is similar to those reported in Cameroon (49 years), Nigeria (48 years) and South Africa (49 years).[18,19,20] Pre-menopausal patients represented 72.1% of the study population, while post-menopausal patients were estimated at 27.9%. This finding is different in developed countries, where breast cancer is detected at an advanced age or in the post-menopausal period.[21] This difference may be linked to race. Various studies have highlighted a certain predisposition of black patients to develop breast cancer at a young age.[22,23] The majority of cases were diagnosed as non-specific infiltrating carcinoma. This histological type is the most common worldwide, with a frequency ranging from 77% to 88% in Africa.[24] Asia registers frequencies ranging from 80 to 94.5%.[25] Nottingham grade II prevailed in this study as reported by several authors.[26,27,28,29] The luminal A molecular subtype was the most frequently observed. This result is in agreement with the work of Effi et al [26] in Côte d'Ivoire, Sahraoui et al.[30] in Tunisia , McCormack et al.[31] in South Africa. This luminal A subtype is also prevalent in Europe, China and the United States, with frequencies ranging from 70% to 73%.[16,32] This high rate of luminal A in the USA and Europe could be justified by the significant use of hormonal contraceptives. The contraceptive prevalence rate in Europe and North America reached 61% in 2019.[33] The triple-negative phenotype was predominant, as reported in Cameroon (42.8%)[18] et au Burkina Faso (52.9%).[27] These discrepancies may be linked to the pre-analytical phase. Over-fixation of the tissue may mask antigenic sites.[15] ASCO recommends fixation of breast biopsies in buffered or neutral formalin diluted to 10 %. ASCO/CAP (College of American Pathologists) prescribes 6 to 72 hours of fixation for hormone receptor analysis and 6 to 48 hours of fixation for HER2 analysis.[15] These differences account for the absence of a uniform, dominant molecular subtype in Africa, due not only to varying fixation times, but also to different dehydration and paraffin impregnation procedures. Luminal type A cancer makes patients eligible for hormone therapy. Hormone therapy has reshaped the management of hormone receptor-positive breast cancers.[34] This therapy consists of preventing estrogen production or competitively blocking its attachment to receptors. It offers a better chance of survival. Indeed, the efficacy of the anti-estrogen tamoxifen has been demonstrated in several studies, with a 41% increase in relapse-free survival and a 34% increase in overall survival.[35,36] The over-expression of HER2 among the participants is consistent with that reported by Hu et al[37] which found over-expression of the oncoprotein in 29.2% of cases. This result is also in line with the literature, which highlights HER2 oncoprotein expression in 15% to 30% of cases.[26,31] Korde et al[38] have highlighted the influence of HER2 over-expression in amplifying breast carcinogenesis and breast cancer progression. The PR gene was most over-expressed in carcinogenic breast tissue, followed by the ER gene. This result differs from that reported by Siddiqui et al[39] who noted over-expression of the ER gene in 65.7% of cases, followed by PR in 57.7%. This difference could be explained by the difference between the black and Indo-Aryan races of the patients in these two studies. The concordance rates for ER mRNA/ER protein, PR mRNA/PR protein and HER2 mRNA/HER2 protein are in line with those reported by Chen et al[5], Sin[7] and Li et al.[3] It is important to emphasize the novel contribution of this study, which was carried out on freshly collected biopsies, in contrast to other work based on previously frozen paraffin embedding blocks. The results of this study showed that it is possible to use two types of packaging for biopsy samples, one reserved for histopathological examination and the other for RT-qPCR. This practice will enable early management of patients, thereby prolonging survival. Despite differences in the identification process, the two methods present concordant results in the determination of genes.[5] The main causes of discrepancies between certain immunohistochemistry and RT-qPCR results have been highlighted by Chen et al.[5] Among these causes are differences in sampling. RT-qPCR uses the whole sectioned tissue, whereas immunohistochemistry analyzes only a few parts of the tissue. Another cause is the low translation efficiency of messenger RNA (mRNA) into proteins.[5]

CONCLUSION

This study highlighted the expression levels of the ER, PR and HER2 genes, as well as the concordance rates between immunohistochemistry and RT-qPCR in breast cancer patients in Côte d'Ivoire. Unlike previous studies, this study was carried out on fresh biopsies, with the advantage of providing patients with results within 24 hours. The PR gene is the most overexpressed in carcinogenic breast tissue. Comparison of gene expression levels with immunohistochemical profiles revealed high concordance rates. Molecular analysis of gene expression levels is proving useful as a substitute for immunohistochemistry, for more accurate results and, consequently, more efficient patient management. An essential step would be to include these molecular analyses in the diagnosis of breast cancers in Côte d'Ivoire, as recommended by ASCO.

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Statements
The ethical approval of the protocol and written consent of the participants were secured. All procedures were approved by the National Ethics Committee of Côte d'Ivoire under the authorization number 142-23/MSHPCMU/CNESVS-km. The anonymity and data confidentiality were ensured.

Conflicts of interest
The authors declare that they have no conflicts of interest**.**

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