Histopathological characteristics of breast cancer and evaluation of ER alpha and Her-2neu using immunohistochemical and RT-PCR techniques

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Abstract *Background and purpose:* Oestrogen receptor (ER) and HER (human epidermal growth factor receptor) family signaling pathways are fundamental to guide treatment and determine prognosis. Categorizing breast cancer tumors as ER and Her-2 positive or negative is usually performed by immunohistochemistry (IHC), however the technique lacks standardization in handling tissues, staining techniques, and scoring systems.

The current study aimed to compare the conventional IHC and the RT-PCR techniques in assessing the ER-alpha status in breast cancer. It also validates the application of RT-PCR technique in detecting the Her-2/neu status.

Subjects and methods: The study included 40 patients with IDC (NOS) (invasive ductal carcinoma; not otherwise specified). Breast tissue specimens were collected at the time of the elective surgery. Specimens were subjected to routine pathological examinations. ER alpha and PR receptor status; assessed by immunohistochemical staining. RNA was extracted, reverse transcribed, and amplified by PCR using ER alpha and HER-2 specific primers. Relative expression was detected

by comparing the expression in normal and tumor samples relative to the housekeeping (β-actin) gene expression.

Results: IHC and RT-PCR techniques showed great discrepancies in detecting ER alpha status which might be due to the presence of ER alpha variants. RT-PCR showed higher sensitivity (72.7%) and accuracy (57.6%) than IHC in detecting ER alpha status. Gene expression of HER-2 was detected (by RT-PCR) in 67.5% (27/40) with overexpression in 25% (10/40) of the cases.

Conclusion: IHC and RT-PCR must be combined for more informative results regarding the ER alpha and HER-2 status. Further studies are mandatory to evaluate the efficacy of anti-estrogens on ER alpha variant expression.

1. Introduction

Breast cancer is the most common form of cancer among women and the second cause of death in childbearing period. The average age of presentation is 10 years lesser than in the west.[1] In Egypt, breast cancer represents 35.1% of all female cancers with 80–90% of cases of advanced stages (III or IV) at presentation.[2]

Breast cancer is characterized by genetic heterogeneity which makes its diagnosis and treatment challenging.[3] Despite having similar histological appearance, individual breast tumors can exhibit tremendous variations in clinical presentation, disease aggressiveness, and treatment response in different patient and ethnic populations.[4] Therefore, breast cancer is currently regarded as a heterogeneous disease that has been classified into various molecular subtypes according to the gene expression profile of ER, PR, and Her-2/neu including: basal cell-like or triple negative (ER-, PR-, and HER2-), Her-2/neu (ER-, PR-, and HER2+), luminal A (ER + and/or PR+, HER2-), luminal B (ER + and/or PR+, HER2+), and normal breast like.[5–8] It is evident now that the breast cancer heterogeneity is more complicated than thought before. Recently, Mosoyan et al.[9] isolated five cell lines (Her2, ER, CK8/18, CD44, and CD24) from a single patient's primary breast cancer tissue. The isolated cells showed variable tumorigenic and metastatic potential when tested in immunocompromised nude mice.

ER is a member of a family of nuclear receptors. It functions as transcriptional regulator that mediates the biological responses to the sex hormone estrogen which is essential for reproduction, cardiovascular, skeletal and nervous systems.[10] Currently there are two different forms of estrogen receptors; α and β receptors that are co-expressed in many cell types. Each encoded by separate genes (ESR1 and ESR2) that are present on different chromosomes. ESR1gene is located on q arm of chromosome 6, while ESR2 gene is located on q arm of chromosome 14.[11–13] ERα and ERβ show significant overall sequence homology with the greatest homology (close to 100%) in their DNA binding domains, and both are composed of seven domains. Hormone activated estrogen receptors form homo-[ERα (αα) or ERβ (ββ)] or heterodimers [ERαβ (αβ)] ERα.[10] ER-α gene (ESR1) comprises eight exons of more than 140 Kb separated by seven introns of ∼ 40 kb. Each exon encodes a certain region of the protein.[14,15]

Researchers have identified 20 ER alpha splice variants in human breast cancers.[16,17] The most abundant variants are created by splicing deletions in one or more exons.[18–22]

Patterns of splice variant expression differ between tumors and are quite heterogeneous. Splice variants could potentially affect the evaluation of ER status, but this has not been proven yet.[23] The generation of human ER alpha mRNA transcripts is a complex process that involves at least seven different promoters and exhibits cell line-dependent promoter usage.[24] Most ER alpha variants differ in the 5′ untranslated region and result in the expression of the full-length 66-kDa form of ER alpha.[24] Among others, 46-kDa isoforms of ER alpha (ER alpha 46) generated from an internal ATG start codon, lacks exon 1 and consequently the N-terminal AF-1 region.[25–27] Wang and co-workers identified and cloned a 36-kDa isoform of ER alpha (named ER alpha 36). ER alpha 36 is generated from a promoter located in the first intron of the ER alpha gene and it lacks both of the two transcriptional activation domains AF-1 and AF-2.[28,29]

In some estrogen-dependent cells and tissues, ER might directly activate G-protein-coupled receptors and/or heterodimeric Her (human epidermal growth factor) receptors, resulting in downstream activation of MAPK and PI3K pathways.[30] In turn, p38 (protein 38), a member of the MAPK family, is capable of phosphorylating and activating ER in a ligand-independent manner. This cross-talk activation of ER results in the loss of inhibitory effects of tamoxifen.[31,32]

Her-2 (human epidermal growth factor receptor-2; also known as c-erb-B2, HER-2/neu, p185, CD340) proto-oncogene is located at the long arm of human chromosome 17 (17q21–q22).[33] It is part of a family of genes that play roles in regulating cell growth. The protein it makes is a tyrosine kinase (TK) growth factor receptor expressed by a number of normal tissues and probably has a role in normal cell function, regulating growth and proliferation.

The full length 4.6 Kb Her-2/neu transcript encodes a 185 kDa transmembrane glycoprotein receptor with intrinsic tyrosine kinase (TK) activity. It contains an N-terminal extracellular domain (ECD), a single transmembrane helix, a TK domain, and an intracellular regulatory domain. The ECD is ∼600 residues long and contains four (I–IV) domains including a site of potential ligand binding between domains I and III. The ECD can undergo proteolytic cleavage in the juxtamembrane region that releases soluble Her-2/neu ECD (90–110 kD).[34] The intracellular domain is ∼500 residues long and consists of a TK domain and an intracellular regulatory domain. The TK domain contains the enzymatic sequences necessary for TK activity. The intracellular domain also contains multiple tyrosine residues which serve as substrates for other TK receptors.[30]

Alternative splicing of Her-2/neu generates a truncated product (100 KDa) consisting of the entire ECD rather than the full-length protein.[35] The 5′ region (2.1 Kb) of the truncated transcript (2.3 Kb) is identical to that of the full length. However, the 3′ end diverges revealing an exonic extension with in frame stop codon and a poly (A) addition site. Thus the truncated transcript differs from the full length in 20 amino acids on the C-terminal.

Her-2/neu gene is amplified in 20–25% of primary breast tumors and has been associated with poor prognosis.[36] Increases in Her-2/neu at the DNA, RNA, and protein levels have been similarly prognostic for poor outcomes in primary breast cancer.[37]

The use of molecular classification of breast cancer is limited in many countries including Egypt. IHC is cheap and widely available. However the reproducibility of ER, PR, and HER2 status is a major issue in daily practice and in clinical investigation. Therefore, the current study was designed to evaluate the accuracy and specificity of IHC and RT-PCR in detecting ER alpha status in IDC (NOS). It also validates the use of RT-PCR in detecting Her-2/neu status.

2. Subjects and methods

The study included 40 patients (sample size was based on previously published work) with IDC (NOS) randomly selected from cases presented to the Cancer Management and Research Department of medical research institute (MRI) (from February 2007 to August 2009). Tumor samples were subjected to pathology assessment and only IDC (NOS) cases were included according to simple random sampling without replacement; lottery method. Clinical diagnosis, treatment, and clinical follow up were performed as described by El-Abd et al.[38] All subjects were recruited according to the ethical rules approved by the ethics committee of the MRI based on Belmont report.[39] Tumor and normal tissue specimens (far from tumor site to serve as self-controls) were collected during the elective surgery and stored at −80 °C until use.

2.1. Pathological investigations

Hematoxylin (Genzyme, England) and Eosin (Chematec, UK) (H&E) stained breast sections (3–4 μm) were examined by the pathologist to determine tumor type, size, grade, stage,[40] and number of the involved lymph nodes. ER alpha, total progesterone receptor (PR) and Her's-2/neu status were detected by IHC using streptavidin–biotin peroxidase labeled specific monoclonal antibodies (Lab Vision, USA)[41,42] and scored according to ASCO-CAP test guideline recommendations (http://www.cap.org/apps/docs/committees/immunohistochemistry/summary_of_recommendations.pdf).

2.2. Molecular investigations

Total RNA was isolated from breast tissue samples using EZ-10 Spin Column Total RNA Minipreps Super Kit (Bio Basic Inc, Canada) according to the manufacturer's instructions. The quantification and purity of the prepared RNA was examined using the spectrophotometer (UNICAM; Helios Delta, England) at wavelengths of 260 nm and 280 nm and by agarose gel electrophoresis. Full-length cDNA prepared from total RNA (5 μg) using revert aid™ H minus first strand cDNA synthesis kit (MBI Fermentas, Germany) and random hexamer primer according to the manufacturer's protocol. Fourth of the reaction was then amplified using 2× PCR Master Mix (MBI Fermentas, Germany) and the sequence specific primers for ERα (5′-ATG AGA GCT GCC AAC CTT-3′ and 5′-AAC AAG GCA CTG ACC ATC T -3′) or Her-2 neu (5′-CCT CTG ACG TCC ATC ATC TC-3′ and 5′-ATC TTC TGC TGC CGT CGC TT-3′) (Metabion International AG, Deutschland). To verify the integrity of the extracted total RNA, all samples were additionally assayed for human β-actin (5′-CAC TGT GTT GGC GTA CAG GT-3′ & 5′-TCA TCA CCA TTG GCA ATG AG-3′) (Metabion International AG, Deutschland). Positive and negative controls were included as appropriate. The Amplification protocol has an initial denaturation for 5–8 min at 95 °C, 35 cycles; 30 s at 95 °C, 90 s at 60 °C for ERα or 56 °C for Her-2 neu and β-actin, and 90 s at 72 °C; plus a final elongation for 7–10 min at 72 °C. The amplified products (ERα; 104 bp, Her-2 neu; 98 bp, and β-actin; 164) were separated by agarose gel electrophoresis (3%), stained with ethidium bromide, and visualized by UV. The relative expression level of ER alpha and Her-2/neu (relative to β-actin expression ratio in both normal and tumor samples) was quantified using the Scion image program for Windows.

2.3. Statistical analysis

Data analysis was performed using SPSS version 11.5. Normality tests showed abnormally distributed continuous variables (K.S < 0.05) except age. Thus, description of abnormally distributed variables was done using the median and range. For age, description was done using the mean and standard deviation (M ± SD). Comparison of continuous variables was done using the Mann–Whitney U test if it was between two groups while Kruskal–Wallis X^2 was used if it was between several groups. Comparison of the age between groups was performed using the student t-test. Regarding qualitative variables, description was done using the percentage and comparison between groups was performed through the Fishers exact test (in case of 2×2 tables) or the Monte Carlo test (in case of rxc tables) due to invalid X^2. The survival curve was constructed using Kaplan–Meier plots and Wilcoxon–Gehan statistics.

3. Results

3.1. Study population

Age of patients ranged between 20 to 74 years (minimum to maximum; Mean ± SD: 50.7 ± 9.8); menstrual status: 55% postmenopausal, 35% premenopausal, and 10% perimenopausal. Patients were followed up clinically for 76 months. During this period, three (3/40; 7.5%) patients had metastasis and 33 (33/40; 82.5%) patients died (Table 1 and Fig. 1).

3.2. ER alpha status

The clinicopathological characteristics of patients with IDC (NOS) are summarized in Table 1. Although great discrepancy

Table 1 Clinicopathological characteristics of patients with IDC (NOS).

Parameter	Number (%)
ERα (as detected by IHC)*	
Negative	11 (27.5)
Positive	29 (72.5)
Weak (+)	13 (32.5)
Moderate (+ +)	11 (27.5)
Strong (+ + +)	5 (12.5)
ERα (as detected by RT-PCR)	
Negative	12 (30)
Positive	28 (70)
*PR (as detected by IHC)**	
Negative	11 (27.5)
Positive	29 (72.5)
Weak (+)	12 (30)
Moderate (+ +)	12 (30)
Strong (+ + +)	5 (12.5)
Her-2/neu gene expression (as detected by RT-PCR)	
Negative	13 (32.5)
Positive	27 (67.5)
LNM	
Negative (0)	10 (25)
Positive	30 (75)
I (1–3)	9 (22.5)
2 (4–9)	11 (27.5)
3 ($\geqslant$10)	10 (25)
Tumor size (cm)	
Minimum–Maximum	1–10
< 2	2 (5)
2–5	31 (77.5)
> 5	6 (15)
Unknown (excision biopsy)	1 (2.5)
Tumor stage	
Tx	1 (2.5)
I	4 (10)
II	11 (27.5)
III	22 (55)
IV	2 (5)
Histological grade	
I	1 (2.5)
II	26 (65)
III	13 (32.5)
Clinical outcome	
Non-metastatic	37 (92.5)
Metastatic	3 (7.5)
Local	1 (2.5)
Distant	2 (5)
Survival	7 (17.5)
Death	33 (82.5)

* Scoring according to CAP-ASCO guidelines.

was noticed in detecting ER alpha status (Table 2), 52.5% (21/40) of cases were positive by both techniques (IHC and RT-PCR) (Table 2, Figs. 2 and 3). Similarly 10% (4/40) were negative for ER alpha status by both techniques (Table 2, Figs. 2 and 3) i.e. the complete match in the results of both IHC and RT-PCR techniques was observed in 62.5% (21/40: positive cases; 4/40: negative cases) and the discrepancy between the two techniques was detected in 37.5% (15/40) of cases.
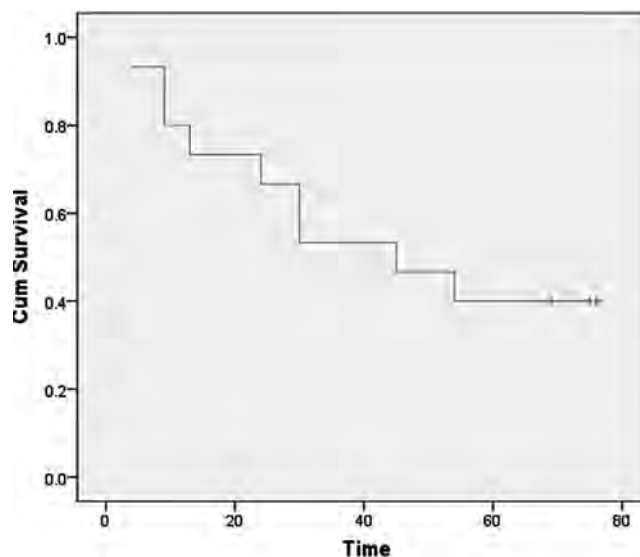
Figure 1 Overall survival curve for breast cancer patients.

Table 2 ERα as detected by both RT-PCR and IHC techniques.

ERα protein (IHC)	ERα gene expression (RT-PCR)		
	Positive	Negative	Total
Positive	21	8	29
Negative	7	4	11
Total	28	12	40

Fifth of cases (8/40; 20%) were ER alpha positive by IHC only (positive by IHC but negative by RT-PCR) (Table 2) and 7 cases (7/40; 17.5%) were ER alpha negative by IHC only (negative by IHC but positive by RT-PCR) (Table 2).

No significant difference was observed between ER alpha status and any of the tumor grade ($p = 0.453$), size ($p = 0.745$), stage ($p = 0.268$), LNM ($p = 0.339$), or the number of the involved LNs ($p = 0.168$).

3.3. PR status

PR was positive in 72.5% of cases as detected by IHC (Fig. 4). PR and ER status differed in one patient only regarding the score of positivity (Table 1).

3.4. Her-2/neu status

Her-2/neu status is not a routine in the pathological protocols in Egypt. It is only examined in ER alpha negative cases that can afford Herceptin (trastuzumab) and/or other tyrosine kinase inhibitor treatment regimens (Figs. 3 and 5). Her-2/neu gene expression was detected (by RT-PCR) in 67.5% (27/40) of tumor specimens (Table 1). Overexpression (relative to β-actin) was detected in 25% (10/40) of the cases.

No significant difference was observed between Her-2 status and any of the tumor grade ($p = 0.714$), size ($p = 0.571$), stage ($p = 0.160$), LNM ($p = 0.693$), the number of the involved LNs ($p = 0.889$), or ER alpha status ($p = 0.354$).
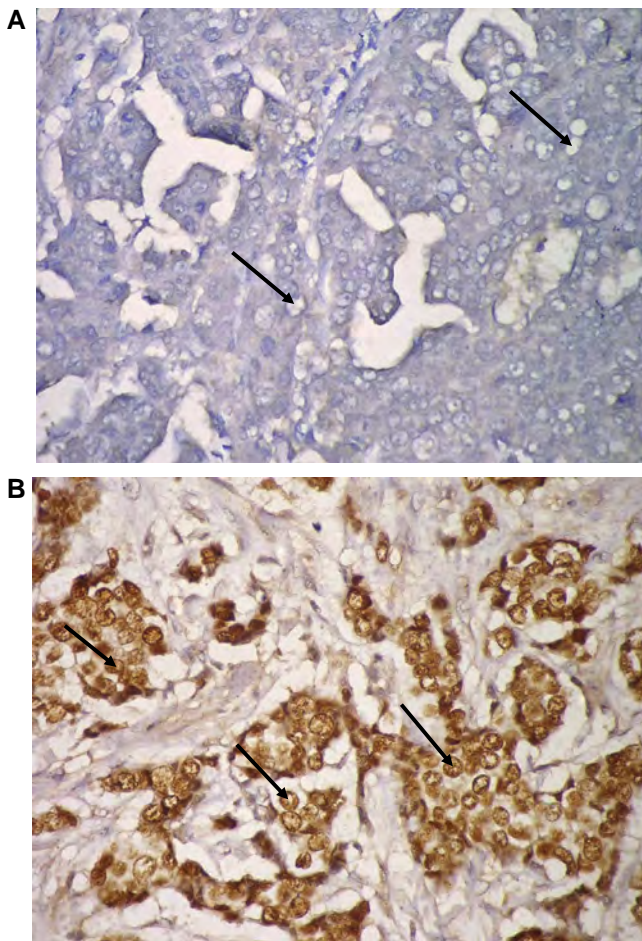
Figure 2 Nuclear staining of ERα by IHC (A: Negative; B: strong positive + + +; ×400).
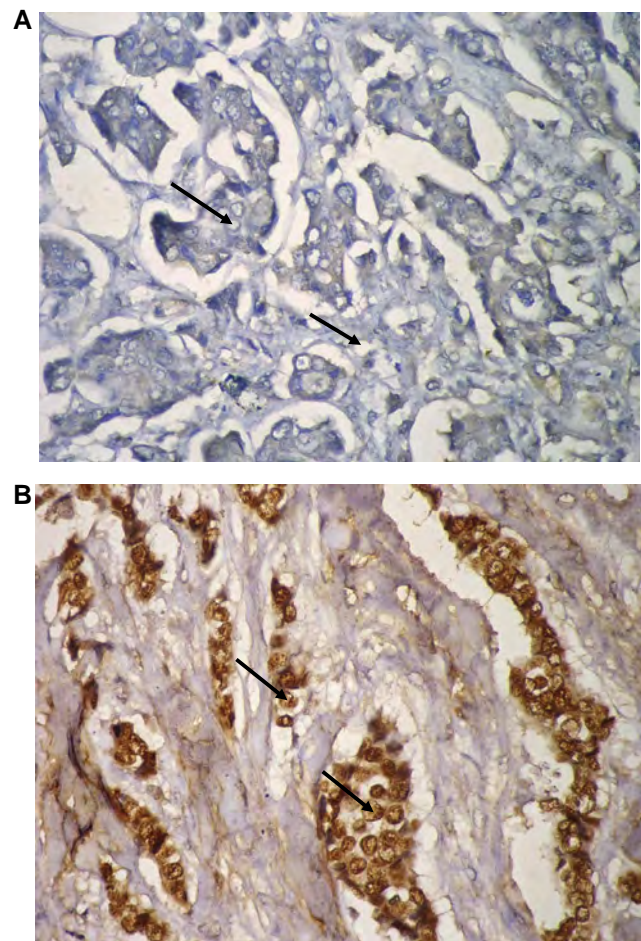

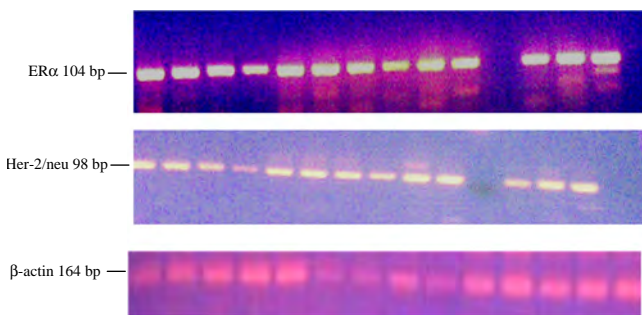**Figure 4** Nuclear staining of PR (total) by IHC (A: Negative; B: strong positive + + +; ×400).

Figure 3 Gel electrophoresis of ERα, Her-2/neu, and β-actin RT-PCR products.

4. Discussion

The current study documents the importance of combining IHC and RT-PCR techniques in detecting ER alpha status at the time of diagnosis and treatment decision.

4.1. Study population

The median age of patients with breast carcinoma in this study (50 years) is almost similar to that detected by Omar et al.[1]

among Egyptian population (30–60 years; median age is 46 years) which is one decade younger than the corresponding age in Europe and North America. Tumor size, grade, histological type, LNM (lymph node metastasis), number of the involved LNs (lymph nodes) and their distribution are also in accordance with disease presentation in Egypt.[1]

4.2. ER alpha Status; controversies between IHC and RT-PCR results

The discrepancies in ER alpha status might indicate technical limitations and/or presence of ER alpha variants. Although the superiority of the predictive value of ER-immunohisto-chemistry (ER-IHC) over ligand-binding techniques has been established to everyone's satisfaction, there remains the controversial issue of immunohistochemical results.[43] Several studies reported controversial results between the IHC and RT-PCR techniques.[44–47]

Although every step in both techniques can affect the results[48]; and thus would account for such variations; other effectors are not ruled out. Taking into consideration that IHC detects the protein and RT-PCR detects the mRNA, one possible cause for discrepancy may be the differential stability between human ER mRNA and protein; variation in translation efficiency or the rate of mRNA and protein
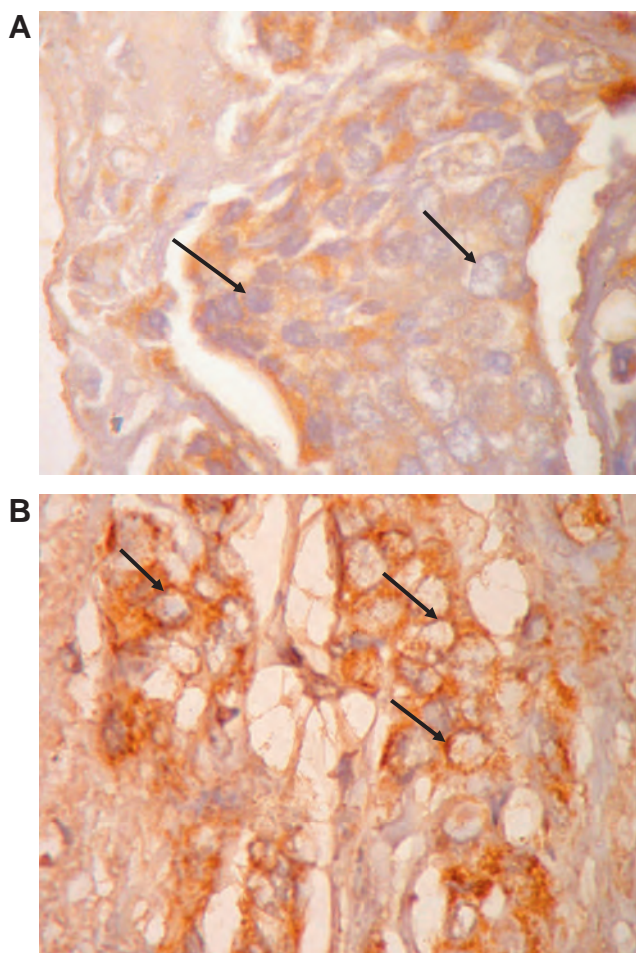
Figure 5 Her-2 neu as detected by IHC (cell membranous staining; A: Negative; B: strong positive + + +; ×1000).

degradation. ERα mRNA usually has a relatively short half-life, which was determined to be approximately 5 h in MCF-7 cell line after actinomycin D treatment.[49] Ligand binding, in addition to altering the conformation of the receptor, has been shown to influence the stability of the receptor. In particular, it has been shown that in the absence of ligand, the half-life of ERα is about 4–5 h, whereas estradiol binding accelerates receptor degradation, reducing its half-life to 3–4 h.[49–53]

4.2.1. RT-PCR negative/ IHC positive cases

Several investigators identified a number of estrogen receptor (ESR) mRNA variants (truncated transcripts, exon deleted transcripts, and larger than wild-type ER mRNA products resulting from nucleotide insertions) in a variety of cancer cell lines and tumors including tissues from human breast cancers.[16,54,55] In 35 ESR-positive tumors,[56] a common profile of variant ESR transcripts was present, with all tumors containing the exon 2-deleted and exon 7-deleted ESR variants, 94% containing the exon 4-deleted ESR variant, and 83% containing the exon 5-deleted ESR variant. Since the primers in our study were designed against a 104 fragment of exon four, variants that lack this exon will not be detected by RT-PCR technique. Several of the variant transcripts generated by an exon skipping mechanism of the primary ER-α pre-mRNA

retain the same reading frame as the full-length transcript, and the corresponding variant proteins have been detected in vivo and in vitro.[22,57–60] Receptors with 'outlaw' function, including both a dominant-positive receptor which was transcriptionally active in the absence of estrogen, and a dominant-negative receptor, which was itself transcriptionally inactive but prevented the action of normal estrogen receptor, were discovered using a yeast transactivation assay and open reading frame analysis.[17,21] The monoclonal antibody SP1 used in our study in detecting ER alpha protein using the IHC technique is designed against 18 merit peptide (SLQKYYITGEAEGFPATV) representing the C-terminal of human ERα protein. Thus, the IHC technique can detect variant products that lack exon-4 provided that the resulting protein has the same affinity toward the antibody, same conformational determinants, and the same recognition epitope(s).

4.2.2. RT-PCR positive/IHC negative cases

ERα 36 variant was detected in colorectal cancers and their matched normal colorectal tissues.[61] In contrast to full-length ERα 66 functional domains, ERα 36 lacks both transactivation domains AF-1 and AF-2 but retains its DNA-binding, ligand-binding domains, partial dimerization and three potential myristoylation sites. It also possesses an extra, unique 27-amino acids' domain to replace the last 5 helixes (helix 8–12) of the 12 helixes in the ERα 66. Providing that ERα 36 variant is dominant in some tumor samples, it would not be detectable by SP1 antibody due to the change in helix 8–12 but will be detectable by RT-PCR.

Due to such controversial issues of IHC and RT-PCR results, it is suggested to combine both techniques in detecting ERα status for breast cancer patients. It is also preferred to follow these recommendations:

1. Proper sample transfer, storage, and processing according to the subsequent technique (IHC or RT-PCR or in situ...etc.).
2. Report should include the sample location, type of antibody or primer, use of standard chemicals and detection kits.
3. Refer to a reference laboratory as a final evaluator.

4.3. Her-2/neu status

Her-2/neu mRNA was detected with variable intensities (degree of positivity) in 67.5% (27/40) and over expression was detected in 10/40 (25%) of cases. Only three cases (3/40; 7.5%) had results using the IHC technique since it is not a routine pathological investigation in Egypt. A result of RT-PCR depends only on the presence (positive) or absence (negative) of bands while both negative (0) and positive one (+) scores in IHC are considered negative. If we apply the same scoring system of the IHC (on protein level) on the RT-PCR (on RNA level) relative expression results, the percentage of positive cases (25%) [0: 13 (32.5%); +: 17 (42.5%); + +: 5 (12.5%); + + +: 5 (12.5%)] will be within the acceptable levels in the Arab countries (22%).[62] It is obvious that results may vary according to the detection technique, molecular detection level, age, race, and tumor stage.[63,64] The RNA expression

analyses show promise as possible independent methods of Her2 assessment[65] that require small amounts of tumor tissues and deliver both semi- and possible quantitative estimates of the Her2 RNA. Further comparative studies to evaluate the usefulness of these techniques; in both diagnosis and prognosis; are required.

No significant correlation was observed between expression of Her-2/neu and any of breast cancer prognostic variables (tumor size, histological grade, lymph node metastasis) or age or menopausal status. These results are in accordance with the research result of Ellsworth et al.[66]

5. Conclusion

We can conclude that it would be advantageous to combine the IHC and RT-PCR techniques in detecting ER alpha status to avoid the false negative results especially for triple negative (TN) patients (ER-, PR-, and HER-2) who usually have poor clinical outcome and no specific systemic treatment. Further studies would be mandatory to estimate the influence of anti-estrogen drugs on ER alpha variants to improve the clinical response in breast cancer patients.

Her-2/neu was detected by RT-PCR, and found in 70% of our cases. Her-2 represents an ideal therapeutic target because it is accessible; as a cell surface receptor; to treatment with chemotherapy. Recently, herceptin, a monoclonal antibody known as trastzumab, has shown to be effective than chemotherapy alone. Therefore, testing Her-2/neu in breast cancer at the time of primary diagnosis is of a prime importance. Thus, a routine examination of Her-2/neu using both RT-PCR and IHC techniques is highly recommended for ensuring proper classification of the patients' Her-2/neu status and tailoring therapy.

Conflict of interest

The authors declare that they have no conflict of interest.

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