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ORIGINAL ARTICLE

qRT-PCR Compliments Immunohistochemistry In Archival Breast Cancer Samples

PRABHU J S *, WANI S [*], KORLIMARLA A, PAYAL K., VICTOR J M , ANAND A, SAHOO R .

ABSTRACT

Background: Molecular characterization of tumour tissue in limited archived formalin fixed, paraffin embedded (FFPE) sample is always challenging. Better molecular characterization will not only help in accurate diagnosis but also in prognosis and guides therapeutic decisions.
Study Design: In the present study we tested the prognostic markers of breast cancer, estrogen receptor (ER) and progesterone receptor (PgR) by immunohistochemistry and quantitative real time polymerase chain reaction (qRT-PCR) on archived FFPE samples.
Results: High concordance was observed between the two methods for ER and PgR.
Conclusion: We conclude qRT-PCR may be used as an alternative method for the study of prognostic factors in archived tissues. Moreover, qRT-PCR can be a high throughput method for evaluation of markers with limited archived tissue.

Keywords: Formalin fixed paraffin embedded *(FFPE),* hormone receptors, breast cancer, IHC, qRT- PCR

Abbreviations

B2M - Beta 2 micro globulin
cDNA - Complimentary deoxyribonucleic acid
EDTA - Ethylene Diamine Tetra acetic acid
ER - Oestrogen receptor
FFPE - Formalin fixed paraffin embedded
GAPDH - Glyceraldehyde 3 phosphate dehydrogenase; µg - microgram
H&E - Haematoxylin and Eosin
HPRT1 - hypoxanthine-guanine phosphoribosyltransferase
IHC - Immunohistochemistry
mRNA - messenger Ribonucleic acid
PgR - Progesterone receptor
qRT-PCR - quantitative reverse trascriptase polymerase chain reaction
RPL13a - Ribosomal protein L13a
RPLP0 - Ribosomal protein Large 0s

Corresponding Author:
Dr.Prabhu J S
Triesta Sciences, India Pvt. Ltd
Institute Of Population Health And Clinical Research Building
St John's Medical College CampusOpp. Bda Complex,
Koramngala,Bangalore-560034
India

Introduction

Archived formalin fixed, paraffin embedded blocks have become valuable for validation of biomarkers in retrospective observational studies as they represent by far, the most abundant supply of solid tissue specimens associated with clinical records.

Until recent past, hormone receptor status in breast cancer was assessed in fresh tissues using ligand-binding assays [1]. Currently immunohistochemistry on formalin fixed paraffin embedded (FFPE) tissue has become the method of choice for determining the biomarkers like hormone receptors which are important regulators of growth and differentiation in the normal mammary gland and are of considerable diagnostic/prognostic value in breast cancer [2]. However it has inherent disadvantages like, intra and inter-observer variation between different labs, dependence on fixation conditions and lack of calibration [2]. In molecular biology, reverse transcription polymerase chain reaction (RT-PCR) is a laboratory technique for amplifying a defined piece of a ribonucleic acid (RNA) molecule. The RNA strand is first reverse transcribed into its DNA complement or complementary DNA, followed by amplification of the resulting DNA using polymerase chain reaction. This can either be a 1 or 2 step process. Polymerase chain reaction itself is the process used to amplify specific parts of a DNA molecule, via the temperature-mediated enzyme DNA polymerase.

In the recent past great advances have been made in the development of other sensitive techniques like quantitative reverse transcriptase polymerase chain reaction (qRT- PCR) for studying gene expression patterns using FFPE blocks [3] in retrospective observational studies. These techniques have the advantage of being robust, quantitative, and accurate and are less amenable to inter observer variation due to automated calibration when compared to subjective assessment in immunohistochemistry (IHC).

To evaluate the feasibility of using FFPE as a substrate for molecular analysis, we explored the utilization of qRT-PCR as a method for determining the hormone receptor status like oestrogen receptor (ER) and progesterone receptor (PgR) in archival breast cancer specimens. Here we report the concordance between conventional techniques like immunohistochemistry and qRT- PCR for hormone receptors like ER and PgR in archival breast cancer specimens.

Materials and Methods

Tissue Specimens

We studied formalin fixed paraffin embedded blocks of 53 patients who had undergone surgery for primary breast cancer. These blocks were obtained from various hospital laboratories after obtaining the approval from the ethics committee of respective hospitals. All 53 patients had undergone surgery between the periods 1994 to 2004. Of these, 46 were diagnosed as Invasive ductal carcinoma, five were invasive lobular carcinoma, and two were papillary carcinomas. Histological analysis was performed on Haematoxylin and Eosin (H&E)-stained slides of the blocks to confirm the diagnosis. One block containing the representative tumour and having more than 50 % of area showing tumour cells was selected for the study in each case.

Immunohistochemistry

5-micron sections were cut from these blocks on Poly-L-Lysine coated slides. The slides were kept in an incubator overnight at 56ºC. Sections were deparaffinised through two changes of Xylene, rehydrated through graded alcohols to distilled water. After blocking endogenous peroxidase activity with 3 % hydrogen peroxide in methanol, sections subjected to antigen retrieval by heating the slides in a pressure cooker in 1 mM ethylene diamine tetra acetic acid (EDTA) buffer, pH 8. Mouse monoclonal anti-ER- α primary antibodies (DAKO M7047) and anti-PgR mouse monoclonal antibody (DAKO M3569) were applied for 60 min at suggested dilutions. DAKO Envision kit was used for application of secondary antibody. All incubations were performed at room temperature. Sections were developed with diaminobenzidine followed by light counter

stain with haematoxylin. Each test batch was run with a known positive and negative control.

Immunostaining Analysis

The stained sections were independently examined by two pathologists and were categorized as positive (antigen present) when more than 10 % of the cells showed brown nuclear staining. Intensity of staining was graded from 1 to 3 according to suggested guidelines.

RNA extraction

Total RNA was extracted from two 20-micron sections taken from each patient's tumour block. After deparaffinization by heat, sections were subjected to overnight digestion using proteinase K (Qiagen #19133). Total RNA was then extracted using a modified TriReagent protocol (Ambion # 9738). Quantitation of RNA was done using the Ribogreen flourimetry system (Molecular probes, Turner Biosystems). On and average each patient sample yielded 5-6 µg of total RNA. 1µg of total RNA was then reverse transcribed using the ABI high capacity cDNA archive kit (ABI # 4322171) as per manufacturer's protocol.

Gene Expression Analysis

Expression levels of two-test genes oestrogen receptor and progesterone receptor were determined along with a panel of 6 reference genes (HPRT1, B2M, RPL13A, β-actin, RPLP0 and GAPDH). Ready to use primer-probe reagents for all genes tested were obtained from Applied Biosystem. The reference genes normalize for any variations that may be introduced through varied sample processing (considering they are from different laboratories) and handling methods which in turn lead to varied levels of RNA preservation in the FFPE blocks. Using 10 ng cDNA per reaction qRT-PCR was done in triplicate using TaqMan™ chemistry on the ABI 7000 sequence detection system. Universal human reference RNA (Stratagene, USA, Cat No# 740000) was also reverse transcribed and 0.5 ng of this was used as a positive control for all genes. Total reaction volume was 20 µl. Pre-incubation and initial denaturation of the template cDNA was performed at 95° for 10 min, followed by amplification for 45 cycles with 95° for 15 sec and 60° for 1 min. The qRT-PCR yielded CT values for the test genes, which were in turn normalized relative to the mean CT value of the six reference genes. Expression levels of the test genes were calculated relative to the Universal human reference RNA.

Gene expression calculations were done using standard and established methods and values greater than or equal to 4 normalized units (Log 2) were considered indicative of over-expression of the gene. Concordance between data of immunohistochemistry and gene expression data was calculated by over all percentage and using Cohen's Kappa statistics [17].

Results

qRT-PCR versus conventional IHC in the detection of the hormone receptors ER and PgR

Parallel analysis of qRT-PCR versus IHC was carried out on 53 breast cancer samples**.**
Concordance between the two methods was compared using Cohen's kappa statistical method [17] [Table/Fig 1a and [Table/Fig 2].

Thirty-six samples were ER positive and 14 were ER negative by both IHC and qRT-PCR. The percentage agreement between the two methods for ER is 94.4 % [Table/Fig 1]. In three samples, there was discrepancy between IHC and qRT-PCR (5.6 %). Of these three samples, one sample was negative by IHC but showed over expression by qRT-PCR (1.8 %). Two samples were positive by IHC but did not show over expression by qRT-PCR (3.7 %).

Of the 53 samples tested, 26 were positive and 18 were negative for PgR by both methods. The percentage agreement between the two methods was high, 83.1 % [Table/Fig 2]. In nine samples, the results of IHC and qRT-PCR for PgR were discordant (16.9%). Of these 9 samples, 7 samples were negative by IHC but showed over expression by qRT-PCR (13.2 %) and 2 samples were positive by IHC but showed no over expression by qRT-PCR (3.7 %).

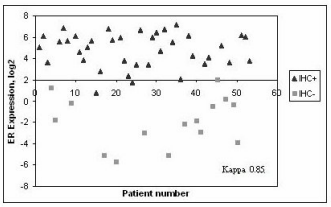
Discussion

Archived formalin fixed paraffin embedded blocks serve as valuable material in observational studies. Although great advances have been made in the development of molecular techniques using FFPE blocks [4], sensitivity of these techniques are highly dependant on the preservation of molecules like protein, RNA and DNA in the archived material. Several factors are known to affect the preservation of these molecules like duration of fixation, type and the amount of fixative used and duration for which the archived material is stored [5],[6] making FFPE a difficult substrate for molecular analysis. Moreover, many studies have also reported chemical modifications induced by formalin such as random base damage in DNA [6] and extensive fragmentation of the RNA [5]. Despite such reports, successful attempts have been made to use mRNA from FFPE tissues for gene expression assays [3],[7].
Though enzyme immunoassays were frequently used in the past [8], immunohistochemistry is considered the gold standard [10] for the evaluation of hormone receptors on FFPE blocks of breast cancer. It is a technique used by most pathology laboratories, as it is simple, relatively inexpensive and does not need highly trained personnel/equipment. It also has the advantage of topoanatomical localization of the antigen in question. Though this technique is best suited for surgical pathology practice, the results are semi quantitative, and limited by subjective interpretation. Therefore, reproducibility and standardization are critical factors in the assay [10]. In addition, many studies have reported a loss of immunostaining intensity, in stored paraffin sections [11], [12] giving false negative results by IHC. We have also observed this loss of immunogenicity in archived FFPE blocks in house.

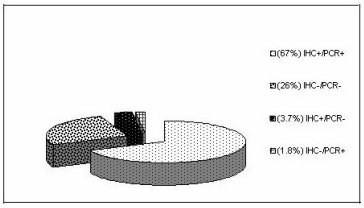
Our results demonstrate that the material extracted from archived FFPE tissues can be used for quantitative RT-PCR, which has the advantage of reproducibility, sensitivity, and quantitation over a dynamic range and hence could be used for confirmation/complementing of IHC results in observational studies. Moreover, qRT-PCR is a high throughput [4], [9] method for mRNA quantitation of many genes using limited archived tissue. Our results of high level of concordance between the two techniques for hormone receptors are in agreement with that seen in other studies [13], [14],[15],[16].

Differences in intracellular stability and biological variations in preservation of different mRNA species may explain variation in concordance levels between oestrogen receptor and progesterone receptor.

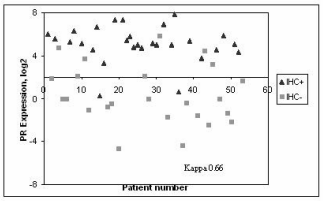
The discrepancies seen in few cases between the two techniques could be explained as follows. Expression of oestrogen receptor in adjacent normal breast tissue could have contributed to high level of expression in qRT-PCR whereas IHC showed negative results. In cases where IHC showed the presence of antigen but qRT-PCR did not show, over expression, is probably due to loss of the tumour tissue in the block after repeated trimming.



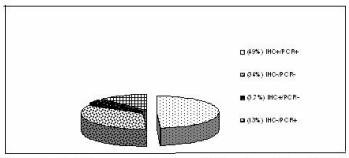
[Table/Fig 1A] Correlation of ER expression by IHC and Real time PCR. Y-axis represents the relative expression (log 2) of mRNA measured by qRT-PCR; X-axis represents number of cases. Each spot on the graph represents one subject. All subjects who were positive by IHC showed a relative expression of more than or equal to log 2 (4 Normalized units).



[Table/Fig 1B]Comparison between IHC and real-time PCR for ER. Both methods show a percentage agreement of 94.4 %.



[Table/Fig 2A] Concordance data for PgR: IHC vs. Real Time: Correlation of PgR expression by IHC and Real time PCR. Y-axis represents the relative expression (log 2) of mRNA measured by qRT-PCR; X-axis represents number of cases. Each spot on the graph represents one subject. All subjects who were positive by IHC showed a relative expression of more than or equal to log 2 (4 Normalized units).



[Table/Fig 2B] Comparison between IHC and real-time PCR for PR. Both methods show percentage agreement of 83 %.

Conclusion

In conclusion, qRT-PCR analysis from FFPE (archived breast tumours) is feasible and results correlate with immunohistochemistry. However, a combined IHC and quantitative RT-PCR approach for determining ER and PgR in archived breast cancer may be an effective and efficient strategy. RNA based techniques may be sufficient for high through put analysis of many markers when tissue is a limiting factor.

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