The Development of a Reverse Transcription Polymerase Chain Reaction System for Detection of Estrogen Receptor in Breast Cancer

Thanongchai Siriapisit, M.D. *
Supornpim Chearskul, M.D.,M.Sc..*
Sirirurg Songsivilai, M.D., Ph.D.#

Abstract : Estrogen receptor (ER) is widely used as an indicator of prognosis and response to endocrine treatment of primary breast cancer. ER phenotypes detected by conventional assays may not reflect their capability on binding specifically to estrogen response elements of target DNA. The reverse transcription polymerase chain reaction (RT-PCR) has been shown to offer the sensitive and specific method for measuring the ER mRNA in breast cancer. The ER-mutant gene which is positively detected in breast tumor by biochemical or immunocytochemical assays may be negatively shown by RT-PCR or vice versa. PCR technique for examining expression of ER mRNA may therefore provide a good screening method for detection of functioning ER that will affect the selection of appropriate treatment and prognosis of breast cancer patients. We have developed RT-PCR assay using β2-microglobulin as internal control for detection and relative-quantitation of ER mRNA in breast cancer tissue. Preliminary results show that the developed assay provides a sensitive and specific method for detection of ER expression in breast tumor. Also, the assay procedure is simple, rapid, non- expensive and required very small amount of breast cancer tissues.

เรื่องย่อ : **การพัฒนาการตรวจตัวรับเอสโตรเจนในมะเร็งเต้านมโดยวิธีรีเวิสทรานสคริปชั่น พีซีอาร์**

ทนงชัย สิริอภิสิทธิ์ พ.บ.*, สุพรพิมพ์ เจียสกุล พ.บ., วท.ม.*, สิริฤกษ์ ทรงศิวไล พ.บ., ปร.ด.**

*ภาควิชาสรีรวิทยา, **ภาควิชาวิทยาภูมิคุ้มกัน, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพมหานคร 10700.

สารศิริราช 2543; 52: 30-36.

**Department of Physiology, **Department of Immunology, Faculty of Medicine Siriraj Hospital, Bangkok 10700, Thailand.*
Received October 20, 1999
Accepted January 24, 2000

การตรวจวัดตัวรับเอสโตรเจน (ER) ในเนื้อมะเร็งเป็นวิธีหนึ่งซึ่งใช้บอกการพยากรณ์โรคมะเร็งเต้านมสตรีและการตอบสนองต่อการรักษาโดยฮอร์โมน การตรวจโปรตีนตัวรับเอสโตรเจนโดยวิธีเดิม อาจมิได้บอกความสามารถที่แท้จริงของการจับระหว่างตัวรับกับส่วนตอบสนองบนสาย DNA ในเซลล์เป้าหมาย เทคนิคของรีเวิสทรานสคริปชั่น พีซีอาร์ (RT-PCR) เป็นวิธีที่มีความไวและมีความจำเพาะของการตรวจสูง สามารถใช้ตรวจวัด mRNA ที่เกิดจากตัวรับเอสโตรเจนในเซลล์มะเร็งเต้านมได้ มิวเตชั่นของยีนตัวรับเอสโตรเจนในเซลล์มะเร็งเต้านมเป็นสิ่งที่พบได้ และในบางครั้งมีผลให้การตรวจโปรตีนตัวรับเอสโตรเจนในมะเร็งเต้านมโดยวิธีทางชีวเคมีหรืออิมมูโนซัยโตเคมีได้ผลตรงข้ามกับผลการตรวจวัด ER mRNA เทคนิค RT-PCR ที่นำมาใช้ตรวจวัด ER mRNA จึงอาจเป็นวิธีการที่ดีสำหรับตรวจตัวรับเอสโตรเจนที่ทำงานจริงได้ ซึ่งจะมีผลต่อการเลือกวิธีรักษาและการพยากรณ์โรคมะเร็งเต้านม คณะผู้วิจัยได้ทำการพัฒนาวิธีการตรวจวัด ER mRNA ในมะเร็งเต้านม โดยใช้เทคนิค RT-PCR และใช้เบต้าไมโครโกลบูลินเป็นตัวควบคุมผลการตรวจ พบว่าวิธีตรวจวัดดังกล่าวนี้เป็นวิธีที่ไวและมีความจำเพาะดีพอสำหรับนำไปใช้วัดระดับ ER mRNA ได้ ทั้งยังเป็นวิธีตรวจที่ทำได้ง่าย, รวดเร็ว, ราคาไม่แพง และสามารถตรวจพบ ER mRNA ในชิ้นเนื้อมะเร็งเต้านมที่มีปริมาณน้อยมากได้

INTRODUCTION

The presence of estrogen receptor (ER) in breast cancer tissue was used as a biologic marker for hormonal responsiveness and the degree of differentiation of the tumor.[1] About 60-70 % of breast cancer with ER- positive phenotype failed to respond to an endocrine therapy and it was suggested that these endocrine-resistant tumors contained dysfunctional ER.[2] Alternatively, 5-10% of apparently ER-negative breast tumors responded to endocrine treatment, suggesting that some of these tumors might contain functional ER that was undetected by classical receptor assay. It may no longer be sufficient to just define that a tumor is ER- positive or ER- negative phenotype but information about its functional behavior is also important.

A variety of techniques have been used to determine ER protein levels in breast tumors. Variation in ER assays is a major factor in the interpretation of ER results and may affect the quality of treatment. The conventional radioreceptor assay and enzyme immunoassay (EIA) require large portion of tumor tissues with high intra- or inter-assay variability. Decreased tumor size due to early detection of breast cancer makes conventional assays become more difficult to study ER protein. The immunocytochemical assay (ICA) yields a higher sensitivity and specificity and is able to perform with smaller pieces of specimen, as well as those from fine needle aspiration. However, ICA requires very specific antibody and relies on subjective interpretation of results. Moreover, measurement of ER content either by EIA or ICA are based on the integrity of the receptor protein's carboxy terminal and steroid recognition domains without regard to structure or functional abnormalities that might exist with its NH_2 -terminal regions including the DNA binding domain. Measurement of the fraction of DNA binding ER may have important prognostic utility.

Recently, the reverse transcription polymerase chain reaction (RT-PCR) for detection of ER

mRNA has come to be research interest. Previous results show that the RT-PCR method offers more rapid and specific tool for detection of ER expression in small amount of breast cancer tissue. Also, ER mRNA expression has been shown to associate with ER phenotype in primary breast cancer as determined by standard quantitative assays.[3-6] Some of the archival specimens were assayed for ER expression by using β_2-microglobulin (β_2M) expression as internal control[5] which made the RT-PCR method be possible for quantitative analysis of ER mRNA. This report will describe the development of RT-PCR assay for relative quantitation of ER mRNA in primary breast cancer in Thai women.

MATERIALS AND METHODS

Tumor tissue

Breast tissues were collected from breast cancer patients treated at Siriraj Hospital, and kept frozen at –70°C until assayed. Positive control was tumor tissue having ER-positive by both EIA and PCR methods while negative control was tumor tissue that gave negative result by these two methods.

RNA isolation

Total RNA was isolated from frozen tumor tissue using modified guanidinium thiocyanate phenol chloroform extraction.[7] Approximately 50 mg of tumor tissue in 0.5 ml solution D (4 M guanidinium thiocyanate, 0.5% lauryl sarcosine, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate pH 7.0) was homogenized. Then 50 µl of 2M sodium acetate pH 4.0, 0.5 ml phenol and 100 µl chloroform : isoamylalcohol (24:1) were added to homogenate before cooling down on ice for 20 minutes. The homogenated tissue was centrifuged at 12,000 g for 20 minutes. The upper phase was transferred to a new tube and 0.5 ml isopropanol was added to precipitate RNA by keeping at –20°C for 2 hours. RNA was pelleted by centrifugation at 12,000 g for 20 minutes. The pellet was washed by 70% ethanol and re-centrifugation at 12,000 g for another 15 minutes. The RNA pellet was collected by discarding the supernatant and dry in room air before dissolving in 25 µl diethylpyrocarbonate treated water. Average amount of RNA concentration obtained from extraction varied from 0.2 to 5 µg/ml.

Primer

ER primers for amplification ER mRNA and internal control primers (β_2M primer) for amplification β_2M mRNA used in the present study were followed those of Chevillard's report.[4] ER upstream primer was 5'ACTCGCTACTGTGCAGTGTGCAATG-3' (cDNA sequence : 776-800), and ER downstream primer was 5'- CCTCTTCGGTCTTTTCGTATCCCAC-3' (cDNA sequence : 1014-990), the product was 239 bp in length, spanning junctions between exons 2 to 3 and 3 to 4. The β_2M upstream primer was 5'-CATCCAGCGTACTCCAAAGA-3' (cDNA sequence : 97-116) and β_2M downstream primer was 5'-GACAAGTCTGAATGCTCCAC3' (cDNA sequence : 242-261), product was 165 bp.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Reverse transcription of RNA and DNA amplification was processed in same tube. Mixture comprised of PCR buffer (50 mM KCl_2, 10 mM Tris-HCl pH 8.3, Perkin Elmer), 1.5 mM MgC, 25 nM of each dNTPs, 100 pmol of each primer (ER upstream, ER downstream, β_2M upstream and β_2M

downstream), 25 U of Molony Murine Leukemia Virus (MuLV) reverse transcriptase (Perkin Elmer), 1.25 U of AmpliTaq DNA polymerase (Perkin Elmer) and total RNA varying from 0.1 μg to 10 μg in a final volume 50 μl. First step incubation of mixture was performed at 42°C for 30 min (DNA thermal cycler 2400, Perkin Elmer) for reverse transcription. The second step containing denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec and extended incubation at 72°C for 60 sec for the total of 35 cycles. Finally the PCR product was incubated at 72°C for 10 min then cooled down to 4°C.

Quantitation of PCR Product

Five μl of PCR product was run on 3% agarose gel, at 100 V for 20 minutes and stained with ethidium bromide in TAE buffer. The image of ethidium bromide stained agarose gel was digitalized by Video Gel Documentation System (GelDoc 1000, BioRad). Intensity of ER band and β_2M band was analyzed by NIH image 1.61 program. ER expression was calculated using ratio between the intensity of ER band over β_2M band. The advanced image-analysis system provides more reliable results thus the subjective interpretation can be avoided. Twelve breast cancer tissues were randomly selected to evaluate for ER mRNA by the developed protocol.

RESULTS

The assay was developed using RT-PCR procedure to detect ER mRNA in frozen breast cancer tissue. ER gene contains eight exons and ER protein has six functional domains, designated A through F. Domain C, which spans exons 2 and 3, binds DNA and domain E, which spans exons 4-8, contains the hormone binding site.[8] The assay was designed in order that DNA binding domain, between exons 2 to 3 and 3 to 4, was amplified. Primers were chosen to amplify a region of both ER gene and β_2M gene across a splice site of both genes to avoid confusion that could arise from amplification of genomic DNA. Since small amount of surgical tissues or fine needle aspiration tissues provided minimal amount of mRNA, modification of RT-PCR assay to achieve good sensitivity and large amplification of ER mRNA in breast cancer is necessary.

Modification of initial amount of total RNA

Same amount of β_2M mRNA was found in every tumor cell thus it could be used as internal control for RNA extraction and calculation of band ratio.[4] If β_2M band was negative in any assay tube the ER-RNA was not collected. Previous report showed that ER/β_2M ratio remained constant when total amount of RNA between 10 ng to 2 μg was used.[4] By varying an amount of total RNA, this developed assay can detect ER band at 0.1 μg total RNA. However, for better intensity of ER band, more than 1 μg of total RNA should be used and 5 μl of initial total RNA obtained from the extraction contained enough RNA template for amplification.

Two-step RT-PCR modification

It was believed that enzyme reverse transcriptase reduced DNA polymerase activity, thus in previous protocol of RT-PCR, two reactions were performed in different tubes.[4] First tube was for a reverse transcription to produce cDNA from mRNA and second tube was for a polymerase reaction to amplify cDNA. Though the original protocol yielded good sensitivity, it had disadvantages of time

consuming and high costs. In this present modified protocol, the reverse transcription and polymerase chain reaction were performed in same tube in order to lessen time and improving the sensitivity of the assay. The results showed that this assay protocol was able to give enough band intensity of both ER and β_2M at only 0.1 µg of initial total RNA. This provides more simple, rapid and less costs to measure ER mRNA expression.

Reduction of enzyme concentration

Following standard protocol of RNA-PCR Kit (Perkin Elmer), 50 U of MuLV reverse transcriptase and 2.5 U of AmpliTaq polymerase were used. The concentration of both enzymes was half reduced in this modified protocol and no significant difference of band intensity between standard and modified protocol was observed. (Figure 1) This modified protocol gave the same band intensity with less non specific product and lower costs than a original protocol.

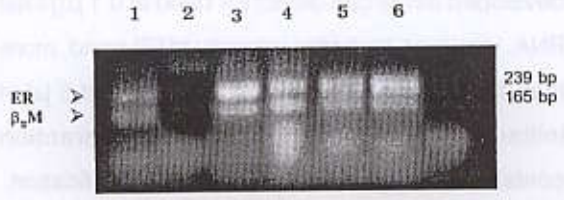

Figure 1. Effects of reduction in concentration of MuLV reverse transcriptase (MuLV-RT) and AmpliTaq polymerase (Taq) enzymes on RT-PCR products of ER mRNA measurement.
lane 1 = positive control
lane 2 = negative control
lane 3 = MuLV-RT 50 U + Taq 2.5 U
lane 4 = MuLV-RT 25 U + Taq 2.5 U
lane 5 = MuLV-RT 50 U + Taq 1.25 U
lane 6 = MuLV-RT 25 U + Taq 1.25 U

Modification of PCR amplification time

Time and temperature used in conventional protocol of PCR (RNA- PCR Kit, Perkin Elmer) was 35 cycles of 60 sec at 94°C for denaturation, 60 sec at 60°C for annealing, and 60 sec at 72°C for extended incubation that took about 3-4 hours total reaction time. The time in standard protocol was modified to become 15 sec at 94°C, 30 sec at 60°C and 60 sec at 72°C for total 35 cycles, followed by extended incubation at 72°C for 10 min. The result showed no significant different of band intensity between conventional and modified protocol.

The results of ER mRNA measurement in 12 breast cancer tissues are shown in Figure 2. Inter-assay reproducibility of a positive control sample has been tested within ten assays and ER mRNA expression was detected in every batch of assays. Figure 3 demonstrated the intra-assay reproducibility when different sample tubes of same ER-positive sample and ER-negative control were simultaneously measured.

DISCUSSION

ER expression is an important prognostic factor of breast carcinoma. Biochemical assays used for measuring ER protein (ER phenotype) have disadvantages of time consuming and need large amount of tissue as well as costly reagents. More importantly, those assays cannot give information about real biological function of the steroid receptor. We have developed RT-PCR assay with internal control amplification to measure ER mRNA available for routine use. This assay is simple, rapid with adequate sensitivity for detecting ER mRNA expression in very small amount of breast cancer tissue (0.1 µg).

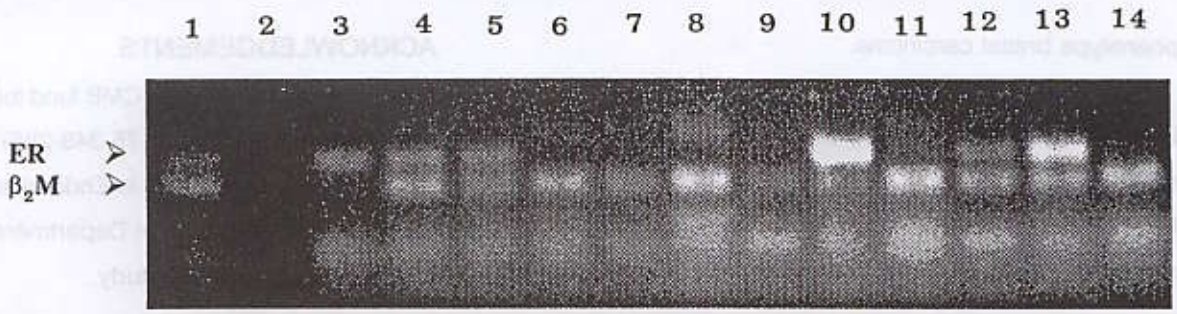

Figure 2. Products obtained from RT-PCR assay for ER mRNA in 12 breast cancer tissues.
lane 1 = positive control
lane 2 = negative control
lane 3,4,5,8,10,12,13 = positive expression of ER mRNA
lane 6,7,9,11,14 = negative expression of ER mRNA

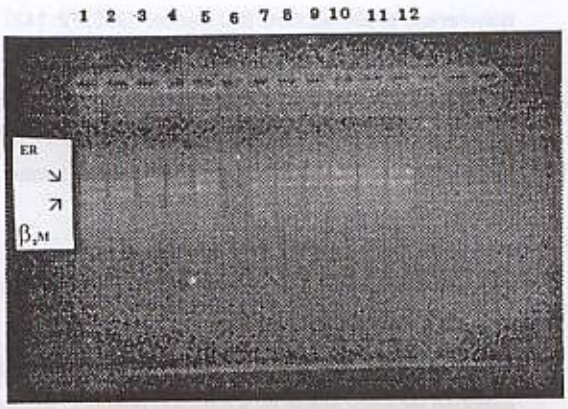

Figure 3. The intra-assay reproducibility of modified RT-PCR assay for detection of ER mRNA in breast cancer. ER mRNA expression was detected in all lanes of positive control sample except in lane 6 which was negative control sample.

Competitive RT-PCR measures the absolute levels of RNA because the amount of competitor RNA added is known.[9] However, competitive RT-PCR is expensive and unsuitable as a routine clinical assay.[10] Chevillard's group successfully developed RT-PCR co-amplification for detection and relatively quantitation of ER mRNA with lower cost than a competitive PCR, and found that it provided information concerning ER expression similar to ER-EIA.[4]

Mutations of ER can result in receptors with varied functions including active receptors, inactive receptors, and dominant negative receptors.[5] Not all tumor cells that had positive ER shown by ICA or EIA, expressed ER mRNA.[11-12] Several variant ER that have deletion were shown to be presented in breast tumor tissues. Some form of variant ER such as ER with exon 7 deletion is unable to function due to transcriptional defect.[13] Alternatively, some study showed that 29% of breast carcinoma with ER-negative phenotype had ER mRNA expression.[5] The result agreed with another work demonstrated in breast carcinoma, an extensive methylation of ER gene 5' CpG island in association with the absence of ER expression.[14] These agreements demonstrated the usefulness of RT-PCR assay for examining expression of ER mRNA especially in ER negative

phenotype breast carcinoma.

Further validation of this assay will be done in order to find the relationship of ER mRNA expression with ER phenotype measured by EIA and/or ICA as well as with other prognostic factors and clinical outcomes of breast cancer.

ACKNOWLEDGEMENTS

We would like to thank Siriraj-CMB fund for supporting this work (grant number 75-348-288). We also wish to thank all technicians in Endocrine Unit, Department of Physiology and in Department of Immunology for their helps in this study.

References :

1. Osborne CK. Receptors. In: Harris JR, Hellman S, Henderson IC, Kinne DL, eds. Breast diseases. Lippincott, Philadelphia,1987: 210-32.
2. McGuire WL. Steroid receptors in human breast cancer. Cancer Res 1978; 38: 4289-91.
3. Fuqua SAW, Falette NF, McGuire WL. Sensitive detection of estrogen receptor RNA by polymerase chain reaction assay. J Natl Cancer Inst 1990; 82: 858-60.
4. Chevillard S, Muller A, Levalois C, Laine-Bidron C, Vielh PH, Magdelenat H. Reverse transcription-polymerase chain reaction (RT-PCR) assay of estrogen and progesterone receptors in breast cancer. Cancer Research and Treatment 1996; 41: 81-89.
5. Carmeci C, de Coninck EC, Lawton T, Bloch DA, Weigel RJ. Analysis of estrogen receptor messenger RNA in breast carcinomas from archival specimens is predictive of tumor biology. Am J Pathol 1997; 150: 1563-70.
6. Henry JA, Nicholson S, Farndon JR, Westly BR, May FEB. Measurement of oestrogen receptor mRNA levels in human breast tumours. Br J Cancer 1988; 58: 600-5.
7. Chomczynski K, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorofrom extraction. Anal Biochem 1987; 162: 156-59.
8. Reichel RR, Jacob ST. Control of gene expression by lipophilic hormones. FASEB J 1993; 7: 427-36.
9. Siebert PD, Lawrrick JW. Competitive PCR. Nature 1992; 359: 557-58.
10. Car M, May FEB, Lennard TWJ, Westley BR. Determination of oestrogen responsiveness of breast cancer by competitive reverse transcription-polymerase chain reaction. Br J Cancer 1995; 72: 1427-34.
11. McCarty KS, Szabo E, Flowers JL, et al. Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. Cancer Res 1986(Suppl): 4244(s)- 4248(s).
12. Walker KJ, Bouzabar N, Robertson J, et al. Immunucytochemical localization of estrogen receptor in human breast tissue. Cancer Res 1988; 48: 6517-22.
13. Fuqua SW, Saundra D, Fitzgerald D, et al. Inhibition of estrogen receptor action by a naturally occuring variant in human breast tumors. Cancer Res 1992; 52: 483-86.
14. Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE. Methylation of estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res 1994; 54: 2552-55.