OVER EXPRESSION OF CYCLOOXYGENASE 2 DETECTED IN MCF-7 BREAST CANCER CELL AND COMPARED WITH LUNG CARCINOMA CELLLINE (A549)

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This article is available online at www.ssjournals.com

ABSTRACT

In advanced studies Cyclooxygenase-2 (COX-2) *mRNA* was determined mostly by *in situ* hybridization or Northern Blot analysis-methods not suitable for absolute quantification of *mRNA* copy numbers. Here we reported that, over expression of COX-2 mRNA was observed and calibrated through highly sensitive externally standardized real-time RT-PCR with Light Cycler instrument. The total *RNA* was isolated from two breast cancer cell lines (MCF-7, ZR-75-1), one lung cancer cell line (A549) and one normal breast cell line (HBL-100). The presence of COX-2 *mRNA* copy numbers was determined in all cell lines through Reverse Transcription PCR by *cDNA* conversion method. In this study, we observed breast cancer cell lines and lung cancer cell lines were showing high expression levels of COX-2 *mRNA* at the same time there was the lowest expression was detected in normal breast cells. This low levels of COX 2 expression due to the tumorogenic action of a normal cells. Thus we evaluated COX-2 expression at different levels in breast and lung carcinoma cell lines. Results of our study provide insight view to the involvement of different carcinoma cells in pathogenesis with respect to COX-2 *mRNA* expression. This Light Cycler technology is currently considered to be the most precise method for nucleic acid quantification and which showed to be a powerful tool for further expression studies on cancer gene pathogenesis.

KEY WORDS: Cyclooxygenase 2; *cDNA* quantification; Carcinoma; Reverse Transcription PCR, Real Time -PCR

1. INTRODUCTION

Cyclooxygenase (COX) is the critical enzyme involved in modulating inflammatory response through the synthesis of prostaglandins[1]. The inducible isoform of the enzyme, COX-2, is over expressed in several malignant and premalignant lesions[1]. Several epidemiological studies also be linked induction as well as over-expression of the cyclooxygenase-2 (COX-2) gene to cancer of the colon[2] esophagus, lung, prostate, skin, bladder and breast[3]. The

cyclooxygenase (COX) genes are involved in the conversion of glycerophosphoric acid into arachidonic acid through COX pathway and the enzyme exists in two isoforms like, COX-1 and COX-2. COX-1 is an enzyme appears to be a responsible for mediating the production of prostaglandins (PGE_2). The isoenzyme COX-2 is primarily associated with inflammatory activity and tumorogenic process[3, 4] . Cytokines and growth factors increases the expression of COX-2, mainly at inflammatory sites, producing prostaglandins and resulting in inflammation, pain and fever[5]. Oncogenes and tumour promoters were also reported in associated with the inflammatory response [4]. Inhibition of the expression of the cyclooxygenase-2 (COX-2) enzyme has been associated with prevention or reversal of cancer development in several organs.[5]

Higher COX-2 expression correlates with a poor clinical outcome in various cancers [5, 6] and selective inhibition of COX-2 suppresses tumour growth[7]. An increased levels of COX-2 expression has been detected in adenocarcinomas (ADC) and squamous cell carcinomas (SQCC) [5, 7] in comparison with normal lung tissue; however, higher levels were observed in SQCC. COX-2 inhibitors are currently being tested in ongoing phase II studies for the chemoprevention of tumor suppression and limiting COX 2 expression at cellular level, based on pre-clinical studies in mice[12].

This study is mainly focused to investigate cyclooxygenase-2 (COX-2) *mRNA* expression in breast, lung when compared with normal cell line like HBL-100. For this study we used Real Time PCR method and we have isolated *mRNA* from the cancer cells. By making *cDNA* conversion with the use of reverse transcriptase through reverse transcription PCR method. These *cDNA* copies were amplified by using specific COX 2 primers, SYBR Green used as detector for the expression amplification of these reactions. SYBR-Green binding that is a quick, reliable, easily optimized and compares well with the other assays. This demonstrates its general applicability by measuring copy number in three different genetic contexts; the quantification of a gene rearrangement (T-cell receptor excision circles (TREC) in peripheral blood mononuclear cells); the detection and quantification of *GLI*, *MYC-C* and *MYC-N* gene amplification in cell lines and cancer biopsies; and detection of deletions in the *OPA1* gene in dominant optic atrophy[25,26]. To investigate expression of Cyclooxygenase-2 (COX-2) in cancer cell lines, RT-PCR and western blot analysis were used. Western blot is one of the most specific methods for expression of COX 2 in all cancer cell lines. It is suggested that COX-2 is expressed in all cancer cell lines, which provides a basis for the chemoprevention of cancer[26].

Here, we reported, a new approach for rapid semi-quantitative multiplex RT-PCR method to study and COX-2 genes relative to a housekeeping gene (β-actin) as a means of analyzing gene expression. With this method, it is possible to rapidly analyze the effect of different expression patterns on Cyclooxygenase genes in multiple cell lines. In this study, multiplex RT-PCR was used to analyze expression of Cyclooxygenase 2 gene expressions in cells derived from breast adenocarcinoma, lung carcinoma and in normal breast cell lines as control for this study. The results were analyzed using the Applied Biosystems software developed by manufacturers. It will be more supportive and highly sensitive

method to enlight all cancer expression of future studies.

2. MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Cell lines were purchased from National Center for Cell Science (NCCS, Pune, India). Sodium bicarbonate, Sodium pyruate, L-glutamine *(Sigma USA)* Glucose, HEPES, Na pyruate, Penicillin, and Streptomycin were purchased *Sigma USA*. The Dulbecco's MEM (Sigma, USA), McCoys 5A Medium, *(HiMedia, Inc)*, Calf insulin, *(HiMedia, Inc)* ,Fetal Bovine Serum *(GIBCO, USA)*, and *RNA* Extraction kit from *BIO BASIC INC Canada*, Primers were designed by using Primer 3 software and primer sequences are procured from *Sigma USA.*

2.2 CELL CULTURE MAINTENANCE

Two breast cancer cell lines, MCF-7 and ZR-75-1,One lung cancer cell line, A549 and normal breast cell line HBL-100 were purchased from NCCS, Pune, India MCF-7 and A549 were maintained in Dulbecco's Modified Eagles medium supplemented with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L Na bicarbonate , 0.1 mM non-essential amino acids, and 1.0 mM of Na pyruate. ZR-75-1 cell was maintained in RPMI 1640 Medium with 2mM L-glutamine.1.5 g/L Na bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM Na pyruate, and HBL-100 cell was maintained in McCoys 5A Medium, *(HiMedia, Inc)* with 2mM L-Glutamine adjusted to contain 1.5 g/L Glucose, 10mM HEPES and 1.0 mM Na pyruate and supplemented with 0.2 Units/ml calf insulin respectively with 10% fetal bovine serum *(GIBCO,USA)*. Penicillin and Streptomycin (100IU/100µg) was adjusted 1 ml per liter. Cells were maintained at 37^0C with 5% CO_2 atmosphere.

2.3. CELL VIABILITY

Cell viability was assessed by trypan blue dye exclusion test as reported by Chakraborty *et al*. (2004). The number of stained and unstained will be counted using haemocytometer. Cell viability was analyzed through 0.4 % Trypan blue dye exclusion, which was always greater than 95%. All cells were showed a very good number of viable cells Figure 1.

2.4. *RNA* EXTRACTION

Cells with logarithmic growth phase were seeded in the 25×cm^2 flask. Approximately 5x 10^6 cells were used for total *RNA* extraction. *RNA* was extracted with commercially available *RNA* preparation kit *BIO BASIC INC Canada*, as per manufacturer's protocol and extracted *RNA* was stored in -80°C for over night[26] .

2.5. REVERSE TRANSCRIPTION PCR

Extracted total *RNA* was reverse-transcribed[29] to *cDNA* using dNTP's 2 µl, random primers. Brifely 1000ng of *RNA*, 200 units of Reverse Transcriptase (25 Units/µl)1µl *(Promega; USA)*, RT buffer 2 µl, template was about 10 µl added and remaining part is nuclease free water mixed with making final volume of 20 µl reactions at 25°c for 10 min, 42°c for 45 min and 95°c for 3 min with only one repeated steps. Measurement of nucleic acid concentrations were done at OD 260 nm on spectrophotometer *(BioPhotometer Eppendorf; Germany)*.

Real Time PCR (RT-PCR) was performed using specific primers for both COX 2 and β- actin with SYBR Green *(Applied Biosystems)* dye as a target detector molecule. Briefly 12.5 µl, Reverse transcriptase (25 Units/µl) 1.0 µl and forward and reverse primers for COX-2 and β- actin 1.0 µl and remaining part was nuclease free water. 5µl of quantified template *RNA* added to make the final reaction volume to 25µl. The primer sequence for COX-2 and β actin is given in table 1.

2.6. PRIMERS SEQUENCES FOR COX 2 AND β - ACTIN

Table 1- Shows primer sequence and size of product for both COX 2 and β- actin used for this study.

The prepared samples were optimized in RT-PCR thermo cycler with standardized cyclic conditions; 95°c for 2 min of initial denaturation, 95°c for 30 sec of denaturation, 61°c for 1 min annealing conditions, 72°c for 1 min of extension and dissociation stage like 95°c for 15 sec, 60°c for 1 min and 95°c for 15 sec for 30 cycles. The cyclic conditions for both the products are similar and the samples were starting to run in the *Applied Biosystems* thermo cycler. Subsequently, a melting curve program was applied with continuous fuorescence measurement.

Western Blotting:

Cyclooxygenase 2 enzyme was treated with specific COX 2 polyclonal antibodies *(Merck, Inc)* synthesized from rabbit, which can be interact with human COX 2 enzyme 1mM concentrations were incubated for 60 min at room temperature. After incubation attached antibodies were washed with blocking buffer for over 45 min, then blocked gels were treated with 0.05% of Tween 20 and immersed was about over night. After that overnight incubated membrane gels were detected by UV Trans illuminator. The Exposed blot was kept to film for 15 sec – 5 min.

3. RESULTS

3.1. DETECTION OF COX 2 AND β ACTIN GENE

The reverse transcripted *cDNA* samples were amplified by using specific primers for the evalution of COX 2 and β actin genes, it was showed the visualized bands of both the genes in all celllines based on their concentration of COX 2 and β actin *cDNA* levels. This indicated to us to proceed Real time PCR study Fig 2 and 3. The *cDNA* concentrations were measured by using spectrophotometer *(BioPhotometer Eppendorf; Germany)*.

3.2. QUANTIFICATION OF COX 2 AND β ACTIN

COX 2 *mRNA* expression was significantly higher in breast cancer cell line (MCF 7), followed by A549, ZR-75-1 and finally normal cell lines (HBL-100) whereas expression of β-actin was normal in all the cancer cell lines followed by MCF 7, A549, ZR-75-1 and HBL 100. MCF 7 amplification was started at the twenty second cycle whereas remaining cancer cell line required slightly higher than MCF7.But Normal cell line amplification started at the twenty sixth cycle. (Table 2).

Table 2. Copy numbers, cyclic amplification and Ct value of both COX 2 and β actin genes in the cancer and normal cell lines.

3.3. REAL TIME PCR EXPRESSION

The isolated RNA was reverse transcribed to *cDNA* and it was subjected for RT PCR evalution of expression

profiles. Whereas all the *cDNA* samples are quantified for their presence of copy numbers in all the cyclic conditions. Thus normal cell line HBL-100 showed very low level of COX 2 expression when compared to other cancer cell lines MCF-7, ZR-751 and A549. The percentage of expression in normal cell was about 61%, at the same time the cancerous cells showed more than 85% of expressions. (Fig 8).

The amplification pattern of all *cDNA* profiles were analysed by using dissociation stages for their amplification curves and their proper amplification. This indicates that proper dissociation gives the better amplification of all *cDNA*.

The amplification plots of COX-2 and β-actin in cancer and normal cell lines are shown in Figure 8.For this expression correlation strategies we used student t-Test for analyse complete expression profiles of *cDNA* in all cancer cell lines.

4. DISCUSSION

In this study over expression of COX 2 was proved by means of RT PCR, western blot analysis and also the presence of *cDNA* copies of all cancer cell lines (Fig. 3, 5, 6, 7, 8). In Fig 3 indicates that amplification pattern of MCF 7 cell line starts it's *cDNA* expression at very earliest cycle of 22nd, at the same time A549 a lung carcinoma stats at 23rd when compared with normal cell line HBL 100. This earliest amplification starting over due to higher expression of COX 2 in MCF 7 when compare with A549. In Estrogen Receptor is one which induces the production of COX 2 in all breast cancer cell lines ans tissues. This induces the HER2 for the activation of COX 2 pathway for prostaglandin synthesis[18.] In due to there was a normal level of all cell lines expresses β actin gene amplification within the nearest range of cycle 23 & 24 Table 2. This gene is house keeping gene for this study and it showed better amplification and dissociation stages are all proper way of amplified Fig 5 & 6. The western blot analysis indicates all the COX 2 genes showed higher intensity of all molecules in the membrane, but there was no longer way of higher expressions in normal cell line Fig 9. Over expression of COX 2 might be the standard reason to prove the higher expression in all cancer cell lines Fig 9.

We have designed primers for both COX 2 and β actin genes. They are having the different sizes. The sizes were confirmed at 386 bp and 540 bp of COX 2 and β actin through the conventional PCR method in agarose gels[13.] The presence of COX 2 enzyme is very high in all types of cancer cells this is the major factor for influencing carcinogenesis in normal cells[13, 14] . The RT-PCR is a recent advanced method for detecting the various kinds of gene expressions in different samples. And this is one of the better methods for getting the correct amplifications at different levels of amplifications[14, 15] . In this study we got expressions in high levels in all cancer cells at the same time the normal cells shows some differences in their expression patterns. This was about the over expression of prostaglandins in cancer cells. We have used β actin gene as a internal control (house keeping) genes for further confirmations of our COX 2 gene expressions. β actin, a house keeping gene was shows better normal level of expression in normal and cancer cell HBL 100. This would Light Cycler RT-PCR was evaluated using different starting amounts of *mRNA* and standard curve.

Compared to traditional methods of studying gene expression such as Northern blot analysis and Ribonuclease Protection Assay, the multiplex RT-PCR is a rapid method and expression of more than one gene can be analyzed in relation to a reference housekeeping gene from a small quantity of *mRNA*[17,18] . Compared to conventional RT-PCR, multiplex RT-PCR has an advantage of sensitivity. Comparing two genes amplified in different reactions, to the same housekeeping gene is less sensitive than comparing both of the genes to a housekeeping gene in a single reaction. In an optimized multiplex reaction, any of the PCR factors that become limiting factor will affect the amplification of each gene equally[18.] The greatest advantage has been the elimination of the limitations imposed by traditional methods, such as gel electrophoresis. These include low sensitivity, non-detection of shoulder peaks, long analysis time with post-analysis visualization and low automation [19] . Also fewer steps to the final data eliminate the loss of sensitivity generated at each step manually or due to the instruments. According to our suggestions whereas the traditional method of slab gel electrophoresis can take up to 5 h to complete, the RT PCR based method can analyze and semiquantitate more samples at the earliest[15]. Considerable savings in reagents, sample size and time, coupled with reduction in size and number of the separation and detection apparatuses needed, has made this technique extremely cost- and time-effective. The molecular mechanisms controlling the expression of COX-2 are not completely understood but are important because this is a crucial component of inflammation and a rate-limiting step in colon carcinogenesis[20]. In this report we demonstrate that a major regulatory point of COX-2 gene expression occurs at the post-transcriptional level

Minnie *et al* showed that cytoplasmic proteins specifically bind to the COX-2 and postulated that this interaction regulates the stability of COX-2 mRNA. Thus, the levels of COX-2 protein are determined by post-transcriptional regulation as well as by transcriptional mechanisms. This level of complexity is consistent with the requirement for tight control of the enzymatic action of COX-2, which has pathogenic effects if its expression is unregulated.

5. REFERENCES

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Table 1: Shows primer sequence and size of product for both COX 2 and β- actin used for this study.

Gene	Region	Sequence	Product Size
COX 2	FP	5'GCTGAGCCATACAGCAAATCC3'	386 bp
COX 2	RP	5'GGGAGTCGGGCAATCATCAG-3'	
β-actin	FP	5'-GTTGCTATCCAGGCTGTGC-3'	540 bp
β-actin	RP	5'GCATCCTGTCGGCAATGC-3'	

Table 2: Copy numbers, cyclic amplification and Ct value of both COX 2 and β actin genes in the cancer and normal cell lines.

Sample Name	Detector	Ct Value	Tm °C	Cycles of amplification	Quantity of mRNA
COX 2 HBL-100	COX 2	12.38	77.8	26	8087 copies/µl
COX 2 MCF-7	COX 2	10.21	75.6	22	18999 copies/ µl
COX 2 ZR-75-1	COX 2	12.01	76	24	10000 copies/ µl
COX 2 A549	COX 2	11.21	74.2	23	13134 copies/ µl
NTC COX 2	COX 2	Undetected	74	NTC	NTC
Beta actin HBL-100	Beta actin	10.92	75	24	50043 copies/ µl
Beta actin MCF-7	Beta actin	11.10	74.7	23	40812 copies/ µl
Beta actin ZR-75-1	Beta actin	12.34	74.8	24	34351 copies/ µl
Beta actin A549	Beta actin	11.78	74	24	32765 copies/ul
Beta actin NTC	Beta actin	Undetected	72	NTC	NTC

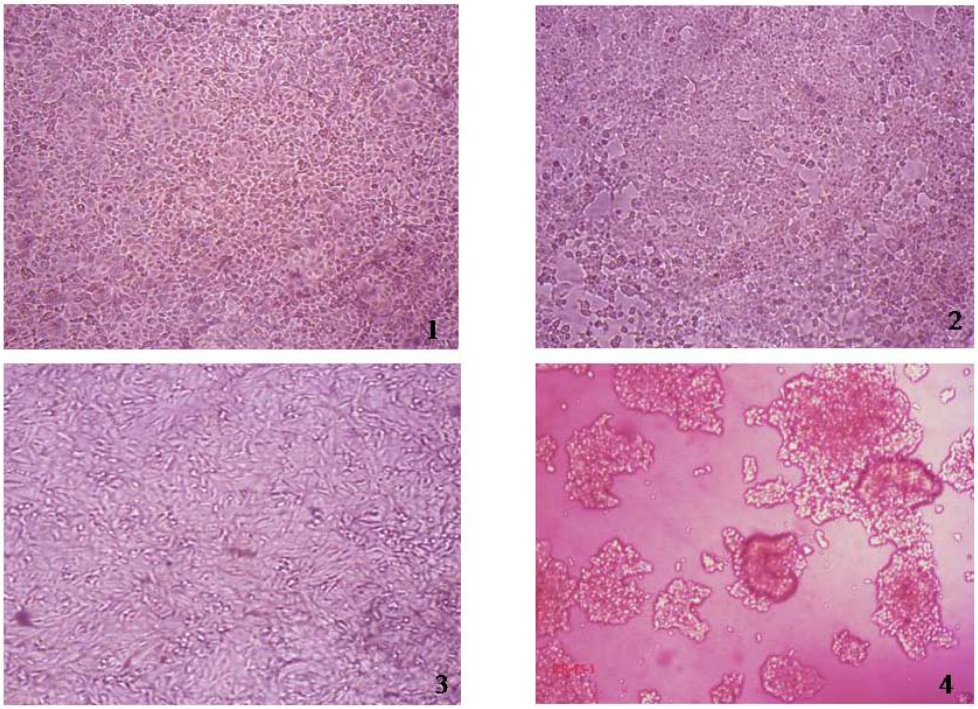

Figure 1: Different types of cell lines and their morphological appearance for specific growth parameters to analysed cell viablity, 1.Normal breast cell HBL-100, 2, Breast adenocarcinom cells MCF-7 and 3, ZR-751, 4, lung carcinoma cells A549.

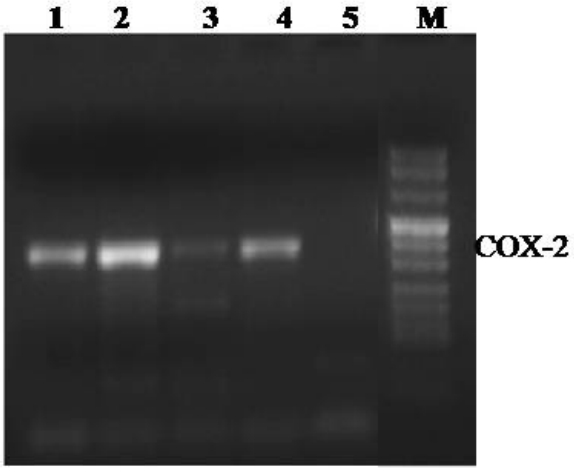

Figure 2: Expression and amplification pattern of COX 2 gene in different cell lines, based on the presence of *cDNA* by Reverse Transcription PCR. Lane 1-4, *cDNA* of COX 2 MW, 386 bp, Lane 1 HBL 100, Lane 2 MCF 7, Lane 3 ZR 751, Lane 4 A549, Lane 5 Negative Control, Lane M 100 bp marker.

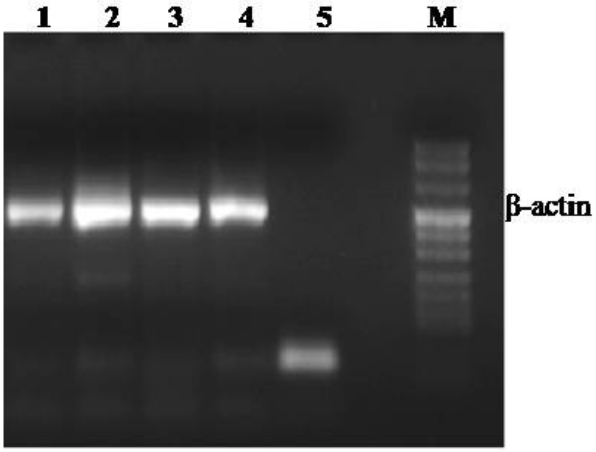

Figure 3: Expression and amplification pattern of β actin gene in different cell lines, based on the presence of *cDNA* by Reverse Transcription PCR. Lane 1-4, *cDNA* of β actin, MW, 540 bp, Lane 1 HBL 100, Lane 2 MCF 7, Lane 3 ZR 751, Lane 4 A549, Lane 5 Negative Control, Lane M 100 bp marker.

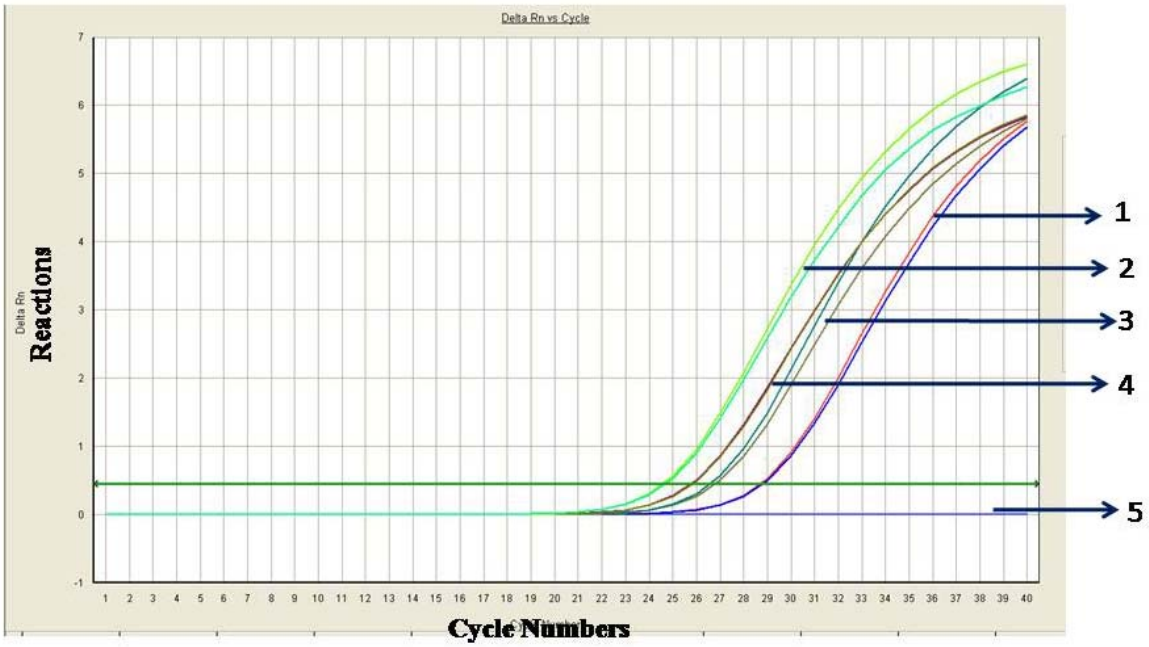

Figure 4: Indicates that COX 2 *cDNA* expression in various cancer cells by RT-PCR method. Here the 1 showing that low level of expression when compare to 2, 3, 4, because this 1 is the normal breast cell and its showing that low expression. The normal cell getting amplification at 26th cycle, the others are starting at the earliest.1. HBL-100, 2. MCF-7, 3. ZR-75-1, 4. A549, 5. Negative Control.

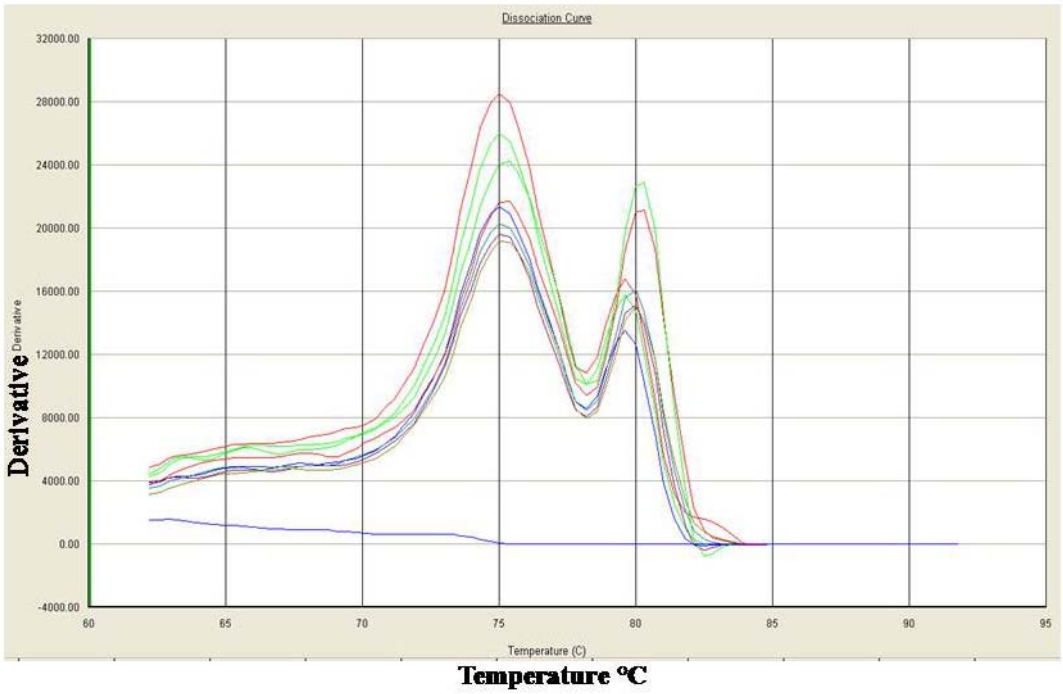

Figure 5: Indicating the dissociation range of COX 2 gene while in the Reverse transcription process.

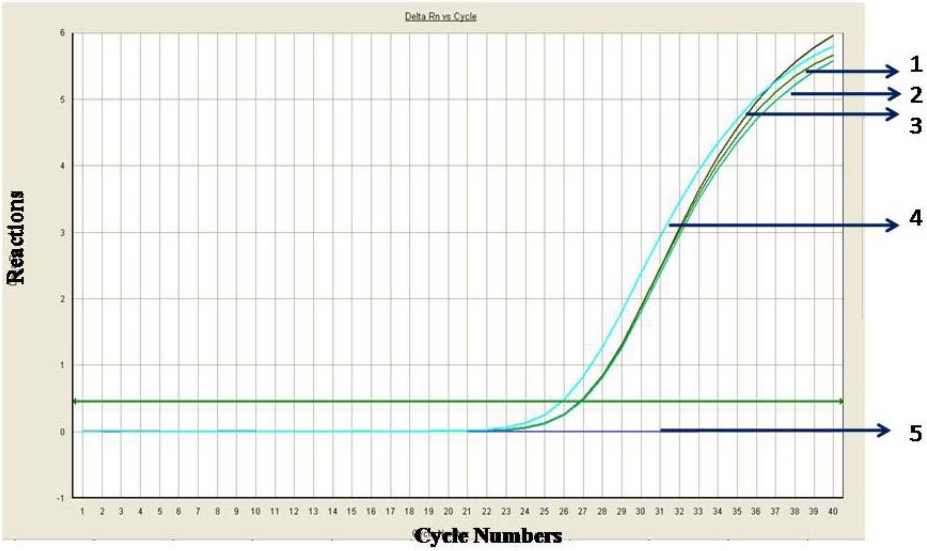

Figure 6: Indicates that β- actin mRNA expression in various cancer cells by RT-PCR method, here all the cell lines are showing that normal level of amplification pattern, when goes to normal cell its getting amplification at earlier of one cycle before cancer cells. The normal cell getting amplification at 23rdcycle, the others are starting at the earliest.1. A549, 2. MCF-7, 3. ZR-75-1, 4. HBL-100, 5. Negative Control.

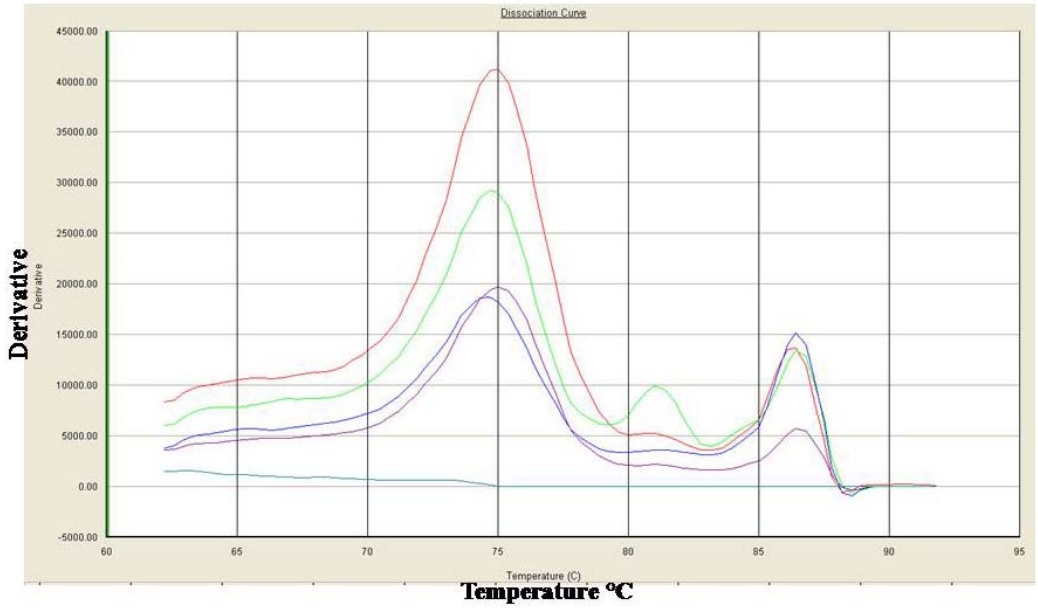

Figure 7: Indicating the dissociation range of β- actin gene while in the Reverse transcription process.

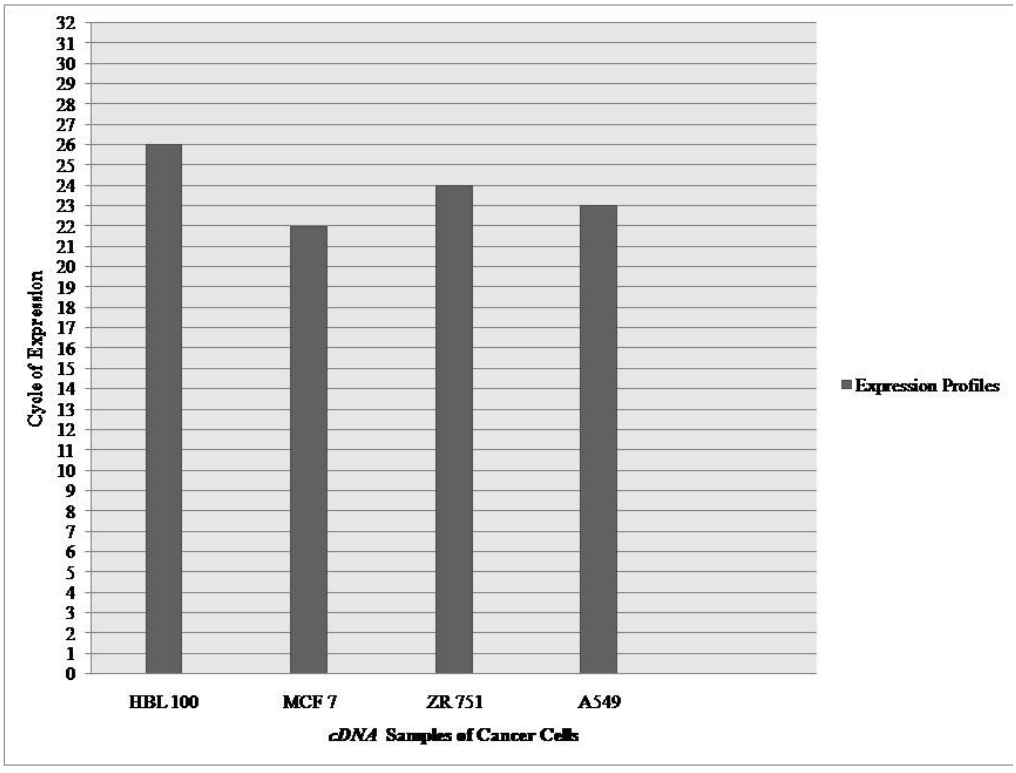

Figure 8: Different kinds of *cDNA* expression analysis and their amplification profiles in Real time PCR process. The MCF 7 *cDNA* expresses earlier amplification when it compare with A549 and HBL 100 normal cell line.

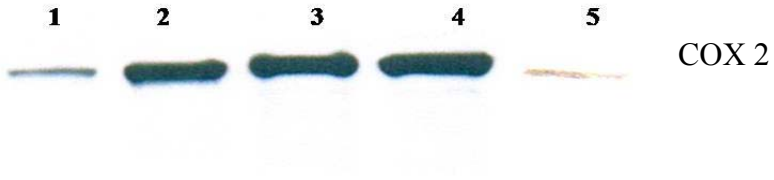

Figure 9: Western blotting results indicates that similar Cyclooxygenase 2 expression in all cell lines like 1. HBL 100, 2, MCF 7, 3, ZR 751, 4, A549. This exhibited over expression of particular protein in MCF 7 when it compared with A549 cell line.