Investigation of PTGS2, MAGE-A3, CALR, KRT19 and TMPRSS4 expressions in HCT116 colon cancer and PC3 prostate cancer cell lines.

Süreyya Bozkurt¹, Filiz Yarımcan², Hüseyin Ayhan¹, Hacer Kotan³, Hüma Tuğçe Sezgin³, Elif Çınar³, Ceren Aynacı³, Remzi Ökan Akar⁴, Veysel Sabri Hançer¹

¹Istinye University, Faculty of Medicine, Department of Medical Biology, Istanbul, Turkey.
²Medipol University, Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkey.
³Istinye University, Institute of Health Sciences, Department of Medical Biology and Genetics, Istanbul, Turkey.
⁴Istinye University, Department of Cancer Biology and Pharmacology, Institute of Medical Sciences, Istanbul, Turkey.

DOI: 10.31383/ga.vol4iss2pp37-42

Abstract

Cancer is a disease arising from DNA alterations that dysregulate gene structure and function. These deregulated genes can also play a role in tumor invasion and metastasis or resistance to treatment. In this study, we determined the gene expression during transcription of PTGS2 (Prostaglandin-endoperoxide synthase 2), MAGE-A3 (Melanoma-associated antigen 3), CALR (Calreticulin), KRT19 (Cytokeratin 19), and TMPRSS4 (Transmembrane protease, serine 4) in HCT116 colon cancer cell line and PC3 prostate cancer cell line. After RNA isolation and cDNA conversion, DNA amplification was performed with Real-Time PCR. We determined the altered transcriptional expression level of those genes. In HCT116 colon cancer cell line, expression of the TMPRSS4 gene, MAGEA3 gene and KRT19 gene was found as increased and expression of the CALR gene and the PTGS2 gene was found as decreased. Especially a 93.70-fold increase in expression of the KRT19 gene was found in HCT116 colon cancer cell line. In PC3 prostate cancer cell lines, TMPRSS4 gene expression and MAGEA3 gene expression were found as increased. But there was 50 fold decrease in PTGS2 gene expression.

Introduction

Cancer is a genome disease arising from DNA alterations that dysregulate gene structure and function (Garraway and Lander, 2013). Damage to the cellular genome or altered expression of genes is a standard feature for virtually all neoplasms. These genes may play a role in cancer formation, tumor invasion, metastasis, or therapy resistance (Negrini et al., 2010). Prostaglandin endoperoxide H synthase 2
PTGS2) converts arachidonic acid to prostaglandins, which are essential inflammatory mediators. It is shown that excessive PTGS2 expression plays a role in different stages of various cancers such as breast, lung, colon, and prostate cancer (Regulsk et al., 2016).

MAGE-A3 gene belongs to the melanoma-associated antigen gene family. MAGE-A3 is a tumor-specific protein and has been identified on many tumors, including melanoma, non-small cell lung cancer (NSCLC), hematologic malignancies. Normal adult cells other than testicular germ cells do not express this gene. The expression of MAGE-A3 by cancer cells was related to a bad prognosis. It may have a role in cancer development (Schcolnik-Cabrera et al., 2019).

Calreticulin (CALR) is a protein found in the endoplasmic reticulum and takes a role in both calcium storage and formation and secretion of glycoproteins. Studies performed show that role of CALR in cancer development is controversial and depends on the type of cancer (Zeng et al., 2016).

Cytokeratin 19 is a member of the cytokeratin family, which are intermediate type filamentous proteins that take a role in forming the cellular skeleton. They are also functional in apoptosis by regulating the cellular response to several stress responses, cell signalization, and organelles' and substrates' intracellular movement. Cytokeratin 19 is heterotrimer and is expressed by the periderm, pancreas, kidney, and digestive system epithelial cells (Mehrpouya et al., 2019; Jain et al., 2010).

The transmembrane protease serine 4 (TMPRSS4) gene encodes for a serine protease, which takes a role in both embryo and cancer development. The overexpression of TMPRSS4 is reported in breast, lung, colorectal, pancreatic, gastric, and liver cancers (Tanabe and List, 2017). Studies show that excessive TMPRSS4 expression is a poor prognostic marker in breast and colorectal cancers (Aberasturi and Calvo, 2015).

Because expression of PTGS2, MAGE-A3, CALR, KRT19, and TMPRSS4 genes are associated with different cancers, in this study, it was aimed to determine the transcriptional expression of PTGS2, MAGE-A3, CALR, KRT19, and TMPRSS4 genes in colon and prostate cancer.

Material and methods

The PC-3 prostate cell line, HCT116 colon cancer cell line, and BEAS2B Epithelial virus-transformed cell type from bronchus used in our study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). BEAS2B Epithelial virus-transformed cell type from bronchus was chosen as a control.

Cell Culture Application

Cells were cultivated in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 6 mM glutamine at 37°C in a humidified incubator (Panasonic) atmosphere containing 5% CO2. Cells were harvested after incubation, and cell pellets were obtained for analysis.

RNA Isolation

To obtain total RNA from cultured 1 x 10⁶ cells, we used the Omega Biotek brand (R6834 catalog number) RNA isolation kit product. RNA isolation steps were carried out according to the steps specified by the manufacturer. RNAs obtained were stored at -80 for use in cDNA transformation. After isolation, amount and purity of RNAs were calculated in Nanodrop and 260/280 ratio higher than 1.8 in all samples.

cDNA Synthesis and Real-Time Polymerase Chain Reaction

QuantiTect Reverse Transcription kit (Qiagen, Hilden- Germany) was used for cDNA synthesis. Following the kit protocol, 10μL total RNA obtained from each cell line was incubated at 65 °C for 5 minutes and then kept on ice for 5 minutes. The solutions used in the reaction buffer are summarized in Table 1. After adding 10 μL of RNA samples to the buffer, the reaction was as 10 minutes at 10 °C, 60 minutes at 50 °C and finally 5 minutes at 85 °C. In the end, the tubes were kept constant at 4 °C. Table 2 shows primers designed for each gene.
DNA amplification was performed on Corbett Rotor-Gene 6000 (Qiagen, Hilden- Germany) Research Real-Time PCR Thermal Cycle device.

The reaction buffer was prepared by adding 10 µL of 2X SYBR Green (HibriGen brand, mg-sybr-01-400 catalog numbered product), 1.5 µL of each of the forward and reverse primers, 4 µL of cDNA sample, 4 µL of nuclease-free water. The samples were run in duplicate. The efficiency of the transcription and PCR was estimated via a standard calibration dilution curve using a conventional RNA (Stratagene) and slope calculation for each assay. Slopes between -3.1 and -3.8 was acceptable, giving reactions efficiency between 90% and 100%. The reaction components are as in Table 3, and the steps of the reaction are as in Table 4.

In the real-time PCR analysis calculations, Ct values of the GAPDH gene were used as a reference. The negative power above two was taken to find the increasing or decreasing coefficient of variation in each replication cycle of the obtained values.

### Table 1. cDNA Reaction Components

| Content                  | Quantity | Concentration |
|--------------------------|----------|---------------|
| 5X Reaction buffer       | 5 µL     | 1X            |
| dNTP                     | 2 µL     | 100 mM        |
| Randomized primers       | 5,25 µL  | 100 µm        |
| RNase inhibitor          | 0,5 M    | 0,5 mM        |
| DTT                      | 1,25 M   | 1,25 mM       |
| RTE                      | 1 M      | 1 M           |
| RNA samples              | 10 µl    | 3 M           |
| Total volume:            | 25 µl    | 25 µl         |

### Table 2. Primer sequences of genes

| Gene  | Primer sequences                  |
|-------|-----------------------------------|
| PTGS2 | Forward: ATCATTCACCAGGCAAATTGC  |
|       | Reverse: GGCTTCAGCATAAAGCGTTTG |
| KRT19 | Forward: CCGGACAAAATCTTGTGCC   |
|       | Reverse: ATCCAGCACCCTGCGAGGCC |
| CALR  | Forward: AAGTTCTACGGTGACGAGGAG |
|       | Reverse: GTCGATGTGTCTGCTAGTGTTC|
| GAPDH | Forward: TGGTGATGGGAGGAGTTAGTAAGT|
|       | Reverse: AACCAATAAAACCTACTCCCTCTTAA|
| TMPRSS4| Forward: CCTGGCGAGATCATCATGTTGT|
|       | Reverse: GATCGTGCTCTTGAGAGGAGGCG|
| MAGE-A3| Forward: AAGCCTGGCCCAGGCTCGGT|
|       | Reverse: GCTGGGCAATGGAGGCCCAC|

### Table 3. PCR reaction components

| Content  | Amount | Final Concentration |
|----------|--------|---------------------|
| 2X SYBR Green | 10 µL  | 1 X                 |
| Forward primer | 1,5 µL | 0,3 µM              |
| Reverse primer | 1,5 µL | 0,3 µM              |
| cDNA      | 4 µL   | <500 ng             |
| Water     | 4 µL   |                     |
| Total     | 21 µL  |                     |

### Table 4. qPCR cycle stages

| Stage  | Temperature | Time |
|--------|-------------|------|
| 1. Denaturation | 95 ºC | 30 sec |
| 2. Annealing  | 58 ºC | 30 sec |
| 3. Elongation | 72 ºC | 30 sec, Fluorescent reading |
| Final        | 72 ºC | 10 min |

X 36 Cycle
Results and Discussion

In quantitative PCR analysis, the expression levels of the PTGS2, KRT19, TMPRSS4, CALR, and MAGEA3 genes were calculated by normalizing them according to the GAPDH gene. Table 5 and Table 6 summarize the values used when calculating the expression levels of the studied genes in HCT116 and PC3 cell lines and the increase and decrease rates of those genes. BEAS-2B cell line was used as a control.

Table 5. Calculation results of Ct values and expression change rate in HCT116 cell line

| Gene     | HCT116 Average ct value | Delta ct (ΔCt) | Gene Expression Change 2^ΔΔCt |
|----------|-------------------------|----------------|-------------------------------|
| GAPDH    | 26.85                   | 4.34           |                               |
| TMPRSS4  | 24.77                   | 2.08           | 4.34                          |
| MAGE-A3  | 20.96                   | 5.89           | 2.82                          |
| KRT19    | 15.1                    | 11.75          | 93.70                         |
| CALR     | 15.96                   | 10.89          | 0.31                          |
| PTGS2    | 26.19                   | 0.66           | 0.17                          |

qPCR analysis for the HCT116 cell line showed a 4.34-fold increase in expression of the TMPRSS4 gene, a 2.82-fold increase in expression of the MAGEA3 gene, a 93.70-fold increase in expression of the KRT19 gene, a 3.22-fold decrease in expression of the CALR gene, and a 5.88-fold decrease in expression of the PTGS2 gene by using the calculation of ΔΔCt compared to the control cell line BEAS-2B. We also calculated the expression levels of PTGS2, TMPRSS4, and MAGEA3 genes in the PC3 prostate cancer cell line. According to the data we obtained in this cell line, there was 50 fold decrease in PTGS2 gene expression, 2.09 fold increase in TMPRSS4 gene expression, and a 6.10 fold increase in MAGEA3 gene expression (compared to the control cell line).

PTGS2, also known as cyclooxygenase (COX), is an enzyme that catalyzes the rate-limiting step in inflammatory prostaglandin synthesis. PTGS2 is an inducible enzyme that converts arachidonic acid to prostaglandins, which play a role in cell proliferation and inflammation (Thongprasert et al., 2016). Studies have shown that PTGS2 also has roles in apoptosis, immune suppression, tumor progression, and metastasis (Hla et al., 1999; Liu et al., 1998). In our study, we found that PTGS2 gene expression decreases as 50 times in the PC3 prostate cancer cell line and 5.5 times in the HCT116 colorectal cell line. Similar to our study, Subbarayan et al. have also found decreased PTGS2 expression in cancer prostate cells than normal prostate cells. Also, in the same study, it was shown that when TNF-α stimulated lower PTGS2 levels in tumor cells, PTGS2 levels increased, leading to tumorigenesis (Subbarayan et al., 2001). Besides the studies showing that PTGS2 has lower expression in prostate cancer cells than normal cells, there are also studies claiming the increased expression of PTGS2 in prostate cancer cells. In their studies of the expression of HER2 and PTGS2 in prostate cancer cells, Edward et al. (2004) have found that PTGS2 expression increases in parallel with the tumor stage (Edwards et al., 2004). Bin et al. (2011), in their studies done in Chinese patients, investigated PTGS2 expression in prostate cancer cells; they determined that PTGS2 is expressed more significantly in tumor cells in comparison to the surrounding tissue cells, and the expression is higher in highly metastatic cell lines (PC-3 M) when compared to low metastatic cell lines (PC-3, DU-145, LNCaP) (Bin et al., 2011). However, as Subbarayan et al. (2001) state, low expression of PTGS2 in prostate cancer does not show that PTGS2 has no role in prostate cancer formation. Subbaryan et al. (2001) demonstrated that although the expression is low in prostate cancer cells, after subsequent TNFα exposure, this protein's increase occurs (Subbarayan et al., 2001). More studies are needed to understand the role of PTGS2 in prostate tumor development, metastasis, and invasiveness.

Table 6. Calculation results of Ct values and expression change rate in PC3 cell line

| Gene     | PC3 Average ct value | Delta ct (ΔCt) | Gene Expression Change 2^ΔΔCt |
|----------|----------------------|----------------|-------------------------------|
| GAPDH    | 28.63                | 2.01           |                               |
| TMPRSS4  | 27.6                 | 1.03           | 2.09                          |
| MAGE-A3  | 21.63                | 7              | 6.10                          |
| PTGS2    | 30.64                | -2.01          | 0.02                          |

While PTGS2 is not found in normal colon mucosa, it is present in 95% of colorectal carcinomas (Brown and DuBois, 2005). In the studies performed, it was
shown that PTGS2 expression decreases the cell-to-cell adhesion, increases angiogenesis, increases cell proliferation, and decreases apoptosis (Kakiuchi et al., 2002). High PTGS2 gene expression increases the PGE2 amount and NF-kB pathway activation. Increased NF-kB pathway activation triggers BCL2 transcription and causes intracellular apoptosis resistance (Park et al., 2006). In our study, however, in contrast to studies showing increased PTGS2 expression in several solid tumors, we found decreased PTGS2 expression in the colorectal cell line. This result shows that PTGS2 can have a different effect than demonstrated so far on colorectal cancer.

The other gene whose effects on colorectal cancer cell lines we studied was calreticulin (CALR). We found 3.22 times decrease in its expression in colorectal cancer. CALR is a multifunctional protein found mainly in the ER and on the cell membrane, nucleus, and cytoplasm. It is the primary calcium regulator in the cell, and it is thought that it has a role in cancer cell invasion and metastasis. In several studies performed on solid tumors such as prostate, breast, and colorectal cancers, the overexpression of CALR was found (Zamanian et al., 2013). Our study found that in the HCT166 colon cancer cell line, CALR gene expression is 3.22 times decreased. There are limited studies on CALR gene expression in colon cancer. Colangelo et al. found that in colon cells, miR27-a suppresses MHC class I expression through CALR downregulation, eventually affecting tumor formation (Colangelo et al., 2016).

Another gene that we studied was KRT19. As an intermediate type filamentous protein, KRT19 is expressed in both normal cells and malignant tumor cells. We found that the KRT19 expression level increases by 93.70 times in HCT-116 colorectal cell line. There are very few studies of colorectal cancer and KRT19. Saha et al. showed that KRT19 expression has a role in cancer development (Saha et al., 2019). We also found 93.70 times increase in KRT19 expression in the HCT 116 colon cancer cell line, contributing to the literature.

A few studies of the TMPRSS4 gene and its effect on prostate and colon cancers are present in the literature. We found the gene expression of TMPRSS4 in PC3 prostate and HCT166 colon cancer cell lines 2.09 and 4.34 times increased, respectively. This result is in parallel with the findings in a few studies in the literature. High TMPRSS4 levels were related to poor prognosis in both colon and prostate cancer (Kim et al., 2019; Huang et al., 2013). Our study contributes to the limited data in the literature.

The last gene whose expression we studied is MAGE-A3, and an increase was found in two cancer cell line types we studied. These findings were similar to the literature data. In the studies performed on both cell lines and patients, for two cancer types, high MAGE-A3 was found to be correlated with disease progression and was used as a bad prognosis marker (Ayyoub et al., 2014).

**Conclusion**

In this study, expression of the TMPRSS4 gene and MAGE-A3 gene was found as increased in both colon and prostate cancer cell line. We found PTGS2 gene expression as decreased in both cell line. Especially in PC3 prostate cancer cell lines, there was 50 fold decrease in PTGS2 gene expression. There is a need for further studies to determine the mechanism by which those genes cause cancer formation.

**Conflict of Interest**

All authors declare that there is no conflict of interest.

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