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

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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, *TMPRS4* 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% CO_2. Cells were harvested after incubation, and cell pellets were obtained for analysis.

RNA Isolation

To obtain total RNA from cultured 1 x 10^6 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.

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

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 estimeted via a standart 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 2. Primer sequences of genes

Gene	Primer sequences
PTGS2	Forward: ATCATTCACCAGGCAAATTGC
	Reverse: GGCTTCAGCATAAAGCGTTTG
KRT19	Forward: CGGGACAAATTCTTGGTGCC
	Reverse: ATCCAGCACCCTGCGCAGGCC
CALR	Forward: AAGTTCTACGGTGACGAGGAG
	Reverse: GTCGATGTTCTGCTCATGTTTC
GAPDH	Forward: TGGTGATGGAGGAGGTTTAGTAAGT
	Reverse:AACCAATAAAACCTACTCCTCCCTTAA
TMPRSS4	Forward: CCTGGCGAGTATCATCATTGTG
	Reverse: GATCGGTCCTTGGAGAGGCG
MAGE-A3	*Forward: AAGCCGGCCCAGGCTCGGT*
	Reverse: GCTGGGCAATGGAGACCCAC

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

Denaturation	94 °C 4 min.
1.Denaturation	95 °C 30 sec.
2. Annealing	58 °C 30 sec.
3. Elongation	72 °C 30 sec, Fluorescent reading
Final	72 °C 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

	HCT116 Average ct value	Delta ct (ΔCt)	Gene Expression Change 2^- ΔΔCt
GAPDH (house- keeping gene)	26,85		
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 *MAGEA* 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 *TMPRS4* 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

	PC3 Average ct value	Delta ct (ΔCt)	Gene Expression Change 2^- ΔΔCt
GAPDH (house keeping gene)	28,63		
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 *MAGEA3* 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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