

# hCAIX Expression and Cell Cycle in Cytokine Stimulated Human Colon Cancer Cells

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## Research article

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# Abstract

**Background:** At the aim of this study, we investigated the effects of different kind of cytokines and the combinations of which on human carbonic anhydrase IX (hCAIX) expression in HT-29 cell selected as colon carcinoma model for different doses and time of exposure to determine role of cytokines for treatment of colon carcinoma cells. **Results:** To sum up, h CA9 expression in the levels of gene and protein increased in HT-29 cells when stimulated with 1000 U/mL TGF- $\beta$  for 24 h. The stimulation of HT-29 cells with IL1  $\alpha$  alone and IL1 $\alpha$ - TGF- $\beta$  combination has not revealed any effect on hCA9 expression in both levels of gene and protein in contrast to, 1000 U/mL IL1 $\alpha$ -TNF $\alpha$  and especially TGF $\beta$ -TNF $\alpha$  have reducing effect on h CA9 expression level in HT-29 cells for time ranges of 24 h, 48 h, 72 h. In addition to this data, it was observed that HT-29 cells at the phase of G0G1 are arrested in cell cycle when stimulated with either of 1000 U/mL IL1 $\alpha$ -TNF $\alpha$  and TGF $\beta$ -TNF $\alpha$ . Moreover, it was observed that h CA9 expression level in HT-29 cells decreases at the phases of synthesis (S) and G2M. **Discussion:** We concluded that combination of TNF $\alpha$ -TGF $\beta$  has created antagonistic effect on hCA9 expression in HT-29 colon cancer cell model. On the other hand, combination of TNF $\alpha$ -IL1 $\alpha$  caused synergistic effect on h CA9 expression level in this cell model. When these results are demonstrated decrease in the cytokine exposed hCA9 expression is a finding to develop a novel approach for anticancer therapy. **Conclusion:** Our finding related to the change in the expression of hCA9 following cytokine stimulate to colon cancer cell line gives an idea about the effect of cytokine stimulation on the expression of this gene which will be a hot spot research in colon carcinogenesis. **Methods:** HT-29 colon cells were chosen as a colorectal adenocarcinoma model. hCA9 gene expression in the level of mRNA was measured in cytokines stimulated HT-29 cells by Quantitative Real-time PCR. Meanwhile, hCAIX expression in the level of protein and cell cycle assay were detected by flow cytometry.

## Background

Colorectal cancer is the most common third type of cancer observed in the world. In the world, colorectal cancer causes 395.000 people to die each year [1, 2]. Therefore, enlightenment of genes which are responsible for colorectal cancer is crucial to contribute a new dimension to conventional recruitment of colorectal cancer.

Human carbonic anhydrase IX (*hCAIX*), which is one of the popular transmembrane protein related with tumor progression and metastasis, is a member of transmembrane carbonic anhydrases. Expression of CAIX is so limited in normal cells. However, hypoxic conditions of the tumor cause to increased CAIX expression and hypoxia inducible factor alpha (HIF1- $\alpha$ ) in different tumor types. These data provide a *hCAIX* is a powerful marker in terms of tumor hypoxia [3, 4]. Moreover, this *hCAIX* enzyme plays a critical function in tumor invasion and metastasis by the way of cell to cell adhesion [5]. Therefore, recent studies about especially *hCAIX* and various types of cancer attract the attention on this carbonic anhydrase subtype [6]. *hCA9* is one of the important genes shown in colorectal tumorigenesis. *hCAIX* expression in colorectal tumors was first demonstrated by Saarnio and et al. along with Ki-67 antigen which is also a major marker for cell proliferation in colorectal adenocarcinoma [6, 7].

Cytokines are secreted peptides which have multiple roles in regulation of cellular interaction and communication and cell proliferation, differentiation, invasion in cancerogenesis. On the side, since these molecules have been used in immunotherapy, studies about cytokines have been getting worse. The fact that these molecules have immunotherapy potential against malignant diseases is the focus of the researchers [8–10].

Immune effector cells capable of destruction of human tumors should be defined as *in vitro* [11]. IL-1 $\alpha$  is the most potent pro-inflammatory cytokines that is generated by macrophages immediately after confronting the inflammatory stimuli. In addition, molecules from IL1 family are mostly expressed in tumor regions and is effective on each stage of tumorigenesis such as tumor invasion and connection between host and immune system in malign cells [12]. Like IL1 $\alpha$ , TGF $\beta$  beta has also pleiotropic roles in tumor cells. TGF $\beta$  supports malign transformation and tumor progression in various types of cell. In studies about colorectal cancer, high level of expression of TGF $\beta$  in primary tumor depends on late stages. Divergent effect of TGF $\beta$  in carcinogenesis depends on differentiation status and proliferative capacity of epithelium cells. TGF $\beta$  has an inhibition effect on well differentiated primary colon carcinoma, TGF $\beta$  makes less differentiated cells invase and proliferate [13, 14]. Therefore, the effect of cytokine on the modulation of cancer-related genes needs to be adressed. In this work, we focused on determining the effect of cytokine on *hCAIX* expressions. There is rather limited study available about cytokines regulation of *hCAIX*. Yildirim and Kockar in 2009 reported that TGF $\beta$  upregulates *hCAIX* expression in liver cell model, Hep3B. It is crucial to know that the knowledge of this type of interaction might be important for improvement of new therapeutic approaches [15].

We hypothesized that if cytokines acting through *caix* expression levels alteration and relation with cell cycle phases in HT-29 cancer cell line. To test this hypothesis, cell line treated with different cytokines and these combinations. Untreated cells were used as control. Our research group previously demonstrated that TNF $\alpha$  decreased the *hCA9* mRNA anf protein expression along with cell cycle arrest in HT-29 cells [16]. Because of the insufficient research about the relation between cytokines and expression of *hCAIX*, this study was conducted in order to investigate contribution of the above mentioned cytokines in regulation of *hCA9* which is a crucial marker in colorectal cancer.

## Methods

### 2.1. Cell culture of HT-29 colon cells

HT-29 colon cell line provided by Sukran Yilmaz, Sap Institue, Ankara, Turkey. HT-29 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and incubated at 37°C in a humidified atmosphere of 95 air and 5% CO<sub>2</sub> incubator. Then, cells were seeded into a flask of 25 cm<sup>2</sup> and following 24 h, these cells were treated with double combinations of these cytokine (TGF $\beta$ , IL1 $\alpha$ ). TNF $\alpha$  cytokine was also included in combination protocol.

### 2.2. Cell cycle analysis of HT-29 colon cells by flow cytometry

The analysis of the cell cycle phase was performed as described previously [16]. Briefly, cells were washed in PBS and then stained with PI using a commercial kit (Beckman Coulter, USA). Cellular DNA content was measured and analyzed on a flow Cytometry System with CXP software (Beckman Coulter FC500 System, USA).

### **2.3. Annexin V staining apoptosis test**

HT-29 colon cells were seeded into a 6-well plate. After 24 h incubation process, each one of 1000U/mL TGF $\beta$ , IL1 $\alpha$  separately and 1000U/mL various combinations of IL1 $\alpha$ -TNF $\alpha$ , IL1 $\alpha$ -TGF $\beta$ , TNF $\alpha$ -TGF $\beta$ .were added to the cells. The follow-up protocols were carried out according to the manufacturer protocol of AnnexinV-FITC kit (Beckman Coulter, USA). Samples were analysed by a flow cytometer and analyzed with CXP software (Beckman Coulter FC500 System, USA).

### **2.4. Total RNA isolation and cDNA synthesis**

Total RNA isolation from the HT-29 cells was done with RNeasy mini kit (Qiagen GmbH., Germany). Reverse transcription for cDNA synthesis was performed in 20  $\mu$ l final reaction volume including 50 U MuLV reverse transcriptase (Applied Biosystems, Foster City, CA), 10X PCR buffer, 5 pmol specific antisense primer, 4 U RNase inhibitor (Roche), 4 mmol/L of each dNTPs, 6.25 mmol/L MgCl $_2$ , and 2  $\mu$ l (1ug) RNA. The RT step was performed as described previously on a Corbett Research Thermocycler at 42°C for 30 minutes followed by 94°C for 5 minutes [16].

### **2.5. *hCA9* gene expression**

*hCA9* (target gene) and TATA binding protein (TBP-housekeeping gene) gene expressions were carried out by quantitative Real-time RT-PCR using the Rotorgene system (Qiagen, Germany) [16]. The primers provided by Primer Design (Primer Design, USA) company designed depending on published information about mRNA sequences in GenBank (sequence Accession Nos., *hCA9*: NM\_001216). For 105 bp amplification product, sense primer was "AGCAGAGGTAGCCGAGACT" (position:1.395) and antisense primer was "ATGAGCAGGACAGGACAGTTA" (position:1.499). For TBP gene, sense primer sequence was "CTGTTTAACTTCGCTTCC" and antisense primer sequence was "CTCTTCTCAGCAACTTCC". The reactions were performed with 5  $\mu$ l cDNA template in a final volume of 25  $\mu$ l, containing 12.5  $\mu$ l Sybr Green master mix (Qiagen, Germany), with 15 pmol primers for whole genes in a Rotor-Gene Real-time PCR instrument (Qiagen, Germany). Initial denaturation was 1 min at 94°C, 40 cycles of specific annealing temperature for 2 sec, extension at 72°C for 10 sec and denaturation at 94°C. Melting curve analysis was carried out in the temperature range of 55 to 95 °C. We calculated the relative quantification using the ratio between *hCA9* and TBP mRNA. Arbitrary copy numbers were analyzed using Rotor-Gene v.5 software (Qiagen, Germany). A melt curve was obtained from 65-99°C for the specificity of the reaction. Three technical and biological reactions were performed for each transcript and negative control was used.

## 2.6. *hCAIX* protein expression

Briefly, after cell culture procedures, cells were scraped with cell scraper and then  $1 \times 10^5$  cells suspended in PBS. Human anti-CAIX antibody was added in each test tube for 45 minutes in ice bath and then washed with PBS to remove unbound antibodies. The assay was carried out in triplicate. The expression of *hCAIX* was monitored and analyzed on a Beckman Coulter FC500 Flow Cytometry System with CXP software (Beckman Coulter, USA).

## 3. Statistical analysis

Each data was the mean of three independent values. All data were expressed as mean  $\pm$  S.D. and analyzed one-way ANOVA Test by SPSS 11.5 (SPSS Inc., Chicago, IL, USA) to determine the significance of differences between groups (untreated control cells and treated cells).  $P < 0.05$  was considered statistically significant.

# Results

## 4.1. Protein expression

HT-29 colon cells were seeded into a 6-well plate and incubated for 24 h. After incubation process, either of 1000 U/mL TGF $\beta$  and IL1 $\alpha$  and various combinations of 1000 U/mL of IL1 $\alpha$ -TGF $\beta$ . were seeded to the cells and incubated for 24 h, 48 h and 72 h. We previously demonstrated that TNF-alpha caused the decrease CAIX mRNA, the combinations of TNF $\alpha$ -TGF $\beta$  and IL1 $\alpha$ -TNF $\alpha$  were included to the analysis. Firstly, we investigated the influence of different doses (100 U/mL, 500 U/mL, 1000 U/mL) and exposure times (24 h, 48 h and 72 h) of TGF $\beta$  and IL1 $\alpha$  and various combinations of 1000 U/mL IL1 $\alpha$ -TNF $\alpha$ , IL1 $\alpha$ -TGF $\beta$ , TNF $\alpha$ -TGF $\beta$  on *hCA9* protein expression level. In order to determine this stimulation effect in HT-29 cells, flow cytometry technique was utilized. While analyzing experimental data, all results evaluated relative to untreated control group. As a first result, we observed that TGF $\beta$  has nearly 26% increase on CAIX protein expression level at a concentration of 1000 U/mL for 24h. Secondly, when the effect of IL1 $\alpha$  was investigated, any significant influence has not been detected. As in previous results, we could not observe the effect of 1000 U/mL IL1 $\alpha$ -TGF $\beta$  combination on *hCA9* protein expression. In contrast, the other combinations of cytokines (IL1 $\alpha$ -TNF $\alpha$  and TNF $\alpha$ -TGF $\beta$ ) have revealed decreasing effect on *hCA9* protein expression level at the concentration of 1000 U/mL for 24 h, 48 h and 72 h when compared to the untreated control cells. For IL1 $\alpha$ -TNF $\alpha$  combination, *hCAIX* protein expression level decreased nearly 22%, 8% and 16% for 24 h, 48 h and 72 h, respectively. For TNF $\alpha$ -TGF $\beta$  combination set, we only observed significant decrease for 72 h incubation time. As shown in figure 2, the level of the *hCAIX* protein expression dropped nearly 45%, for 72 h. The results were obtained by performing the experiment in triplicate.

## 4.2. mRNA expression of *hCAIX*

The effect of these cytokines on *hCA9* mRNA expression level was evaluated relative to untreated control cell group. Firstly, HT-29 cells were exposed to TGF $\beta$  for 24 h, it was observed that *hCA9* mRNA expression level pointed out nearly  $2.5\pm 0.8$ -fold increase when compared to untreated control cells as shown in figure 1. This result is consistent with the data mentioned on *hCAIX* protein expression part. No significant effect of TGF $\beta$  on *hCA9* mRNA expression was observed in HT-29 cells for 48h and 72 h (data not shown). Secondly, we investigated the effect of IL1 on *hCA9* mRNA expression for different doses (100 U/mL, 500 U/mL, 1000 U/mL) and incubation periods (24 h, 48 h, 72 h) in HT-29 cells. However, we could not detect any significant effect of IL1 $\alpha$  on *hCA9* expression level for different doses and periods. Thirdly, we studied the effect of IL1 $\alpha$ -TGF $\beta$  combination at 1000 U/mL concentration for different incubation times. However, we could not observe any effect of this combination set on *hCA9* expression level as in previous case. Although the use of IL1 $\alpha$  alone and together with TGF $\beta$  has not revealed any acceptable effect on the *hCA9* expression level, the combination of TGF $\beta$ -IL1 $\alpha$  significantly influenced the *hCA9* expression level. 1000U/mL of IL1 $\alpha$ -TNF $\alpha$  combination caused to  $3.9\pm 1.2$  fold reduction of *hCA9* expression level for 48 h. As the incubation time increased to 72h for the same combination set,  $15.7\pm 7.9$ -fold decrease in *hCA9* expression level was observed as shown in figure 2. Lastly, the effect of TNF $\alpha$ -TGF $\beta$  combination on CA9 expression level was studied for 24 h, 48 h and 72 h. The similar change in CA9 mRNA expression level in terms of incubation time was observed for this combination set of cytokines. As similar to the effect of previous combination, TNF $\alpha$ -TGF $\beta$  combination led to  $2.4\pm 0.7$ ,  $3.4\pm 2.1$  and  $7.0\pm 0.4$ -fold decrease in *hCA9* expression level for 24 h, 48 h and 72 h, respectively as shown in figure 2. All experiment was performed in triplicate.

### 4.3. Cell cycle analysis and apoptosis

Flow cytometry method is easily used for cell cycle studies. PI staining was used to detect cell cycle phases (G0G1, S, G2M) with or without cytokines (1000 U/mL TGF $\beta$ , IL1, IL1 $\alpha$ -TNF $\alpha$ , IL1 $\alpha$ -TGF $\beta$ , TNF $\alpha$ -TGF $\beta$ ) treatment in HT-29 cells. We determined the statistically significant G0G1 arrest with concomitant depletion of S and G2M phases in HT-29 cells for 24 h, 48 h and 72 h at a highest concentration of 1000 U/mL TNF $\alpha$ -TGF $\beta$  cytokine combination. For 1000 U/mL IL1 $\alpha$ -TNF $\alpha$  combination, we observed similar effect as in cell cycle phases in HT-29 cells for 72 h. The results are shown in table 1. No apoptotic effect was detected for any cytokine set in HT-29 colon cancer cells. All data obtained from three independent assay and  $p<0.05$  value was considered statistically significant for all experiment.

## Discussion

The expanding drugs and inhibitors have been used that inhibition of *hCAIX* against various cancer and metabolic disorders as a molecule [17–19]. This knowledge prompted us to investigate the effects of different cytokines that crucial proteins for immunotherapy on expression of *hCAIX* critical proliferation mechanism of cancer cells. HT-29 model colon cells are utilized at many studies about colorectal carcinoma. These cells are important in many studies since they have most of characteristic colonic epithelium [20]. Several investigators reported the *hCAIX* expression in colon cells. When normal mucosa in which hypoxic response is observed early *hCAIX* is compared with colorectal cancers, it is observed

that there is an increase in *hCAIX* expression in colorectal cancers [6, 21, 22]. According to the study demonstrated by Adrián (2003), expression of carbonic anhydrase IX was increased under hypoxic conditions in xenograft nude mice model that were created a colorectal tumor using HT-29 colon carcinoma cells [23]. For this purpose, we selected HT-29 colon carcinoma cell line to point out relationship between *hCA9* expression and cytokines. In this study, two cytokines (IL1 $\alpha$  and TGF $\beta$ ) were used since these cytokines have been implicated in cancer. TNF $\alpha$ , previously demonstrated decreasing effect on *hCA9* gene expression, was also included into the study. This study, the association between cytokines and CA9 which is a gene related with tumor, was investigated due to the lack of information about this subject. The data obtained in our research attracts more attention because there was limited study conducted before about this topic. TGF $\beta$ , one of these cytokines, causes malign transformation and tumor progression in various types of cell. Moreover, production of TGF $\beta$  tends to increase in various cancer including colon cancer [24]. These cancer cells on which oncogenes are dominant activate transcription of TGF gene [25, 26]. In studies about colon and colorectal cancer, it was shown that high level of expression of TGF in primary tumor depends on late stages. There are various types of mechanisms affecting development of tumor. TGF has inhibition effect on primary colon carcinoma differentiated well and causes the invasion and proliferation of less differentiated cells. Divergent effect of TGF in carcinogenesis depends on differentiation status and proliferative capacity of epithelium cells. After the transformation, TGF supports viability, invasion and metastasis of tumor cells [27]. This interaction is important for improvement of new therapeutic approaches. In the study, we tried to determine whether the level of *hCA9* expression in HT-29 cells changes or not when stimulated with different cytokines for different doses (100 U/mL, 500 U/mL, 1000 U/mL) and exposure times (24 h, 48 h, 72 h). Moreover, we investigated cell cycle phases and apoptosis in HT-29 cells at the same conditions. When expression of *hCAIX* in HT-29 cells exposed to 1000 U/mL TGF $\beta$  was investigated for 24 h, the increase in expression of *hCAIX* was observed in the level of protein and mRNA. The increase in *hCA9* expression level in HT-29 colon cancer cells stimulated with TGF $\beta$  was shown in this study at first time. This observation can be regarded as significant finding since many common tumor cells have enhanced levels of TGF $\beta$  [28]. In another study, it was observed that the effect of TGF $\beta$  on *hCAIX* also has similar effect on Hep3B cells [15]. The results of these two studies have supported each other. These findings are crucial to develop new approaches for recruitment of cancer. In this context, it is important to investigate expression level of *hCAIX* used as biomarker. It has also active role in regulation of cell cycle for cell proliferation. In some various types of tumors, because of CA location and the fact that this molecule has a role in regulation of cell growth at other locations *hCAIX* expression in fast proliferating cells of tumor shows an increase [29].

IL1 $\alpha$  and TNF $\alpha$  are pro-inflammatory cytokines and inductions of which by inflammation are effective on expression of lots of genes. Cytokines such as IL1 $\alpha$  and TNF $\alpha$  taking place in both of carcinogenesis and development and invasion of tumors which induct chemokines attracting neutrophils playing active role in both of production of reactive types of oxygen and carcinogenesis [30]. There are so many investigation indicating the relation between cancer development and expressions of oncogenes. Besides this, existence of the relation between chronic inflammation and cytokine production brings additional

mechanism in the carcinogenesis process. In cancer, NFK $\beta$  activation has essential role in various tumor [31]. This activation is also essential for regulation of genes acting on immune response. However, the association between expression of oncogene and pro-inflammatory cytokines could still not have been raised. Molecules from IL1 family are mostly expressed in tumor regions and which are potential cytokines affecting tumorigenesis. IL1 is effective on each stage of tumorigenesis such as tumor invasion and connection between host and immune system in malign cells [12]. Immune effector cells capable of destruction of human tumors are defined as *in vitro*. Monoclonal antibodies identifying antigens related with tumors were produced in most of vertebrate cells including human being too [11].

Not only the effect of TGF $\beta$  on level of *hCAIX* expression was investigated, but the effect of IL1 $\alpha$  and TGF $\beta$  combinations of cytokines were also investigated in order to determine synergistic and antagonistic effects of cytokines. Effects of these cytokines on expression of *hCAIX* in rank both of protein and mRNA were investigated by introducing 1000 U/mL cytokines for different time ranges (24 h, 48 h and 72 h). It was seen that there is no remarkable effect of this combination of IL1 $\alpha$ -TGF $\beta$  on *hCA9* protein and mRNA expression level and on cell cycle phases of colon cancer cells. It could not be found that there is an effect on viability and necrosis. Moreover, the relations among *hCAIX*, cell cycle and apoptosis in model of colon carcinoma were clarified by considering parameters of cell cycle and apoptosis important for tumor development. When 1000 U/mL IL1 $\alpha$ -TNF $\alpha$  and TNF $\alpha$ -TGF $\beta$  combinations were introduced into HT-29 cells at 24 h, 48 h and 72 h, it was observed that the expression of *hCAIX* at the level of protein and mRNA sharply decreases as time dependent manner. This inhibition effect of TNF $\alpha$  combinations is significant and this fact was indicated in this study at first time. Our results have been correlated with the study in which the effect of TNF on histondeasetilase 3 related with tumors was investigated [32]. By considering this results, we can claim that combination of TNF $\alpha$ -TGF $\beta$  has created the antagonistic effect on CA9 expression in HT-29 colon cancer cell model. In the other hand, combination of TNF $\alpha$ -IL1 $\alpha$  caused synergistic effect on *hCA9* expression level in this cells. In addition to this, all TNF $\alpha$  combinations lead to arrest the colon cells in G0/G1 phase of cell cycle at. Furthermore, This combinations caused cell necrosis supported by several literatures [32–35] while colon cells at GOG1 stage, TNF $\alpha$  also lead to decrease in *hCAIX* expression in colon cells.

## Conclusions

This finding reflects the functionally multiple nature of these cytokines and represent a useful paradigm to study the complex cellular targets such as CAIX that regulate colon cancerogenesis. Therefore, immunotherapy potential of these cytokines against malign disease is always focus for researchers.

## Abbreviations

***hCAIX***: Human carbonic anhydrase IX

**TNF $\alpha$** : Tumor necrosis factor alpha

**IL1 $\alpha$** : Interleukin 1 alpha

**TGF $\beta$** : Transforming growth factor beta

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and material

Not applicable

### Competing interests

The authors declare that they have no competing interest.

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### Authors' contributions

RIS and FK constructed hypothesis and planned methodology equally for research. They contributed to write equally for manuscript. RIS contributed to all experimental studies, data analysis and presentation of results. SY contributed to cell culture studies. UM and AS contributed to intellectual content. All authors have read and approved this manuscript.

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## Tables

Table 1. Flow cytometry analysis of cell cycle phases of HT-29 colon cells treated with different combinations of cytokines for different times.

Cell cycle parameters			
	G0G1 (%)	S (%)	G2M (%)
<b>Control group (24 h)</b>	79,7±1,4	7,5±0,2	13,2±0,8
<b>IL1α-TNFα (24 h)</b>	84,9±0,4	4,9±0,2	10,3±0,2
<b>IL1α-TGFβ (24 h)</b>	83,3±0,8	5,8±0,5	10,9±0,2
<b>TNFα-TGFβ (24 h)</b>	89,7±0,7	3,1±0,2	7,4±0,4
<b>Control group (48 h)</b>	87,0±0,7	6,5±2,8	7,2±3,1
<b>IL1α-TNFα (48 h)</b>	89,4±0,2	3,7±1,1	6,7±0,8
<b>IL1α-TGFβ (48 h)</b>	87,3±4,2	3,8±0,2	6,3±0,8
<b>TNFα-TGFβ (48 h)</b>	90,5±0,4	3,2±1,0	6,3±0,6
<b>Control group (24 h)</b>	75,2±1,0	10,9±0,4	13,4±1,2
<b>IL1α-TNFα (24 h)</b>	82,0±0,9	5,9±0,7	11,9±0,3
<b>IL1α-TGFβ (24 h)</b>	78,9±0,3	5,8±1,3	15,1±1,1
<b>TNFα-TGFβ (24 h)</b>	86,2±5,8	5,7±0,7	11,5±0,2

## Figures

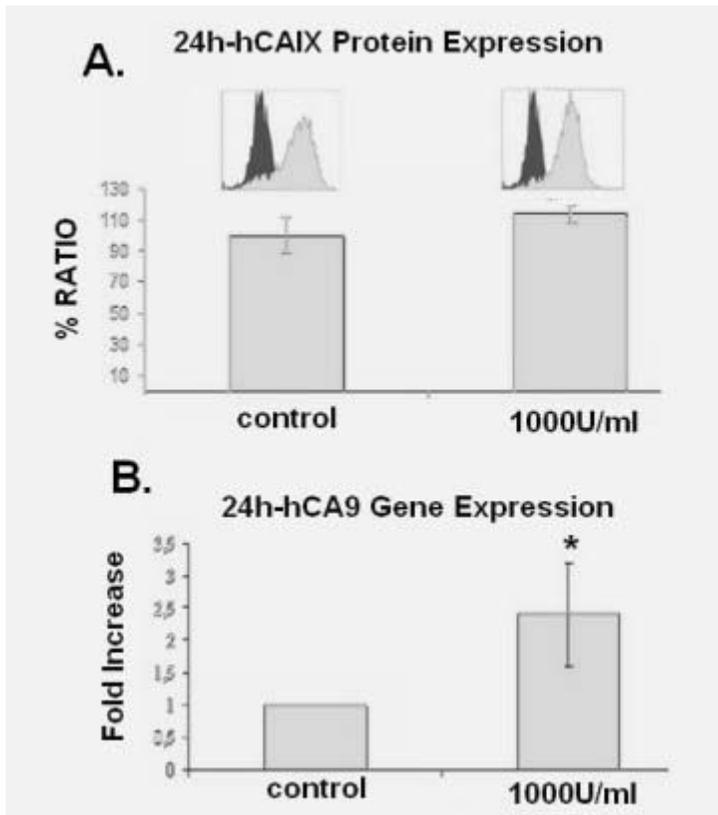


Figure 1

The graph of hCAIX expression level in TGFβ treated and untreated HT-29 cells for 24 h. A: The increase ratio of hCAIX protein expression at the highest concentration of TGFβ (1000 U/mL) exposed HT-29 colon cells for 24 h. B: Fold increase of hCA9 gene expression in 1000 U/mL TGFβ exposed HT-29 colon cells for 24 h.

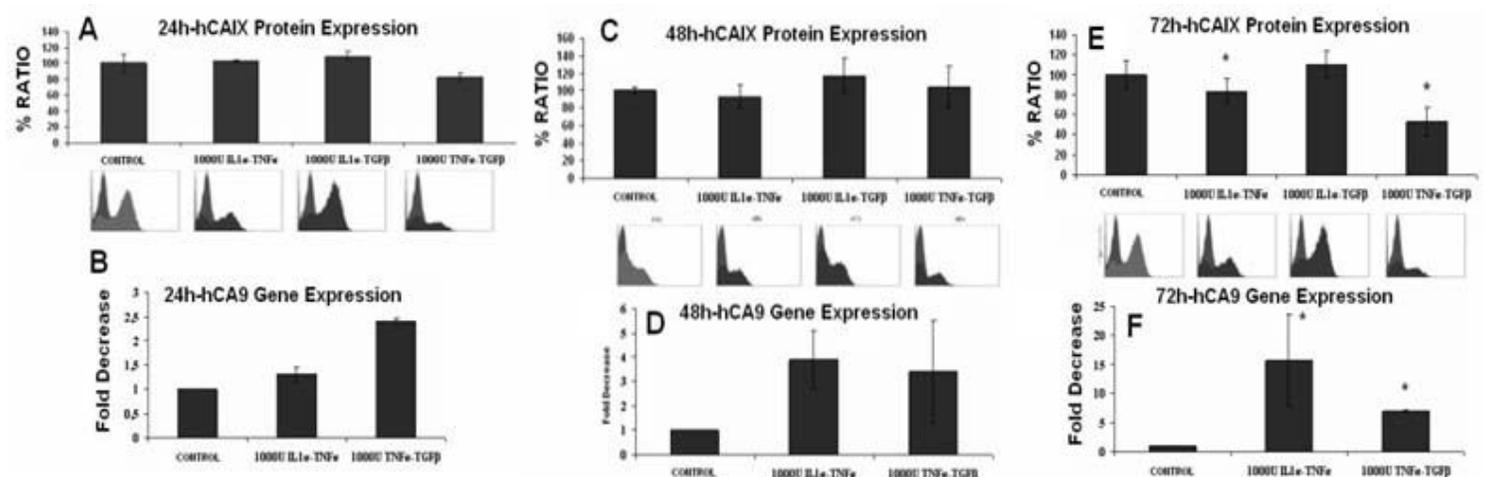


Figure 2

Flow cytometry analysis of hCAIX expression and Quantitative real time PCR analysis of CA9 gene in cytokine combination treated and untreated HT-29 colon cells. A: The decrease ratio of CA9 protein expression treated with IL1α-TNFα, IL1α-TGFβ, TNFα-TGFβ at concentration of 1000 U/mL for 24 h. B.

Fold decrease of hCA9 expression in the level of gene treated with IL1 $\alpha$ -TNF $\alpha$ , IL1 $\alpha$ -TGF $\beta$ , TNF $\alpha$ -TGF $\beta$  at concentration of 1000U/mL for 24 h. C: The decrease ratio of hCA9 protein expression treated with IL1 $\alpha$ -TNF $\alpha$ , IL1 $\alpha$ -TGF $\beta$ , TNF $\alpha$ -TGF $\beta$  at concentration of 1000 U/mL for 48 h. D: Fold decrease of hCA9 expression in the level of gene treated with IL1 $\alpha$ -TNF $\alpha$ , IL1 $\alpha$ -TGF $\beta$ , TNF $\alpha$ -TGF $\beta$  at concentration of 1000 U/mL for 48 h. E: The decrease ratio of hCA9 protein expression treated with IL1 $\alpha$ -TNF $\alpha$ , IL1 $\alpha$ -TGF $\beta$ , TNF $\alpha$ -TGF $\beta$  at concentration of 1000 U/mL for 72 h. F: Fold decrease of CA9 expression in the level of gene treated with IL1 $\alpha$ -TNF $\alpha$ , IL1 $\alpha$ -TGF $\beta$ , TNF $\alpha$ -TGF $\beta$  at concentration of 1000 U/mL for 72 h.