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Association of mRNA Levels of CB1R and/CB2R Genes in Leukocytes with Different Types of Cancer

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ABSTRACT

ECS can modulate several signaling pathways causing anti proliferative effects, whereas its activation can accelerate the progression of tumor. CB1 and CB2 receptors are members of ECS. Accumulated evidence elucidates that CB receptors have the ability to regulate several signaling pathways that they are critical to survive and growth cell. The main idea of this project is to evaluate the expression level of CB genes including (CB1 and CB2) in different types of patients with cancer. As well as to answer weather there is any association between mRNA levels of these receptors. The peripheral blood samples were collected from patients of different type of cancers and samples of control were studied. The level expression was measured using Real-time polymerase chain reaction. All types of cancer were expressed CB receptors compared with control group. CB1 and CB2 genes significantly expressed within prostate and cervical cancers. In contrast, they expressed with low level in lymphoma cancer. These findings suggest a possible function of CB genes in cancer disease. The expression levels of CB1and CB2 genes are not very well correlated. However, current study represents the first one in Iraqi cancer patients. Thus, further studies are required to study these genes functions and their roles in cancer progression.

Introduction

Endocannabinoid system comprises of endogenous cannabinoids (ligands), cannabinoid receptors (CBR) and enzymes, which implicated in regulating endocannabinoids synthesis, transport them and their degradation as well as signaling pathways that regulated by the receptors activation (Fraguas-Sánchez *et al.*, 2016). ECS involved in multiple physiological functions including anxiety, stress responses, energy balance, embryogenesis and immune response (Fraguas-Sánchez *et al.*, 2018). These physiological processes triggered by binding endogenous lipid, which produced from phospholipids cell membrane, by CB receptors (Lakiotaki *et al.*, 2015). CB receptors are organs of largest trans membrane family protein that important to transduce extracellular signals into several types of intercellular responses (Moreno *et al.*, 2019). Two types (CB1R and CB2R) differently distributed in human tissues (Miller and Devi, 2011).

In the human genomic DNA, cannabinoid receptor gene 1 (CNR1) composed of 4 exons and is located in the long arm of chromosome 6 (6q15). This gene encodes three isoforms of CB1R protein (Moreno *et al.*, 2019). CB1 receptor is mainly detected in adipocytes, pancreas, liver, T-lymphocytes, skeletal muscle and central nervous system (Miller and Devi, 2011). The human cannabinoid receptor gene 2 (CNR2) composed of three exons and is existed in short arm of chromosome 1(p36.11). This gene encodes multiple isoforms of CB2R protein (Moreno *et al.*,

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2019). CB2 receptor located in immune system, cerebrum micro vascular and central nervous system cells like microglia and astrocytes (Pertwee *et al*., 2010).

Both (CB1and CB2) receptors are implemented in regulating vital processes of liver, lungs, kidneys, skin, bones, lymph nodes and reproductive system as well they are expressed in the periphery (Moreno *et al*., 2019). Breast tissue has been found to contain cannabinoid receptors; CB1 was detected in patients with breast cancer (Qamri *et al.*, 2009). As well as, CB2 genes transcript was identified in a number of cell lines including breast cancer (MDA-MB-468, EVSA-T, SkBr3 and T-47D,) and breast cancer tissues (Melck *et al*., 2000; Ligresti *et al*., 2006; Sarnataro *et al.*, 2005). Up-regulation of CB transcript level may be linked to the cancer aggressiveness (Moreno *et al*., 2019). Endogenous CB1 agonists and 2-Arachidonoylglycerol (2-AG) were decreased cells invasion through reducing phosphokinase activity and the dependent inhibition of CB1 receptor (Nithipatikom *et al*., 2004). CB2 receptor expression was dysregulated in endometrial cancer for example, its level expression was higher in AN3CA (Uterus, Endometrium) human cell line when it is transfected with cDNA of CB2 receptor (Guida *et al*., 2010). This research aims to measure the levels of CB1 and CB2 genes mRNA in various carcinoma patients.

Materials and methods

Blood Samples collection of patients

Human peripheral blood samples (40 samples) were obtained from patients with cancer including (prostate, cervical, colon and lymphoma cancer), at least three samples for each type of cancer were collected at Al -Diwaniya hospital. Samples of human peripheral blood (40 samples) were obtained from healthy volunteers that used as a control group. 3ml of each sample were kept in EDTA tube and the samples were kept at 4 °C until used to extract RNA samples.

RNA extraction, cDNA creation

Total RNA of cells was extracted using organic RNA extraction methods by adding 1m of Trizol reagent into blood cells (Bioneer Auzol™, Irans Zolvp, Tran) according the manufacture's instruction. After that RNA samples were quantified using a NanoDrop. The first strand of cDNA was synthesised following manufacture's protocol of kit (Easy Script® one- step gDNA removel and cDNA synthesis super mix (Trans)).

Quantitative PCR

Created complementary DNA (cDNA) and specified primers were utilized to detect RNA transcripts of a given gene. Cycle threshold (Ct) value was detected using the Step One Plus™ Real-Time PCR system (Strata gene Agilent technologies, Germany).

Statistical analysis

Real time PCR results are represented as cycle threshold (Ct) value comparative to a housekeeping gene (human glyceraldehyde-3- phosphate dehydrogenase (GAPDH)) that represents ΔCt. $2^{-\Delta\Delta Ct}$ formula of Livak and Schmittgen, 2001 was applied to calculate fold change of mRNA target gene expression where ΔΔCt = ΔCt (patient)- ΔCt (control). ANOVA (One-way) and T-test were applied to identify the significance of qPCR results. The significance was characterized according to P-value if it is equal 0.05 or less than this value.

Results

Cannabinoid receptor 1 (CB1) gene expression

Relative CB1gene expression was significantly expressed in whole blood samples of cancer samples (median ΔCt = 11.06±0.28, $p \leq 0.05$) and in all healthy samples (median ΔCt 12.09±0.35). The calculated fold change of CB1mRNA of patients cancer increased up to 2.05

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times compared with control samples (Table 1).

Genes	Groups	C_T of GAPDH	C_T Mean CB genes	ΔC_T	$\Delta\Delta C_T$	Fold change
CB1R	Control	27.16±0.35	39.26±0.22	12.09±0.35	- 1.03	2.05
	Cancer samples	26.26±0.18	37.32±0.22	11.06±0.28*		
CB2R	Control	27.74±0.53	36.64±0.46	8.89±0.64	-1.65	3.13
	Cancer samples	26.03±0.29	33.28±0.29	7.24±0.38*		

Table (1) CB genes expression in cancer patients healthy groups. *Results show cycle threshold (Ct) for GAPDH, CB1 and CB2. ΔCt values represent the difference between Ct of GAPDH and Ct of CB1 or CB2 genes. ΔΔCt shows fold change. Results showed as a mean ±slandered error mean.*

Riser expression of CB1 gene was significantly identified with cervical carcinoma ($p<0.05$) and prostate cancer. However, there is no significant transcript of CB1 was observed in colon and lymphoma cancers as shown in figure (1).

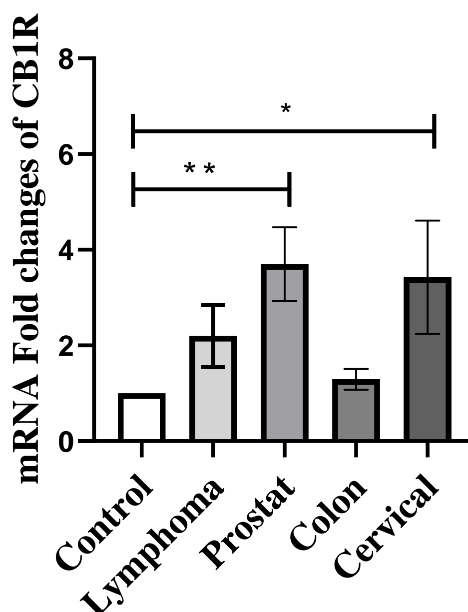

Figure (1) CB1 gene relative expression of patients samples compared with control group. *The results offered as mRNA fold change relative to control group. Error bars refer to ±SEMs from three technical replicates. The significant was detected depending ANOVA (One-way) test.*

Cannabinoid receptor 2 (CB2) gene expression

CB2 receptor expression was determined in healthy blood samples (median ΔCt = 8.89±0.64) as well as its expression was significantly increased in blood samples of cancer patients (median ΔCt = 7.24±0.38, $p\leq0.05$) (Table 1). Additionally, the calculated fold change of CB2mRNA was increased up to approximately three times in cancer patients compared with control group (Table 1).

Quantitative PCR was performed to measure CB2 mRNA gene in different samples of cancer. As shown in figure (2), CB2 mRNA level was characterized in all studied types of cancer. The lowest expression level of CB2 receptor was detected in lymphoma cancer. However, the heights level of mRNA transcript of CB2 gene was in cervical cancer ($p<0.0001$). Colon cancer

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significantly expressed CB2 mRNA at $p<0.01$. In addition, CB2 transcript level was significantly detected in prostate cancer (Figure 2, $p <0.01$).

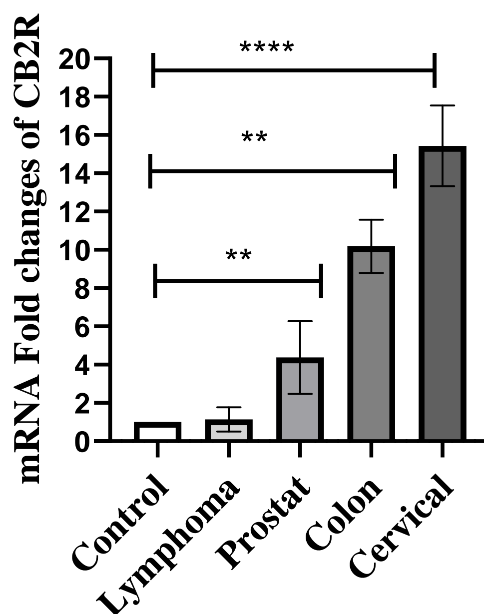

Figure (2) CB2 receptor gene relative expression of patients cancer compared with control group. *Error bars identified as ±SEMs from three technical replicates. The significant assessed According to ANOVA (One-way) test.*

All types of cancer that included in current study (lymphoma, prostate, colon and cervical cancers) expressed cannabinoid receptors (CB1 and CB2) mRNA (figure 3). ΔCt method was applied to assesse relative transcript level of CB1 and CB2 genes, using GAPDH as a calibrator gene. CB2 gene expression was characterised as higher level than the expression level of CB1 gene in all studied types of cancer. The higher level of CB receptors expressed in cervical cancer while, the low level of their expression was detected in Lymphoma cancer.

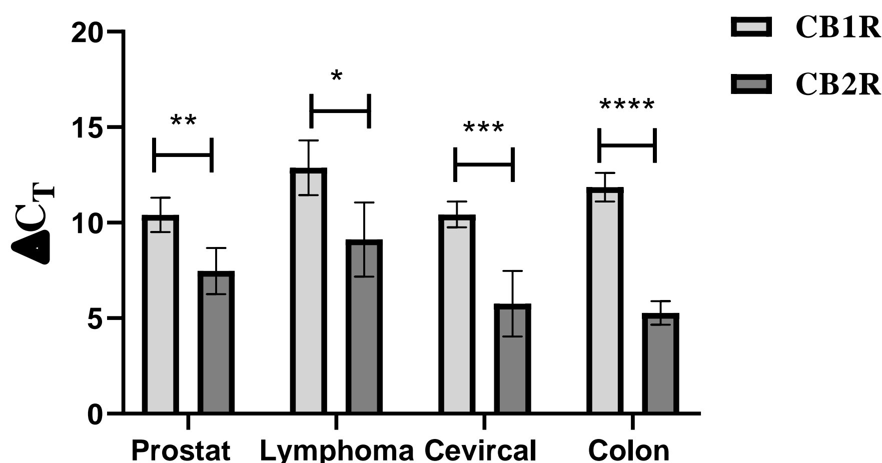

Figure (3) CB1 and CB2 gene expression association with different types of cancer. *ΔCt was calculated against geometric mean of GAPDH. Statistically significant was characterised using T-test depending P-value equal or 0.05 or less than 0.05.*

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Discussion

In the last decade, ECS has a great interesting deal in terms of its involvement in clinical medicine and this is due to their implication in broad physiological functions such as cell cycle regulation (Fraguas-Sánchez *et al.*, 2018). Cell cycle phases regulation disorder can lead to cause cancer, resulting in unregulated cell division and reducing cell death. Endocannabinoids impact differentiation, proliferation cells and cell survival in numerous types of cancer and this is lead to antitumor effects (Bifulco *et al.*, 2008). Therefor these genes were studied to measure their expression in several types of carcinoma samples. In current study, CB genes expression increased in carcinoma samples compared with control group (Table 1). These results are corresponding with previous study that refers to general up-regulation in cannabinoid receptors expression in tumor samples (Caffarel *et al*., 2006; Malfitano *et al.*, 2011). Reduced expression of CB receptors in normal cells acts as a defense mechanism against some ligands of CB receptors. Increased CB receptors expression could be linked to various cancer aggressiveness characteristics (Pyszniak *et al.*, 2016).

CB ligands and their receptors have been found in several types of carcinoma and they are considered as a therapeutic components according to their functional role in cell cycle (Xu *et al*., 2006). CB1and CB2 genes expressed significantly in peripheral blood samples that obtained from patients with prostate cancer. These findings are corresponding with findings of Sarfaraz *et al*. (2005) when they detect high levels of CB receptors expression in (DU145, LNCap) prostate carcinoma cell lines compared with their nonmalignant counterparts. Other research on prostate carcinoma found elevated expression of CB1and CB2 genes in carcinoma cells compared to their non- prostate cancer cell lines, yielding similar results(Chung *et al*., 2009; De Jesús *et al.,* 2010). Cervical cancer is the other type that significantly expressed CB receptors in current findings, which they are corresponding with findings of Contassot *et al.*, 2004. They characterized the effect of AEA on Cervical cell line and the results were intriguing. AEA was found to be toxic to studied cell lines, causing DNA fragmentation, cell cycle inhibit, and apoptosis (Armstrong *et al.*, 2015). Interestingly, specific CB receptors antagonists increased the toxicity of AEA, implying the functional role of CB receptors in protective AEA causing cell apoptosis (Armstrong *et al.*, 2015).

In contrast, lymphoma cancer was expressed CB genes with low levels as shown in figures (1and 2). Cannabinoids are thought to be efficient in killing carcinoma cells with abundant of CB receptors but could expanse metastasis and tumor growth or suppress tumors cells toxicity with low level expression of CB receptors, such as breast cancer with the possible mechanism that includes the inhibition of an anticancer immune response (Ayakannu *et al.,* 2018). Down regulation of CB genes expression correlated with increasing cell apoptosis (Gustafsson *et al.,* 2008).

According to the current results, CB receptors transcript was characterized to be low in other types of cancer like colon tumor that expressed CB1resepter with low level (figure1). Proposing that during the arrest of cell cycle, prevention of metastasis, tumor's inhibition of neoangiogenesis and promotion cell transformation. Active CB receptors could block several forms of cancer cell proliferation (Prescott and Majerus, 1983). Additionally, endocannabinoids prevent tumorogenesis and promote cell apoptosis in colorectal cancer patients by pathways including both CB receptors (Ayakannu *et al*., 2018). Another study agrees with our results in finding that low level expression of CB1 receptor as shown figure (1). They identified the methylation status within CB1 promoter in CPG islands (Wang *et al.,* 2008). Colon cancer was significantly expressed CB2 receptor in current study but CB1 expression was reduced in this type of cancer. Current findings correspond with the finding of (Wang *et al.,* 2008). However, CB2 receptor could be a good marker for this type of cancer and use CB2 receptor agonists as a target to treat colon cancer.

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All types of cancer in current study were expressed CB receptors some of them significantly expressed while the others expressed in low level as shown figures (1 and 2). According to ΔCt value, CB2 receptor was expressed significantly higher than CB1receptor in all studied cancer. In Corresponding to current findings, CB1 and CB2 genes expression in tumour cells and their transcript level in derived cell from the same tissue frequently do not correspond well (Fernández-Ruiz *et al.*, 2007; Velasco *et al.*, 2012). However, It is possible those distinct malignant tissues require different CB receptors for survival, or that these tissues require a missing cofactor implicated in the signaling pathway of cannabinoid receptor. Several studies have revealed that ECS components express themselves differently in cancer, however the degree and direction of this variety is not always the same (Larrinaga *et al*., 2013; Wang *et al.*, 2008).

In conclusion, these discrepancies show that CB gene mRNA and prognostic usefulness in neoplasms of human are largely depending on the kind of malignancy.

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