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Endocannabinoid system expression in ovarian epithelial tumors according to the dualistic model of ovarian carcinogenesis

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Abstract. – OBJECTIVE: Our study aimed to confirm the expression of the endocannabinoid system in the human epithelial ovarian tumors, assessing the immunohistochemical expression of Cannabinoid Receptor Type 1 and Fatty Acid Amide Hydrolase in benign, borderline and malignant tumors.

MATERIALS AND METHODS: Cannabinoid Receptor Type 1 and Fatty Acid Amide Hydrolase immunohistochemical expression was determined in 118 epithelial ovarian tumors sequentially treated during the last decade in our department: 36 benign, 34 borderline and 48 malignant neoplasms. Cannabinoid Receptor type 1 and Fatty Acid Amide Hydrolase expression resulted predominantly weak-moderate in the benign and borderline forms.

RESULTS: concerning malignant tumors, Cannabinoid Receptor Type 1 expression resulted predominantly moderate-strong in Type I tumors and negative-weak in Type II tumors. Fatty Acid Amide Hydrolase expression resulted, instead, independent by the tumor types. Furthermore, there was no significant difference in the Cannabinoid Receptor Type 1 and Fatty Acid Amide Hydrolase expression relatively to the tumoral stages.

CONCLUSIONS: The present study confirmed a variable expression of the endocannabinoid system in human ovarian tumors. Cannabinoid Receptor Type 1 expression was significantly different in malignant epithelial ovarian tumors according to dualistic model of ovarian carcinogenesis. Thus, in the most aggressive types II ovarian tumors, Cannabinoid Receptor Type 1 expression resulted predominantly negative or weak.

Key Words:
Endocannabinoid system, Epithelial ovarian tumors, Cannabinoid receptor, Fatty acid amide hydrolase, Kurman's dualistic model.

Introduction

The endocannabinoid system (ECS) is a complex endogenous signaling system with numerous – and only partially known – functions, influencing multiple metabolic pathways essential for the homeostasis of the organism[1]. ECS is composed of transmembrane endocannabinoid receptors, their endogenous ligands and the enzymatic system involved in biosynthesis, transporting, degradation and signaling[2,3]. Endocannabinoids are a group of unsaturated fatty acid derivatives, including anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), acting autocrine or paracri-

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ne roles[4]. The two most known endocannabinoid receptors are named Cannabinoid Receptor Type 1 (CB1R) and Cannabinoid Receptor Type 2 (CB2R), belonging to the Gi/o family of seven trans-membrane G-protein-coupled receptors 4. Cannabinoids are metabolized to arachidonic acid and ethanolamine by the enzyme Fatty Acid Amide Hydrolyse FAAH[1]. The ECS is almost ubiquitously present throughout several cell types, playing neuro-protective, anti-inflammatory, analgesic and antioxidant functions[5]. In particular, the presence of the ECS was also demonstrated in the human ovary, where it regulates folliculogenesis, oocyte maturation, and ovulation[6]. As the knowledge of the physiological role of ECS has improved, the correlation between ECS and ovarian pathologies became more evident. ECS is actually hypothesized to be involved in the pathogenesis of polycystic ovarian syndrome, through an effect on insulin hypersecretion and insulin resistance[7]. Recent studies[8,9] investigated the presence of ECS in tumors, but the role of the system in the neoplastic tissues is still poorly understood. Increasing experimental data demonstrated that ECS might modulate cell survival and cell proliferation, suggesting a correlation between ECS and cancer. Indeed, ECS induces apoptosis, cell cycle arrest and the inhibition of angiogenesis in normal cells[10-13]. However, the role of ECS in the neoplasms is still poorly understood since ECS could play different roles in different neoplasms, maybe due to the variable distribution of the cannabinoid receptors in the tissues. Neoplastic cells derived from different neoplasms breast carcinoma, melanoma, lymphoma, pancreas carcinoma and thyroid carcinoma have shown increased sensitivity to the endocannabinoids when compared with normal healthy counterparts[14-16]. Furthermore, elevated levels of AEA, 2-AG and FAAH have been documented in prostate adenocarcinoma[17,18]. An anti-neoplastic role of ECS has been hypothesized and the inhibition of tumor growth in several types of neoplastic cells (including glioma, glioblastoma, breast cancer, prostate cancer, thyroid cancer, colon carcinoma, leukemia and lymphomas) has been demonstrated by endocannabinoids, endocannabinoids analogs, endocannabinoid transport inhibitors and endocannabinoid degradation inhibitors[19]. In particular, ECS seems to have an anticancer effect through the perturbation of several signaling pathways involved in tumorigenesis and progression, including p38, MAPK, cAMP, PI3K-PKB[19-22]. Nevertheless, ECS expression in epithelial ovarian tumors (EOTs) is still poorly investigated. EOTs are the most frequent ovarian neoplasms, including benign, borderline and malignant histological types. According to the widely accepted Kurman's dualistic model of ovarian carcinogenesis, malignant EOT are classified in two types[22]. Type I carcinomas include endometrioid, clear cell and low grade serous and mucinous carcinomas, deriving from endometrial tissue, fallopian tube tissue and transitional epithelium. Type II carcinomas are largely composed of high-grade serous carcinoma, deriving from fallopian tube tissue. These two types of carcinomas show different clinical behaviour and biological features 19. In our previous study, we reported that malignant EOTs showed an increased expression of CB1R compared to benign and borderline EOTs[23]. Our current study aims to confirm the expression of the ECS in human EOTs, assessing the trend of CB1R and FAAH expression according to the histological types.

Materials and Methods

Patients and Immunohistochemical Analysis

This study included 118 patients affected by EOTs, sequentially treated during a decade in our Department of "Women, Child and General and Specialized Surgery", University of Campania "Luigi Vanvitelli" (Naples, Italy). In particular, the inclusion criteria in our series were: (1) unilateral or bilateral ovarian mass with S (2) documented ovarian epithelial origin. Thus, the series were composed by 36 benign epithelial neoplasms, 34 borderline neoplasms and 48 malignant neoplasms. The 36 benign EOTs included 19 benign serous tumors, 14 mucinous tumors and 3 Brenner's tumors. The 34 borderline EOTs included 23 serous tumors and 11 mucinous tumors. The 48 malignant EOTs included 34 high grade papillary serous carcinomas, 8 endometrioid carcinomas, 5 clear cell carcinomas and 1 mucinous carcinoma. All cases were revised by two different pathologists, which evaluated histological type and grading of the neoplasms.

Each patient signed a generic informed consent related to the use of examination results and/or biological material. Our departmental institutional review board committee and Institutional Ethical Committee approved the study. Data about the staging of malignant tumors were obtained by

the database of Our University. The staging was based on International Federation of Gynecology and Obstetrics [FIGO] cancer staging system.

Three tissue microarrays (TMA) were built using the paraffin blocks of 118 cases. Two representative areas of each tumor were selected on the H&E-stained slides. Tissue cylinders (1 mm diameter), as many as the different selected histologic patterns, were punched from each 'donor' tissue block and brought into one recipient paraffin block (3×2.5 cm). Immunohistochemical staining was carried out on TMAs slides using the primary antibodies anti-FAAH and anti-CB1. Paraffin slides were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed with slides heated in EDTA buffer [pH 9.0] in a bath for 20 min at 97°C. After antigen retrieval, the slides were allowed to cool. The slides were rinsed with Tris Buffered Saline [TBS] and the endogenous peroxidase was inactivated with 3% hydrogen peroxide. After protein block (BSA 5% in PBS 1×), the slides were incubated with polyclonal goat anti-rabbit (anti-CB1R: #ab209550; Calbiochem, Merck, KGaA, Darmstadt, Germany; Anti-FAAH; Cayman Chemical, IDS Ltd; Boldon, Tyne and Wear, UK; dilution, 1:400) at 4°C overnight. The sections were incubated with biotinylated anti-rabbit antibody for 60 minutes at room temperature. Immunoreactivity was visualized by means of avidin-biotin-peroxidase complex kit reagents (Novocastra, Newcastle, UK) as the chromogenic substrate. Finally, sections were weakly counterstained with hematoxylin and mounted.

Two different and independent operators, who were blind to the clinical data, evaluated the immune-histochemical expression. Inter-observer agreement was 97%. Both percentage of positive cells and intensity of expression were evaluated and scored from 0 to 3. Percentage of positive cells was evaluated as: 0 [no positive cells]; 1 [≤ 10% positive cells]; 2 [10-50% positive cells]; 3 [> 50% positive cells]. Intensity of expression was evaluated as: 0 [no positivity]; 1 [barely perceptible positivity]; 2 [distinctly recognizable positivity]; 3 [intense positivity]. The final scoring resulted from the sum between the two scores: 0-1: negative expression; 2: weak expression; 3-4: moderate expression; 5-6: strong expression. Figure 1 shows some examples of different expression scores in EOTs. The scoring system is schematized in Table I.

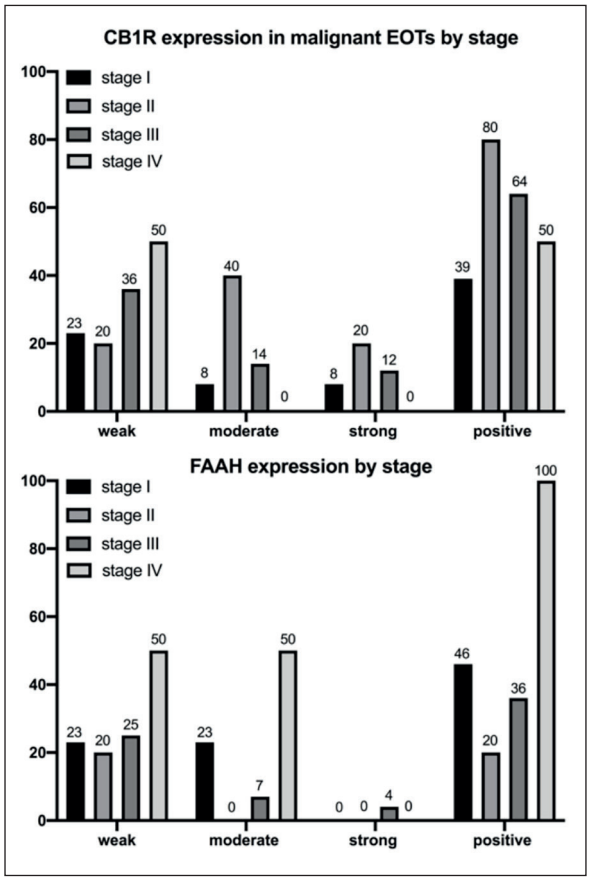

Figure 1. Immunohistochemical expression of Cannabinoid receptor type 1 [CB1R] and Fatty Acid Amide Hydrolase [FAAH] in epithelial ovarian tumors.

The Fisher-Freeman-Halton test and the Pearson X-square test were performed to determine the association of histopathological features with proteins expression score. $p<0.05$ [2-sided] was considered to be statistically significant. Data

Table I. Immunohistochemistry interpretation scoring system.

Percentage of expression		Intensity of expression		Final score	
0	No positive cells	0	No positivity	0-1	Negative
1	≤ 10% positive cells	1	barely perceptible	2	Weak
2	10-50% positive cells	2	distinctly recognizable	3-4	Moderate
3	> 50% positive cells	3	intense	5-6	Strong

analysis and summarization were conducted using statistical package built into the GraphPad Prism computer program (GraphPad Software Inc., San Diego, CA, USA).

Results

Clinic-Pathological Features of the Patients

The main clinic-pathological features were reported in the Table II.

CB1R expression was evaluated in 118 EOTs, including 36 benign tumors, 34 borderline tumors and 48 malignant tumors (Figure 1). 22/118 (19%) cases resulted negative, 44/118 (37%) showed a weak expression, 38/118 (32%) showed a moderate expression, 14/118 showed a strong expression. Concerning benign EOT, the expression resulted weak in 14/36 cases (39%), moderate in 21/36 cases (58%) and strong in 1/36 case (3%). Concerning borderline EOT, the expression resulted negative in 2/34 cases (6%), weak in 15/34 cases (44%), moderate in 10/34 cases (29%) and strong in 7/34 (21%). Concerning malignant EOT, the expression resulted negative in 20/48 (42%) cases, weak in 15/48 (31%) cases, moderate in 7/48 (15%) and strong in 6/48 (12%). FAAH expression in 104 EOT, including 22 benign tumors, 34 borderline tumors and 48 malignant tumors was evaluated. FAAH expression resulted negative in 41/104 (39%) cases, weak in 39/104 (38%) cases, moderate in 17/104 (16%) cases and strong in 7/104 (7%) cases. Concerning benign tumors, the evaluation resulted negative in 8/22 (36%) cases, weak in 11/22 (50%) cases, moderate in 3/22 (14%). No benign EOT showed a strong expression of FAAH. Concerning borderline tumors, the evaluation resulted negative in 2/24 (6%) cases, weak in 17/34 (50%) cases, moderate in 8/34 (23%) cases and strong in 7/34 (21%) cases. Malignant tumors showed a negative expression in 31/48 (65%) cases, a weak expression in 11/48 (23%) cases and a moderate expression in 6/48 (12%) cases. Strong expression of FAAH was not observed in malignant tumors.

According to Kurman's dualistic model of ovarian carcinogenesis, EOT are classified in Type I and Type II tumors. Our series included 14 Type I tumors (constituted by 7 endometrioid carcinomas, 5 clear cell carcinomas, 1 endometrioid carcinoma and 1 mucinous carcinoma) and 34 Type II tumors (constituted by 34 cases of high grade papillary serous carcinoma). Concerning CB1R expression, Type I tumors showed weak expression in 2/14 (14%) cases, moderate expression in 5/14 (36%) cases, strong expression in 6/14 (43%) cases. 1/14 (7%) Type I tumors resulted negative. Type II tumors showed weak expression in 13/34 (38%) cases, moderate expression in 2/34 (6%) cases. 19/34 (56%) cases resulted negative, and none showed strong expression. Concerning FAAH expression, Type I tumors resulted negative in 9/14 (64%) cases and showed weak expression in 3/14 (22%) cases, moderate expression in 2/14 (14%). Strong FAAH expression was not observed in Type I tumors. Instead Type II tumors showed weak expression in 8/34 (23%) cases, moderate expression in 4/34 (12%) cases. 22/34 (65%) cases resulted negative, and none showed strong expression.

Table II. Clinic-pathological features of the series.

Age	< 60 yrs	81/118 (68%)
	> 60 yrs	27/118 (32%)
Laterality	Right	57/118 (48%)
	Left	49/118 (41%)
	Bilateral	12/118 (11%)
Type	Benign	36/118 (30%)
	Malignant	48/118 (41%)
	Bordeline	32/118 (29%)
Histotype	Serous	76/118 (65%)
	Mucinous	26/118 (22%)
	Clear cell	5/118 (4%)
	Endometrioid	8/118 (7%)
	Brenner	3/118 (2%)
Stage	I	13/118 (11%)
	II	5/118 (4%)
	III	28/118 (24%)
	IV	2/118 (1%)
	NS	60/118 (51%)

NS Not stageble because benign or borderline.

Statistical Analysis

Fisher-Freeman-Halton exact test showed significant difference of the expression of both CB1R and FAAH between malignant and not malignant (benign and borderline) EOTs ($p<.01$). The expression of CB1R and FAAH by tumor type is summarized in Figure 2 and Table III.

In addition, Fisher-Freeman-Halton exact test showed significant difference of the expression of CB1R between Type I and type II malignant EOTs ($p<.01$), while no significant difference of FAAH expression was observed between the two groups ($p=.97$). Figure 3 and Table IV summarized CB1R and FAAH expression in Type I and Type II malignant EOTs.

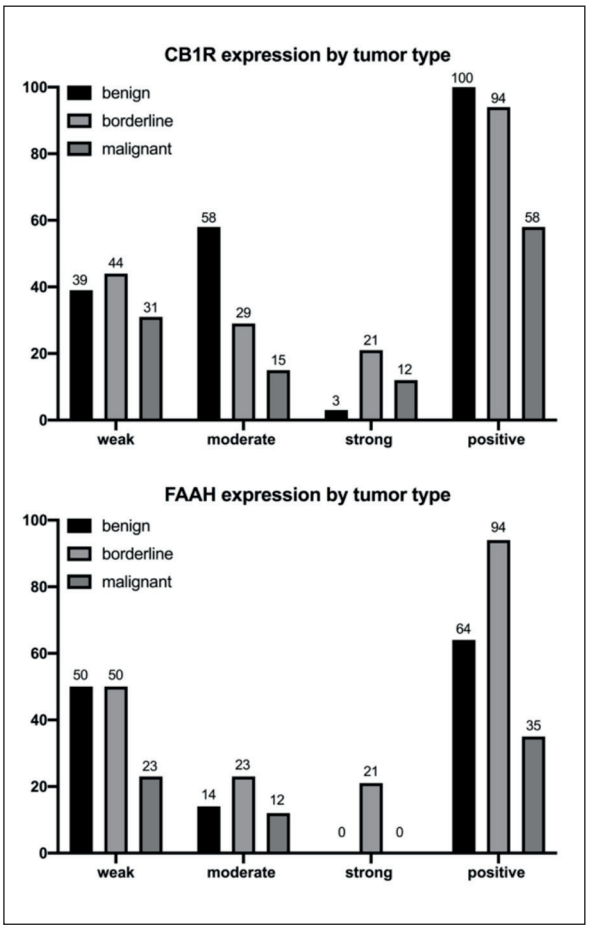

Figure 2. Cannabinoid receptor type 1 [CB1R] and Fatty Acid Amide Hydrolase [FAAH] expression in epithelial ovarian tumors, by tumor type. Positive cases are less frequent in the group of malignant tumors than in the groups of benign and borderline tumors.

In brief, 13/48 malignant EOT were Stage I neoplasms, 5/48 were Stage II neoplasms, 28/48 were Stage III neoplasms, 2/48 were Stage IV neoplasms. Fisher-Freeman-Halton exact test showed no difference of CB1R and FAAH expression in malignant EOTs depending on stage. The expressions of CB1R and FAAH in malignant EOTs by stage are detailed in the Table V.

Discussion

ECS has been recently related to several types of neoplasms. Although the current data seem to suggest that levels of CB1 and CB2 are on average higher in neoplastic cells than in normal cells[24], the eventual role of ECS in development and survival of neoplasms is still poorly understood. One of our previous study[23] showed a different expression of CB1R in EOTs, depending on biological behavior of the neoplasm. In particular, the CB1R immunohistochemical expression resulted more intense in invasive malignant EOTs than in benign and borderlines EOTs[23]. In the present study, we investigated the immunohistochemical expression of CB1R and FAAH in a larger series of EOTs. Our results confirmed a variable expression of the ECS in human EOT. In particular, CB1R expression resulted significantly different in malignant EOTs according to Kurman's dualistic model of ovarian carcinogenesis. Indeed, we observed that immunohistochemical expression of both CB1R and FAAH tends to decrease moving from benign to malignant EOT, considered as a whole, but we

Table III. CB1R and FAAH expression by tumor type.

	Tumor type				
CB1R Expression	**Benign n = 36**	**Borderline n = 34**	**Malignant n = 48**	**Total n = 118**	***p*-value***
Negative	0 (0%)	2 (6%)	20 (42%)	22 (19%)	< 0.1
Weak	14 (39%)	15 (44%)	15 (31%)	44 (37%)	
Moderate	21 (58%	10 (29%)	7 (15%)	38 (32%)	
Strong	1 (3%)	7 (21%)	6 (12%)	14 (12%)	
FAAH expression	**Benign n = 22**	**Borderline n = 34**	**Malignant n = 448**	**Total n = 104**	***p*-value***
Negative	8 (36%)	2 (6%)	31 (65%)	41 (39%)	< 0.1
Weak	11 (50%)	17 (50%)	11 (23%)	39 (38%)	
Moderate	3 (14%)	8 (23%)	6 (12%)	17 (16%)	
Strong	0 (0%)	7 (21%)	0 (0%)	7 (7%)	

CB1R: cannabinoid receptor type 1; FAAH: Fatty Acid Amide Hydrolase. *Fisher-Freeman-Halton exact test.

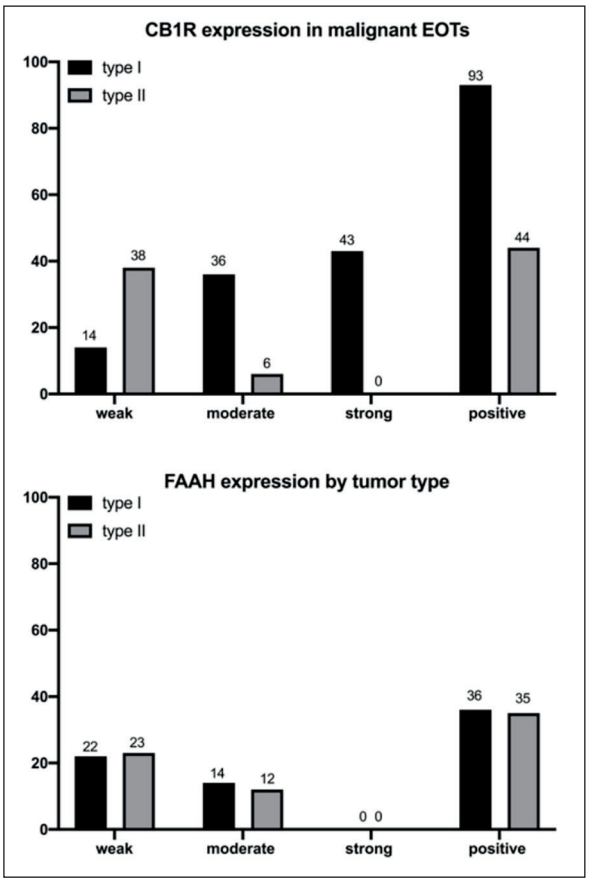

Figure 3. Cannabinoid receptor type 1 [CB1R] expression in malignant epithelial ovarian tumors [EOTs]. Positive cases are less frequent in the group of type II malignant EOTs then in the group of type I malignant EOTs.

observed a different pattern of expression in the malignant epithelial ovarian tumors, when considered according to the dualistic model of ovarian carcinogenesis proposed by Kurman et al[22]. Thus, Type I tumors showed a moderate-strong expression of CB1R 79% – 11/14, while Type II tumors showed a negative-weak expression 94% – 32/34, with statistically significant difference $p<.01$. This different expression is very interesting, because it provides important insights into the different biology of the two groups of tumors. Since Type I tumors have a less aggressive clinical behavior than Type II tumors, it could be hypothesized that ECS, and particularly CB1R, could play a role in limiting tumor aggressiveness. However further studies are necessary to really understand these data. FAAH expression trend was independent from tumor types according to the dualist model and a significantly different expression of CB1R and FAAH, regardless of tumor type, was recorded $p=.018$. Probably, as shown by Bagavandoss et al[25] in the rat ovary, the components of the endocannabinoid signaling system are differentially expressed in time and space in specific cell types. Recently, Pirone et al[26] came to the same conclusions showing a differential expression of both CB1R and FAAH during different stages of ovarian function in the cat ovary and oviduct.

Conclusions

In summary, this study showed a variable expression of ECS in human EOTs. The most interesting observation is the statistically significant difference of CB1R expression in malignant tumors, according to dualistic model of ovarian carcinogenesis proposed by Kurman et al[22]. The differences that we observed could imply a reduction

Table IV. CB1R and FAAH expression according to the Dualistic Model of Ovarian Carcinogenesis.

	Tumor type		
CB1R expression	**Type I n = 14**	**Type II n = 34**	***p*-value***
Negative	1 (7%)	19 (56%)	< 0.1
Weak	2 (14%)	13 (38%)	
Moderate	5 (36%)	2 (6%)	
Strong	6 (43%)	0 (0%)	
FAAH expression	**Type I n = 14**	**Type II n = 34**	***p*-value***
Negative	9 (64%)	22 (65%)	= .97
Weak	3 (22%)	8 (23%)	
Moderate	2 (14%)	4 (12%)	
Strong	0 (0%)	0 (0%)	

CB1R: cannabinoid receptor type 1; FAAH: Fatty Acid Amide Hydrolase. *Fisher-Freeman-Halton exact test.

Table V. CB1R and FAAH expression in malignant EOT by stage.

	Malignant EOT				
CB1R Expression	**Stage I n = 13**	**Stage II n = 5**	**Stage III n = 28**	**Stage IV n = 2**	***p*-value***
Negative	8 (61%)	1 (20%)	10 (36%)	1 (50%)	> 0.5
Weak	3 (23%)	1 (20%)	10 (36%)	1 (50%)	
Moderate	1 (8%)	2 (40%)	4 (14%)	0 (0%)	
Strong	1 (8%)	1 (20%)	4 (12%)	0 (0%)	
FAAH expression	**Stage I n = 13**	**Stage II n = 5**	**Stage III n = 28**	**Stage IV n = 2**	***p*-value***
Negative	7 (54%)	4 (80%)	18 (64%)	0 (0%)	> 0.5
Weak	3 (23%)	1 (20%)	7 (25%)	1 (50%)	
Moderate	3 (23%)	0 (0%)	2 (7%)	1 (50% <)	
Strong	0 (0%)	0 (0%)	1 (4%)	0 (0%)	

EOT: epithelial ovarian tumors; CB1R: cannabinoid receptor type 1; FAAH: Fatty Acid Amide Hydrolase. *Fisher-Freeman-Halton exact test.

of ECS expression in tumor progression of EOTs or different ECS roles in the pathogenesis of the two Kurman's groups. Further studies are necessary to define the cellular and molecular mechanisms of ECS pathway in EOTs and to evaluate the prognostic significance of ECS expression. Pathways stimulated by ECS in cancer cells involve several tyrosine kinases, AMPK and cAMP regulating cancer cell survival, inflammation and drug resistance[27,28]. As summarized in Figure 4, ECS are able to stimulate type 1 and type 2 cannabinoid receptors that regulate angiogenesis and apoptosis. Specifically, AEA and 2-AG, the most studied endocannabinoids in human tissues, are agonists of cannabinoids receptors CB1 and CB2, both expressed in several cancer cells. Activation of CB1 inhibits Phosphoinositide 3-kinases PI3K /Protein kinase B PKB or AkT pathways leading

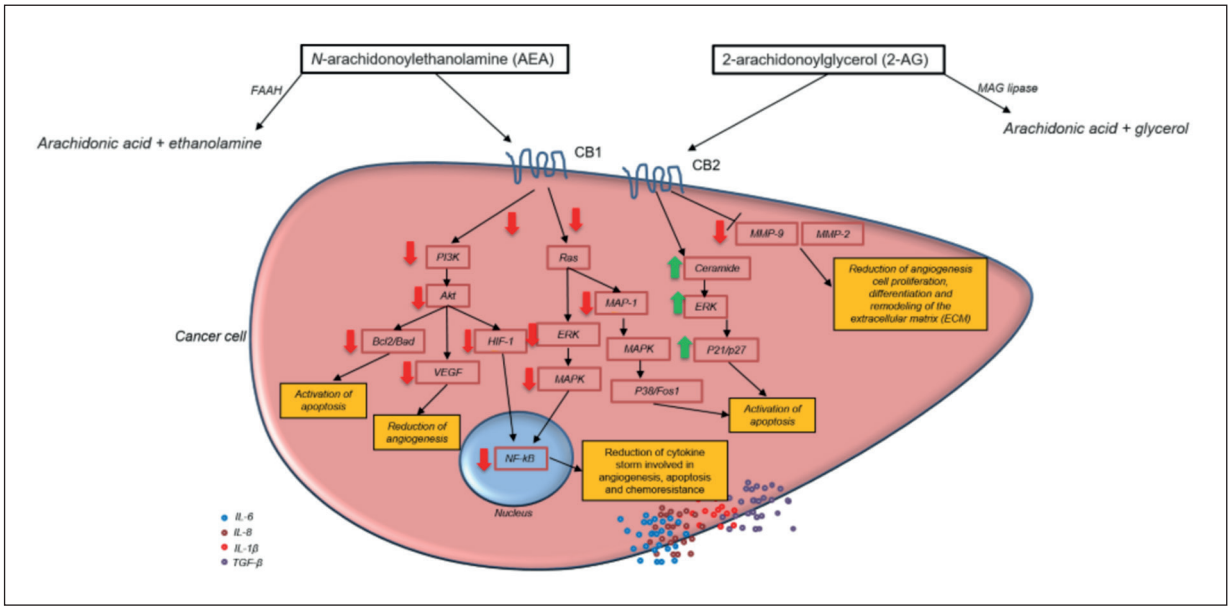

Figure 4. Signaling pathways induced by endocannabinoids in ovarian cancer cells. Endocannabinoids affect many essential cellular processes and signaling pathways which are crucial for tumor development. For example, they can promote apoptosis, inhibit proliferation, migration and angiogenesis of cancer cells.

to activation of apoptosis through B-cell lymphoma 2 Bcl2/ Bcl-2-associated death promoter Bad and inhibition of cellular angiogenesis through Vascular endothelial growth factor VEGF and Hypoxia-inducible factors HIF related pathways. Stimulation of CB1 reduces Ras/ERK/MAPK pathways leading to anti-inflammatory effects mediated by inhibition of NF-kB. Other pro-apoptotic effects of ECS in cancer cells are mediated by the activation of ceramides that in turns activates ERK/p21/p27 axis increasing intracellular concentration of pro-apoptotic proteins. Another pathway involved in CB2-mediated anticancer effects is the inhibition of metalloproteases MMP[29]; indeed, AEA and 2-AG decreases MMP-9 and MMP-2 expression in cancer cells leading to a reduction of angiogenesis, cell proliferation, differentiation and remodeling of the extracellular matrix ECM.

Our preliminary results underline a potential different role of ECS, not only considering benign vs malignant, but also in relation to categories proposed by Kurman et al[22]. Thus, the control of neoplastic growth through ECS shows different profiles in Type I and Type 2 EOC. In such view the CB1 absent expression could be related to a worst prognosis. The data should be validated on a prognostic series.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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