mRNA expression of CRF family members in urothelial bladder cancer

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Abstract. The corticotropin-releasing factor (CRF) gene family includes the three urocortins (UCN1, 2 and 3) and the two receptors (CRFR1 and 2), which play a significant role in the physiology of various organs. The expression of the CRF family of genes and its receptors are shown to participate in the pathogenesis of inflammation and even tumorigenesis. However, data regarding the human urinary tract, especially the bladder, are scarce. To the best of our knowledge, no studies are currently available on the CRF system and bladder cancer. The primary goal of the present study was to investigate the mRNA expression of the CRF family members in bladder cancer. The secondary aim was to analyze the differences with the expression of the same mRNAs in normal bladders. From August 2018 to July 2021, 43 recruited patients were divided into three groups. Group A included healthy patients, group B included patients with bladder cancer and group C included patients with a history of cancer from whom samples were taken from the normal bladder mucosa. Detection of mRNA of the CRF family of genes was performed using reverse transcription-quantitative PCR. The mRNA of the three urocortins, CRF and the two receptors were predominantly expressed in all three groups of patients. Statistical analysis using the Kruskal-Wallis test showed that UCN1 was downregulated in patients with bladder cancer and those with possible cancer compared with the healthy group (mean rank group A=24.3 vs. mean rank group B=12.58; P=0.006) and (mean rank group A=24.3 vs. mean rank group C=8.88; P=0.001). The present experiments showed that mRNA of the CRF family of genes was amplified in normal and cancer bladder tissues. Downregulation of the UCN1 gene may be associated with bladder cancer, contributing to the prognosis, diagnosis or therapy of urothelial malignancies.

Introduction

Corticotropin-releasing factor (CRF) is a 41-amino acid peptide (1), which plays an essential role in maintaining homeostasis (2). The CRF family of peptides consists of four members, including CRF and urocortins (UCN1,2,3). The latter have their amino acid sequence homologous to CRF and also play a role in the process of homeostasis by modulating, for example, the cardiovascular, endocrine, immune, and reproductive systems (3). CRF and UCNs exert their actions on target cells through the activation of CRF receptors [Corticotropin-Releasing Factor Receptors (CRFRs) type 1 (CRFR1) and type 2 (CRFR2)] (3). CRFRs belong to the G-protein-coupled receptor family, have seven transmembrane domains, are encoded by two different genes and exhibit distinct pharmacological properties and selectivity compared to agonists (3). CRF family peptides and their receptors are expressed in the central nervous system and other systems, such as the urogenital system (4). Activation of CRFRs appears to have different biological effects on each tissue (5). For example, in the gastrointestinal system, CRFR1 activation appears to increase gut motility, whereas CRFR2 activation decreases it (6). While UCN2 and UCN3 have a selective affinity for CRFR2, UCN1 has a high affinity for both receptors (7). In addition to its central expression, UCN1 has also been found in peripheral tissues such as adipose tissue, heart, thymus, spleen, skin, testis, kidney, adrenal gland and gastrointestinal tract (8). The CRF system plays a significant role in the physiology and pathophysiology of various systems (esophagus, intestine, skin, etc.). However, very little is known about its role in the urogenital system, particularly in the bladder (4). CRF, UCN1 and CRFRs have been identified in the bladder of experimental animals (4,9). Studies have shown that CRF, UCNs and CRFRs are expressed in both normal and inflammatory (cystitis) feline bladder mucosa (urothelium), showing functional differences (4). CRF and CRFR2 also show increased expression in the bladder of adult mice with cyclophosphamide-induced chemical cystitis. (9). CRFR1 has also been found in the bladder of adult mice and even more strongly in various inflammatory conditions (10). Recently, a

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study has shown for the first time the presence of CRFR1 and CRFR2 peptides in the human bladder, using western blot and immunochemistry (11). However, it is not clear what occurs with the expression of UCN1.

Moreover, the expression of CRF family peptides and their receptors has also been associated with different types of cancer in humans, playing a possible role in their progression (12). Indeed, in the urogenital system, the expression of CRF family genes has been studied in prostate and renal cancer, where it has been shown that the degree of growth or apoptosis of cancer cells can be modified by an agonist or antagonist of CRFRs (13-15).

It is well known that most bladder tumours are of epithelial origin, up to 99.5% of cases (16), highlighting the importance of developing cancer therapies, especially local treatments (e.g. intravesical instillations), in the early stages of the disease. Theoretically, intravesical administration of an antagonist of a receptor whose upregulation is associated with bladder cancer could be applicable. Similarly, administering an agonist of a receptor whose downregulation was associated with carcinogenesis could provide a field of investigation. To our knowledge, no reports about the mRNA expression levels of CRF family genes in bladder cancer are available. This research aimed to investigate the mRNA expression of CRF family members in the normal human bladder and urothelial carcinoma, using quantitative real-time polymerase chain reaction (qPCR) analysis and compare the expression of the genes between healthy individuals and cancer patients.

Materials and methods

Study population. Our study protocol was approved by the ethical committee of the University General Hospital of Heraklion, Greece (Protocol no. 20/25-07-2018, 804). All patient candidates signed the informed consent before any intervention under the Helsinki Declaration (17). From August 2018 to July 2021, we enrolled 43 patients according to our research protocol. We divided the patients into three groups. Group A included 14 healthy subjects (control group), group B included 20 patients with bladder cancer, and group C included nine patients with a history of bladder cancer, from whom we obtained tissue samples from normal mucosa. Samples from healthy individuals were obtained during prostatectomy or endoscopic ureterolithotripsy using cold cup biopsy forceps. Samples were taken only from the dome and not from the bladder triangle. In group B, tissues were taken from the surface of the tumour with cold biopsy forceps and in group C, from normal mucosa away from the neoplasm site before transurethral resection of the bladder tumour (TURBT). The exclusion criteria of the study are summarized in Table I. We recorded the patients' demographic and clinical data, including age, sex, type of surgery, grade of tumour, t stage of the tumour, and risk of progression in patients with cancer. The grade was classified according to the world health organization (WHO) 2004/2016 system, and the risk of tumour progression was based on the study by Sylvester *et al* (18). We excluded two patients with muscle-invasive bladder cancer and one with a clear cell type variant from group B.

RNA extraction. After the surgical procedure, the samples were immediately stored at -80°C until used. All the tissues had 2-3 mm thickness. Total RNA was extracted from the tissues with TRIzol reagent (Invitrogen Life Technologies), and cDNAs were synthesized using TAKARA PrimeScript 1st strand cDNA synthesis kit (Takara Bio) (19). Expression of each gene of interest was determined using SYBR Green master mix (Kapa Biosystems) containing a specific set of primers in a final volume of 10 μl. Amplification conditions included denaturation at 95°C for 2 min followed by 40 cycles at 95°C for 30 secs and at 60°C for 30 sec. To verify the accuracy of qPCR (melting curves and PCR products), we ran 1.5-2% agarose gel. All samples were initially tested for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Additionally, we used the ribosomal protein S23 (RPS23) gene, which seemed to be the most suitable and stably expressed housekeeping gene in bladder samples (20). Therefore, the calculations are done according to the formula RQ=E^-(min Cq-sample Cq) (21), (where Cq is the cycle threshold and E the primer's efficiency) based on the geometric mean between the relative quantities (RQ) of GAPDH and RPS23. Using two reference genes also balanced the problems of expression variation between pathological and normal tissue. We performed the experiments in triplicate to allow for statistical assessment. We also created a standard curve from 5-point cDNA dilution series finding the slope and the primer's efficiency.

Primers. Primers for housekeeping and CRF family genes are shown in Table II.

Statistical analysis. We used the Kolmogorov-Smirnov test to check the normality of the data. We compared the relative gene expression between groups using the Kruskal-Wallis and ANOVA tests followed by Dunn's-Bonferroni and Tukey HSD post hoc analysis, respectively (SPSS for Linux, version 23). In addition, we examined the correlation of patients' data (tumour grade, tumour stage, tumour progression risk and age) with gene expression using Spearman analysis. Finally, we performed an ANOVA test regarding patients' age between the groups. Thus, to the extent possible, we have excluded potential confounding factors affecting the exposure and the outcomes. P<0.05 was considered to indicate a statistically significant difference.

Results

Initially, all tissues were investigated for the presence of the GAPDH reference gene. Of the 14 normal tissues, GAPDH expression was observed in 11. Of the remaining 17 cancerous tissues, GAPDH expression was observed in 13. Finally, of the nine patients with a history of bladder cancer, the expression was observed in 8. In addition, we decided to study the more suitable and stably expressed housekeeping gene, RPS23 (20). The main characteristics of the patients per group who were studied are presented in Table III. UCN1 mRNA expression was identified in 10 of 11 samples in group A, 12 of 13 in group B and in all samples (n=8) from group C. The average Cq value ± standard deviation (SD) of UCN1 mRNA expression in groups A, B, and C was 24.86±1.01, 28.64±2.42, and 28.94±2.75, respectively. The product of qPCR, UCN1, was selectively confirmed by running agarose gel (Fig. 1).

Table I. Exclusion criteria for the study protocol.

Patients with cancer	Patients without cancer (controls)
Age <18 years	Age <18 years
History of neurogenic bladder	History of neurogenic bladder
Urinary tract infection (sterile urine culture) required preoperatively	Urinary tract infection (sterile urine culture) required preoperatively
History of bladder catheterization, lithiasis and intravesical treatment (BCG or chemotherapy)	History of bladder catheterization or lithiasis
History or suspicion of prostate cancer, renal cancer and other malignancy	History or suspicion of prostate, renal, bladder cancer and other malignancy
Immunosuppression, immunodepression	Immunosuppression, immunodepression
Pregnancy or breastfeeding	Pregnancy or breastfeeding
Muscle-invasive bladder cancer, metastatic disease, second malignancy and other than typical papillary bladder cancer	Endoscopic findings: Detrusor hypertrophy, ulcers and inflammatory lesions
Participation in another research protocol	Participation in another research protocol

BCG, Bacillus Calmette-Guérin.

Table II. PCR primers used in the present study.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')	Size (bp)
CRF	CAC-CCT-CAG-CCC-TTG-GAT-TTC	GCC-CTG-GCC-ATT-TCC-AAG-AC	413
UCN1	CAG-GCG-AGC-GGC-CGC-G	CTT-GCC-CAC-CGA-GTC-GAA-T	146
UCN2	AGA-CCA-CAG-GAC-AGT-AGT-GC	GTG-AGG-TCA-GGC-GCC-AC	90
UCN3	TGC-TGC-TCC-TGC-TGC-TGC-TC-3'	GTG-TCC-TGG-CGT-GGC-TTT-CCC-3'	310
CRFR1	GGC-AGC-AGC-TAG-TGG-TTC-GGC-C	TCG-CAG-GCA-CCG-GAT-GCT-C	272
CRFR2	ATG-GAC-GCG-GCA-CTG-CTC-CA	CAC-GGC-CTC-TCC-ACG-AGG-G	342
RPS23	TGG-AGG-TGC-TTC-TCA-TGC-AA	AAT-GGC-AGA-ATT-TGG-CTG-TTT-G	76
GAPDH	CTG-CAC-CAC-CAA-CTG-CTT-AG	GGG-CCA-TCC-ACA-GTC-TTC	120

CRF, corticotropin-releasing factor; UCN1, urocortin 1; UCN2, urocortin 2; UCN3, urocortin 3; CRFR1, corticotropin-releasing factor receptor 1; CRFR2, corticotropin-releasing factor receptor 2; RPS23, ribosomal protein S23; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Subsequently, statistical analysis and comparison of relative UCN1 mRNA expression levels between groups were performed using the Kruskal-Wallis test as the levels did not follow a normal distribution (Kolmogorov-Smirnov test, P<0.001). UCN1 mRNA expression levels were statistically lower in group B compared to A (test statistics 11.717, mean rank A=24.3, mean rank B=12.58, P=0.006) and statistically lower in group C compared to A (test statistics 15.425, mean rank C=8.88, P=0.001). Between groups B and C, there was no difference (P=1.000) (Fig. 2A, B). The ANOVA test showed no differences in the age of the patients between the groups (P=0.112, f=2.38). Finally, in the Spearman's correlation test, the relative UCN1 mRNA expression and the average raw Cq values did not seem to relate to the age and sex of the patients, to the grade and the T stage of tumour or to the risk of cancer progression (All correlations showed P>0.05).

Regarding UCN3 mRNA expression, although the average raw Cq values were higher in group A (26.29±1.83 in group A and 24.44±1.94 in group B), no statistically significant difference was observed (Kruskal-Wallis test for relative UCN3 mRNA expression, P=1.000). UCN3 mRNA expression was identified in four of eight samples screened in group A, seven of twelve in group B and all (n=8) from group C.

Regarding CRF, UCN2, CRFR1 and CRFR2 mRNAs, no significant difference was observed between the groups (Kruskal-Wallis test P-value for CRF and UCN2 0.429, and 0.514 respectively, ANOVA P-value for CRFR1, and CRFR2 0.523 and 0.640, respectively). Overall, the expression of all CRF family genes was identified in most samples (from 50 to 96.77%). The average raw Cq values ± SD and the percentage of mRNA detection per group and total are presented in Table IV.

Discussion

Several studies have shown that the expression of CRF family genes is involved in either the development or progression of cancer (12). Therefore, investigating their presence in different organs, such as the bladder, is of great interest. The present

Table III. Main characteristics of the patients per group.

A, Group A

Samples	Age (years)	Sex	T stage	Grade[a]	Risk[b]	Surgery
1	65	Female	-	-	-	URS
2	45	Male	-	-	-	URS
3	61	Female	-	-	-	URS
4	79	Male	-	-	-	TURP
5	79	Male	-	-	-	TURP
6	73	Male	-	-	-	URS
7	80	Male	-	-	-	TURP
8	61	Male	-	-	-	TURP
9	70	Male	-	-	-	URS
10	65	Male	-	-	-	URS
11	41	Male	-	-	-	URS

B, Group B

Samples	Age (years)	Sex	T stage	Grade[a]	Risk[b]	Surgery
1	79	Male	T1	High	High	TURBT
2	78	Male	Ta	Low	Low	TURBT
3	40	Male	Ta	High	Low	TURBT
4	56	Male	T1	High	Intermediate	TURBT
5	67	Male	Ta	High	Intermediate	TURBT
6	74	Male	Ta	Low	Low	TURBT
7	74	Male	T1	High	High	TURBT
8	64	Male	T1	High	Intermediate	TURBT
9	87	Male	T1	High	High	TURBT
10	51	Male	Ta	High	Intermediate	TURBT
11	70	Male	Ta	High	Intermediate	TURBT
12	77	Male	Ta	Low	Low	TURBT
13	72	Male	Ta	High	Low	TURBT

C, Group C

Samples	Age (years)	Sex	T stage	Grade[a]	Risk[b]	Surgery
1	79	Male	Ta	High	Intermediate	TURBT
2	77	Male	Ta	High	Intermediate	TURBT
3	65	Male	Ta	High	Intermediate	TURBT
4	84	Male	T1	High	High	TURBT
5	81	Female	T1	High	High	TURBT
6	71	Male	Ta	High	Intermediate	TURBT
7	81	Male	T1	High	High	TURBT
8	85	Male	Ta	High	Intermediate	TURBT

[a]Grade of tumour according to the WHO 2004/2016 classification system (18). [b]Risk of tumour progression is based on the study by Sylvester *et al* (18). Group A included patients with normal bladder (control group). Group B included patients with bladder cancer. Group C included patients with a history of bladder cancer in which the biopsies were taken from a non-cancer site. TURBT, transurethral resection of bladder tumour; TURP, transurethral resection of the prostate; URS, ureteroscopy.

study shows that the mRNAs of the three UCNs, CRF and the two CRF receptors are expressed in the normal human bladder. The second finding was that the CFR family mRNAs are expressed in the samples received by the bladder of cancer
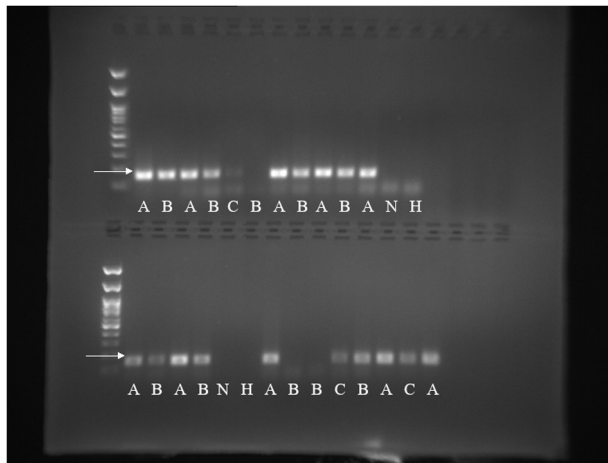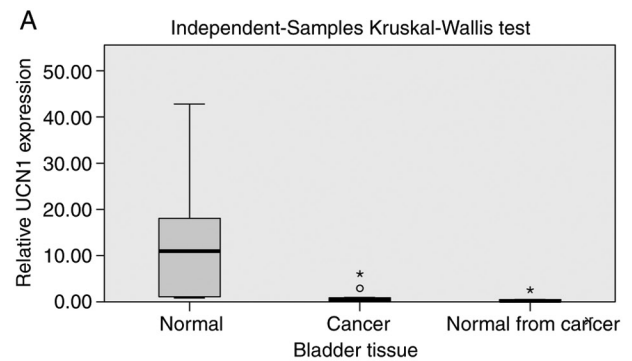
Figure 1. Agarose gel of samples after qPCR analysis of UCN1 gene. Samples from (A) group A, (B) group b and (C) group C participants. N and H represent samples with no RT and H_2O, respectively. The predicted size of human UCN1 is 146 bp, as highlighted in the figure (white arrows). qPCR, quantitative PCR; RT, reverse transcription. UCN1, urocortin 1.

patients. Several cancer-related studies have shown that UCN1 is expressed in the following human tissues: gastric adenocarcinoma, pancreatic adenocarcinoma, liver carcinoma, endometrial carcinoma, renal clear cell carcinoma and prostate adenocarcinoma (22). It is also expressed in the following cell lines: glioblastoma, pituitary adenoma, malignant melanoma, insulinoma, pheochromocytoma, thyroid carcinoma, adrenal carcinoma and breast cancer (22). However, to the extent of our knowledge, this is the first study showing the expression of these genes in urothelial cancer tissues.
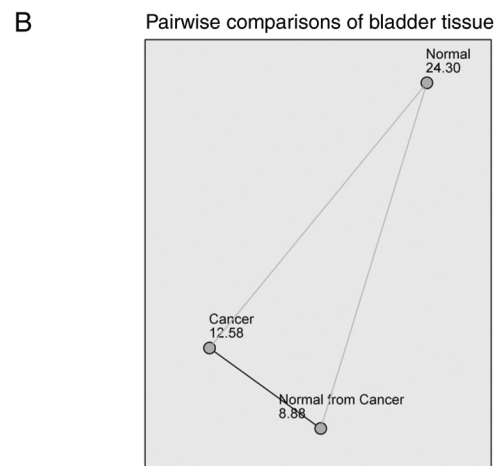
We also found that UCN1 mRNA levels were downregulated in tissues taken from bladder cancer compared to the tissues received from the healthy group. Similar results were found in the group of bladder cancer patients where samples were taken from normal urothelium.

Downregulation of UCN1 mRNA expression in cancerous and potentially cancerous tissues does not necessarily represent a cause-effect relationship. It could be the effect rather than the cause of carcinogenesis or a chance event. If the change in UCN1 expression results from urothelial cancer, it could be investigated further as a biomarker. As a chance event, it could be presented due to the non-study of possible confounding and interactive factors related to the patient group. For example, patients' exposure to certain chemicals could cause alterations in UCN1 expression in parallel and independently of carcinogenesis. Similar results to our study, i.e. reduced expression of UCN1, have been reported in endometrial cancer (23). The decreased expression of UCN1 in endometrial carcinoma could occur through the activation of estrogen receptor a (23). Additional data showed that cell proliferation in endometrial carcinoma was inhibited through the activation of the cAMP-protein kinase A (PKA) pathway by CRF/UCN1 (24). In another study, UCN1 inhibited differentiation in melanoma cell lines by regulating intracellular Ca^{2+} homeostasis (25). Furthermore, UCN1 controlled hepatocellular cell carcinoma migration by decreasing the expression of the calcium-independent phospholipase A2 enzyme (iPLA2) (23). Finally, UCN1 could inhibit carcinogenesis by the cessation of

Figure 2. Analysis of UCN1 relative gene expression. (A) Box plots show relative UCN1 expression for Group A (normal), Group B (cancer) and Group C (normal from cancer). ° indicates an outlier and * indicates an extreme outlier. (B) Pairwise comparisons between Group A and Group B (P=0.006), Group A and Group C (P=0.001) and Group B and Group C (P=1.000). UCN1, urocortin 1.

angiogenesis (26). It is well known that UCN1 has a strong affinity for both receptors (CRFR1 and CRFR2) (27), which could explain the conflicting results of several cancer-related studies. Thus, activation of CRFR1 by UCN1 could promote anticancer effects related to the PKA pathway or intracellular Ca^{2+} signaling (24,25).

Table IV. Average means raw Cq values and the percentage of gene detection in groups A, B, C and total.

Gene	Group A Average mean Cq value ± SD value	Group A Detection of the gene (%)	Group B Average mean Cq value ± SD	Group B Detection of the gene (%)	Group C Average mean Cq value ± SD	Group C Detection of the gene (%)	Total population Detection of the gene (%)
CRF	32.84±2.96	8/8 (100)	30.56±4.01	7/9 (77.78)	32.65±3.94	6/7 (85.7)	21/24 (87.5)
UCN1	24.86±1.01	10/11 (90.9)	28.64±2.42	12/13 (92.3)	28.94±2.75	8/8 (100)	30/32 (93.75)
UCN2	30.96±2.22	10/11 (90.9)	29.72±2.2	12/12 (100)	29.89±2.31	8/8 (100)	30/31 (96.77)
UCN3	26.29±1.83	4/8 (50)	24.44±1.94	7/12 (58.3)	27.26±1.43	8/8 (100)	19/28 (67.9)
CRFR1	35.08±4.68	2/7 (28.57)	30.22±3.62	7/9 (77.78)	32.82	1/2 (50)	10/18 (55.56)
CRFR2	32.86±4.08	4/7 (57.14)	31.47±4.36	3/9 (33.33)	31.29±0.61	2/2 (100)	9/18 (50)

CRF, corticotropin-releasing factor; UCN1, urocortin 1; UCN2, urocortin 2; UCN3, urocortin 3; CRFR1, corticotropin-releasing factor receptor 1; CRFR2, corticotropin-releasing factor receptor 2.

On the other hand, activation of CRFR2 may favor cancer migration (23). Activating CRFR2 by UCN1 could inhibit angiogenesis (26), one of the significant mechanisms in oncogenesis. Also, the reduction in the expression of iPLA2, which inhibits hepatocellular carcinoma migration, occurs via the activation of CRFR2 (23).

All the mentioned mechanisms seem to be relevant to bladder cancer. In particular, activation of the PKA pathway appears to control cancer invasion by modulating Microtubule-associated protein 4 (MAP4) (28). Goto and Miyamoto studied the association of estrogen receptors in the pathogenesis of urothelial carcinoma by analyzing their possible involvement. Thus, bladder cancer could be associated with the endocrine system (29). Furthermore, there is evidence that intracellular Ca^{2+} homeostasis plays a role in bladder cancer (30). About iPLA2, the observation of Cai *et al* was representative as its activity was elevated in bladder cancer, while, as mentioned before, its decrease controls cancer migration (31). Finally, angiogenesis seems to be involved in the progression and recurrence of bladder cancer (32).

The notable limitations of our work were the small number of samples and the inability to use a second method, such as western blot and immunohistochemistry, to confirm the specific cells expressing UCN1. Unfortunately, several samples were either inadequate or inappropriate due to the lack of expression of the housekeeping gene. In addition, the exclusion criteria and the invasive nature of our study made collecting large numbers of tissues difficult. As for the second limitation, the experiments were repeated three times to be as accurate as possible. Regarding the cells expressing UCN1, we consider that the samples did not include muscle layers as they were taken with cold biopsy forceps (tissue thickness 2 to 3 mm). Also, the normal samples were taken from the bladder dome. As regards the cancerous samples, the biopsy was superficial from the tumour margin. As it is known in the literature, only the lamina propria in the dome region can be thick up to 3.1 mm (33). Also, the muscularis mucosae layer is mainly located in the dome and constitutes the firm boundary before the muscle layer (33).

Our study nature is mostly exploratory. Thus, our results put the basis for future studies addressing the pathophysiology of bladder cancer related to CRF family genes. In our ongoing research, we could investigate whether the administration of a UCN1 affects the progression and migration of urothelial cancer cells in vitro. Also, a larger number of samples would probably result in a statistically significant difference in the expression of the other genes of the CRF family as UCN3, which may be expressed to a greater extent in cancer samples. Finally, whether altered gene expression would contribute to the prognosis or diagnosis of urothelial cancer as a biomarker should be examined.

In summary, our novel findings show that some of the mRNAs of the CRF gene family are likely expressed in human bladder cancer. Although no specific pathogenic mechanism was investigated, UCN1 gene downregulation seems to be associated with urothelial carcinoma. Further studies with a larger sample number could investigate the relationship of CRF genes with cancer development, invasion, progression and migration and their use as biomarkers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CMav, CMam, MV and GL conceptualized the project. MV and ED designed the experiments. CMav, MD, ED and MV performed the experiments. CMav, ED, MV, GL and CMam contributed to data acquisition, analysis and interpretation. MV, GL and CMam provided the resources. CMav and CMam performed the biopsies. CMav, ED, MD and MV supplied the computer software used in the study. CMav wrote the original draft. MV, MD, ED, GL, CMav and CMam reviewed, edited and wrote the final draft. All authors read and approved the final version of the manuscript. MV and ED confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the University General Hospital of Heraklion, Greece (approval no. 20/25-07-2018, 804), as a research project. All patient candidates signed the informed consent before any intervention under the Helsinki Declaration.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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