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Involvement of Corticotropin-releasing Hormone-related Peptides in Cellular Stress Caused by Anticancer Drugs in Colorectal Cancer

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Abstract. *Background/Aim: The expression of corticotropin-releasing hormone (CRH)-related peptides involved in stress response in colorectal cancer has been reported. We examined the involvement of CRH-related peptides in cellular stress caused by anticancer drugs in colorectal cancer. Materials and Methods: Changes in the expression levels of CRH-related peptides and their receptors in HCT116, DLD-1, and SW480 cell lines after fluorouracil (5-FU) loading were evaluated. Effects of the receptor antagonist against DNA synthesis disorder caused by 5-FU and SN-38 were evaluated using the ^{3}H-labeled deoxyribonucleoside incorporation assay. Results: No changes in the mRNA expression levels of CRH-related peptides (UCN and UCN2) -and their receptors (CRHR1 and CRHR2) were observed. Addition of antagonists to cells with DNA synthesis disorder caused by 5-FU and SN-38 showed no differences in the incorporation of ^{3}H-labeled deoxyribonucleoside. Conclusion: CRH-related peptides showed no effect on the stress response to anticancer drugs nor on DNA synthesis disorder in colorectal cancer.*

Colorectal cancer is the third most fatal cancer worldwide, over 1.8 million new cases and 881,000 deaths are estimated to have occurred in 2018 (1). It has been reported that the median survival time (MST) of patients with metastatic colorectal cancer without chemotherapy is approximately 8 months (2). With the advancement of recent chemotherapy (including FOLFOX, FOLFIRI, and molecular targeted therapy), the MST has been significantly improved to 30 months (3-5); however, complete recovery remains a challenge.

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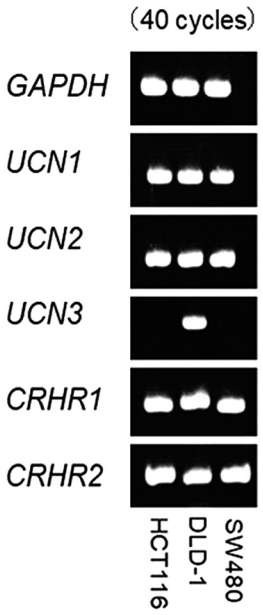
Key Words: Colorectal cancer, corticotropin-releasing hormone, stress, anticancer drug.

The expression of corticotropin-releasing hormone (CRH)-related peptides and their receptors in various cancer types (6-17), including colorectal cancer (18-21), has been reported in recent years. CRH and CRH-related peptides are physiologically peptide hormones that play a role in various stress responses (22-25). CRH is secreted in the hypothalamus in response to numerous types of stress, causing various stress responses in the biological body through the hypothalamic–pituitary–adrenal axis (26). CRH-related peptides exhibit homologies in amino acid sequences with CRH. Three types of CRH-related peptides, urocortin (UCN), UCN2, and UCN3, are known (22). They are widely distributed in not only the central nervous system, but also peripheral tissues, and their involvement in various stress responses has been indicated. Additionally, two types of receptors for CRH and CRH-related peptides (CRHR1 and CRHR2) are known (22, 23). CRH and CRH-related peptides act as ligands that are involved in stress response through these receptors. CRH has a high affinity to CRHR1, activating the hypothalamus-pituitary-portal axis *via* CRHR1 located in the pituitary and contributing to an increase in adrenocorticotropic hormone and glucocorticoids. UCN has an affinity to both CRHR1 and CRHR2, while UCN2 and UCN3 have an affinity for CRHR2. These CRH-related peptides are involved in stress responses that are not mediated by the hypothalamus-pituitary-portal axis in the body, and are associated with cardioprotective action against stress-induced myocardial ischemia (27), stress-induced gastrointestinal motility disorders (28-30), and regulation of immune function (31).

It has been reported that these peptides and receptors may influence the proliferation, migration, and invasion potencies of cancer cells (18, 19, 32-35). However, different results have been shown and no consensus has yet been reached. Additionally, although it is possible that the physiological function of these CRH-related peptides may be in stress response to chemotherapy and modification of therapeutic effects of anticancer drugs, no such research on chemotherapy has been reported.



qRT-PCR (Cq value) mean±standard deviation

	HCT116	DLD-1	SW480
GAPDH	17.6±0.8	16.2±0.6	17.4±0.4
UCN	21.9±1.1	24.7±1.2	23.9±1.1
UCN2	22.2±0.5	25.3±0.5	24.4±0.1
UCN3	ND	35.2±0.2	ND
CRHR1	32.5±0.8	32.2±0.6	30.5±0.3
CRHR2	30.4±0.7	29.2±1.3	30.5±1.3

Figure 1. *The quantitative reverse transcriptase-polymerase chain reaction values of basal urocortin (UCN), UCN2, UCN3 corticotropin-releasing hormone-related peptides receptors CRHR1, and CRHR2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression at the mRNA level in the HCT116, DLD 1, and SW480 cell lines. ND: Not detected.*

In this study, we examined whether CRH-related peptides acted *via* autocrine/paracrine mechanisms in colorectal cancer cell lines to influence DNA synthesis. Moreover, we reviewed whether CRH-related peptides played a role in the response to cellular stress caused by anticancer drugs and affected the effects of anticancer drugs (DNA synthesis disorder).

Materials and Methods

Three human colorectal cancer cell lines, HCT116, DLD-1, and SW480 (American Type Culture Collection, Rockville, MD, USA) were used.

UCN, UCN2, UCN3, *CRHR1*, and *CRHR2* expression at the mRNA level in colorectal cancer cell lines was examined. After extracting total RNA from untreated cells using TRIZOL® Reagent (Invitrogen, Carlsbad, CA), RNAs were treated with RNase inhibitor and RNase-free recombinant DNase I (Takara Bio, Ohtsu, Japan). Then cDNA was prepared using TAKARA PrimeScript™ RT Master Mix (Takara Bio). mRNA expression was examined using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) by the LightCycler® 96 System (Roche Diagnostics, Mannheim, Germany) at 95˚C for 30 s followed by 40 cycles at 95˚C for 5 s and then at 60˚C for 30 s (CYBR Premix Ex TaqII) for each of the triplet samples. The quantification cycle value (Cq) of the target genes and internal reference gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were recorded.

The products were also analyzed by 10% polyacrylamide gel and visualized by ethidium bromide.

Forward/reverse primers used for genes: *UCN*: 5'-GTCCATTGACC TCACCTTTCA-3'/5'-GTCGAATATGATGCGGTTCTG-3'; *UCN2*: 5'-CTGACCTCACGATGACCAGG-3'/ 5'-AGCTGGAAGGTTGGG ATAGGG-3'; *UCN3*: 5'-AGGCACCCGGTACAGATACG-3'/5'-GAGGGACAGGGTGAACTTGG-3'; *CRHR1*: 5'-GAACCCTGGA GACAGAAGTCA-3'/5'-AGTGCCA CAGTGCCAGTAAG-3'; *CRHR2*: 5'-CCTCTCTTGGGAGACC CCTT-3'/5'-CACGATGCAAC TGGTTGACTA-3'; *GAPDH*: 5'-GAGCACAAGAGGAAGAGA GAGAC-3'/5'+TCTAC ATGGCAACTGTGAGGAG-3'.

Using fluorouracil (5-FU), the most widely used chemotherapy agent for colorectal cancer, we examined the changes in CRH-related peptide expression levels at the mRNA level in response to 5-FU-induced cellular stress for cell line HCT116. HCT116 cells were seeded (2×10^6 cells/25 cm^2 flask) and cultured for 24 h. Then 5-FU was added at 2.8 μM, the half-inhibitory concentration (IC_{50}) as determined by the methylene blue assay. The expression of *UCN, UCN2, CRHR1,* and *CRHR2* at the mRNA level was examined before 5-FU (Sigma-Aldrich, Saint Louis, MO, USA) administration and 15 min, 30 min, and 1, 2, 4, 6, 12, 24, and 48 h after administration. We determined ΔCq ($Cq_{target\ gene}-Cq_{GAPDH}$) and plotted the relative values after setting the expression level (before 5-FU addition) to 1(ΔΔCq method). Values are the means of experiments conducted in triplicate.

Colorectal cancer cells (HCT116, DLD-1, SW480) were seeded in 96-well flat bottom plates at 1×10^4 cells/well and cultured for 24 h. CRHR1 antagonist antalarmin and CRHR2 antagonist astressin 2B were added at 1 μM and cells were further cultured for 24 h. Controls without antagonists for each cell lines were also prepared. Since dimethyl sulfoxide was contained in the stock solution of

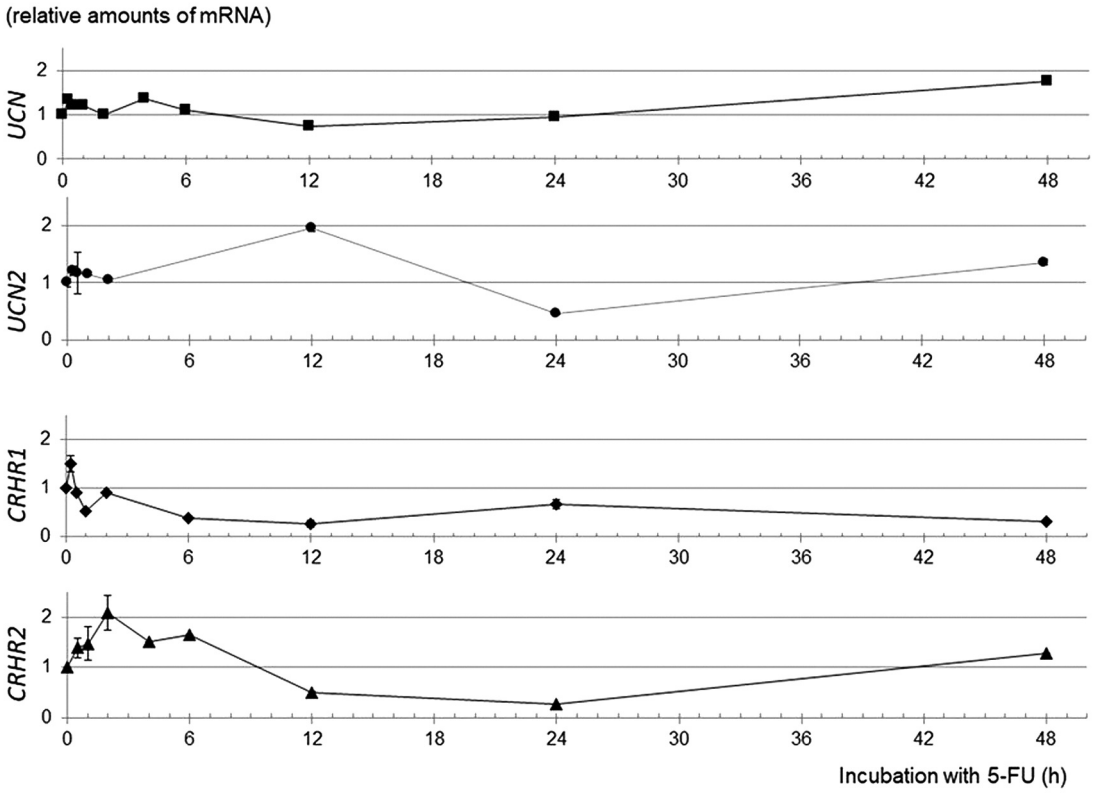


Figure 2. *Expression of urocortin (UCN), UCN2, UCN3 corticotropin-releasing hormone-related peptides receptors CRHR1 and CRHR2 at the mRNA level in the HCT116 cell line before and after adding 5-fluororouracil (5-FU).*

antalarmin, dimethyl sulfoxide was added at the same concentration to the controls and astressin 2B-added samples. Then anticancer agents 5-FU (Sigma-Aldrich) or SN-38(Toronto Research Chemicals, Toronto, Canada) at the IC_{50} was added (5FU: 2.8, 9.9, 48.9 μM and SN-38: 23, 397, 141 nM for HCT116, DLD-1 and SW480, respectively), and ^{3}H-labeled deoxyribonucleoside was added at 18.5 μCi/well for incubation 1 h after administration of anticancer agent. Cells were collected onto a Unifilter 96 GF/B® using Cell Harvester® after 8 h incubation, and MicroScint 20® was added (all PerkinElmer, Waltham, MA, USA). Then the amount of ^{3}H-labeled deoxyribonucleoside taken up was calculated using a Hidex Sense Microplate Reader® (Hidex, Lemminkaisenkatu, Finland). Experiments were conducted in triplicate. Statistical analysis was performed by two sample *t*-test.

Results

UCN, UCN2, CRHR1, and *CRHR2* (PCR product: 100 bp) expression at the mRNA level was recognized in all three cell lines (HCT116, DLD-1, and SW480). *UCN3* was detected only in DLD-1. The Cq values are indicated in Figure 1.

In the HCT116 colorectal cancer cell line, no significant change in *UCN, UCN2, CRHR1,* and *CRHR2* expression levels at the mRNA level was observed before/after 5-FU addition, as shown in Figure 2.

The results of the ^{3}H-deoxycytidine uptake assay, including treatment with 5-FU, are shown in Figure 3. No significant difference was observed in DNA synthetic ability (^{3}H-deoxycytidine uptake) with and without CRHR1 antagonist antalarmin and CRHR2 antagonist astressin 2B under 5-FU treatment.

The results of ^{3}H-thymidine uptake assay, including treatment with SN-38, are shown in Figure 4; no significant difference was observed in the DNA synthesis ability with and without antalarmin and astressin 2B under SN-38 treatment.

Discussion

CRH-related peptide expression in various malignant tumors has been reported. *UCN, UCN2, CRHR1,* and *CRHR2* expression at the mRNA level was observed in the HCT116, DLD-1, and SW480 cell lines in this study. It has been reported that CRH-related peptides are involved in various stress responses under physiological conditions (28, 36, 37). Therefore, we examined whether CRH-related peptide expression levels in cancer cell line (HCT116) changed in response to cellular stress caused by the addition of an

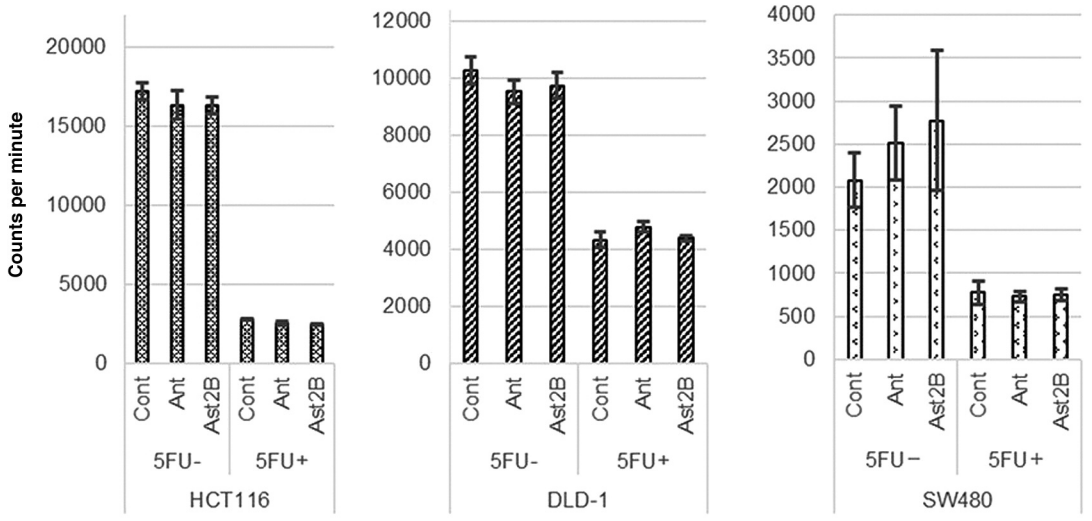


Figure 3. *The results of the ^{3}H-deoxycytidine uptake assay in cells with and without 5-fluororouracil (5-FU) treatment. Cont: Control; Ant: antalarmin, corticotropin-releasing hormone-related peptide receptor 1 antagonist; Ast2B: astressin 2B, corticotropin-releasing hormone-related peptide receptor 2 antagonist.*

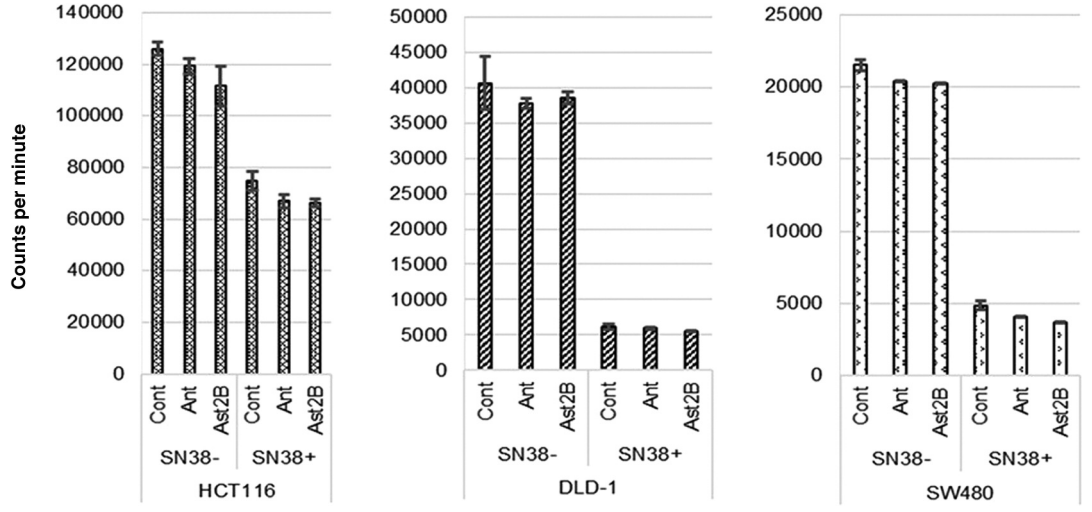


Figure 4. *The results of ^{3}H-thymidine uptake assay in cells with and without SN-38 treatment. Cont: Control; Ant: antalarmin, corticotropin-releasing hormone-related peptide receptor 1 antagonist; Ast2B: astressin 2B, corticotropin-releasing hormone-related peptide receptor 2 antagonist.*

anticancer drug. No change in the amounts of *UCN, UCN2, CRHR1,* and *CRHR2* mRNA with 5-FU treatment was observed.

There are many reports on the potency of CRH-related peptides in cancer cell proliferation (18, 38, 39). Previous reports indicated that UCN suppressed cellular proliferation through CRHR2 in colorectal cancer (18), UCN and UCN2 promoted cellular proliferation through CRHR2 in stomach cancer (24), and UCN inhibited cellular proliferation in hepatocellular cancer (6). The effects of CRH-related peptides on the proliferation potency of cancer cells remain controversial. In this study, we examined whether CRH-related peptides act *via* autocrine/paracrine mechanisms and affect cell proliferation and evaluated the DNA synthesis ability of the cells based on their uptake of ^{3}H-labeled deoxyribonucleoside. No significant difference was observed in the ^{3}H -labeled

deoxyribonucleoside uptake in each cell line with or without the addition of the CRHR1/CRHR2 antagonists, and hence, no difference in the DNA synthesis ability was observed. Hence, it is doubtful that CRH-related peptides affect the DNA synthesis ability of cells *via* autocrine/paracrine mechanisms.

Furthermore, we examined whether CRH-related peptides are involved in the resistance to cellular stress caused by anticancer drugs using 5-FU and SN-38. SN-38 is the active form of irinotecan. No significant differences in the ^{3}H-labeled deoxyribonucleoside uptake with and without the addition of the CRHR1/CRHR2 antagonists were observed, and hence, no difference in the inhibitory effects of anticancer drugs on DNA synthesis was observed. Therefore, this result suggests that it is doubtful that CRH-related peptides affect the inhibitory effects of anticancer drugs on DNA synthesis *via* autocrine/paracrine mechanisms.

This study has several limitations based on the relationship between CRH-related peptides and DNA synthesis inhibition by anticancer drugs. Firstly, we examined the action of CRH-related peptides only *via* autocrine/paracrine mechanisms in this study. There is a possibility that CRH-related peptides control gene expression *via* non-autocrine/paracrine mechanism, such as peptide translocation into the nucleus to control gene expression related to cell proliferation (10). In the future, studies using cells overexpressing CRH-related peptides or cells in which *UCN* expression has been knocked down by small interfering RNA are required. Secondly, since the CRHR1/CRHR2 expression levels in the cell lines used in this study were low, it is possible that the effects of the peptides *via* autocrine/paracrine mechanisms were not recognized. Additional studies using cells with high CRHR1/CRHR2 expression obtained by gene transfer are required.

Conclusion

The expression levels of CRH-related peptides UCN and UCN2, and their receptors, CRHR1 and CRHR2, in colon cancer cell lines did not change in response to cellular stress caused by anticancer drugs 5-FU and SN-38. CRH-related peptides showed no effect on DNA synthesis in response to cellular stress caused by anticancer drugs.

Conflicts of Interest

The Authors declare they have no conflicts of interest regarding this study.

Authors' Contributions

GK and YM designed the study. GK carried out all experiments and drafted the article. KI and YM supervised all experiments performed. GK prepared the final article. As the principal investigator, YM supervised the study. All Authors read and approved the final article.

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